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Synthesis of Bone-like Nanocomposites Using Multiphosphorylated Peptides

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Abstract

There is a great need for novel materials for mineralized tissue repair and regeneration. Two examples of such tissue, bone and dentin, are highly organized hierarchical nanocomposites in which mineral and organic phases interface at the molecular level. In contrast, current graft materials are either ceramic powders or physical blends of mineral and organic phases with mechanical properties far inferior to those of their target tissues. The objective of this study was to synthesize composite nanofibrils with highly integrated organic/inorganic phases inspired by the mineralized collagen fibrils of bone and dentin. Utilizing our understanding of bone and dentin biomineralization, we have first designed bioinspired peptides containing 3 Ser-Ser-Asp repeat motifs based on the highly phosphorylated protein, dentin phosphophoryn (DPP) found in dentin and alveolar bone. We demonstrate that up to 80% of serines in the peptide can be phosphorylated by casein kinases. We further tested the ability of these peptides to induce biomimetic calcium phosphate mineralization of collagen fibrils. Our mineralization studies have revealed that in the presence of these phosphorylated peptides, mineralized collagen fibrils structurally similar to the mineralized collagen fibrils of bone and dentin were formed. Our results demonstrate that using phosphorylated DPP-inspired peptides, we can successfully synthesize biomimetic composite nanofibrils with integrated organic and inorganic phases. These results provide the first step in the development of biomimetic nanostructured materials for mineralized tissue repair and regeneration using phosphopeptides.

Introduction

Collagenous mineralized tissues such as bone and dentin are unique, hierarchical nanocomposites [1]. They comprise ~70% w/w carbonated apatite, 20–25% w/w organic matrix, and 5–10% w/w water. While collagen fibrils are the major organic component of these tissues, other non-collagenous proteins (NCPs) and glycoproteins accounting for less

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than 10% of total organic content, play very important roles in the regulation of mineralization [2, 3], cell signaling [4–7] and the mechanical performance of the tissue [8–10].

The basic building blocks of bone and dentin are mineralized collagen fibrils, comprising the first level of structural hierarchy of these tissues [1]. Mineralized collagen fibrils contain stacks of plate-shaped crystallites of carbonated apatite. These crystallites are only 3–5 nm thick, 50 to 100 nm in two other dimensions, and are aligned with their crystallographic c-axes along the fibril axis. It has been shown that the mineral component in these fibrils has almost 2 times greater strain than geologic or synthetic apatite while their organic component is much stiffer than nonmineralized collagen [11, 12]. These differences are due in part to the nanoscopic dimensions of the crystallites; their plate-like shape leads to insensitivity of these nanocrystals to flaws [13] and extremely high surface-to-bulk ratio translates to high strain values [14]. Furthermore, the interlaced structure of the mineralized collagen fibrils creates intimate interactions of the mineral crystallites with collagen triple helices leading to the unique mineral-organic interface at the molecular level [11, 15, 16]. This complex organization and the unique mechanical properties of the mineralized tissues are in a stark contrast to the contemporary composite bone-grafting materials, which are simple physical blends of organic and mineral phases [17, 18]. It is therefore highly desirable to design novel nanomaterials modeled after the mineralized tissues. Bioinspired approaches, namely applying our knowledge of the basic mechanisms of collagen mineralization to materials design, can provide new strategies to such nanomaterials.

