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FULL PAPER

Synthesis of Phospho-Polypeptides via Phosphate-Containing N-Carboxyanhydride: Application in Enzyme-Induced Self-Assembly, and Calcium Carbonate Mineralization

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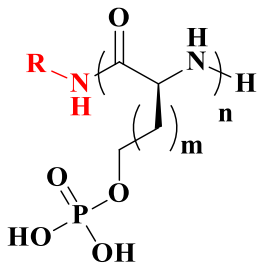
Abstract: An easy synthetic strategy was developed to synthesize phosphate functionalized amino acid N-carboxyanhydride (NCA), using simple primary amine initiators to obtain homo and block phospho-polypeptides with controlled molecular weight and molecular weight distribution. The methodology was extended for the synthesis of end-functionalized homo polypeptide (15 to 50 repeat unit) and block co-polypeptides with PEG (0.7K, 2K, and 5K) and glycopolypeptide (15 unit mannose glycopolypeptide) as one of the blocks. The deprotected fully water-soluble anionic phosphate-based polypeptides displayed pH-dependent helical conformation with a helical content of 20%, which further changed to β-sheet upon addition of the enzyme alkaline phosphatase(ALP) due to dephosphorylation. The block co-polypeptide containing PEG as one of the blocks led to its self-assembly into colloidal structures, such as vesicles with hydrodynamic diameter of ~250 nm, due to the formation of amphiphilic block co-polymer upon dephosphorylation. The nature of the colloidal structures formed can be temporally controlled by the amount of dephosphorylation. Finally, the phospho-polypeptides were able to act as a template for the mineralization of calcium carbonate with varying polymorphs and morphologies.

Introduction

Protein phosphorylation is a reversible post-translational modification (PTM) of protein, in which amino acid side chains such as serine, threonine, tyrosine, and histidine of protein is phosphorylated by the addition of a phosphate group [1-3]. It is a crucial step in all signaling cascades involved in almost every cellular process, including growth, apoptosis, differentiation, and immunity[4-6]. In addition to that, phosphorylated proteins play a significant structural role during biomineralization processes for the development of hard tissues like bone and teeth [7-13]. Due to this immense biological importance of phosphorylation, research efforts have focused on mimicking the features of natural phosphoproteins[14]. In this regard, researchers attempted the synthesis of phospho-polypeptides analogous to natural phosphoprotein *via* the ring-opening polymerization (ROP) of α-amino acid N-carboxyanhydride (NCA) bearing phosphate or phosphonate side chains. Homopolymers of L-phosphoserine and L-phophothreonine were prepared *via* ROP of NCAs bearing phosphate side chains [15-18]. However, extremely slow reaction, the requirement of expensive catalyst, no control over chain length, and absence of end-group functionality limited the practical utility and applicability of this methodology. Our group also attempted earlier to synthesize phosphoserine analog, which yielded a controlled chain length and end-functionalized water-soluble phosphono polypeptide [19]. Nonetheless, we were unable to synthesize the fully water-soluble phosphate analog since the final deprotection step in our strategy to produce water-soluble peptide led to dephosphorylation. Concurrently, Deming *et al.* also reported a strategy to obtain phosphono polypeptides with controlled chain lengths, which displayed conformation change in response to a change in pH [20-24]. Although these phosphono polypeptides can be an isosteric analog of poly(L-phosphoserine), the phosphonate nature of the pending moiety (contaning a C-P bond instead of O-P bonds in phosphates) makes it insensitive towards enzymes that regulate phosphorylation. The use of such phosphorylation and de-phosphorylation reactions have triggered the disintegration and modulation of polymeric assemblies and nanoparticles [25-30]. In addition to that, phosphonate, in comparison to phosphate, may regulate mineral deposition differently during biomineralization due to its somewhat differing geometry [31-37]. Taken together, direct carbon-phosphorous linkage in phosphono polypeptides may limit their ability to mimic natural phosphoproteins [15-19]. Recently, Lu *et al.* reported a controlled synthesis of poly(L-phosphotyrosine) that is structurally identical to its natural analog and found it to be responsive towards the enzyme alkaline phosphatase (ALP) [38]. However, the serine analog poly(L-phosphoserine), a vital component of all phosphoproteins, remain inaccessible. Thus, many important aspects of synthetic poly(L-phosphoserine) such as enzyme responsiveness and biomineralization, among others, remain unexplored. We envisioned that an easy and efficient synthetic strategy for the appropriate poly(L-phosphoserine) analog could address this problem for the advancement of this field.



R= Hexyl, Propargyl, PEG, Azido PEG

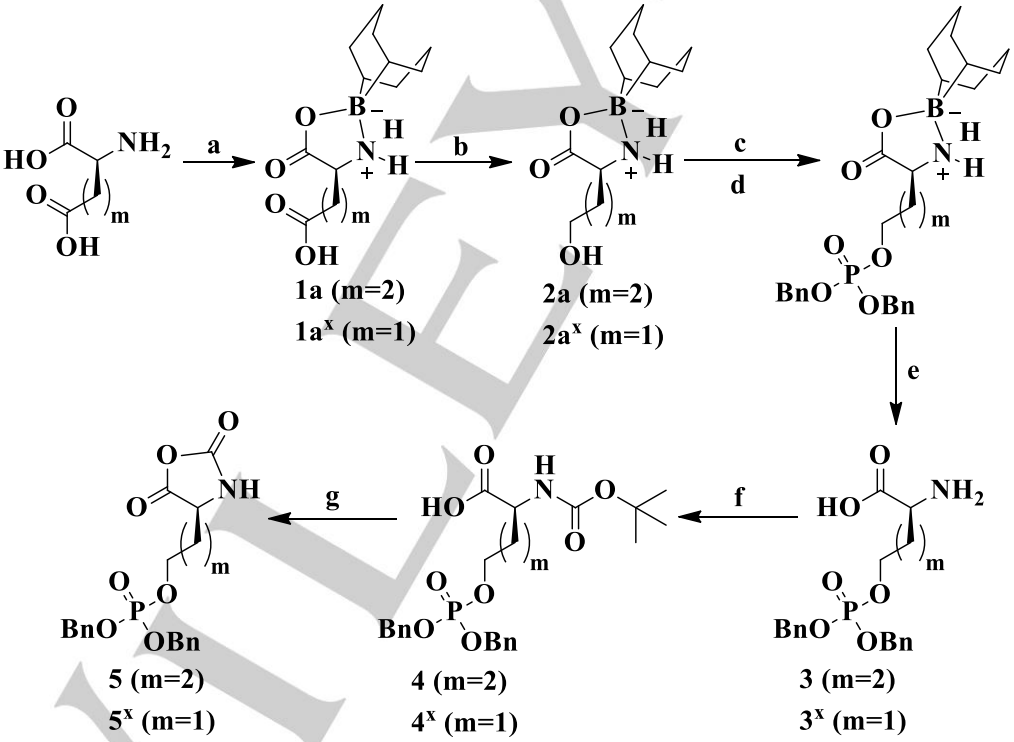
Figure 1: General chemical structures of phosphate functionalized polypeptides derived from the amino acids

Herein we have developed a straightforward methodology for the efficient synthesis of phospho-polypeptides with low polydispersity and controlled molecular weight using the ROP of phosphate functionalized NCA derived from carboxylate containing amino acids. Using this methodology, we were also able to synthesize block copolypeptides, including a polymer that included both the glycopolypeptide and phospho-polypeptide block.

Interestingly, the completely water-soluble phospho-polypeptide displayed a considerable amount of helical content at neutral pH. Unlike phosphono polypeptides reported earlier[19,21,38], this phospho-polypeptide is responsive to the enzyme alkaline phosphatase (ALP), which induces self-assembly of block co-polypeptide containing phospho-peptide into various colloidal structures that can be temporally controlled. We also demonstrate preliminary experiments for the biomineralization of calcium carbonate using these polypeptides as a template.

Results and Discussion

An efficient synthetic strategy for the synthesis of fully water-soluble phospho-polypeptides seeks for easy installation of the protected phosphate group to the side chain of amino acid monomer and subsequent removal of the protecting groups after polymerization without undergoing dephosphorylation.



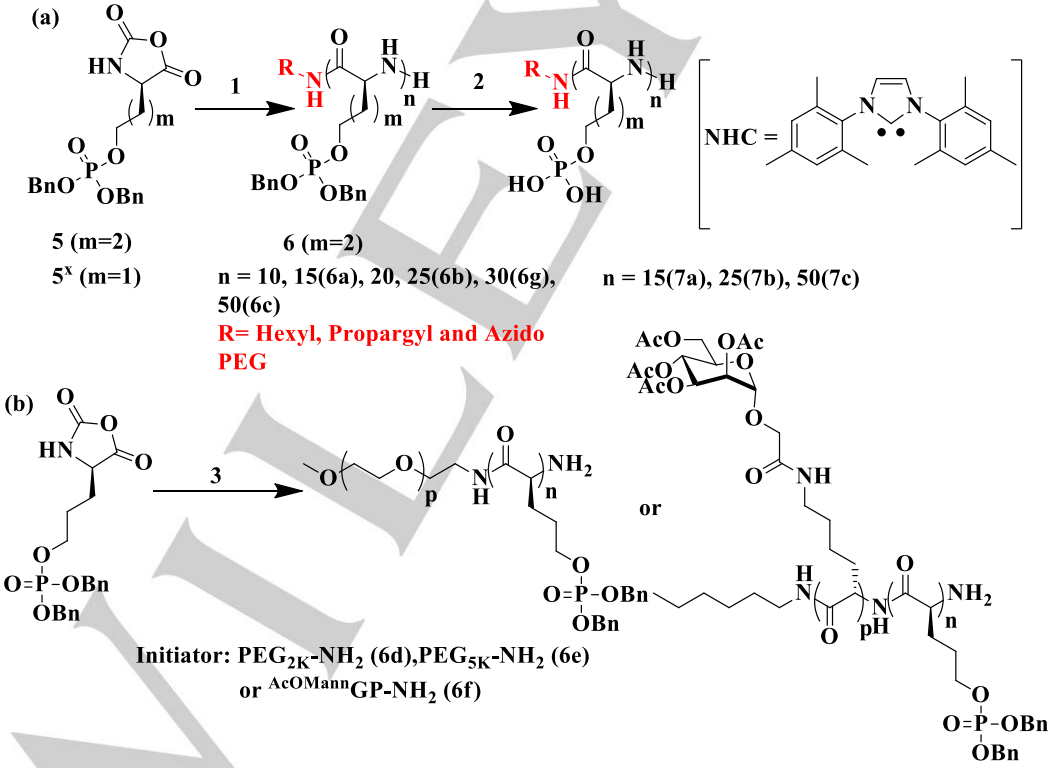
[a] Reagent and condition: (a) 9-BBN Dimer, Dry Methanol, 55 °C, 3 h; (b) BH_3-DMS, Dry THF, 0 °C-rt, 6 h; (c) dibenzyl N, N-diisopropyl phosphoramidite,1-H Tetrazole, Dry ACN, 0 °C-rt, 30 min; (d) ^{t}Bu-OOH, Dry ACN, 15 min; (e) $CHCl_3$: MeOH (5:1), rt, 24 h; (f) BOC_2O, THF, 12 h; (g) Ghosez's reagent, Na Dried THF, 3 h

Scheme 1: General synthetic scheme for the synthesis of $(OBn)_2P(O)$-pent-NCA/$(OBn)_2P(O)$-but-NCA.

