13

Designing Bio-Inspired Composite Materials for Medical Applications

Oana Craciunescu and Lucia Moldovan
Department of Cellular and Molecular Biology,
National Institute R&D for Biological Sciences, Bucharest,
Romania

1. Introduction

Composite materials are multi-phased combinations of two or several components, which acquire new characteristic properties that the individual constituents, by themselves, cannot obtain. A composite material typically consists of a certain matrix containing one or more fillers which can be made up of particles, sheets or fibers. When at least one of these phases has dimensions less than 100 nm, the material is named a nanocomposite and offers in addition a higher surface to volume ratio. There are natural composite materials, like wood and plant leaves, in the vegetal kingdom and bird feathers, silky threads spun by the spider and shells, in the animal kingdom. Also, connective tissues from animal and human body are natural composite materials due to their composition and structure. Connective tissues are the major supporting tissues of the body. They are named after their main function, i.e. packing and binding other structures together, and also providing a framework for the body. Connective tissues are generally soft tissues (e.g., skin, cartilage, cornea, etc), excepting bone which is a dense connective tissue.

Similar to other natural composite materials, bone consists of an organic part that forms the matrix and an inorganic part representing the filler. Bone matrix is a framework mainly composed of collagen fibers which together with small quantities of other non-collagenous proteins, proteoglycans, lipids, peptides and water form a hydrogel (~ 30 % from bone dry weight). The filler, that reinforces bone matrix, is formed of nano-sized crystals of carbonated calcium phosphate apatite (~ 70 % from bone dry weight). This natural nanocomposite material has superior strength and toughness than its individual components. Bone matrix is a source for nourishing bone cells, such as osteoclasts, osteoblasts and osteocytes, which grow inside it. It also increases cell biological activities like adhesion, proliferation and differentiation. Bone has key functions as skeletal tissue of the body, including support of softer tissues, mechanical protection for many internal organs and storage of minerals. In the development phase, bone attains the most suited structure to resist the forces acting upon it. During life time, bone is subjected to various diseases that are inherited (*osteogenesis imperfecta*) or caused by metabolism disturbances, such as osteoporosis, osteosarcoma, osteoarthritis. Bone is also affected by traumas, i.e. fractures, micro-fractures. Each year, millions of people are treated in hospitals for fractures presenting risk of developing into delayed union or nonunion. Also, progressive aging of

population and the related pathologies lead to loss of variable quantities of bone that have to be replaced. The demand for bone substitutes is extremely large in orthopedic clinics from all over the world. That is why a growing interest in different aspects of creation, characterization, testing and application of composite materials for biomedical applications is registered.

Composite materials for medical application are developed for pathologies of osseous tissues from different parts of the body, like long bones, vertebrae, cartilage and teeth. Two-dimensional and three-dimensional structures are fabricated and commercialized as composite materials or combined with therapeutic organic substances (drugs, growth factors, etc) (table 1). There are several treatments for bone repairing: patching, replacing the missing tissue using allografts or xenografts, or self-healing initiated by materials containing signal molecules for tissue remodelling. The existing methods and techniques for treatment of large bone defects, as a result of trauma or tumor, do not satisfactory restore bone tissue. The classic technique for bone repair consists in autologous bone implantation, but is limited by the availability of transplanted material, the morbidity of the donor, difficulties in harvesting, longer hospitalization period and higher treatment costs. The last decade, initiated the utilization of resorbable materials, tailored with structures having controlled porosity, as medical devices for *in vivo* tissue regeneration (Silva et al., 2005; Patterson et al., 2008). The structure of the composite material has a role in the transport of nutrients, metabolites and regulator molecules towards and from the cells. New rapid prototyping techniques, like 3-D printing, selective laser sintering, stereolithography, allow the development of desired structures, similar to natural bone, having reproducible, well-defined shapes and controlled pore morphology and density. Composite material has a microporosity referring to the free spaces remaining between ceramic material particles bound on the polymer and a macroporosity meaning the pores larger than 100 μm from its structure. The morphological characteristics have a direct impact on the uniform distribution of cells within the porous material. An optimal pore size and interconnectivity facilitate cell colonization into the construct and influence the geometry of the new developed tissue. As for material properties, there is a need to improve its mechanical characteristics, in order to obtain a controlled biodegradability and good biocompatibility.

The main constituent of bone matrix – collagen, can be prepared using standardized techniques and purified at high levels (>90 %, w/w). For better mimicking natural bone composition, chondroitin sulfate is added into the collagen matrix. Chondroitin sulfate represents the glycanic part of the small proteoglycan, named biglycan, found in bone. It is a glycosaminoglycan having a linear chain of repeating units of anionic, acidic sugars. Chondroitin sulfate plays an important role in the regulation of mineralization process and in repair of bone defects in animals (Douglas et al., 2008). Also, chondroitin sulfate carries negative charges which are known to enhance osseous cell proliferation (Ohgaki et al., 2001).

The other main component of composite materials intended for bone substitution is one of the several constituents of calcium phosphate minerals class (Barrere et al, 2006). Calcium hydroxyapatite, ($Ca_{10}(PO_4)_6(OH)_2$) and tricalcium phosphate (TCP), ($Ca_3(PO_4)_2$) are the most commonly investigated ceramics for biomedical applications because they possess the ability to improve new bone formation, showing osteoconductive properties (Laurencin et al, 2006). When their crystal size is similar to the nanometer size of the apatite from the natural bone, an increase in protein adsorption and osteoblast adhesion is expected (Webster et al., 1999). Both ceramics are thoroughly used in bone substitutes, but it is demonstrated a better conductivity, osteocompatibility and resorption rate for tricalcium

phosphate than for highly crystalline, sintered hydroxyapatite (Vaccaro, 2002). The latter is limited in use because of its brittleness and difficult processing (Fujita et al, 2003). Bone apatite also contains various trace elements, such as magnesium, copper, zinc, silicate, fluoride, which are showed to have an effect on bone quality. Magnesium ion is the most abundant in the development phase of cartilage and bone tissues and sharply decreases when the bone is mature. Magnesium depletion alters bone and mineral metabolism which results in bone loss and is a risk factor for osteoporosis (Rude et al., 2009).

Product	Conditioning form	Composition	Delivered substance	Target tissue	Reference
MinerOss	Powder	Bone allograft	-	Periodontal	Gapski et al., 2008
Cortoss	Injectable	Resin with glass ceramic particles	-	Spinal	Bae et al., 2010
rh-BMP-2 (development)	Sponge	Collagen and titanium mesh	rhBMP-2	Spinal	Mulconrey et al., 2008
Healos	Sponge	Collagen type I with hydroxyapatite coating	rhGDF-5, gentamicin, marrow aspirate	Bone	Magit et al., 2006 Carter et al., 2009 Furstenberg et al., 2010
ProOsteon	Sponge	Sea coral with hydroxyapatite	-	Bone	Jensen et al., 2007
Immix	Microsphere	Polylactic acid/glycolic acid	-	Bone	Chenite & Chaput, 2010
Carticel	Hydrogel	Synthetic and natural polymers	growth factors, cells	Cartilage	De Bie, 2007

(rh-BMP-2 is recombinant human bone morphogenetic protein-2, rhGDF-5 is recombinant human growth and differentiation factor-5)

Table 1. Several composite products for bone repair

A series of collagen-calcium phosphate composite materials are tailored and used as temporary scaffolds in studies on animals and humans for tissue regeneration (Wang et al., 2004; Chen et al., 2009). The addition of collagen to a ceramic material provides many advantages for medical applications: shape control, spatial adaptation and ability for clot formation (Scabbia & Trombelli, 2004). Collagen could also serve as efficient bonding agent for ceramic particles. Conversely, the addition of calcium phosphates to collagen scaffolds improves the osteoconductive properties of the material (Takahashi et al., 2005; Kretlow et al., 2007). Composite properties are strongly dependent on synthesis conditions, like the calcium phosphate/collagen ratio, temperature and pH. The cohesion between the two materials is based on the interaction of calcium ions from ceramic material and the carboxyl groups from collagen (Zou et al., 2005).

The main advantage of a composite material tailored from collagen and calcium phosphate is the excellent biocompatibility property, due to collagen and its ability to allow bone cell attachment and differentiation. The crystals of synthetic calcium phosphate remain undistorted, for a long period after material implantation, sustaining the formation of new tissue.

The main difficulty in using these devices is that they are easily degraded and reabsorbed by the body. After hydration, they don't possess strength and their mechanical properties are relatively low in comparison to bone (Matsuno et al., 2006). The problem to be solved is to obtain a controlled degradation of the composite material so that to ensure as long as possible a scaffold where cells could deposit new bone tissue. Cross-linking could be used in

order to control composite biodegradation rate and its mechanical characteristics, but it might compromise the biocompatibility.
Several bone regeneration therapies use a combination of collagen-ceramic composite materials, cell population and signal substance delivery to initiate more rapidly the healing process (Arinzeh et al., 2005; Guo et al., 2006). It were tested various growth factors, like transforming growth factor-beta, basic fibroblast growth factor or bone morphogenetic proteins (McKay et al., 2007; Evans, 2010).
This chapter describes the design of bio-inspired composite materials, as bone substitutes, choosing the most appropriate composition and structure to fulfill the unique morphological characteristics and biological properties of natural bone. Several aspects regarding the fabrication of a composite material that mimic porous bone structure are discussed in this chapter. A key aspect is composite cross-linking and a discussion on carbodiimide advantages is presented. *In vitro* experimental models on cell cultures are used for composite material biocompatibility evaluation. A bioactive implant consisting of osteoblast cells injected into the composite material and cultivated *in vitro* is analyzed for osteogenic properties by cell adhesion assay and osteoblast-specific marker expression.

2. Preparative methods

At present, there are known 29 types of collagen, having various structural and functional properties, depending on the connective tissue where they are found. Therefore, collagen extraction from different tissues it is not a standard procedure. Collagen can be obtained in its insoluble form, acid soluble form, neutral salt soluble form or its denatured form – gelatin using three types of extraction methods with neutral salt solutions, dilute acid solvents, chemical agents (acids or bases) with proteolytic enzymes. The neutral salt and dilute acid extraction methods are efficiently applied only to extract collagen from young animal tissues. For mature tissues, chemical reagents and proteolytic enzymes are used together to yield triple-helical molecules of collagen. Collagen type I can be extracted from animal tissues like skin, tendon and cornea and is commercialized in its insoluble form as an acidic solution. Tendon contains a high quantity of collagen (86 % from dry weight mass) which is made up of 97 % collagen type I. There are technologies applied for collagen extraction that aim to obtain soluble, but non-denatured collagen molecules with an intact triple-helix conformation. The chemical and enzymatic processes used in these technologies remove the non-helical polypeptidic ends (telopeptides) from the collagen molecule and break up the intermolecular cross-links. The enzymatic reaction does not succeed in completely cleavage of these chemical bonds present in all the three-dimensional structure of collagen. Therefore, a collagenous extract containing more than 70 % intact atelocollagen macromolecules is obtained. When natural conditions are induced (temperature 37 °C, pH 7.4), these intact triple-helical macromolecules are able to spontaneously aggregate to form fibers.

2.1 Tissue processing

Bovine tendons were obtained from the local abattoir after animal slaughtering. They were immediately rinsed in cooled water (4 °C) or phosphate-buffered saline (PBS) (pH 7.4). After transportation to the lab, in a cooler box (4 °C), they were peeled from adherent tissues with a scalpel and washed in cold tap water. The tendons were minced in 1-2 mm^3 pieces, and kept at -18 °C until processing.

