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Differentiation of human mesenchymal stem cells on niobium-doped fluorapatite glass-ceramics

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Abstract

Objectives—Our goal was to characterize the response of human mesenchymal stem cells (hMSCs) to a niobium-doped fluorapatite-based glass-ceramic (FAP).

Methods—The glass was prepared by twice melting at 1525°C for 3h, and cast into cylindrical ingots later sectioned into discs and heat-treated to promote crystallization of fluorapatite submicrometer crystals. Tissue culture polystyrene (TCP) was used as control. The surface of the FAP discs was either left as-heat treated, ground or etched. Initial cell attachment was assessed at 3h. Proliferation and alkaline phosphatase (ALP) expression data was collected at days 1, 4, and 8. Cell morphology was examined using SEM, at days 2 and 4. Mineralization was evaluated by Alizarin Red staining and SEM.

Results—Initial cell attachment on as heat-treated, etched, or ground surfaces was similar to that of the positive control group ($p>0.05$). The percentage of area covered by living cells increased significantly on as heat-treated, etched, or ground surfaces between days 1 and 8 ($p<0.05$). There was no significant difference amongst groups in cell coverage at day 8, compared to TCP control. SEM revealed well spread polygonal cells with numerous filopodia, either attached to the ceramic surface or connected to neighboring cells. ALP expression at day 8 was significantly higher in osteogenic media compared to growth media on both FAP and control. FAP discs stained positively with alizarin red and calcium-rich mineralized granules associated with fibrils were observed by SEM at day 35.

Significance—hMSCs displayed excellent attachment, proliferation, and differentiation on niobium-doped FAP glass-ceramic.

Keywords

Fluorapatite; niobium; glass-ceramic; surface topography; Mesenchymal stem cells; biocompatibility; mineralization; adhesion; proliferation; differentiation

Introduction

Currently available materials for bone replacement include autologous bone, allografts, xenografts, and various synthetic materials. Autologous bone, is considered a gold standard but presents some drawbacks, such as donor site morbidity and, in some cases, limited availability [1]. Both allografts and xenografts possess shortcomings of potential disease transmission, unpredictable resorption rate, and graft rejection [2]. To overcome these challenges, synthetic materials are being developed, amongst which bioactive glass-ceramics have raised considerable interest. A biomaterial is described as bioactive when “an interfacial bond forms between the tissue and the implant” [3]. Bioactive glass-ceramics encompass a wide variety of crystalline phases, ranging from micas to calcium silicates and various apatites [4]. Fluorapatite glass-ceramics containing mica have been commercially available for use as inter-vertebral spacers and ossicles of the middle ear since the early 1990's [5].

When selecting materials for bone replacement, surface topography is considered to be critical in determining the fate of early peri-implant healing [6]. It is well established that topographical features provide cues through focal adhesions that can interact with cell proliferation and differentiation. This is true at various scales from macrotopographical features to nanotopographical features. The placement of an implant at a surgical site triggers a cascade of molecular and cellular activities that leads to new tissue formation on the biomaterial surface. Briefly, fibrin from the blood is adsorbed on the implant surface, to which the platelets adhere and get activated. These activated platelets then release cytokines to recruit undifferentiated cells on implant site and trigger their osteogenic differentiation (osteoinduction). The combination of recruitment and migration of osteogenic cells (osteoconduction) and bone formation by these cells on the implant surface is known as contact osteogenesis [7–9]. This phenomenon can be favorably assisted by the presence of a microtopographically complex implant surface by increasing the available surface area for fibrin attachment, leading to enhanced platelet activation followed by the recruitment of osteogenic cells. Additionally, efficient bone binding may occur through inter-digitation of newly formed mineralized matrix [10]. Titanium implants modified by nano-crystalline deposition have been shown to improve osteoconduction *in vitro* [11]. Acid-etching of titanium implants was also shown to lead to enhanced endosseous integration in a rabbit model [12]. Moreover, a gradually increasing body of preclinical and clinical findings associates topographic features at the nanometer and submicrometer levels and improved implant integration [13–15].

