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Methionine sulfoxide and phosphonate containing double hydrophilic block copolypeptides and their mineralization of calcium carbonate

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ABSTRACT: In effort to address challenges in the efficient synthesis of highly functional block copolypeptides, we report use of a combination of functional monomer polymerization and post-polymerization modification to obtain new double hydrophilic block copolypeptides with desirable properties. We prepared copolymers that contain discrete hydrophilic, non-ionic poly(L-methionine sulfoxide) and Ca^{2+} ion binding poly(L-phosphonohomoalanine) segments. The facile and selective post-polymerization conversion of inexpensive, readily prepared poly(L-methionine) segments into non-ionic, hydrophilic poly(L-methionine sulfoxide) segments reduces the need for use of combinations of protecting groups. The complex copolypeptides prepared using this strategy were able to promote formation of $CaCO_3$ microspheres with tunable polymorphism.

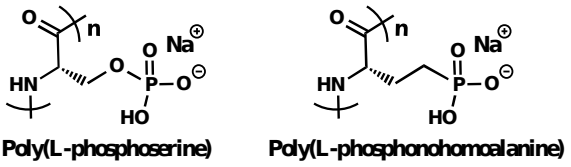
KEYWORDS: block copolypeptides, hydrophilic polypeptides, stimuli-sensitive polymer, calcium carbonate, microspheres.

INTRODUCTION

The ability to readily prepare complex copolymers that contain a diverse array of side-chain functional groups is an ongoing goal in the field of polymer synthesis.[1-3] Desirable functionalities include those that impart stimuli responsiveness,[4] ability to bind to select molecules,[5] as well as undergo efficient reactions with specific partners.[2,6] As the desire for increasingly complex functional polymers continues, obtaining compatibility between functional groups and polymerization chemistries has become a challenging problem. This is especially true for the polypeptide field, where high monomer reactivity limits the types of functional side-chain groups that can be used, and often requires use of cumbersome protecting group strategies.[1-3] Here, we report the use of a combination of functional monomer polymerization and post-polymerization modification to obtain new, highly functional, double hydrophilic block copolypeptides with desirable properties. This strategy provides an efficient method to prepare complex copolypeptides that were used to promote formation of $CaCO_3$ microspheres with tunable polymorphism.

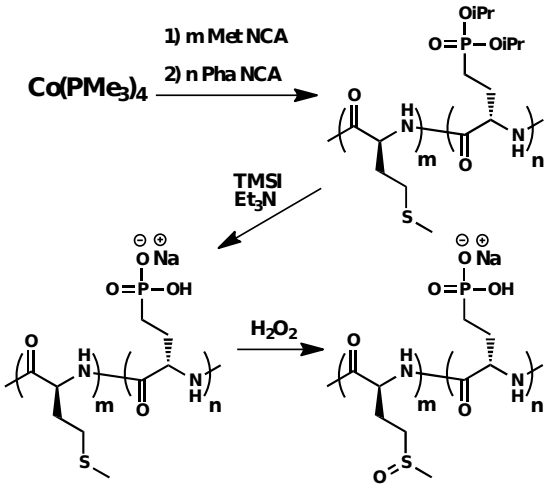
Inorganic materials of specific shape, size, and polymorph are valuable in the fields of nano-materials,[7] ceramics,[8] electronics,[9] and medicine.[10] Biological systems have been expertly forming minerals for millennia, mainly through use of organic molecules as control agents.[7] Much effort has gone into mimicking these natural processes using synthetic polymers, which have successfully been used to control many aspects of mineralization processes.[11,12] $CaCO_3$, in particular, has received considerable attention in these studies due to its importance as a biomineral in many organisms, its use in paper and paint industries, and since it is a major component in scale