2.2 Collagen extraction

Small pieces of tissue were put in a one-liter Berzelius glass and 0.5 M acetic acid containing pepsin (E.C. 3.4.23.1, Sigma), in a weight ratio of 1:10 (w/w) enzyme:dry tissue was added. The extraction process was conducted at 4 °C, with gentle stirring, for 24 h. The obtained gel was filtered and the remaining tissue was again extracted as above. The two viscous solutions were combined in the same glass and a precipitation-step was achieved by slowly adding 0.7 M NaCl in the gel and leaving the mixture at 4 °C, for 20 h. The precipitate was separated by centrifugation at 4000 rpm, for 20 min and it was dissolved in acetic acid 0.5 M by homogenizing on a magnetic stirrer at 500 rpm, for 2 h. The purified collagen type I solution was dialyzed against distilled water using cellulose tubes (molecular mass cut-off 12,400) for one week, renewing the outer solution three times a day. All extraction steps were performed at 4 °C in order to prevent denaturation of collagen.

The obtained collagen solution was characterized by analytical techniques and the results indicated 9.98 % hydroxyproline content, 84.94 % collagen, 88.40 % total protein content, 10.80 % hexosamines and pH 6.0. The value of its average molecular weight, determined by viscosimetry (Turkovski et al., 2008) was 308 kDa, comparable to that of tropocollagen (300 kDa). This observation indicates that the used enzymatic extraction is a non-denaturing method, which preserves the native triple helix structure of collagen. At the same time, the method eliminates collagen telopeptides to yield a non-immunogenic polymer. The purity analysis, conducted by SDS-polyacrylamide gel electrophoresis (Miller & Rhodes, 1982), revealed the presence of five distinct bands, corresponding to α constituent chains of collagen type I (α_1 and α_2), two β dimers and a γ trimer, having identical mobilities to the control collagen (Biocolor, UK) (fig. 1). The ratio between the specific α_1(I) and α_2(I) chains was very close to the natural value of 2:1, that confirmed the native structure of extracted collagen type I, $[\alpha_1(I)]_2\alpha_2(I)$. It was showed by electron microscopy that collagen obtained by this method and used to prepare composite scaffolds is mostly organized as fibril aggregates and a few fibers having the 67-nm characteristic banding pattern (Zarnescu et al., 2010).

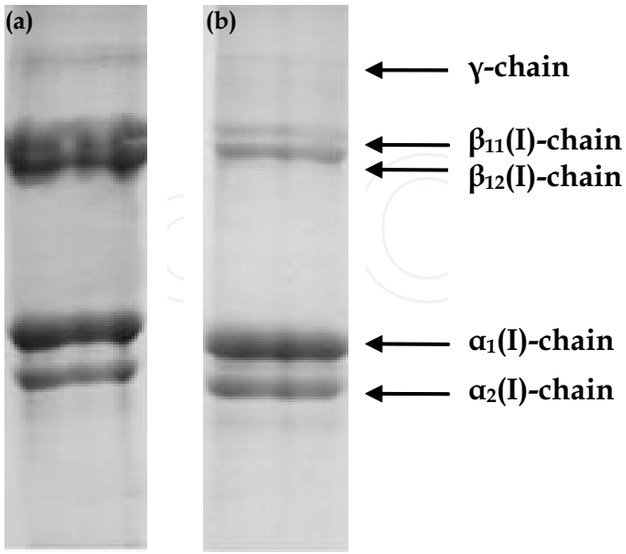


Fig. 1. SDS-polyacrylamide gel electrophoresis of collagen type I from control (a) and extracted from bovine tendon (b) showing the presence of two constituent α-chains, α1(I) and α2(I), small quantities of dimer (β) and trimer (γ) chains, clearly separated

2.3 Collagen/n-β-TCP composite material preparation

Several ceramics were investigated for their role in bone regeneration, but they lack structural stability and it is difficult to maintain them at the defect site. Therefore, TCP must be included into a polymeric scaffold of collagen.

Nanopowder of β-TCP (n-β-TCP) was obtained from the Ceramic lab of INCDIE ICPE-CA Bucharest, Romania. The particles had unit cell parameters similar to ASTM data and their diameter was lower than 84 nm. Nano-sized β-TCP powder presented a good *in vitro* biocompatibility in cell culture (Tardei et al., 2010). The dimension of ceramic crystals is an important factor involved in the first phase of cell-biomaterial interactions.

A nanocomposite material, collagen/n-β-TCP, consisting of the two main components of natural bone was prepared from a 0.8 % (w/w) collagen type I solution and β-TCP nanopowder mixed in a ratio of 50:50 (w/w) and homogenized with a manual speed-stirrer (Xenox, Germany) at 6000 rpm, at room temperature. In the next step, the mixtures were poured into glass molds (15 mm diameter) and were frozen at – 20 °C, overnight.

The polymeric matrix of natural collagen was not only used to achieve a stable composite, but to prevent rapid release of calcium ions in surrounding medium and to improve the interaction with osteoblast cells. Both components, collagen type I and calcium phosphate stimulate osteoblastic differentiation in cell cultures (Xie et al., 2004) and together, in composite materials, accelerate osteogenesis and allow the achievement of mechanical and biological properties, superior to their individual ones (Wahl and Czernuszka, 2006). Fang et al. (2009) showed that nanocomposites containing biomimetic HA deposited from simulated body fluid facilitate adhesion and spreading of human mesenchymal stem cells. The majority of temporary bone substitutes developed in the last decade are resorbable composite materials consisting of fibrils of collagen type I and calcium phosphate crystals, mimicking the composition and tissue structure (Yamauchi et al., 2004).

3. Composite material design

Designing an artificial bone substituent involves a process of optimizing its composition and structure that influence the osteoconductive properties and interaction with cells. Recent scientific progress in material science and engineering evolved in a biomimetic approach for bone substitute fabrication. A biomimetic composite material can be any artificial material designed to mimic one or several features of the natural one. Natural bone biomimetism implies using collagen type I and apatite in the form of nanoparticles. The osteoinductive property of collagen combined with the bioactivity and osteoconductive property of calcium phosphates give a high biocompatibility to the composite material and favor cell growth (John et al., 2001).

A collagen sponge prepared by freeze-drying (fig. 2A) is similar in structure to trabecular bone, where mineralized fibrils are arranged in a network of trabeculae and voids which are filled by bone marrow *in vivo* (Fantner et al., 2006). Attachment of calcium phosphate particles doesn't significantly modify collagenous network porosity as they are tightly bond to the collagen fibrils, wrapping the skeleton with a fine layer (fig. 2B).

Collagen-calcium phosphate composites can be conditioned in various shapes and forms. Sheets are obtained by mixture drying at room temperature or electrospinning, a technique that yields nanostructures. Cells could adhere to these two-dimensional materials, but proliferation is restricted in comparison to three-dimensional constructs. Composites conditioned as hydrogels are preferred as injectable form for tissue repair in order to avoid complicated operation; they are three-dimensional structures, but they lack mechanical strength (Hunt & Grover, 2010).

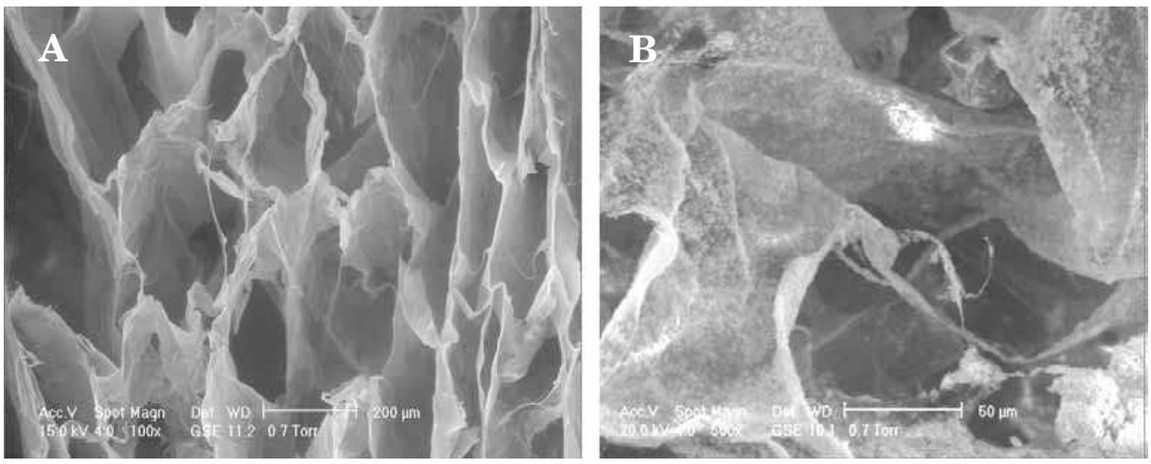


Fig. 2. Scanning electron micrograph of a freeze-dried collagen material presenting a network of fibrils and voids (A) and a detail of a freeze-dried collagen/TCP 50/50 (w/w) composite material showing the collagenous skeleton wrapped with a fine layer of tricalcium phosphate particles (B)

Collagen-calcium phosphate composites can be conditioned in various shapes and forms. Sheets are obtained by mixture drying at room temperature or electrospinning, a technique that yields nanostructures. Cells could adhere to these two-dimensional materials, but proliferation is restricted in comparison to three-dimensional constructs. Composites conditioned as hydrogels are preferred as injectable form for tissue repair in order to avoid complicated operation; they are three-dimensional structures, but they lack mechanical strength (Hunt & Grover, 2010).
Selection of bone substituent composition indicates an optimum ratio between collagen and calcium phosphate of 50:50 (w/w). A higher calcium phosphate concentration in a composite with 50:100 (w/w) ratio between the two components led to an intense loaded collagenous network having less strength, a lower value of porosity and decreased biocompatibility (Moldovan et al., 2009).

3.1 Freeze-drying of composite material

Freeze-drying technique is based on two processes: first, a solution is frozen and then, the solvent, which is usually water, is removed under vacuum, at low temperatures by sublimation. The solvent crystals formed during freezing have the same size and morphology as the pores of the material after drying. Therefore, parameters like the rate and temperature of freezing, concentration and pH of the solution, and the presence or absence of other macromolecules influence pore morphology and size in the final product.
The mixture, consisting of collagen type I and n-β-TCP particles, was subjected to freeze-drying using a freezing temperature of -35 °C. The programme of the freeze-dryer (Christ, Germany) continued with a 0 °C-step, at 0.26 mbar, for 17 h and drying at + 30 °C. This process yielded a nanocomposite material, conditioned as porous scaffold, which was sealed in a plastic bag and exposed to UV-radiation, for 8 h, in a sterilization cabinet (Scie-Plas, UK). A collagen solution was identically processed and used as control material.
All the operations for collagen/n-β-TCP composite material fabrication are summarized in fig. 3.

