Preparation of chitosan/calcium phosphate based injectable system for guided bone regeneration and its properties investigation

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The main objective of the work was to design and fabricate an injectable biomaterial with osteoconductive properties that can be used in dental applications in peri-implant therapy concerning guided bone regeneration. For that purpose, a self-setting biomaterial consisting of chitosan/tricalcium phosphate microparticles and sodium alginate was formulated.

The obtained material was characterized regarding microsphere and formed agglomerates morphology and microstructure. Physical properties, relating to setting time and mechanical properties, were also investigated. Finally, *in vivo* response to implanted biomaterial was studied on a rat model and compared with commercially available alloplastic material. The results showed that designed injectable biomaterial fulfilled main requirements for guided bone regeneration application.

Keywords and phrases: chitosan, alginate, β-tricalcium phosphate, injectable systems, guided bone regeneration, in vivo studies, biomaterial.

Introduction

Guided bone regeneration (GBR) is a dental technique utilizing various materials for missing bone regeneration (for example ridge reconstruction, sinus-lift procedure). The therapy also requires using an additional membrane acting as a barrier preventing soft tissue ingrowth into regenerated area. Bone substitute materials should ensure non-toxic, non-immunogenic, osteoconductive, bioresorbable and biodegradable properties. As "osteoconductive" we consider biomaterial which possesses ability to recruit osteoblast to the material surface and enhance proliferation of the cells and mineralization process. For GBR procedure and bone augmentation oral surgeons have a choice between allogenic (from patient's bone tissue), xenogenic (animal origin) or alloplastic materials (made of polymers and ceramics). However, each of them inherits disadvantages coming from the type and properties of the graft, that is employed in bone tissue reconstruction [1–2]. For example: usage of allogenic materials requires excision of bone fragments from patients, xenogenic materials are neither biodegradable nor bioresorbable and can cause Creutzfeldt-Jakob disease. Therefore, recently researchers are focused on making alloplastic osteoconductive materials which could be manufactured and state an alternative solution to allogenic and xenogenic bone grafts [3–4]. These materials are generally non-immunogenic, resorbable, easy for application and their properties might be adjusted regarding the surgeon's and patient's needs. The goal of presented work was to develop formulation of injectable, biodegradable material acting as a bone substitute that could support new bone tissue ingrowth. Evaluation of physical and biological properties relating to designed self-setting biomaterial are discussed herein.

Materials and Methods

Materials

The presented material was designed as a biphasic system where solid phase was consisting of chitosan/β-tricalcium phosphate (TCP) particles and a liquid phase constituted of 2 wt% solution of alginate salt. Chitosan has been chosen due to its non-immunogenic, non-toxic and resorabable properties [5–6]. The injectable system formation relied on calcium ions release from chitosan/TCP particles into liquid phase that tends to gelling due to calcium-alginate gel formation phenomenon [7].

Chitosan (~95% degree of deacetylation) was purchased from Medical Heppe GmbH, β-tri-calcium phosphate from Sigma Aldrich and alginic acid sodium salt from brown algae was purchased from Fluka.

Chitosan/β-tri-calcium phosphate beads were prepared by hydrodynamic formulation of chitosan/ceramics solution in drop forming rate and were being collected into precipitation bath consisting of NaOH solution. Chitosan/β-TCP solution was prepared by suspending TCP powder in 2 wt% chitosan in 2 wt% acetic acid solution. The homogenous suspension was being placed in 50 ml syringe (BD Perfusion) and pressed out through the plastic nozzle using infusion pump (AP22, Ascor) into continuously stirring (rpm = 800) 2 wt% NaOH bath. Afterwards, the formulated beads were washed with distilled water and dehydrated with the use of 96% ethanol. In the next step, the granules were dried at room temperature.

Dried microspheres were immersed in $CaCl_2$ solution in order to enrich chitosan/TCP particles with Ca^{2+} ions, so that the microspheres could constitute a carrier and source of cross-linking agent for alginate gel formation. Calcium chloride concentration and exposition time to the calcium solution were considered and studied in the hereby presented studies.

