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# Supramolecular Nanoassemblies for the Separation and Mass Spectrometric Analysis of Peptides and Modified Proteins

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# SUPRAMOLECULAR NANOASSEMBLIES FOR THE SEPARATION AND MASS SPECTROMETRIC ANALYSIS OF PEPTIDES AND MODIFIED PROTEINS

A Dissertation Presented

by

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## ABSTRACT

### SUPRAMOLECULAR NANOASSEMBLIES FOR THE SEPARATION AND MASS SPECTROMETRIC ANALYSIS OF PEPTIDES AND MODIFIED PROTEINS

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Protein post-translational modifications (PTMs) play key roles in cellular physiology and disease, and identifying their locations on proteins can be beneficial for understanding more deeply protein chemistry. The methods applied for PTM analysis are most often based on mass spectrometry (MS). In the past few years, considerable progress has been made in developing MS-based proteomics technologies for global PTM analysis. Novel mass spectrometric peptide sequencing and analysis technologies allow for modification site mapping at molecular level. However, detecting PTMs on proteins and peptides by MS is challenging because of their low abundance and heterogeneity. Therefore, separation prior to MS analysis is typically required. This dissertation describes the use of supramolecular nanoassemblies, formed by amphiphilic polymers, as novel enrichment tools for the detection and analysis of peptides and proteins that are phosphorylated and glycosylated. The selectivity of the amphiphilic nanoassemblies were changed by loading metal ions for the enrichment of phosphopeptides and by incorporating hydrazide functional groups for the enrichment of glycopeptides. In addition to developing new nanoassemblies for the enrichment of phosphopeptides and glycopeptides, we also explored how supramolecular systems could be tuned to enhance extraction selectivity and

efficiency via structural variations to the amphiphilic polymers. The utility of these materials for the enrichment of phosphopeptides and glycopeptides from complexed samples is also demonstrated.

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# CHAPTER I

# ANALYSIS OF POST-TRANLATIONS MODIFICATIONS BY MASS SPECTROMETRY

## Introduction

### Post-Translational Modifications and Clinical Relevance

Cells need to detect and react to changes that are from inside and outside. One strategy is by chemically modifying proteins. Proteins are the chief actors within the cell, serving enzymatic, signaling and structural functions.[1] They are formed by the end products of gene expression, which may then undergo post-translational modifications (PTMs), referring to the covalent cleavage or addition of modifying groups to amino acids. These processes play key roles in sensing and responding to the conditional chemical changes. A single PTM can re-establish the entire downstream trafficking transforming the protein function and cell fate. Hence PTMs contribute significantly to cellular physiology and disease.[2-5]

Post-translational modifications can happen at any step of the protein lifespan in diverse ways, which may include modification short after translation to mediate proper folding, confer stability, or translocate proteins, such as proteolysis, glycosylation, phosphorylation, etc.[6-8] Other modifications may occur after the folding and localization for activation or inactivation of catalytic activity and functions, like the reversible phosphorylation and the acetylation, etc.[8-10] Proteins can also be post-translationally tagged by ubiquitin for degradation.[11] Besides these most common PTMs (Figure 1.1),[12] there are

as many as 300 PTMs of proteins are known to occur physiologically, and their importance in biological processes and disease has led them be the focus of clinical and pharmaceutical research as potential drug targets.[13-16]

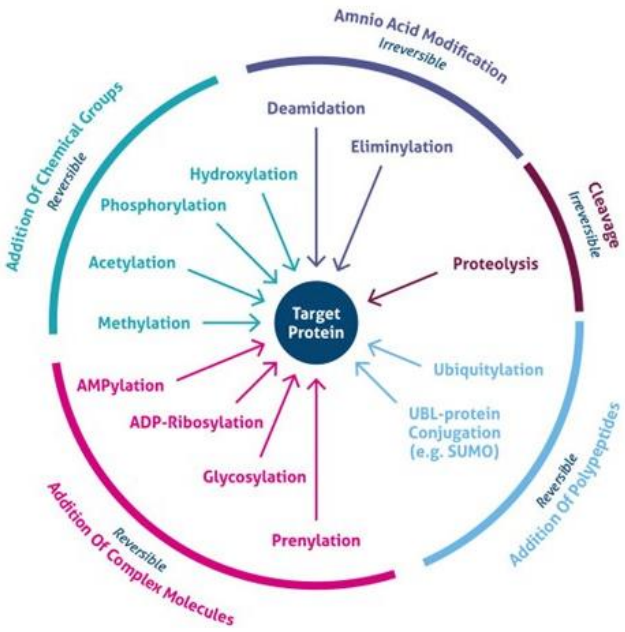


**Figure 1.1.** Types of post-translational modifications (PTMs).[12]

Since PTMs determine the protein-protein interactions and form the basis of some cellular signaling pathways, which also regulate the cell–cell and cell–matrix interactions, so they have comprised the basis of several drug targeting strategies and diagnostic tests and been reported to have clinical relevance in disease of inflammation, host-pathogen interactions, immune modulation, and degenerative and proliferative disorders.[17-22] Among those phosphorylation and glycosylation are the most common and well-studied post-translational modifications (Figure 1.2).[23]

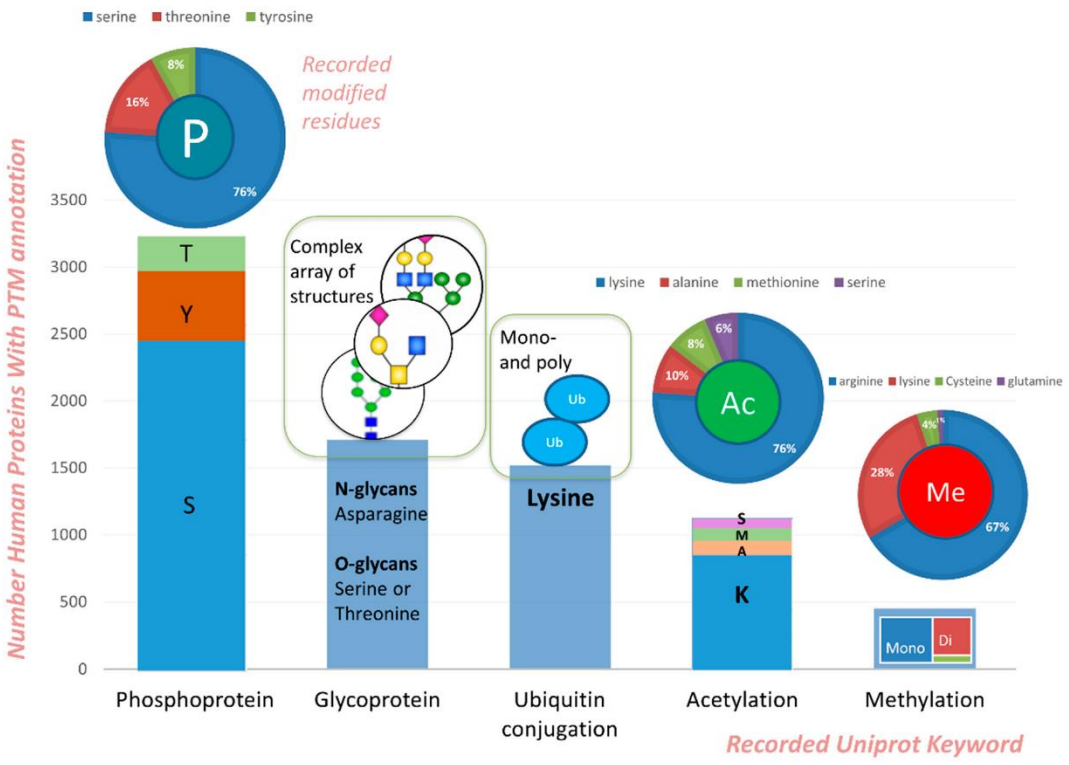


**Figure 1.2.** Human proteins with PTM currently available in the UnitProt database.[23]

**Phosphorylation.** Protein phosphorylation is one of the most common and important post-translational modifications.[24] It occurs through kinases adding one phosphate group ($PO_4$) to the serine (Ser), threonine (Thr) or tyrosine (Tyr) residues. As a result, the protein becomes hydrophilic polar, providing the flexibility of conformational change when interacting with other molecules. The

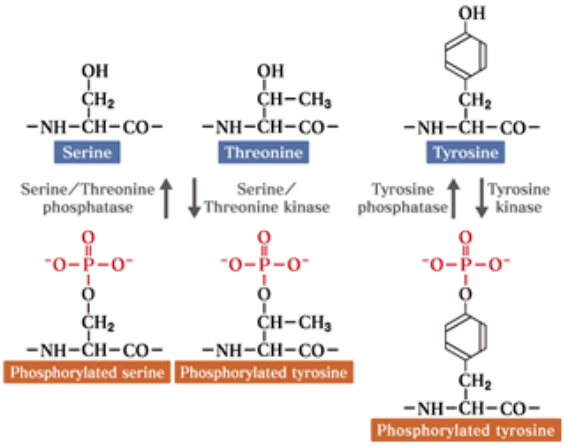


**Figure 1.3.** Mechanism of phosphorylation.

reversibility of protein phosphorylation by kinase and dephosphorylation by phosphatase makes this type of PTM ideal for protein function regulation and signal transduction, as it allows cells to respond rapidly to intracellular and extracellular stimuli.[23-25] The PhosphoSite Plus database has listed over 200,000 known human phosphorylation sites. It is estimated that over one-third of the 21,000 proteins encoded by the human genome are to be phosphorylated, and more than 350 phosphorylated proteins are under disease conditions.[23] Indeed, the phosphorylation events plays critical roles in the control of biological processes such as differentiation, proliferation and apoptosis.[26-28] Numerous examples have been found. For example, in chromic myeloid leukemia, the retinoblastoma (pRb), a kinase generated by the translocation of Philadelphia chromosome was found to be always "on", which leads to the proliferation of tumor cells.[29] In fact, the kinase regulated signaling pathways contribute to the start and progression of almost all types of cancer. Another common concern is Alzheimer's disease (AD). Back in 1980s, hyperphosphorylated neurofibrillary tangles (NFT) was reported to be found in brains with Alzheimer disease.[30] And the detection of Tau phosphorylation in the cerebrospinal fluid suggests the pathological mechanisms under this disease.[31] In addition, the phosphorylation of α-synuclein at S129 has been used a biomarker in dementia and Parkinson's disease (PD).[32] Because of all these and other negative modulation of the phosphorylation, the study of the signaling pathways regulated by kinases with blocking possibility has become the major drug target for treatment in clinical and pharmaceutical research.

**Glycosylation.** Glycosylation, the attachment of sugar moieties to specific amino acids, is another common PTM. It has been found in almost all the living organisms that have been studied and is estimated that half of all the proteins are glycosylated.[33] There are

two major types of glycosylation: N-linked and O-linked (Figure 1.4).[34] However, the diversity that the two types of glycosylation add to the proteome to a level that is unparalleled by any other PTMs, which allow the glycoproteins to adapt to various functions in a wide range of biological processes, including molecule transportation, enzyme production, cell attachment recognition, etc.[35,36]

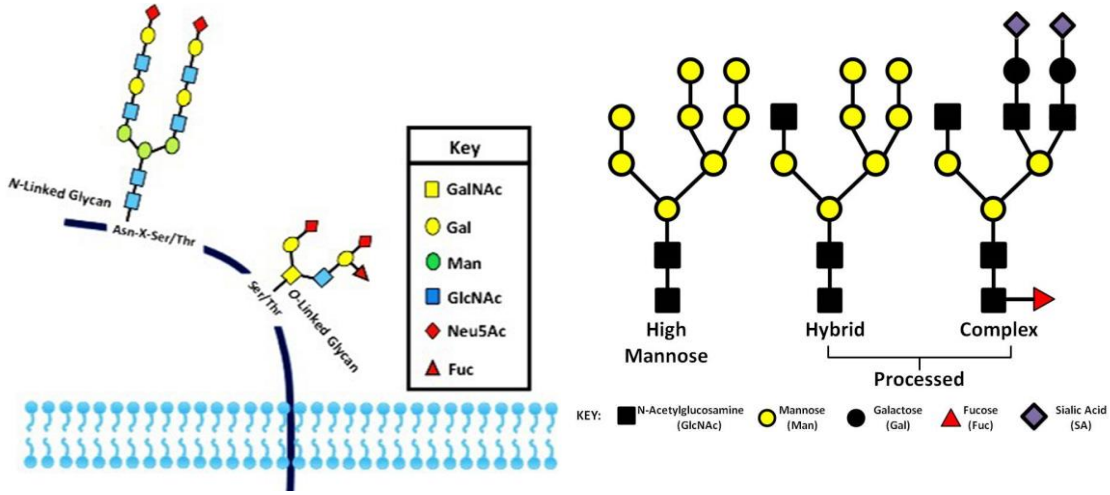


**Figure 1.4.** The two major types of protein glycosylaiton (left) and N-linked glycosylation (right).[34]

Numerous glycoproteins are N-linked that the glycans are covalently attached to the asparagine (Asn) residue of the protein. It mostly happens when the newly translated proteins are transported into the ER. Not all Asn can be glycosylated, but only when the amino acid motif be Asn-X-Ser/Thr, where X can be any amino acid except Pro, then the Asn could accept an N-glycan. All N-glycans share a common core sequence, and be classified into three types: high mannose, hybrid and complex depending on the difference of types of sugar units (Figure 1.4).[37] There are also a variety of glycoproteins that are O-linked, which means that short glycan chains are attached to the proteins on Ser and/or Thr

residues. Therefore, the result of protein glycosylation is a complex array of monosaccharides or glycan structures with different glycosidic linkages (sugar-peptide bond), glycan composition (types of sugars), glycan structure (branched or unbranched sugar chains) and glycan length (short or long chain of oligosaccharides).

Glycoproteomics is a rapidly emerging field and its clinical relevance have been made clear in recent years. For example, in neurodegenerative disease, the glycosylation of acetylcholinesterase or butyrylcholinesterase has been reported as a maker of disease progression in AD patient,[38] and specific glycosylation pattern was observed in frontal cortex in PD patient.[39] Also, glycan changes have also been found in breast cancer patient mediating the disease progression,[40] and glycated haemoglobin changes have been used as diagnostic test in diabetes.[41] Therefore, glycoproteomics has been widely used for diagnosis of various diseases. Since protein modifications influence and even define cell functions, identifying PTMs is critical for the understanding of cell biology. Many modification sites are implicated in the catalytic function of key enzymes, which suggests identification of the modification sites helps the understanding of the mechanistic details of protein-protein interactions and how PTMs modulate the protein functions.