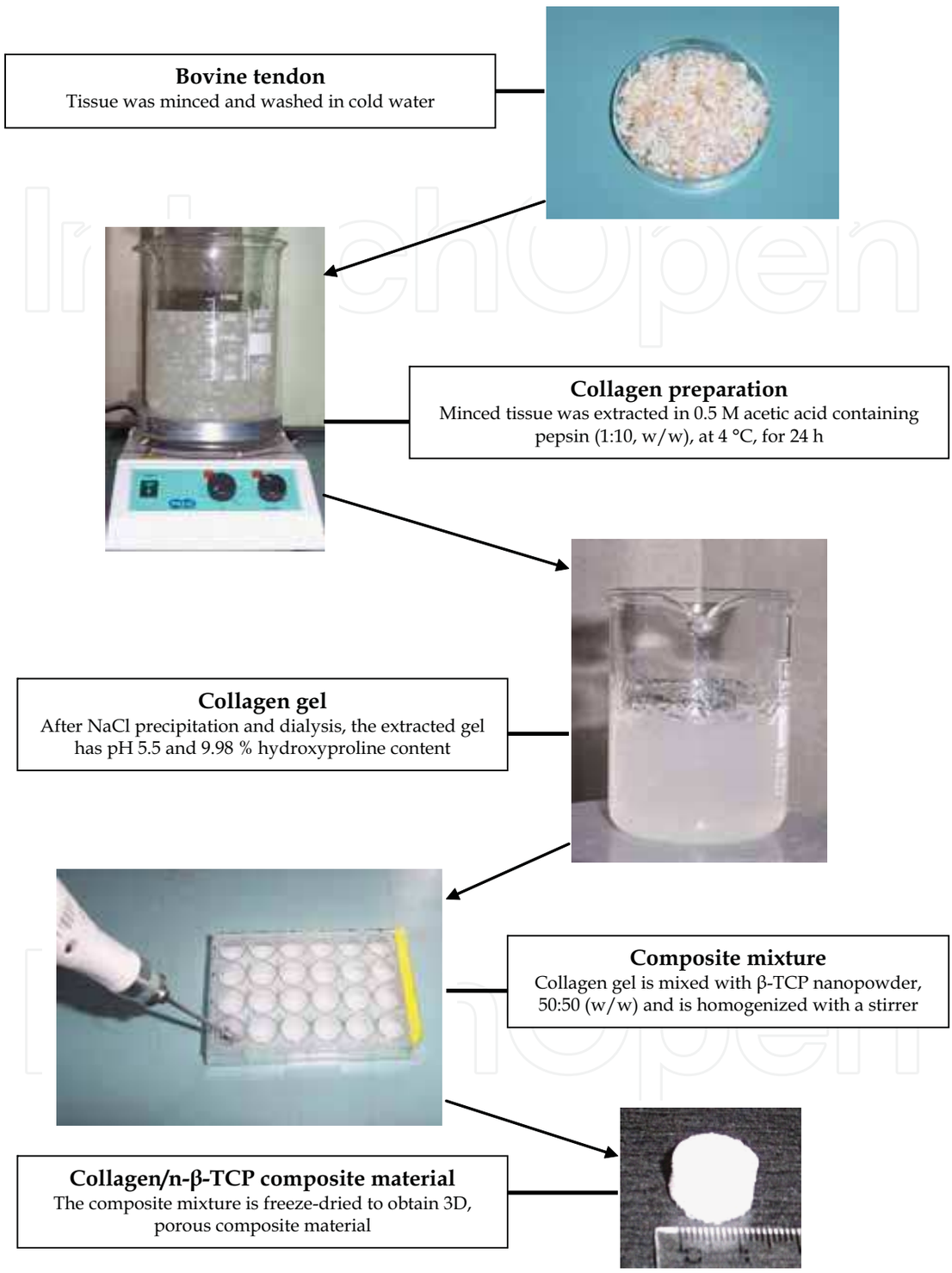


Fig. 3. Schematic diagram showing the experimental procedures used to prepare collagen/n-β-TCP nanocomposite material from collagen type I solution and β-TCP nanopowder by freeze-drying technique

Freeze drying is a technique used for the fabrication of porous materials (Schoof et al., 2001). The final porosity of three-dimensional composites can be controlled by varying the freezing temperature at -20 °C, -78 °C or -196 °C, respectively; namely, the lower the temperature, the smaller the pore size (Karageorgiou & Kaplan, 2005). Still, a temperature of -196 °C gives too small pores for a medical implant and its mechanical properties are altered. Porous materials having pores in the range of 50-1500 μm can be obtained by freeze-drying (Li, 2000).
The materials having high porosity are preferred because they have a high void volume within cells can grow and form new tissue. A network formed from well defined and interconnected pores is necessary in view of viable implant development. The porosity value for a composite material used in bone tissue engineering must be above 70 % in order to allow cell growth and proliferation (Boland et al., 2004). The size of the pores from bone substitutes used as cell scaffolds must have at least 100 μm because osteoblast cells have sizes in the range of 10-60 μm, depending on the species and cell line (Xu & Simon, 2004).

3.2 Cross-linking of composite material

Collagen-based composite materials used in bone repair mimic the ultrastructure of native extracellular matrix, but possess high sensitivity to enzymatic degradation. Therefore, new covalent bonds must be introduced in collagen structure in order to register less biodegradability. Cross-linking of collagen-based composite materials must be a compulsory step in their fabrication in order to control collagen biodegradation rate and their mechanical characteristics. However, depending on the used reagent, composite material biocompatibility might be compromised.
Chemical cross-linking is a technique that involves the formation of covalent bonds between two different or identical protein molecules. It uses bifunctional reagents, containing reactive groups that react with functional groups present on the side chains of amino acid residues, such as the amino group of lysine, arginine, glutamine and asparagine, or sulfhydryl from cysteine. The commonly used cross-linking agents for collagen-based materials, including glutaraldehyde, formaldehyde and epoxy compounds can be used by directly mixing with the protein or in a vapour chamber. They are cytotoxic owing to reactive moieties covalently coupled between neighbour collagen fibrils (Badylak, 2002). A neutralization of the cytotoxic residues after cross-linking is achieved using 10 mM sodium borohydride, at 4 ^{0}C, for 24h prior to implantation. Glutaraldehyde is also involved in development of calcification that occurs subsequently to implantation (Schoen & Levy, 2005). An efficient cross-linking method uses the heterobifunctional carbodiimide, 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloride (EDC), so-called zero-length agent because it does not incorporate itself into the polymer macromolecules, thus improving the biocompatibility of the material. This is an important advantage over other chemical cross-linking agents. EDC reagent is suited for use with collagen type I materials as showed Pieper et al. (1999) and electrospun collagen type II materials as showed Barnes et al. (2007). At present, EDC is used to cross-link composites like collagen-glycosaminoglycan skin substitutes (Powell & Boyce, 2006), collagen-elastin-glycosaminoglycan vascular scaffolds (Daamen et al., 2008) or gelatin-hydroxyapatite for bone repair (Chang & Douglas, 2007). Polyethylene glycol is used in biomedicine as dispersing agents, solvents, ointment, and suppository bases and is currently tested as cross-linking agent (Popescu et al., 2009).
Physical methods of cross-linking, such as ultraviolet irradiation (Lee et al., 2001), photo-oxidation (Turek & Cwalina, 2010) and dehydrothermal treatment (Haugh et al., 2009) have

a mild effect on collagenous materials, but they obtain an improved enzymatic resistance and biocompatibility.

Recently, natural plant polyphenolic compounds, like tannic acid (Isenburg et al., 2005), genipin (Bi et al., 2011), proanthocyanidins (Chen et al., 2008), catechin (Madhan et al., 2005), and riboflavin (Ashwin & McDonnell, 2010) have been shown to stabilize collagen structure through hydrogen bonding and hydrophobic interactions, while preserving its cytocompatibility.

Enzymatic cross-linking using transglutaminase (EC 2.3.2.13) is applied to denatured collagen-based composite materials, conditioned as films or gels, leading to formation of covalent amidic bonds between carboxyl group of glutamine residues and ε-amino group of lysine residues (Chen et al., 2003). The process results in irreversible network junctions, similar to that formed by chemical agents. Microbial transglutaminase, a calcium-independent enzyme, has a higher specific activity that stimulated new applications, especially in food industry (Collighan et al., 2002; Garcia et al., 2007).

Three cross-linking protocols using different agents (EDC, glutaraldehyde and catechin) were comparatively evaluated in terms of efficiency on collagen porous material. First, the cross-linking process was carried out in ethanolic solution of EDC, at pH 5.5, by slowly shaking on a platform, at room temperature, for 18 h (Pieper et al., 2002). Alternatively, collagen sample was treated with EDC/N-hydroxysuccinimide (NHS) solution, in the same conditions and was shaken for 4 h. After cross-linking, several washing steps of the samples were carried out in order to eliminate any unreacted intermediates. The samples were washed in solutions of 0.1 M sodium phosphate (pH 9.1), 1 M and 2 M NaCl. After the final washing in distilled water, samples were once again lyophilized.

The second cross-linking process used glutaraldehyde and took place in a special chamber in which the solution does not contact the material sample. A solution of 3 % (w/w) glutaraldehyde was put at the bottom of the chamber and collagen material was exposed to the vapors, at room temperature, for 18 h. The sample was then washed in distilled water, renewed every 1 h.

The third method of cross-linking used catechin, a natural polyphenol from green tea and was carried out by simply immersion of collagen sample in 10 mM catechin solution and shaking at 300 rpm, at room temperature, for 18 h.

The cross-linking degree can be assayed using physical or chemical methods for the determination of shrinkage and denaturation temperature, content in amino free groups, *in vitro* enzymatic digestion or mechanical properties. In order to compare the cross-linking degree of collagen materials, it was calculated the percentage of free amino groups lost during the process by spectrophotometric assay using 2,4,6-trinitrobenzene sulfonic acid (TNBS) (Barnes et al., 2007). The degree of cross-linking was expressed as percentage loss in free amino groups after cross-linking and was calculated as follows:

$$\%\ \text{Cross}-\text{linking degree} = 1 - \left(\text{ABS}_{\text{CL}} / \text{MASS}_{\text{CL}}\right) / \left(\text{ABS}_{\text{NCL}} / \text{MASS}_{\text{NCL}}\right) \quad (1)$$

where ABS is absorbance at 346 nm, MASS is sample weight, CL is the cross-linked sample and NCL is the non-cross-linked sample.

The results showed that there is a similar cross-linking degree of collagen materials treated with 30 mM EDC and EDC/NHS, compared to 3 % glutaraldehyde vapors and 10 mM catechin (fig. 4). The cross-linking of collagen porous material was achieved at a rate above 50 % for all used agents.

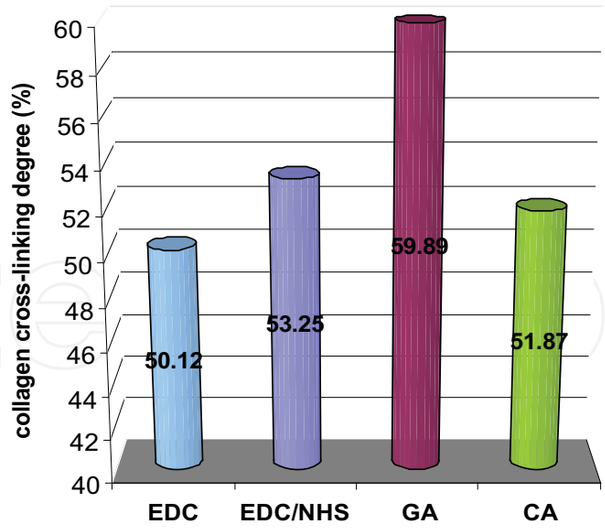


Fig. 4. Variation of cross-linking degree for porous collagen material treated with EDC, EDC/NHS, glutaraldehyde (GA) and catechin (CA)

EDC cross-linker has a mechanism of action consisting of reaction with carboxyl groups converting them into O-acylisourea intermediates that further react with lysine residues or other available primary amines. The amines exert a nucleophilic attack on the intermediate, resulting in a stable amide bond (see reaction on Thermo Fisher Scientific). EDC is released as a soluble urea derivative and can be removed by several washings (Pieper et al., 1999, 2002).
NHS is often included in EDC coupling protocols to improve efficiency. The reaction of NHS with carboxyl groups is mediated by EDC and results in formation of an NHS ester intermediate. The advantage of NHS is the higher stability of its intermediate, compared to EDC intermediate. This property allows a more efficient conjugation to the primary amines. The amide bond is formed in shorter periods of time, compared to EDC cross-linking and NHS is released.
EDC cross-linking is an efficient process when takes place in acidic conditions (pH 5.5) and a suitable buffer for carbodiimide reaction is 4-morpholinoethanesulfonic acid buffer. A higher pH (up to 7.2) could lower the efficiency of the reaction but can be compensated by increasing the amount of EDC. Still, a high quantity of EDC could prevent cross-linking reaction. The optimum molar ratio between protein and EDC reactive groups is 1:1, as earlier established in studies on collagenous materials (Olde Damink et al., 1996). NHS cross-linking reaction is optimal at physiologic or slightly alkaline pH (up to 8.5); thus, phosphate, bicarbonate or carbonate, HEPES or borate buffers are commonly used. The optimal temperature for EDC reaction is room temperature and higher than 40 °C for NHS reaction. The final washings are important to eliminate intermediary products formed during the reaction and to avoid cytotoxicity.
In the particular case of collagen cross-linking, there are aspartic and glutamic acid available residues within the α chains that interact with lysine and hydroxylysine residues from the same chain, the neighbor chain or from other collagen molecule/fibril, forming intra- or intermolecular covalent cross-links. When cross-linking is applied to porous collagenous structure, ethanol is recommended to prevent pore morphological changes. Ethanol reduces dipolar forces and allows bond changes due to its lower dielectric constant of 35, compared to 81 for water (Barnes et al., 2007). Also, ethanol molecules could act as proton donors in reaction with EDC, improving cross-linking yield.

