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Understanding the influence of phosphorylation and polysialylation of gelatin on mineralization and osteogenic differentiation

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ABSTRACT

Post-translational modifications such as phosphorylation and sialylation impart crucial functions such as mineral deposition and osteogenic differentiation to non-collagenous bone matrix proteins. In this work, the influence of phosphorylation and polysialylation of gelatin on mineralization in simulated body fluid (SBF) and on osteogenic differentiation of mesenchymal stem cells (MSC) was studied. It was observed that increase in phosphorylation could be directly correlated with the mineralization ability of phosphorylated gelatin in SBF. The total calcium and phosphate deposited increased with increase in degree of phosphorylation and was >3 fold higher on the highest degree of phosphorylation. Whereas, polysialylation did not have any significant influence on mineral deposition in SBF. On the other hand, when MSCs were cultured on polysialylated surfaces they showed relatively higher cell elongation with 1.5 fold higher cell aspect ratio, higher alkaline phosphatase activity and 3 fold higher mineral deposition when compared to control and phosphorylated gelatin surfaces. In conclusion, phosphorylation and polysialylation of gelatin show a significant influence on mineralization and osteogenic differentiation respectively which can be advantageously used for bone tissue engineering.

1. Introduction

Development of biomaterials for improved bone repair/regeneration is one of the major requisites in the area of bone tissue engineering. In this pursuit, biomimetics has been one of the commonly followed strategies. In such strategies a better understanding of the native tissue can enable improved approaches. While bulk of the bone matrix consists of collagen type I and hydroxyapatite, a milieu of non-collagenous matrix proteins are also secreted by the cells during bone development and homeostasis. These non-collagenous proteins include molecules like bone sialoprotein (BSP), osteopontin (OPN), osteocalcin (OCN) and dentin sialophosphoprotein (DSPP) [1]. Unlike collagen I which is largely a structural protein of the bone, these proteins are biological modulators of cell fate and mineral deposition in bone [2]. While there is little sequence similarity amongst these non-collagenous bone matrix proteins there are certain conserved features that are present in most of them. These features include, (i) presence of Arg–Gly–Asp (RGD) motif; (ii) presence of regions rich in acidic amino acids like Asp and Glu; and (iii) similar post-translational modifications including high degree of phosphorylation and glycosylation [3]. The RGD motif in these proteins mediates cell–matrix interactions through integrin receptors and initiates the downstream signaling pathways. Whereas, the stretches of acidic amino acids and acidic post-translational modifications (phosphorylation, glycosylation with acidic sugars) play a crucial role in sequestering calcium ions, and thus, these features act as crucial factors in nucleation and growth of hydroxyapatite mineral of bone [3]. In fact, it has been demonstrated previously that dephosphorylation of some phosphoproteins abrogates the mineral deposition abilities of these proteins [4]. Amongst the various glycosylations, sialic acid is a prominent sugar that is present in these modifications and can play a role in both mineral deposition and cellular response [3]. Another striking feature common to these proteins includes — absence of stable 3D structure; as these proteins lack secondary structures and are largely composed of unstructured regions or random coils [5]. More importantly, it has also been indicated that the highly disordered structure of these proteins is crucial for the regulated growth of mineral phase during ossification [6]. Therefore, we hypothesized, that combining functional features of non-collagenous bone matrix proteins (namely RGD motif, acidic amino acids, phosphorylation and glycosylation) in materials can mimic the non-collagenous bone matrix protein function and can hence facilitate matrix mineralization and osteogenesis for bone regenerative engineering. The inability to isolate purified non-collagenous proteins and the lack of appropriate post-translational modification in recombinant versions further gives an impetus to mimic these molecules synthetically for therapeutic purposes [7]. It is interesting to note that even though the functional role of sialic acid in non-collagenous proteins is multifaceted including calcium binding and enabling better osteogenic differentiation [8,9], there are no reports which talk about the use of sialic acid based polymers for bone tissue engineering.