We envisaged the introduction of the benzyl protected phosphate moiety through phosphoramidite chemistry based on our previous reports [39]. Hence NCA monomer synthesis was started with the simple aspartic/glutamic acid by protecting its α amino-acid using 9-

BBN, the manipulation of which is much more straight-forward than the much-used Boc and benzoyl groups. Then carboxylic moiety of 9-BBN protected amino acid was reduced to corresponding alcohol using BH_3-DMS. The presence of the hydroxyl group in the side chain of amino acids allowed the easy installation of the phosphate group, as shown below (Scheme 1). For example, with the glutamic acid precursor, the resulted 9-BBN protected 2-amino-5-hydroxypentanoic acid was converted into the precursor phosphine analog using N, N-dibenzyl phosphoramidite, which was subsequently oxidized to the desired phosphate in high yield (90 % after two steps). Removal of the 9-BBN followed by (scheme 1) Boc protection of the 2-amino-5-((bis(benzyloxy)phosphoryl)oxy)pentanoic acid (NH_2-$(OBn)_2P(O)$-pent-COOH) and sub-cyclization in the presence of Ghosez's reagent produced a new $(OBn)_2P(O)$-pent-NCA in good yield (80 % overall yield) (Scheme 1). The purified NCA was characterized by FT-IR, ^1H, ^{13}C, and ^{31}P NMR spectroscopy. In FT-IR spectra presence of two anhydrides stretching at 1786 cm^{-1} and 1854 cm^{-1}, indicating the formation of cyclic N-carboxyanhydride. In ^{13}C NMR, two peaks at 151.87 ppm and 169.95 ppm indicated two carbonyl carbons present in NCA while the phosphorus peak at -2.42 ppm in ^{31}P NMR indicated that the phosphate moiety remained intact during NCA formation. This methodology was further extended for the synthesis of the analogous $(OBn)_2P(O)$-homoserine-NCA starting from aspartic acid (Figure S61-S65).

Early attempts to polymerize $(OBn)_2P(O)$-pent-NCA in the glove box using hexylamine as the initiator (monomer: initiator = 30:1) in all the three solvents (DMF, DCM, and Dioxane) were unsuccessful and did not show any disappearance of the anhydride stretch in FT-IR even after 96 h (Scheme 2 & Figure S1). This unsuccessful polymerization attempt contrasts with our earlier reports with the corresponding ethyl protected phosphonate/phosphate-containing NCA, where polymerization was observed in DMF solvent using a primary amine initiator, albeit slow polymerization rates (36 h to obtain a DP of 25). Upon comparison, this dibenzyl-phosphate functionalized glutamic-NCA is significantly bulkier than the previously reported ethyl protected phosphate due to the presence of the benzyl groups and was likely the reason for the unsuccessful polymerization reaction. Recently the use of N-heterocyclic carbene (NHC) to accelerate NCA polymerization of the related O-diethylphospho L-tyrosine NCA was reported [40-43]. Upon using catalytic amounts of NHC (1,3-bis(2,4,6-trimethylphenyl)-imidazolium) for the dibenzyl-phosphate functionalized glutamic-NCA polymerization, more than 85 % monomer conversion was observed after 24 h by FT-IR spectroscopy in both DCM and DMF solvents. The DP of the resulting polymer in DCM matched closer to the M/I ratio used during polymerization than that performed in DMF (Figure 2). Therefore, all subsequent polymerization of dibenzyl-phosphate functionalized glutamic-NCA was carried out only in DCM.



[a] Reagent and condition: 1) R-NH_2, NHC, Dry DCM, 24 h; 2) Pd-C/H_2, MeOH, 12 h or TFA in DCM (yield 90 %); (3) Initiator, NHC, Dry DCM, 24 h.

Scheme 2: General synthetic scheme for the polymerization of $(OBn)_2P(O)$-pent-NCA/$(OBn)_2P(O)$-but-NCA [a]

The poly((BnO)$_2$P(O)-pent) obtained after polymerization was purified by reprecipitation in diethyl ether, and the structure of polymers characterized by FT-IR, ^{1}H and ^{31}P NMR spectroscopy (Supporting Information). Polymer molecular weights (M_n) were calculated from the relative intensity of the peak at 0.89 ppm due to characteristic proton present in the hexylamine initiator (methyl –C$\underline{H}_3$) with the aromatic protons (benzyl group –C$_6\underline{H}_5$) present in the (BnO)$_2$P(O)-O moiety of the polymer (7.21-7.38 ppm). For the polypeptide 6g (DP 30) formed in DCM, the M_n was estimated from ^{1}H NMR to be 12.3 kDa (in DMF M_n of 9.8 kDa was estimated). In ^{31}P NMR, broadened and slight upfield shifted phosphorous peak of the polymer relative to the phosphorous peak in NCA suggested the formation of the polymer (Figure S23 and S25). SEC analysis performed to determine the PDI and molecular weight distribution of the polymers indicates that the molecular weight of the

Synthesized poly((BnO)$_2$P(O)-pent) polymer had a close to that calculated from ^{1}H NMR. Further, the molecular distribution (M_w/M_n) was monomodal and reasonably narrow (~1.1) (Figure 2a). To investigate if chain length could be controlled during polymerization, polypeptides with varying molecular weights (M/I = 10, 15, 25, 30, 50) were obtained using hexyl amine as the initiator in DCM (Figure S2). Upon increasing the monomer/initiator (M/I) ratios from 10/1 to 50/1, the molecular weight (M_n) of the resulting poly((BnO)$_2$P(O)-pent) increased linearly, and the PDI (M_w/M_n) ranged between 1.10 and 1.17; indicating that well-defined polypeptides were formed. Notably, using monomer to initiator ratio M/I = 50, the obtained M_n of the resulted polymer was close to the expected molecular weight (Table 1) with low polydispersity. Similarly, the (OBn)$_2$P(O)-homoserine-NCA was polymerized in dry DCM with hexylamine to afford well-defined phosphor-homoserine polypeptide with low PDI (Figure S2). These results illustrate the broader scope of this methodology.

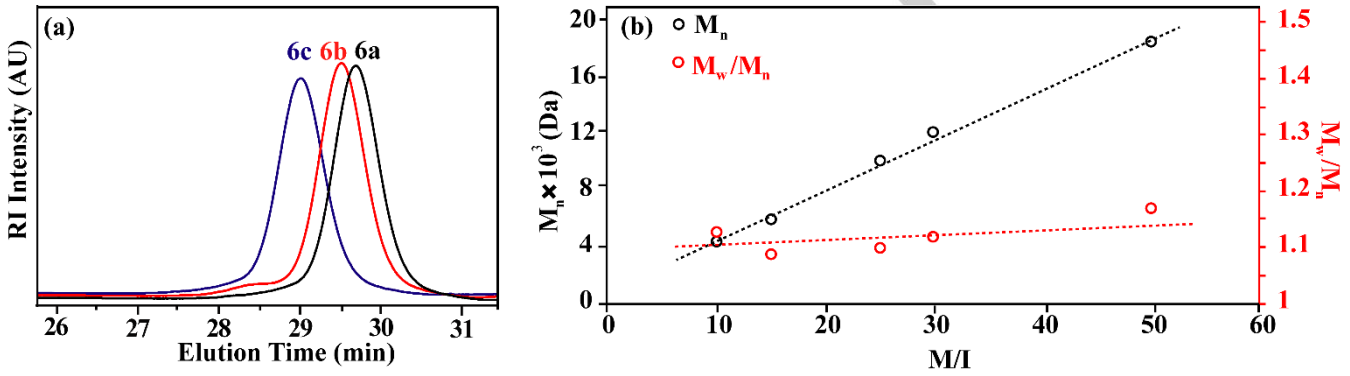


Figure 2: Size exclusion chromatogram of poly((BnO)$_2$P(O)-pent) with hexyl amine initiator (a) GPC trace of the polymers (b) M_n (determined by ^{1}H NMR) vs. M/I and M_w/M_n (determined by GPC analysis) vs. M/I graph. GPC measurements were carried at 25 °C using chloroform as the mobile phase with polystyrene standards.

