Oral Tissue Engineering of Complex Tooth Structures on Biodegradable DLPLG/β-TCP Scaffolds

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1. INTRODUCTION

Research for new technologies and biomaterials improving orofacial implantation and regeneration has evolved at a fast-pace[1, 2, 4, 13-16]. Teeth are essential for survival in many vertebrates, and missing or misplaced teeth can have fatal consequences, causing some species to be unable to make use of available food supplies. Tooth loss due to periodontal disease, dental caries, trauma, or a variety of genetic disorders is one of the most severe human health problems. It is critical, therefore, that the dentition develops correctly, with the required number and type of teeth developing in specific positions in the jaws. A biological tooth substitute that could replace lost teeth would provide a vital alternative to currently available clinical treatments[5]. The purpose of this study leads to new composite constructs to be shapable, resorbable, and biocompatible multifunctional bone equivalent for applications in eriodontology, oral surgery and trauma of the teeth and periodontal tissues, including bone. The goal of our compiled projects is tooth organ engineering. In order to organize such a complex project, work has been divided in three phases. Starting phase deals with experimental tools for *in vitro* and *in vivo* tooth engineering. Second phase would be selection, collection and preservation of high number of healthy teeth

extracted for orthodontic reasons, and the final phase will be to treat organizational aspects of a bank for tooth tissue. Tooth germ is an accessible source of high-quality human postnatal stem cells for research and growth factor delivery systems (GFDS). This phase requires a much better understanding of stem cells, cell-proliferation and differentiation *in vitro*. We used the approach of tooth tissue replacement utilizing culturing cells seeded onto extracellular matrices. The bioactive scaffolds (surfaces), for example, are natural or synthetic bone equivalent and/or dental implants, with bound proteins and cells on surface. The development of scaffolds and implants made of biomaterials that are not only permissive for cell growth but also equipped with a plethora of active functions is being observed: attracting or repelling cells, providing compartmentalization, having 3-D memory, allowing monitoring and modulation, regulating growth and differentiation, to name a few. Model of tooth morphogenesis is a wonderful model for studying how genes and molecules interact to create complex patterns. It is a model system for comparative studies on normal and pathological growth and differentiation of epithelial (ameloblastoma), epithelial-ectomesenchymal (ameloblastic fibroma) and ectomesenchymal (odontogenic myxoma) tissues[5,7]. Of particular interest to maxillo-facial applications are multifunctional biodegradable scaffolds. Poly(lacticacid)-based composites due to their biocompatibility and bioresorbability are used for bone plates or temporary internal fixation of damaged bone[9,22,20,24]. In the future, the availability of shapeable, biodegradable, biocompatible and bioactive constructs might be of great interest in order to avoid the inconvenient surgical insertion of large implants. Recently, the development of bio-hybrid systems (composites, copolymers, complexes, hydrogels, blends, etc.), based on natural and synthetic polymers, and their wide range of applications in biomaterial science has received tremendous attention[25]. Results from these studies will benefit scientific and clinical community by: a) organization of tooth bank, b) enabling continuous source of tooth germ pluripotent stem cells, and c) stimulating development of very specific bioactive scaffolds for tempo-spatial regulation of whole tooth differentiation which are crucial for 3-D organ engineering in general.

2. MATERIAL AND METHODS

2.1 Composites Scaffolds

β-TCP was prepared by modified protocol of Jain[6]. Spherical granules were dried-calcined at 1,100°C/12 h. The heating and cooling rates were

Anka Letić-Gavrilović: Oral Tissue Engineering Of Complex Tooth Structures On Biodegradable DLPLG/ß-TCP Scaffolds.
In Advances in Experimental Biology. Biomaterials, 2004

each 10°C/min. Evaluation of chemical composition and purity was carried out by Fourier Transform-Infrared Spectroscopy (FTIR), recorded using the KBr pellet method by Perkin Elmer 782 spectrometer. Porosity was assessed from the N_2 desorption isotherms (BET method). Three particle sizes of β-TCP (<100 µm, >100 µm and >300 µm) were used to prepare three main composite formulations, with the solvent evaporation technique. After D,L-poly(glycolic acid) DLPLG, (50:50, MW 40, 000-75, 000; Sigma-Aldrich) was dissoluted in acetone (0.5 copolymer/5 mL acetone), the solution was stirred at 100 rpm/5 h. Ceramic particles of three given sizes: a) 25-100 µm (or <100µm) b) 150-300 µm (or >100 µm) and c) >300 µm, were added to the polymer solution, until reaching the ratio (by weight) of 80/20 acetone/copolymer, was reached leading to a broad scale of the final DLPLG/βTCP ratio from 90/10-30/70. The material was mixed at 50 rpm/h. The mixture was cast in the desired shaped using chilled Teflon moulds without any pressure. For this study, discs of ϕ5 mm were prepared. After solvent evaporation, the samples were air dried (48 h), freeze-dried (24 h), lyophilized and sterilized with ETO. The control biomaterials (only copolymer) were processed using the same method. By adopting the same process and changing a few parameters (e.g. temperature, pressure and time), the biomaterial could be produced with various compressive strengths and porosity. Porosity of bulk composites was assessed by BET test. Structure, mechanical properties and *in vitro* degradation were measured using various techniques.