Fluorapatite (FAP) is structurally and chemically similar to hydroxyapatite, and is known to be more chemically stable and easier to synthesize as a stoichiometric compound [16, 17]. Additionally, FAP can provide fluoride release at a controlled rate [18] and several studies have demonstrated the stimulating effect of fluoride on bone formation [19–21]. FAP-based glass-ceramics have long attracted considerable interest [22]. Depending on the composition of the base glass, the crystallization temperature has been shown to vary with the Ca:P ratio and particle size of the glasses, reaching a minimum value for the glasses with the apatite stoichiometry of 1.67 [23]. *In vitro* studies showed that addition of up to 40 wt. % fluorapatite to thermally sprayed hydroxyapatite coatings led to elevated expression of several proteins involved in bone metabolism regulation in osteoblast cultures [24]. Similarly, fluorapatite glass-ceramic coatings on alumina elicited greater proliferation response of osteoblast-like cells compared to uncoated alumina [25]. *In vivo* studies have shown that fluorapatite coatings on implants in non-weight-bearing models were more chemically stable than hydroxyapatite coatings [26, 27]. Previous work in our laboratory revealed that doping fluorapatite glass-ceramic compositions with small amounts of niobium oxide promoted the formation of spherical submicrometer fluorapatite crystals (200–300 nm

in diameter, while the undoped composition exhibited long needle-shaped crystals (5 μm in length) [28]. The introduction of niobium oxide is thought to decrease the diffusivity of the glassy matrix, thereby limiting the growth of fluorapatite crystals to a sub-micrometer level. Subsequent cytotoxicity experiments with human gingival fibroblasts on these niobium-doped fluorapatite glass-ceramics demonstrated their good cytocompatibility [28], prompting the need for a more complete assessment of cell attachment, proliferation and differentiation on this material.

Mesenchymal stem cells in the bone marrow stroma are a well-established source of osteoblasts *in vivo* [29]. Moreover, these newly formed osteoblasts are majorly responsible for *de novo* bone formation at the time of injury or on implant surface [30]. The purpose of this study was to investigate the behavior of human mesenchymal stem cells (hMSCs) on a niobium-doped fluorapatite glass-ceramic. This experimental glass-ceramic has many potential uses; we anticipate that it could be used as a coating, as bulk part in non-load bearing implants or as a macroporous ceramic scaffold for bone grafts. Our experimental design therefore includes several groups with different surface topographies (as heat-treated, etched or ground) in the initial attachment, cell morphology and cell proliferation studies, with a polished group as negative control in the initial cell attachment evaluation. These surface finishes are directly applicable to bulk implants and coatings. Meanwhile, our research plan focuses on tridimensional macroporous scaffolds, these sponge-like cellular structures will be either left as heat-treated or chemically etched to reveal the unique microstructural features of this material. The cell differentiation studies are thus targeted at chemically etched substrates. The hypotheses tested were that the expression of submicrometer topographical features via various surface treatment techniques could affect initial cell attachment and that these features would provide an enhanced environment for cell proliferation, protein expression, and differentiation. Our long-term goal is to evaluate this glass-ceramic as a bone substitute in order to support osteo-specific differentiation through nanoscale disorder of fluorapatite isometric crystals.

Materials and Methods

Specimen Preparation

A fluorapatite-based glass composition doped with 1 wt.% of niobium oxide was prepared by mixing reagent grade oxides and carbonates as previously described [28]. The batch ingredients were melted at 1525°C for 3 hours in platinum crucibles. After quenching, the glass frit was powdered and melted again at 1525°C for 3 hours. The glass was cast into pre-heated cylindrical stainless steel molds to form 12×60 mm rods. Rods were furnace-cooled to room temperature and cut into discs (12 mm in diameter, 1.5 mm thick) using a low speed diamond saw. The glass discs were then heat treated at 950°C for one hour to promote nucleation and crystallization of spherical submicrometer fluorapatite crystals, as previously established [28]. Three types of experiments were conducted: 1/ initial cell attachment and cell morphology, 2/ cell proliferation and 3/ alkaline phosphatase (ALP) activity and cell differentiation. Glass-ceramic discs were randomly divided into three groups ($n=8$ per group): specimens were either left as-heat treated, etched with a diluted hydrofluoric solution for 60 seconds or ground with a 600-grit diamond disc under water irrigation. Additional specimens for initial cell attachment experiments were polished to a 1- μm finish and served as negative controls. As mentioned earlier, ALP activity and cell differentiation experiments were conducted on the etched group only, as most revealing of the submicrometer FAp crystallites. Glass-ceramic specimens were sterilized in a steam autoclave at 134°C for 30 minutes. Tissue culture polystyrene (TCP) was used as a positive control for all cell culture experiments.