Next, we attempted the synthesis of block co-polypeptide by the polymerization of (OBn)$_2$P(O)-pent-NCA monomer using PEG-amine (M_n = 2 kDa and 5 kDa respectively) as the macroinitiator in DCM. These macroinitiators also generated polypeptides in high yields and with low PDIs. The incorporation of the PEG block was confirmed by ^{1}H NMR spectroscopy and SEC analysis (Figure S2). The molecular weight (M_n) of the PEG-*b*-poly((BnO)$_2$P(O)-pent) block co-polypeptide determined by end group analysis from ^{1}H NMR was found to be similar to the molecular weight obtained from SEC (Figure S43, S47 & S50). Similarly, block co-polypeptides were also prepared by the polymerization of (OBn)$_2$P(O)-pent-NCA using the 15-mer glycopolypeptide ^{AcOMann}GP$_{15}$-Amine as the macroinitiator [44]. The resulted copolymerization afforded block co-polypeptide ^{AcOMann}GP$_{15}$-*b*-poly((OBn)$_2$P(O)-pent)$_{25}$ in high yield (>80 %) and with moderate PDI (1.2) (Figure S4 & S55). The orthogonal selection of protecting groups enabled us to obtain fully water-soluble co-polypeptide containing both the glyco- and phospho-polypeptide block (Figure S53). This presentation of phosphate and sugar moieties on the side-chain of a single polypeptide mimics the features of the small integrin-binding ligand N-linked glycoproteins known as SIBLING proteins [45]. Finally, clickable end group functionality in a polypeptide was introduced by using azido-PEG-amine as the initiator. The FT-IR spectra of the resulting polymer from azido-PEG-amine initiator displayed an organo-azide peak at 2108 cm^{-1}, which confirmed the incorporation of azide containing initiator into the polypeptide (Figure S55). The incorporation of the azido-PEG initiator was further confirmed by ^{1}H NMR spectroscopy (Figure S53). In a similar approach, the alkyne end-functionalized polymer was obtained by using a propargyl amine initiator. These clickable phospho-polypeptides can participate in copper-catalyzed azide-alkyne "click chemistry," which would be very convenient for attachment of these polypeptides onto various surfaces as has been shown before [46-51]. Completely water-soluble poly((HO)$_2$P(O)-pent) polymer was obtained by deprotection of the benzyl protection groups under hydrogen (balloon pressure) in the presence of Pd/C as the catalyst in methanol solvent (Scheme 2). Within 2 h, complete deprotection was achieved, and the resulting polymer was purified by dialysis. The disappearance of the benzyl peaks in the ^{1}H NMR spectrum suggested the complete removal of the protecting group (Figure S26). The shift of the phosphorous peak from -0.90 ppm to 0.17 ppm in ^{31}P NMR (Figure S27) further confirmed the complete deprotection of the benzyl group of the phosphoesters. However, no detectible dephosphorylation occurred during the deprotection, since a single phosphorous peak in ^{31}P NMR was observed for poly((HO)$_2$P(O)-pent) polymer.

Table 1: Synthesis of Phospho-polypeptides at different monomer to initiator ratio.

M/I[a]	Initiator	Polymer	DP[b]	M_n [c](10^{-3} g/mol)	M_w/M_n [d]	Yield [e] (%)
15	Hexyl Amine [**6a**]	poly((OBn)$_2$ P(O)-pent)$_{15}$	17	6.2	1.1	89
25	Hexyl Amine [**6b**]	poly((OBn)$_2$ P(O)-pent)$_{25}$	27	9.1	1.1	90
50	Hexyl Amine [**6c**]	poly((OBn)$_2$ P(O)-pent)$_{50}$	52	18.7	1.2	83
25	PEG$_{2K}$-Amine [**6d**]	PEG$_{2K}$-*b*-poly((OBn)$_2$ P(O)-pent)$_{25}$	26	11.6	1.1	88
25	PEG$_{5K}$-Amine [**6e**]	PEG$_{5K}$-*b*-poly((OBn)$_2$ P(O)-pent)$_{25}$	28	13.7	1.15	87
25	^{AcOMann}GP$_{15}$-Amine [**6f**]	^{AcOMann}GP$_{15}$-*b*-poly((OBn)$_2$ P(O)-pent)$_{25}$	27	17.5	1.2	81

[a] M/I = monomer to initiator ratio [b] Degrees of polymerization (DP) were calculated from ^{1}H NMR; [c] Calculated from NMR [d] Calculated from gel permeation chromatography using RI detector in chloroform at 25 °C using chloroform as eluent; [e] Total isolated yield of the phospho-polypeptides

The conformation of water-soluble phospho-polypeptides (homo and block) was studied using circular dichroism (CD) spectroscopy. We expected that the phosphate groups would mostly stay deprotonated at pH 7.2, and hence the ionic repulsion of the side chains phosphates would confer a coiled structure of the phospho-polypeptide.To our surprise, solutions of poly((HO)$_2$P(O)-pent)$_{25}$ in water or neutral pH buffer displayed a CD spectrum having minima at 208 and 222 nm that indicated secondary structures with a helical content of 20%. To the best of our knowledge, this is the first report of synthetic phospho-polypeptides having some (20%) helical content at neutral pH. As expected, the helical content of the polymer increased with decreasing pH. Organophosphates having molecular formula RO-P(O)(OH)$_2$ contain two ionizable groups having pKa of ~1 and ~7. pH titration of the polypeptide poly((HO)$_2$P(O)-pent)$_{25}$ showed the second pK_a to be at 6.8 (Figure S60). Thus, at pH 2, more protonation of the –OH groups would decrease the ionic repulsion among the side chains and increase the helical content in the polypeptide as compared to pH 7.4. In contrast, at pH 10, the –OH groups of phosphate would be deprotonated, and the polypeptide would favor an extended chain conformation due to the repulsive ionic interactions between the negatively charged phosphate side chains. As a result, the CD spectra (shows two minima at 215 and 220 nm compare to helix which has two minima at 208 and 222 nm and sheet has only minima at 215 nm) of poly((HO)$_2$P(O)-pent)$_{25}$ at pH 10 display the formation of a non helical structure (Figure 3). A similar result was also obtained for the PEG$_{5k}$-b-poly((HO)$_2$P(O)-pent)$_{25}$ block copolymer (figure S70).

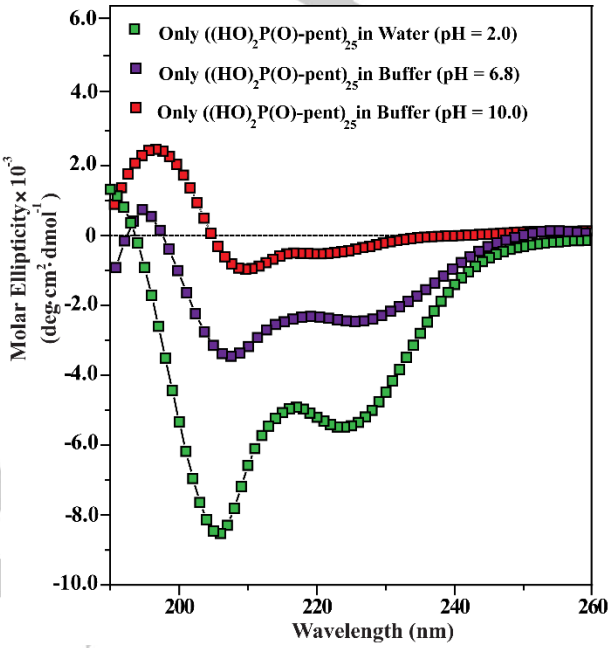


Figure 3: Circular Dichroism Spectra of poly((HO)$_2$P(O)-pent)$_{25}$ polymer in water or buffer at different pH

The phosphate-containing polypeptides are synthetic mimics of natural phosphoprotein, which undergo enzymatic dephosphorylation by alkaline phosphatase (ALP) to constitute an essential process in cell signaling. Therefore, ALP sensitive synthetic phospho-polypeptide could have potential in stimuli-responsive biomaterials. The enzyme responsive property was examined by treating the 10 mg of phospho-polypeptide (poly((HO)$_2$P(O)-pent)$_{25}$) with ALP (5 µL from 1ng/mL stock of ALP) at physiological pH. The reaction was monitored by ^{31}P NMR, where the shifting of the phosphorus peak to 0 ppm indicated dephosphorylation leading to free phosphate formation (Figure 4a). The ^{31}P NMR spectra suggested that complete dephosphorylation took place in 48 h. However, time-dependent ^{31}P NMR data indicated that about 80 % dephosphorylation occurred within the first 20 h. We believe that the enzymatic removal of highly water-soluble phosphate moiety leaves behind the peptide backbone with butanol side chains that could undergo aggregation due to the amphiphilic

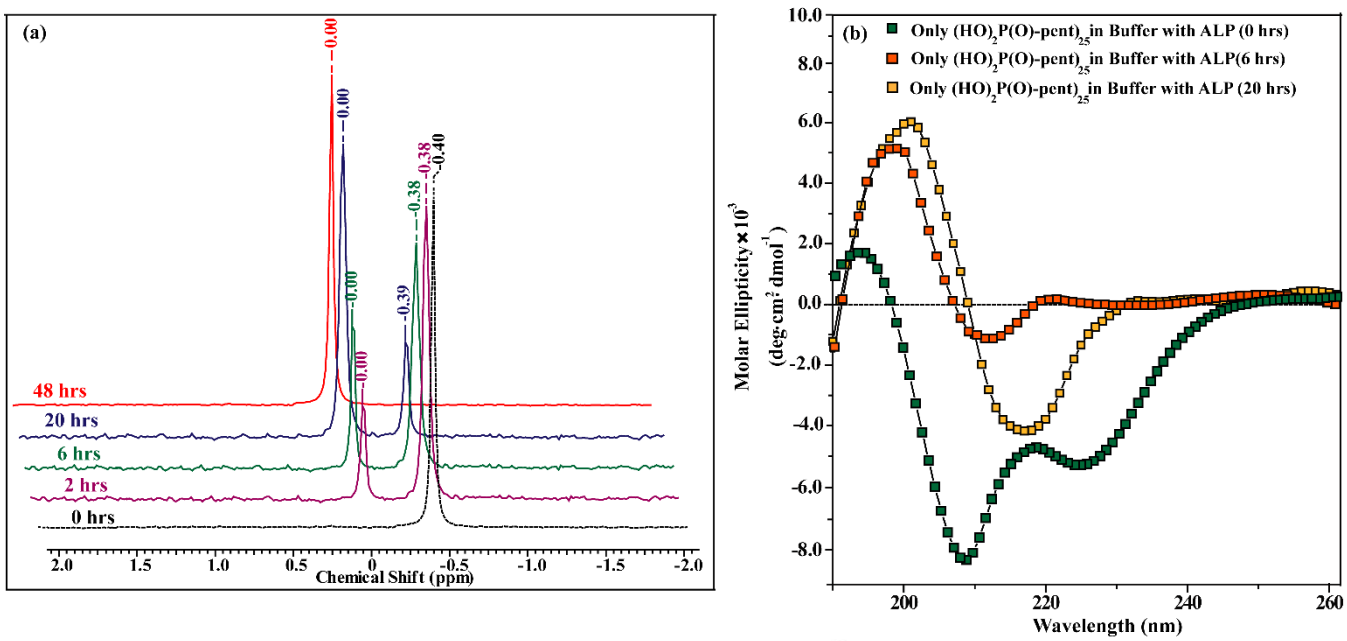


Figure 4: Enzyme responsive property of poly((HO)$_2$P(O)-pent)$_{25}$ polymer (a) time dependent phosphorus NMR and (b) time dependent circular dichroism spectra in the presence of ALP

nature of the resultant polypeptide. As a result, some of the phosphates are probably buried inside the aggregated structures and are not easily accessible by ALP. Time-dependent CD spectra of the ALP treated phospho-polypeptide (Figure 4b) also indicated a loss in its helical content and formation of β-sheet after 20 h of incubation. These results described above demonstrate the enzyme responsive secondary conformation switching of the peptide backbone.