2.2 Mechanical testing of composites

Rectangular sheets of 2 mm thickness were made in Teflon moulds by press moulding at room temperature and dumbbell-shaped specimens for mechanical testing were cut from the sheet with a cutting die (ISO R37). The mechanical properties were determined in dry state. The elastic modulus, tensile strength and elongation at break were determined in a Houndsfield testing machine at a testing speed of 50 mm/min at room temperature. Ten specimens were used for each testing.

2.2.1 Degree of Swelling of the Composites

For swelling tests six samples/group/five experiments were cut from hot press sheets with a size of $1\times1\times0.2$ cm^3. The swelling test was carried out in distilled water at room temperature (23±1.5 °C). Swelling was assessed every 20 h for eight days. The degree of swelling of the composites was:

$$S_w = (W_t - W_o) / W_o$$

Anka Letić-Gavrilović: Oral Tissue Engineering Of Complex Tooth Structures On Biodegradable DLPLG/ß-TCP Scaffolds.
In Advances in Experimental Biology. Biomaterials, 2004

where S_w is the swelling degree in a given time, W_t the weight of the tested specimens after immersion in water for a time *t*, and W_o the weight of the tested specimens at the beginning of the test. Each data point represents the mean±SD. Copolymer without ceramic was control.

2.2.2 Hydrolytic Degradation

For studying degradation for two months, 14 specimens per three groups were used in addition to two controls with polymer only, one at pH 3.7 and one at pH 7.3. They were placed into 70 small flasks filled with phosphate buffer. The flasks were allowed to stand in a thermostatic oven for predetermined periods of time. For degradation at 37° C, the pH value 3.7 was selected. Two specimens were withdrawn from the aging media at each degradation time (every ten days), and washed with distilled water. Each data point represents the mean ± SD of two measurements/five experiments. Copolymer without ceramic was control.

2.3 Animals

Male rats of an inbred AO strain were obtained from the Institute for Experimental medicine, Military Medical Academy (Belgrade, Yugoslavia). All animals were used at 3 or 4 months of age, and had ad libitum access to rat chow and water. Mandibles from 20 rats were soaked in butadiene for 15 min to prevent contamination and then rinsed in phosphate-buffered saline (PBS) before being transferred to a sterile environment.

2.3.1 Purified Low Molecular Weight Protein (10kDa) Extracts

About 20 rats were killed by cervical dislocation and the molar tooth buds free of connective tissues were collected in five volumes of cold saline solution. Tissue samples allocated for digestion were treated with collagenase (1 mg/mL) for 12 h in an incubator at 37°C and 5% CO_2 .The suspension was filtrated and washed in PBS. Five mL was plated into 25 cm^2 polystyrene tissue culture flasks and 2 mL was plated onto 35x10 mm^2 Petri dishes for staining purposes. After they were minced in a mixer and centrifuged at 7,000 g for 30 min, supernatants were collected, and then re-centrifuged at 11,000 g for 30 min at 4°C. After lipids were discarded, supernatants were collected and filtered through a 0.45 µm filter. They were used as crude extracts of the tooth buds. Ultrafiltrates of 100 kDa, 30 kDa, and 10 kD (Advantec, Toyo, Ultrafilter) were collected and then sterilized by passage through a 0.22 um filter and stored at -20°C. In all fractions, the

Anka Letić-Gavrilović: Oral Tissue Engineering Of Complex Tooth Structures On Biodegradable DLPLG/ß-TCP Scaffolds. In Advances in Experimental Biology. Biomaterials, 2004

concentration of protein was determined by the Lowry[17] method. Only the 10 kDa fraction of ultrafiltrates was lyophilized (Speed Vac Concentrator) and was re-constituted with saline solution at a four-fold higher concentration. Gel-filtration. The 10 kDa fraction was filtered through Sephadex G-25. Experimental conditions were as follows: the column was 35 cm x 2.6 cm, gel 30.9 cm, pressure 34.5 mL/h, and flow rates 35 mL/h. Elution was done with a 0.1 M phosphate buffer at pH 8. After application of the 10 kDa fraction, each1.16 ml fraction was collected and three tubes were combined. After sterilization by passage through a 0.22 µm filter, fractions were kept at -20°C. RP-HPLC chromatography. All separations were carried out at 30°C and at a constant flow rate of 1 mL/min. Operating parameters were controlled by use of a LKB Ultrachrom GTI System. A LKB-BROMA liquid chromatograph equipped with 2156 solvent conditioner, 2152 LC controller with 2155 HPLC Column Oven, 2151 Variable Wavelength Monitor and Fraction Collector FRAC-100 (Pharmacia Fine Chemicals), were used. The column was Sepherisorb ODS-2 (5 µm), and was developed with a 35 min linear gradient from 0 to 100 per cent B at a flow rate of 1 mL/min, where the primary solvent (A) was water and the secondary solvent (B) was 70% acetonitrile /30% water. The system was fitted with an injection loop for a 1 ml sample. The column elute was monitored by absorbance at 280 nm. Pressure was 134 bar and chart speed 4 mm/min. Each fraction of 1.5 mL per tube was collected automatically.