formation.[13-15] Numerous reports have described use of double hydrophilic block copolymers (DHBC)[16-18] as well as polypeptides[19-22] to influence $CaCO_3$ formation. Most of these polymers employed multiple carboxylate side-chain groups,[16,19-22] although phosphate[16] and phosphonate[18] containing polymers were also studied since these groups can interact more strongly with Ca^{2+} ions.[11,12] In particular, Yamamoto's lab reported the use of phosphoserine and phosphothreonine polymers as additives to modify $CaCO_3$ formation.[23,24] An ongoing challenge in use of these phosphorylated polymers has been their synthesis and purification, which is complicated by the chemistry of phosphate and phosphonate functional groups.[23-26] We recently reported a more stable, isosteric analog of poly(L-phosphoserine), poly(L-phosphono-homoalanine), **Pha**, (Scheme 1), which allows for facile syntheses and incorporation into block copolypeptides.[27]



Scheme 1. Structures of Phosphorylated Polypeptides

To demonstrate the utility of **Pha**, we pursued its incorporation it into block copolymers to impart Ca^{2+} binding properties. A common non-interacting hydrophilic segment used in DHBC is PEG, which is non-ionic and considered essentially inert in $CaCO_3$ mineralization.[11] To create a fully degradable copolymer, we sought to replace PEG with a non-ionic polypeptide segment. We chose poly(L-methionine sulfoxide), $\mathbf{M^O}$, as a candidate inert segment since it is water soluble, possesses a disordered conformation, is enzymatically degradable, and can be incorporated using an inexpensive natural amino acid in a facile synthetic process.[28,29] Hence, we designed poly(L-methionine sulfoxide)$_m$-*b*-poly(L-phosphono-homoalanine)$_n$, $\mathbf{M^O_mPha_n}$, (Scheme 2), as new DHBCs for Ca^{2+} binding.



Scheme 2 Synthesis of $M^O_mPha_n$ Diblock Copolypeptides.

EXPERIMENTAL

Materials

Unless otherwise stated, reactions were carried out in oven-dried glassware under an atmosphere of N_2 using anhydrous solvents. Tetrahydrofuran (THF), hexanes, DCM (dichloromethane), and toluene were dried by passage through activated alumina-packed columns under N_2. All chemicals were purchased from Aldrich, Spectrum, Alfa Aesar, Bachem, or CombiBlokcs and were used as received. Deionized (DI) water (18 MΩ-cm) was obtaining by passing in-house deionized water through a Millipore Milli-Q Biocel A10 purification unit.

Instrumentation

^{1}H NMR spectra were recorded at 500MHz on Bruker instruments; ^{13}C NMR spectra were recorded on 125MHz Bruker instruments. All Fourier Transform Infrared (FTIR) spectra were recorded on a Perkin Elmer RX1 FTIR spectrometer. Circular dichroism spectra were recorded on an OLIS RSM CD spectrophotometer. SEM images were recorded on a JEOL SM-71010 scanning electron microscope. The samples were imaged using a

Zeiss Axiovert 200 DIC/Fluorescence Inverted Optical Microscope. Powder X-ray data were obtained on a Panalytical X'Pert Pro X-ray Powder Diffractometer.

Polymer Synthesis

Poly(L-methionine)$_n$-block-PEG$_{22}$, M$_n$PEG$_{22}$.