**MS Detection of PTMs**

For the analysis of protein modifications, traditional strategies, such as radioactive labeling and western blotting, can be specific and relatively quantitative, but they require prior knowledge of the type of modification and are limited by antibody availability and specificity. Mass spectrometry (MS), as a fast and sensitive analytical tool, become the method of choice. It can rapidly identify proteins and provide universal information of their

primary structures and modification without the need for prior knowledge.[42-46] Currently, there are two major complementary strategies for MS analysis of proteins PTM identification: the bottom-up and top-down approaches (Figure 1.5).[47]

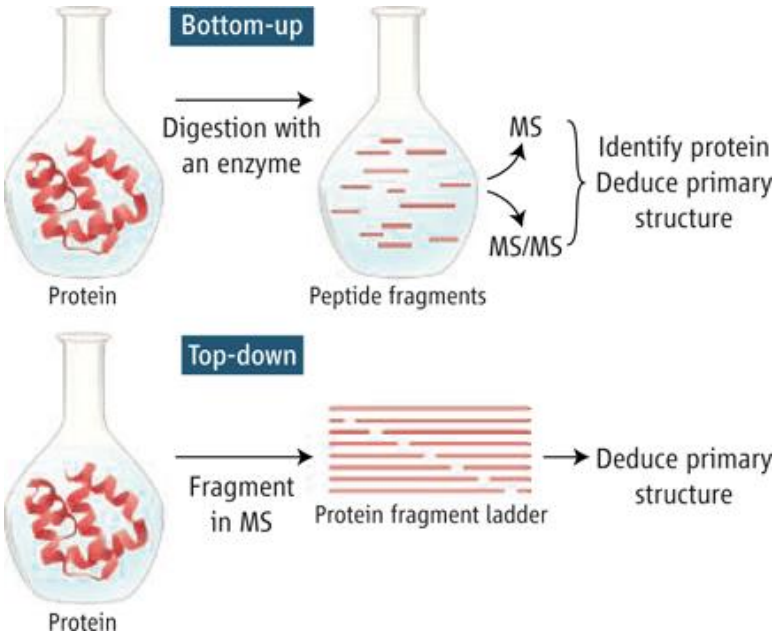


**Figure 1.5.** Protein primary structure determination by mass spectrometry.[47]

**Bottom-Up Strategy.** The bottom-up approach is the traditional proteomic approach and has been widely used to identify proteins and determine the details of the sequence and the PTMs.[45] In this approach, proteins of interest are digested with an enzyme like trypsin into small peptides. These peptides will first be ionized by matrix-assisted laser desorption/ionization (MALDI) or electrospray ionization (ESI). These "soft ionization" techniques allow peptide ions to be put into gas phase with minimal to no fragmentation for subsequent MS analysis. Then the MALDI- or ESI-MS can provide two types of information of the peptides. First, the peptide masses can be determined, and then the detailed information about peptide sequence and modifications can be achieved by

fragmenting the peptide ions in the gas phase. The bottom-up approach is especially useful for protein identification as peptides are easily solubilized, separated and detected by MS, which is not easy for parent proteins. Also, many peptides can be easily fragmented and analyzed to provide enough information for the parent protein determination. Though bottom-up strategy provides higher sensitivity than top-down (in later discussion), there are some limitations in using bottom-up approach characterizing protein modifications. First, there is only a fraction of the peptides that is normally detected, so the modification in the unrecovered sequence will remain undiscovered. In addition, because the sequence digested by trypsin are relatively small, there might be a loss of correlation that is between modifications on different parts of the protein.[45,47]

**Top-Down Strategy.** The top-down approach is a more powerful way to analyze unknown protein modifications as the intact protein is injected and fragmented subsequently inside the mass spectrometer without digestion, so the molecular masses of both the protein and all the fragment ions can be acquired and analyzed. Therefore, it is possible to get full information of the primary structure, all the modifications of the protein, and the relationship between amino acid sequence and modifications belonging to a specific proteoform. This relationship cannot be preserved in bottom-up approaches characterizing peptides after proteolytic digestion. However, it's very important for the understanding of biological system as different proteoforms can function very differently. Although this strategy has shown being able to measure the molecular masses of intact protein, it has proved challenging for top-down approach to produce extensive gas-phase fragmentation of intact protein, especially for large proteins. Great effort has been put into the method developments for fragmenting large proteins or peptides.[47,48]

It is also worth mentioning that middle-down proteomics, as a variant of the top-down strategy, has recently emerged as a high throughput strategy in PTM characterization.[49] In this approach, the proteins are proteolyzed to large peptides, so to preserve more sequence and modification information, and then be fragmented and analyzed like top-down. Because of the different challenges in bottom-up and top-down approaches and depending on the different questions that researchers try to answer, the two approaches are still coevolving.[47]

**Mass Spectrometry Fragmentation Strategies.** In MS-based protein detection, tandem mass spectrometry (MS/MS) is the key technique for protein and peptide sequencing and PTM analysis.[50] It is essential to have proper fragmentation to produce enough fragment ion information for the sequence identification and modification site localization. There are several fragmentation methods available, including collision-induced dissociation (CID), electron-capture dissociation (ECD), electron-transfer dissociation (ETD) and higher energy collisional dissociation (HCD).[51]

CID, also known as collisionally activated dissociation (CAD), has been the most widely used MS/MS technique. In this dissociation method, gas phase peptide or protein cations are heated by collisions with rare gas atoms, resulted in fragmentation of C-N bond in the peptide backbone, producing a series of b- and y-fragment ions. This method is not friendly for labile PTMs because of the slow-heating and high energy required in the fragmentation process. Other internal fragmentation and neutral loss of $H_2O$ or $NH_3$ can also occur. And this technique usually works for small peptides (< 15 amino acids).[52,53]

A later developed fragmentation technique, ECD, works by capturing a thermal electron causes the protonated peptide or protein cation be fragmented on the N-Cα bond,

resulting in a series of c- and z-fragments ions. ECD can preserve the labile modifications and provide more extensive fragmentation and higher sequence coverage, thus become a powerful technique but mostly constrained to the expensive and sophisticated FTICR instruments.[54]

ETD is a similar technique to ECD but could be more commonly used in bench top mass spectrometers. In this method, the electrons transfer from radical anions with low electron affinity to protonated peptide or protein cations, to produce fragmentation resulting in c- and z-types of ions. It is very complementary to conventional CID in analyzing very large peptides because of its higher efficiency and has shown great potential in labile PTM analysis.[55]

**PTM Enrichment Strategies**

The bottom-up proteomics, such as shotgun proteomics, provide high sensitivity in characterizing modifications after protein proteolysis. Although the mass spectrometers have evolved dramatically in recent decades, it is still impossible to directly identify most of the PTMs in a standard MS analysis from complex protein digests. The general workflow for protein PTM analysis always includes efficient peptide enrichment prior to MS detection because of the low abundance of modified proteins and low stoichiometry of PTMs. The presence of huge amount of unmodified peptides significantly suppresses the detection of modified peptides.[56,57]

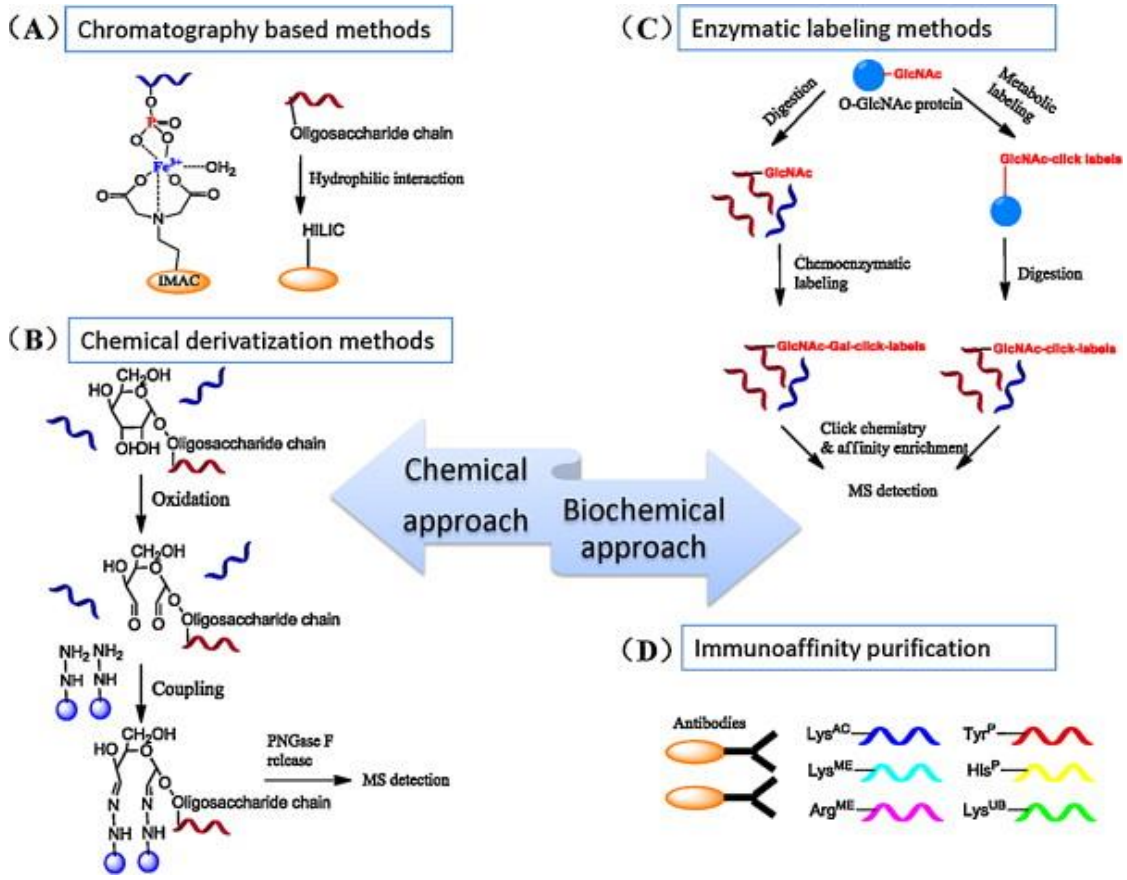


**Figure 1.6.** Major strategies for enrichment of PTM peptides.[56]

Various available enrichment methods can be divided into two types: chemical approaches and biochemical approaches (Figure 1.6),[56] where chemical approaches make use of the different chemical properties of modified and unmodified peptides while biochemical approaches utilize the specific interactions between biomolecules. In the first approach, for example, chromatography-based methods, such as immobilized metal ion affinity chromatography (IMAC) and hydrophilic interaction chromatography (HILIC), enrich phosphopeptides and glycopeptides because that phosphate groups have higher affinity to metal ions and glycosylated peptides are more hydrophilic than non-modified peptides. Also, glycopeptide enrichment can be enriched by hydrazide chemistry because

oxidized glycans can react with hydrazide groups while non-glycopeptides cannot. Biochemical approaches, however, are based on the specific recognition of enzyme and substrate, such as enzymatic labelling methods, of antibody and antigen, like immunoaffinity purification. The enzymatic labeling methods have been used for enrichment of peptides with O-GlcNAc and S-glutathionylation, and immunoaffinity purification has been applied for a bunch of PTM peptide enrichment, such as acetylation, methylation, ubiquitination and phosphorylation. Chemical approaches, providing more flexibility and universality, has been kept developing and optimizing to acquire more in-depth PTMs information, which can be clearly seen in the evolution of phosphopeptide enrichment (Figure 1.7).[56]

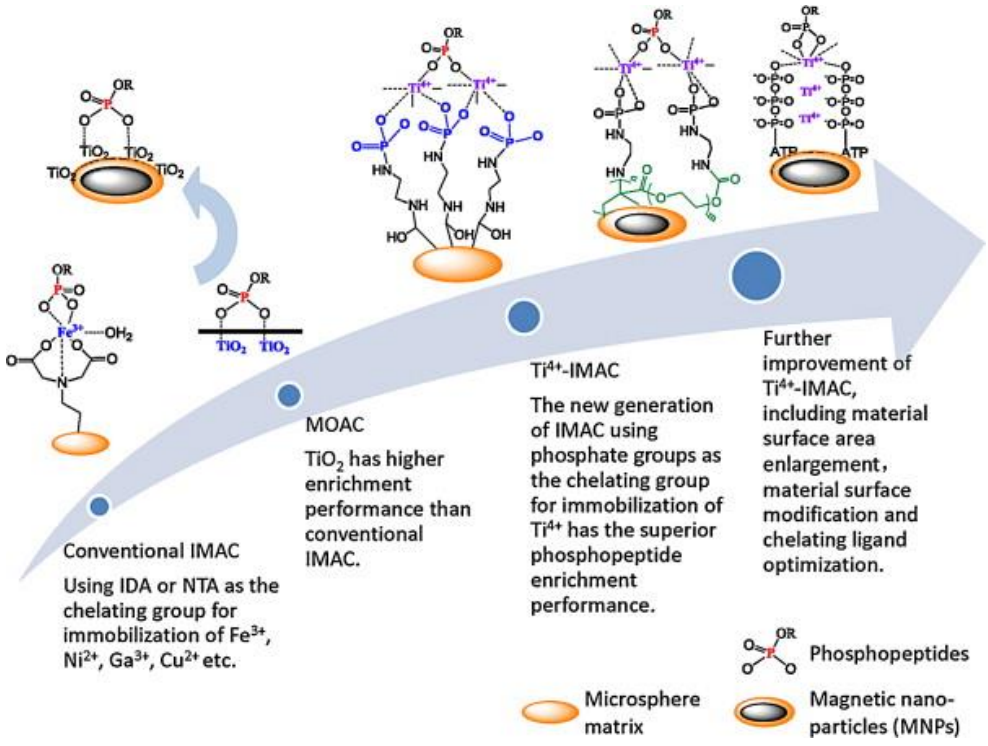


**Figure 1.07.** Evolution of phosphopeptide enrichment methods.[56]

Conventional IMAC for the enrichment of phosphopeptides is based on the chelating interactions between phosphate group and metal ions that are immobilized on solid beads with iminodiacetic acid (IDA) or nitrilotriacetic (NTA) as the chelating groups. $Fe^{3+}$ was the initial metal ions, and then a lot of ions, such as $Ni^{2+}$, $Ga^{3+}$, $Gu^{2+}$, $Co^{2+}$, $Al^{3+}$, etc. have been applied to IMAC.[58] This conventional IMAC has been developed for a long time but still suffer a great challenge of non-specific binding from Glu and Asp. This problem was tried to be overcome by the methyl esterification of carboxyl groups on Asp and Glu prior to phosphopeptide enrichment.[59] However, this method was rarely used because of the incomplete esterification, side reaction and possible peptide loss, then optimizing loading buffer became a more practical and simple way to improve the enrichment specificity. Higher selectivity could be achieved by protonating the carboxylic groups on Asp and Glu, while leaving the phosphate groups deprotonated in the loading buffer of pH 2-2.5 as the pKa of E and D is around 4 while of phosphate group is about 2.[60] Later the conventional IMAC was mostly replaced by the metal oxide affinity chromatography (MOAC) especially in large-scale phosphoproteome studies, because of the higher enrichment efficiency and easier availability shown by $ZrO_2$ and $TiO_2$.[61] More recently, a new type of IMAC that tries to combine the advantages of IMAC and MOAC has been developed, where $Zr^{4+}$ and $Ti^{4+}$ are used as the chelating metal ions because of the higher specificity between metal (IV) ions and phosphate groups than that of conventional IMAC metal ions. In addition, compared to MOAC, $Zr^{4+}$- or $Ti^{4+}$-IMAC shows higher efficiency because of the flexible arms introduced in IMAC that can reduce the steric hindrance.[62,63] Based on that, some new matrix materials have been developed to further improve their enrichment efficiency. Among those, incorporating nanomaterials

has become the most attractive method as it can provide higher surface area for higher capacity and can be easily modified on the material surface and on the chelating ligands for simple optimization.[64-66]

## Supramolecular Nanoassemblies as Selective Extraction Agents for MS Analysis of PTMs

Supramolecular nanoassemblies, including micelles, reverse micelles, vesicles, polymersomes, and gels, have been widely studied for encapsulation, detection, and targeted delivery of biomolecules.[67,68] A key characteristic of supramolecular systems is their ability to be tuned via changes at the molecular level. Non-covalent interactions, including electrostatic interactions, H-bonding, π-π stacking, metal-ligand coordination, and general host-guest interactions, are often used for this purpose.[69,70] Supramolecular assemblies formed by amphiphilic polymers as enrichment agents for the selective detection of biomolecules through electrostatic interactions have been explored in our group. 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 (Figure 1.8).[71-81]

These nanoassemblies have shown great features for biomarker extraction. 1) They can be easily synthesized and can be widely used by changing the functional groups based on the property of target biomolecules. For example, to target positively-charged peptides or proteins, carboxylate, sulfonate or phosphonate-containing polymers can be synthesized, either as homopolymers or random co-polymers, and for negatively-charged peptides or

proteins, the hydrophilic part of polymer can be replaced as positively-charged quaternary amine groups.[71,72,79,80] 2) After the functional groups recognize and bind with the peptides or proteins of interest via two-phase liquid-liquid extraction, they can be easily collected in the organic phase because they tend to be trapped only in the toluene phase where they are initially assembled. The use of two immiscible phases allows easy isolation of the extracted peptides after centrifugation.[77] 3) Enhanced detection has been observed which is significant for the detection of usually low-abundant biomarkers. The MALDI signal enhancement was found to be due to the donor-acceptor and electrostatic interactions in the supramolecular systems during MS analysis, where the donor-acceptor interactions drive a formation of ternary assembly of the polymer, peptide and matrix, which produces peptide-rich zones or 'hot spots' that maximize the ion signal in the mass spectrum.[76] Detection of peptides of interest can also be enhanced by selective removal of interfering peptides or proteins using the corresponding polymers. For example, abundant acidic proteins in serum can be depleted using positively-charged polymers and so allow for the enhanced detection of higher pI proteins.[78] This depletion efficiency can be further improved by varying the polymer structure, concentration and charge density.[79,80] 4) A systemic study of how polymer structure influences extraction shows that the inherent feature of the functional group of the polymer like its $pK_a$ affects not only selectivity as in free aqueous solution, but also affects the extraction capacity because of the hydrophilic-lipophilic balance (HLB) of the polymer, which allows the materials to be used as a more predictable separation method.[79] These fundamental studies have given us insight that has allowed us to extract peptides with specific pI values,[72,73] generate titration curves for individual peptides in a mixture,[74] efficiently release peptides for quantitative analysis,[81]

enrich biomarkers in human serum and breast milk by specific pI bracketing, and reduce sample complexity with concurrent pI bracketing for high sensitive MS detection of the biomarkers of interest.[75,78]