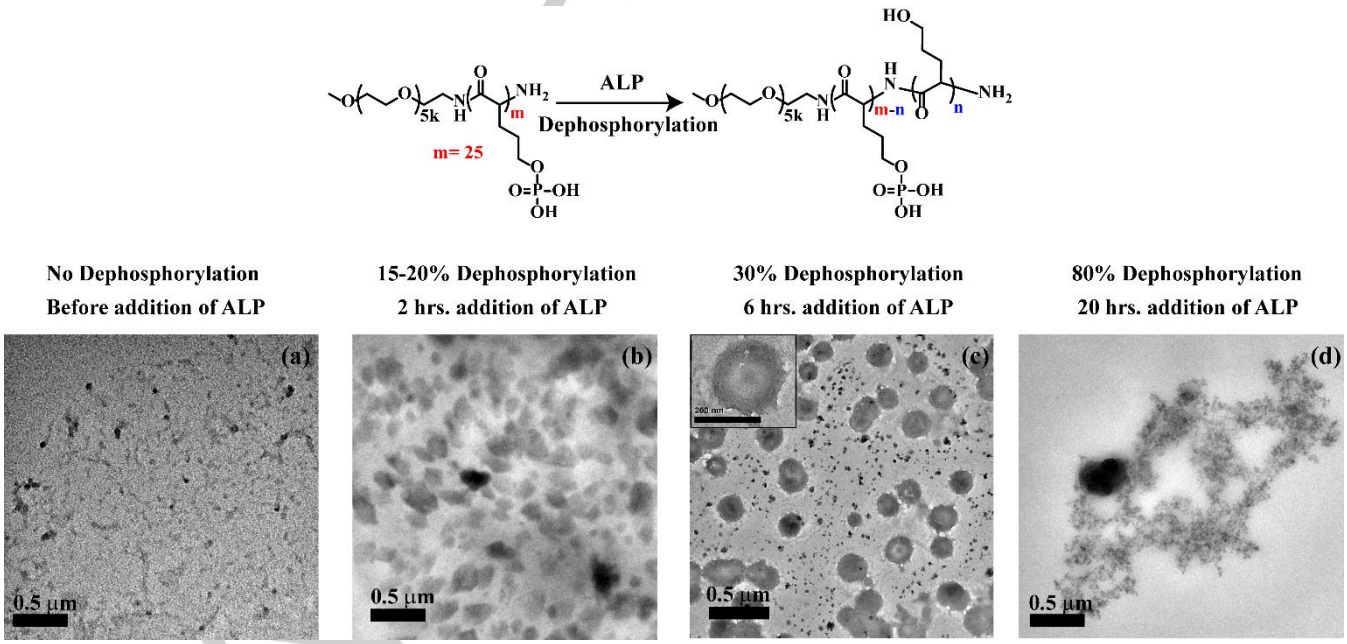


Figure 5: Enzyme responsive assembly formation of PEG$_{5K}$-*b*-poly((HO)$_2$P(O)-pent)$_{25}$ block co-polypeptide. TEM images: (a) in absence of ALP; (b) after 2 hrs. ALP treatment; (c) after 6 hrs. ALP treatment; and (d) after 20 hrs. ALP treatment.

This encouraged us to study the effect of dephosphorylation on the corresponding double hydrophilic PEG$_{5K}$-*b*-poly((HO)$_2$P(O)-pent)$_{25}$ block copolymer (6e) which is expected exist in a roughly coil-helix conformation with a highly

charged phosphate side-chain in aqueous solutions. Incubation of PEG_{5K}-*b*-poly$((HO)_2P(O)\text{-pent})_{25}$ block co-polypeptide with ALP is expected to dephosphorylate the phosphate side-chain, thus rendering this block-copolymer

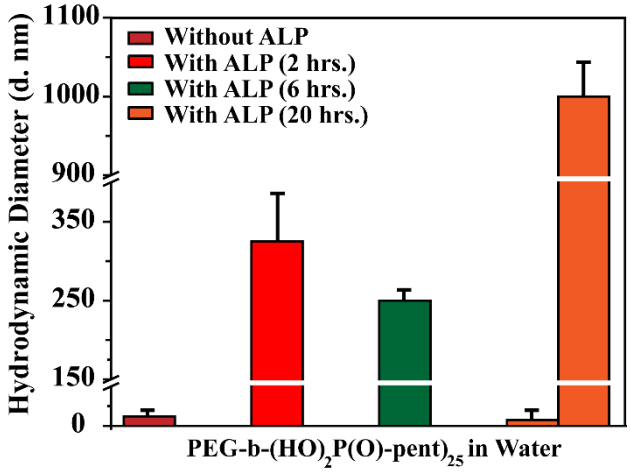


Figure 6: Enzyme responsive assembly formation of PEG_{5K}-*b*-poly$((HO)_2P(O)\text{-pent})_{25}$ block co-polypeptide (DLS analysis; in the absence of ALP and in the presence of ALP at different time interval).

amphiphilic. This, in turn, would lead to its self-assembly into colloidal structures in water. Since the nature of the colloidal structure formed depends on the extent of amphiphilicity of the block copolymer (which is dependent on the extent of the dephosphorylation upon addition of ALP), we hypothesized that the nature of the colloidal structure formed upon self-assembly could be temporally controlled. We thus monitored the colloidal structures formed upon the addition of ALP to the PEG_{5K}-*b*-poly$((HO)_2P(O)\text{-pent})_{25}$ polymer as a function of time using ^{31}P NMR, CD, DLS, and TEM imaging. After 2 h of ALP incubation, 15-20 % of dephosphorylation was observed (^{31}P NMR; Figure S6a), which induced some hydrophobicity into the block co-polypeptide and led to the partial aggregation of the polypeptide (TEM, Figure 5b and DLS, Figure 6). However, no regular morphology like a micelle or vesicle was observed. After 6 h incubation, ~30 % dephosphorylation was observed (^{31}P NMR; Figure S6a), which resulted in further aggregation to form spherical particles with a diameter of ~250 nm (TEM, Figure 5c and DLS, Figure 6). The CD spectra of this sample showed a significant loss in helicity and appearance of features, which indicate a non-helical structure (Figure S6b). Upon further incubation (20 h), ~80 % dephosphorylation was observed (Figure S6a), which significantly reduced the hydrophilicity of the polypeptide resulting in the formation of large unstructured aggregates (TEM, Figure 5d and DLS, Figure 6). The loss of 80 % phosphate group also lead to complete loss of helicity, and the corresponding formation of β sheet-like structures was observed. Hence the morphology of the aggregate formed after self-assembly can be controlled over time by the addition of ALP enzyme. Thus we conclude that the nature of colloidal structure formed upon self-assembly could be temporally controlled.

To further showcase the utility of these phospho-polypeptides in biomineralization, crystallization of $CaCO_3$ in the presence of phospho-polypeptides was examined. Phosphate-containing polymers have attracted attention for their utility, such as tooth replacement, dental adhesives, and bone regeneration. Therefore, it would be advantageous to explore the ability of these polymers to mimic the features of natural phosphoproteins. Biomineralization of $CaCO_3$ was initiated by the addition of varying amounts of either poly$((HO)_2P(O)\text{-pent})_{25}$ or PEG_{5K}-*b*-poly$((HO)_2P(O)\text{-pent})_{25}$ to a $CaCO_3$ solution (1 M) in water. The molar ratio of $CaCO_3$:polypeptide was varied in between 1:0.05 (Ca^{2+}/polypeptide=20) to 1:0.00625 (Ca^{2+}/polypeptide=160). After 15–20 days of the growth, mineralized $CaCO_3$ was washed with water and characterized by SEM (Figure S57 & S58) and WXRD (Figure S60 & S61)technique. It was observed that the presence of higher concentration phospho-polypeptides (Ca^{2+}/polypeptide=20) induced $CaCO_3$ crystallization in a sheet morphology whereas, at low concentrations (Ca^{2+}/polypeptide=160) mineralized $CaCO_3$ having spherical morphology was observed in SEM (Figure 8 & Figure S57). WXRD study indicated the formation of CaCO3 crystals with varying polymorphs morphology (Figure S60) and the polymorphs are mostly a mixture of stable calcite and aragonite.Control experiments where no polypeptide was added yielded amorphous $CaCO_3$, (Figure S57 & S58)). To investigate the role of the phosphate side chain, the interaction of the phosphate side chain of the polypeptide and added Ca^{2+} ions were investigated using ^{31}P NMR and CD spectroscopy. A downfield shift in the ^{31}P peak was observed upon the addition of Ca^{2+}, which indicated the interaction between Ca^{2+} ion and phosphate moiety (Figure 7b). The CD spectra also showed an increase in helicity of the PEG_{5K}-*b*-poly$((HO)_2P(O)\text{-pent})_{25}$ polymer upon the addition of Ca^{2+} ions (Figure 7a). The increase in helicity arises as a result of the binding of Ca^{2+} to phosphate. This binding neutralizes the side chain negative charge, which leads to an increase in helicity. Finally, to exclude any possibility that the interaction between polypeptide backbone and Ca^{2+} was responsible for biomineralization, experiments were carried out with the polypeptide that had been treated with ALP for 48 h (to ensure that complete dephosphorylation occurs before the Ca^{2+} ions were added). All the dephosphorylated polypeptides afforded (Ca^{2+}/dephosphorylated polypeptide=20 to 160) amorphous $CaCO_3$ particles that were similar to what was observed for control mineralization experiments (without the use of polypeptide) (Figure S59). All these data strongly imply that only the pending phosphate group interacted with $CaCO_3$ and strongly influenced their crystallization. Finally, the block co-polypeptide PEG_{5K}-*b*-poly$((HO)_2P(O)\text{-pent})_{25}$ block copolymer was also used as a template to investigate its effect in the $CaCO_3$ biomineralization process. Interestingly, the polymorphs of the $CaCO_3$ crystal found (Figure S61) here was similar (stable calcite and aragonite) that was observed for the homopolymer at a similar Ca^{2+}/polypeptide ratio, but the polymorphos morphology are different (Figure 8 & Figure S58). For Ca^{2+}/polypeptide=20, the block co-polypeptide displayed a dendritic pattern, and with lowering the concentration of polypeptide, it formed a spherical morphology.