The polymerization of L-methionine N-carboxyanhydride, Met NCA,[1] was performed in a N_2 filled glove box using anhydrous, N_2 purged solvents. To a solution of Met NCA (200 µl, 50 mg/ml, 5.7x10^{-5} mol) in THF was added the desired volume of Co(PMe$_3$)$_4$ (10 mg/ml) in THF.[3] The polymerization was allowed to proceed for 2 hours, at which point all NCA was consumed as confirmed by FTIR. The reaction was then treated with an excess of □-methoxy-□-isocyanoethyl-poly(ethylene glycol)$_{22}$, PEG$_{22}$-NCO, MW = 1000 Da, 3 eq per Co(PMe$_3$)$_4$).[4] The resulting solution was allowed to react for 3 hours. The reaction was then brought outside of the glove box and was precipitated into 1.0 M HCl (1.0 ml); the pellet was collected by centrifugation and was washed two times with DI H_2O (1.0 ml). The polymer was collected by centrifugation and the residual water was removed by lyophilization to give the product. Integration of the methionine CH_2 resonance at δ 2.71 versus the PEG resonance at δ 3.93 was used to obtain poly(L-methionine) length. ^{1}H NMR of Poly(L-methionine)$_{50}$-*block*PEG$_{22}$ (500 MHz, *d*TFA) δ 4.88 (CH, 47H, br s), 3.93 (OCH_2CH_2O, 90H, br s), 2.71 ($SC\underline{H}_2CH_2$, 100H, br s), 2.19 ($SCH_2C\underline{H}_2$ + SCH_3, 245H).

General procedure for synthesis of M$_m$(iPr$_2$Pha)$_n$.

The copolymerizations were performed in a N_2 filled glove box using anhydrous, N_2 purged solvents. To a solution of Met NCA (200 µl, 50 mg/ml, 5.7x10^{-5} mol) in THF was added the desired volume of Co(PMe$_3$)$_4$ (10 mg/ml) in THF. The polymerization was allowed to proceed for 2 hours, at which point all NCA was consumed as confirmed by FTIR. A desired amount of L-diisopropylphosphonohomoalanine N-carboxyanhydride, Pha NCA[2] (50 mg/ml) in THF (see Table 1) was then added and the polymerization was allowed to proceed overnight. The next day, consumption of the second NCA was confirmed by FTIR and the polymer was isolated by precipitation into 1.0 M HCl (1.0 ml per 10 mg of combined NCAs), followed by resuspension in water (1.0 ml per 10 mg of combined NCAs) and centrifugation to collect the pellet. The residual water was removed by lyophilization to give the polymer as a white, stringy solid. Integration of the methionine CH_2 resonance at δ 2.77 versus the iPr$_2$Pha CH_3 resonances at δ 1.41 was used to obtain Pha segment lengths. ^{1}H NMR of M$_{50}$(iPr$_2$Pha)$_5$ (500 MHz, *d*TFA) δ 4.76-5.03 (CH + POCH, 67H), 2.77 ($SC\underline{H}_2CH_2$, 100H, br s), 1.97-2.43 (SCH_3 + $SCH_2C\underline{H}_2$ + $PC\underline{H}_2C\underline{H}_2$, 273H), 1.41 (($C\underline{H}_3)_2CH$, 58H). FTIR (THF): 1644, 1550 cm^{-1}. 92% yield.

General Procedure for synthesis of M^O$_m$Pha$_n$.

A sample of **M$_m$(iPr$_2$Pha)$_n$** was dissolved in anhydrous dichloromethane (20-30 mg/ml) and treated with iodotrimethylsilane (10 eq. per iPr$_2$Pha residue) and triethylamine (8 eq. per iPr$_2$Pha residue). The reaction was sealed under N_2 and stirred at 50 °C overnight. The next day, the solution was allowed to cool to room temperature and was treated with isopropanol (1 ml per ml of reaction mixture). The resulting solution was then precipitated into hexane (3 ml per ml of reaction mixture) and the precipitate formed was collected by centrifugation. The pellet was stripped of volatiles under high vacuum and the residue was suspended in 0.1 M HCl (1 ml per 10 mg copolymer) and then collected by centrifugation once again. The pellet was resuspended in DI water (~1 ml per 20 mg copolymer). This suspension was treated with 30% hydrogen peroxide (3 µl per mg copolymer) and was stirred at 38 °C for 2 hours. Next, the resulting clear solution was cooled to 0 °C and treated with concentrated sodium thiosulfate to quench excess hydrogen peroxide. The solution was then transferred to a 2000 MWCO dialysis bag and was dialyzed against 0.1 M sodium sulfite followed by 0.1 M NaCl, followed by water for 3 more days with twice daily water changes. Once dialysis was

completed, the water was removed by lyophilization to afford the product as a fluffy, stringy solid. ^{1}H NMR of $M^{O}_{65}Pha_{20}$ (500 MHz, D_2O) δ 4.49 (66H, br s), 2.90-3.05 (137H, br s), 2.73 (195H, br s), 2.27 (70H, br s), 2.19 (64H, br s), 1.26 (82H, br s), 0.88 (35H, br s). 89% yield.

