Biomaterials 32 (2011) 1291–1300

Contents lists available at ScienceDirect

Biomaterials

journal homepage: www.elsevier.com/locate/biomaterials

Hierarchical and non-hierarchical mineralisation of collagen

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ARTICLE INFO

Article history:
Received 23 September 2010
Accepted 10 October 2010
Available online 30 October 2010

Keywords:
Biomineralisation
Non-protein molecules
Hierarchical intrafibrillar mineralisation
Crossbanding
Non-hierarchical intrafibrillar mineralisation
Extrafibrillar mineralisation

ABSTRACT

Biomineralisation of collagen involves functional motifs incorporated in extracellular matrix protein molecules to accomplish the objectives of stabilising amorphous calcium phosphate into nanoprecursors and directing the nucleation and growth of apatite within collagen fibrils. Here we report the use of small inorganic polyphosphate molecules to template hierarchical intrafibrillar apatite assembly in reconstituted collagen in the presence of polyacrylic acid to sequester calcium and phosphate into transient amorphous nanophases. The use of polyphosphate without a sequestration analogue resulted only in randomly-oriented extrafibrillar precipitations along the fibrillar surface. Conversely, the use of polyacrylic acid without a templating analogue resulted only in non-hierarchical intrafibrillar mineralisation with continuous apatite strands instead of discrete crystallites. The ability of using simple non-protein molecules to recapitulate different levels of structural hierarchy in mineralised collagen signifies the ultimate simplicity in Nature's biomineralisation design principles and challenges the need for using more complex recombinant matrix proteins in bioengineering applications.

1. Introduction

Nature utilises hierarchical structures in intriguing ways to create multifunctional materials. Type I collagen represents an example of Nature's bottom-up approach [1] in self-assembling molecules at the nanoscopical scale to produce highly-ordered macromolecular structures at the microscopical and macroscopical dimensions. For organic-inorganic nanocomposites such as bone, this self-assembly process defines the framework and spatial constraints for nucleation and propagation of the reinforcing mineral phase [2]. During initial biomineralisation, no seed crystallites are available within the organic scaffold to serve as nidi for heterogeneous nucleation [3]. Type I collagen by itself is insufficient to induce nucleation of carbonated apatite from transient amorphous calcium phosphate (ACP) phases [4]. Hence, alternative matrix-mediated mineralisation mechanisms that involve kinetically-driven steps are required to overcome the thermodynamically-unfavourable energy barrier in homogeneous nucleation [3,5]. Noncollagenous matrix macromolecules contribute to these kinetically-driven steps in biomineralisation by stabilising ACPs in the form of nanoprecursors (sequestration motif), and for initiating nucleation and hierarchical assembly of apatite within the collagen scaffold (templating motif) [6–8].

Because of the mineralisation potential of natural non-collagenous matrix proteins, recombinant versions of these molecules or their critical domains are employed in bioengineering [9–11]. Owing to their limited availability and high cost of production, others resort to using polyanionic electrolytes to mimic the functional domains of these proteins. Polycarboxylic acids such as polyacrylic acid or polyaspartic acid are often employed as biomimetic analogues to simulate the sequestration functional motif of matrix proteins [12–15]. When stabilised as ACP nanoparticles, the latter demonstrate mouldable, fluid-like characteristics which enable them to infiltrate the internal water compartments of a collagen fibril [16]. However, the use of only a polycarboxylic acid sequestration analogue results in intrafibrillar mineralisation that lacks the hierarchy of apatite assembly (i.e. overlapping platelets that produce cross-banding patterns) in naturally mineralised collagen [14,15].

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doi:10.1016/j.biomaterials.2010.10.018

Inorganic polyphosphates play important roles in biomineralisation [17,18]. Sodium trimetaphosphate (STMP) is a chemical phosphorylation agent for food proteins [19], carboxymethylcelluose [20] and type I collagen [21]. However, when this simple cyclic polyphosphate molecule is used as a templating analogue without a sequestration agent to stabilise ACP in the form of nanoprecursors, either large extrafibrillar mineral spheres are produced in the vicinity of the collagen fibrils [22] or extrafibrillar apatite crystals are precipitated in a non-oriented manner along the fibrillar surface [23]. Extrafibrillar mineralisation with randomised large crystals deposited over an organic matrix does not reproduce the mechanical properties exhibited by natural mineralised tissues at the nanoscale level [24].

Recent studies on biomimetic collagen mineralisation appear to indicate that both the sequestration and templating functional motifs of matrix proteins involved in biomineralisation have to be reproduced before hierarchical intrafibrillar mineralisation of a collagen matrix can be realised [25,26]. Here we report a polyphosphate-based biomimetic collagen mineralisation strategy using a low molecular weight polyacrylic acid to replicate the sequestration functional motif of the N-terminal fragment of dentine matrix protein-1 (DMP-1) [6], and a small inorganic polyphosphate to replicate the templating functional motif of the C-terminal fragment of DMP-1. Two polyphosphates were employed: sodium trimetaphosphate (STMP) and sodium tripolyphosphate (TPP), a linear tripolyphosphate to demonstrate the temporal and spatial events that lead to the appearance of cross-banding in unstained mineralised collagen. Sodium trimetaphosphate exhibits strong affinity to type I collagen via irreversible binding under alkaline conditions [27]. As TPP may be used directly in lieu of STMP for chemical phosphorylation of food proteins [28], this linear polyphosphate was also investigated to determine its potential for replacing recombinant matrix proteins for hierarchical intrafibrillar apatite assembly within a collagen matrix.