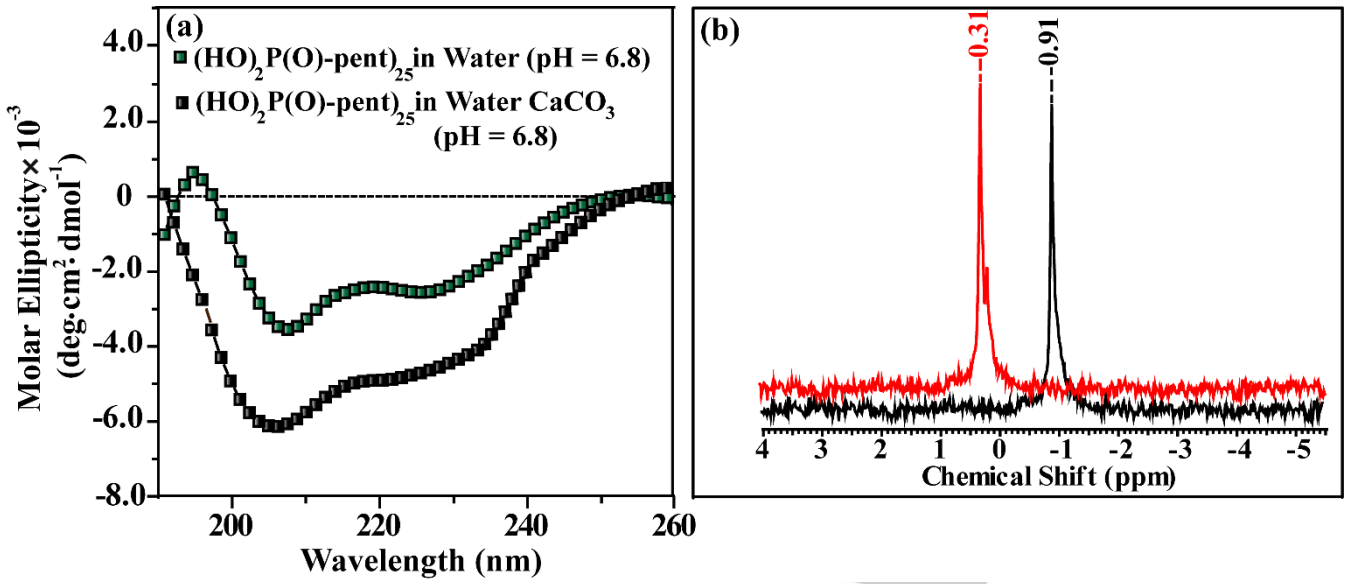


Figure 7: Interaction of poly((HO)$_2$P(O)-pent)$_{25}$ polymer with $CaCO_3$ (a) circular dichroism spectra and (b) phosphorus NMR spectra before the addition of $CaCO_3$(black) and after addition of $CaCO_3$ (red).

This is in contrast to amorphous morphology obtained for the homopolymer under similar conditions. This result suggests phospho-polypeptide could also have some effect on the $CaCO_3$ crystallization process and likely change the outcome of crystal morphology.

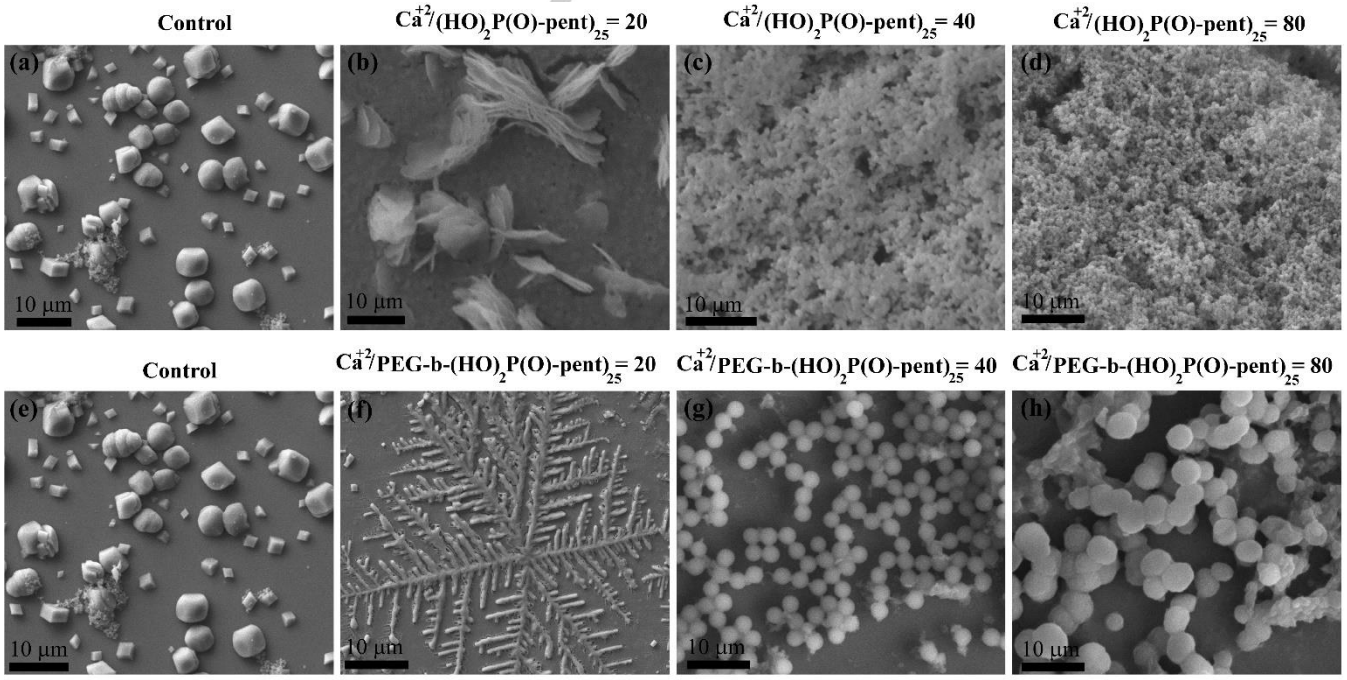


Figure 8: Biomineralization of $CaCO_3$ in the presence of polypeptides poly((HO)$_2$P(O)-pent)$_{25}$ (a, b, c, and d) and PEG$_{5K}$-*b*-poly((HO)$_2$P(O)-pent)$_{25}$ (e, f, g, and h) respectively.

Conclusion

In summary, we have successfully developed an easy route for the synthesis of the water-soluble, end-functionalized phosphate-containing polypeptide with controlled molecular weight and low polydispersity. The method was extended for the synthesis of phosphate-containing block co-polypeptides with PEG and glycopolypeptide as one of the blocks. The glycol-b-phospho-polypeptide mimics SIBLING proteins, which are essential in dentine and bone formation. These phospho-peptides adopt a helical conformation at neutral pH, unlike their polyglutamate analogs, which also bear negatively charged side-chains. Changes in the secondary structure were observed upon change of pH with the polypeptide showing more helicity at low pH. These phosphopolypeptides were also responsive to the enzyme alkaline phosphatase due to the dephosphorylation of the side chain. This leads to the formation of a polypeptide with $–(CH_2)_4$-OH side chains, which are inherently more hydrophobic. This enzyme-responsiveness was utilized to dephosphorylate the PEG-*b*-phospho-polypeptide copolymer, which self-assembled into colloidal structures due to the formation of an amphiphilic block copolymer. We demonstrated that the nature of the colloidal structures formed could be temporally controlled, which was a function of the extent of dephosphorylation that occurred. Finally, upon the addition of calcium carbonate solution of these phospho-polypeptides, biomineralization was observed, leading to the formation of mineralized calcium carbonate with varying polymorphs and morphologies that can be controlled by the amount of polypeptide added as the template. We believe that these homo phospho-polypeptides and block co-polypeptide can be used for several biomedical applications like drug delivery and tissue engineering. Such efforts are underway in our laboratory.

Experimental Section

Materials and Methods

Unless otherwise noted, materials were obtained from commercial suppliers and were used without further purification. Removal of the solvent *in vacuo* refers to distillation using a rotary evaporator attached to an efficient vacuum pump. Products obtained as solids or syrups were dried under a high vacuum. 9-BBN dimer, Ghosez's Reagent, 10% Pd/C, tert-Butyl hydrogen peroxide, BH_3-DMS, $CDCl_3$ was purchased from Sigma Aldrich. All other chemicals used were obtained from Merck, India. Diethyl ether, petroleum ether (60−80 °C), ethyl acetate, dichloromethane, and tetrahydrofuran were purchased from Merck, India; dried by conventional methods and stored in the glove box. Analytical thin-layer chromatography was performed on pre-coated silica plates (F_{254}, 0.25 mm thickness); compounds were visualized by UV light or by staining with Ninhydrin spray. ^{1}H NMR spectra were recorded on Bruker Spectrometers (200 MHz, 400 or 500 MHz) and reported relative signals according to the deuterated solvent used. ^{13}C NMR and DEPT spectra were recorded on Bruker Spectrometer (50, 100, or 125 MHz), and reported relative signals according to the deuterated solvent used. ^{31}P NMR spectra recorded on Bruker Spectrometer (202.46 MHz) using an internal 85 % aqueous H_3PO_4 as reference. FT-IR spectra were recorded on a Perkin-Elmer FT-IR spectrum GX instrument by making KBr pellets. Pellets were prepared by mixing 3.0 mg of the sample with 97.0 mg of KBr. The molecular weights of polypeptides were determined using gel permeation chromatography (GPC). GPC measurements were carried on a Thermo Quest (TQ) GPC at 25 °C using chloroform as the mobile phase. The analysis was carried out at a flow rate of 1 mL/min using a set of five μ-Styragel HT Columns (HT-2 to HT-6) and a refractive index (RI) detector. Columns were calibrated with polystyrene standards, and the molecular weights are reported in comparison to polystyrene. Circular dichroism measurement of all polypeptides has been performed to show the conformation of polypeptides. All the polypeptide solutions were filtered through 0.22 μm syringe filters. CD (190−250 nm) spectra of the polypeptides (1.0−0.25 mg/mL in water or buffer) were recorded by JASCO J-815 CD spectrophotometer in a cuvette with 1 mm path length. All the spectra were recorded for an average of three scans, and the spectra were reported as a function of molar ellipticity [θ] versus wavelength. The molar ellipticity was calculated using the standard formula, $[\theta]=(\theta \times 100 \times Mw)/(C \times l)$, where θ = experimental ellipticity in millidegrees, Mw and C was molecular weight and concentration of the amino acid repeat unit, and l = path length in cm. The % α helicity was calculated by using the formula: % α helicity = $[(-[\theta]_{222}$ nm + 3000)/39 000] × 100.