$CaCO_3$ mineralization studies

Preparation of $CaCO_3$ in the presence of polypeptides

A desired concentration of copolymer was dissolved in 10 ml of Millipore water in a 50 ml conical centrifuge tube. 160 µl of 0.5 M Na_2CO_3 were then added and the solution pH was lowered to 10 using 1.2 M HCl. The solution was then vortexed and 160 µl of 0.5 M $CaCl_2$ were added in a single portion. The solution was vortexed for another 2 minutes and was left undisturbed for 24 hours. The precipitated solids were then collected by centrifugation, washed twice with Millipore water, and then freeze dried.

Light microscopy of $CaCO_3$ samples.

$CaCO_3$ samples were imaged either in wet or dry state. For dry imaging, a small amount of sample was transferred onto a microscope slide. Sample was spread across the slide using a piece of filter paper and was directly imaged. For higher magnification, a drop of water was added onto the sample containing slide and the resulting suspension capped with a cover slip. A drop of oil was then placed on top of the cover slip to obtain images using an oil immersion objective.

Extraction of copolypeptide from $CaCO_3$ sample.

A sample of $CaCO_3$ microspheres prepared using $\mathbf{M^{O}_{65}Pha_{20}}$ was dissolved in 1.2 M HCl. The resulting solution was transferred into a 2000 MWCO dialysis bag and was dialyzed against DI water for 3 days with twice daily water changes. The water from the sample was removed by lyophilization and the resulting stringy solid was weighed and confirmed to be $\mathbf{M^{O}_{65}Pha_{20}}$ by ^{1}H NMR. >90% recovery of copolypeptide from the $CaCO_3$ spheres was obtained.

RESULTS AND DISCUSSION

Since the Ca^{2+} binding properties of phosphonohomoalanine residues had not been previously studied, we performed initial investigations using $\mathbf{Pha_{20}}$ homopolymer.[27] We found that upon exposure to soluble Ca^{2+} ions, i.e. $CaCl_2$ in water, solutions of $\mathbf{Pha_{20}}$ at 20 °C formed insoluble polymer aggregates over time (see SI, Figure S1). Precipitates of $\mathbf{Pha_{20}}$ formed upon addition of $CaCl_2$, but not after addition of either NaCl or $MgCl_2$. These observations suggest that $\mathbf{Pha_{20}}$ precipitation stems from binding to Ca^{2+} ions, and not due to salting out. Circular dichroism (CD) spectra have shown that $\mathbf{Pha_{20}}$ adopts a disordered conformation in water at neutral pH and an α-helical conformation in strongly acidic solution (pH < 2).[27] Addition of $CaCl_2$ to $\mathbf{Pha_{20}}$ under dilute conditions gave solutions that could be analyzed by CD spectroscopy. A change in polypeptide conformation upon addition of $CaCl_2$ was observed, but was still indicative of a highly disordered conformation (see Figure S1). In the solid-state, FTIR analysis of precipitate formed from $CaCl_2$ and $\mathbf{Pha_{20}}$ revealed predominant Amide I and II bands at 1620 and 1535 cm^{-1}, which indicated the presence of significant β-sheet structure (see Figure S2).[30] This change in conformation from the protonated form of $\mathbf{Pha_{20}}$ is likely due to the different steric environment that results from Ca^{2+} ion binding and possible bridging of phosphate groups. These observations show that the phosphonate groups of **Pha** are able to bind well to Ca^{2+} ions, similar to the phosphate groups of poly(L-phosphoserine),[23,24] and that **Pha** may be useful in controlling $CaCO_3$ mineralization.

