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Innovative methodology for developing a bone grafting composite biomaterial starting from the seashell of *Rapana thomasiana*

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ABSTRACT

Bone grafts are used in a wide array of clinical settings to augment bone repair and regeneration. This article reports a new method for the elaboration of a hybrid biomaterial in the form of sponge based on collagen gel, $CaCO_3$ from recycled *Rapana thomasiana* seashell, and $Na_2HPO_4 \cdot 2H_2O$. Practically, collagen acts as a matrix through which calcium and phosphate ions are diffusing during in situ hydroxyapatite synthesis. The organic–inorganic interactions among biomaterial components have been studied by infrared spectroscopy, and the surface morphology was investigated by scanning electron microscopy technique. Moreover, the developed biomaterials were studied for in vitro biocompatibility with MG63 human osteoblasts. The results obtained demonstrated that the developed hybrid material does not exhibit a significant cytotoxicity and supports cell proliferation. Consequently, it holds great promise for applications in bone tissue engineering.

1. Introduction

The field of bone tissue engineering (BTE) [1,2] was initiated three decades ago to induce new functional bone regeneration [3]. Engineered bone grafts have been shown to have capacity for osteogenesis, osteoconduction, osteoinduction, and osteointegration [4]. In this context, a wide range of materials have been developed and identified as biomaterials for BTE. These materials can be classified into three categories: natural [5] or synthetic polymers [6], ceramics such as calcium phosphates and bioactive glasses or glass ceramics, and composites [7]. Despite the huge research efforts worldwide in this field, it is difficult to obtain a biomaterial able to satisfy most of the requirements need for effective bone regeneration. A better choice for BTE is to use a composite system [8,9], which confers not only mechanical properties but also osteoconduction and osteointegration.

Given its chemical similarity to the main structural proteins found in the extracellular matrix of several hard tissues, type I collagen has been tested in bone regenerative studies because of its ability to replicate the three-dimensional bone structure [10]. It facilitates a natural biological potential by creating a favorable environment for cellular adhesion, cellular migration, and cell growth [11]. Chemical cross-linking of collagen can modify its mechanical and physical properties, and also composite system of collagen and ceramic improves its mechanical properties and enhances bone conductivity [12,13].

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On the other hand, hydroxyapatite (HA, $Ca_{10}(PO_4)_6(OH)_2$), one of few materials classified as bioactive, osteoconductive, and biocompatible with hard tissues, is widely used in dental implants. Different chemical procedures for HA synthesis use precursors such as $CaCO_3$ [14], $Ca(NO_3)_2$, or $Ca(OH)_2$ as the source of calcium ions and $(NH_4)_2HPO_4$, H_3PO_4, or Na_2HPO_4 as the source of phosphate ions. However, commercially available HA is relatively expensive because of the use of high purity reagents. The HA derived from natural materials provided by marine organisms such as corals [15], seashells [16], eggshells [17], snails [18] has an important advantage like better tissue response owing to its chemical and structural similarity with the inorganic constituents of biological hard tissues and strong bonds with the hard tissues.

It is known that a part of marine species, like corals, is limited and protected, whereas, on the contrary, there are a variety of available and abundant materials that have not been used for different applications till now. The worldwide availability, the low production cost of *Rapana thomasiana* (RT) shell [19], its biological-natural origin, and mechanical properties similar to human bone are important characteristics that classify this shell as an ideal candidate for preparing calcium phosphate materials used in biomedicine. RT, a predatory gastropod considered an environmental threat [20] contains 95–99% by weight of $CaCO_3$, which allows to be used in various applications. Several studies have been previously reported for synthesis of collagen/HA composites for BTE.

