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Bio-mimetic mineralization potential of collagen hydrolysate obtained from chromium tanned leather waste

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ABSTRACT

Hydroxyapatite (HA) ceramics serve as an alternative to autogenous-free bone grafting by virtue of their excellent biocompatibility. However, chemically synthesized HA lacks the strong load-bearing capacity as required by bone. The bio-mimetic growth of HA crystals on collagen surface provides a feasible solution for synthesizing bone substitutes with the desired properties. This study deals with the utilization of the collagen hydrolysate recovered from leather waste as a substrate for promoting HA crystal growth. Bio-mimetic growth of HA was induced by subjecting the hydrolysate to various mineralization conditions. Parameters that would have a direct effect on crystal growth were varied to determine the optimal conditions necessary. Maximum mineralization was achieved with a combination of 10 mM of $CaCl_2$, 5 mM of Na_2HPO_4, 100 mM of NaCl and 0.575% glutaraldehyde at a pH of 7.4. The metal–protein interactions leading to formation of HA were identified through Fourier-transform infrared (FTIR) spectroscopy and x-ray diffraction (XRD) studies. The crystal dimensions were determined to be in the nanoscale range by atomic force microscopy (AFM) and scanning electron microscopy (SEM). The size and crystallinity of bio-mimetically grown HA indicate that hydrolysate from leather waste can be used as an ideal alternative substrate for bone growth.

1. Introduction

Bone is a biological nano-composite comprising of fibrous type I collagen as a scaffold, upon which hydroxyapatite crystals [$(Ca)_{10}(PO_4)_6(OH)_2$] are deposited. Bone growth is highly organized and is characterized by a high degree of control over crystal location and orientation that eventually contributes to the strength and rigidity of the resultant product [1]. The inorganic components of this bio-composite confer the osteo-conductivity and bending abilities while the substratum polymer offers the design flexibility and stability to achieve high porosity and surface area [2]. Collagen, the organic polymer of bone is not only indispensible in supporting its architecture but also acts as the base for mineralization nucleation for HA growth [3].

Bone grafts are used to correct bone defects including bone restoration or stabilization of a broken joint [4]. Autografts and allografts are commonly used techniques for bone transplantations but are associated with their own unique advantages and disadvantages. Autograft harvesting can result in donor site morbidity and allograft tissue is limited by donor availability along with an increased risk of immunogenic response, disease transmission and infection. Such limitations have motivated extensive research in the area aimed at commercial product development of suitable synthetic bone graft substitutes [5–7].

Since HA is a major component of bone and HA crystals synthesized in vitro are biocompatible with living systems, numerous techniques have been used either to synthesize HA or to extract it from natural sources, including corals and bovine bones [8–12]. Despite its excellent biocompatibility, pure HA's brittle nature and low fracture toughness have limited its biomedical use to only non-load bearing applications [13,14]. Some success has been achieved in strengthening the final product by synthesizing crystalline HA doped with iron, chromium or nickel. However, a comparatively in-expensive and promising technique for producing HA is the bio-mimetic approach, which mimics the in vivo synthesis of bone through in vitro mineralization [15,16].

The process of biomineralization, through which bone is formed, involves a series of molecular events in which the surface interaction between the inorganic phase and specific amino acid sequences of the organic phase regulates the morphology of the final composite product with high precision. Functional groups such as $-COO^-$, $-OH$, $-SH$, $-CHO$ and $-NH_2$, from the organic substratum function as anchors for inorganic crystals to nucleate and consequently, develop into a biomineral composite [17,18]. The first step in crystal formation is believed to involve the adsorption of ions onto a protein substrate leading to the nucleation and formation of particles in nanometer range. This nucleation occurs at precise sites and the substrate seems to orient the crystal growth pattern. The ability to trigger nucleation and initiate biomineralization could possibly be restricted to small conserved regions in the large polypeptide chain [19].

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Bio-mimetic preparation of HA crystals on pure collagen necessitates the isolation and purification of collagen from an animal source. Chrome shavings, an underutilized chromium-containing leather industry waste, mostly disposed off as landfill can provide itself as an alternative cheap source of collagen [20,21]. The protein can be extracted in the form of a hydrolysate after the removal of chromium and can subsequently be used for several industrial applications [22]. The purpose of this study is to evaluate the potential of the extracted collagen hydrolysate as a bio-mimetic substrate for HA growth. This would ultimately lead to enhanced utilization of this inexpensive collagen rich waste and reduction in environmental pollution. The basic hypothesis behind the investigation focuses on the fact that utilization of collagen hydrolysate as the organic matrix instead of the intact protein might significantly facilitate the biomineralization process. Hydrolysis is expected to result in a diverse array of collagen peptides with consequently higher amount of unmasked or exposed small nucleation sequences with side chain carboxylic groups thus resulting in increased biomineralization efficiency.

The chrome shavings were degraded by a patented protocol using a chromium tolerant proteolytic bacteria and the chromium was removed to obtain the protein-rich hydrolysate [22]. The bio-mimetic growth of HA was induced by subjecting the hydrolysate to mineralization process. Several physiological parameters influencing crystal growth were varied to determine the optimum conditions required for maximum biomineralization.

2. Materials and methods

Chrome shavings were obtained from pilot tannery, Central Leather Research Institute, Chennai, India. Sodium chloride (ACS grade), calcium chloride (ACS grade), disodium hydrogen phosphate heptahydrate (ACS grade) and KBr (FTIR grade) were bought from Sigma Aldrich, St. Louis, USA. Tris salt, formalin and 23% glutaraldehyde (EM grade) were bought from Himedia, India.