It is widely accepted that NCPs play a critical role in collagen mineralization [2, 3]. A unique characteristic of NCPs is the disproportionately large percentage of acidic amino acids such as Asp, Glu and Ser^(P) [2, 19]. For example, the major noncollagenous protein in dentin and craniofacial bones, phosphophoryn (DPP) [20–23], comprises primarily Ser-Ser-Asp repeat motifs with more than 90% of serines phosphorylated [24]. Although protein phosphorylation is one of the most common post-translational modifications, the vast majority of phosphorylated proteins contain only a handful of phosphorylation sites adjacent to kinase-specific recognition motifs [25, 26]. Kinase recognition sites are characterized by the presence of clusters of acidic residues in the positions between –2 and +5 relative to the target for CK2 and between clusters of acidic amino acids ending in the position –3 or Ser^(P) in the position –3. In contrast, DPP has a limited number of kinase recognition sites and its precise mechanism of phosphorylation is still poorly understood [27]. It has been proposed that casein kinases (CK1 and CK2) phosphorylate DPP intracellularly in the endoplasmic reticulum [28]. According to the hypothesis by Veis et al., it occurs via a chain or sequential reaction in which once the first serine adjacent to the CK recognition site is phosphorylated, it becomes part of the successive CK recognition site, leading to subsequent phosphorylation of new serines [29]. Since DPP sequence primarily consists of DSS repeats, the sequential model can explain the high level of DPP phosphorylation *in vivo*. Casein kinases transfer γ -phosphate of ATP (or GTP) to the hydroxyl group of serine or threonine or to the phenolic hydroxyl on tyrosine residues in proteins.

In our recent *in vitro* mineralization study, the presence of two phosphorylated NCPs (DPP and dentin matrix protein 1(DMP1)) leads to formation of highly organized mineralized

collagen fibrils, similar to those found in bone and dentin [30]. In contrast, in the presence of nonphosphorylated DPP and DMP1, no organized mineralization of collagen fibrils was observed. These experiments clearly demonstrate that phosphorylation is essential for proper bone mineralization and this has inspired us to use phosphorylated peptides, modeling the NCPs for synthesis of bioinspired nanostructured materials based on mineralized collagen fibrils.

A number of peptides mimicking NCPs have been synthesized [31–33]. In these studies, however, phosphorylated amino acids were introduced during the synthesis phase. This approach for synthesis of bioinspired peptides has several limitations [33]. Importantly, introducing any single phosphorylated amino-acids during peptide synthesis leads to a significant decrease in the yield, thereby limiting the total number of phosphorylated amino acids that can be added to a peptide. To overcome this problem, we sought to develop an approach of post-synthesis phosphorylation by adapting biological phosphorylation strategies for highly phosphorylated NCPs.

The goal of this study was twofold: 1) to obtain new insight into how high a degree of phosphorylation of DPP is achieved and to test the hypothesis of sequential or chain phosphorylation in serine high-density sequences proposed by Veis et al. [25]. These studies are anticipated to inspire new approaches toward synthesis of phosphopeptides with multiple phosphate groups; and 2) to test the ability of these highly phosphorylated bioinspired peptides to induce the organized mineralization of collagen fibrils -- a key step toward the development of bioinspired nanostructured hierarchical composites for mineralized tissue repair.

Materials and Methods

Peptide Synthesis

The designed peptide: RRRDEDESSDSSDSSDDEG-amide (R-SSD3) (the letters correspond to standard one letter codes for amino acids), molecular weight 2142.91 Da, at 92.26% purity) was synthesized by 21st Century Biochemicals (Marlboro MA). Another peptide containing proline residue between the CK2 recognition site and SDD repeat motif, called RSSD3P, was synthesized to test the hypothesis that the disruption of the recognition site will reduce the extent of phosphorylation. The peptide sequences are presented in Figure 1. The purity of the peptides was determined using mass spectrometry and HPLC by the company prior to shipment. 3 N-terminal arginines (R) were added to create a cluster of positive charges at the N-terminus (Figure 1); this addition counterbalances the high negative charge of the rest of the peptide, thus making the purification and handling of the peptide easier.

Peptide Phosphorylation

Casein Kinase 1 active (CK1) (0.2mg/ml), Casein Kinase 2 active (CK2), (0.1mg/ml) Mg/ATP cocktail, and P81 phosphocellulose paper were obtained from Upstate Cell Signaling Solutions (Lake Placid, NY). Assay Dilution Buffer I and CK2 substrate peptide (1mM), and active CK1 (0.25mg/ml) were obtained from (Millipore, Billerica, MA).