Cell Culture

Human Mesenchymal Stem Cells (hMSCs, Lonza, Walkersville, MD) were cultured in growth medium (Mesenchymal Stem Cell Growth Medium Bullet Kit, Lonza, Walkersville, MD), passaged by trypsinizing (0.05% trypsin/EDTA) and sub-cultured at a density of 2500–5000 cells/cm². Cells between the third and fifth passage were used for all experiments. The cell cultures were maintained under standard culture conditions (37°C, 95% relative humidity, and 5% CO₂). hMSC Osteogenic Bullet Kit (Lonza, Walkersville, MD) was selected for osteogenic induction during experiments. Normal Human Osteoblasts (NhOsts, Lonza, Walkersville, MD) served as positive controls for analysis of extracellular matrix secretion. NhOsts were seeded in growth medium (Osteoblast Growth Medium Bullet Kit) and mineralization was induced by osteogenic medium (Osteogenic bullet Kit, Lonza, Walkersville, MD).

Initial Attachment

Discs from each experimental group ($n=4$ per group) were seeded with 3000 cells. Tissue culture polystyrene (TCP) surface was used as positive control. At 3 hours, the discs were stained with green calcein dye (Invitrogen, Carlsbad, CA). In order to count the number of live cells on each substrate, digital micrographs were taken at three random locations on each disc. NIH Image J (version 1.440) software was used for digital image analyses [31]. The experiment was performed in duplicate.

Cell Proliferation

A live/dead assay was used to assess cell proliferation at days 1, 4 and 8. This well-established assay uses a membrane-permeant calcein-AM dye, which is cleaved by esterases in a live cell, yielding green fluorescence, and a membrane impermeant ethidium homodimer-1, which binds to nucleic acids in membrane, leading to a red fluorescence in compromised cells. For each time point, three steam-sterilized discs from each group were seeded with 3000 cells. TCP was used as positive control. At days 1, 4 and 8, the cell culture medium was replaced with Dulbecco's phosphate-buffered saline (pH 7.2) containing 0.002 mmol/L calcein-AM and 0.002 mmol/L ethidium homodimer-1 (Invitrogen, Carlsbad, CA). After 30 minutes of incubation at room temperature, the cells were observed using epifluorescence microscopy (Olympus, BX 51, Tokyo, Japan). Digital micrographs were taken at five random locations on each disc. Images were analyzed using Image J software. The amount of cell attachment was measured on each micrograph as the percentage of specimen surface area covered by live cells.

Early Cell Morphology

In order to examine the cell-glass ceramic interaction and early cell morphology, hMSCs were cultured on steam-sterilized glass-ceramic discs for 2 to 4 days and prepared for examination by scanning electron microscopy (SEM). Specimens were rinsed with phosphate buffered saline and fixed in formaldehyde, followed by graded ethanol dehydrations up to final dehydration in absolute ethanol. Specimens were then dried in hexamethyldisilazane (HMDS)/ethanol solution series and gold-coated prior to SEM examination in secondary electron imaging and ultra high-resolution mode (Sirion, FEG scanning electron microscope, FEI Company).

Alkaline Phosphatase (ALP) Activity

ALP is an enzyme expressed by cells during osteogenesis and is well established as a differentiation marker. A colorimetric *p*-nitrophenol phosphate assay (Stanbio, Boerne, TX) was used to measure ALP expression. hMSCs were seeded on etched glass-ceramic and TCP discs in either osteogenic or control growth medium. At days 1, 4, and 8 cells were

lysed using a mammalian protein extraction reagent (Thermo Scientific, Rockford, IL). Cell lysates were assayed for ALP activity using the manufacturer's protocol. ALP activity was normalized to total protein content using a Bradford assay (Thermo Scientific, Rockford, IL).