Material morphology

The morphological evaluation of composite CH/TCP particles and formed injectable system were performed by optical microscope (Nikon eclipse 80i) and SEM analysis (Zeiss Supra).

FTIR/ATR spectroscopy

FTIR/ATR spectra of the pure chitosan, pure β-tri-calcium phosphate and chitosan/β-tri-calcium phosphate/alginate agglomerates were obtained using Thermo Scientific Nicolet 6700 FT-IR spectrometer with ATR pickup. All samples were dry and pressed with potassium bromide into a pellet (d = 15 mm). Chitosan/β-tri-calcium phosphate agglomerates were milled in pebble mill prior to pellet formation.

Setting time

The gelling time of injectable system was evaluated with the use of a double plunger syringe (Fig. 1). In order to determine the setting time liquid alginic phase was forced through the composite granule phase which was previously placed in the syringe. The system was assumed as gelled when resistance of liquid phase flow, due to increasing density of gel, was observed and measured as setting time with the use of timer. To conduct investigation 1,00 g of composite granules were placed into syringe and 2 ml of alginic acid sodium salt was added. Both, the influence of calcium chloride concentration (1 wt%, 2 wt%, 10 wt%) and time (15 min and 24 h) of soaking the solid phase in the solution of calcium chloride were evaluated during the study.

Mechanical properties

Mechanical properties concerning compression tests were studied on cylindrical shape samples. There were two generations of the biomaterials tested: the specimens completely dried in the room temperature for 48 h (n = 6) and "wet" samples which were tested in their "wet" form, just after material formation (n = 6). The universal electromechanical testing set-up MTS Q/test 10 with work parameters: load 10 kN and constant speed of crosshead displacement 0,1 mm/s was used. Young modulus values were calculated from the slope of linear part of stress-strain curves and compressive strength was obtained from the first maximum of stress visible in the curves.

In vivo studies

The studies were conducted on the rat model. Both, fabricated material, and commercial product based on TCP microparticles were implanted into experimentally created critical-size (7 mm) defects in rats skull. There were 40 rats subjected to the surgical procedure. Histological evaluation after implantation was conducted after 4 and 12 weeks.

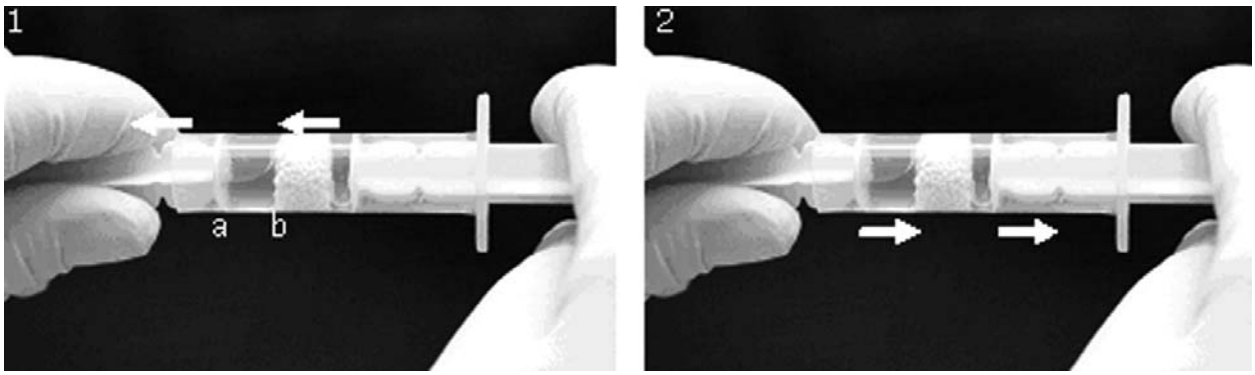

Fig. 1. Setting time evaluation; a — liquid phase, b — solid phase (beads).