2.1. Preparation of the hydrolysate

The collagen hydrolysate was extracted from the tanned leather waste using a patented protocol involving hydrolysis of the chrome shavings by the bacterium *Alcaligenes odorans* over a 5 day period [23]. The hydrolysate was collected and the chromium was removed through alkaline precipitation. The process was repeated multiple times to ensure complete removal of chromium. The metal-free hydrolysate was dialyzed in 0.05 M phosphate buffer at pH 7 at 4 °C for 6 h with frequent buffer changes to remove neutral salts. The salt-free hydrolysate was used as the organic component for the biomineralization.

2.2. Degree of hydrolysis and total free carboxyl groups

Formol titration was performed to determine the number of free carboxyl groups in the hydrolysate according to the protocol given by Denis et al. [24]. 5 ml of the hydrolysate sample (protein content, 'P' in g) was added to 15 ml of deionized water in a 100 ml conical flask. The pH of the sample and the formalin solution was adjusted to 7.4 ± 0.1 with 0.05 M NaOH just before use. 5 ml of formalin was added to 15 ml of the test solution and titrated to pH 9.2 ± 0.1 with 0.01 M NaOH. The volume (V in ml) of NaOH required was noted.

The total free carboxyl groups, N (mmol g^{-1}) was calculated according to the formula,

$$N = 0.01 \times V/P.$$

The degree of hydrolysis (DH) of the protein was calculated from the following formula given by Angeles Navarrete del Toro et al. [25]:

$$DH = V \times N_B \times 1.04 \times (1/P) \times (1/h_{tot}) \times 100$$

where N_B denotes the molarity of the base, 1.04 is the calibration factor at 30 °C taken from standard and h_{tot} is 11.1 (in meq g^{-1} for collagen, standard content of peptide bonds).

2.3. Bio-mimetic growth of HA crystals

The HA crystal growth was carried out according to the protocol of Gafni et al. [26]. A known amount of the hydrolysate was suspended in 100 mM of tris buffer (pH 7.4) containing disodium hydrogen phosphate and sodium chloride and incubated at 28 °C. After 24 h, calcium chloride solution buffered at pH 7.4 was added to the suspension and the total volume was adjusted to 5 ml with 100 mM of tris buffer. The mixtures were incubated for 21 days at 28 °C to allow for biomineralization.

Among the various factors that affect mineralization in vitro, five parameters that have been recognized to influence crystal growth, viz., calcium and phosphate concentrations, protein content in the hydrolysate, pH of the medium, cross-linker concentration and ionic strength of the reaction medium [27–29] were chosen for biomineralization studies. To determine the optimum conditions for the crystal growth, one component was varied while keeping the others constant and each optimized parameter in a particular reaction system was applied to the next set of experiments. For samples 16–20, glutaraldehyde (stock: 23%) has been added in various volumes to create cross-linker concentrations ranging from 0.0028 to 1.15% in the reaction mixture. From sample 21 onwards, 125 μl of 23% glutaraldehyde was added to maintain a concentration of 0.575%. The various experimental reaction systems with the altered parameters in each case are listed in Table 1. After the incubation period, the crystals were washed twice with deionized water and dried. The dried solid was crushed to a fine powder, desiccated and analyzed by FTIR spectroscopy and XRD. The size of the pure HA crystals was determined through SEM and AFM analyses and the composition was determined through SEM-EDX.

Table 1
Reaction systems for optimization of biomineralization conditions. Reaction systems numbered from 1–30 were used for creating variation in parameters. Triplicate experimental set-ups were used for each reaction system. The Ca/P ratio in solution is given in the last column.

System	$CaCl_2$ mM	Na_2HPO_4 mM	NaCl mM	Hydrolysate mg/ml	Glutaraldehyde (%)	pH	Ca/P ratio in solution
1	10	0	50	0.5	0	7.4	–
2	10	5	50	0.5	0	7.4	2.59
3	10	10	50	0.5	0	7.4	1.29
4	10	50	50	0.5	0	7.4	0.259
5	10	100	50	0.5	0	7.4	.012
6	0	5	50	0.5	0	7.4	–
7	5	5	50	0.5	0	7.4	1.29
8	10	5	50	0.5	0	7.4	2.59
9	50	5	50	0.5	0	7.4	12.98
10	100	5	50	0.5	0	7.4	25.9
11	10	5	50	0	0	7.4	2.59
12	10	5	50	0.25	0	7.4	2.59
13	10	5	50	0.5	0	7.4	2.59
14	10	5	50	0.75	0	7.4	2.59
15	10	5	50	1	0	7.4	2.59
16	10	5	50	0.5	0.0028	7.4	2.59
17	10	5	50	0.5	0.0575	7.4	2.59
18	10	5	50	0.5	0.2875	7.4	2.59
19	10	5	50	0.5	0.575	7.4	2.59
20	10	5	50	0.5	1.15	7.4	2.59
21	10	5	50	0.5	0.575	2	2.59
22	10	5	50	0.5	0.575	4	2.59
23	10	5	50	0.5	0.575	6	2.59
24	10	5	50	0.5	0.575	8	2.59
25	10	5	50	0.5	0.575	10	2.59
26	10	5	0	0.5	0.575	7.4	2.59
27	10	5	25	0.5	0.575	7.4	2.59
28	10	5	50	0.5	0.575	7.4	2.59
29	10	5	100	0.5	0.575	7.4	2.59
30	10	5	200	0.5	0.575	7.4	2.59