General procedure for the synthesis of 9-BBN protected amino acids

In a 250 mL round-bottom flask, 1.05 eq. of 9-BBN dimer (12.5 mmoL) was taken at room temperature under argon in 150 mL of dry methanol. The mixture was heated at reflux until the 9-BBN was completely dissolved (30 min) and to this solution, 25 mmol of the amino acid (L-Glutamic acid/L-Aspartic acid) was added. The resultant reaction mixture was heated for an additional 3 h until gas evolution ceased, and the suspension became a clear homogenous solution. The methanol was removed on the rotary evaporator, and the residue dissolved in hot THF (100 mL), filtered and the filtrate was concentrated to afford a white gummy residue of 9-BBN protected amino acids (L-glutamic acid/L-aspartic acid). Excess of 9-BBN was removed by washing with hot hexane and then subjected to a high vacuum for 1 h, during which time it became an amorphous solid. This material was used without any further purification for the subsequent reaction (quantitative yield for both 1a and 1a[x])

Compound 1a: ^{1}H NMR (200.13 MHz, DMSO-D_6): δ(ppm) 0.46-0.51 (1H, d, *J*=0.05), 1.47-1.87 (14H, m), 2.01-2.11 (1H, m), 3.55-3.62 (2H, m), 5.88-5.98 (1H, t), 6.44-6.53 (1H, m); ^{13}C NMR (50.32 MHz, DMSO-D_6): δ(ppm) 13.95, 14.18, 18.74, 20.86, 23.98, 24.33, 25.77, 30.17, 30.79, 30.85, 31.29, 34.75, 53.75, 50.90, 60.50, 173.61, 174.40

General procedure for the Synthesis of 9-BBN protected 2-amino-5-hydroxypentanoic acid/9-BBN protected 2-amino-4-hydroxybutanoic acid

To a suspension of 9-BBN protected amino acids (L-glutamic acid/L-aspartic acid) (18.6 mmol) in dry THF (75 mL), BH_3-DMS (18.6 mmol) was added dropwise through an addition funnel over 20 min at 0 ºC under argon atmosphere. After stirring 1 h at 0 ºC, the reaction mixture was stirred overnight at room temperature. After completion of the reaction, the solvent was evaporated by applying a vacuum. The residue obtained was purified by silica gel column chromatography using ethyl acetate as eluent to get 9-BBN protected 2-amino-5-hydroxypentanoic acid/9-BBN protected 2-amino-4-hydroxybutanoic acid as white solid (yield: 4.2 gm, 89.7 % for 2a and 83 % for $2a^x$).

Compound 2a: ^{1}H NMR (200.13 MHz, DMSO-D_6): δ(ppm) 0.47-0.57(d, 2H, *J*=0.04), 1.57-1.84(m, 14H), 3.39-3.53(m, 3H), 4.63(br, 1H), 5.82-5.92(m, 1H), 6.36-6.46 (m, 1H); ^{13}C NMR (50.32 MHz, DMSO-D_6): δ(ppm) 22.40, 23.99, 24.33, 25.21, 27.41, 28.84, 30.79, 30.84, 31.30, 54.50, 60.40, 67.11, 173.89.

Synthesis of 1,1-dichloro-N,N-diisopropylphosphinamine

Phosphorous trichloride (5 mL, 57.3 mmol) was dissolved in 50 ml of dry hexane under N_2, and the mixture was cooled to -78 ºC. A solution of freshly distilled di-isopropylamine (16 mL, 114.6 mmol) in 50 mL of dry hexane added to the reaction mixture over 1h using an addition funnel. Following the addition, the reaction was stirred at -70 ºC for an additional hour [25]. The cooling bath was then removed, and the reaction was stirred at room temperature for another 4 h. The reaction was then filtered through a glass frit under positive N_2 pressure, and the solvent with excess phosphorus trichloride was removed by short-path distillation at 40 ºC with a high vacuum. The product was obtained as a clear, colorless liquid and was stored at -22 ºC (yield: 10 gm, 88%), which transformed into crystalline solid at low temperature.

Compound 1b: ^{1}H NMR (200.13 MHz, $CDCl_3$): δ(ppm) 1.27-1.30(d, 12H), 3.84-4.07(m, 2H); ^{31}P NMR (202.46 MHz, $CDCl_3$): δ(ppm) 169.57

Synthesis of dibenzyl N, N-diisopropyl phosphoramidite

1,1-dichloro-N, N-diisopropylphosphinamine (10 gm, 50.1 mmol) was dissolved in 100 mL of dry diethyl ether under N_2, and the mixture was cooled to -10 ºC. A solution of benzyl alcohol (11.45 mL, 110.2 mmol), triethylamine (15.35 mL, 110.2 mmol), and 100 mL of dry diethyl ether was added to the reaction vessel through an addition funnel over the course of 1.5 h. After completion of the addition, the cooling bath was then removed, and the reaction was stirred at room temperature for 6 h. The reaction was filtered through a glass frit, and the solvent was removed under reduced pressure yielding yellowish oil. The crude oil was then purified by column chromatography. The column was packed with silica gel (100-200 mesh) using 2.5 % triethylamine in hexane until the smell of triethylamine persisted. After loading the crude reaction mixture, the desired product was eluted using 2 % ethyl acetate-2.5 % triethylamine in hexane as the eluent. The fractions containing the product were combined and concentrated under reduced pressure during which the volatiles was removed under vacuum, yielding clear and colorless oil (yield: 15.5 gm, 90 %).

Compound 2b: ^{1}H NMR (200.13 MHz, $CDCl_3$): δ(ppm) 1.24-1.27 (d, 12H), 3.69-3.85(m, 2H), 4.69-4.94(m, 4H), 7.29-7.43(m, 10H); ^{31}P NMR (202.46 MHz, $CDCl_3$): δ(ppm) 146.68

General procedure for the synthesis of 2-amino-5-((bis(benzyloxy)phosphoryl)oxy)pentanoic acid (NH_2-$(OBn)_2$P(O)-pent-COOH)/2-amino-4-((bis(benzyloxy)phosphoryl)oxy)butanoic acid ($(NH_2$-$(OBn)_2$P(O)-but-COOH)

9-BBN protected 2-amino-5-hydroxypentanoic acid/2-amino-4-hydroxybutanoic acid (16.6 mmol), and dibenzyl diisopropyl phosphoramidite (29.8 mmol) were evaporated twice with dry MeCN (30 mL) and then dissolved in dry MeCN (75 mL). In another RB, 29.8 mmol of 1H-tetrazole (dried by twofold evaporation with MeCN) was dissolved in 20 mL of MeCN. The 1H-tetrazole solution was then added dropwise to the reaction mixture on an ice bath with continuous stirring. After addition, the ice bath was removed, and the reaction mixture was stirred at room temperature for 30 mins upon which precipitation of white solid was observed. Precipitated white crystalline side product was filtered, discarded, and the filtrate was concentrated on a rotary evaporator. The crude product obtained after removal of solvent was used further without any purification.

The crude product was dissolved in MeCN and (6M) ^{t}Bu-OOH (16.6 mmol) was added dropwise at 0 ºC. Then the reaction mixture was stirred till reaction completed, monitored by TLC. The solvent was evaporated to dryness, and the crude product was purified by silica gel column chromatography using 2 % MeOH in ethyl acetate as eluent. The fractions containing the product were combined and concentrated under reduced pressure, and the volatiles was removed under vacuum, yielding a clear and colorless oil 9-BBN protected 2-amino-5-((bis(benzyloxy)phosphoryl)oxy)pentanoic acid (yield: 8 gm, 94 % for 3 and 90 % for 3^x).

9-BBN protected 2-amino-5-((bis(benzyloxy)phosphoryl)oxy)pentanoic acid/9-BBN protected 2-amino-4-((bis(benzyloxy)phosphoryl)oxy)butanoic acid was dissolved in MeOH:$CHCl_3$ (1:10) mixture and stirred it for 24 h at room temperature. After complete cleavage of 9-BBN complex, the reaction mixture was concentrated and treated with hot petroleum ether to remove excess 9-BBN. The crude product was directly subjected to the next reaction without any further purification.

Compound 3: ^{1}H NMR (200.13 MHz, $CDCl_3$): δ(ppm) 1.62-2.03(m, 6H), 3.92(s, 3H), 4.89(s, 2H), 4.93(s,2H), 7.29(10H), 8.31(2H); ^{13}C NMR (50.32 MHz, $CDCl_3$): δ(ppm) 25.35, 25.41, 28.01, 56.88, 66.82, 66.88, 69.71-69.81(1C), 127.95-128.11(4C), 128.60-128.84(4C), 135.38-135.43(1C), 151.87, 169.95; ^{31}P NMR (202.46 MHz, $CDCl_3$): δ(ppm) -2.42.