Results and Discussion

Material morphology

Particles obtained by drop forming method are shown in the Fig. 2a and Fig. 3e. Due to incorporated inorganic calcium phosphate phase they tend to possess rather rough surface with well-developed microstructure what can be easily found by considering SEM microphotographs presented in Fig. 2b.

Formed into cylindrical shape, previously dried, CH/TCP/Alg biomaterial was also analyzed by SEM and the obtained pictures are presented in the Fig. 2 c, d. When compared with CH/TCP beads, alginic layer seems to possess smooth surface that tends to be fractured when material completely dried. Fig. 3e, f and g present images obtained by optical microscope. From the left hand side respectively, morphology of CH/TCP microspheres (e) and formed injectable system covered with the layer of alginate gel (f, g).

FTIR/ATR spectroscopy

These peaks are almost the same as those of the TCP but higher. FTIR/ATR analysis (Fig. 4) showed

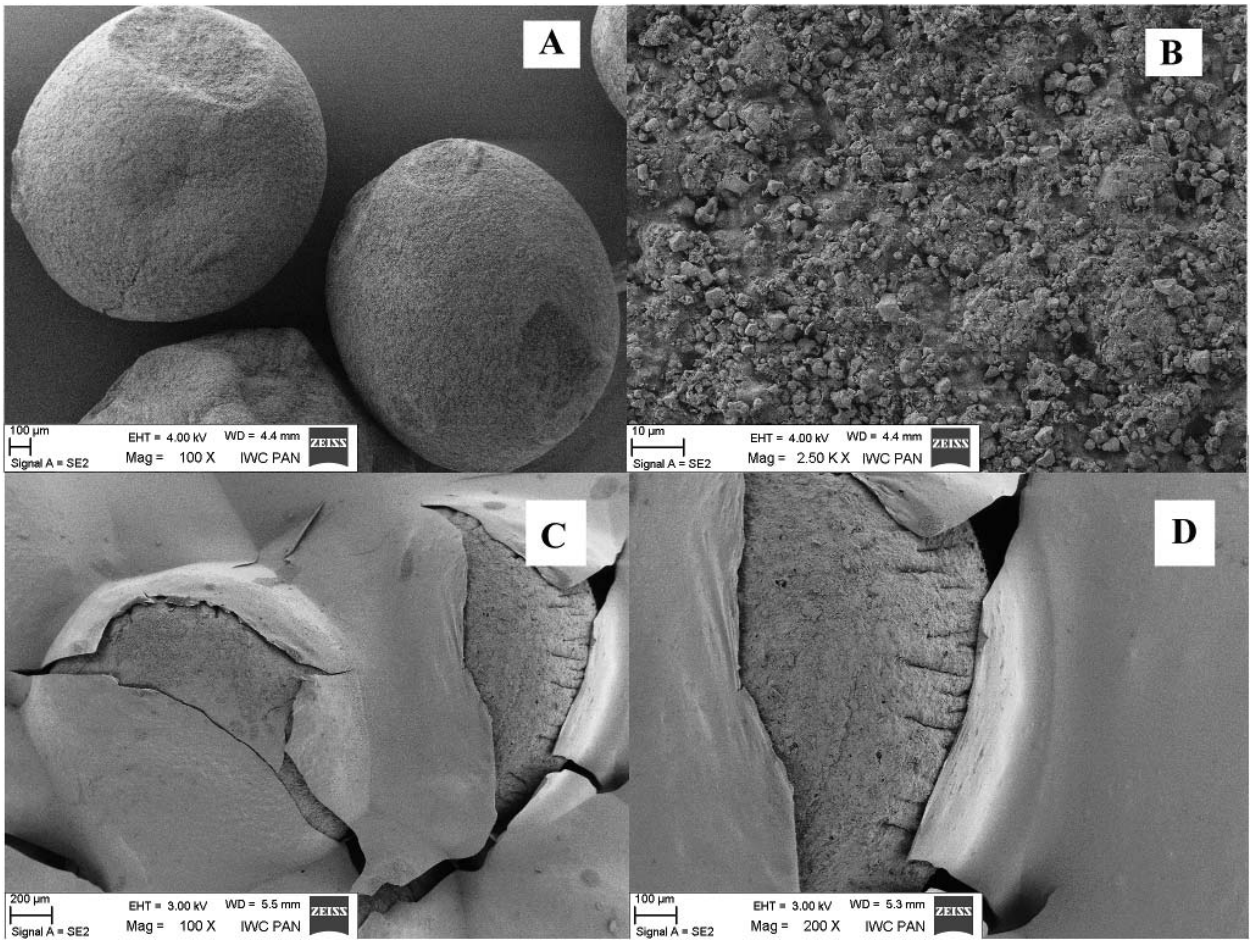

Fig. 2. Morphology and topography of CH/TCP particles obtained by SEM analysis (a, b); SEM microphotography of formed CH/TCP/Alg biomaterial (c, d).

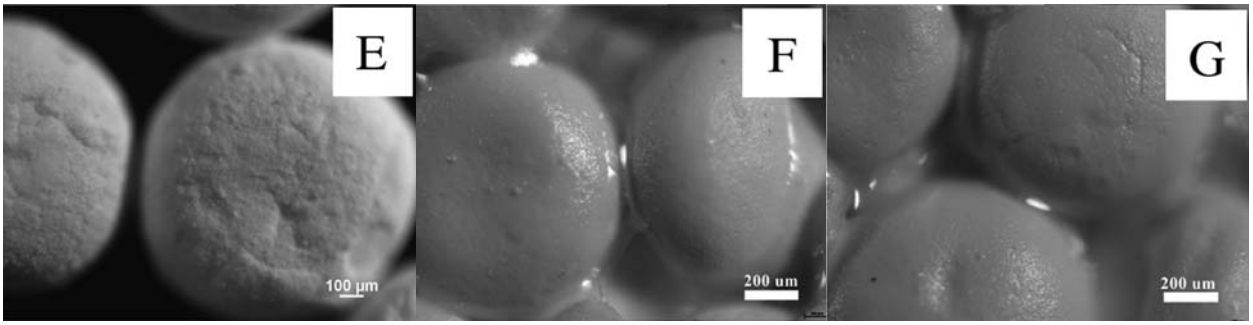

Fig. 3. Morphology of biomaterial observed by optical microscope (e, f, g).

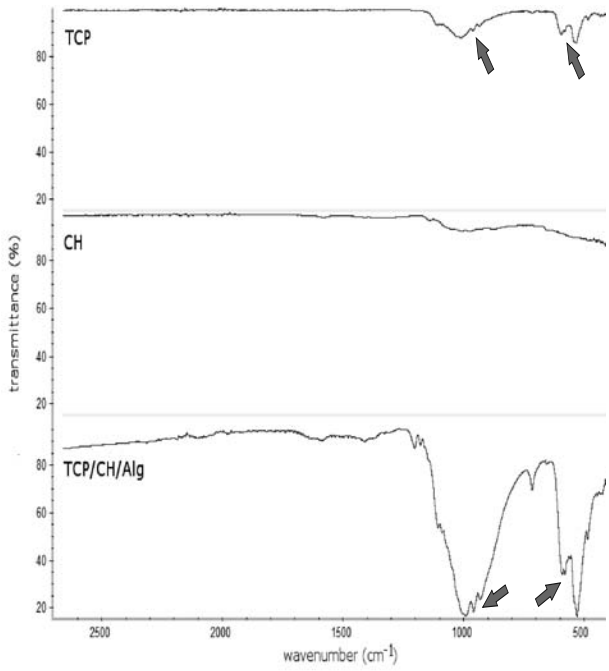

Fig. 4. FTIR spectra of pure β-tri-calcium phosphate (TCP), pure chitosan (CH) and investigated chitosan/β-tri-calcium phosphate agglomerates (TCP/CH/Alg).

that our material consists mainly β-tri-calcium phosphate.

Setting time

From setting time studies it became apparent that duration of particle soaking in specified calcium chloride solution does not influence setting time. Investigation also confirmed assumption that gelling time is decreasing with increasing $CaCl_2$ solution concentration.