Adenosine 5' – triphosphate, Ultratide /Isobluestabilized (ATP- $\gamma^{32}\text{P}$) (with a specific activity of 800 Ci/mmol) was purchased from MP Biomedical (Solon, OH). 16.5% Tris-Tricine gel, 10 $\times$ Tris-Tricine/SDS buffer, and Tricine sample buffer were purchased from Bio Rad Laboratories (Hercules, CA). ACS-grade phosphoric acid (85%), ScintiVerse Scintanalyzer, and acetone were purchased from Fisher Scientific (Pittsburgh, PA). Micro 1000 MWCO Tube-O-DIALYZER was purchased from G-Biosciences (St. Louis, MO).

R-SSD3 was phosphorylated *in vitro* by CK1, CK2, or a mixture of both kinases. ATP was used as a source of phosphate and [$\gamma^{32}\text{P}$] ATP was used for the scintillation counter experiments. The standard assay mixture consisted of 50 mM Tris-HCl, pH 7.4, 5 mM MgCl_2 , 1 mM EGTA, and 10 mM β -glycerophosphate. The assay was carried out in a total volume of 40 μl , containing substrate peptide (10 μl of 1mg/ml solution), 10 μl of Assay Dilution Buffer I (ADBI with a composition of 20mM MOPS, pH 7.2, 25mM β -glycerol phosphate, 5mM EGTA, 1mM sodium orthovanadate, 1mM dithiothreitol), and 10 μl of active casein kinase (1 or 2). The samples were vortexed and kept for 20 minutes at room temperature before being transferred into a water bath of 30°C. The reaction was started by adding 10 μl of ATP (cold Mg-ATP and 1 μCi of [$\gamma^{32}\text{P}$] ATP) and the reaction mixture was incubated at 30°C in a shaker for 1 hour. To assess the kinetics of the reactions, phosphorylation was allowed to proceed for 20 minutes, 1 hour, 4 hours, and 24 hours before the reaction was stopped. The reaction was terminated by adding 40 μl of loading Tricine sample buffer (200 mM Tris-HCl, pH 6.8, 2% SDS, 40% glycerol, 0.04% Coomassie G-250 dye). The samples were then boiled in a water bath for five minutes and subjected to electrophoresis on 16.5% Tris-Tricine gel run using Tris-Tricine/SDS buffer (containing 100 mM Tris, 100 mM Tricine, 0.1% SDS, pH 8.3) at 100V for 1.5 hours.

For mass spectrometry (MS) analysis, the reaction was performed as above; radiolabeled ATP, however, was not used and the reaction mixture was transferred into a Tube-O-DIALYZER with a MWCO of 1000 and dialyzed overnight.

Characterization of Phosphorylation

Recombinant casein kinase 1 (CK1; Cat #14-112) and casein kinase 2 (CK2; Cat #14-197) were obtained from Millipore Inc. ATP and [$\gamma^{32}\text{P}$] ATP were obtained from Sigma-Aldrich (St. Louis, MO), and Perkin Elmer (Shelton, CT), respectively.

In Vitro Phosphorylation Assay

In vitro phosphorylation assay was performed as described elsewhere [27, 34]. 25 μl reactions containing 1 μg of R-SSD3 peptide, in the presence and absence of 300 ng of either CK1 or CK2 or both containing 10 μCi ^{32}P γ [ATP]. The reactions were performed in triplicate at 30°C for 1 hour, 4 hours, and 24 hours using one batch of radiolabeled ATP and enzymes from a single lot.