Cell Differentiation

Mineral formation was evaluated by seeding both hMSCs and NhOsts (as control) on etched glass-ceramic discs. hMSCs and NhOsts were allowed to proliferate for a week. Osteogenesis was then induced by replacing growth medium with osteogenic and mineralization medium, respectively. At 21 days some hMSCs specimens were stained with Alizarin Red to visualize calcium-rich nodules. Briefly, discs were washed with PBS and fixed with ice-cold ethanol at 4°C for 1 hour. The fixed cells were soaked in 1% Alizarin Red (Sigma) solution for 5 minutes at room temperature (pH 4.0) and washed with water to remove the residual dye, prior to examination by optical microscopy. Specimens were fixed for SEM at 21 and 35 days as described earlier, and gold-coated prior to examination. The cell morphology, as well as nodules and fibril formation was investigated in ultra-high resolution mode.

Statistical Analysis

All experiments were performed twice with at least three specimens per treatment condition for a total of 6 to 8 specimens per group. Graphical results are presented as mean $\pm$ standard deviation (SD). A *p*-value of less than 0.05 was considered statistically significant. ANOVA was used to detect differences between group means. Tukey's simultaneous tests were used for comparison between groups.

Results

Initial Attachment

The cell counts on glass-ceramic surfaces after 3 hours are displayed in Figure 1. The mean number of cells attached to as-heat-treated, etched, or ground glass-ceramic surfaces was similar to that of the positive control TCP surface, and significantly higher than on polished surfaces ($p < 0.001$). For clarity and due to the poor performance of the polished group for initial cell attachment, this group was not included in subsequent cell proliferation and differentiation experiments.

Cell Proliferation

As shown in Figure 2, cells were well dispersed on all surfaces at day 1. At day 4, an increase in the number of cells per unit area was clearly noted for all surfaces. By day 8, the cells covered the entire surface, achieving confluence for all treatment groups. Due to the overlapping morphology of spread cells, we chose to measure the area covered by living cells as an indicator of cell proliferation, rather than attempting to count the number of cells. Results are displayed in Figure 3. The area covered by living cells increased significantly between day 1, day 4 and day 8 for all surfaces ($p < 0.05$). Meanwhile, there were no significant differences amongst surfaces or TCP control at either day 1, 4, or 8.

Early Cell Morphology

Scanning electron micrographs of hMSCs on as-heat treated, etched, ground or polished surfaces at days 2 or 4 are shown in Figure 4 (A through D). With the exception of the polished group, cells appeared well spread, exhibiting distinct osteoblast-like morphology, i.e. polygonal in shape, with numerous thin filopodia either attached to the glass-ceramic surface or connecting to neighboring cells. On both as-heat treated and etched surfaces, it is

worth noticing that some cells exhibited extended filopodia displaying preferred attachment to sub-micrometer spherical fluorapatite crystallites (insets Figure 4A and 4B). Meanwhile cells on polished surfaces appeared less active and more compact (Figure 4D).

Since no significant difference in the amount of cell proliferation was observed between groups at any time point, all subsequent experiments, including ALP activity and cell differentiation, were conducted on etched surfaces as this type of surface treatment is applicable to tridimensional macroporous scaffolds and most revealing of the microtopography created by the submicrometer fluorapatite crystals.

Alkaline Phosphatase (ALP) Activity

Results of ALP activity measurements for cells cultured on TCP control and etched glass-ceramic discs are graphically displayed in Figure 5. These results were normalized to the amount of protein per sample ($\mu\text{mol}/\text{min}/\text{g}$). A significant increase in ALP activity was observed for cells cultured in osteogenic medium over time compared with cells cultured in growth medium. At day 8, the ALP activity on the etched glass-ceramic was 8-fold higher in osteogenic medium (0.256 ± 0.028) compared to growth medium (0.037 ± 0.004 ; $p < 0.001$). Similarly, ALP activity on TCP control was significantly higher in osteogenic medium (0.252 ± 0.051) compared to growth medium (0.088 ± 0.025 ; $p < 0.001$). Remarkably, no significant differences were found between ALP activity on TCP control surface and glass-ceramic surfaces in osteogenic medium ($p > 0.05$).