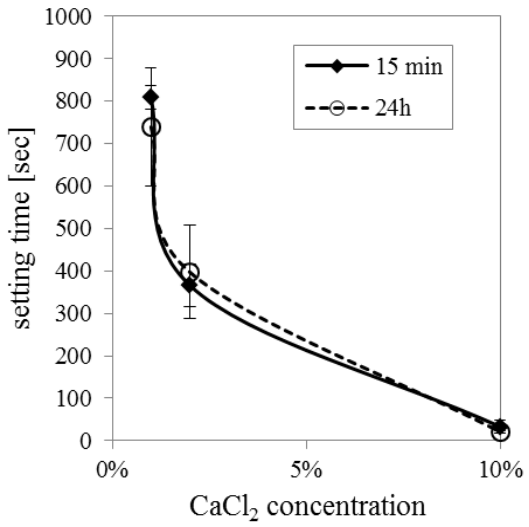

Fig. 5. Effect of calcium chloride concentrations (1 wt%, 2 wt% and, 10 wt%) and immersion times (15 min, 24 h) on the setting time of injectable system.

Mechanical properties

Two generations of the cylinder-shaped biomaterials were tested in order to evaluate mechanical properties of the injectable system. Both, dried samples, and newly formed scaffolds wetted by alginic layer were subjected to the study. It was clearly found that "dry" specimens tend to possess stiffer architecture due to dried alginic phase covering beads surfaces which during drying process lost its gel-like elastic structure. Young modulus for "dry" materials reached out 16,19±2,35 MPa and the compressive strength amounted to 1,97±0,77 MPa. In case of "wet" samples both elastic modulus and compressive strength obtained possessed lower values and reached out 9,54±0,62 MPa and 0,94±0,07 MPa respectively.

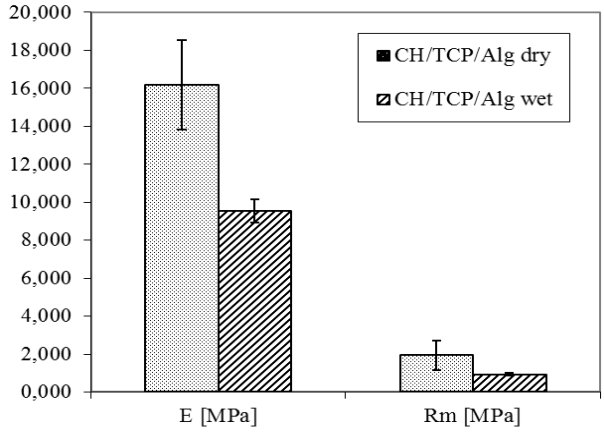

Fig. 6. Mechanical properties; E — Young modulus, Rm — compressive strength.

In vivo studies

In vivo studies on the rat model revealed that after a month and three months from implantation there was newly formed bone observed around biomaterial. In comparison commercially available alloplastic material did not exhibit such good properties and revealed high inflammatory response without osteogenic activity (Fig. 7).

Conclusions

Formulation procedure of biomaterial with osteoconductive properties for guided bone regeneration was successfully designed. It was established that formulated system can be potentially applied for new bone tissue formation in dental application. The studies revealed that micro- and macrostructures setting time and mechanical strength of obtained material are suitable for application in guided bone