The reaction products were purified using SDS-PAGE, visualized by autoradiography, and band intensities were quantified using a Kodak 1D 3.6 imaging system. After autoradiography, the gel was overlaid on the autoradiogram, the individual protein bands were excised, and ^{32}P incorporation was determined using a liquid scintillation counter

(Beckman LS6500 system). To calculate the number of moles of phosphates transferred to 1 μg of peptide (0.466 pM) in the kinase reaction, the mean counts per minute obtained in the kinase reactions from triplicates (minus blank) were divided by the specific activity of the $^{32}\text{P}\gamma[\text{ATP}]$ in the kinase assay.

$$\text{Number of pM of P32 transferred/pM of peptide} = \frac{\text{Mean CPM of phosphorylated peptides}}{\text{Specific activity of } ^{32}\text{P added in the kinase reaction}} \times \frac{1.00}{0.466}$$

MALDI-TOF MS and LC-ESI MS Analyses

For MALDI-TOF-MS analysis, the peptides and CHCA matrix (α -cyano-4-hydroxycinnamic acid, 10 $\mu\text{g}/\mu\text{L}$, in 50% acetonitrile containing 0.1% trifluoroacetic acid) solutions were premixed in a small Eppendorf tube, spun down, and remixed three times, then applied directly to the sample plate. Once applied to the target, the sample was allowed to air dry. The samples were analyzed using an Applied Biosystems (Foster City, CA) Voyager DE Pro or 4700 Proteomics Analyzer TOF/TOF instrument. The samples were also analyzed by LC-MS/MS on a ThermoFischer (Waltham, MA) Surveyor LC System coupled to a ThermoFischer LCQ Deca XP Plus mass spectrometer equipped with a nanospray ion source and also a ThermoFischer LTQ-XL instrument. The LC system contained a sample trap followed by a C18 column (BioBasic C18 PicoFrit column, 10 cm $\times$ 75 μM , New Objective, Inc., Woburn, MA). The elution gradient for chromatographic separation of the peptides was obtained with two solvents: solvent A (100% water with 0.1% formic acid) and solvent B (100% acetonitrile with 0.1% formic acid). During the elution process, solvent B was increased from 5% to 50% over 25 minutes, and then further increased from 50% to 98% over 5 minutes. The elution continued at 98% solvent B for 5 minutes, reduced from 98% to 5% solvent B over 5 minutes, then finished at 5% solvent B for 10 minutes. The flow rate of the LC system was 160 nL/minute.

Upon elution off the C18 column, the analyte was ionized by nano-capillary ESI. Ions were produced in positive mode (ESI voltage at 1.6 kV; heated capillary at 180°C). A full MS scan was done (m/z 300–2000 AMU), followed by three MS/MS scans on the three most intense peaks with dynamic exclusion. The MS/MS spectra were analyzed with BioWorks 3.2 Browser with the Sequest search engine. The search used the UniProtKB/ Swiss-Prot human protein knowledgebase, which was first indexed for a trypsin digest, no missed cleavages, and three modifications (oxidation of methionine, carboxyamidomethylation and methylation of cysteine). The search results that were accepted contained cross correlation scores (Xcorr) for singly charged peptides >1.5 , doubly charged peptides >2.0 , and triply charged peptides >3.0 .

Mineralization Experiments

Mineralization experiments were carried out using a modification of an “on grid” mineralization setup developed in our lab and published elsewhere [30, 35–37]. The tail tendons of 4–8 weeks old rats were collected and washed in protease inhibitor buffer [38] rinsed in deionized water (DDW), homogenized and solubilized in 2mM HCl (pH 2.8) at 4°C with stirring. The solution was centrifuged at 25000 $\times$ g to remove aggregates. Collagen

was then purified by salt precipitation in 5N KCl followed by acid dissolution as reported elsewhere [39–41]. The purity of the isolated collagen was tested by SDS-PAGE electrophoresis.

