Altering the Peptide Binding Selectivity of Polymeric Reverse Micelle Assemblies via Metal Ion Loading

Meizhe Wang,‡ Bo Zhao,‡ Jingjing Gao,‡ Huan He,‡ Laura J. Castellanos,‡ S. Thayumanavan,*†,‡,§ and Richard W. Vachet§,*†,‡

†Department of Chemistry, ‡Center for Bioactive Delivery—Institute for Applied Life Sciences, and §Molecular and Cellular Biology Program, University of Massachusetts, Amherst, Massachusetts 01003, United States

ABSTRACT: Supramolecular reverse micelle assemblies, formed by amphiphilic copolymers, can selectively encapsulate molecules in their interiors depending on the functional groups present in the polymers. Altering the binding selectivity of these materials typically requires the synthesis of alternate functional groups. Here, we demonstrate that the addition of Zr(IV) ions to the interiors of reverse micelles having phosphonate functional groups transforms the supramolecular materials from ones that selectively bind positively charged peptides into materials that selectively bind phosphorylated peptides. We also show that the binding selectivity of these reverse micelle assemblies can be further tuned by varying the fractions of phosphonate groups in the copolymer structure. The optimized reverse micelle materials can selectively transfer and bind phosphorylated peptides from aqueous solutions over a wide range of pH conditions and can selectively enrich phosphorylated peptides even in complicated mixtures.

INTRODUCTION

Supramolecular polymeric systems, including micelles, reverse micelles, vesicles, polymersomes, and gels, have been widely studied for the encapsulation, detection, and targeted delivery of biomolecules.1−7 A key characteristic of supramolecular systems is their ability to be tuned via changes at the molecular level. Noncovalent interactions, including electrostatic interactions, H-bonding, π−π stacking, metal−ligand coordination, and general host−guest interactions, are often used for this purpose. Metal−ligand coordination and host−guest complexation are particularly useful in constructing supramolecular systems.8−10 Metal−ligand interactions offer various coordination geometries, strong yet tunable binding abilities,11,12 and unique electronic or magnetic properties.13−15 For example, strands of DNA can be self-assembled in the presence of copper ions, and controlling the spacing between the ions can impart ferromagnetism to the materials.16 In addition, polymeric materials assembled with reversible metal−ligand interactions can be made to have impressive self-healing properties.17 Host−guest interactions can also endow materials with good selectivity and adaptive capability, especially in response to external stimuli such as pH, ligand binding, or light.18−28 As an example, polymeric nanogels with acetol- and ketal-based linkers show a variable encapsulation/release ability for guest molecules via pH-dependent cross-linker degradation.21

Given the promising attributes of supramolecular systems, our group has been exploring supramolecular assemblies formed by amphiphilic polymers as enrichment agents for the selective detection of biomolecules in complex mixtures.29−35 These amphiphilic polymers, either homopolymers or copolymers, consist of hydrophobic and functional hydrophilic moieties that can self-assemble into reverse micelles in apolar solvents. Once assembled, they act as nanocontainers that selectively enrich biomolecules from aqueous solution into their interiors by bringing them across the solution−solution interface. This feature has allowed them to be used to extract peptides with specific pI values, generate titration curves for individual peptides in a mixture, and enrich biomarkers in human serum for high-sensitivity detection by mass spectrometry (MS).30−35 Although these supramolecular materials have been effective at enriching molecules based on complementary charge, altering the selectivity of these promising materials is an important goal that typically requires the synthesis of new functional polymers. In this work, we have explored changes in the enrichment selectivity of these polymeric reverse micelles via the addition of metal ions. We find that by loading the reverse micelles with Zr(IV) ions we can dramatically change the selectivity of the materials so that they specifically bind phosphopeptides (Scheme 1). Further tuning of the selectivity and efficiency of the enrichment process can be accomplished by varying the polymer architecture. The resulting materials can selectively enrich phosphorylated peptides, which are present in low levels in protein mixtures, thereby offering a potentially new approach for studying protein phosphorylation, which is important for a variety of biological phenomena.36−40

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Scheme 1. Schematic Illustration of Polymeric Reverse Micelles Having Phosphonate Functional Groups Loaded with Zr(IV) That Can Selectively Bind Phosphopeptides

■ EXPERIMENTAL METHODS

Materials. α-Casein, β-casein, chicken ovalbumin, bovine serum albumin (BSA), lysozyme, dithiothreitol (DTT), iodoacetamide (IAM), phosphoric acid (H₃PO₄), and zirconium(IV) oxychloride octahydrate (ZrOCl₂·8H₂O) were obtained from Sigma-Aldrich. 2,5-Dihydroxybenzoic acid (DHB), Tris hydrochloride, toluene, and tetrahydrofuran (THF) were purchased from Fisher Scientific. Trypsin was obtained from Promega. Urea was purchased from MP Biomedicals. Ammonium bicarbonate (NH₄HCO₃) was obtained from Biomedicals. Trypsin was obtained using a Milli-Q water purification system (Millipore, Bedford, MA). All other chemicals were obtained from commercial sources.