General procedure for BOC protection of the NH_2-$(OBn)_2P(O)$-pent-COOH/ NH_2-$(OBn)_2P(O)$-but-COOH

To a solution of 2-amino-5-((bis(benzyloxy)phosphoryl)oxy)pentanoic acid/2-amino-4-((bis(benzyloxy)phosphoryl)oxy)butanoic acid (15.6 mmol) in 50 mL of THF, triethyl amine was suspended at 0 °C. Then Boc_2O (18.72 mmol) was added dropwise to that suspension, and the reaction mixture was stirred overnight at room temperature. After completion of the reaction, acidified with 2(N) HCL and worked up by DCM, water. The organic part was washed with a brine solution and then dried over sodium sulfate. The organic part was evaporated to dryness in rotavapor to get the BOC protected NH_2-$(OBn)_2P(O)$-pent-COOH/ NH_2-$(OBn)_2P(O)$-but-COOH, used further without any purification (yield: 6.8 gm, 88 % for 4 and 83 % for 4^x).

Compound 4: ^{1}H NMR (200.13 MHz, $CDCl_3$): δ(ppm) 1.26 (m, 2H), 1.42-1.47 (m, 9H), 1.64-1.70 (m, 3H), 1.84-1.85 (m, 2H), 3.97-4.00 (m, 2H), 4.27-4.29 (m, 1H), 5.00-5.02 (m, 4H), 7.32-7.34 (m, 10H); ^{13}C NMR (50.32 MHz, $CDCl_3$): δ(ppm) 25.46, 26.04, 26.11, 28.19, 28.72, 31.93, 52.68, 52.34, 67.26, 67.79, 67.30, 127.88, 127.47, 135.52, 135.59, 155.31, 155.47, 172.33, 172.42; ^{31}P NMR (202.46 MHz, $CDCl_3$): δ(ppm) -2.44.

General procedure for the synthesis of $(OBn)_2P(O)$-pent-N-carboxyanhydride/$(OBn)_2P(O)$-but-N-carboxyanhydride (NCA)

To a solution of BOC protected NH_2-$(OBn)_2P(O)$-pent-COOH*/$(OBn)_2P(O)$-but-N-carboxyanhydride* (2.03 mmol) in freshly distilled anhydrous tetrahydrofuran (10 mL) was Ghosez's reagent (4.06 mmol) in Glove Box and the reaction mixture was stirred for 2.5 h at room temperature in Glove Box. It was then evaporated to dryness in a high vacuum under argon atmosphere and purified by silica gel column chromatography using ethyl acetate as eluent. The resulting fractions were collected and evaporated to get the $(OBn)_2P(O)$-pent-N-carboxyanhydride/$(OBn)_2P(O)$-but-N-carboxyanhydride under high vacuum at 50 °C (yield: 0.68 gm, 80 % for 5 and 75 % for 5^x).

Compound 5: ^{1}H NMR (200.13 MHz, $CDCl_3$): δ(ppm) 1.62-2.03(m, 6H), 3.92(s, 3H), 4.89(s, 2H), 4.93(s,2H), 7.29(10H), 8.31(2H); ^{13}C NMR (50.32 MHz, $CDCl_3$): δ(ppm) 25.35, 25.41, 28.01, 56.88, 66.82, 66.88, 69.71-69.81(1C), 127.95-128.11(4C), 128.60-128.84(4C), 135.38-135.43(1C), 151.87, 169.95; ^{31}P NMR (202.46 MHz, $CDCl_3$): δ(ppm) -2.42.

General Procedure for the polymerization of $(OBn)_2P(O)$-pent-NCA/$(OBn)_2P(O)$-but-NCA

To a solution of $(OBn)_2P(O)$-pent-N-carboxyanhydride/$(OBn)_2P(O)$-but-N-carboxyanhydride (100 mg/mL) in dry $CDCl_3$ was treated with NHC (0.3 equiv to monomer) as an additive and hexyl-amine (0.5 M stock solution) as the initiator inside the glove box. The progress of the polymerizations was monitored by FT-IR spectroscopy by comparing it with the intensity of anhydride stretching at 1785 cm^{-1} and 1858 cm^{-1} of the parent NCA. The reactions were generally completed within 24 h. Aliquots for GPC analysis were picked periodically until completion of polymerization. Finally, the solvent was removed under reduced pressure from the reaction mixture. The resulting residue was re-dissolved in dichloromethane, and the polymer was precipitated by the addition of diethyl ether. The precipitated polymer was collected by centrifugation and dried to afford polypeptides in greater than 90 % yield as a white powder.

Polymer 6a: ^{1}H NMR (200.13 MHz, $CDCl_3$): δ(ppm) 0.83 (3H), 1.70-2.01, 3.96, 4.88-5.03, 7.19-7.32; ^{31}P NMR (202.46 MHz, $CDCl_3$): δ(ppm) -0.91

Polymer 6b: ^{1}H NMR (200.13 MHz, $CDCl_3$): δ(ppm) 0.95-1.00, 1.31-1.34, 1.76-2.27, 3.50(3H), 3.57-3.62, 3.76-3.77, 4.08-4.09, 4.95-5.16, 7.33-7.46; ^{31}P NMR (202.46 MHz, $CDCl_3$): δ(ppm) -0.90

Polymer 6c: ^{1}H NMR (200.13 MHz, $CDCl_3$): δ(ppm) 1.22-1.80, 3.50(3H), 3.77, 4.96-5.15, 737-7.45; ^{31}P NMR (202.46 MHz, $CDCl_3$): δ(ppm) -0.88

General Procedure for the Synthesis of block-co-polymer

To a solution of $(OBn)_2P(O)$-pent-N-carboxyanhydride (100 mg/mL) in dry $CDCl_3$ was treated with NHC (0.3 Equiv to monomer) as an additive and PEG_{2K}, PEG_{5k}, $^{AcOMann}GP_{15}$ amines as the macroinitiator inside the glove box. The progress of the polymerizations was monitored by FT-IR spectroscopy by comparing it with the intensity of anhydride stretching at 1785 cm^{-1} and 1858 cm^{-1} of the parent NCA. The reactions were generally completed within 24 h. Aliquots for GPC analysis were picked periodically until completion of polymerization. Finally, the solvent was removed under reduced pressure from the reaction mixture. The resulting residue was re-dissolved in dichloromethane, and the polymer was precipitated by the addition of diethyl ether. The precipitated polymer was collected by centrifugation and dried to afford polypeptides in greater than 90 % yield as a fluffy white compound.

Polymer 6d: ^{1}H NMR (200.13 MHz, $CDCl_3$): δ(ppm) 0.95-1.00, 1.31-1.34, 1.76-2.27, 3.50(3H), 3.57-3.62, 3.76-3.77, 4.08-4.09, 4.95-5.16, 7.33-7.46; ^{31}P NMR (202.46 MHz, $CDCl_3$): δ(ppm) -0.82

Polymer 6e: ^{1}H NMR (200.13 MHz, $CDCl_3$): δ(ppm) 1.22-1.80, 3.50(3H), 3.77, 4.96-5.15, 737-7.45; ^{31}P NMR (202.46 MHz, $CDCl_3$): δ(ppm) -0.82

Polymer 6f: ^{1}H NMR (200.13 MHz, $CDCl_3$): δ(ppm) 0.87 (3H), 1.10-1.11, 1.23-1.26, 1.41-1.44, 1.64-1.65, 1.98, 2.04-2.24, 2.93, 3.10, 3.03, 3.96-3.98, 4.10-4.18, 4.87-5.04, 5.30-5.34, 7.23-7.34; ^{31}P NMR (202.46 MHz, $CDCl_3$): δ(ppm) -0.94

General Procedure for the deprotection of Polypeptides

The phosphate deprotection was performed either by hydrogenation or by using TFA-DCM depending on the requirement. Hydrogenolysis of polymers was carried out using 10 % Pd/C in MeOH at balloon pressure for 12 h. After completion of the reaction, the reaction mixture was filtered and concentrated under reduced pressure to afford benzyl deprotected phospho-polypeptide in almost quantitative yield. The resulting compounds were directly used for the next step without any further purification.

Polymer 7a: ^{1}H NMR (200.13 MHz, D_2O): δ(ppm) 0.85, 1.02-1.03, 1.70, 1.81-2.06, 3.65-3.91, 4.19-4.38; ^{31}P NMR (202.46 MHz, D_2O): δ(ppm) 0.31

Polymer 7b: ^{1}H NMR (200.13 MHz, D_2O): δ(ppm) 0.79-0.81, 1.19-2.09, 3.53-4.18; ^{31}P NMR (202.46 MHz, D_2O): δ(ppm) 0.17

Polymer 7c: ^{1}H NMR (200.13 MHz, D_2O): δ(ppm) 0.99-1.00, 1.61-2.19, 3.29, 3.32, 3.65-3.67, 3.87-4.37; ^{31}P NMR (202.46 MHz, D_2O): δ(ppm) 0.14

General Procedure for the Enzyme Responsive Studies

Enzymatically triggered self-assembly process was performed at 37 °C under aerobic conditions. Alkaline Phosphatese (ALP) was added to a solution of poly($(HO)_2$P(O)-pent)$_{25}$ or PEG_{5K}-*b*-poly($(HO)_2$P(O)-pent)$_{25}$ block co-polypeptide polymer in DI water at pH 6.8. In a typical experiment, 2 µL of 1 ng/mL ALP was added to 4 mg of phospho-block co-polypeptide PEG_{5k}-b-(poly($(HO)_2$P(O)-pent)$_{25}$). The formation of PEG_{5K}-*b*- poly($(HO)_2$P(O)-pent)$_{25}$ block co-polypeptide self-assembly was monitored using dynamic light scattering and transmission electron microscopy techniques in a different time interval.

General Procedure for the $CaCO_3$ Biomineralization

Biomineralization of calcium carbonate in the presence of phosphate functionalized homo and block co-polypeptides was performed in the physiological environment. Typically, $CaCO_3$ solution was prepared by mixing an equimolar solution of $NaHCO_3$ and hydrated $CaCl_2$ in DI water. [18] To this solution, phospho-polypeptide was added to obtain a Ca^{+2}/phosphor polypeptide ration between 1:20-160. In a typical experiment, 0.05 mmol of polypeptide or block co-polypeptide was added to 1 mmol of $CaCO_3$ solution. The mixed solution was kept for 15 to 20 days to obtain biomineralized $CaCO_3$ crystals. Biomineralized calcium carbonate was characterized by optical microscopy, SEM, and WXRD technique.