The first study on applying EDC and EDC/NHS cross-linking methods to collagen/n-β-TCP 50/50 (w/w) composite material was performed to compare their efficiency. The protocols were identical to those applied to collagen material (see above). The results showed that the cross-linking process resulted in a decrease of the free amine group content relative to non-cross-linked composite material. The value for the composite cross-linking degree was 25.64 % using EDC. A stronger capability of cross-linking was registered for EDC/NHS agent, 28.01 % (fig. 5). An increase in TCP quantity (50/100 ratio, w/w) resulted in a decrease of composite cross-linking degree to values of 20.76 % for EDC and 24.34 % for EDC/NHS. According to TNBS assay, the cross-linking degree for collagen material was significantly higher than for composite material, taking into account that the same quantity of collagen was used to prepare simple and composite materials. This result might indicate that the presence of β-TCP nanoparticles on collagen fibril surface partially hindered lysine interactions with aspartic and glutamic acid residues within the three α-chains of collagen fibrils. A similar action was reported for 1,4-butanediol diglycidyl ether used as cross-linking agent, namely the process was more evident for collagen without mineral phase than for the composite (Tampieri et al., 2008).
Composite material cross-linking influences its structure, biodegradability, calcium release and biocompatibility.

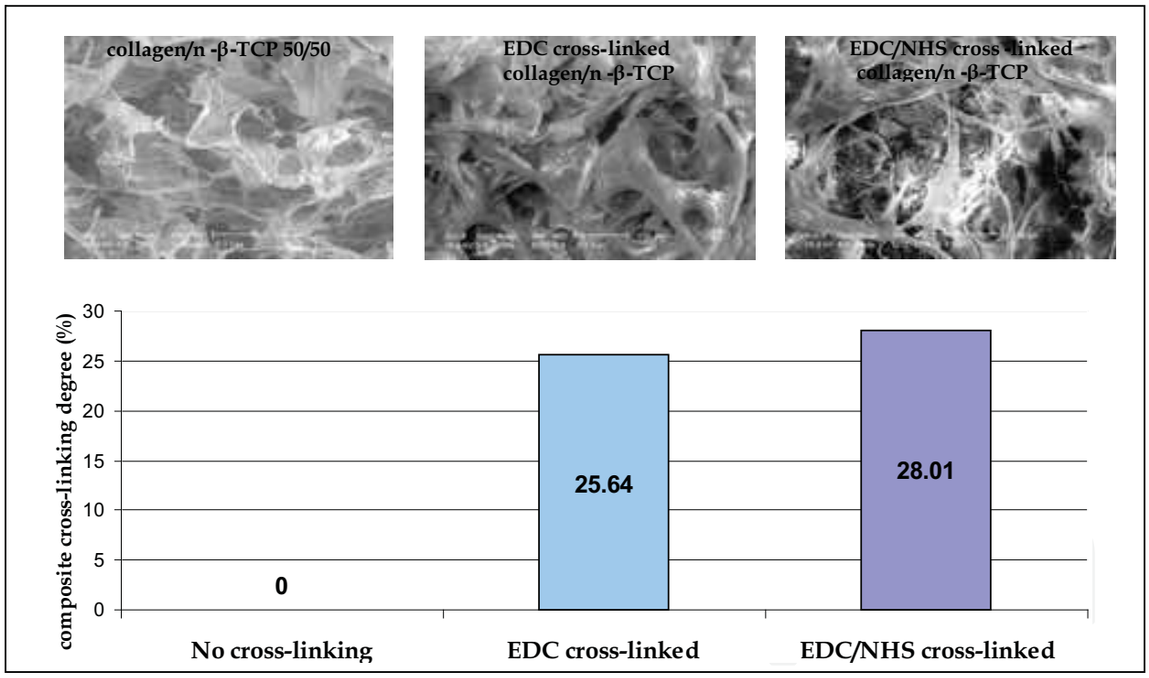


Fig. 5. Micrographs of scanning electron microscopy of collagen/n-β-TCP 50/50 (w/w) composite material before and after cross-linking with EDC and EDC/NHS and their percentage of cross-linking degree assayed with TNBS

4. Morphological characterization of cross-linked composite material

4.1 Scanning electron microscopy of cross-linked composite material

The microstructure of the obtained composite material before and after cross-linking was examined by environmental scanning electron microscopy. The sample was mounted on

carbon pads attached to aluminium stubs and visualized at an ESEM apparatus (Quanta 400, FEI, Philips, Holland) using the low vacuum mode. Micrographs showed white deposits of β-TCP nanoparticles disposed on the surface of collagen fibrils (fig. 5). Images of non-cross-linked composite variant showed a typical structure of lyophilized collagenous materials, with regular pores, which favor a good biological behavior. Pore morphology was affected by the cross-linking process. The ordered structure with interconnected pores of non-cross-linked composite material was replaced by a structure with unevenly sized pores, ranging from 20 to 200 μm in the EDC and EDC/NHS cross-linked materials. Sample rehydration and the additional lyophilization process that occur during the cross-linking treatment could induce a slightly collapse of pore network and led to a dense material.

4.2 Porosity of cross-linked composite material

The porosity (ε) of composite materials was measured by water displacement method (Zhang et al., 2003). The following equation was used to calculate the porosity value:

$$\varepsilon = \left(v_1 - v_3\right) / \left(v_2 - v_3\right) \times 100 \quad (2)$$

where v_1 is a known volume of water in a graded test tube, v_2 is the total volume of water plus the water-impregnated composite material sample, after 3h of incubation to allow water to penetrate and fill the pores, v_3 is the residual volume of water after removing the water-impregnated composite material from the test tube.

In comparison to the non-cross-linked composite material, having a porosity of 94.83 %, the values for cross-linked composite materials decreased according to the used method of cross-linking and the degree of cross-linking. Thus, the porosity value for the EDC-treated material was 83.76 % and 78.25 % for the EDC/NHS-treated material. Composite material porosity decrease, registered after EDC or EDC/NHS cross-linking could be due to the newly formed cross-links. Results showed that the used freeze-drying process, with freezing temperatures of -35 °C, led to composites with a porosity of at least 78 %, a value that allows a good infiltration of cells.

5. Biochemical and biological properties of cross-linked composite material

5.1 Collagenase degradation of cross-linked composite material

Bone regeneration takes place over duration of several months. It is important for a composite material used for tissue repair to degrade in a controlled fashion while new tissue is formed. An *in vitro* experimental model using bacterial collagenase mimics the enzymatic attack on the collagenous composite material implanted *in vivo*. This enzyme acts specific on the amino bond of glycine from the peptidic repetitive sequence, -X-Gly-Pro-, from the helical region of collagen. This model shows if the covalent cross-links introduced in the collagen molecule by carbodiimide treatment could hinder and protect the cleavage site, block bacterial collagenase action and reduce material degradability.

To quantify collagen/n-β-TCP composite degradation, each sample of material was weighed and pre-incubated in TES buffer, pH 7.4, containing 50 mM $CaCl_2$, at 37 °C, for 30 min. In the next step, 100 μl bacterial collagenase type IA (Sigma-Aldrich) in TES buffer were added and the degradation took place in a water bath, at 37 °C, for different periods of time (6h, 12h, 18h and 24 h). At the end of each incubation period, the reaction was stopped with EDTA, at 0 °C and the protein content of the supernatant was assayed by ninhydrin method. The percentage of biodegradation was calculated using the equation:

$$\% \text{ biodegradation} = \left(\mu M \text{ aminoacids}_{CL} / w_{CL}\right) / \left(\mu M \text{ aminoacids}_{NCL} / w_{NCL}\right) \times 100 \quad (3)$$

where w is the material weight in grams, CL is the cross-linked sample and NCL is the non-cross-linked sample which is completely degraded after 6h of incubation (control).
Fig. 6 compares the biodegradability of collagen/n-β-TCP composite before and after the cross-linking treatment. The non-cross-linked composite material had been thoroughly degraded after incubation in collagenase solution for only 6 h (100 % biodegradability). After cross-linking treatment, the biostability of the material was enhanced according to the cross-linking agent. The EDC-cross-linked material was only 58.17 % degraded in 24 h. The EDC/NHS-cross-linked material had a better ability to resist collagenase degradation (max. 43.41 %) due to its higher cross-linking degree. These results reveal that both cross-linking methods improve collagen/n-β-TCP material biostability, but EDC/NHS treatment is faster and more efficient. The cross-linking treatment lowered the biodegradability of the composites which were less susceptible towards collagenase attack. It was observed a good correlation between the cross-linking degree and the biodegradability of each sample. Composites with a higher degree of cross-linking yielded a smaller quantity of degraded collagen. These results indicated a better stability of EDC/NHS cross-linked composite material over EDC cross-linked one and both values over untreated sample.