Cell Differentiation

The ability of cells to form calcium-rich mineralized nodules, on the surface of etched glass-ceramic discs, was examined by both SEM and Alizarin red calcium staining under optical microscopy. An unexpected outcome was that SEM examination revealed the presence of spherical nodules ($0.5\text{--}0.8\text{ }\mu\text{m}$ in diameter) associated with hMSCs cultured in growth medium (Figure 6A). These nodules appeared either well aligned as if undisturbed by SEM preparation, or gathered in formations. Fibril-like material was also observed at the cell surfaces. Overall, these nodules were fewer and smaller in growth medium than in osteogenic medium with corresponding fainter alizarin red staining (Figure 6A inset). In osteogenic medium, larger nodules ($0.4\text{--}1.5\text{ }\mu\text{m}$ in diameter) appeared located both above and below the cell surface (Figure 6B), suggesting that there may be more nodules covered by layers of cells that were not entirely visible by SEM. Alizarin red staining confirmed that these nodules were mineralized and calcium-rich (Figure 6B inset). Interestingly, at 5 weeks, fibrils with very similar appearance were found associated with mineralized nodules in both hMSCs culture (Figure 7A) and NhOst culture (Figure 7C). Higher magnification highlights the similarity in fibril morphology between both cell lines (Figure 7B and 7D). This morphology is reminiscent of the morphology of collagen fibers with a distinct banding pattern (Figure 7D, bar = 67 nm).

Discussion

The results showed that hMSCs attached, spread and proliferated as well on niobium-doped fluorapatite glass-ceramic surfaces as they did on the positive control TCP material. As mentioned earlier, the initial phase of cell-biomaterial interaction is critical for peri-implant healing, because the ability to attach and spread conditions the proliferation and differentiation of cells on any biomaterial [32]. After 3 hours of culture, the number of cells attached to fluorapatite glass-ceramic surfaces was similar to that on control TCP surfaces, a gold standard for *in vitro* tissue culture (Figure 1). As expected, initial cell adhesion on polished surfaces was significantly lower than on TCP. These results are in good agreement with previous *in vitro* studies with hMSCs, demonstrating a higher level of cell adhesion and

proliferation on topographically complex surfaces compared to smooth surfaces [33]. Electrochemically etched silicon surfaces have been shown to increase protein adsorption resulting in enhanced cell adhesion and proliferation in four different mammalian cell lines, compared to unetched substrates [34].

After 24 hours, cells had fully spread to attain a characteristic polygonal morphology. The surface area covered by cells gradually increased up to day 8, to reach a complete colonization of the surfaces, indicating that the niobium-doped glass-ceramic tested was not only cyto-compatible but readily promoted cell proliferation. In addition, the absence of dead cells on the live/dead images through day 8 indicated that all attached cells were viable [35], i.e. the material exhibited excellent biocompatibility. SEM examination confirmed the excellent spreading of cells on glass-ceramic surfaces, particularly on as heat-treated and etched surfaces. Previous work has shown that niobium oxide additions in this glass-ceramic system promoted the development of sub-micrometer spherical fluorapatite crystallites. In contrast, the crystallization of large needle-shaped crystals was observed in the corresponding undoped glass-ceramic composition [28]. This unique microstructure of spherical microcrystals can be revealed by etching the ceramic surface, and prompted the selection of this particular composition for further biocompatibility studies. Indeed, well spread hMSCs were observed in the present study, with extended filopodia preferentially attached to these submicrometer crystallites. Moreover, polished specimens that were devoid of this niobium-induced microtopography displayed significantly lower initial attachment and poor interaction with the glass-ceramic. These results concur with previous findings by Gough *et al.*, where, osteoblasts cultured on roughened 45S5 bioactive glass displayed more filopodia than those cultured on smoother surfaces [36]. Doping with niobium oxide appears therefore to indirectly play a key role on cell response by determining the microstructure of this glass-ceramic. It is speculated that this microtopography favors cell adhesion by enhancing protein adsorption *in vitro* [37].

Close examination of the SEM micrograph of cells on a polished surface also revealed microscopic alterations around the attached cell. It is hypothesized that the local pH change at the cell material interface (due to the metabolic activity of cells) also leads to a cell-mediated dissolution of superficial inorganic components. Solution-mediated surface modification has been documented by Dias *et al.*, where calcium phosphate glass-ceramic discs were presoaked in growth medium prior to cell seeding [38]. This surface instability at the material-media interface is required for bone bonding *in vivo* [39]. It is believed that this cell and solution-mediated surface modification may result in the three dimensional micro-complexity *in vivo* which is critical to contact osteogenesis at the tissue-implant interface [10].