The collagen fibrils were reconstituted on carbon-coated Cu grids, mesh #400 (EMS, Hatfield, PA) from an acidic rat tail collagen solution by increasing pH and temperature. The pH of ice-cold collagen solution was adjusted to 7.5 and the grids were placed on top of the 50 μ L droplets of the solution in a humidity chamber placed on ice. The chamber was transferred to a 37°C incubator and left there for 90 to 120 min in order to induce assembly of the collagen fibrils. The grids were briefly rinsed in DDW and air dried. Mineralization experiments were conducted in the mineralization solutions prepared by mixing concentrated 10 $\times$ PBS (mono- and disodium phosphate totaling 40 mM and 1.5 M NaCl), peptide stock solutions, deionized distilled water (DDW) to obtain final concentrations of 4 mM PBS, 1.67 mM Ca and 15 μ g/ml peptide. Prior to the experiments, the pH of concentrated 10 $\times$ PBS was adjusted such that upon mixing, the pH of the final mineralization solution was 7.8. The TEM grids coated with reconstituted collagen fibrils were floated on 50 μ L droplets of the mineralization solution at 100% humidity for 16–24 h at 37°C. After the mineralization reactions the grids were briefly rinsed with DDW, blotted against filter paper, and air dried.

Transmission Electron Microscopy (TEM)

TEM and selected area electron diffraction (SAED) studies were conducted using JEOL 1210 operated at 100 kV. The micrographs were recorded using an AMT CCD camera (AMT, Danvers, MA). An aluminum-coated TEM grid (EMS Hatfield, PA) was used as a standard for the calibration of SAED patterns for d-spacing calculations. The micrographs were analyzed using an ImageJ 1.38 software package (Bethesda, MD).

Electron Tomography

Tomography tilt series of mineralized samples were acquired at nominal magnification 26,000, using Tecnai 12 transmission electron microscope (FEI, Hillsboro, OR) equipped with a LaB6 filament at 120 kV at the beam density of $\sim 300 \text{ e}^-/\text{\AA}$. The micrographs were recorded automatically using bottom-mounted Gatan 2000 CCD camera (2048 $\times$ 2048 pix, with the physical pixel size of 14 μ m. The micrographs were taken in a tilt range from -60° to 60° with of 1° increment from -45° to 45° and 0.5° increment from -60° to -45° and from 45° to 60° . Because of the strong contrast of mineralized samples, the images were aligned using fiducial-less procedure in IMOD reconstruction package (University of Colorado, Boulder, CO) [42]. Three-dimensional density maps were reconstructed from the tilt series images using Chimera software (University of California, San Francisco, CA [43]).

Statistical Analysis

T-tests and two way ANOVA test have been performed on the phosphorylation data using Origin Pro 8.6 package (Northampton, MA).

Results

Peptide Design

We have chosen 3 Ser-Ser-Asp (SSD3), a motif which comprises the major portion of DPP, as a basis for our model peptide (Figure 1) [29]. The SSD3 motif was flanked on the C-terminal end by a DEG-CK2 recognition motif and on the N-terminal side with DEDE-CK1 recognition motif (Figure 1). Finally, 3 N-terminal arginines (R) have been added to create a cluster of positive charges at the N-terminus (Figure 1). This addition counterbalances the high negative charge of the rest of the peptide, thus making the purification and handling of the peptide easier. The resulting peptide was named R-SSD3.

Phosphorylation

We have conducted phosphorylation studies in the presence of CK1, CK2, and the combination of these two enzymes. A visual analysis of the autoradiographs clearly indicates that in all three experiments, the degree of phosphorylation increased over 24 hours from the beginning of the reaction (Figure 2).