Previous reports have demonstrated that chemical moieties in the gelatin hydrogel microenvironment are crucial for nucleation and

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growth of minerals. In this direction, the bio-mineralization potential of gelatin hydrogels has previously been modulated by including both organic and inorganic additives such as poly-aspartate polypeptides and magnesium respectively to promote biomineralization [10]. Free glutamic acid is another additive that has also been used in gelatin hydrogel environment. A recent report suggested that addition of glutamic acid led to plate shaped and mushroom-like biomimetic brushite crystals which bears a strong resemblance to the brushite kidney stones [11]. In addition to peptides and amino acids, recent studies also support the point that novel polysaccharides can be used as backbones for deposition of bone like calcium phosphates. Two polysaccharides which have been used for this purpose include iota carrageenan and starch, the key precursor of HAp *i.e.* brushite of varying morphology could be synthesized using these two carbohydrate moieties [12,13].

Moreover, a recent study evaluated the relative mineral induction capacity of phosvitin (an egg derived phospho-glyco-protein — bone non-collagenous protein mimic), chondroitin sulfate (a highly sulfated and carboxylated polysaccharide) and poly-L-lysine using a layer-by-layer system. The study clearly demonstrated that phosvitin performed significantly better in terms of hydroxyapatite deposition indicating the potential of acidic, phosphorylated glycoproteins to facilitate mineralization [14]. Other methods that have been employed for inducing acidic polarity on surfaces include Ar/O_2 and NH_3/C_2H_4 plasma pre-treatment and enzymatic hydrolysis [15,16].

In this work, we used gelatin type B as the base material due to its flexible and unstructured nature (high content of random coils) and abundant presence of Asp, Glu (acidic amino acids) and RGD motifs. Phosphorylated and polysialylated forms of gelatin were chemically synthesized and characterized as a mimic of the various bone/dentin phosphoproteins and sialoproteins respectively. The modified gelatin molecules were then evaluated for their mineral inducing ability and for inducing osteogenic differentiation *in vitro*.

2. Materials and methods

2.1. Synthesis and characterization of phosphorylated gelatin

Phosphorylated gelatin was synthesized using a modification of the protocol previously reported for casein as per the reaction mechanism shown in Fig. 1A [17]. Briefly, varying amounts of 20% $POCl_3$ in CCl_4 was added dropwise to a solution of gelatin type B (Sigma-Aldrich, St louis, MO, USA) (5 mg/ml in PBS) maintained at low temperature using an ice bath. During this reaction the pH was continuously monitored using a pH indicator and maintained between pH 6 to 8 by the addition of 5 N NaOH. After complete addition of $POCl_3$ the reaction was allowed to go to completion (till no change in pH). The aqueous fraction of reaction mixture containing the modified gelatin was then separated and dialysed (dialysis membrane cut off 13 kDa — Himedia Labs, India) against 0.1 M KCl followed by deionized water for 24 h and 48 h respectively. Finally, the solution was freeze dried to obtain dried phosphorylated gelatin. $POCl_3$ was used at three ratios — 6.25, 12.5 and 25 mg per mg of gelatin to obtain gelatin with varying degrees of phosphorylation, which were referred to as PG1, PG2 and PG3 respectively.

The degree of phosphorylation of PG1, PG2 and PG3 was calculated by quantifying the total inorganic phosphate content after sequential acidic (5 N HCl) and alkaline (5 N NaOH) hydrolysis at 85 °C for 2 h each. The difference between inorganic phosphate content after and before hydrolysis was used to calculate the degree of phosphorylation. Total inorganic phosphate was quantified using malachite green assay as reported previously [18].