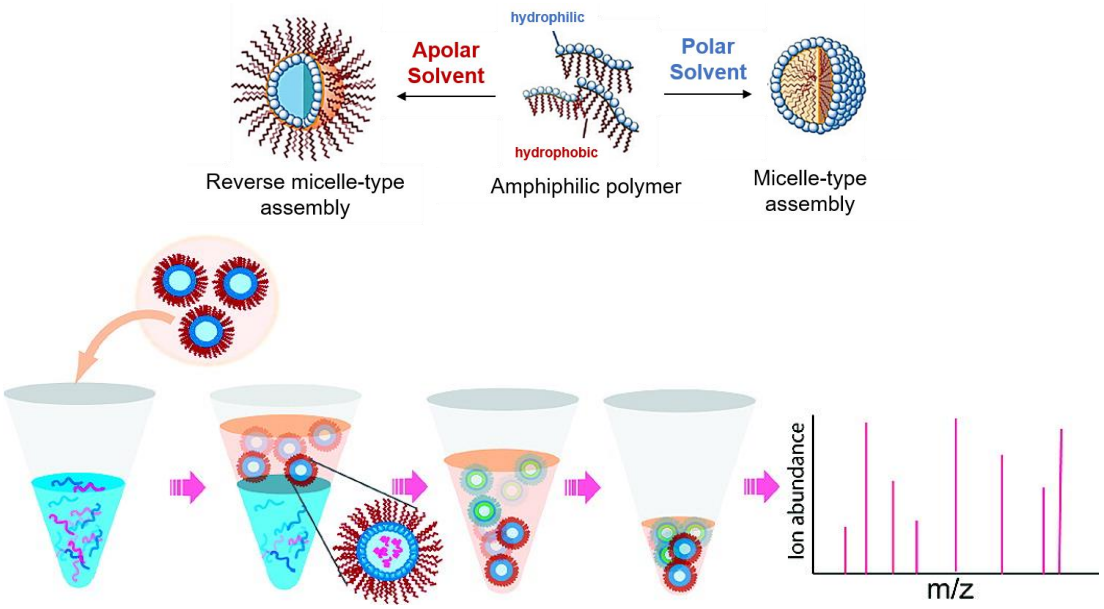


**Figure 1.8.** Amphiphilic polymers self-assemble into micelle-type assembly or reverse micelle-type assemblies, which can be used for two-phase liquid-liquid extraction of peptides prior to MS analysis.[72]

While these supramolecular materials have been effective at enriching molecules based on complementary charge, the electrostatic interaction-based affinity cannot satisfy the need for the selectivity of extracting modified peptides. To study a wider range of biomolecules, altering the selectivity of these promising materials is an important goal in this dissertation. In chapter II, a simple approach for changing the enrichment selectivity of these materials instead of synthesizing new functional polymers has been explored, which is via the addition of metal ions. A series of polymer with varying phosphonate architecture have been synthesized and loaded with different amount of $Zr^{4+}$ and their

extraction performance have been studied. With the best selectivity and efficiency achieved by certain polymer and ratio to metal ions, these materials are used for selectively enrich phosphorylated peptides present in low levels in protein mixtures.

Although non-covalent interactions have proved successful in extraction, the selectivity achieved by non-covalent bond cannot be competitive to that of covalent bonds, as non-specific interactions can be washed off thoroughly. In chapter III, we used covalent binding via hydrazide groups to achieve high selectivity for glycopeptide. At the same time, a non-covalent interaction was incorporated to catalyze this normally slow covalent binding reaction, thereby improving extraction efficiency. We find that in the confined environment of the nanoassembly the reaction of hydrazide with glycans is faster than in existing approaches. Moreover, the extraction efficiency can be further increased by placing functional groups in the assemblies that catalyze the reaction of hydrazide moieties with glycans.

In chapter IV, another effective way of expanding the scope of the material's selectivity is demonstrated. Besides of selective extraction using the nanomaterials, we add another dimensional control over the peptide separation, which is controlled release. The release behavior of the peptides from nanoassemblies into stripping buffer has been studied, and it shows great potential in isolating desired peptides after peptide synthesis.

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# CHAPTER II

## SUPRAMOLECULAR NANOASSEMBLIES FOR PHOSPHOPEPTIDE ENRICHMENT

The majority of this chapter was published in Wang, M.; Zhao, B.; Gao, J.; He, H.; Castellanos, L.J.; Thayumanavan, S.; Vachet, R. W. Altering the Peptide Binding Selectivity of Polymeric Reverse Micelle Assemblies *via* Metal Ion Loading. *Langmuir* **2017**, *33*, 14004-14010.

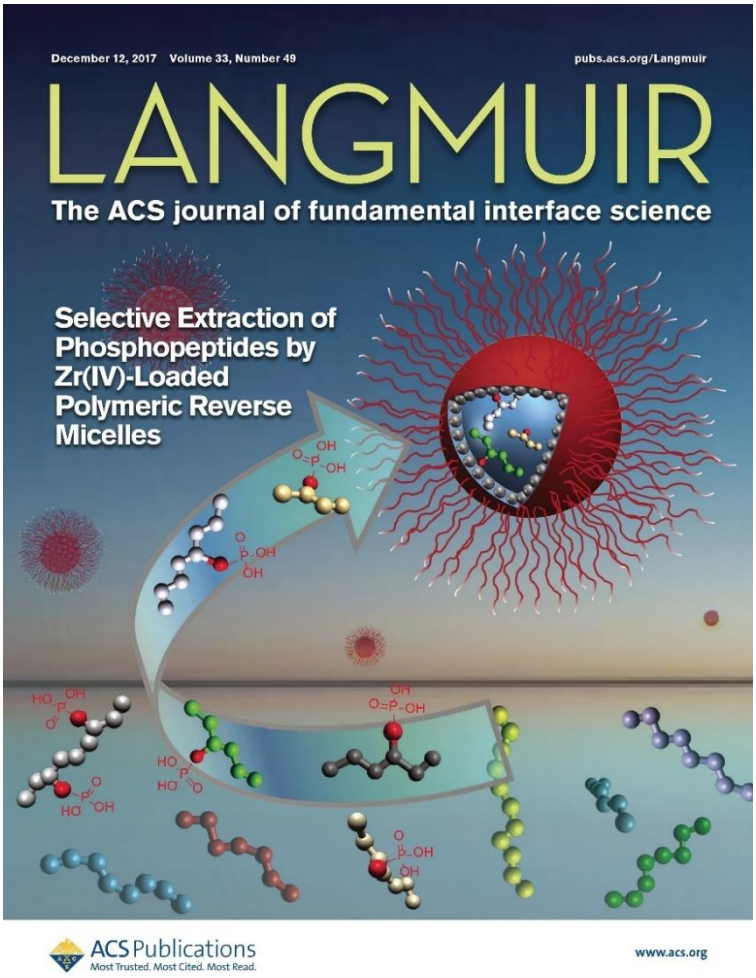



**Figure 2.1.** Langmuir journal cover of selective extraction of phosphopeptides by Zr(IV)-loaded polymeric reverse micelles.

## Introduction

Supramolecular nanoassemblies formed by amphiphilic polymers have been proved very effective at enriching molecules based on complementary charge, [1-6] altering the selectivity of these promising materials is an important goal that typically requires synthesis of new functional polymers. In this chapter, we have explored a simpler approach for changing the enrichment selectivity of these polymeric reverse micelles via the addition of metal ions. We find that by loading the reverse micelles with Zr ions, we can dramatically change the selectivity of the materials so that they specifically bind phosphopeptides (Figure 2.1). 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.[7-11]

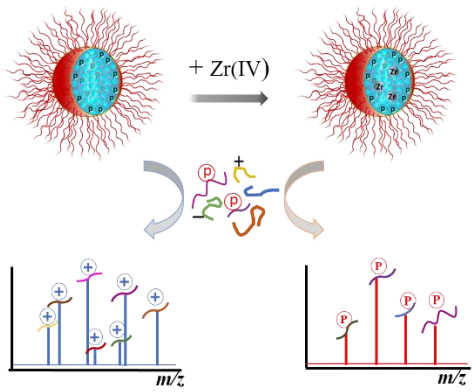
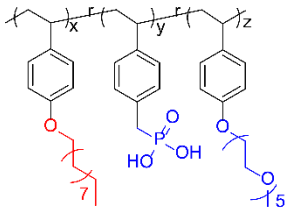


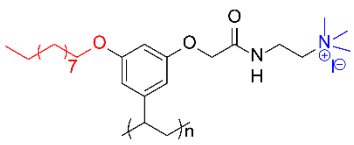

**Figure 2.2.** Schematic illustration of polymeric reverse micelles having phosphonate functional groups loaded with Zr(IV) that can selectively bind phosphopeptides.

## Results and Discussion

An aqueous phase peptide mixture created by digestion of the proteins α-casein, β-casein, chicken ovalbumin, bovine serum albumin (BSA), and lysozyme was extracted using reverse micelles of polymer **P1**, containing phosphonate moieties (Figure 2.2), and analyzed by MALDI-MS (Figure 2.3). Before extraction, the mass spectrum (Figure 2.3a and Table 2.1) is dominated by peptides with low pI values, which is consistent with the fact that four of the five proteins are acidic. After extraction using polymer **P1**, peptides with pI values close to or above the aqueous solution pH of 7 are detected (Figure 2.3b and Table 2.2), which is consistent with our previous work that showed reverse micelles of negatively charged polymers extract positively charged peptides.[1-6]



**P1** x:y:z = 43:37:20
**P2** x:y:z = 48:16:36
**P3** x:y = 53:47
**P4** x:z = 61:39



**P5**

**Figure 2.3.** Chemical structures of amphiphilic random copolymers **P1**-**P4** and amphiphilic homopolymer **P5**.

The goal of the current work is to test whether the selectivity of these polymers could be varied by the simple addition of something to the reverse micelle solution. To test this idea, we loaded the reverse micelle interiors with Zr(IV), which has 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 2.3c and Table 2.3), indicating that the driving force for extraction has been converted from electrostatic interactions to Zr(IV)-phosphate interactions. It should be stated that the extraction with the Zr(IV)-loaded polymer is selective for phosphopeptides and not just acidic peptides, as positively charged polymer P5 extracts acidic peptides, including non-phosphorylated ones (Figure 2.3d and Table 2.4).

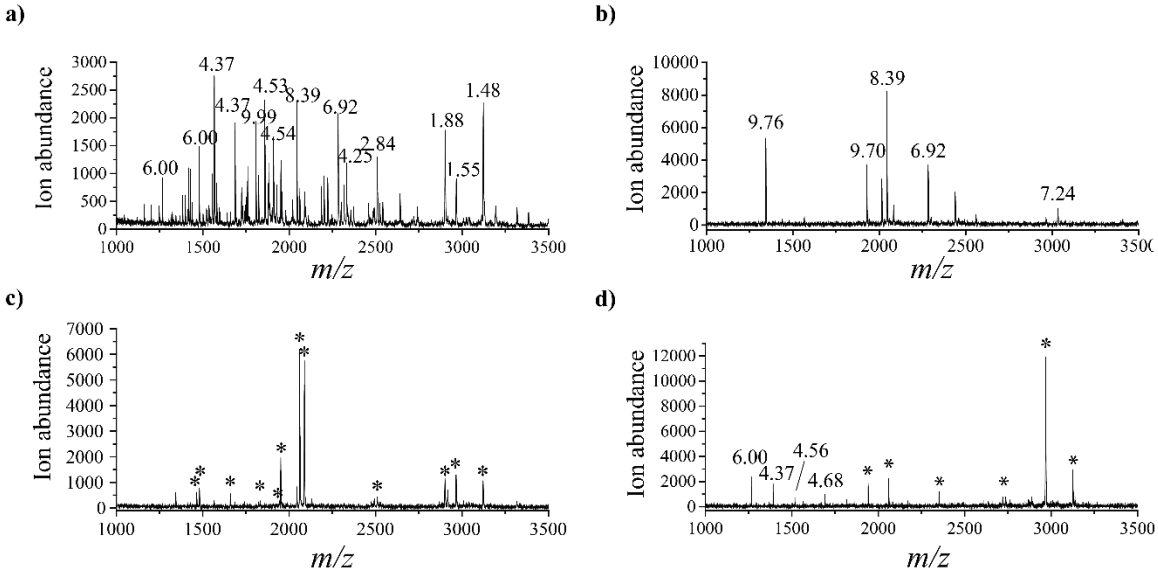



**Figure 2.4.** MALDI mass spectra of a protein digest mixture of α-casein, β-casein, chicken ovalbumin, bovine serum albumin (BSA) and lysozyme each at 0.5 μM, acquired in the negative ion mode (a) before enrichment, (b) after enrichment at pH 7 using reverse micelles of polymer P1, (c) after enrichment at pH 7 using reverse micelles of polymer **P1** loaded with Zr(IV), and (d) after enrichment at pH 7 using reverse micelles of polymer **P5**. The numbers above the peaks indicate the calculated pI values of the peptides, and the asterisks indicate the phosphorylated peptides.

**Table 2.1.** Most abundant peptides detected from protein mixture consisting of α-casein, β-casein, chicken ovalbumin, BSA and lysozyme by MALDI-TOF-MS analysis in negative mode before enrichment.

| Peptide sequence | pI | $[M-H]^-$ (Da) | Protein |
|---|---|---|---|
| YLGYLEQLLR | 6.00 | 1265.7 | α-casein |
| FFVAPFPEVFGK | 6.00 | 1382.7 | α-casein |
| SLHTLFGDELCK | 5.30 | 1417.7 | BSA |
| DAFLGSFLYEYSR | 4.37 | 1565.7 | BSA |
| GGLEPINFQTAADQAR | 4.37 | 1685.8 | ovalbumin |
| DSTRTQINKVVRFDK | 9.99 | 1805.0 | ovalbumin |
| ELINSWVESQTNGIIR | 4.53 | 1857.0 | ovalbumin |
| LFTFHADICTLPDTEK | 4.54 | 1905.9 | BSA |
| RHPYFYAPELLYYANK | 8.39 | 2043.0 | BSA |
| VTEQESKPVQMMYQIGLFR | 6.92 | 2282.1 | ovalbumin |
| LPGFGDSIEAQCGTSVNVHSSLR | 2.84 | 2509.1 | ovalbumin |
| FDKLPGFGDSIEAQCGTSVNVHSSLR | 1.88 | 2889.3 | ovalbumin |
| ELEELNVPGEIVESLSSSEESITR | 1.55 | 2963.3 | β-casein |
| RELEELNVPGEIVESLSSSEESITR | 1.48 | 3120.5 | β-casein |

**Table 2.2.** Positively charged peptides detected from protein mixture consisting of α-casein, β-casein, chicken ovalbumin, BSA and lysozyme by MALDI-TOF-MS analysis in negative mode after enrichment using polymer **P1** at pH 7.

| Peptide sequence | pI | $[M-H]^-$ (Da) | Protein |
|---|---|---|---|
| HIATNAVLFFGR | 9.76 | 1343.7 | ovalbumin |
| YLGYLEQLLRLKKYK | 9.70 | 1926.1 | α-casein |
| RHPYFYAPELLYYANK | 8.39 | 2043.0 | BSA |
| VTEQESKPVQMMYQIGLFR | 6.92 | 2282.1 | ovalbumin |
| VHHANENIFYCPIAIMSALAMVYLGAK | 7.24 | 3031.5 | ovalbumin |

**Table 2.3.** Phosphopeptides detected from protein mixture consisting of α-casein, β-casein, chicken ovalbumin, BSA and lysozyme by MALDI-TOF-MS analysis in negative mode after enrichment using Zr loaded polymer **P1** at pH 7.

| Peptide sequence | No. of phosphorylation sites | $[M-H]^-$ (Da) | Protein |
|---|---|---|---|
| TVDMESTEVFTK | 1 | 1464.6 | α-casein |
| TVD[Mo]ESTEVFTK | 1 | 1480.6 | α-casein |
| VPQLEIVPNSAEER | 1 | 1658.8 | α-casein |
| YLGEYLIVPNSAEER | 1 | 1830.9 | α-casein |
| DIGSESTEDQA[Mo]EDIK | 2 | 1941.7 | α-casein |
| YKVPQLEIVPNSAEER | 1 | 1950.0 | α-casein |
| FQSEEQQQTEDELQDK | 1 | 2059.8 | β-casein |
| EVVGSAEAGVDAASVSEEFR | 1 | 2087.9 | ovalbumin |
| LPGFGDSIEAQCGTSVNVHSSLR | 1 | 2509.1 | ovalbumin |
| FDKLPGFGDSIEAQCGTSVNVHSSLR | 1 | 2889.3 | ovalbumin |
| ELEELNVPGEIVESLSSSEESITR | 4 | 2963.3 | β-casein |
| RELEELNVPGEIVESLSSSEESITR | 4 | 3120.5 | β-casein |

[Mo], Oxidation on methionine; S: phosphorylated residue.