The present article reports a newly developed method for obtaining a highly porous collagen/HA composite for bone tissue regeneration. This new biomaterial produced in the form of sponge is obtained from collagen gel (CG) combined with $CaCO_3$ originated from recycled RT seashells and $Na_2HPO_4 \cdot 2H_2O$. In this case, the collagen acts as a matrix for diffusion of calcium and phosphate ions to perform in situ HA synthesis. To confirm the conversion of the recycled seashell of RT into HA, a comparative CG/commercial HA sponge was prepared. The differences between biomaterial characteristics and their potential application have been examined by spectroscopic study and surface morphology investigations. Also, in vitro studies have been performed with MG63 human osteoblasts to establish the cell behavior in terms of cell survival and proliferation status.

2. Experimental part

2.1. Materials

Stranded individuals of RT were collected from the beach of Cape Midia (N44.34507°, E28.69201°), Black Sea, Romania. Type I CG of bovine origin with an initial concentration of 2.11% and approximate pH of 2.3 was provided by Collagen Department of National Research & Development Institute for Textiles and Leather, Bucharest, Romania. Glutaraldehyde (GA; Merck, Germany) as a cross-linking agent and commercial Fluka HA were used. $Na_2HPO_4 \cdot 2H_2O$ (Sigma-Aldrich Co., Germany) as a source of phosphate ions and commercial Fluka HA (Sigma-Aldrich Co., Germany) were used. Analytical grade NaOH 1 M was used for the pH adjustment.

2.2. RT shell preparation

The RT shell was first washed in distilled water and dried in oven at a temperature of 105 °C. The fragments obtained were milled using a pestle and a mortar followed by ball milling with zirconium oxide balls (Retsch GmbH, Haan, Germany) in a horizontal ball mill (9VS; Pascall Engineering Co. Ltd, Suffolk, UK) for 6 h. The shell powder ($CaCO_3$) was left for 3 h in acetone solution to remove the organic matter and afterward rinsed with distilled water, oven dried at 105 °C for 1 h, and sieved in the Analysette 3-pro shaker (Fritsch GmbH, Germany) to segregate the particle to specific sizes less than 45 μm.

2.3. Preparation of marine organism and natural polymer based biomaterials

The RT powder (< 45 μm) was added to the CG and mixed under stirring for 5 h at 25 °C, with a required volume of an aqueous solution of $Na_2HPO_4 \cdot 2H_2O$ to set the stoichiometric molar ratio of Ca/P equal to 1.667 appropriate for HA formation. The main conversion is running according to the following reaction:

$$10\,CaCO_3 + 6\,Na_2HPO_4 + 2\,H_2O \rightarrow Ca_{10}(PO_4)_6(OH)_2 + 6\,Na_2CO_3 + 4\,H_2CO_3$$

The increase in temperature and the low pH of the CG promoted the binding between collagen fibers and HA, which was obtained in situ through the precipitation of $CaCO_3$ microparticles in $Na_2HPO_4 \cdot 2H_2O$ solution. One percent of a GA solution was added to facilitate cross-linking and was mixed for another 60 min at 25 °C. The obtained homogenous mixture with an adjusted pH between 7.4 and 7.6 was transferred to a well culture plate and prefrozen at −15 °C for 24 h and freeze-dried at 0.12 mbar starting from −40 to +30 °C during 40 h to obtain the porous HA/composite biomaterial. The resultant sponge (sample A) had a form of a disc with a diameter of 15 mm and thickness of 10 mm (Fig. 1).

A similar type of sponge (sample B) based on commercial synthetic HA and CG was prepared by using the same procedure. For reference, a sponge based on CG without any ceramic material was prepared (sample C).

The gravimetric ratio between dry substance of CG and HA, or between CG and RT with $Na_2HPO_4 \cdot 2H_2O$ was 1:5, and 0.5% between GA and CG with HA, or between GA and CG with RT and $Na_2HPO_4 \cdot 2H_2O$.

2.4. Characterization techniques

Both raw materials and newly prepared biomaterials were characterized for their composition and morphology. Fourier transform infrared (FT-IR) spectroscopy was performed using a Perkin Elmer Spectrum 100 FT-IR spectrophotometer in the attenuated total reflection mode. The spectra were collected in the range 4000–600 cm^{-1} at a