The extent of phosphorylation of the peptides was assessed using scintillation counter quantification. Based on the scintillation data, we have calculated a number of phosphate groups per peptide molecule in all three experiments after 1, 4, and 24 hours of incubation (Table 1, Figure 3). The results of the phosphorylation analysis show that after one hour, a very small fraction of serines, between 0.1% and 2% are phosphorylated. At this stage, the degree of phosphorylation in the presence of CK1 is significantly lower than in the presence of combined CK1+CK2 ($p=0.018$). At the same time, no significant differences in the degree of phosphorylation between CK1 and CK2 groups were observed (Supplementary Table 1). Four hours into the reaction, the degree of phosphorylation has slightly but statistically significantly increased, with 2% to 6% of all serines phosphorylated (Table 1, Figure 3). In all experiments, the difference in the degree of phosphorylation between 1 hour and 4 hour time-points was statistically significant (Supplementary Table 1). At the 4 hour time-point, the degree of phosphorylation was significantly higher in the reaction in the presence of CK2 or a combination of CK1+CK2 than with CK1 alone (Supplementary Table 1). The degree of phosphorylation between CK2 and CK1+CK2 groups was not statistically different (Supplementary Table 1). By the 24 hour time-point, the peptides in all groups had attained a significant degree of phosphorylation (with an average of 1.8 phosphates (30% of all serines) per peptide in the CK1 group, 4.4 phosphates (73% of all serines) in the CK2 group, and 3.5 phosphates (58% of all serines) in CK1+CK2 group). Again, as in the four hour samples, the degree of phosphorylation in the presence of CK1 was significantly less than in the two other groups while no significant differences were observed between the CK2 and CK1+CK2 groups (Table 1, Supplementary Table 1). When 2-way ANOVA was performed on the whole dataset, significant differences in the degree of phosphorylation were only detected between different time-points ($p=2.8 \times 10^{-7}$) but not between different kinases ($p=0.07$). However, when CK1+CK2 group was removed from the analysis, significant differences were observed between CK1 and CK2 treatments ($p=0.03$). These results further support the results of the t-tests which show that no significant differences in

the degree of phosphorylation exist between CK2 and CK1+CK2 treatments at any given time-point.

Overall, our data indicate that CK2 phosphorylates the peptide at a much higher rate than CK1 and that the addition of CK1 to CK2 does not affect the phosphorylation rate. Furthermore, these results indicate that multiple phosphorylation of the synthetic peptide can be achieved post-synthetically, demonstrating the feasibility of using this technique for the manufacturing of peptides with multiple phosphorylation sites. To further test the hypothesis that DPP phosphorylation is sequential, we designed a modified peptide with a proline residue (RRRDEDESSDSSDSSPDDEG) to disturb the first recognition motif of CK2. In phosphorylation assays we observed a significant decrease in the degree of phosphorylation by CK2 of the modified R-P-SSD3 peptide compared to that of the original peptide (Figure 4). In contrast, this modification did not alter the degree of CK1 phosphorylation (Figure 4). These data indicate that CK2 phosphorylation is sequential: once the CK2 recognition motif is disturbed near a critically-placed serine, then the overall phosphorylation of the peptide is dramatically reduced.

Mass Spectrometry (MS)

To quantify the number of phosphorylated serines, MS analysis revealed that the maximum number of phosphates incorporated onto the R-SSD3 peptide is four (out of six possible) when the *in vitro* phosphorylation of the peptide was carried out for one hour with a mixture of CK1 and CK2, as shown in Figure 5.

Mineralization of Collagen Fibrils

When reconstituted collagen fibrils were mineralized in the presence of nonphosphorylated R-SSD3, SAED indicated that randomly oriented crystals formed throughout the grid; no preferential co-localization and co-orientation of these crystals with the fibrils was observed (Figure 6 A). In contrast, in the presence of the phosphorylated peptides, organized mineralization of collagen fibrils has been observed (Figure 6 B, C). The areas of low mineral density were selected for Figure 6 in order to demonstrate organization of individual crystals in the fibrils; however the majority of fibrils were mineralized (see Supplementary Figure 1). These mineralized collagen fibrils contained bundles of apatitic crystallites. Electron diffraction analysis confirmed that the mineral crystals had lattice parameters of hydroxyapatite and their optical *c*-axes were aligned with the axes of the fibrils. In order to better assess the organization of mineral particles in the mineralized collagen fibrils, we have conducted an electron tomography of our sample. The results of this study clearly demonstrate that mineral particles are organized into parallel arrays along the axis of collagen fibril (Figure 7, Supplementary Movie). These structural features and alignments are the hallmark of the mineralized collagen fibrils of bone and dentin [2]. Importantly, almost no mineral crystals were observed outside of the collagen fibrils indicating that R-SSD3 suppressed mineral nucleation outside of the collagen fibrils. Furthermore, the diameters of mineralized and nonmineralized portions of the fibrils were of the same size, suggesting that the mineral was formed intrafibrillarly [30]. These attributes of collagen mineralization in the presence of phosphorylated R-SSD3 are similar to what has been

observed in the experiments with phosphorylated DPP [30], suggesting similarities in the mechanisms of regulation of mineralization by DPP and its model peptide R-SSD3.