2. Materials and methods

2.1. Self-assembly of collagen

A single-layer of type I collagen fibrils was reconstituted over formvar-and-carbon-coated 400-mesh Ni TEM grids (Electron Microscopy Sciences, Hatfield, PA, USA) by neutralising a 0.15 mg/ml collagen stock solution with ammonia vapour for 4 h [26]. For preparation of collagen stock solution, lyophilised type I collagen powder derived from calf skin (Sigma–Aldrich) was dissolved in 0.1 M acetic acid (pH 3.0) containing phenol red at 4 °C overnight. The neutralised collagen solution was left to gel by incubation at 37 °C for 3–5 days. To stabilise the structure of the reconstituted collagen fibrils, collagen cross-linking was further performed with 0.3 M 1-ethyl-3-(3-dimethylaminopropyl)-carbodiimide (EDC)/0.06 M N-hydroxysuccinimide (NHS) (Thermo Scientific Pierce, Rockford IL, USA) for 4 h. Thereafter, the collagen-coated grids were dipped in and out of deionised water and air-dried [26].

2.2. Amorphous calcium phosphate

Composite disks were prepared from a light-polymerisable hydrophilic resin blend containing set white Portland cement powder and silanised silica [26]. Set

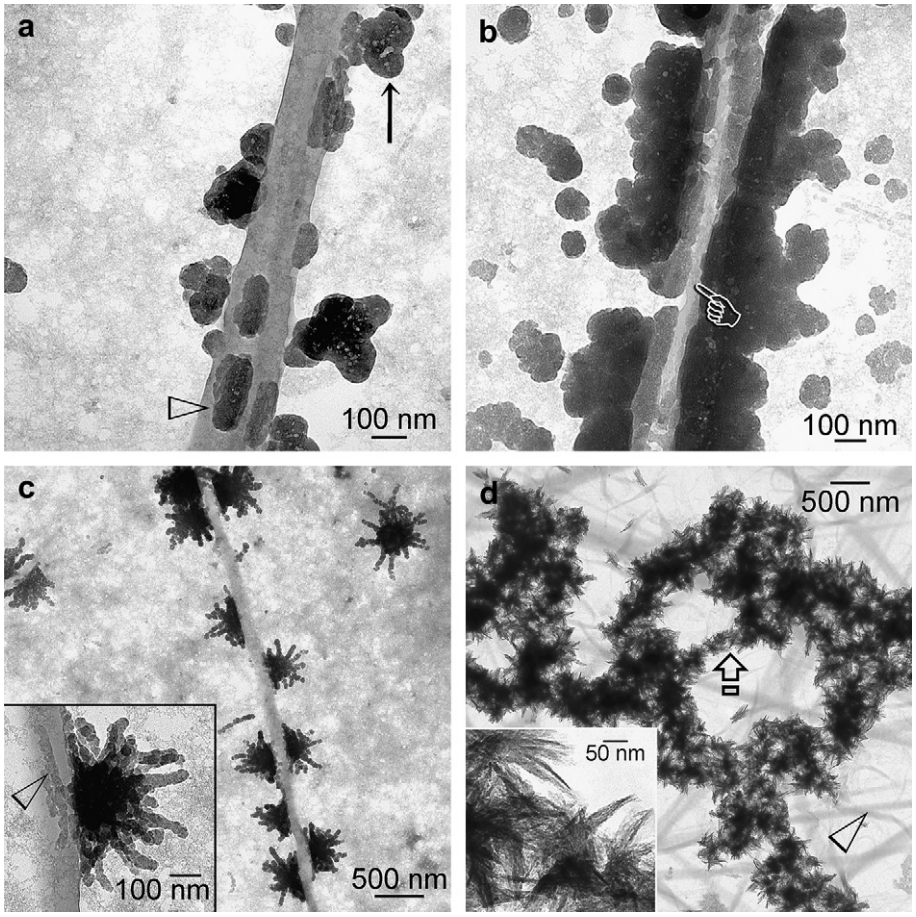

Fig. 1. Unstained TEM showing only extrafibrillar mineralisation in the templating analogue control (no sequestration analogue in mineralisation medium). (a) After 24 h, partially-coalesced ACPs (arrow) were attached to the fibril surface (open arrowhead) but were too large to penetrate the collagen fibril. (b) An unmineralised collagen fibril (pointer) with heavier ACP surface aggregation. (c) Some ACP phases were transformed into immature finger-like immature apatite (inset). (d) After 72 h, spherules of needle-shaped apatite crystallites (inset; >100 nm long) coated the surface (open arrow) of unmineralised collagen fibrils (open arrowhead).