2.3.2 Cell cultures

Neonatal AO rat calvaria steoblasts (RCO) were isolated via sequential collagenase digestions. Cells were grown to confluence in standard media (Dulbecco's Modified Eagle's Medium, [DMEM], Life Technologies, Gaitersburg, MD, USA) containing 15% heat inactivated fetal calf serum (FCS; HyClone, Logan; UT, USA) and penicillin and streptomycin (P/S; Life Technologies) on our 3-D scaffolds. All cells were cultured in a humidified 5% CO_2, 37°C incubator, and seeded in flasks. Thirty samples of three composites with various granule sizes were placed in 24-well culture plates, and the cell suspension (1×10^4 cell/mL/100 µL) was applied onto each sample. The control was cells in empty wells. Cells were allowed to attach for 2 h. At the end of the experiment, RCO were characterized for phenotypes: Alkalinephosphatase activity (ALP, Sigma, USA), Lactatedehydrogenase (LDH, Sigma, USA), calcium (Ca, Sigma), phosphorus (P, Sigma), and all tests as indicators of osteoblast activity. The MTT test (Sigma) was performed to assess cell proliferation.

Anka Letić-Gavrilović: Oral Tissue Engineering Of Complex Tooth Structures On Biodegradable DLPLG/ß-TCP Scaffolds. In Advances in Experimental Biology. Biomaterials, 2004

2.3.3 Histology and Scanning Electron Microscopy

Mandibular tooth buds and representative samples of bulk-composites and cells attached were processed for microscopy. Morphological and element analysis (KEVEX) were performed by Etec Autoscan (Etec-Haywood, CA, USA).

2.4 Statistics

All measurements were done in five separate experiments and expressed as mean ±SD of mean. A two-tailed unpaired t-test for the statistical significance was used. The values of cell culture between the groups were compared using analysis of variance (ANOVA) and Scheffé *post hoc* multiple comparison test.

3. RESULTS

3.1 Physicochemical Characteristics of β-TCP and DLPLG/β-TCP Composites

The characteristics of β-TCP particles were as follows: 25-100 μm , 150-300 μm and >300 μm were 100% pure with 70% interconnected porosity. The Specific Surface Area (SSA) of the particles obtained by BET, yielded 1.07 m^2/g. Electron-microscopy image of bulk-material showed a uniform distribution of the βTCP particles in composites Fig.1.A. On higher magnification porosity and interconnectivity of 20 % are visible Fig.1.B.

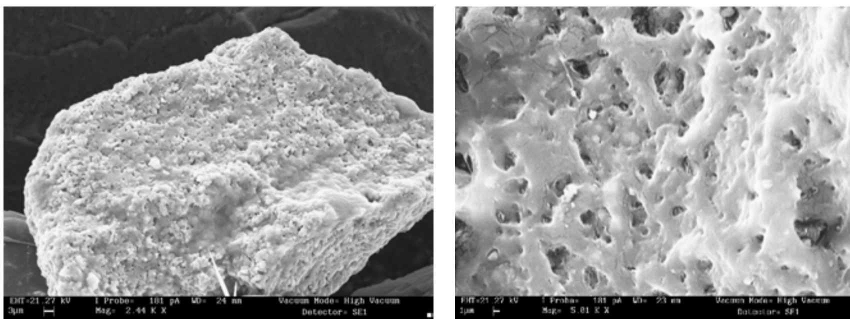

Figure 1. Bioresorbable composites (DLPLG/βTCP) with granule size <100 μm used in experiments. Micrographs of composites with porosity (<20%) and interconnectivity (bar 1μm); A) bar 3 μm, B) bar 1 μm

Anka Letić-Gavrilović: Oral Tissue Engineering Of Complex Tooth Structures On Biodegradable DLPLG/ß-TCP Scaffolds. In Advances in Experimental Biology. Biomaterials, 2004

3.1.1 Mechanical Properties of the Composites

The mechanical properties of composites were affected by the three different sized granules of β-TCP filler. Quantity and size of granules ranging from <100 μm to >300μm, improved effectively elastic modulus of composites formulation (Table 1A). Comparatively, tensile strength and percent of elongation were reduced with increasing the granule size. The tensile tests showed that, although the elastic modulus of the composites was increased by the incorporation of bigger granules, the tensile strength and elongation at break were not changed significantly. Porosity and inter-connectivity of composites processed without any pressure yielded 20%.

3.1.2 Degree of Swelling

The stability of the composite structure in water was studied for eight days. The results are shown in Fig. 2B. All three formulations tested were saturated with solvent for 1 h. In time the swelling degree increased up to the value 0.6%. The composite retained a compact structure and disintegration was not observed, even after experiment.