**Table 2.4.** Negatively charged non-phosphorylated peptides detected from protein mixture consisting of α-casein, β-casein, chicken ovalbumin, BSA and lysozyme by MALDI-TOF-MS analysis in negative mode after enrichment using positively charged polymer **P5** at pH 7.

| Peptide sequence | pI | $[M-H]^-$ (Da) | Protein |
|---|---|---|---|
| YLGYLEQLLR | 6.00 | 1265.7 | α-casein |
| TVMENFVAFVDK | 4.37 | 1397.7 | BSA |
| DAFLGSFLYEYSR | 4.37 | 1565.7 | BSA |
| LKPDPNTLCDEFK | 4.56 | 1517.7 | BSA |
| DAFLGSFLYEYSR | 4.37 | 1565.7 | BSA |
| AEFVEVTKLVTDLTK | 4.68 | 1690.9 | BSA |

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 2.4a)

that change from about 90 nm to 120 nm upon adding Zr. These sizes are consistent with the sizes of polymeric reverse micelles that have been studied previously.[12,13] Measuring the sizes of the assemblies after extraction of the aqueous phase is complicated by the formation of a interfacial layer between the phases. Second, complexation of Zr by the phosphonate groups in polymer P1 was confirmed by FT-IR measurements (Figure 2.4b). After loading metal, the P=O stretch of the phosphonate group shifts from 1241 cm-1 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.[14,15] The disappearance of the 993 cm-1 band and appearance of a new band around 948 cm-1 in the Zr-loaded polymer suggests the replacement of P-O-H with P-O-Zr. Third, the concentration of Zr in the polymeric assemblies, as assessed by ICP-MS (Table 2.5), confirm that (i) Zr is present in the polymer assemblies, as Zr itself is not soluble in toluene alone, and (ii) the Zr-phosphonate stoichiometry can be varied by changing the amount of Zr that is added.

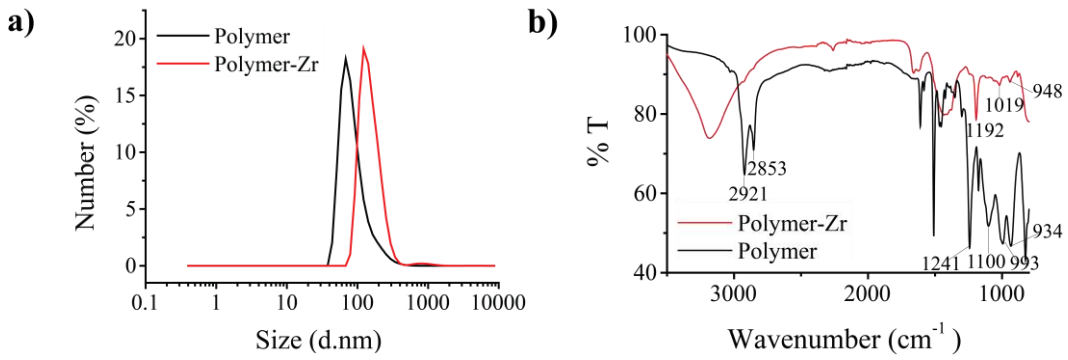



**Figure 2.5.** (a) DLS of polymer **P1** (black) and Zr loaded polymer **P1** (red) in toluene at concentrations of 0.1 mM in terms of phosphonate groups. (b) FT-IR of polymer **P1** (black) and Zr loaded polymer **P1** (red) after drying the solutions from toluene.

**Table 2.5.** ICP-MS determined Zr concentrations of the polymer-containing toluene phase upon adding 0.07 mg/mL of polymer **P1** (100 μM phosphonate groups) and different molar ratios of Zr.

| Added Zr/P ratio | Measured Zr/P ratio |
|---|---|
| 0 | 0* |
| 0.1 | 0.0187 ±0.0008 |
| 0.2 | 0.058 ±0.002 |
| 0.5 | 0.239 ±0.005 |
| 1 | 0.58 ±0.01 |
| 2 | 1.21 ±0.02 |
| 5 | 2.69 ±0.05 |

* Zr concentrations are below detectable levels.

We next studied how Zr(IV) loading influenced phosphopeptide binding selectivity and efficiency for polymer **P1**. Selectivity is reported as the percentage of detected peptides that are phosphopeptides and provides a measure of the specificity of the enrichment process. We used total phosphopeptide intensity as an indicator of the efficiency of the enrichment process, as 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 2.5a). 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 2.5b), 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 extraction efficiency. This effect might arise due

to MALDI-MS signal suppression in the presence of higher Zr concentrations or overloading of the reverse micelles that lead to 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,[16] 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 aqueous phase when the Zr/P ratio is between 0.5 and 3.0.

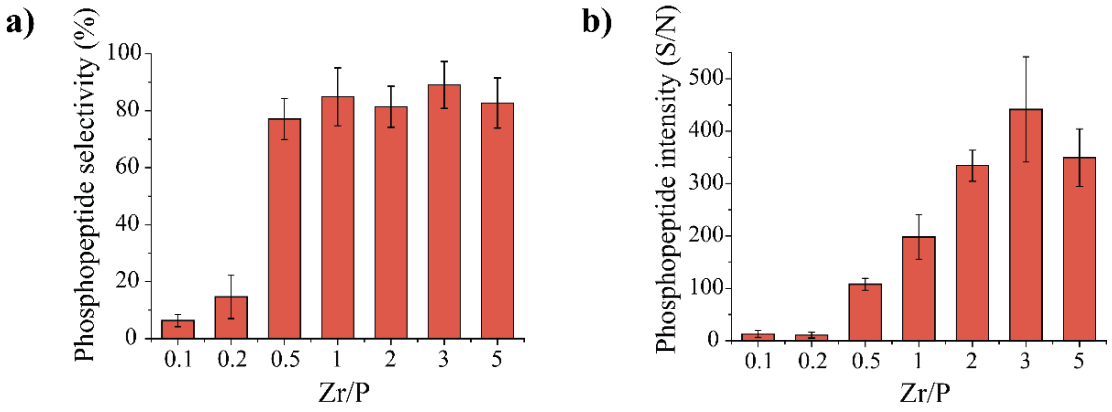



**Figure 2.6.** (a) Selectivity and (b) efficiency of extracting phosphopeptides from a protein digest mixture of α-casein, β-casein and chicken ovalbumin each at 0.5 μM at pH 7 using polymer **P1** loaded with Zr at different molar ratios of Zr/P.

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** (Figure 2.2) having different percentages of phosphonate and PEG groups. The PEG groups were chosen to act as weak Zr coordination sites that could be replaced by phosphopeptides.

Phosphopeptide enrichment selectivity and efficiency was found to be the highest when the random co-polymers had both phosphonate and PEG groups (**P1** and **P2**), rather than just phosphonate groups or just PEG groups (Figure 2.6). 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 co-monomer 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 2.6b). The relatively low enrichment selectivity and efficiency of polymer **P3** are attributed to the inability of some of the polymer phosphonate groups to be displaced from Zr(IV) inside the reverse micelles upon exposure to phosphopeptides, resulting in inefficient phosphopeptide capture. 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 Zr(IV) coordination strength and the ability to open up coordination sites for entering phosphopeptides. 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.[16]

Another interesting feature of these materials is that their selectivity and efficiency are somewhat independent of pH (Figure 2.6), 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 work well under acidic conditions.[7-11] Perhaps the confined environment inside the reverse micelles limits hydroxide levels, thereby minimizing hydrolysis.

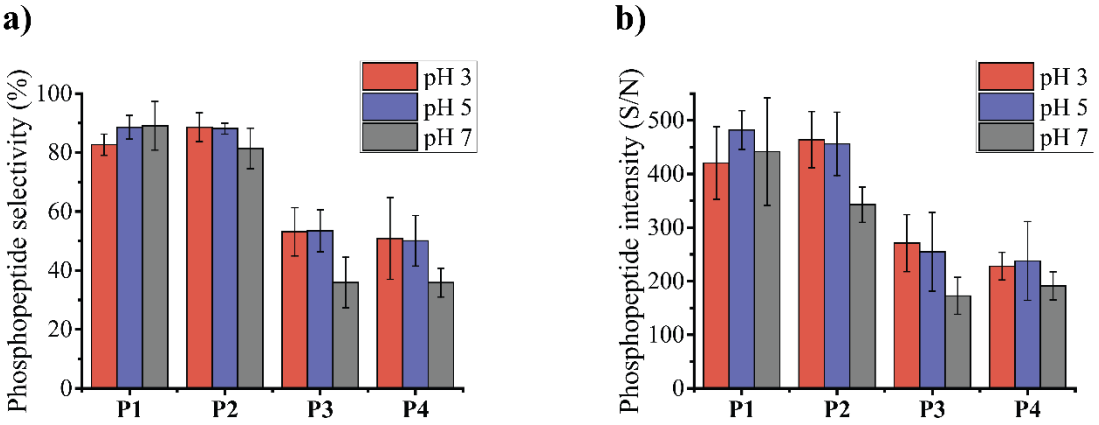



**Figure 2.7.** Phosphopeptide enrichment (a) selectivity and (b) efficiency using the polymers **P1**-**P4** in Scheme 2 to extract a three-protein digest mixture from an aqueous phase pH of 3, 5 and 7.

Upon better understanding 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 non-phosphorylated peptides originating from BSA (Figure 2.7a). The presence of exclusively BSA-related peptides is not surprising as digestion of this protein can produce more than 200 peptides, while β-casein typically produces only 20 peptides, of which only 3 are phosphorylated. Upon using polymer P1 or **P2** to enrich the phosphopeptides of β-casein, MALDI-MS spectra are now much simpler with 3 or 2 phosphopeptides detected (Figure 2.7b and c, respectively). These results highlight the high degree of selectivity possible when Zr(IV) is loaded into these polymeric reverse micelles.

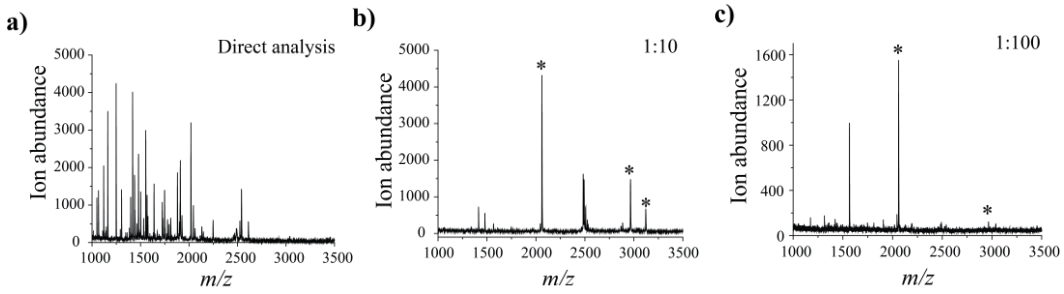



**Figure 2.8.** MALDI mass spectra of protein digests of β-casein and BSA (a) before enrichment; (b) after enrichment using Zr loaded polymer **P1** and a β-casein: BSA ratio of 1:10 (β-casein 50 nM; BSA 0.5 μM) at pH 7; (c) after enrichment using Zr loaded **P2** and a β-casein: BSA ratio of 1:100 (β-casein 10 nM; BSA 1 μM) at pH 3. The peaks labeled with asterisks indicate the phosphorylated peptides.

## 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 micelle 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 so that it can be used to improve the detection of phosphopeptides, which could make them valuable materials for phosphoproteomics studies.

## Experimental Methods

### Materials and Reagents

α-Casein, β-casein, chicken ovalbumin, BSA, lysozyme, DL-dithiothreitol (DTT), iodoacetamide (IAM), phosphoric acid ($H_3PO_4$), and zirconium(IV) oxychloride octahydrate ($ZrOCl_2 \cdot 8H_2O$) 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 ($NH_4HCO_3$) was obtained from Fluka. Water was purified using a Milli-Q water purification system (Millipore, Bedford, MA). All other chemicals were used as obtained from commercial sources.

### Polymer Synthesis

The amphiphilic random copolymers bearing hydrophobic p-alkoxy moieties and hydrophilic variable phosphonate, pentaethylene glycol monomethyl ether (PEG), or carboxylate groups that were used in this study are shown in Figure 2.2. 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).

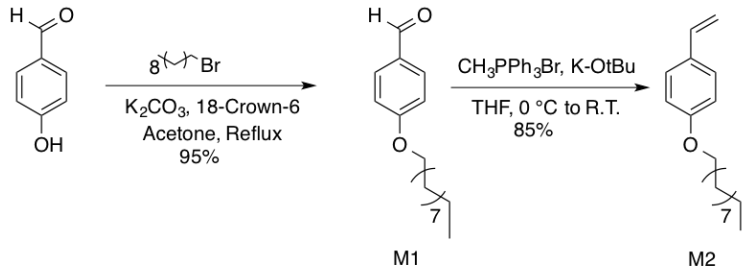



**Figure 2.9.** Synthetic scheme for monomer M2.

**Synthesis of Monomers. Synthesis of compound M1**: To a solution of acetone mixed with $K_2CO_3$ (11.84 g, 85.65 mmol) and 18-crown-6 (1.13 g, 4.28 mmol), 4-hydroxybenzaldehyde (5.23 g, 42.83 mmol) was added and stirred for 5 min. To this mixture, 1-bromodecane (14.21 g, 64.24 mmol) was added and stirred at reflux for 20 h. The reaction mixture was then cooled to room temperature and filtered to afford the crude product in acetone solution. The solvent was evaporated to dryness and purified by silica gel column chromatography (8-10% ethyl acetate in hexanes) to obtain 10.5 g (95% yield) of **M1**. $^1$H NMR (400MHz, $CDCl_3$) δ 9.86 (s, 1H), δ 7.80-7.82 (d, 2H), δ 6.96-6.99 (d, 2H), δ 4.00-4.04 (t, 2H), δ 1.76-1.83 (quint, 2H), δ 1.47-1.26 (m, 14H), δ 0.85-0.89 (t, 3H); ESI-MS (expected: $[m+H]^+$= 263.2, obtained: $[m+H]^+$= 263.2).

**Synthesis of monomer M2**: Methyltriphenylphosphonium bromide (6.58 g, 25.11 mmol) and potassium tert-butoxide (3.94 g, 35.15 mmol) were mixed in a round bottom

flask, and dry THF (20 mL) was added to the mixture. The mixture was stirred under argon atmosphere in an ice bath for 15 min to yield a bright yellow solution. **M1** (6.58 g, 25.11 mmol) was slowly added to the mixture. The reaction mixture was further stirred for 5 h. After the reaction, saline and ethyl acetate were added for extraction. The combined organic layer was separated and washed with saline (3 x). The organic layer was evaporated to dryness and purified by silica gel column chromatography (3-5% ethyl acetate in hexanes) to afford 5.5 g (85% yield) of **M2**. $^{1}$H NMR (400MHz, $CDCl_3$) δ 7.31-7.33 (d, 2H), δ 6.83-6.85 (d, 2H), δ 6.61-6.68 (q, 1H), δ 5.57-5.61 (d, 1H), δ 5.09-5.12 (d, 1H), δ 3.93-3.96 (t, 3H), δ 1.73-1.80 (quint, 2H), δ 1.27-1.46 (m, 14H), δ 0.86-0.89 (t, 3H); ESI-MS (expected: $[m+H]^+$= 261.2, obtained: $[m+H]^+$= 261.2).

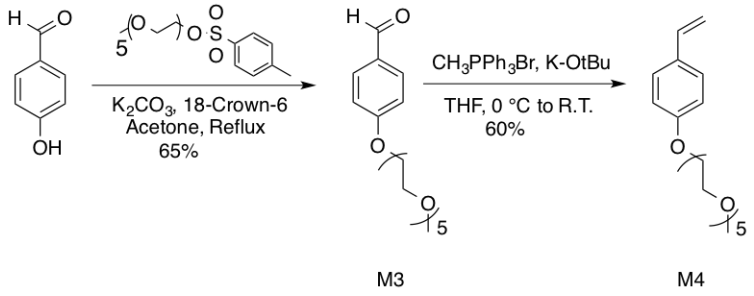



**Figure 2.10.** Synthetic scheme for monomer M4.

**Synthesis of compound M3**: To a solution of acetone mixed with $K_2CO_3$ (1.17 g, 8.45 mmol), and 18-crown-6 (0.56 g, 2.11 mmol), 4-hydroxybenzaldehyde (0.52 g, 4.23 mmol) was added and stirred for 5 min. To this mixture, tosylate of pentaethyleneglycol monomethyl ether (2.06 g, 5.07 mmol) was added and stirred with reflux for 20 h. The reaction mixture was then cooled to room temperature and filtered to afford the crude product in acetone solution. The solvent was evaporated to dryness and purified by silica gel column chromatography (3-5% MeOH in DCM) to obtain 1.0 g (65% yield) of **M3**. $^{1}$H

NMR (400MHz, $CDCl_3$) δ 9.88 (s, 1H), δ 7.82-7.84 (t, 2H), δ 7.01-7.03 (d, 2H), δ 4.20-4.22 (t, 2H), δ 3.88-3.90 (t, 2H), δ 3.53-3.73 (m, 16H), δ 3.37 (s, 3H); ESI-MS (expected: $[m+H]^+$= 357.2, obtained: $[m+Na]^+$= 379.2).