TABLE 1 Synthesis of Diblock Copolypeptides

	First monomer	Second monomer	First segment[b]		Diblock copolymer[c]		
Sample	Met NCA [a]	Pha NCA [a]	M_n	DP [d]	M_n	DP[d]	Yield (%)[e]
$M_{50}(iPr_2Pha)_5$	22	4	6 600	50	7 800	55	92
$M_{50}(iPr_2Pha)_{13}$	22	7	6 600	50	9 800	63	90
$M_{50}(iPr_2Pha)_{20}$	22	11	6 600	50	11 500	70	91
$M_{50}(iPr_2Pha)_{29}$	22	15	6 600	50	13 800	79	92
$M_{65}(iPr_2Pha)_{20}$	28	11	8 600	65	13 500	85	96

[a] Number of equivalents of monomer per $Co(PMe_3)_4$ initiator. [b] Molecular weight of first segments determined for PEG_{22} end-capped samples using ^1H NMR. [c] Molecular weight of diblock copolymers determined using ^1H NMR. [d] DP = degree of polymerization. [e] Total isolated yield of purified, protected diblock copolypeptides.

Based on previous studies, it is known that DHBC containing a Ca^{2+} ion binding segment are capable of directing formation of $CaCO_3$ microspheres.[16,17,20] Hence, we synthesized copolypeptides of **$M^O_mPha_n$** (Scheme 2), which contain discrete hydrophilic, non-ionic **M^O** and Ca^{2+} ion binding **Pha** segments. Initially, we prepared a series of precursor copolypeptides composed of poly(L-methionine)$_{50}$-*b*-poly(L-diisopropylphosphonohomo-alanine)$_n$, **$M_{50}(iPr_2Pha)_n$** (n = 5 – 29) (Table 1) using conditions previously shown to give living polymerization of both monomers.[27,29] Confirmation of controlled growth of initial **M** segments of desired length was obtained by quantitative end-capping of chains with isocyanate terminated PEG and endgroup analysis by ^1H NMR (see SI).[31] Confirmation of successful diblock copolypeptide formation and copolymer compositions were obtained by comparison of **M** and **Pha** resonances in ^1H NMR spectra of purified copolymers (see SI). Since **M** polymers are not soluble in common solvents used for GPC,[29] polydispersity indices could not be determined for these samples. Efficient deprotection of **iPr_2Pha** segments was carried out using iodotrimethylsilane (TMSI),[27] and **M** segments were then fully oxidized to **M^O** using dilute H_2O_2 in water at 37 °C (Scheme 2).[29] The order of deprotection and oxidation is important, since TMSI can reduce sulfoxides to thioethers.[32,33] No change in copolymer composition after deprotection, oxidiation and purification was observed, indicating lack of any chain cleavage. Use of **M** as a hydrophilic segment precursor offers many advantages including no need for protection, compatibility with many deprotection conditions, and facile conversion to hydrophilic **M^O** under mild conditions.[29]

The ability of **$M^O_{50}Pha_n$** copolypeptides to affect $CaCO_3$ mineralization was studied using a modified literature procedure.[20] The copolymers were separately dissolved in 0.5 M Na_2CO_3 that was adjusted to pH 10 using 1.0 M HCl. A solution of 0.5 M $CaCl_2$ was quickly added to each sample with vortexing, which was continued for 2 min. Solids formed over a 24 h period, and were then washed with DI water and collected by centrifugation. The solids were lyophilized and then examined by light microscopy. All **$M^O_{50}Pha_n$** copolypeptides gave $CaCO_3$ precipitates, yet **$M^O_{50}Pha_{13}$** and **$M^O_{50}Pha_{20}$** were found to be good compositions for consistent microsphere formation. **$M^O_{50}Pha_5$** yielded only irregular precipitates. Similar to observations made with other DHBCs, the phosphonate segments of **$M^O_{50}Pha_n$** bind with Ca^{2+} ions in solution, likely causing a local supersaturation.[11,16] The **M^O** segments can act as steric stabilizers to limit aggregation of **Pha** segments, which constrains mineral growth.