Alkaline phosphatase is an enzyme expressed by cells during early stages of osteogenic differentiation, and is a well-characterized marker. Increased levels of ALP activity (Figure 5) over time are indicative of the differentiation of hMSCs in osteogenic medium towards osteogenic lineage. No significant difference was observed between ALP levels on glass-ceramic and control TCP surfaces. These results compare well with published data. Reilly *et al.* reported comparable ALP expression for hMSCs cultured in osteogenic medium on TCP and bioglass surfaces at 7 days [40].

Moreover, the cells successfully differentiated and produced calcium-rich mineralized nodules associated with fibrils, when cultured on fluorapatite glass-ceramic discs. SEM micrographs revealed formation of confluent cell multi-layers with islands of mineralized granules, on the glass-ceramic surface. Numerous calcium-rich nodules were observed on hMSCs cultured in osteogenic medium. After five weeks, fibrils were also observed in association with the mineralized nodules. These fibrils exhibited a defined banding pattern

reminiscent of collagen fibers [41]. Rohde *et al.* reported similar results, with bone marrow stromal cells from chick embryos. It was shown that upon osteogenic induction, mineralized nodules are first deposited on culture surface (day 18–21), while collagen fibrils are later produced with increased culture duration (days 25–30) [42]. In the present study, Normal Human Osteoblasts (NhOsts) were also cultured in mineralization medium as optimized culture conditions, conducive for secretion of bone-like extracellular matrix, and served as controls. As seen in Figure 7, the morphology of the fibrils is comparable in both hMSCs (Figure 7B) and NhOsts (Figure 7D) cell lines. This is indicative that hMSCs successfully differentiated towards osteogenic lineage, and produced extracellular matrix consisting of calcium-rich mineralized nodules, associated with fibrils, similar to differentiated osteoblasts. More remarkably, calcium-rich nodules were also found on hMSCs cultured in growth medium, suggesting some degree of differentiation towards osteoblastic lineage without addition of osteogenic supplements.

Conclusions

Our results showed that hMSCs attached, spread and proliferated as well on fluorapatite glass-ceramic surfaces as they did on the positive control TCP material. The presence of surface microtopography in the form of submicrometer fluorapatite crystals on as-heat treated, etched or ground surfaces clearly promoted initial cell adhesion, compared to polished surfaces.

Moreover, cells successfully differentiated and produced calcium-rich mineralized nodules associated with fibrils, when cultured in osteogenic medium on etched fluorapatite glass-ceramic discs. The morphology of these nodules and fibrils was very similar to that of nodules and fibrils produced by human osteoblasts cultured on the glass-ceramic.

Interestingly, mineralized nodules were also observed after hMSCs culture in growth medium only, without osteogenic medium, suggesting some degree of differentiation towards osteogenic lineage. Further studies are underway to characterize this differentiation process and ascertain the nature of the fibrils observed after 35 days.

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Figure 1. Normalized number of cells adhering to substrate after 3 hours ($n=8$ per group). Horizontal line indicates no statistically significant difference at $\alpha=0.05$.

Figure 2. Fluorescence micrographs of hMSCs stained with live/dead assay on heat-treated (A-C), etched (D-F), and ground (G-H) glass-ceramic surfaces. (Scale bar is 500 micrometers)

Figure 3. Percentage of area covered by living cells ($n=6$ per group). Horizontal line indicates no statistically significant difference.

Figure 4.
SEM micrographs of hMSCs grown for 2 to 4 days on (A) heat-treated (B) etched (C) ground and (D) polished fluorapatite glass-ceramic surfaces.

Figure 5.
ALP activity of hMSCs on etched fluorapatite glass-ceramic and control TCP surfaces
(Identical letters denote no statistically significant difference).

Figure 6. Scanning electron micrographs of hMSCs cultured on etched glass-ceramic surfaces for 21 days in (A) Growth medium and (B) Osteogenic medium. Arrows in (A) indicate nodules. Insets show corresponding optical micrographs of Alizarin red staining (scale bar is 500 micrometers).

Figure 7. Scanning electron micrographs of hMSCs (A, B) and NhOsts (C, D) cultures on etched fluorapatite glass-ceramic for 35 days. Green scale bar in 6 (D) is 67 nm.