**Synthesis of monomer M4**: Methyltriphenylphosphonium bromide (1.35 g, 3.79 mmol) and potassium tert-butoxide (0.42 g, 3.74 mmol) were mixed in a round bottom flask, and dry THF (15 mL) was added to the mixture. The mixture was stirred under argon atmosphere in an ice bath for 15 min to yield a bright yellow solution. **M3** (0.9 g, 2.53 mmol) was slowly added to the mixture. The reaction mixture was further stirred for 5 h. After the reaction, saline and ethyl acetate were added for extraction. The combined organic layer was separated and washed with saline (3 x). The organic layer was evaporated to dryness and purified by silica gel column chromatography (30-40% ethyl acetate in hexanes) to afford 0.54 g (60% yield) of **M4**. $^1$H NMR (400MHz, $CDCl_3$) δ 7.31-7.32 (d, 2H), δ 6.85-6.86 (d, 2H), δ 6.61-6.67 (q, 1H), δ 5.57-5.61 (q, 1H), δ 5.09-5.12 (q, 1H), δ 4.10-4.12 (t, 2H), δ 3.83-3.85 (t, 2H), δ 3.53-3.72 (m, 16H), δ 3.37 (s, 3H); ESI-MS (expected: $[m+H]^+$= 355.2, obtained: $[m+Na]^+$= 377.2).

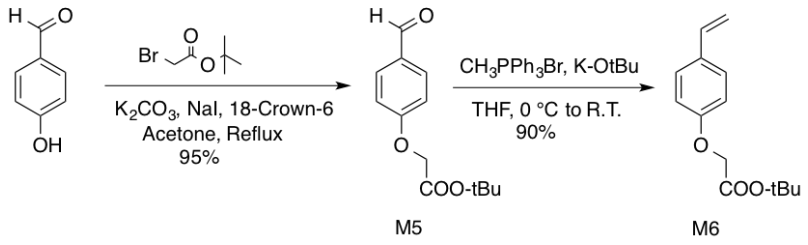



**Figure 2.11.** Synthetic scheme for monomer M6.

**Synthesis of compound M5:** To a solution of acetone mixed with $K_2CO_3$ (6.79 g, 49.13 mmol), NaI (7.36 g, 49.13 mmol) and 18-crown-6 (0.65 g, 2.46 mmol), 4-

hydroxybenzaldehyde (3.00 g, 24.57 mmol) was added and stirred for 5 min. To this mixture, tert-butyl-bromoacetate (9.58 g, 49.13 mmol) was added and stirred with reflux for 20 h. The reaction mixture was then cooled to room temperature and filtered to afford the crude product in acetone solution. The solvent was evaporated to dryness and purified by silica gel column chromatography (10-13% ethyl acetate in hexanes) to obtain 5.5 g (95% yield) of **M5**. $^{1}$H NMR (400MHz, $CDCl_3$) δ 9.88 (s, 1H), δ 7.82-7.84 (d, 2H), δ 6.97-6.99 (d, 2H), δ 4.59 (s, 2H), δ 1.47 (s, 9H); ESI-MS (expected: $[m+H]^+$= 237.1, obtained: $[m+Na]^+$= 259.1).

**Synthesis of monomer M6:** Methyltriphenylphosphonium bromide (7.94 g, 22.24 mmol) and potassium tert-butoxide (2.50 g, 22.24 mmol) were mixed in a round bottom flask, and dry THF (15 mL) was added to the mixture. The mixture was stirred under argon atmosphere in an ice bath for 15 min to yield a bright yellow solution. **1c** (3.5 g, 14.83 mmol) was slowly added to the mixture. The reaction mixture was further stirred for 5 h. After the reaction, saline and ethyl acetate were added for extraction. The combined organic layer was separated and washed with saline (3 x). The organic layer was evaporated to dryness and purified by silica gel column chromatography (3-5% ethyl acetate in hexanes) to afford 3.1 g (90% yield) of **M6**. $^{1}$H NMR (400MHz, $CDCl_3$) δ 7.33-7.35 (d, 2H), δ 6.84-6.87 (d, 2H), δ 6.63-6.68 (q, 1H), δ 5.60-5.64 (q, 1H), δ 5.13-5.15 (q, 1H), δ 4.51 (s, 2H), δ 1.49 (s, 9H); ESI-MS (expected: $[m+H]^+$= 235.1, obtained: $[m+Na]^+$= 257.1).

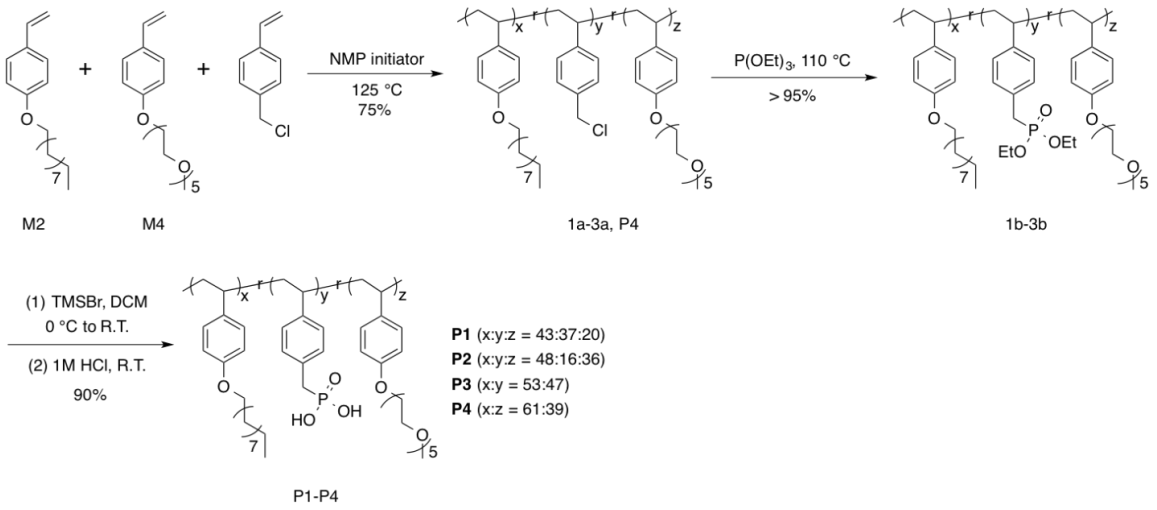



**Figure 2.12.** Synthetic scheme for random copolymer **P1**-**P4**.

**Synthesis of Polymers.** Synthesis of polymers **1a**-**3a** and **P4**: A mixture of the compound **M2** (200 mg, 0.77 mmol), **M4** (109 mg, 0.31 mmol), commercial available compound 4-vinylbenzyl chloride (70 mg, 0.46 mmol) and *N*-*tert*-Butyl-*N*-(2-methyl-1-phenylpropyl)-*O*-(1-phenylethyl)hydroxylamine (NMP initiator, 10 mg, 0.031 mmol) were degassed by three freeze/thaw cycles, sealed under argon, and heated at 125 °C under argon for 12 h. After the reaction cooled down to room temperature, the reaction mixture was dissolved in DCM, and dialyzed against DCM/MeOH (v/v= 6/1) for 2 days. The solution was collected and dried under vacuum to yield 290 mg (75% yield) of **1a**. GPC (PMMA/THF): $M_n$= 12K Da, Đ=1.5; Same method was applied for polymers **2a, 3a** and **P4** except for feeding ratios. For **2a**, compound **M2** (200 mg, 0.77 mmol), **M4** (163 mg, 0.46 mmol), 4-Vinylbenzyl chloride (47 mg, 0.31 mmol) were added. For **3a**, compound **M2** (200 mg, 0.77 mmol), 4-Vinylbenzyl chloride (117 mg, 0.77 mmol) were added. For **P4**, compound **M2** (200 mg, 0.77 mmol), **M4** (117 mg, 0.77 mmol) were added.

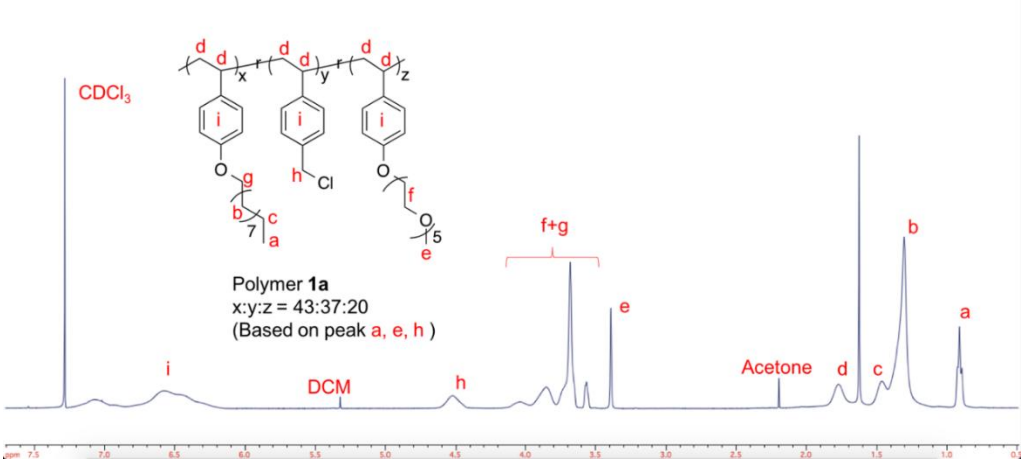



**Figure 2.13.** 1 H NMR Spectrum of polymer 1a.

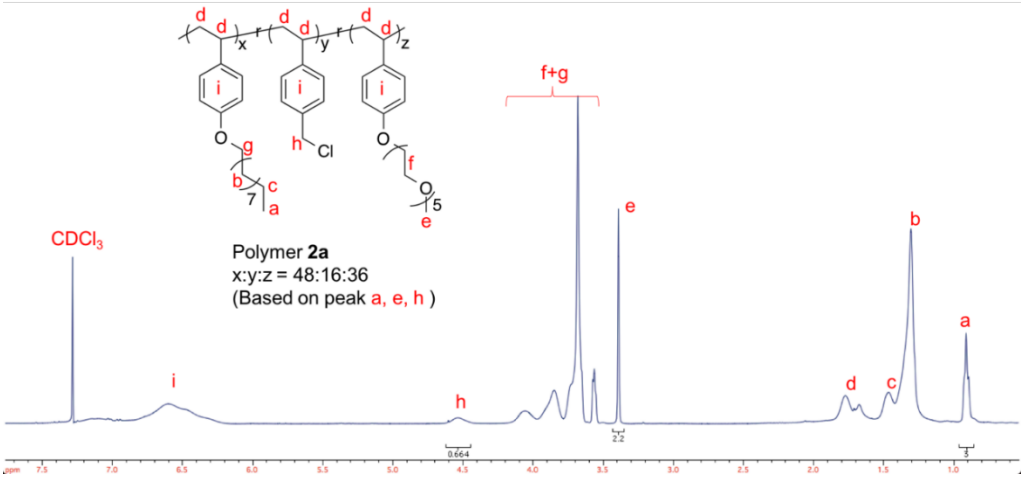



**Figure 2.14.** 1 H NMR Spectrum of polymer 2a.

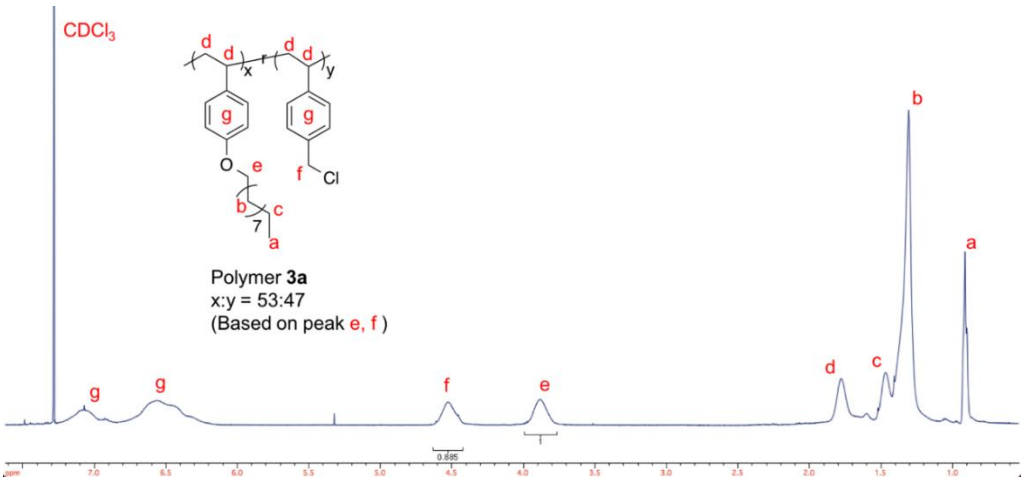



**Figure 2.15.** 1 H NMR Spectrum of polymer 3a.

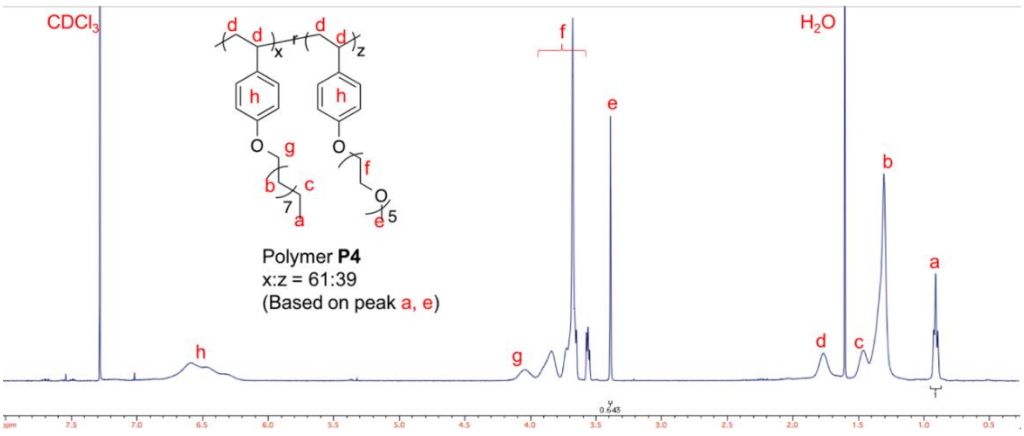



**Figure 2.16.** 1 H NMR Spectrum of polymer P4.

**Synthesis of polymers 1b-3b**: Polymer precursors **1a** was added to 2mL of triethylphosphite in a round bottom flask and stirred with reflux at 110 °Cfor 24 h. The reaction mixture was then cooled to room temperature and dialyzed against DCM/MeOH (v/v= 6/1) for 2 days to remove excess triethylphosphite. The solution was collected and dried under vacuum to yield **1b**. $^{1}$H NMR indicates that there is a quantitative conversion from benzyl chloride to benzyl phosphonate functional group. Same method was applied for polymers **2b** and **3b**.

