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Effects of crystalline phase on the biological properties of collagen–hydroxyapatite composites

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ABSTRACT

The objective of this study was to investigate the effects of spatial structure and crystalline phase on the biological performance of collagen–hydroxyapatite (Col–HA) composite prepared by biomineralization crystallization. Two types of Col–HA composites were prepared using mineralization crystallization (MC composites) and pre-crystallization (PC composites), respectively. Structural characteristics were analyzed by scanning electron microscopy and transmission electron microscopy. Surface elemental compositions were measured by electron spectroscopy for chemical analysis (ESCA). These composites were used in in vivo repair of bone defects. The effects of the crystalline phase on the biological performance of Col–HA composites were investigated using radionuclide bone scan, histopathology and morphological observation. It was observed that in MC composites, HA was located on the surface of the collagen fibers and aggregated into crystal balls, whereas HA in PC composites was scattered among the collagen fibers. ESCA showed that phosphorus and calcium were 8.99% and 17.56% on MC composite surface, compared with 4.39% and 5.86% on the PC composite surface. In vivo bone defect repair experiments revealed that radionuclide uptake was significantly higher in the area implanted with the PC composite than in the contralateral area implanted with the MC composite. Throughout the whole repair process, the PC composite proved to be superior to the MC composite with regard to capillary-forming capacity and the amount of newly formed bone tissue. So it could be concluded that HA placement on collagen fibers affected the biological performance of Col–HA composites. Pre-crystallization made HA scattered among collagen fibers, creating a better structure for bone defect repair in comparison with MC Col–HA composites.

1. Introduction

Great efforts have been made to develop biomaterials capable of repairing bone defects resulting from various causes. Bone tissue is a particularly complex composite because it contains multiple levels of organic and inorganic nanophases. At the lowest level of this hierarchy is a three-dimensional composite made up of collagen triple helices, and the hydroxyapatite crystals grow in some different form: the majority of the mineral lies in such a way that its *c*-axes are oriented along the long axes of the fibrils (called intra-fibrillar mineral) [1–3], some lies between the fibrils, the *c*-axes of which are perpendicular to the collagen molecular axis (called inter-fibrillar mineral or extra-fibrillar mineral) [4,5].

Many researchers have attempted to construct composites of hydroxyapatite and collagen (Col–HA) for bone defect repair. Self-assembly and biomineralization have been used recently in biology for the fabrication of bone-repairing materials [6–8]. The core issue in the preparation of Col–HA composite lies in its structure, in particular the relative phase relationship between the two materials. But there are also a few hints of a fresh theme emerging from this work: the design of artificial nanostructures which can interact with and replace natural and biological materials [9]. It is still difficult to design supramolecular structures, particularly starting with designed molecules and forming bone-like objects between nanoscopic and macroscopic dimensions, which affects the composite's properties, especially its mechanical properties [10].

Bone repair materials prepared by biomineralization differ from natural bones in the crystalline phase [11]. During mineralization of natural bones, organic matter like collagen is first secreted in areas where osteogenesis occurs. No local formation of hydroxyapatite crystals happens in the early stage of osteogenesis. Consequently, collagens form a cross-linking network in an interference-free environment, while association between collagen fibers is enhanced by proteoglycans. This allows collagen fibers in a relatively stable state, i.e., bone matrix gel, before deposition of hydroxyapatite. Meanwhile, a large number of negatively charged proteoglycans

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exist among and on the collagen fibers, providing a basis for chemical and electrical bonding. These proteoglycans serve as crystal nuclei during hydroxyapatite deposition [12,13], inducing HA crystallization along the length of collagen fibers [14]. However, in the preparation of Col–HA composites by in vitro biomineralization and self-assembly, no pre-cross-linking of collagen fibers is introduced to form bone matrix gel. To maintain the stability of collagen sol during reaction, cross-linking or bonding should be avoided between collagen fibers. When hydroxyapatite crystals precipitate in the collagen swelling solution, hydroxyapatite crystals grow using polar groups near the ends of collagen fibers as crystal nuclei. Since crystal nuclei localize at the ends of collagen fibers, association between collagen fibers is impaired; meanwhile, aggregation and elongation of the crystals themselves are also affected [15]. Precursor HA crystals deposit on the polar groups of Col fibers in the composites, which may block cell adhesion sites (such as RGD binding sites), thus changing the biological properties of Col–HA composites [16].

Based on the above analysis, this work presents a method of producing Col–HA composites making use of pre-crystallization. Prior to precipitation of collagen fibers, nano-HA crystals with independent crystal nuclei were prepared. These crystals were absorbed among collagen fibers by electric bonding rather than aggregated at the ends of collagen fibers. This process exposes collagen fibers, which have higher biocompatibility, on the composite exterior. Col–HA composites formed by pre-crystallization and mineralization crystallization were also characterized and then their biological performance in bone defect repair was compared both in vivo and in vitro to observe the crystalline phase on the biological properties of Col–HA composites.