Sample Preperation of Scanning Electron Microscopy

The polymeric solutions were casted on silicon wafers and kept undisturbed till the solvent evaporated completely. Then the samples were kept in a desiccator and dried in high vaccum to remove moisture completely. After that the samples were analyzed by scanning electron microscopy.

Sample Preparation for Transmission Electron Microscopy

Drops of the polymeric solutions (10 micro L) were casted on carbon coated 400 mesh Cu grid and kept for 15-20 min. Then the excess solvent was soaked by Whattman filter paper and the grids were negatively stained by 0.2 wt% uranyl-acetate for 10 seconds, and excess solvent was soaked by Whattman filter paper. Next the grids were washed with deionised water 2-3 times to remove unbound u-a from the grids. After that, the grids were kept in a desiccator and dried in vacuum to remove the moisture completely. Then, the samples were analysed by transmission electron microscopy (TEM).

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References:

[1] P. Cohen, *Nature* **1982**, *296*, 613–620.
[2] P. Donald Voet, J. G. Voet, W. Charlotte, Fundamentals of biochemistry: life at the molecular level (2. ed.). *Hoboken, NJ: Wiley* **2006.**
[3] H. Lodish, A. Berk, S. L. Zipursky, "17.6, Post-Translational Modifications and Quality Control in the Rough ER". *Molecular Cell Biology (4th ed.). New York: W. H. Freeman* **2006**
[4] X. Li, M. Wilmanns, J. Thornton, M. Köhn, *Sci Signal* **2013**, *6*, 10.
[5] F. Sacco, L. Perfetto, L. Castagnoli, G, Cesareni, *FEBS Lett* **2012**, *586*, 2732–2739.
[6] B. Alberts, A. Johnson, J. Lewis, M. Raff, K. Roberts, P. *Walter* Molecular Biology of the Cell. Anderson M and Granum S (eds). 5th edition. *Garland Science, New York, NY* **2007**, 175.
[7] C. S. Rubin, O. M. Rosen, *Annu. Rev. Biochem.* **1975**, *44*, 831–887.
[8] E. ed. Bauerlein, Biomineralization: From Biology to Biotechnology and Medical Application, *Wiley-VCH, Weinheim* **2000**.
[9] M. G. Herring (1972) in The Biochemistry and Physiology of Bone (Bourne, G. H., ed) 2nd Ed, pp. 127-189, Academic Press, New York.
[10] W. T. Bulter, *Eur. J. Oral Sci*. **1998**, *106*, 204.
[11] A. George, J. Hao, *Cells Tissues Organs* **2005**, *181*, 232– 240.
[12] J. Sodek, B. Ganss, M. D. McKee, *Crit. Rev. Oral Biol. Med*. **2000**, *11*, 279–303.
[13] T. Wang, N. Nakabayashi, *J. Dent. Res.* **1991**, *70*, 59–66.
[14] K. Ohkawa, S. Hayashi, N. Kameyama, H. Yamamoto, M. Yamaguchi, S. Kimoto, S. Kurata, H. Shinji, *Macromol. Biosci*. **2009**, *9*, 79–92.
[15] H. Yamamoto, A. Saitoh, K. Ohkawa, *Macromol. Biosci*. **2003**, *3*, 354–363.
[16] K. Ohkawa, A. Saitoh, H. Yamamoto, *Macromol. Rapid Commun*. **1999**, *20*, 619–662.
[17] S. Hayashi, K. Ohkawa, Y. Suwa, T. Sugawara, T. Asami, H. Yamamoto, *Macromol. Biosci*. **2008**, *8*, 46–59.

[18] S. Hayashi, K. Ohkawa, H. Yamamoto, *Macromol. Biosci*. **2006**, *6*, 228–240.
[19] S. Das, M. Kar, S. Sen Gupta, *Polym. Chem*. **2013**, *4*, 4087–4091.
[20] I. Yakovlev, T. J. Deming, *J. Am. Chem. Soc*. **2015**, *137*, 4078–4081.
[21] I. Yakovlev, T. J. Deming, *ACS Macro Lett*. **2014**, *3*, 378–381.
[22] I. Captain,T. J. Deming, *Journal of Polymer Science Part A: Polymer Chemistry* **2016**, *54*, 3707–3712.
[23] H. Lu, J. Wang, Y. Bai, J. W. Lang, S. Liu, Y. Lin, J. Cheng, *Nat. Comm.* **2011**, *2*, 1–9.
[24] Z. Song, N. Zheng, X. Ba, L. Yin, R. Zhang, L. Ma, J. Cheng, *Biomacromolecules* **2014**, *15*, 1491–1497.
[25] R. J. Amir, S. Zhong, D. J. Pochan, C. J. Hawker, *J. Am. Chem. Soc*. **2009**, *131*, 13949–13950.
[26] P. F. Caponi, P. X. Qiu, F. Vilela, F. M. Winnik, R. V. Ulijn, *Polym. Chem*. **2011**, *2*, 306–308.
[27] H. Kuhnle, H. G. Borner, *Angew. Chem., Int. Ed*. **2009**, *48*, 6431–6434.
[28] A. J. Harnoy, I. Rosenbaum, E. Tirosh, Y. Ebenstein, R. Shaharabani, R. Beck, R. J. Amir, *J. Am. Chem. Soc*. **2014**, *136*, 7531−7534.
[29] Z. Feng, T. Zhang, H. Wang, B. Xu, *Chem. Soc. Rev*. **2017**, *46*, 6470.
[30] A. Tanaka, Y. Fukuoka, Y. Morimoto, T. Honjo, D. Koda, M. Goto,T. Maruyama, J. Am. Chem. Soc. **2015**, *137*, 770−775.
[31] S. Hayashi, K. Ohkawa, H. Yamamoto, M. Yamaguchi, S. Kimoto, S. Kurata, H. Shinji, *Macromol. Mater. Eng*. **2009**, *294*, 315–322.
[32] D. Ziwei, J. M. G. Habraken, M. Peeters, A. Heise, G. de With, A. J. M. N. Sommerdijk, *Soft Matter* **2011**, *7*, 9685–9694.
[33] C. R. Navarro, K. Kudłacz, Ö. Cizerc, E. R. Agudo, *Cryst Eng Comm.* **2015**, *17*, 58–72.
[34] M. Nia, B. D. Ratner, *Surf. Interface Anal*. **2008**, *40*, 1356–1361.
[35] Q. Shen, H. Wei, Y. Zhou, Y. Huang, H. Yang, D. Wang, D. Xu *J. Phys. Chem. B.* **2006**, *110*, 2994–3000.
[36] R. Löbbicke, M. Chanana, H. Schlaad, C. Pilz-Allen, C. Günter, H. Möhwald, A. Taubert, *Biomacromolecules* **2011**, *12*, 3753–3760.
[37] A. Shkilnyy, J. Brandt, A. Mantion, O. Paris, H. Schlaad, A. Taubert, *Chem. Mater*. **2009**, *21*, 1572–1578.
[38] Y. Sun, Y. Hou, X. Zhou, J. Yuan, J. Wang, H. Lu, *ACS Macro Lett.* **2015**, *4*, 1000–1003.
[39] S. Das, N. Parekh, B. Mondal, S. Sen Gupta, *ACS Macro Lett*. **2016**, *5*, 809−813.
[40] Z. Song, N. Zheng, X. Ba, L. Yin, R. Zhang, L. Ma, J. J. Cheng, *Biomacromolecules* **2014**, *15*, 1491−1497.
[41] L. Guo, D. Zhang, *J. Am. Chem. Soc*. **2009**, *131*, 18072–18074.
[42] H. Lu, Y. Bai, J. Wang, N. P. Gabrielson, F. Wang, Y. Lin, J. J. Cheng, *Macromolecules* **2011**, *44*, 6237–6240.
[43] N. E. Kamber, W. Jeong, R. M. Waymouth, *Chem. Rev*. **2007**, *107*, 5813−5840.
[44] P. M. Rendle, A. Seger, J. Rodrigues, N. J. Oldham, R. R. Bott, J. B. Jones, M. M. Cowan, B. G. Davis, *J. Am. Chem. Soc.* **2004**, *126*, 4750–4751.
[45] K. A. Staines, V. E. MacRae, C. Farquharson, *J. Endocrinol* **2012**, *14*, 241–55.
[46] P. L. Golas, K. Matyjaszewski, *Chem. Soc. Rev*. **2010**, *39*, 1338−1354
[47] M. Kar, P. Minios, K. Sharma, G. Kumaraswamy, S. Sen Gupta, *Langmuir* **2011**, *27*,12124
[48] M. Kar, B. Malvi, A. Das, S. Panneri, S. Sen Gupta, *J. Mat. Chem*. **2011**, *2*, 6690.
[49] B. Malvi, S. Sen Gupta, *Chem. Comm*. **2012**, *48*, 7853.
[50] V. Dhaware, A.Y. Shaikh, M. Kar, S.Hotha, S. Sen Gupta, *Langmuir* **2013**, *29*, 5659.
[51] D. Pati, S. Das, N. Patil, N. Parekh, D. Anjum, V. Dhaware, A. V. Ambade, S. Sen Gupta, *Biomacromolecues* **2016**, *17*, 466.

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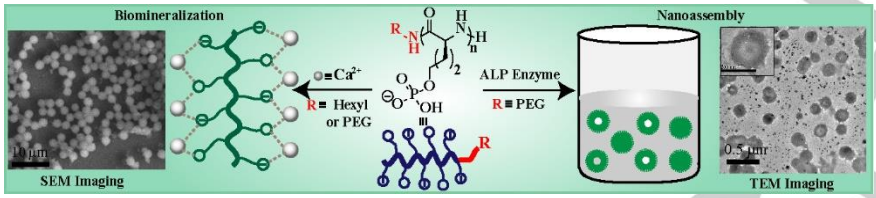

An easy synthetic strategy was developed to synthesize phosphate functionalized polypeptide from amino acid N-carboxyanhydride (NCA). The deprotected phosphate-based polypeptides displayed pH-dependent helical conformation, which further changed to β-sheet upon addition of the enzyme alkaline phosphatase(ALP) due to dephosphorylation. The block co-polypeptide led to its self-assembly into temporally controlled colloidal structures due to the formation of amphiphilic block co-polymer upon dephosphorylation. Finally, the phospho-polypeptides induce the mineralization of calcium carbonate with varying polymorphs and morphologies.