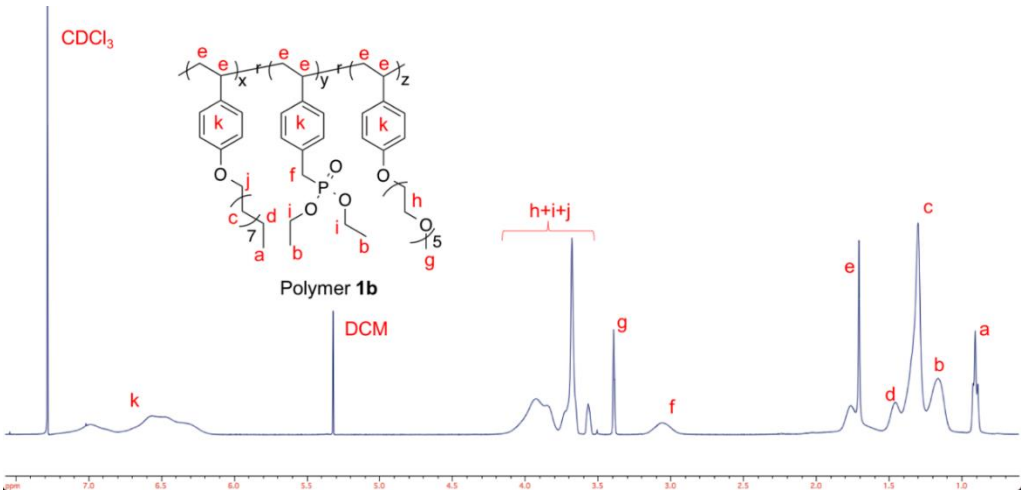



**Figure 2.17.** 1 H NMR Spectrum of polymer **1b**.

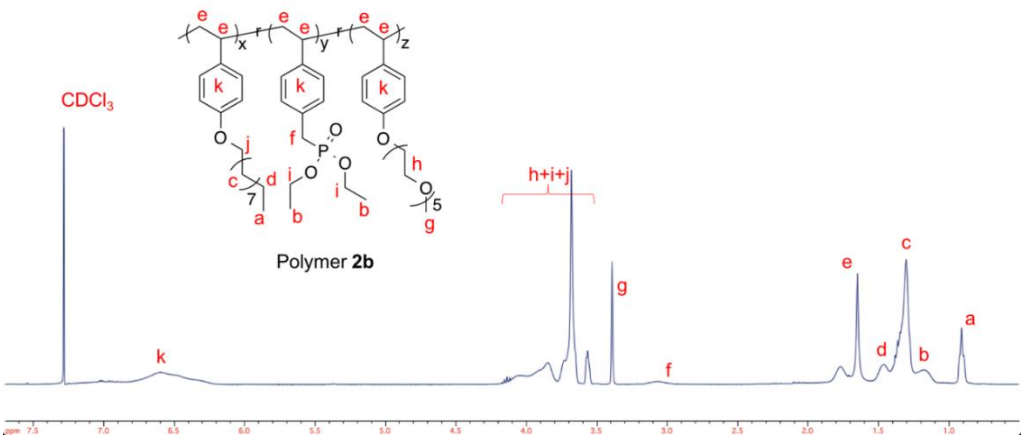



**Figure 2.18.** 1 H NMR Spectrum of polymer **2b**.

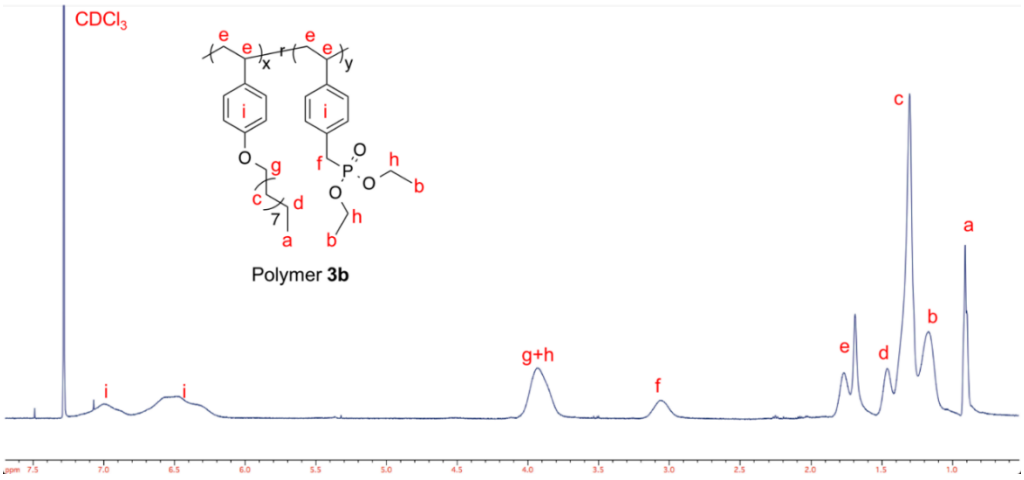



**Figure 2.19.** 1 H NMR Spectrum of polymer **3b**.

**Synthesis of polymers P1-P3**: Polymer **1b** was dissolved in 3 mL of DCM in a round bottom flask and stirred in an ice bath for 15 min. Bromotrimethylsilane (0.27 mL, 2.07 mmol) was slowly added to the solution. The reaction mixture was further stirred for 12 h. After the reaction, the solvent and excess bromotrimethylsilane was evaporated to obtain dark yellow solids. 3 mL DCM was added to re-dissolve the compounds and 1 M HCl aqueous solution (1 mL) was added. The reaction mixture was stirred at room temperature for 1 hour. After the reaction, DCM was evaporated and water was removed

by lyophilizing to obtain the final polymer **P1**. Same method was applied for polymers **P2** and **P3**.

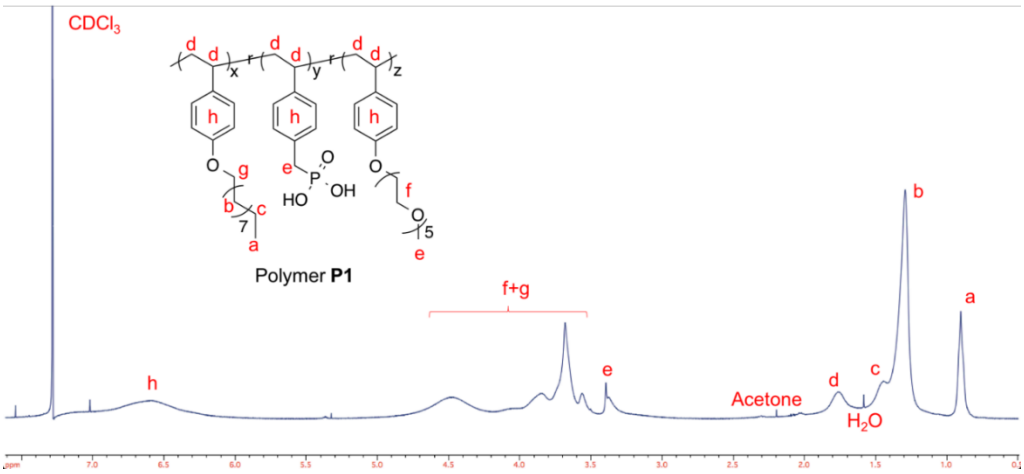



**Figure 2.20.** 1 H NMR Spectrum of polymer **P1**.

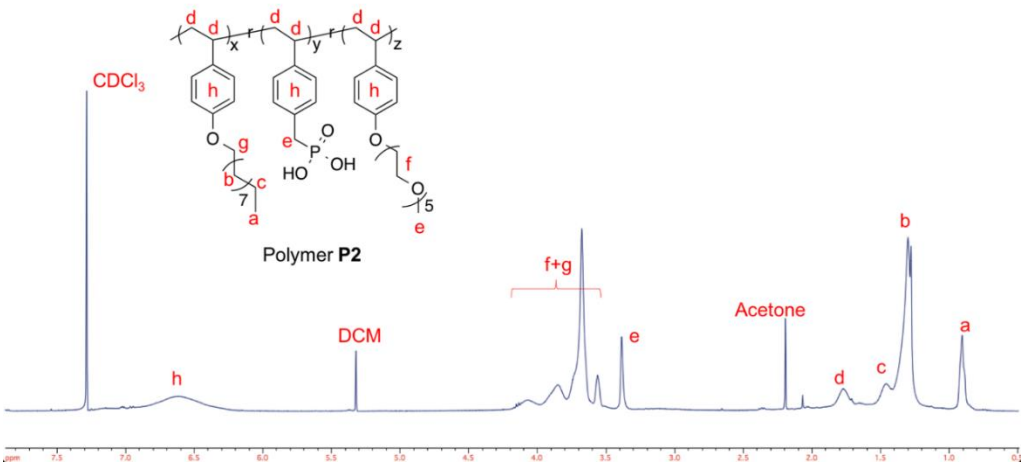



**Figure 2.21.** 1 H NMR Spectrum of polymer **P2**.

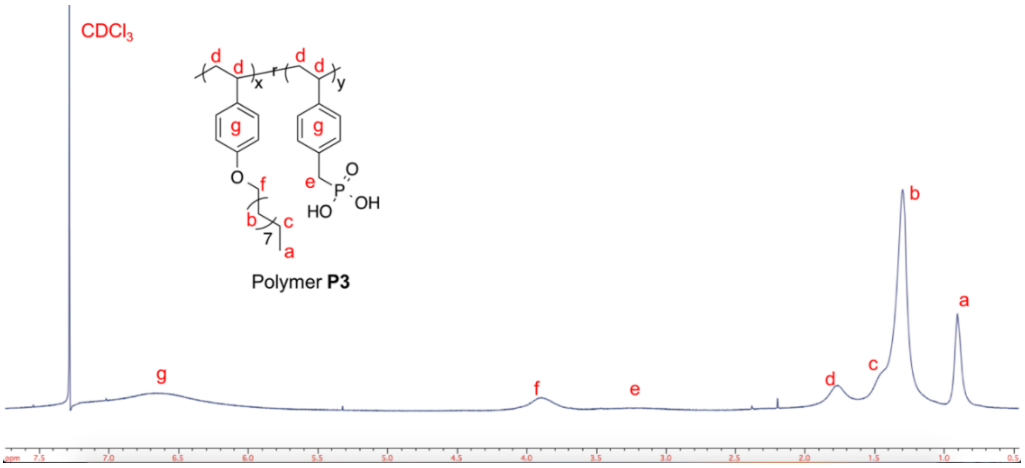



**Figure 2.22.** 1 H NMR Spectrum of polymer **P3**.

## 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 the phosphonate functional group concentration of 100 µM. $ZrOCl_2 \cdot 8H_2O$ was dissolved in water and added into the toluene solution at different molar ratios of Zr to 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 of phosphonate or PEG groups. These solutions were used for the liquid-liquid extraction.

## Preparation of Protein Digests

50 µM of α-casein and β-casein were dissolved in 50 mM $NH_4HCO_3$ (pH 8.2) and digested for 12 h with trypsin at an enzyme-to-protein ratio of 1:100 (wt/wt). 50 µM of chicken ovalbumin, BSA and lysozyme were dissolved in 50 mM $NH_4HCO_3$ (pH 8.2) containing 8 M urea. DTT was added to a final concentration of 5 mM and the mixture was incubated at 37 ºC 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 over-alkylation. The solution was diluted with 50 mM $NH_4HCO_3$ 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 ºC.

**Liquid-Liquid Extractions**

Protein digests were diluted with 50 mM Tris buffer and adjusted to the desired pH using HCl or $NH_4OH$. 200 μL of the polymeric reverse micelle solution was added to 1 mL of the peptide solution and vortexed vigorously for 1.5 hours. Centrifugation at 15,000 rpm for 30 minutes 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 $N_2$ gas. This dried residue was re-dissolved in 20 μL of THF and mixed with 20 μL of a DHB matrix solution (25 mg/mL in 70% (vol/vol) acetonitrile containing 1% (wt/vol) $H_3PO_4$). 1 μL of this solution was directly spotted on the 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. ICP-MS data were obtained on a PerkinElmer Nexion 300 ICP mass spectrometer. Zr

concentrations loaded into polymer P1 were determined by adding 0.5 mL of fresh aqua regia for 30 min after evaporation of toluene in 10 μL of polymer solutions. Zr concentrations in aqueous phase after extraction were measured by directly adding 0.5mL of fresh aqua regia into 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 calibration of ICP-MS measurements. $^{1}$H-NMR spectra were recorded on a 400 MHz NMR spectrometer using residual proton resonance of the solvents as internal standard. Chemical shifts are reported in parts per million (ppm).

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# CHAPTER III

# SUPRAMOLECULAR NANOASSEMBLIES FOR GLYCOPEPTIDE EXTRACTION AND RELEASE FOR MASS SPECTROMETRIC DETECTION

## Introduction

Protein glycosylation plays an important role in biological process, such as cell recognition,[2] signaling pathways[3] and development of genetic disorders.[4] Also, it has recently gained attention in the pharmaceutical industry as part of monoclonal antibody drug characterization.[5–7] The methods applied for glycosylation analysis are most often based on mass spectrometry (MS), but because of the low abundance and heterogeneity of glycosylated sites, separation prior to MS analysis is typically required.[8–10]

Efforts have been made to improve the separation of glycosylated proteins or peptides through noncovalent and covalent interactions.[9,11] Noncovalent interactions include hydrophilic, lectin and chelation interactions that are relatively easy to incorporate into online analysis before MS detection.[11–14] Covalent binding using beads with boronic acid or hydrazine/ hydrazide functional groups are also widely used because of their higher specificity, which is essential for complex mixtures.[15–20] For better selectivity and efficiency in extraction, covalent and noncovalent interactions have also been used complementarily. For example, Wang et al. used boronic acid nanoparticles for specific binding with glycopeptides and poly(methyl methacrylate) nanobeads for non-specific interactions with non-glycopeptides to achieve better selectivity.[21] Also, glycoprotein enrichment based on lectin affinity chromatography and on hydrazide chemistry have been

used separately by Song, et al. to determine and confirm glycoprotein/glycopeptide biomarkers in human blood serum more efficiently.[22] Based on the continued need for both high selectivity and efficiency in glycopeptide enrichment approaches, we designed a new strategy that is inspired by the use of covalent and non-covalent interactions. Instead of using both covalent and non-covalent interactions to target peptides, we used covalent binding via hydrazide groups to achieve high selectivity for glycopeptide and a secondary non-covalent interaction to catalyze this normally slow covalent binding reaction, thereby improving extraction efficiency. We employ this strategy in a two-phase liquid-liquid extraction platform because of its advantageous ability to extract, purify and concentrate in one step.[23,24] To achieve this two-phase extraction, we have designed a supramolecular nanoassembly that can be readily tuned for optimal extraction efficiency. The supramolecular nanoassembly described here is based on an amphiphilic polymer design that we recently developed to selectively enrich phosphopeptides and other peptides according to their isoelectric point.[25–30] We find that in the confined environment of the nanoassembly the reaction of hydrazide with glycans is faster than in existing approaches. Moreover, the extraction efficiency can be further increased by placing functional groups in the assemblies that catalyze the reaction of hydrazide moieties with glycans.

## Results and Discussion

### Glycopeptide Enrichment Using Hydrazide-Containing Nanoassemblies

The extraction procedure for glycopeptides by the hydrazide-containing nanoassemblies is illustrated in Figure 3.1. IgG1, with its four well characterized glycans on Asn292,[30] was used as a model protein to evaluate the extraction performance of

nanoassemblies of polymer **P6** (Figure 3.2). Before enrichment, the two most abundant glycopeptides from the IgG1 digest are detected (Figure 3.3a), while the other two glycopeptides are not detected. After mild oxidation, enrichment with the nanoassemblies and release, only the four glycopeptides are detected (Figure 3.3b). Some heterogeneity in the detected glycopeptides is observed due to the oxidation reaction, but most of the glycan information is retained after enrichment (Figure 3.4). The extraction selectivity is further confirmed by PNGase F treatment of the enriched/released glycopeptides. Only a single peptide containing the glycosylated residue Asn292 is measured after deglycosylation (Figure 3.3c), indicating that only glycopeptides are extracted.

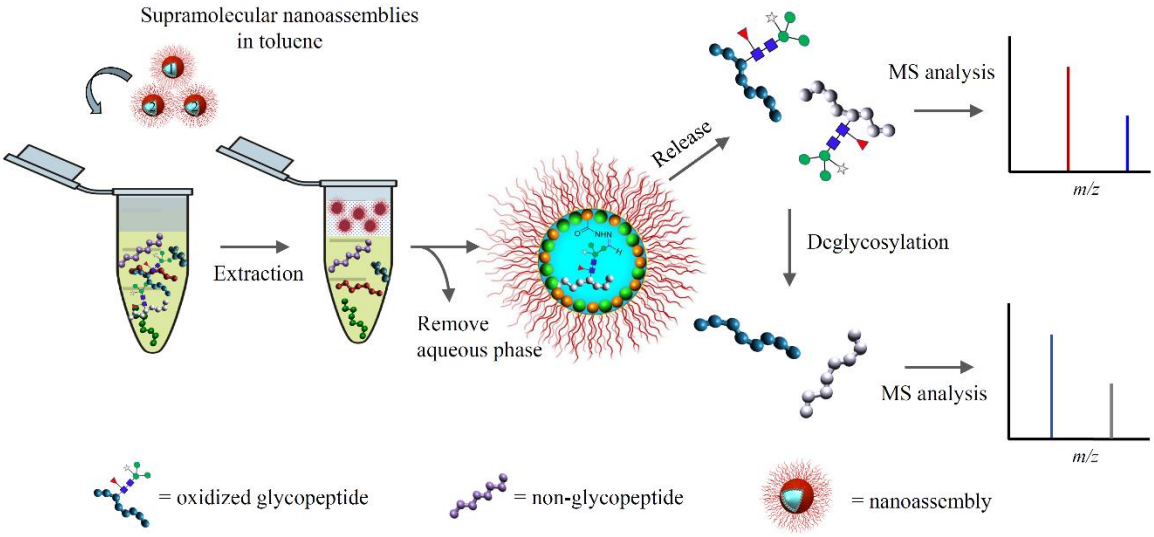



**Figure 3.1.** Workflow of glycopeptide enrichment using supramolecular nanoassemblies and analysis using mass spectrometry.