Discussion

Phosphorylation

We have successfully demonstrated that casein kinases 1 and 2 can phosphorylate multiple phosphorylation sites in the R-SSD3 synthetic peptide, modeled after motifs in the highly-phosphorylated, noncollagenous protein, DPP. These findings support the hypothesis that casein kinases 1 and 2 phosphorylate this protein *in vivo* [27, 28]. The fact that CK2 has the higher phosphorylation potential is in agreement with the data from *in vivo* studies showing that CK2 is essential for the phosphorylation of DPP [27]. Furthermore, we have clearly demonstrated the feasibility of achieving high phosphorylation levels in synthetic peptides using the post-synthesis phosphorylation approach.

The results of our phosphorylation experiments with the modified peptide R-SSD3P point toward the possibility that the phosphorylation of R-SSD3 peptides occurs via a chain or sequential mechanism. There are numerous CK recognition sites described in the literature [28], however they share a common characteristic: namely, they consist of a stretch of negatively-charged amino acids in a close proximity to serine or threonine amino acid. It was therefore proposed that in the case of DPP, phosphorylation of serine next to the kinase recognition site leads to the formation of negatively charged phosphoserine. This phosphoserine becomes a part of a CK recognition site, allowing for the subsequent phosphorylation of additional serines (Figure 8) [29]. The results of our phosphorylation experiments with R-SSD3 demonstrate that both CK1 and CK2 can phosphorylate multiple serines in (DSS)_n sequence, thus confirming this hypothesis. Furthermore, in the experiments with site-modified RDSSP3, the amount of phosphorylation significantly decreased when the recognition site was disturbed, indicating that the phosphorylation by CK2 progresses sequentially from the C-towards the N-terminus. These data support the hypothesis of sequential phosphorylation proposed by Veis et al. [29].

Mineralization

Our mineralization experiments with phosphorylated R-SSD3 demonstrate the same trends observed in the experiments using full-length DPP molecules [16], indicating that phosphorylated DSS repeats are involved in the regulation of mineralization by DPP. Indeed, in both cases, the phosphorylated molecules induced organized mineralization of collagen fibrils, structurally similar to the mineralized collagen fibrils of bone and dentin, while nonphosphorylated molecules did not have any significant effect on collagen mineralization. The fact that in the presence of R-SSD3, crystal formation outside of the collagen fibrils was inhibited and collagen mineralization occurred intrafibrillarly, suggests that the mechanism of mineralization by highly acidic proteins such as DPP and poly-L-Asp [30, 35, 44–46] share some similarities. A recent cryoEM study by Nudelman et al. [44] using poly-L-Asp as a model for acidic noncollagenous proteins has shown that this peptide can stabilize calcium phosphate prenucleation clusters which condense into amorphous aggregates and prevent crystalline mineral phase formation outside of collagen fibrils. This

work further demonstrated that negatively charged complexes of poly-L-Asp and prenucleation clusters condense in the gap regions of collagen fibrils attracted by a concentration of positive charge and then transform into apatitic crystals [44]. Based on the similarities of the mineralization reaction products between poly-L-Asp and phosphorylated DPP and R-DSS3, we believe that these molecules regulate collagen mineralization in a similar manner. Similarly-organized mineralized collagen fibrils have been achieved using synthetic additives such as polyphosphate and polyacrylate [47, 48]. Furthermore, strategies for biomimetic remineralization of dental tissues using such additives have been proposed [49–51]. We anticipate that our bioinspired peptide designs will lead to the development of strategies for highly controlled biomimetic peptide-based mineralized tissue regeneration and the eventual development of novel nanocomposite materials.