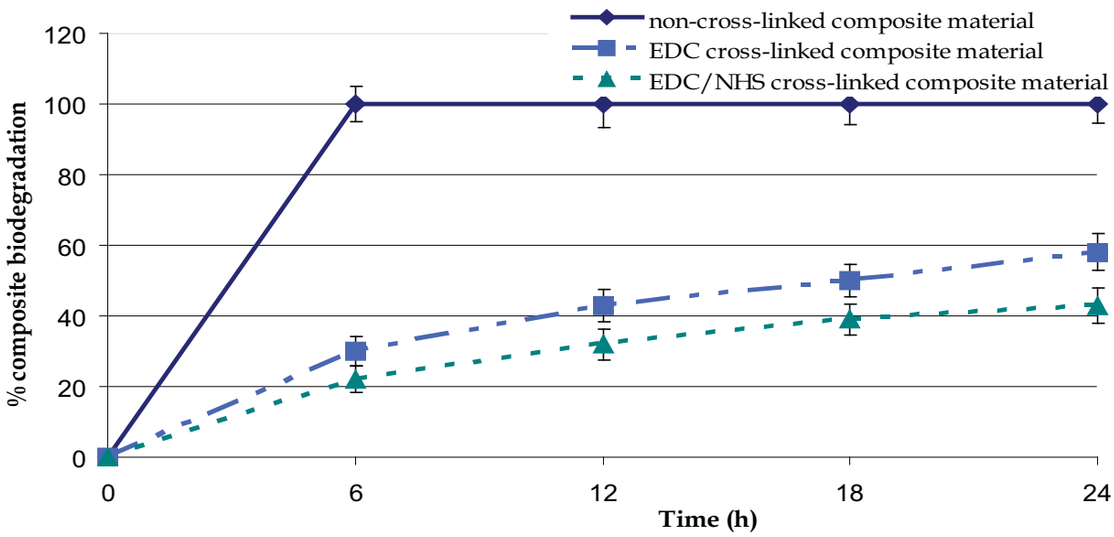


Fig. 6. The biodegradation degree of collagen/n-β-TCP 50/50 (w/w) composite material before and after cross-linking with EDC and EDC/NHS, after incubation with bacterial collagenase, for 24 h

5.2 *In vitro* calcium release from cross-linked composite material

In vivo dissolution of ceramic particles takes place by a decrease in crystal size and increase in macroporosity and microporosity (LeGeros et al., 2003). When ceramics are soaked in buffer solution, a dissolution reaction leads to increasing calcium and phosphate ion concentrations in the solution. A decrease of calcium concentration in the medium is registered when the reprecipitation reaction occurs (Wang et al., 2004).
The dissolution of β-TCP nanoparticles attached to collagen fibrils in composite material was analyzed by assessment of calcium ions released in solution, in physiological

conditions. Samples of material were incubated in phosphate buffer saline, pH 7.4, for 5 days. At each 24 h, 5 µl of supernatant were transferred to a 96-well plate and the same volume of fresh PBS was added to the reaction tube. The calcium content of the supernatant was determined using the QuantiChrom Calcium Assay kit (BioAssay Systems, USA), according to the instructions. After 3 min of incubation with reaction reagent, the optical density was read at 612 nm using a plate-reader (Tecan, Austria). The concentration of calcium was calculated using a standard curve in the range 0-200 µg/ml. The dynamic of the dissolution behavior of n-β-TCP particles from non-cross-linked and cross-linked material variants is shown in fig. 7. The calcium quantity released from the non-cross-linked material increased in the first 48 h and was higher than the value for the cross-linked ones after 5 days. The cross-linked composite materials had a similar pattern for calcium release, regardless of the cross-linking method, having a maximum value of calcium ions after 24 h of incubation. It was concluded that the cross-linking process is beneficial for collagen/n-β-TCP composite materials because the calcium release takes place in a controlled-fashion, in comparison to the non-treated composite materials.

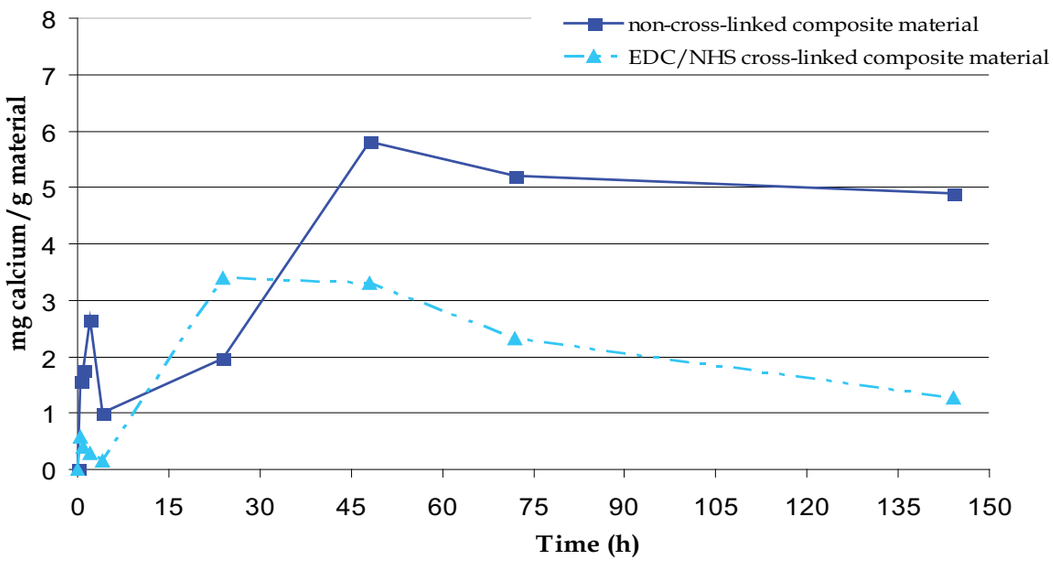


Fig. 7. Variation of calcium ion concentration released from non-cross-linked and EDC/NHS cross-linked collagen/n-β-TCP composite materials in 0.1 M PBS, pH 7.4

All the above tested properties and the obtained results lead us to conclude that EDC/NHS cross-linking protocol allows to fabricate a stable collagen/n-β-TCP composite material, with a controlled release of calcium and a porous microstructure adequate for cell infiltration and proliferation.

5.3 Preliminary *in vitro* biological testing of cross-linked composite material

In the last decade, porous composite materials prepared from synthetic and/or natural polymers combined with a ceramic component are tailored and tested for their efficacy in regeneration of wounded tissue (Ge et al., 2008). These composites serve as scaffolds for cell cultivation *in vitro* or as temporary bone substitutes *in vivo* guiding cell proliferation and new extracellular matrix formation (Leong et al., 2003). Composite materials designed for medical applications must be first tested for *in vitro* cytotoxicity on cell cultures, before *in*

vivo pre-clinical and clinical trials. According to the International Standard ISO 10993-5, there are several specific methods to analyze the cytotoxicity of medical devices, which involves different aspects of cell function, like viability and proliferation, loss of membrane integrity, decrease in cell adhesion, cell morphology. The viability of cells cultured in the presence of a medical device could be assayed using MTT assay (Mossman, 1983). This is a colorimetric assay based on the reduction of yellow soluble salts of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) to dark purple insoluble formazan crystals by dehydrogenases from cell mitochondria. Since conversion of MTT takes place only in metabolically active cells, the level of enzymatic activity measured as optical density is directly proportional to the viability of the cells.

The standard extract protocol consisted in sterile samples of cross-linked and non-cross-linked collagen/n-β-TCP composite material immersed in the culture medium DMEM supplemented with 10 % fetal calf serum (FCS) and incubated in a humidified atmosphere of 5 % CO_2 and 95 % air, for 24 h. The used ratio between the surface area of the composite and the volume of culture medium was 1 cm^2/ml, ranging between 0.5-6.0 cm^2/ml, the values from ISO standard. The conditioned medium, named extract, was used for MTT assay.

Fibroblasts from NCTC cell line (ECACC) were seeded in the wells of a 24-well culture plate, at a density of $5x10^4$ cells/ml and cultivated in DMEM containing 10 % FCS and 1% antibiotic mixture. The plate was placed into an incubator, with 5 % CO_2 atmosphere, at 37 °C, for 24h, to allow cell adhesion. The culture medium was, then, replaced with the same volume of extract and the plates were incubated in humidified atmosphere with 5 % CO_2, at 37 °C, for 48h.

In order to assay the viable cells after *in vitro* culture with the extracts, the medium was removed and fresh medium with MTT solution, in a 10:1 (v/v) ratio was added. The plates were incubated at 37 °C, for 3h. The medium was removed and 500 µl isopropanol were added to each well and the plate was gently shaken on a platform, for 3 h, to dissolve the formazan crystals. The colored solution was transferred to another 96-well plate and the optical density was read at 570 nm, with reference settled at 630 nm, using a microplate reader (Sunrise Tecan, Austria). The cells cultured with complete culture medium were negative control (nontoxic) and cells cultured in the presence of hydrogen peroxide were positive control (toxic). The experiment was performed with three samples per each group (n=3). The results were calculated as mean values ± standard deviation for cells cultured with the control or the material extract, respectively and expressed as percentage from the negative control, considered to be 100 % viable cells.

The extract method allows the evaluation of possible toxic compounds released from the material in the medium and that could modulate the cellular activity. As shown in fig. 8, the viability of cells cultured with composite material extracts was superior approx. 1.2-fold to the negative control, after 48h of cultivation. The extract of cross-linked material induced a higher viability to NCTC fibroblasts in comparison to the non-cross-linked material extract. These values indicated that cross-linked composite was a bioactive material, able to control and stimulate the cellular activity, better than the non-cross-linked variant. Other collagen-based composite materials were shown to modulate fibroblast activity in culture (Zhang et al., 2003; Jantova et al., 2009).

Other 24-well plate was seeded with NCTC fibroblasts and cultivated in the presence of composite extract using the same protocol as for the extract method (described above). After 48 h of incubation, cells cultured on polystyrene plate were fixed in methanol and Giemsa stained. The morphology of cells grown in extract medium was observed by light microscopy. The micrographs showed that cells maintained their normal phenotype, presenting euchromatic nuclei with 1-2 nucleoli and a clear cytoplasm (fig. 8, right).

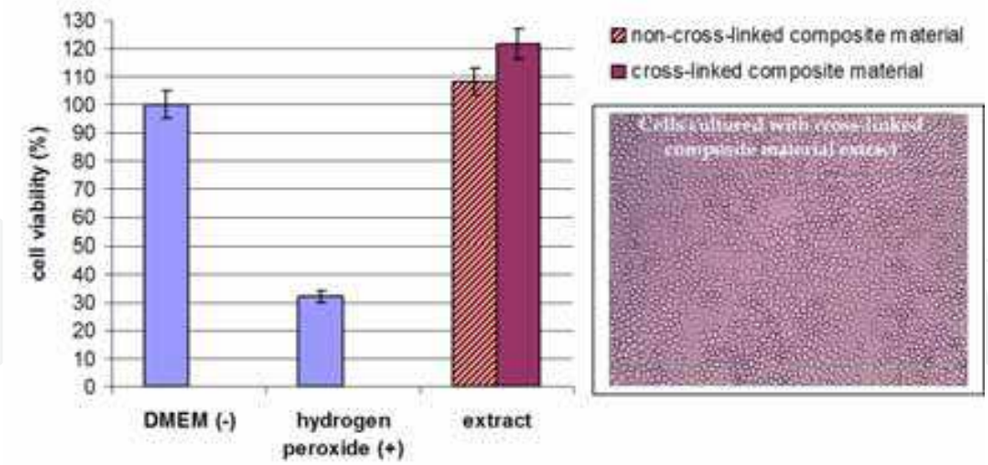


Fig. 8. Cell viability measured by MTT assay after 48h in culture with DMEM (negative control), DMEM containing hydrogen peroxide (positive control) and collagen/n-β-TCP composite extract. On the right a light micrograph showing fibroblast cell morphology after 48h of cultivation in extract of collagen/n-β-TCP cross-linked composite material.

The culture media removed from the MTT test was analyzed by lactate dehydrogenase (LDH) assay. LDH assay is based on the reduction of nicotinamide adenine dinucleotide (NAD) by LDH. The resulting NADH is used to convert a tetrazolium salt to a colored formazan, which strongly absorbs in the visible range 490-520 nm. The quantified LDH activity is an indicator of cell viability as only lysed cells are able to release this enzyme from the cytosol into the medium. The NCTC fibroblast membrane integrity was assayed in this experiment as a function of the amount of cytoplasmic LDH released into the culture medium, according to the instructions of the kit (Cayman Chemical Co., USA). After an incubation of the centrifuged culture medium with reagent mixture with gentle shaking, at room temperature, for 30 min, the optical density was measured at 490 nm using a microplate reader (Sunrise Tecan, Austria). LDH activity (mU/ml) was calculated from the standard curve plotted with standard LDH in the range 0-1 mU. Results were reported in arbitrary units, the negative control being considered equal to 1.
No increase in LDH leakage was observed for cells cultivated with composite material extracts for 48 h (non-cross-linked-0.95; cross-linked-1) when compared to the negative control. This indicates that composites have no cytotoxicity related to NCTC fibroblasts. The LDH analysis in the culture medium from the same experiment with cells analyzed by MTT allows meaningfully comparison of the results. Both experiments confirmed a high *in vitro* biocompatibility of collagen/n-β-TCP that is an important property for a polymeric composite material of medical utility, intended to support tissue repair.