3.1.3 Hydrolytic Degradation In Vitro

The first signs of degradation were recorded after 10 days Fig. 2A. Hydrolytic degradation generally occurred in two steps. In the first step, the structure became saturated and showed a very slowly increasing concentration of free lactic acid, attributable to the breakage of its chemical bonds, which was particularly marked in the lactic acid part of copolymer. In the second step, the degree of degradation was slightly lower, with no significant difference between the groups and the control.

3.2 Purification of the Molar Tooth Bud Extracts

The crude extracts of the molar tooth germs (Fig. 2) from the AO rats were ultrafiltered and 100 kDa, 30 kDa, and 10 kDa ultrafiltrates were obtained. The protein concentrations of these fractions were markedly decreased as the molecular weight of ultrafiltrates became smaller. The 10 kDa fraction was gelfiltered by Sephadex G-25 chromatography and yielded five fractions. Fraction with highest protein content was further analyzed by RP-HPLC and used to incorporated into composite scaffolds. Fraction with the highest protein content was incorporated into the three composite formulations for cell culturing.

Anka Letić-Gavrilović: Oral Tissue Engineering Of Complex Tooth Structures On Biodegradable DLPLG/ß-TCP Scaffolds.
In Advances in Experimental Biology. Biomaterials, 2004

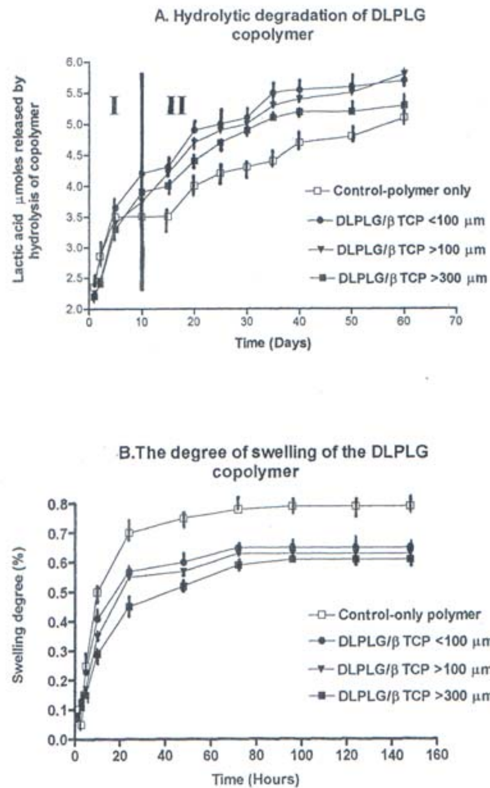

Figure. 2. A) Biodegradation of composites by hydrolysis. The first phase (I) occurred within 10 days and second phase (II) from 10th to 70th day. Control was polymer without any ceramic. Degradation characteristics were measured for two months, 14 specimens per three groups, plus two controls with polymer only. For degradation at 37° C, the pH value 3.7 was selected. Two specimens were withdrawn from the aging media at each degradation time (every ten days), and washed with distilled water. Each data point represents the mean±SD of two measurements per five experiments. B) The degree of swelling of the composites. Note that the degree of swelling nearly reached equilibrium after 24 h immersion in water. For swelling tests six samples/group were cut from hot press sheets with a size of $1\times1\times0.2$ cm^3. The swelling test was carried out in distilled water at room temperature (23±1.5 °C). Swelling were assessed every 20 h during eight days. DLPLG copolymer without ceramic was control. Each data point represents the mean±SD of six samples/group/measurements per five experiments.

Anka Letić-Gavrilović: Oral Tissue Engineering Of Complex Tooth Structures On Biodegradable DLPLG/ß-TCP Scaffolds.
In Advances in Experimental Biology. Biomaterials, 2004

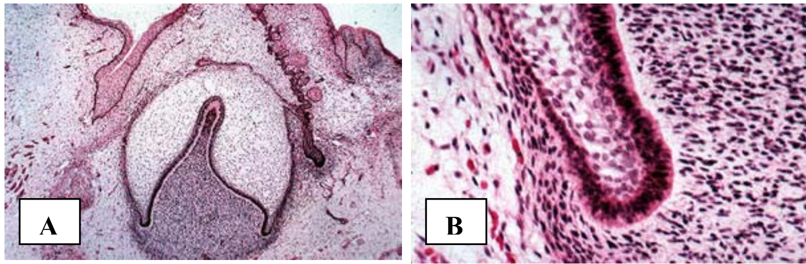

Figure.3. Histological section of molar tooth bud from AO male rat.

3.3 Cell Culture and Biochemical Testing

Results showed that, during the incubation of osteoblast-like cells on the three formulations of composites, no signs of cytotoxicity appeared, (Table 1.B). In fact, the LDH measured in sample cultures of the materials matched those of the control cultures. The ALP values showed that the presence of bone substitute biomaterials did not negatively interfere with osteoblast activity. Ca and P determined in the supernatant were significantly lower than that in the controls. The MTT test revealed that cell proliferation significantly improved in the presence of biomaterials with respect to the control.