Polymer Synthesis. Amphiphilic random copolymers P1−P4 bearing hydrophobic p-alkoxy moieties and hydrophilic variable phosphonate, pentaethylene glycol monomethyl ether (PEG), or carbosilane groups that were used in this study are shown in Scheme 2. All monomers were synthesized through Wittig reactions of the corresponding aldehydes, and the polymerizations were carried out using nitroxide-mediated radical polymerization (NMP). The molecular weight of each polymer was measured by gel permeation chromatography (GPC). The ratios of repeating units in each polymer were calculated by nuclear magnetic resonance (NMR). Details of the synthesis and characterization of these polymers can be found in the Supporting Information. Amphiphilic homopolymer P5 was synthesized as previously described¹ and used for control experiments.

Preparation of Zirconium(IV) Ion-Loaded Polymeric Reverse Micelles. Reverse micelle solutions of polymer P1 were prepared by dissolving 0.7 mg of polymer P1 in 10 mL toluene to obtain a phosphonate functional group concentration of 100 μM. ZrOCl₂·8H₂O was dissolved in water and added to the toluene solution at different molar ratios of Zr to the phosphonate group (0.1, 0.2, 0.5, 1, 2, 3, and 5). Sonication was conducted until the solution became optically clear. Zr-loaded reverse micelle solutions of polymer P2−P4 were prepared in the same way to obtain 100 μM phosphonate or PEG groups. These solutions were used for the liquid−liquid extraction.

Preparation of Protein Digests. α-Casein and β-casein were dissolved in 50 mM NH₄HCO₃ (pH 8.2) to a concentration of 50 μM each and digested for 12 h with trypsin at an enzyme-to-protein ratio of 1:100 (w/w). Chicken ovalbumin, BSA, and lysozyme were dissolved to concentrations of 50 μM each in 50 mM NH₄HCO₃ (pH 8.2) containing 8 M urea. DTT was added to a final concentration of 5 mM, and the mixture was incubated at 37 °C for 1 h with gentle agitation to reduce the disulfide bonds in the proteins. IAM was added to a final concentration of 10 mM in the solution and was incubated at RT for 30 min in the dark to alkylate the reduced disulfide bonds. DTT was added to obtain a final concentration of 5 mM again and incubated at RT for 30 min in the dark to stop overalkylation. The solution was diluted with 50 mM NH₄HCO₃ to reduce the urea concentration to 1.6 M. Trypsin was added at an enzyme-to-protein ratio of 1:100 and incubated for 12 h at 37 °C.

Liquid−Liquid Extractions. Before extraction, the protein digests that were prepared as described above were diluted at least 100-fold with 50 mM Tris buffer and adjusted to the desired pH using HCl or NH₄OH. Two hundred microliters of the polymeric reverse micelle solution was added to 1 mL of the peptide solution and vortex mixed vigorously for 1.5 h. Centrifugation at 15 000 rpm for 30 min was employed to break the resulting emulsion and separate the two phases. The aqueous phase was removed, and the organic phase was dried by blowing N₂ gas. This dried residue was redissolved in 20 μL of THF and mixed with 20 μL of a DHB matrix solution (25 mg/mL in 70% (v/v) acetonitrile containing 1% (w/v) H₃PO₄). One microliter of this solution was directly spotted on the matrix-assisted laser desorption/ionization (MALDI) target for analysis.

Instrumentation. MALDI-MS analyses were performed on a Bruker Autoflex III time-of-flight mass spectrometer. All mass spectra were obtained in negative linear mode and represent an average of 400 shots acquired at 34% laser power with an accelerating voltage of 19 kV. Dynamic light scattering (DLS) measurements were performed using a Malvern Zetasizer. FT-IR spectra were recorded on a PerkinElmer Spectrum 100 FT-IR spectrometer. Inductively coupled plasma (ICP)−MS data were obtained on a PerkinElmer Nexion 300 ICP mass spectrometer. Total Zr concentrations loaded into polymer P1 were determined by adding 0.5 mL of fresh aqua regia for 30 min after the evaporation of toluene in 10 μL of polymer solutions. Total Zr concentrations in the aqueous phase after extraction were measured by directly adding 0.5 mL of fresh aqua regia to 100 μL of the aqueous solutions for 30 min. The resulting solutions were then diluted to 10 mL with deionized water for the ICP−MS measurements. A series of Zr standard solutions (0, 0.2, 0.5, 1, 2, 5, 10, and 20 ppb) were prepared in 5% aqua regia for the calibration of ICP−MS measurements. ¹H NMR spectra were recorded on a 400 MHz NMR spectrometer using residual proton resonance of the solvents as an internal standard. Chemical shifts are reported in parts per million (ppm).