5.4 Osteogenic properties of cross-linked composite material

Many cell types need an appropriate adhesion surface in order to maintain their proliferation ability and specific or differentiated functions. Cell adhesion is an important factor influenced by the surface characteristics of the material. Material efficacy in fullfilling these requirements depends mainly on the chemical characteristics of the surface, that determines cell-substrate interaction, but also on cell morphology and the relation between the cells and the material.

It was demonstrated that this organizatoric role can be successfully played by porous composite materials using an *in vitro* experimental model, i.e. rat osteoblasts from a primary culture cultivated into the three-dimensional, porous collagen/n-β-TCP 50/50 composite material. The cell-composite construct, cultured for different periods of time, yield a bioactive implant that can be used in tissue repair. In order to assess its medical utility, cell proliferation and adhesion was evaluated by total DNA quantification. The primary cell culture of rat osteoblasts was established from parietal and frontal bones by enzymatic method, as described by Gu et al. (2002), according to the international guidelines for care and use of laboratory animals. Cells were cultivated in DMEM supplemented with 0.05 mM ascorbic acid (Sigma) and 10 mM β-glycerophosphate (Sigma), for 21 days and alkaline phosphatase activity and calcium phosphate deposits were histochemically detected, to confirm cell osteoblast phenotype (Oprita et al., 2008).

Sterile samples of collagen/n-β-TCP cross-linked composite material (5x5x5 mm^3) were placed into the wells of a 24-well culture plate. Rat osteoblasts in 200 µl DMEM supplemented with 10 % FCS, at a density of $4x10^6$ cells/cm^3, were injected into the samples and the plates were incubated in humidified atmosphere, with 5 % CO_2, at 37 °C. After 4h, 0.5 ml of the same medium were added into each well to cover the cell-composite construct and they were incubated at 37 °C, for 6 days. Cell culture medium was renewed twice a week.

Total DNA content was fluorimetrically assayed in cell lysate. After 1 day and 6 days of cultivation, respectively, each cell-composite construct was washed three times in phosphate-buffered solution and was frozen at -80 °C until analysis. After thawing, the constructs were cut in very small pieces and the fragments were incubated with saline-sodium citrate buffer, pH 8.5 containing 0.02 % SDS, at 37 °C, with occasional stirring, for 1 h. After centrifugation at 10000 g, an aliquot of 10 µl of cell lysate was transferred to a test tube to determine the DNA content, with Quant-iT dsDNA HS Assay kit (InVitrogen, USA) on a Qubit fluorometer (InVitrogen, USA). Results were reported as cell number, assuming a standard quantity of 8 pg DNA per cell (Ahlfors & Billiar, 2007).

The number of adhered cells after 1 day of cultivation was approx. 54 % related to the seeded cell number. An approx. 1.4-fold increase in cell number was observed from $2.7x10^5$ cells in day 1 to $3.8x10^5$ cells in day 6. The cross-linked composite material allowed osteoblast adhesion and proliferation. As Anselme (2000) reported, rat osteoblast adhesion does not occur preferentially to ceramic crystals or to collagen fibrils and it is independent of the roughness of the material surface. Other authors demonstrate that collagen-based composites, organized as three-dimensional scaffolds, enhance the contact guidance process of osteoblasts inoculated onto the material surface (Rodrigues et al, 2003). It is known that collagen has binding sites that promote cell attachment through focal contacts and adhesion plaques, providing an increased cell adhesion to composites designed for tissue regeneration (Douglas et al., 2008).

In another experiment, osteoblast-composite constructs were cultivated in normal medium (DMEM) and osteogenic medium (DMEM supplemented with 0.05 mM ascorbic acid, 10 mM β-glycerophosphate and 100 nM dexamethasone), for 21 days. After 7, 14 and 21 days of cultivation, the constructs were washed twice in PBS and frozen at -80 °C. After thawing, cell lysis was conducted as described above. The alkaline phosphatase activity was analyzed using an artificial substrate, p-nitrophenylphosphate reagent, pH 9.8, freshly prepared. The color developed after incubation of the lysate with substrate reagent was read at 410 nm, using a microplate reader (Sunrise Tecan, Austria). The standard curve was constructed

using p-nitrophenol in the range of 0-1.0 mM concentration, as the resulting product from the enzymatic reaction. The results were expressed as micromoles of p-nitrophenol per min reported to the total protein content.

Rat osteoblasts injected into composite materials expressed alkaline phosphatase during the 3 weeks of cultivation. Temporal expression of alkaline phosphatase showed a gradually increase in the first 14 days of culture, peaked around day 14 and then, a decrease to day 21 (fig. 9). Comparing the culture conditions, it were registered higher values of alkaline phosphatase activity for constructs cultured in DMEM than in osteogenic medium.

Expression of alkaline phosphatase activity demonstrated maintenance of the osteoblastic phenotype after cell cultivation into collagen/n-β-TCP cross-linked composite material and its osteogenic properties. The increase in alkaline phosphatase activity indicates the presence of mature osteoblasts and its decrease corresponds to their differentiation into osteocytes (Heinemann et al., 2008).

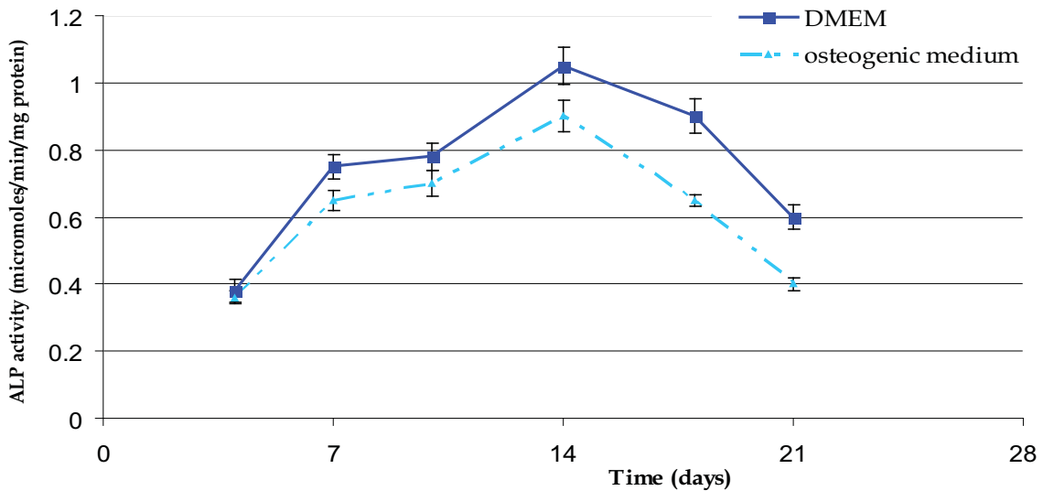


Fig. 9. Temporal expression of alkaline phosphatase (ALP) activity by rat osteoblasts injected into collagen/n-β-TCP cross-linked composite material and cultured in DMEM (straight line) and osteogenic medium (dot line), for 21 days

6. Applications

Bone is the second most transplanted tissue in the body after blood transfusions. Autologous bone transplantation is limited by the transplantable quantity and the damages produced in healthy body parts after harvesting. The cell-composite constructs cultured *in vitro* for different periods of time yield bioactive implants that can be used in tissue-engineered bone transplantation, a new medical technology used for the regeneration of bone and joints. Therefore, cooperation between a human or veterinary clinic and a research institution with experienced cell culture laboratory and biomaterial engineers must be established. A flow of clinical application research was verified as showed in fig. 10.

The bone tissue of new born rats was transferred from a veterinary clinic to the clean room of the cell culture facility where was processed in culture flasks using cell culture medium to obtain a primary osteoblast culture that multiplied. These cells were seeded into a three-dimensional, porous collagen/n-β-TCP cross-linked composite material, biomimetic to bone and cell biocompatible. The construct was cultured for three weeks in cell culture medium

to achieve a bioactive implant. The cells maintained their osteogenic phenotype during *in vitro* culture into the composite material, as indicated by osteoblast specific marker identification. This tissue-engineered artificial bone has to be transferred back to the clinic for implantation in adult rats to test its inflammatory response. Implant ability to induce new bone formation is verified in animal defect experimental models and comparison to existent treatments is necessary.

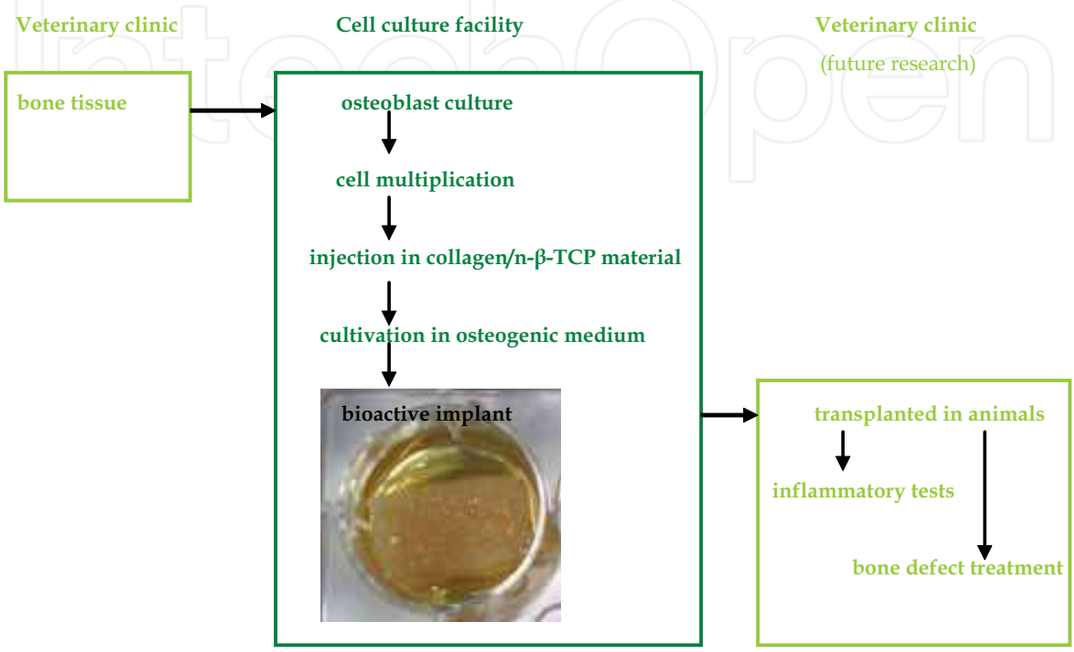


Fig. 10. Biotechnological research of a cell culture facility in collaboration with a veterinary clinic for achievement of a bioactive implant after osteoblast cell seeding into collagen/n-β-TCP cross-linked composite material and *in vitro* cultivation for three weeks

7. Conclusions and future research

A composite material mimetic to trabecular bone was prepared by freeze-drying a mixture of β-TCP nanopowder and collagen type I solution. It was reported a fast and efficient chemical cross-linking method using EDC/NHS. The cross-linked composite material had a porosity near 80 %. It was 50 % more stable than the non-cross-linked variant in the presence of bacterial collagenase and released calcium ions in a controlled manner. The cross-linked composite material was tested in a cell line of NCTC fibroblasts and showed a good biocompatibility after 48h of cultivation. An *in vitro* experimental model using rat osteoblasts from a primary cell culture showed that collagen/n-β-TCP cross-linked composite material allowed cell adhesion to its walls and cell proliferation. It was also observed that osteoblasts from the three-dimensional cell-composite construct maintained their phenotype after 21 days in culture. All these results demonstrate that collagen/n-β-TCP cross-linked composite material designed as a three-dimensional porous scaffold is useful in cell culture studies or bioactive implant development for bone tissue engineering.
These observations supply a basis for future studies regarding osteoblast activity and differentiation when cultivated in collagen/n-β-TCP composite material to achieve bioactive

implants for bone repair. The evaluation is associated with the synthesis of extracellular matrix components (collagen type I, osteocalcin, etc). In order to examine collagen/n-β-TCP cross-linked composite material ability to induce bone repair, *in vivo* experimental models are necessary.