3.4 Scanning Electron Microscopy

After culturing on the composites with smaller granule size (<100µm) RCO osteoblast cells displayed extremely long and thin processes (Fig. 4.A and B). Numerous cells proved to have close relations with the substrate. Additionally, these cells gave rise to many filamentous processes towards an extracellular matrix substrate. The long lamellar-granular structures in the extracellular spaces were closely related to the substrate. The small granular structures in the extracellular spaces were demonstrated to have close relations with the cells. Morphologically, the cells cultured on second size substrate (>100 µm) were elongated with a large number of processes (Figs. 4.C and D). These cells made numerous contacts with one another and were firmly and strongly attached to the substrates. On the cell surface, a high number of short/thick or long/thin extensions were detected. On these cells, attached particles were observed and appeared to have high affinity towards

Anka Letić-Gavrilović: Oral Tissue Engineering Of Complex Tooth
Structures On Biodegradable DLPLG/ß-TCP Scaffolds.
In Advances in Experimental Biology. Biomaterials, 2004

the third formulation with the largest granules (>300μm) showed a wide variety of forms and appearance (Figs. 4.E and F).

Table. 1 Various mechanical (A) and biochemical (B) characteristics of composites DLPLG/β-TCP with three different granule sizes. A. Mechanical properties in dry state; B. Biochemical values of osteoblast-like cells cultured on composites.

A. Mechanical properties of the Composites* with three different granule size in the dry state (Mean±SD)

COMPOSITES	**Elastic modulus (MPa) (n=10)**	**Tensile strength (MPa) (n=10)**	**Elongation (%) (n=10)**
Polymer only (n=10)	30.5±0.1	7.0±0.2	370±100
Composites (DLPLG/βTCP) <100 μm	49.3±1.3*	6.8±0.5	250±73
Composites >100 μm	56.0±4.7*	6.0±0.2	220±53*
Composites >300 μm	89.6±8.4*	5.1±0.3*	180±24*

B. Biochemical values of osteoblast-like cells after 48 h of culturing with composites DLPLG/β-TCP with three different granule size (Mean ± SD)

Osteoblast culture	**LDH (U/L) (n=6)**	**ALP (U/L) (n=6)**	**Ca (mg/dL) (n=6)**	**P (mg/dL) (n=6)**	**MTT OD550nm) (n=6)**
Control without composites	3.3±0.07	18.5±0.7	7.3±0.26	3.17±0.2	0.3±0.05
Composites (DLPLG/βTCP) <100μm	3.4±0.14	19.3±1.1	3.0±0.4*	1.12±0.1*	0.5±0.03*
Composites >100 μm	3.3±0.01	18.3±0.6	5.1±0.5	1.77±0.1*	0.5±0.03*
Composites >300 μm	3.3±0.07	23.5±1.7*	2.9±0.3*	1.35±0.3*	0.5±0.04*
ANOVA F	**0.44, *ns***	**1.25, *ns***	**113.51, $p<0.0005$**	**85.89, $p<0.0005$**	**21.27, $p<0.0005$**

Scheffé *post hoc* multiple comparison test: DMEM-control vs other composite materials, $p< 0.001$;

* DLPLG/βTCP; n=experimental samples per group

Anka Letić-Gavrilović: Oral Tissue Engineering Of Complex Tooth Structures On Biodegradable DLPLG/ß-TCP Scaffolds. In Advances in Experimental Biology. Biomaterials, 2004