2. Materials and methods

2.1. Materials

Type I collagen (Col) was obtained from Department of Biomedical Engineering, Peking Union Medical College and Chinese Academy of Medical Science. H_3PO_4 and $Ca(OH)_2$ used in this study were of analytical pure grade, obtained from Tianjin Chemical Reagent Company (Tianjin, China).

2.2. Preparation of Col–HA composites

2.2.1. Preparation of Col–HA composites by mineralization crystallization

Five grams of type I Col was added to a solution of H_3PO_4 (59.7 mM). The mixture was agitated to ensure uniform distribution. Meanwhile, 199.1 mmol $Ca(OH)_2$ was dispersed in 2 dm^3 of distilled water and thoroughly mixed. Next, the $Ca(OH)_2$ solution was added to the solution of swollen collagen at a ratio of 1:4 (Col:HA). The resultant mixture was allowed to react for 12 h. The precipitate was then freeze-dried using a lyophilizer under vacuum. Later, a solution of 0.25% glutaraldehyde was added to the precipitate. The cross-linking reaction proceeded for 2 h at room temperature. Subsequently, the cross-linked composites were washed with distilled water ten times and then relyophilized to yield a product referred to hereafter in this study as MC composite.

2.2.2. Preparation of Col–HA composites by pre-crystallization

First, 199.1 mmol $Ca(OH)_2$ was added to 2 dm^3 of distilled water and mixed to ensure even distribution. Next, a solution of 59.7 mM H_3PO_4 was added to the $Ca(OH)_2$ solution. The mixture was ultrasonicated and allowed to react for 2 h at room temperature, yielding a white solid precipitate. The precipitate was harvested by centrifugation at 800 rpm for 10 min and then transferred to a lyophilizer to freeze dry under vacuum for 24 h, producing white solid HA. The HA was added to a 5% Col swollen solution (5.0 g) at a ratio of 1:4 (Col:HA). The mixture was agitated and allowed to react for 12 h. After that, the precipitate was placed in a lyophilizer to freeze dry under vacuum. Subsequently, a solution of 0.25% glutaraldehyde was added to facilitate the cross-linking reaction for 2 h at room temperature. The cross-linked material was washed with double distilled water 10 times and then relyophilized to yield a composite referred to hereafter in this study as PC composite.

2.3. Structural characterization

2.3.1. Scanning electron microscopy (SEM)

MC and PC composites were sectioned, covered with gold and then examined with a Hitachi S-3500N scanning electron microscope to determine their surface features.

2.3.2. Transmission electron microscopy (TEM)

To investigate the mineralization properties of the materials, samples were stained, embedded in epoxy resin and then sectioned. A JEM 2010 TEM was used with the positive stain uranyl acetate, which preferentially stains acidic groups. TEM showed higher electron density at the periphery of the fiber, revealing donut-shaped patterns indicating that only the outer portion of the fiber was stained.

2.4. Physicochemical characterization

2.4.1. Electron spectroscopy for chemical analysis (ESCA)

The surface elements of MC and PC composites were characterized by electron spectroscopy for chemical analysis using a magnesium anode (Mg K = 1253.6 eV) with a survey scan range of 0–1000 eV. All electron binding energies were referenced to the C1s hydrocarbon peak at 284.6 eV.

2.4.2. Water absorption of the Col–HA composites

After weighing (W_d), three samples from each of the MC and PC composites were immersed in PBS solution (pH 7.2–7.4) at room temperature. The samples were extracted after 2 h and placed on filters to remove excess water. Subsequently, the scaffolds were weighed (W_h), and the amount of water absorbed (W_a) by each sample was calculated according to the equation:

$$W_a = \frac{W_h - W_d}{W_d} \times 100\% \quad (1)$$

2.5. Evaluation of in vitro biological performance

2.5.1. Separation and seeding of bone marrow stromal stem cells

According to the methods previously described [17,18], bone marrow stromal stem cells (BMSC) were isolated from the long bone of 6-week-old Wistar rats. Isolated cells were cultured in an incubator at 37 °C in an atmosphere of 5% CO_2. The culture medium was replaced after 24 h of culture and then changed every 2 or 3 days. Four to five days later, when cells reached 80–90% confluence, cells were digested with 0.25% trypsin and passaged.

Samples from the MC and PC composites (each with a diameter of 16 mm and a thickness of ~3 mm) were each placed into a 24-well culture plate. Third or fourth generation BMSC in the exponential growth phase were digested with 0.25% trypsin and centrifuged. The pelleted cells were resuspended in DMEM culture medium for cell counting and trypan blue cell viability assays. The cell suspension was then adjusted to a concentration of 1.0×10^6 cells ml^{-1}. Subsequently, 1 ml of BMSC suspension was added to each well containing a composite or pure collagen. Cells