8. Acknowledgement

This work was supported by Projects No. 61012 and No. 62059 from Romanian Research Programme 4 - *Partnership in Strategic Domains*.

9. References

Ahlfors J.E.W. & Billiar K.L. (2007). Biomechanical and biochemical characteristics of a human fibroblast-produced and remodeled matrix, *Biomaterials*, Vol. 28, pp. 2183-2191

Anselme K. (2000). Osteoblast adhesion on biomaterials, *Biomaterials*, Vol. 21, pp. 667-681

Arinzeh T.L., Tran T., Mcalary J. & Daculsi G. (2005). A comparative study of biphasic calcium phosphate ceramics for human mesenchymal stem-cell-induced bone formation, *Biomaterials*, Vol. 26, pp. 3631-3638

Ashwin P.T. & McDonnell P.J. (2010). Collagen cross-linkage: a comprehensive review and directions for future research, *British Journal of Ophthalmology*, Vol. 94, pp. 965-970

Badylak S.F. (2002). The extracellular matrix as a scaffold for tissue reconstruction, *Seminars in Cell and Developmental Biology*, Vol. 13, pp. 377-383

Bae H., Shen M., Maurer P., Peppelman W., Beutler W., Linovitz R., Westerlund E., Peppers T., Lieberman I., Kim C. & Girardi F. (2010). Clinical experience using Cortoss for treating vertebral compression fractures with vertebroplasty and kyphoplasty: twenty four-month follow-up, *Spine*, Vol. 35, pp. E1030-E1036

Barnes C.P., Pemble IV C.W., Brand D.D., Simpson D.G. & Bowlin G.L. (2007). Cross-linking electrospun type II collagen tissue engineering scaffolds with carbodiimide in ethanol, *Tissue Engineering Part A*, Vol. 13, pp. 1593-1605

Barrere F., van Blitterswijk C.A. & de Groot K. (2006). Bone regeneration: molecular and cellular interactions with calcium phosphate ceramics, *International Journal of Nanomedicine*, Vol. 1, pp. 317-332

Bi L., Cao Z., Hu Y., Song Y., Yu L., Yang B., Mu J., Huang Z. & Han Y. (2011). Effects of different cross-linking conditions on the properties of genipin-cross-linked chitosan/collagen scaffolds for cartilage tissue engineering, *Journal of Materials Science: Materials in Medicine*, Vol. 22, pp. 51-62

Boland F.D., Espy P.G. & Bowlin G.L. (2004). Tissue engineering scaffolds, In: *Encyclopedia of Biomaterials and Biomedical Engineering*, G.L. Bowlin and G. Wnek (Eds.), pp. 2828-2838, Marcel Dekker Inc., New York, USA

Carter J.D., Swearingen A.B., Chaput C.D. & Rahm M.D. (2009). Clinical and radiographic assessment of transforaminal lumbar interbody fusion using HEALOS collagen-hydroxyapatite sponge with autologous bone marrow aspirate, *The Spine Journal*, Vol. 9, pp. 434-438

Chang M.C. & Douglas W.H. (2007). Cross-linkage of hydroxyapatite/gelatin nanocomposite using imide-based zero-length cross-linker, *Journal of Materials Science: Materials in Medicine*, Vol. 18, pp. 2045-2051

Chen T., Embree H.D., Brown E.M. & Taylor M.M. (2003). Enzyme-catalyzed gel formation of gelatin and chitosan: Potential for *in situ* applications, *Biomaterials*, Vol. 24, pp. 2831-2841

Chen K.Y., Shyu P.C., Chen Y.S. & Yao C.H. (2008). Novel bone substitute composed of oligomeric proanthocyanidins-crosslinked gelatin and tricalcium phosphate, *Macromolecular Bioscience*, Vol. 8, pp. 942-950

Chen Y.S., Kuo S.M. & Yao C.H. (2009). A review for gelatin used for artificial nerve and bone implants, *BioMedical Engineering-Applications Basis Communications*, Vol. 21, pp. 233-238

Chenite A. & Chaput C. (2010). Injectable *in situ* self-forming mineral-polymer hybrid composition and uses thereof, *Patent US 20100021545*

Collighan R., Cortez J. & Griffin M. (2002). The biotechnological applications of transglutaminases, *Minerva Biotechnologies*, Vol. 14, pp. 143-148

Daamen W.F., Nillesen S.T., Wismans R.G., Reinhardt D.P., Hafmans T., Veerkamp J.H. & van Kuppevelt T.H. (2008). A biomaterial composed of collagen and solubilized elastin enhances angiogenesis and elastic fiber formation without calcification, *Tissue Engineering Part A*, Vol. 14, pp. 349-360

De Bie C. (2007). Genzyme: 15 years of cell and gene therapy research, *Regenerative Medicine*, Vol. 2, pp. 95-97

Douglas T., Hemper U, Mietrach C., Viola M., Vigetti D., Heinemann S., Bierbaum S. & Worch H. (2008). Influence of collagen-fibril-based coatings containing decorin and biglycan on osteoblast behavior, *Journal of Biomedical Materials Research*, Vol. 84, pp. 805-816

Evans C.H. (2010). Gene therapy for bone healing, *Expert Reviews in Molecular Medicine*, Vol. 12, pp. e18-e26

Fang B., Wan Y.Z., Tang T.T., Gao C. & Dai K.R. (2009). Proliferation and osteoblastic differentiation of human bone marrow stromal cells on hydroxyapatite/bacterial cellulose nanocomposite scaffolds, *Tissue Engineering Part A*, Vol. 15, pp. 1091-1098

Fantner G.E., Rabinovych O., Schitter G., Thurner P., Kindt J.H., Finch M.M., Weaver J.C., Golde L.S., Morse D.E., Lipman E.A., Rangelow I.W. & Hansma P.K. (2006). Hierarchical interconnections in the nano-composite material bone: fibrillar cross-links resist fracture on several length scales, *Composites Science and Technology*, Vol. 66, pp. 1202-1208

Fujita R., Yokoyama A., Nodosaka Y., Kohgo T. & Kawasaki T. (2003). Ultrastructure of ceramic-bone interface using HA and beta–tricalcium phosphate ceramics and replacement mechanism of beta–tricalcium phosphate in bone, *Tissue & Cell*, Vol. 35, pp. 427–440

Furstenberg C.H., Wiedenhofer B., Putz C., Burckhardt I., Gantz S., Kleinschmidt K. & Schroder K. (2010). Collagen hydroxyapatite (Healos) saturated with gentamicin or levofloxacin. *In vitro* antimicrobial effectiveness - a pilot study, *Orthopade*, Vol. 39, pp. 437-443

Gapski R., Misch C., Stapleton D., Mullins S., Cobb C., Vansanthan A. & Reissner M. (2008). Histological, histomorphometric and radiographic evaluation of a sinus augmentation with a new bone allograft: a clinical case report, *Implant Dentistry*, Vol. 17, pp. 430-438

Garcia Y., Collighan R., Griffin M. & Pandit, A. (2007). Assessment of cell viability in a three-dimensional enzymatically cross-linked collagen scaffold, *Journal of Materials Science: Materials in Medicine*, Vol. 18, pp. 1991-2001

Ge Z., Jin Z. & Cao T. (2008). Manufacture of degradable polymeric scaffolds for bone regeneration, *Biomedical Materials*, Vol. 3, pp. 022001-022011

Gu Q., Zhu H.M. & Zhang X.J. (2002). Apoptosis of rat osteoblasts in process of calcification *in vitro*, *Acta Pharmacologica Sinnica*, Vol. 23, pp. 808-812

Guo X., Zheng Q., Kulbatski I., Yuan Q., Yang S., Shao Z., Wang H., Xiao B., Pan Z. & Tang S. (2006). Bone regeneration with active angiogenesis by basic fibroblast growth factor gene transfected mesenchymal stem cells seeded on porous beta-TCP ceramic scaffolds, *Biomedical Materials*, Vol. 1, pp. 93-99

Haugh M.G., Jaasma M.J. & O'Brien F.J. (2009). The effect of dehydrothermal treatment on the mechanical and structural properties of collagen-GAG scaffolds, *Journal of Biomedical Materials Research*, Vol. 89, pp. 363-369

Heinemann C., Heinemann S., Bernhardt A., Worch H. & Hanke T. (2008). Novel textile chitosan scaffolds promote spreading, proliferation and differentiation of osteoblasts, *Biomacromolecules*, Vol. 9, pp. 2913-2920

Hunt N.C. & Grover L.M. (2010). Cell encapsulation using biopolymer gels for regenerative medicine, *Biotechnological Letters*, Vol. 32, pp. 733-742

Isenburg, J.C., Simionescu, D.T. & Vyavahare, N.R. (2005). Tannic acid treatment enhances biostability and reduces calcification of glutaraldehyde fixed aortic wall, *Biomaterials*, Vol. 26, pp. 1237-1245

Jantova S., Letasiova S., Theiszova M. & Palou M. (2009). Comparison of murine fibroblast cell response to fluor-hydroxyapatite composite, fluorapatite and hydroxyapatite by eluate assay, *Acta Biologica Hungarica*, Vol. 60, pp. 89-107

Jensen T.B., Overgaard S., Lind M., Rahbek O., Bunger C. & Soballe K. (2007). Osteogenic protein-1 increases the fixation of implants grafted with morcellised bone allograft and ProOsteon bone substitute: an experimental study in dogs, *Journal of Bone and Joint Surgery*, Vol. 89, pp. 121-126

John A., Hong L., Ikada Y. & Tabata Y. (2001). A trial to prepare biodegradable collagen-hydroxyapatite composites for bone repair, *Journal of Biomaterials Science, Polymer Edition*, Vol. 12, pp. 689-705

Karageorgiou V. & Kaplan D. (2005). Porosity of 3D biomaterial scaffolds and osteogenesis, *Biomaterials*, Vol. 26, pp. 5474-5491

Kretlow J.D., Klouda L. & Mikos A.G. (2007). Injectable matrices and scaffolds for drug delivery in tissue engineering, *Advanced Drug Delivery Reviews*, Vol. 59, pp. 263-273

Laurencin C., Khan Y. & El-Amin S.F. (2006). Bone graft substitutes, *Expert Review of Medical Devices*, Vol. 3, pp. 49-57

Lee J.E., Park J.C., Hwang Y.S., Kim J.K., Kim J.G. & Suh H. (2001). Characterization of UV-irradiated dense/porous collagen membranes: morphology, enzymatic degradation and mechanical properties, *Yonsei Medical Journal*, Vol. 42, pp. 172-179

LeGeros R.Z., Lin S., Rohanizadeh R., Mijares D. & LeGeros, J.P. (2003). Biphasic calcium phosphate bioceramics: preparation, properties and applications, *Journal of Materials Science: Materials in Medicine*, Vol. 14, pp. 201-209

Leong K.F., Cheah C.M. & Chua C.K. (2003). Solid freeform fabrication of three-dimensional scaffolds for engineering replacement tissues and organs, *Biomaterials*, Vol. 24, pp. 2363-2378