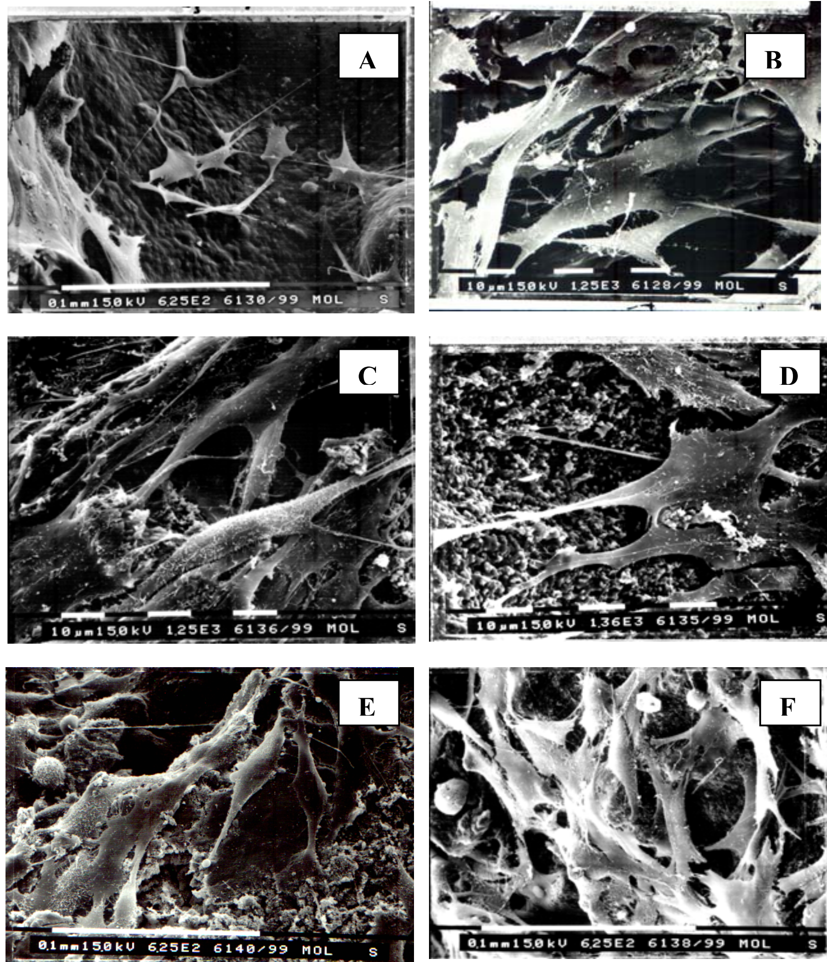

***Figure.* 4.** Electron-micrograph of osteoblast-like cells cultured on DLPLG/βTCP composites with three granule sizes. A and B) After cultivation on the composites with granules <100 µm osteoblast-like cells show extremely long and thin cell processes. The numerous cells make close relations with substrate by cell processes and by cell bodies. Bar: A- 0.1 mm; B -10 µm; C and D) Cell morphology of osteoblast-like cells cultivated on composites with granules > 100 µm shows great number of short, thick (C) and some very long and very thin (D) cytoplasmic continuations on cell surface. Bar: C, D - 10 µm. E and F) The osteoblast-like cells cultivated on composites with granules > 300µm show a great variety of forms and cellular appearance. On this substrate osteoblasts are abundant, highly proliferated, extremely connected and firmly attached to the substrate. Significantly increased number of cells (F), on the small surface area, indicates a high degree of confluence. Bar: E, F - 0.1 mm

RCO osteoblasts were abundant, highly proliferative, closely connected and firmly attached to the substrate. A sharp increase in the number of cells in a small area was indicative of high degree of confluence. These cells were centrally widened and had long cell processes on both poles, although there were cells with numerous processes. A high number of long and short processes were firmly attached to the composite substrate. In extracellular spaces, rough-globular structures of the substrate were attached to or were in close relation with the cells.

4. DISCUSSION

Tooth loss due to periodontal disease, dental caries, trauma, or a variety of genetic disorders continues to affect most adults adversely at some time in their lives. A biological tooth substitute that could replace lost teeth would provide a vital alternative to currently available clinical treatments. To pursue this goal, we dissociated rat molar tooth buds into single-cell suspensions and seeded them onto biodegradable polymers. The target of the study was to design and develop novel multi-functional composites as a bone alternative for use in three-dimensional bone regeneration thanks to its biocompatibility, mouldability and biodegradability. The research activity included technological processes for creating resorbable composite consisting of polymer poly(D,L-lactide-co-glycolide) (DLPLG) and ceramic beta-tricalcium phosphate (β-TCP), and then physicochemical, mechanical and biological analyses *in vitro* to evaluate the biocompatibility of the composite as substrates for new bone deposition. We designed and developed a multifunctional bone equivalent by reinforcing poly(D,L-lactide-co-glycolide) (DLPLG) substrate with microparticles of osteoconductive ceramic: β-tricalcium phosphate (β-TCP) with various particle sizes. The performance of the composites were evaluated by physicochemical tests and in vitro culturing with osteoblast-like cells (RCO). The results showed that the composites had excellent mechanical and physicochemical characteristics, including elastic modulus, tensile strength, swelling, biodegradation and had positive effects on RCO. This indicates that the composites are suitable materials as a scaffold for new bone ingrowth, enabling better functional and aesthetic results in patients treated for cranial-facial tumors, malformations and traumas. We decided to design composites of DLPLG/βTCP, because both components are bioresorbable in the shortest time possible and in this case a reconstruction of hard tissue is synchronised with degradation of composite components and the biomaterial starts to be porous during the healing process. Regarding porosity and/or density of composites, reconstruction of hard tissue is synchronised with degradation of the biomaterial and starts to augment its porosity during this

Anka Letić-Gavrilović: Oral Tissue Engineering Of Complex Tooth Structures On Biodegradable DLPLG/ß-TCP Scaffolds.
In Advances in Experimental Biology. Biomaterials, 2004

process. Therefore, our material being biodegradable does not need to be porous at the beginning of experiment, as it will become porous soon after insertion into dynamic *in vivo* conditions. The question on porosity is crucial only for non-biodegradable biomaterials. DLPLG is the biodegradable polymer with a high starting strength, which lasts for some time, actually a rather short time of up to a few months, and produces nontoxic products during degradation[3], [18]. We tested degradation rate in low pH, which was chosen for *in vitro* "static" conditions where the buffer solution is not replaced over the whole testing period. This results in a dramatic drop in the pH of the solution due to the release of the acids from the device. Although this low pH is not very probable in dynamic *in vivo* conditions in which the products of degradation are transported away from the implantation site, this situation would be possible when large bulky implants degrade at a very high rate, as it is the case with our composites.