Based on these initial results, we prepared a larger batch of diblock copolypeptide of similar

composition ($\mathbf{M^{o}_{65}Pha_{20}}$, **1**, Table 1) for more detailed studies. Copolymer **1** has good water solubility and possesses a disordered conformation in solution, as expected for both the $\mathbf{M^{o}}$ and **Pha** segments (see Figure S3).[27,29] $CaCO_3$ microspheres were also obtained when **1** was used as an additive in the mineralization procedure described above. The product solids were imaged using both SEM and DIC light microscopy, which showed that only microspheres were formed at 0.1 mg/ml copolymer (Figure 1a,b). More irregular particles were formed at higher compolymer concentrations and prismatic $CaCO_3$ crystals were formed at lower copolymer concentrations (see Figure S4). Control experiments with no copolymer (see Figure S5) or $\mathbf{M^{o}_{50}}$ both yielded only prismatic $CaCO_3$ crystals (Figure 1c), showing that $\mathbf{M^{o}}$ does not affect with mineral growth. A mineralization experiment using $\mathbf{Pha_{20}}$ homopolymer gave irregular, large fused spheres of $CaCO_3$ (Figure 1d). These results showed that both domains of diblock copolypeptide **1** were necessary for the selective formation of $CaCO_3$ microspheres.

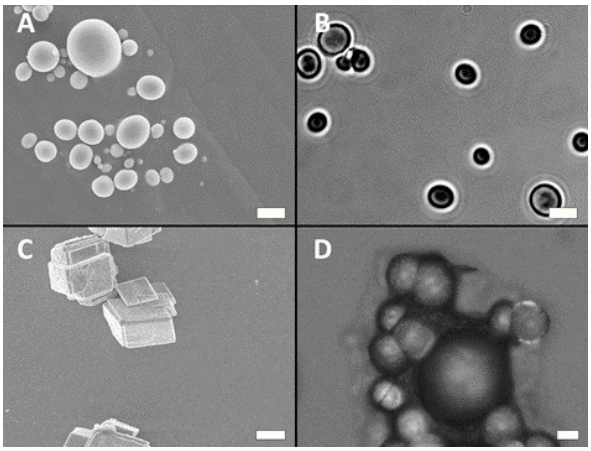


Figure 1. Images of $CaCO_3$ prepared in the presence of different polymers. (A) SEM image of $CaCO_3$ microspheres prepared using **1**. (B) DIC light microscopy image of $CaCO_3$ microspheres prepared using **1**. (C) SEM image of $CaCO_3$ prepared using $\mathbf{M^{o}_{50}}$. (D) DIC light microscopy image of $CaCO_3$ prepared using $\mathbf{Pha_{20}}$. All polymer concentrations were 0.1 mg/ml. All scale bars = 5 μm.