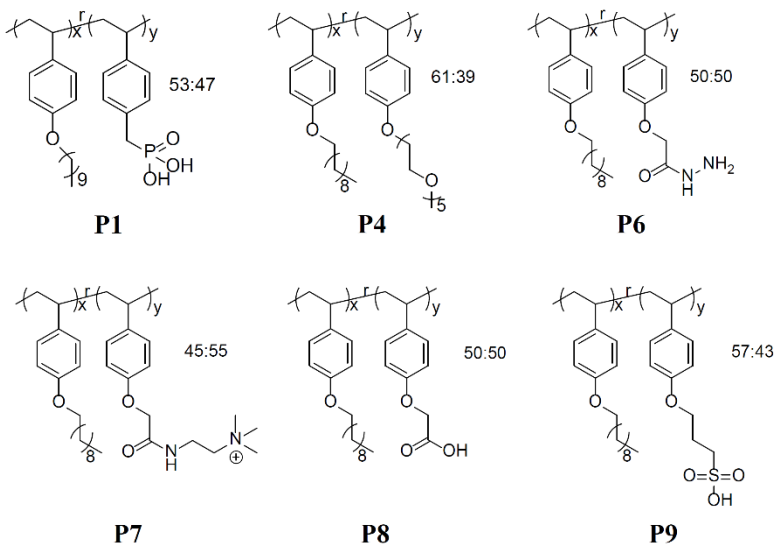



**Figure 3.2.** Structural features of polymer **P1**, **P4**, **P6-P9**.

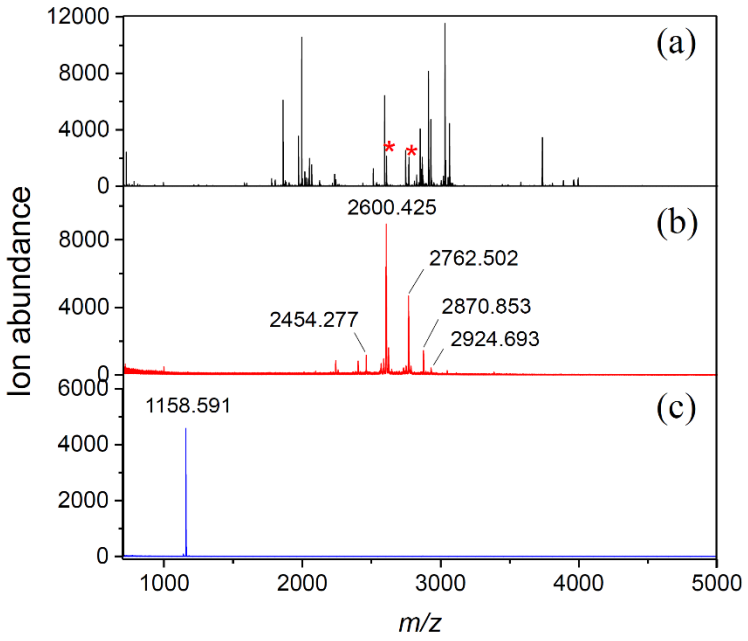



**Figure 3.3.** MALDI-TOF mass spectra of the oxidized IgG1 tryptic digests before and enrichment. (a) Mass spectrum before enrichment; (b) mass spectrum after enrichment using nanoassemblies of the hydrazide polymer **P1** at pH 4; and (c) mass spectrum after enrichment and deglycosylation by PNGase F.

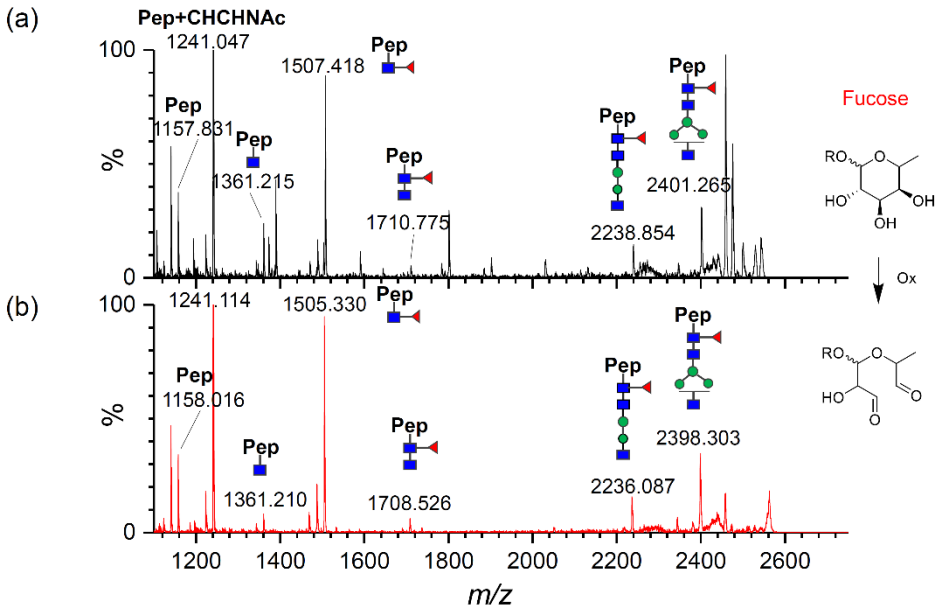



**Figure 3.4.** Glycopeptide analysis before and after enrichment with polymer **P6**. (a) Tandem mass spectrum of the precursor ion at *m/z* 2602.1 from the IgG1 digest and (b) Tandem mass spectrum of m/z 2600.4 after oxidation and enrichment (b). Symbol: (■) GlcNAc (●) Mannose (◀)Fucose.

**Improved Enrichment Efficiency via Proximity-Assisted Reactivity**

For more heterogeneous glycoproteins, enrichment using polymer **P6** can sometimes lead to relatively inefficient detection of some low level glycopeptides. As an example, enrichment of HRP using nanoassemblies of **P6** can enable the selective detection of all 7 known glycosylation sites (Figure 3.5a), even though most are undetectable before enrichment (Figure 3.5c), but some of the glycopeptides have low relative abundance. To improve the enrichment efficiency, we hypothesized that a copolymer with both hydrazide and weak acid functional groups would facilitate the binding in the nanoassemblies as the hydrazide-aldehyde conjugation reaction is known to be catalyzed by an acid and conjugate base.[31] We reasoned that a nearby acid and conjugate base in the local microenvironment would accelerate the reaction via a proximity-based effect. We tested this idea using mixed

nanoassemblies with 10 nm diameters (Figure 3.5d) of polymer **P6** and **P1** (Figure 3.2). A polymer containing phosphonate groups (i.e. polymer **P1**) was chosen because it is a polyprotic acid with $pK_a$ values of 2.5 and 7.5 that could provide both acidic and conjugate base functionality at the enrichment pH of 5.0. Upon extraction of the HRP digest by the mixed nanoassembly, we find a significant intensity increase in the total ion intensity of the glycosylated peptides (Figure 3.5b), and even detect new glycopeptides that are due to enzymatic mis-cleavages that were not detected upon enrichment with nanoassemblies of **P6** (Figure 3.5a).

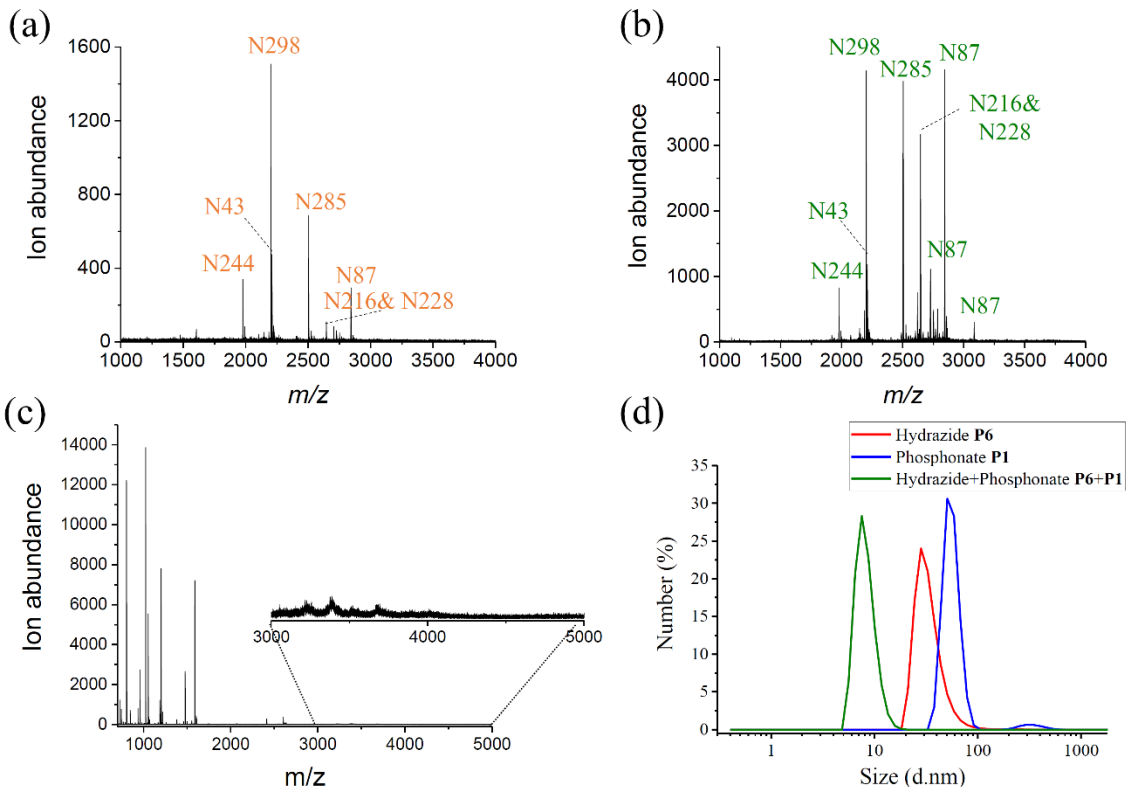



**Figure 3.5.** Enhanced glycopeptide detection after proximity-assisted enrichment. (a) MALDI-TOF mass spectrum of an oxidized HRP tryptic digest after enrichment using nanoassemblies of the hydrazide polymer **P6**; (b) MALDI-TOF mass spectrum after enrichment using nanoassembly mixtures of hydrazide polymer **P6** and phosphonate polymer **P1**; (c) MALDI spectrum of oxidized HRP digests before enrichment. No glycopeptides are detected; (d) Dynamic light scattering data for nanoassemblies formed by polymers **P6**, **P1** and **P6**+**P1**.

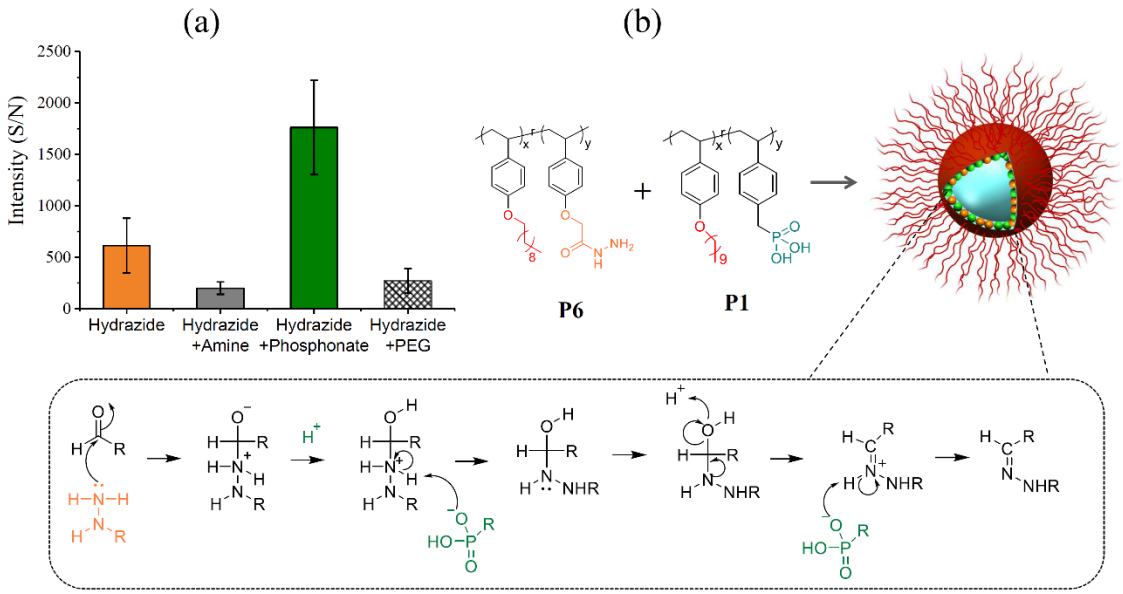



**Figure 3.6.** Enhanced glycopeptide detection after proximity-assisted enrichment. (a) Total intensity of glycopeptides extracted by nanoassemblies of **P6** and of **P6**+**P1**, **P6**+**P7** and **P1**+**P4**; and (d) proposed proximity effects that improve the extraction efficiency of glycopeptides.

Two sets of control experiments were employed to further test our hypothesis that a nearby acid and conjugate base improve enrichment efficiency. Mixed nanoassemblies of **P6** and (i) a quaternary amine containing polymer, **P7**, that does not yield a proton or conjugate base or (ii) an ethyleneglycol-containing polymer, **P4**, without acidic or basic functionality were also studied. Using total glycopeptide intensity as an indicator of the enrichment efficiency, we find that mixed assemblies with the phosphonate polymer **P1** *increase* glycopeptide signal by almost a factor of 2 compared to **P6** alone, while mixed assemblies with polymers **P7** and **P4** *decrease* glycopeptide signal by factors of 3 and 2.3, respectively (Figure 3.6a). The increased glycopeptide signal caused by the presence of **P1** in the nanoassemblies together with the decreased signal in the presence of the other polymers supports our hypothesis that nearby proton donors and acceptors are responsible

for the improved enrichment efficiency. We postulate that the improved efficiency is due to proximity effects that accelerate the hydrazide-glycan conjugation reaction (Figure 3.6b).

To further understand how acidic functional groups in the polymer nanoassemblies influence the enrichment efficiency, we synthesized polymers with carboxylate (**P8**) and sulfonate (**P9**) groups (Figure 3.2) and tested their enrichment efficiencies as a function of pH. These functional groups, along with the phosphonate functional groups, provide $pK_a$ values that range from ~ -1 ($-SO_3H$) to 7.5 ($-PO_3H^-$), providing a test of the importance of both the acid and conjugate base functionality in enhancing the enrichment reaction. We find that the enrichment efficiency is improved by forming mixed assemblies with all the acidic polymers when the extraction is done at low pH (Figure 3.7). At higher pH values, however, the enrichment efficiencies become more similar to the hydrazide assembly alone,

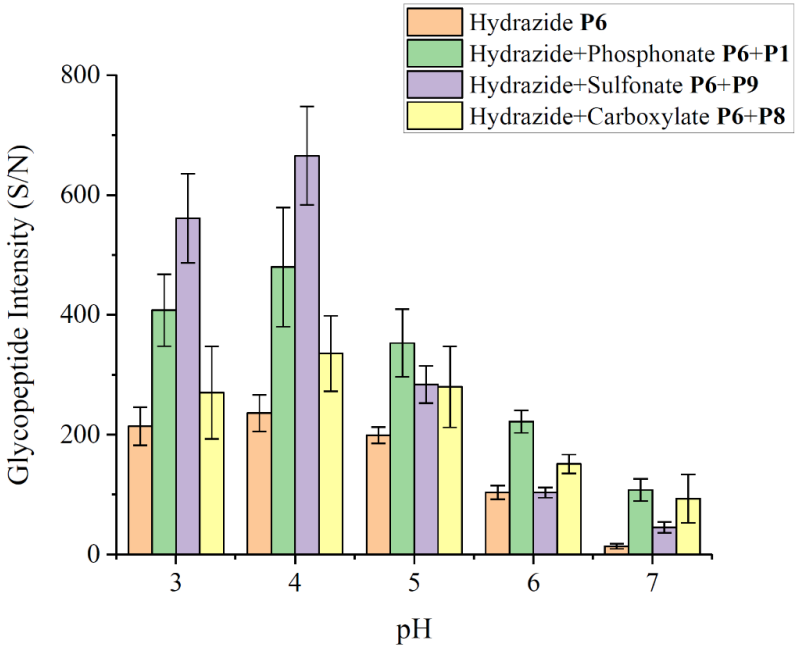



**Figure 3.7.** Total intensity of glycopeptides extracted from tryptic digests of oxidized HRP at pH values ranging from 3 to 7 using nanoassemblies of **P6**, **P6**+**P1**, **P6**+**P9** and **P6**+**P8**.

suggesting that deprotonation of the acidic group leads to less enhancement. Interestingly, the drop in the enhancement as the pH is increased is more extensive for the sulfonate polymer (**P9**), which has the lowest $pK_a$ and is the least extensive for the phosphonate polymer (**P1**), which has the highest $pK_a$. Indeed, the phosphonate group has two acidic protons causing its enhancement to exist over a wider range of pH values. As a whole, these results provide additional evidence for a proximity-based effect in the nanoassemblies that improve glycopeptide enrichment. These improvements, together with previous work from our group using similar materials, highlights the advantages of using supramolecular nanomaterials for peptide enrichment in general.