While the specific requirements and characteristics of a biodegradable biomaterial depend on a specific application, there is a general set of criteria for "a good biodegradable polymeric biomaterial" and we believe that our composite formulations fulfill these criteria. Further more, DLPLG/β-TCP is excellent due to its good tensile strength and improved elastic modulus at the beginning of experiments. However, diverse factors like the molecular weight, copolymer composition (lactide to glycolide ratio), crystallinity, and β-TCP granule size affect the mechanical strength of the composition[8]. The modulus of elasticity for copolymer is relatively low compared to that of natural cortical bone[12]. To meet that requirement introduction of a certain amount of osteoconductive ceramic such as β-TCP, and change the size and quantity of used particles and the modulus would be improved effectively as was shown in our experiments and others[20]. With our composites formulations, the mechanical properties and degradation time of polymers showed to be adjustable by adding ceramics[11,12,26]. The experimental results of this study demonstrate that all three formulations of β-TCP/DLPLG composites were biocompatible *in vitro*. These formulations have an outstanding propensity to be colonized with osteoblasts, thereby promoting their osteogenic activity. These results are comparable with the others[22, 19]. The results of our *in vitro* study provide strong evidence of good osteocompatibility of the proposed composite formulations. ALP, Ca, P and MTT as indicators of bone cell differentiation seem to be increased when measured in RCO cultured on the composites with the biggest granule size >300 μm. This is in agreement with literature reports on the impact of matrix geometry on osteogenesis[10] and also with our results regarding other biochemical parameters and microscopic observations. Cellular attachment to the implant surface is an important step in the process of tissue-implant interaction and thus long-term successful implantation[21].

Anka Letić-Gavrilović: Oral Tissue Engineering Of Complex Tooth Structures On Biodegradable DLPLG/ß-TCP Scaffolds. In Advances in Experimental Biology. Biomaterials, 2004

Tissue engineered (TE) product formulation proposed in this study will have significant clinical advantages for bone and tooth-like tissue regeneration. The proposed TE formulations (Patent Pending, Number) have a very important potential as scaffolds for cell transfer, thus offering also the possibility of rapid tissue regeneration[5,14-16,23,27]. Also, depending on future developments in TE, it may be multi-functional, i.e. used both as a valuable biomaterial and as an in vitro and in vivo drug delivery device of growth factors. Thus, our future goal is to promote combined clinical, pharmaceutical and TE research efforts to obtain bone equivalent products for successful cranio-facial reconstructions based on tooth-germ epithelial-mesenchymal interactions.

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REFERENCES

1. Alsberg, E., Hill, E.E., and Mooney, D.L., 2001, Craniofacial Tissue Engineering. *Crit Rev Oral Biol Med* 12:64-75.
2. Davin, J.E., Attawis, M.A., and Laurencin, C.T., 1996, Three-dimensional degradable porous polymer-ceramic matrices for use in bone repair. *J Biomater Sci Polym Ed* 7: 661-669.
3. Dunn, A.S., Campbell, P.G., and Marra, K.G., 2001, The influence of polymer blend composition on the degradation of polymer/hydroxyapatite biomaterials. *J Mater Sci: Mater Med* 12: 673-677.
4. Hench, L.L., and Polak, J.M., 2002, Third-Generation Biomedical Materials. *Science* 295: 1014-1017.
5. Young, C.S., Terada, S., Vacanti, J.P., Honda, M., Barlett, J.D., and Yelick, P.C., 2002, Tissue Engineering of Complex Tooth Structures on Biodegradable Polymer Scaffolds. *J Dent Res* 81(10): 695-700.
6. Jain, R.A., 2000, The manufacturing techniques of various drug loaded biodegradable poly(lactide-co-glycolide) (PLGA) devices. *Biomaterials* 21:2475-2490.
7. Glasstone, S., 1967, Development of Teeth in Tissue Culture. *J Dent Res* v. 46; Supp. 5.
8. Kasuga, T., Ota, Y., Nogami, M., and Abe, Y., 2001, Preparation and mechanical properties of polylactic acid composites containing hydroxyapatite fibers. *Biomaterials* 22: 19-23.
9. Kikuchi, M., Suetsugu, Y., Tanaka, J., Akao, M., 1997, Preparation and mechanical properties of calcium phosphate/copoly-L-lactide composites. *J Mater Sci* 8:361-364.
10. Kuboki, Y., Saito, T., Murata, M., Takita, H., Mizuno, M., Inoue, M., Nagai, N., Pool, and R., 1995, Two distinctive BMP-carriers induce zonal chondrogenesis and membranous ossification, respectively; geometrical factors of matrices for cell-differentiation. *Connect Tissue Res* 32: 219-226.
11. Kurashina, K., Kurita, H., Kotani, A., Kobayashi, S., Kyoshima, K., and Hirano, M., 1998, Experimental cranioplasty and skeletal augmentation using an alpha-tricalcium

Anka Letić-Gavrilović: Oral Tissue Engineering Of Complex Tooth Structures On Biodegradable DLPLG/ß-TCP Scaffolds. In Advances in Experimental Biology. Biomaterials, 2004

phosphate/dicalcium phosphate dibasic/tetracalcium phosphate monoxide cement: apreliminary short-term experiment in rabbits. *Biomaterials* 19:701–706.