Scheme 2. Chemical Structures of Amphiphilic Random Copolymers P1−P4 and Amphiphilic Homopolymer P5

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The goal of the current work is to test whether the selectivity of these polymers could be varied by simply adding metal ions to the reverse micelle solution. To test this idea, we loaded the reverse micelle interiors with Zr(IV), which has a high affinity for phosphate groups. We hypothesized that the addition of this metal would convert the interior from being negatively charged to an interior that presented coordinated Zr(IV) ions capable of selectively binding phosphopeptides. We were indeed pleased to find that upon extracting the same protein digest, which contains three phosphoproteins (i.e., α-casein, β-casein, and ovalbumin), the polymer assembly’s selectivity changes dramatically. The mass spectrum is now dominated by phosphopeptides (Figure 1c and Table S3), indicating that the driving force for extraction has been converted from electrostatic interactions to Zr(IV)−phosphate interactions.

The Zr(IV)−polymer assemblies were characterized in several ways to assess the nature of the resulting materials. First, DLS of the polymers in toluene with and without Zr(IV) demonstrate the formation of assemblies with narrow size distributions (Figure 2a) that change from about 90 to 120 nm upon adding Zr. These sizes are consistent with the sizes of polymeric reverse micelles that have been studied previously. Measuring the sizes of the assemblies after extraction of the aqueous phase is complicated by the formation of an interfacial layer between the phases. Second, the complexation of Zr by the phosphonate groups in polymer P1 was confirmed by FT-IR measurements (Figure 2b). After loading metal, the P−O stretch of the phosphonate group shifts from 1241 to 1192 cm$^{-1}$, which is consistent with coordination between Zr(IV) and the phosphonate group, causing the formation of longer P−O bonds in the polymer. The disappearance of the 993 cm$^{-1}$ band and the appearance of a new band around 948 cm$^{-1}$ in the Zr-loaded polymer suggest the replacement of P−O−H with P−O−Zr.

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We next studied how Zr(IV) loading influenced the phosphopeptide binding selectivity and efficiency for polymer P1. Selectivity is reported as the percentage of detected peptides that are phosphopeptides, as opposed to non-
phosphorylated peptides, and it provides a measure of the specificity of the enrichment process. We used the total phosphopeptide intensity as an indicator of the efficiency of the enrichment process in which a greater number of enriched phosphopeptides should give rise to higher MALDI-MS ion signals. It was found that the selectivity levels off when the polymer is loaded with a 0.5 Zr/P ratio (Figure 3a). Presumably, this amount of Zr is the minimum amount necessary to shield the effect of the negatively charged phosphonate groups in the reverse micelles such that positively charged peptides are no longer selectively extracted. Interestingly, further increases in the Zr/P ratios up to about 3 lead to greater enrichment efficiencies (Figure 3b), suggesting that increases in Zr concentrations lead to more open coordination sites for phosphopeptide binding. Increases in Zr/P ratios beyond 3 cause decreases in the extraction efficiency. This effect might arise from MALDI-MS signal suppression in the presence of higher Zr concentrations or overloading of the reverse micelles that lead to the leaching of Zr back into the aqueous phase during the two-phase extraction process. In the latter case, presumably free Zr in the aqueous phase could form complexes with phosphopeptides, thereby decreasing the efficiency with which they are extracted into the reverse micelles. Support for this idea is found upon measuring the Zr concentration in the aqueous phase after extraction. When the Zr/P ratio is 5, 12.0 ± 0.4% of the added Zr ends up back in the aqueous phase, whereas only 0.21 ± 0.01 to 3.00 ± 0.03% of the added Zr is found in the aqueous phase when the Zr/P ratio is between 0.5 and 3.0.

We then investigated whether the binding selectivity could be further tuned by varying the phosphonate composition of the polymer. We hypothesized that there would be an optimal number of phosphonate groups that would yield the proper balance between immobilizing Zr inside the reverse micelle assemblies while providing replaceable coordination sites for phosphopeptide binding. To test this idea, we designed a series of random copolymers P1–P4 (Scheme 2) having different percentages of phosphate and PEG groups. The PEG groups were chosen to act as weak Zr coordination sites that could be displaced to allow binding by extracted phosphopeptides.