Conclusions

We have successfully synthesized nanocomposite mineralized fibrils mimicking mineralized fibrils of bone and dentin using phosphorylated peptides (R-SSD3) modeled after the major NCP of dentin DPP.

We have also achieved the phosphorylation of R-SSD3 peptides at multiple serine sites. These results demonstrate a feasibility of manufacturing highly phosphorylated peptides via post-synthesis phosphorylation approach. Our data supports the hypothesis of sequential phosphorylation of the NCPs. Knowledge obtained in these studies provides an important step toward bioinspired nanocomposite materials modeled after the collagenous mineralized tissues.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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List of Common Abbreviations

ANOVA	analysis of variance
CCD	Charge Coupled Device
CPM	counts per minute
EDTA	Ethylenediaminetetraacetic acid
EGTA	Ethylene glycol tetraacetic acid
HPLC	High Pressure Liquid Chromatography
MALDI TOF	Matrix-assisted laser desorption/ionization Time of Flight
MS	Mass Spectrometry

MOPS	3-(N-morpholino)propanesulfonic acid
PBS	Phosphate buffer saline
TRIS	Tris(hydroxymethyl)aminomethane
Tricine	N-(2-Hydroxy-1,1-bis(hydroxymethyl)ethyl)glycine
SDS-PAGE	Sodium dodecyl sulfate polyacrylamide gel electrophoresis

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Figure 1.

Peptide design schematics. R-SSD3 sequence contains 1) 3 Arg amino acids at its N-terminus end; 2) followed by 4 amino acids forming the CK1 recognition site and 3) three Ser-Ser-Asp repeats. CK2 recognition motif is located at the C-terminus end of the peptide. R-SSD3P has an identical sequence as R-SSD3 with the exception of a proline residue inserted within the CK2 recognition motif.

Figure 2.

In vitro phosphorylation of R-SSD3 by CK1, CK2 and CK1+CK2 using $^{32}\text{P}[\gamma\text{ATP}]$.
Autoradiogram shows the phosphorylation of R-SSD3 at 1, 4 and 24 hours

Figure 3.

Quantitative analysis of $^{32}\text{P}[\gamma\text{ATP}]$ incorporation per mole of R-SSD3 peptides at 1, 4 and 24 hours. The bar graph shows the extent of peptide phosphorylation by CK1 (blue bar) – CK2 (red bar) and CK1+CK2 (green bar).

Figure 4.
In vitro phosphorylation of R-SSD3 compared to R-SSD3P where the CK2 site was modified. The arrow points to the radioactively labeled peptides.

Figure 5.
Mass spectrometry identification of phosphates incorporated onto R-SSD3 at one hour

Figure 6. TEM micrographs of collagen mineralization in the presence of nonphosphorylated (A) and phosphorylated (B, C) R-SSD3. Insets contain diffraction patterns from the corresponding bright field images.

Figure 7.

Electron tomographic reconstruction of collagen fibril mineralized in the presence of phosphorylated RSSD3. Panel A) bright field TEM micrograph of the collagen fibril which was used for tomography. Panels B through D are reconstructed projections of the fibril in panel A at 0, -20 and 20 degrees. Supplementary movie includes the complete reconstruction of the fibril in Panel A.

Figure 8.
Schematic describing the CK2 sequential phosphorylation.

Table 1

Phosphorylation kinetics based on scintillation analysis. The values in the table represent an average number of phosphates per peptide. Standard deviation is given in parenthesis.

	1 hour	4 hours	24 hours
CK1	0.006 (0.002)	0.139 (0.103)	1.778 (1.196)
CK2	0.0148 (0.015)	0.435 (0.099)	4.354 (0.957)
CK1 and CK2	0.0191 (0.007)	0.343 (0.124)	3.4555 (1.755)