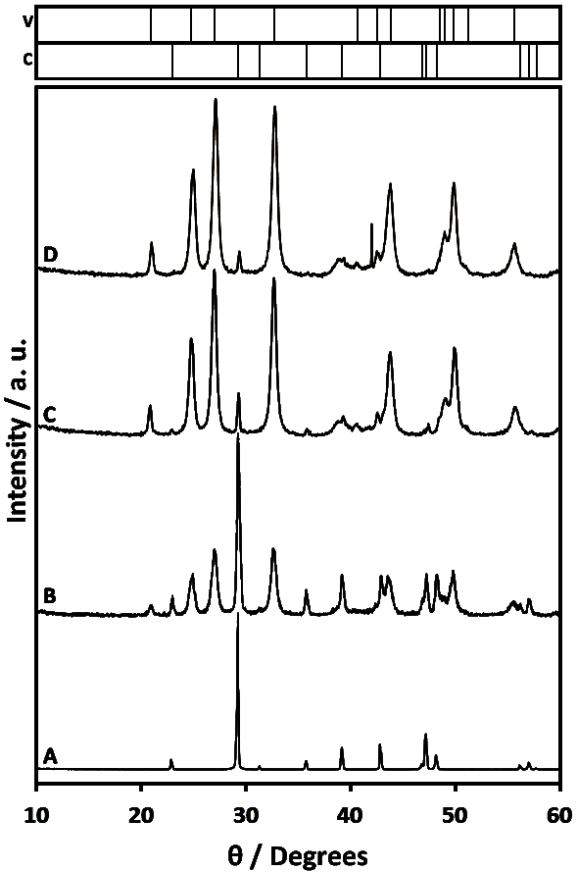


Figure 2 Wide-angle X-ray diffractograms of $CaCO_3$ samples prepared using different rates of $CaCl_2$ addition to solutions of **1**. (A) Control with no copolymer **1**. (B) $CaCl_2$ added all at once. (C) $CaCl_2$ added over one minute. (D) $CaCl_2$ added over two minutes. Diffraction patterns for different polymorphs of $CaCO_3$ are shown at top of figure: vaterite (v) and calcite (c).

Powder wide-angle X-ray diffraction experiments on the mineralized products confirmed that crystalline $CaCO_3$ was obtained under all mineralization conditions (Figure 2). Without any copolymer additive, the most stable polymorph, calcite, was obtained as expected (Figure 2a).[13-15] The $CaCO_3$ microspheres obtained in the presence of **1** were comprised of a mixture of calcite and vaterite polymorphs (Figure 2b-d). The rate of addition of $CaCl_2$ into solutions of **1** was found to greatly affect the ratio of these two polymorphs. Rapid mixing favored increased calcite formation (Figure 2b), but slow addition

of $CaCl_2$ favored the less stable vaterite (Figure 2c,d). Addition of $CaCl_2$ over a two minute period gave microspheres composed primarily of vaterite (Figure 2d). Formation of polycrystalline vaterite microspheres has also been observed with other DHBC systems.[16,21] To confirm the incorporation of **1** in the microspheres, a sample of $CaCO_3$ spheres was dissolved in 1.2 M HCl and the resulting solution was dialyzed against DI water for 3 days. Following lyophilization, copolymer **1** was recovered from the sample in 90% yield.

CONCLUSIONS

In summary, we have developed an efficient synthetic method for preparation of double hydrophilic block copolypeptides with new functionality. This method utilizes the facile and selective post-polymerization conversion of inexpensive, readily prepared **M** segments into non-ionic, hydrophilic $\mathbf{M^o}$ segments, which lessens the need for, and concerns of compatibility among, multiple protecting groups. The combination of non-ionic and phosphonate containing segments in **1** are unprecedented, and provide a new structural motif for polypeptides, beyond glutamate and aspartate polymers,[19-22] that can promote the formation of $CaCO_3$ microspheres with tunable polymoprhism.

ACKNOWLEDGEMENTS

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GRAPHICAL ABSTRACT

Ilya Captain and Timothy Deming

Methionine sulfoxide and phosphonate containing double hydrophilic block copolypeptides and their mineralization of calcium carbonate

Double hydrophilic methionine-phosphonohomoalaine peptide block copolymers were prepared. The facile and selective post-polymerication conversion of inexpensive, readily prepared poly(L-methionine) segments into non-ionic, hydrophilic poly(L-methionine sulfoxide) segments reduces the need for protecting group use. The complex copolypeptides prepared using this strategy were able to promote formation of $CaCO_3$ microspheres with tunable polymorphism.

GRAPHICAL ABSTRACT FIGURE