Li S.T. (2000). Biologic biomaterials: tissue-derived biomaterials, In: *The biomedical engineering handbook*, 2nd ed., Vol. I, J.D. Bonzino (Ed.), pp. 42.1-42.20, CRC Press, Springer-Verlag, Boca Raton, Florida, USA

Madhan B., Subramanian V., Rao J.R., Nair B.U. & Ramasami T. (2005). Stabilization of collagen using plant polyphenol: Role of catechin, *International Journal of Biological Macromolecules*, Vol. 37, pp. 47-53

Magit D.P., Maak T., Trioano N., Raphael B., Hamouria Q., Polzhofer G., Drespe I., Albert T.J. & Grauer J.N. (2006). Healos/recombinant human growth and differentiation factor-5 induces posterolateral lumbar fusion in a New Zealand white rabbit model, *Spine*, Vol. 31, pp. 2180-2188

Matsuno T., Nakamura T., Kuremoto K., Notazawa S., Nakahara T., Hashimoto Y., Satoh T. & Shimizu Y. (2006). Development of beta-tricalcium phosphate/collagen sponge composite for bone regeneration, *Dental Materials Journal*, Vol. 25, pp. 138-144

McKay W.F., Peckham S.M. & Badura J.M. (2007). A comprehensive clinical review of recombinant human bone morphogenetic protein-2, *International Orthopaedics*, Vol. 31, pp. 729-734

Miller E.J. & Rhodes R.K. (1982). Preparation and characterization of the different types of collagen, In: *Methods in Enzymology*, L.W. Cunninghaam & D.W. Frederiksen (eds.), pp. 33-64, Academic Press, New York, USA

Moldovan L., Craciunescu O., Oprita E.I., Balan M., Zarnescu O. (2009). Collagen-chondroitin sulfate-hydroxyapatite porous composites: preparation, characterization and *in vitro* biocompatibility testing, *Romanian Biotechnological Letters*, Vol. 14, pp. 4459-4466

Mossman T. (1983). Rapid colorimetric assay for cellular growth and survival: application to proliferation and cytotoxic assays, *Journal of Immunological Methods*, Vol. 65, pp. 55-63

Mulconrey D.S., Bridwell K.H., Flynn J., Cronen G.A. & Rose P.S. (2008). Bone morphogenetic protein (rhBMP-2) as a substitute for iliac crest bone graft in multilevel adult spinal deformity surgery: minimum two-year evaluation of fusion, *Spine*, Vol. 33, pp. 2153-2159

Ohgaki M., Kizuki T., Katsura M. & Yamashita K. (2001). Manipulation of selective cell adhesion and growth by surface charges of electrically polarized hydroxyapatite, *Journal of Biomedical Materials Research*, Vol. 57, pp. 366-373

Olde Damink L.H.H., Dijkstra P.J., van Luyn M.J.A., van Wachem P.B., Nieuwenhuis P. & Feijen J. (1996). Cross-linking of dermal sheep collagen using a water-soluble carbodiimide, *Biomaterials*, Vol. 17, pp. 765-773

Oprita E.I., Moldovan L., Craciunescu O. & Zarnescu O. (2008). *In vitro* behaviour of osteoblast cells seeded into a COL/beta-TCP composite scaffold, *Central European Journal of Biology*, Vol. 3, pp. 31-37

Patterson T.E., Kumagai K., Griffith L. & Muschler G.F. (2008). Cellular strategies for enhancement of fracture repair, *The Journal of Bone and Joint Surgery*, Vol. 90, pp. 111-119

Pieper J.S., Oosterhof A., Dijkstra P.J., Veerkamp J.H. & van Kuppevelt T.H. (1999). Preparation and characterization of porous cross-linked collagenous matrices containing bioavailable chondroitin sulphate, *Biomaterials*, Vol. 20, pp. 847-858

Pieper J.S., van der Kraan P.M., Hafmans T., Kamp J., Buma P., van Susante J.L.C., van den Berg W.B., Veerkamp J.H. & van Kuppevelt T.H. (2002). Crosslinked type II collagen matrices: preparation, characterization and potential for cartilage engineering, *Biomaterials*, Vol. 23, pp. 3183-3192

Popescu R.G., Morega A., Iordachescu D. & Demetrescu I. (2009). Ternary biopolymeric films based on collagen gel, polyethylene glycol and hydroxyapatite, *Materiale Plastice*, Vol. 46, pp. 140-143

Powell H.M. & Boyce S.T. (2006). EDC cross-linking improves skin substitute strength and stability, *Biomaterials*, Vol. 27, pp. 5821-5827

Rodrigues C.V.M., Serricella P., Linhares A.B.R., Guerdes R.M., Borojevic R., Rossi M.A., Duarte M.E.L. & Farina M. (2003). Characterization of a bovine collagen-hydroxyapatite composite scaffold for bone tissue engineering, *Biomaterials*, Vol. 24, pp. 4987-4997

Rude R.K., Singer F.R. & Gruber H.E. (2009). Skeletal and hormonal effects of magnesium deficiency, *Journal of the American College of Nutrition*, Vol. 28, pp. 131-141

Scabbia, A. & Trombelli, L. (2004). A comparative study on the use of a HA/collagen/chondroitin sulphate biomaterial (Biostite) and a bovine-derived HA xenograft (Bio-Oss) in the treatment of deep intra-osseous defects, *Journal of Clinical Periodontology*, Vol. 31, pp. 348-355

Schoen F.J. & Levy R.J. (2005). Calcification of tissue heart valve substitutes: progress toward understanding and prevention, *The Annals of Thoracic Surgery*, Vol. 79, pp. 1072-1080

Schoof H., Apel J., Heschel I. & Rau G. (2001). Control of pore structure and size in freeze-dried collagen sponges, *Journal of Biomedical Materials Research*, Vol. 58, pp. 352-357

Silva G.A., Marques A.P., Gomes M.E., Coutinho O.P. & Reis R.L. (2005). Biodegradable Systems in *Tissue Engineering and Regenerative Medicine*, CRC Press, Reis, R.L., San Roman, J. (eds), pp. 339-361, Boca Raton, Florida, USA

Takahashi Y., Yamamoto M. & Tabata Y. (2005). Enhanced osteoinduction by controlled release of bone morphogenetic protein-2 from biodegradable sponge composed of gelatin and beta-tricalcium phosphate, *Biomaterials*, Vol. 26, pp. 4856–4865

Tampieri A., Sandri M., Landi E., Pressato D., Francioli S., Quarto R. & Martin I. (2008). Design of graded biomimetic osteochondral composite scaffolds, *Biomaterials*, Vol. 29, pp. 3539-3546

Tardei C., Moldovan L. & Craciunescu O. (2010). Hybrid bioresorbable composites with high bioactivity properties, *Romanian Journal of Materials*, Vol. 40, pp. 41-49

Thermo Fisher Scientific, www.piercenet.com/products/browse.cfm?fldID=CE4D6C5C-5946-4814-9904-C46E01232683

Turek A. & Cwalina B. (2010). Some irradiation-influenced features of pericardial tissues engineered for biomaterials in *New Developments in Biomedical Engineering*, InTech, pp. 543-563, Vienna, Austria

Turkovski I.I., Paramonov B.A., Antonov S.F., Kozlov D.N., Klimova O.A. & Pomorski K.P. (2008). Comparative evaluation of the depth of collagen and hyaluronic acid

hydrolysis *in vitro* by collagenase and hyaluronidase preparations, *Bulletin of Experimental Biology and Medicine*, Vol. 146, pp. 81-82

Vaccaro A.R. (2002). The Role of the Osteoconductive Scaffold in Synthetic Bone Graft, *Orthopedics*, Vol. 25, pp. 571–578

Wahl D.A. & Czernuszka J.T. (2006). Collagen-hydroxyapatite composites for hard tissue repair, *European Cells & Materials*, Vol. 11, pp. 43-56

Wang X., Grogan S.P., Rieser F., Winkelmann V., Maquet V., Berge M. L. & Mainil-Varlet P. (2004). Tissue engineering of biphasic cartilage constructs using various biodegradable scaffolds: an *in vitro* study, *Biomaterials*, Vol. 25, pp. 3681-3688

Webster T.J., Siegel R.W. & Bizios R. (1999). Osteoblast adhesion on nanophase ceramics, *Biomaterials*, Vol. 20, pp. 1221-1227

Xie J., Baumann M.J. & McCabe L.R. (2004). Osteoblasts respond to hydroxyapatite surfaces with immediate changes in gene expression, *Journal of Biomedical Materials Research*, Vol. 71, pp. 108-117

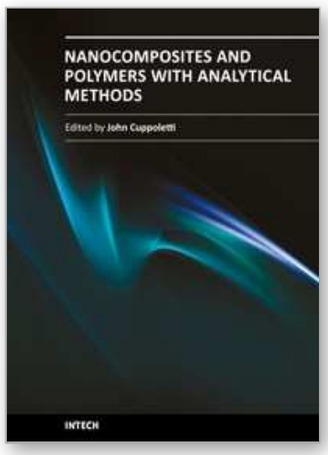
Xu H.H. & Simon C.G. (2004). Self-hardening calcium phosphate cement-mesh composite: reinforcement, macropores and cell response, *Journal of Biomedical Materials Research*, Vol. 69, pp. 267-278

Yamauchi K., Goda T., Takeuchi N., Einaga H. & Tanabe T. (2004). Preparation of collagen/calcium phosphate multilayer sheet using enzymatic mineralization, *Biomaterials*, Vol. 25, pp. 5481-5489

Zarnescu O., Craciunescu O. & Moldovan L. (2010). Collagen-chondroitin sulphate-hydroxyapatite porous composites: a histochemical and electron microscopy approach, *Microscopy & Microanalysis*, Vol. 16, pp. 137-142

Zhang S.M., Cui F.Z., Liao S.S., Zhu Y. & Han L. (2003). Synthesis and biocompatibility of porous nano-HA/collagen/alginate composite, *Journal of Materials Science: Materials in Medicine*, Vol. 14, pp. 641-645

Zou C., Weng W., Deng X., Cheng K., Liu X., Du P., Shen G. & Han G. (2005). Preparation and characterization of porous beta-tricalcium phosphate/collagen composites with an integrated structure, *Biomaterials*, Vol. 26, pp. 5276-5284



Nanocomposites and Polymers with Analytical Methods
Edited by Dr. John Cuppoletti

ISBN 978-953-307-352-1
Hard cover, 404 pages
Publisher InTech
Published online 09, August, 2011
Published in print edition August, 2011

This book contains 16 chapters. In the first part, there are 8 chapters describing new materials and analytic methods. These materials include chapters on gold nanoparticles and Sol-Gel metal oxides, nanocomposites with carbon nanotubes, methods of evaluation by depth sensing, and other methods. The second part contains 3 chapters featuring new materials with unique properties including optical non-linearities, new materials based on pulp fibers, and the properties of nano-filled polymers. The last part contains 5 chapters with applications of new materials for medical devices, anodes for lithium batteries, electroceramics, phase change materials and matrix active nanoparticles.

INTECH
open science | open minds

InTech Europe
University Campus STeP Ri
Slavka Krautzeka 83/A
51000 Rijeka, Croatia
Phone: +385 (51) 770 447
Fax: +385 (51) 686 166
www.intechopen.com

InTech China
Unit 405, Office Block, Hotel Equatorial Shanghai
No.65, Yan An Road (West), Shanghai, 200040, China
中国上海市延安西路65号上海国际贵都大饭店办公楼405单元
Phone: +86-21-62489820
Fax: +86-21-62489821

IntechOpen