Phosphopeptide enrichment selectivity and efficiency were found to be the highest when the random copolymers had both phosphonate and PEG groups (P1 and P2) rather than just phosphate groups or just PEG groups (Figure 4). For example, at pH 7 polymers P1 and P2 are very selective with percentages of 89 ± 8 and 81 ± 7, respectively, whereas polymers P3 and P4 (without the PEG-based comonomer and the phosphonate-based monomer in the polymer respectively) have selectivity percentages of 36 ± 9 and 36 ± 5, respectively. Likewise, polymers P1 and P2 more efficiently extract phosphopeptides as indicated by the higher phosphopeptide intensities (Figure 4b). The relatively low enrichment selectivity and efficiency of polymer P3 are attributed to the inability of some of the polymer phosphate groups to be displaced from Zr(IV) inside the reverse micelles to allow phosphopeptides to bind and remain captured. The low enrichment selectivity and efficiency for polymer P4 might be caused by the poor coordinating ability of the PEG groups such that Zr(IV) does not remain stably bound inside the reverse micelles upon exposure to the phosphopeptides, thereby preventing efficient peptide capture. Evidence for this idea comes from ICP–MS measurements of the aqueous phase after extraction. When P4 is used for extraction, 16.5 ± 0.5% of the added Zr is found in the aqueous phase, whereas for polymers P1, P2, and P3 3.00 ± 0.03, 2.73 ± 0.07, and 1.64 ± 0.02%, respectively, are found in the aqueous phase. Overall, polymers P1 and P2 seem to provide the right balance of coordination strength to maintain Zr(IV) inside the reverse micelles and the ability to open up coordination sites for entering phosphopeptides so that they can remain encapsulated. It should be noted that an analogous effect has been observed in immobilized metal affinity chromatography, where an optimum coordination number for the immobilized metal leads to more efficient extractions.

Another interesting feature of these materials is that their selectivity and efficiency are somewhat independent of pH.
Figure 4. Phosphopeptide enrichment (a) selectivity and (b) efficiency using polymers P1−P4 in Scheme 2 to extract a three-protein digest mixture from an aqueous phase at pH values of 3, 5, and 7.

(Figure 4), emphasizing the role that Zr−phosphate interactions play in the binding selectivity. Moreover, this behavior contrasts with most immobilized metal affinity approaches that suffer from Zr(IV) hydrolysis at higher pH values and thus only works well under acidic conditions.36−40 Perhaps the confined environment inside the reverse micelles limits hydroxide levels, thereby minimizing hydrolysis.

With a better understanding of the polymer features that influence phosphopeptide binding, we explored the scope of the binding specificity by extracting phosphopeptides from digests of the phosphoprotein β-casein in the presence of 10- and 100-fold molar excesses of BSA. Before extraction of the protein digest mixture, analysis by MALDI-MS reveals a spectrum dominated by numerous nonphosphorylated peptides originating from BSA (Figure 5a). The presence of exclusively BSA-related peptides is not surprising because the digestion of this protein can produce more than 200 peptides, whereas β-casein typically produces only 20 peptides, of which only 3 are phosphorylated. Upon using either polymer P1 or P2, which both have similar extraction abilities, to enrich the phosphopeptides of β-casein, MALDI-MS spectra are now much simpler with three or two phosphopeptides detected (Figure 5b,c, respectively). These results highlight the high degree of selectivity possible when Zr(IV) is loaded into these polymeric reverse micelles.

■ CONCLUSIONS

We have developed a simple method of varying the binding selectivity of polymeric reverse micelles by changing the chemistry of their interiors via the addition of Zr(IV) ions. Metal addition resulted in reverse micelles capable of selectively enriching phosphopeptides from protein digest mixtures. We further tuned the selectivity of these materials by varying the ratio of hydrophilic functional groups in the reverse micelle interior and found that a combination of PEG groups and phosphonate groups provided the optimum binding selectivity and efficiency. Finally, the optimized polymer structure, loaded with Zr(IV) ions, allowed us to selectively bind phosphorylated peptides that are present at very low levels in a more complicated sample. This study demonstrates that supramolecular materials based on polymeric reverse micelles can be readily designed to selectively target biomolecules of interest. Future work will further develop these Zr(IV)-loaded polymers and apply them for the detection of phosphorylated peptides in cell lysates and other more complicated mixtures, which could make them valuable materials for phosphoproteomics studies.

■ ASSOCIATED CONTENT

5 Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acs.langmuir.7b02488.

Synthesis protocols and characterization data for all compounds, NMR spectra, and mass lists of detected peptides (PDF)
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AUTHOR INFORMATION

Corresponding Authors

*E-mail thai@chem.umass.edu.

*E-mail rrwachet@chem.umass.edu.

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