12. Kurashina, K., Kurita, H., Hirano, M., Kotani, A., Klein, C.P., and de Groot, K., 1997, *In vivo* study of calcium phosphate cements: implantation of an alpha-tricalcium phosphate/dicalcium phosphate dibasic/tetracalcium phosphate monoxide cement paste. *Biomaterials* 18:539–543.
13. Letic-Gavrilovic, A., Scandurra, R., and Abe, K., 2000, Genetic potential of interfacial guided osteogenesis in implant devices. *Dent Mater J* 19(2): 99-132.
14. Letic-Gavrilovic, A., Crudo, V., and Abe, K., 2002, Microspheres-based tissue engineering product for craniofacial bone reconstruction, (abstract). *Tissue Engineeering* 8(6): 1234.
15. Letic-Gavrilovic, A., Crudo, V., and Abe, K., 2002a, Tooth-germ morphogenesis signals for 3D tissue engineering scaffolds in oral reconstructions (abstract). *Tissue Engineeering* 8 (6): 1165.
16. Letic-Gavrilovic, A., Piattelli, A., and Abe, K., 2003, Nerve growth factor β (NGFβ) delivery via a collagen/hydroxyapatite (Col/HAp) composite and its effects on new bone ingrowth. *J Mat Sci: Mater Med* 14: 95-102.
17. Lowry, Q.H., Rosebrough, N.J., Farr, A.L., and Bandall, R.J., 1951, Protein measurements with the Folin Phenol reagent. *J Biol Chem* 193:265-275.
18. Merolli, A., Gabbi, C., Cacchioli, A., Ragionieri, L., Caruso, L., Giannotta, L., Tranquilli Leali, P., 2001, Bone response to polymers based on poly-lactic acid and having different degradation times. *J Mater Sci:Mater Med* 12:775-778.
19. Neo, M., Herbst, H., Voigt, C.F., and Gross, U.M., 1998, Temporal and spatial patterns of osteoblast activation following implantation of beta-TCP particles into bone. *J Biomed Mater Res* 39:71–6.
20. Rejda, B.V., Peelen, J.G., and de Groot, K., 1977, Tri-calcium phosphate as a bone substitute. *J Bioeng* 1: 93–97.
21. Warren, S.M., Fong, K.D., Chen, C.M., Loboa, E.G., Cowan, C.M., Lorenz, H.P., and Longaker, M.T., 2003, Tools and Techniques for Craniofacial Tissue Engineering. *Tissue Engineering* 9(2): 187-200
22. Shikinami, Y., and Okuno, M., 1999, Bioresorbable devices made of forged composites of hydroxyapatite (HA) particles and poly-L-lactide (PLLA): Part I. Basic characteristics. *Biomaterials* 20:859-877.
23. Shikinami, Y., and Okuno, M., 2001, Bioresorbable devices made of forged composites of hydroxyapatite (HA) particles and poly-L-lactide (PLLA): Part II. Practical properties of miniscrews and miniplates. *Biomaterials* 22: 3197–3211.
24. Tada, H., Hatoko, M., Tanaka, A., Kuwahara, M., Mashiba, K., Yurugi, S., Iioka, H., and Niitsuma, K., 2002, Preshaped hydroxyapatite tricalcium-phosphate implant using three-dimensional computed tomography in the reconstruction of bone deformities of craniomaxillofacial region. *J Cranio Surg* 13(2): 287-292.
25. Wang, M., Chen, J., Ni, J., Weng, J., and Yue, C.Y., 2001, Manufacture and evaluation of bioactive and biodegradable materials and scaffolds for tissue engineering. *J Mater Sci: Mater Med* 12: 855-860.
26. Wiltfang, J., Merten, H.A., Schleger, K.A., Schultze-Mosgau, S., Kloss, F.R., Rupprecht, S., and Kessler, P., 2002, Degradation characteristics of alpha and beta tri-calcium-phosphate (TCP) in minipigs. *J Biomed Mater Res* 63: 115-121.
27. Zerbo, I.R., Bronckers, A.L.J.J., de Lange, G.L., van Beek, G.J., Burger, E.H., 2001, Histology of human alveolar bone regeneration with a porous tricalcium phosphate. A report of two cases. *Clin Oral Impl Res* 12: 379-384.