2.2. Synthesis and characterization of polysialylated gelatin

Polysialic acid (PSA) with terminal aldehyde group was obtained as a gift sample from Serum Institute of India, Pune, India. Polysialylation of gelatin was performed using reductive amination reaction (Fig. 1B) in the presence of sodium cyanoborohydride as has been reported previously for other proteins [19]. The reaction was performed at final concentration of 5 mg/ml of gelatin, 5 or 10 mg/ml PSA and 5 mg/ml sodium cyanoborohydride in PBS at room temperature for 3 days. After the completion of reaction the protein was precipitated using excess ammonium sulfate and washed thrice with saturated ammonium sulfate solution. The precipitated product was dissolved in water and dialysed (dialysis membrane cut off 13 kDa — Himedia Labs, India) against deionized water for 48 h. The amount of conjugated PSA was quantified in the final product using a thiobarbituric acid based spectrophotometric assay as reported previously [20]. PSA was used at two ratios — 1 and 2 mg per mg of gelatin to obtain gelatin with two degrees of polysialylation, which were referred to as SG1 and SG2 respectively.

The conjugation of PSA to gelatin was confirmed using polyacrylamide gel electrophoresis. The gels were stained with toluidine blue O followed by coomasie blue staining after complete destaining of toluidine blue O. Trinitrobenzenesulfonic acid assay was used to determine total amine content in modified and control gelatin molecules as reported previously [21].

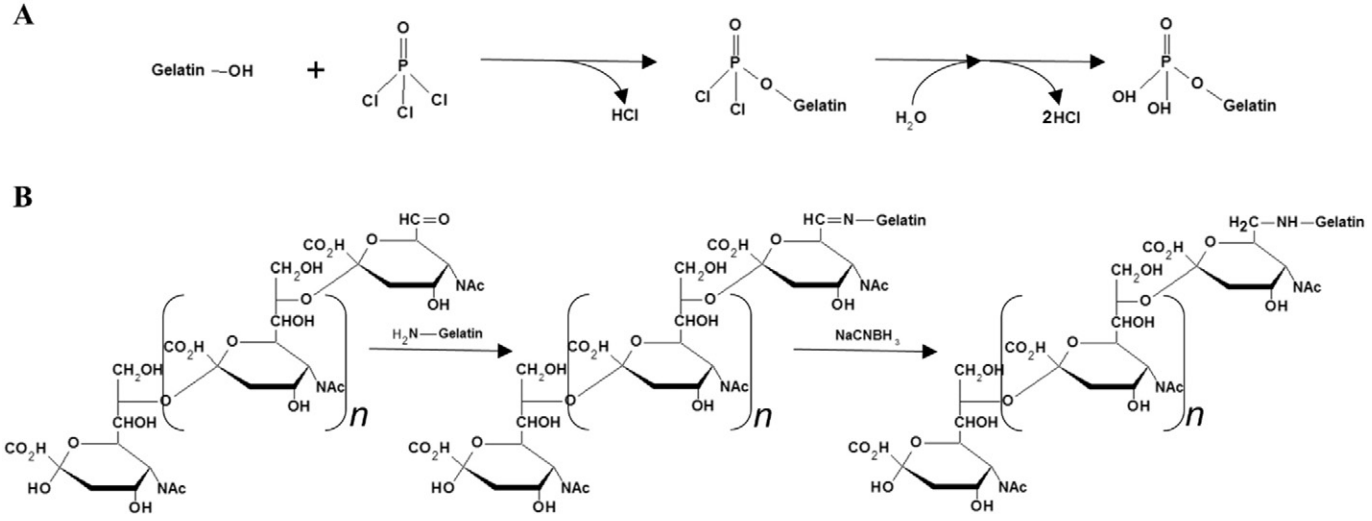

Fig. 1. Schematic depicting mechanism of reaction for phosphorylation and polysialylation of gelatin. (A) Phosphorous oxychloride reacts with hydroxyl groups of gelatin to form a phosphate ester of gelatin; and (B) polysialic acid aldehyde reacts with amine groups of gelatin to form a Schiff's base which is reduced in the presence of sodium cyanoborohydride to form polysialylated gelatin.