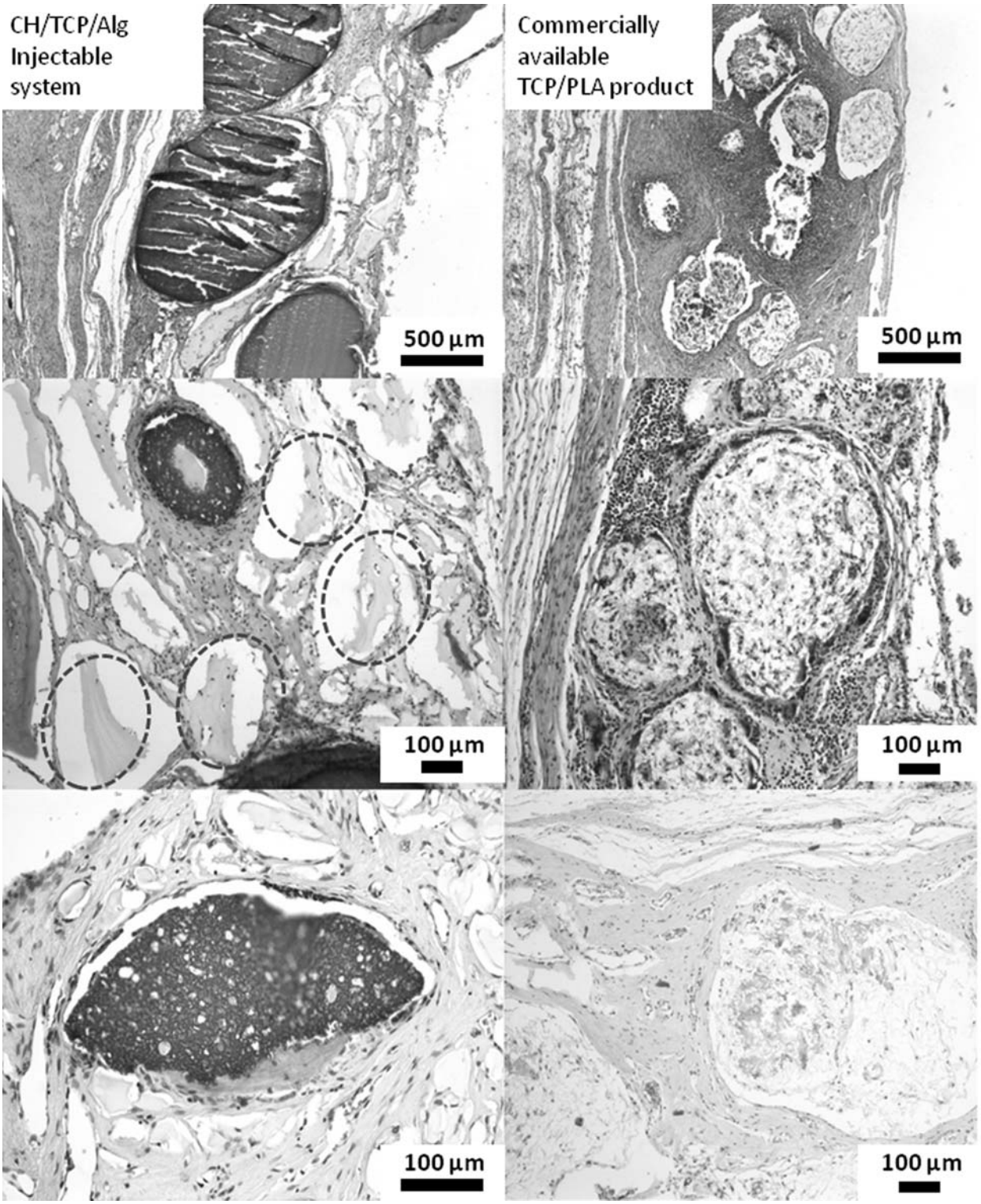

Fig. 7. Histological evaluation after 4 and 12 weeks from biomaterials implantation; a, c, e — CH/TCP/Alg injectable system, b, d, f — commercially available alloplastic material (TCP/PLA), respectively. (c) newly formed bone tissue around fabricated biomaterial observed after 4 weeks, sites marked by circles (d) lack of bone formation in case of commercial product, inflammatory response, after 4 weeks; e, f — results obtained after 12 weeks, (e) newly formed, calcified bone tissue growing onto material surface, (f) slight bone formation around commercial material.

regeneration. Presented material might be also used as a carrier of growth factors or human cells. In comparison with commercially available product satisfactory histological analysis results were obtained. Proper ridge reconstruction on a large animal model can be expected.

Acknowledgements

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