**Extraction of Glycopeptides from Complexed Samples**

With a better understanding of the polymer features that influence enrichment efficiency, we then tested the ability of mixed nanoassemblies to extract trace-level glycopeptides in a mixture. For this purpose, IgG1 and HRP were digested in the presence of 100-fold molar excesses of BSA. Before enrichment of the protein digest mixture, no glycopeptides for IgG1 or HRP can be detected (Figure 3.8a and d). After using nanoassemblies of **P6** (Figure 3.8b) or mixed nanoassemblies of **P6** and **P9** (Figure 3.8c), the glycopeptide from IgG1 is selectively detected after release and deglycosylation, with higher signal being observed for the mixed nanoassemblies. A similar effect is observed for the glycopeptides from HRP. The mixed nanoassemblies containing **P6** and **P9** allow the selective detection of more glycopeptides (Figure 3.8f) than the nanoassembly with hydrazide polymer (**P6**) alone (Figure 3.8e). These results highlight the high degree of selectivity possible with these supramolecular nanoassemblies. Future work will explore the ability of these materials to selectively enrich even more complicated mixtures.

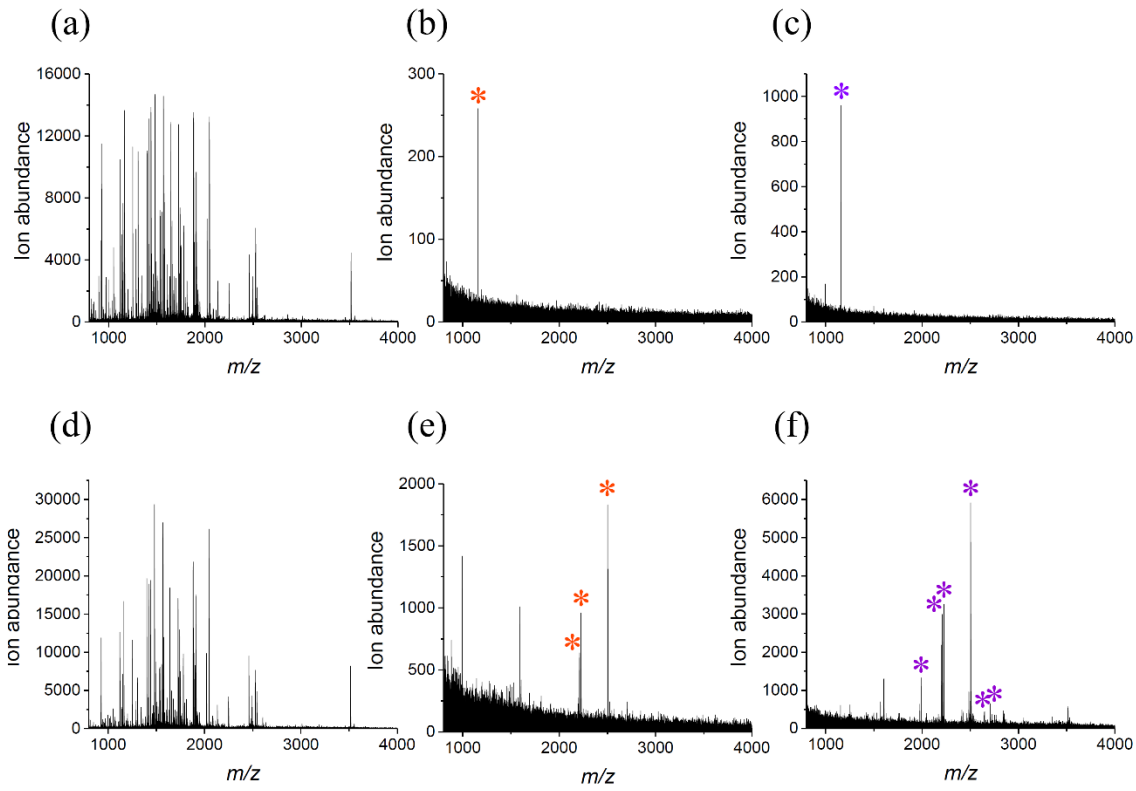



**Figure 3.8.** Enrichment and sensitive detection of trace-level glycopeptides in protein digest mixtures. (a) MALDI-TOF mass spectra of a tryptic digest of IgG1 (10 nM) and BSA (1 μM) before enrichment; (b) after enrichment using nanoassemblies of polymer **P6** at pH 4 followed by release and deglycosylation; and (c) after extraction using mixed nanoassemblies of polymers **P6** and **P9** at pH 4 followed by release and deglycosylation. (d) MALDI mass spectra of a tryptic digest mixture of HRP (50 nM) and BSA (5 μM) before enrichment; (e) after enrichment using nanoassemblies of polymer **P6** at pH 4 followed by release and deglycosylation; and (f) after extraction using mixed nanoassemblies of polymers **P6** and **P9** at pH 4 followed by release and deglycosylation.

## Conclusions

We have developed a simple method to improve glycopeptide enrichment efficiency and MS detection using supramolecular nanoassemblies based on amphiphilic polymers with hydrazide functional groups. The hydrazide-containing polymer itself forms nanoassemblies that are highly selective for oxidized glycopeptides. Co-assembly of the hydrazide polymer together with acidic polymers further improve enrichment efficiency via a proximity effect that catalyzes the hydrazide-glycan conjugation reaction. Using the

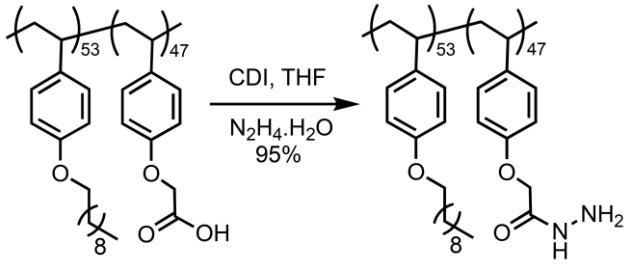
nanoassembly mixtures allow the selective and efficient enrichment and detection of glycopeptides that are present at low levels in mixtures. This study further demonstrates the value of designer supramolecular materials based on amphiphilic polymers as a platform for enriching and detecting biomolecules of interest. Future work will apply these nanoassemblies for the detection of glycosylated peptides or proteins in cell lysate, which would make them valuable materials for glycoproteomics studies.

## Experimental Section

### Materials and Reagents

Horseradish peroxidase (HRP), bovine serum albumin (BSA), DL-dithiothreitol (DTT), iodoacetamide (IAM), trifluoracetic acid (TFA) and potassium acetate were obtained from Sigma-Aldrich. The monoclonal antibody (IgG1) was purchased from Waters. PNGase F was obtained from New England Biolabs. Trypsin was purchased from Promega. Sodium periodate, 2, 5-dihydroxybenzoic acid (DHB), MOPS, acetic acid, toluene, tetrahydrofuran (THF) and acetonitrile (ACN) were purchased from Fisher Scientific. Urea was purchased from MP Biomedicals. Ammonium bicarbonate ($NH_4HCO_3$) was obtained from Fluka. THF was distilled before use. Water was purified using a Milli-Q water purification system. All chemicals were used as received from commercial sources.

**Polymer Synthesis, Self-assembly and Co-assembly in Toluene**




**P0** **P6**

**Figure 3.9.** Synthesis of amphiphilic random copolymer **P6**.

**Synthesis of Polymer**. Polymer **P0** was prepared according to previous procedures.[27] **P0** (100 mg) and carbonyldiimidazole (61 mg, 2 equivalents of carboxylate group) were dissolved in 5 mL tetrahydrofuran and stirred at room temperature for 2 hours. Then, this mixture was added to a hydrazine monohydrate/THF (2 mL/2 mL) solution dropwise and allowed to reflux overnight. The solvent was removed by a rotavapor, and the crude oil was purified by precipitating it 3 times in methanol. Yield: 90%, GPC (THF) Mn: 11.5 K. Đ: 1.08. $^{1}$H NMR (400 MHz, $CDCl_3$): δ 6.57-6.2, 4.53, 3.89, 1.77, 1.48-1.26, 0.90. $^{13}$C NMR (100 MHz, $CDCl_3$) δ 157.1, 128.5, 114.2, 67.9, 31.9, 29.6, 29.3, 29.1, 26.2, 25.5, 22.7, 14.1. From $^{1}$H NMR, integration of the proton peaks at 0.90 ppm and 4.53 ppm confirmed the molar ratio of these two components to be 53:47.

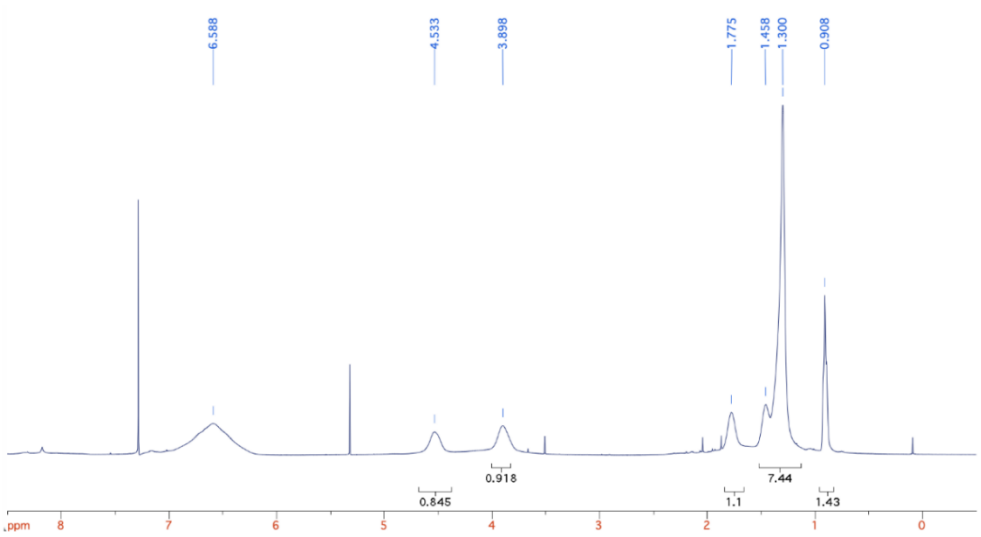



**Figure 3.10.** 1 H NMR Spectrum of polymer **P6**.

The hydrazide functional group attachment was confirmed by IR (Figure 3.10) showing a broad peak at 3319 $cm^{-1}$ corresponding to N-H bond, C=O bond shift from 1731 $cm^{-1}$ to 1693 $cm^{-1}$ due to the conversion of -OH to -$NHNH_2$.

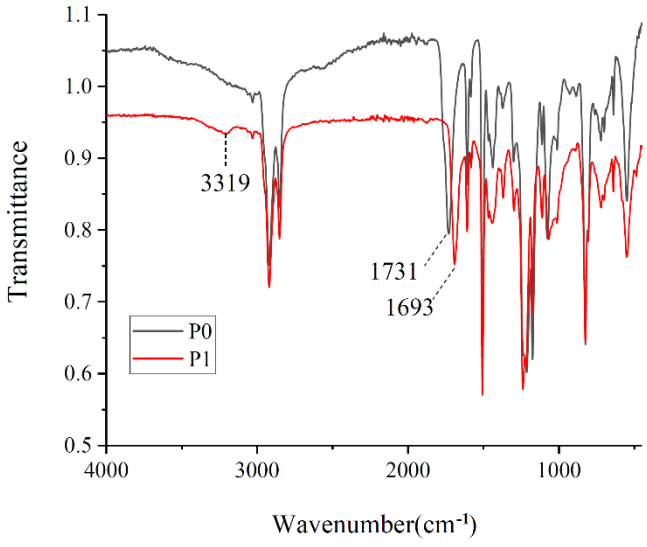



**Figure 3.11.** FT-IR of polymer carboxylate polymer **P0** and hydrazide polymer **P6**.

Polymers **P1, P4** and **P7-P9** were synthesized as previously described.[25,28,29] Nanoassembly solutions of individual polymers were prepared by dissolving solid polymers in toluene. Nanoassemblies of co-assembled polymers were prepared by mixing their already prepared nanoassembly solutions. The concentration of **P6** is 0.3 mg/mL and of **P1, P4** and **P7-P9** are 0.6 mg/mL. Dynamic light scattering on the resulting polymer solutions after self-assembly and co-assembly were conducted (Figure 3.5d).

**Protein Oxidation and Digest**

To facilitate enrichment of the glycosylated peptides by the hydrazide-containing nanoassemblies, samples of 0.5 mg of IgG1 or 1 mg of HRP were mildly oxidized with 10 mM sodium periodate in 1 mL of 0.1 M sodium acetate buffer at pH 5.5 to produce free aldehydes capable of reacting with hydrazide. Samples were incubated in the dark at room temperature for 30 min and then were washed 3 times and desalted by a 10 kDa molecular weight cutoff filter to remove excess periodate before digestion. To prepare the samples for proteolytic digestion, they were dissolved in 500 µL denaturing buffer containing 8 M urea, 50 mM $NH_4HCO_3$ and 5 mM DTT and incubated for 1 h at 37 °C with gentle agitation to reduce the disulfide bonds in the proteins. The protein solutions were brought to room temperature and 10 mM IAM was added into the solutions, before incubating in the dark at room temperature for 30 min to alkylate the reduced disulfide bonds. 5 mM DTT was added again, and the samples were incubated in the dark at room temperature for another 30 min to stop overalkylation. The solutions then were diluted with 50 mM $NH_4HCO_3$ to reduce the urea concentration to 1.2 M. Trypsin was added at an enzyme-to-protein ratio of 1:50 and incubated for 12 h at 37 °C. BSA was digested in the same manner as the other proteins.

**Liquid-Liquid Extraction and Release**

Before extraction with the nanoassemblies, the protein digests were diluted to the desired pH and concentration. 200 µL of the nanoassembly solution was added to 500 µL of the protein digest and vortex mixed vigorously for 30 min. The sample was incubated in a sand bath at 50 °C for 1 h, vortexed again for 30 min, and then incubated again at 50 °C for 1 h. After extraction, centrifugation at 14000 rpm for 30 min was used to separate the two phases. The aqueous phase was removed, and 500 µL of fresh acetate buffer was added to wash the organic phase by mixing the two phases for 30 min and removing the aqueous phase after centrifugation. The organic phase was dried by vacuum centrifuge. 300 µL of THF was added to break the supramolecular assembly and 200 µL of MOPS buffer (pH 7) was added to wash the polymer thoroughly. Then, the THF was evaporated and aqueous phase was removed. To release the glycopeptides from the polymer, the dry residue was re-dissolved in 300 µL of fresh THF and 200 µL of 0.1 M HCl was added to hydrolyze the hydrazone group between the polymer and the glycopeptides. The mixture was incubated for 1 h at 60 °C to complete peptide release. The aqueous phase with released glycopeptides was collected after centrifugation. Before adding PNGase F, vacuum centrifugation was used to remove the remaining THF, and the pH was adjusted to 8.4 in 50 mM $NH_4HCO_3$. 1 µL of PNGase F was then added to 20 µL of the peptide solution and incubated at 37 °C with gentle agitation for 12 h.

**Mass Spectrometry Analysis**

The peptide solutions resulting from nanoassembly enrichment before and after deglycosylation were analyzed by MALDI-MS. 0.5 µL of peptide solution was mixed with 0.5 µL of DHB matrix solution (25 mg/mL in 70% ACN, 29% $H_2O$ and 1% TFA) and

spotted on MALDI target for analysis. MALDI analysis was performed on a Bruker UltrafleXtreme MALDI-TOF/TOF. Spectra were obtained in positive ionization mode using reflectron detection with a repetition rate of 2 kHz and an acceleration voltage of 20 kV. Peptides were identified using MS/MS using the LIFT mode[33] at a 1 kHz laser repetition rate, applying 7.5 kV for initial acceleration of ions and 19 kV for reacceleration of the product ions in the LIFT cell. 5000 laser shots were accumulated per spectrum.

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# CHAPTER IV

# SELECTIVE CAPTURE AND CONTROLLED RELEASE OF PEPTIDES USING AMPHIPHLIC NANOASSEMBLIES

## Introduction

Peptide-based drugs fill a gap between traditional small molecule therapies and more recent protein therapies and biologics. Compared with small molecules, peptides have higher efficacy, safety and tolerance in the human body, and their production complexity and cost are lower than protein-based biopharmaceuticals.[1-4] Therefore, peptides are becoming more and more attractive as potential drug candidates. Whether peptides are made by solid phase synthesis, solution phase synthesis or purified from natural sources, most peptides begin as part of complex mixtures that require purification.[5-8] Although solid-phase synthesis reactions can be carefully controlled, impurities are inevitably formed, and often include missing, truncated or chemically modified sequences resulting from cleavage of adducts or other by-products formed during processing.[9-12] For peptide therapeutics, purity and target peptide yield are key factors to be considered, which offer great challenges in the development of peptide separation methods. Often, the goal of a given separation strategy is to rapidly purify peptides with the highest degree of purity. For typical laboratories that synthesize many different peptides, it is useful to establish a standard purification protocol that can be used for most samples while allowing for simple optimization when necessary.

Reversed-phase chromatography is by far the most popular peptide purification method due to its reliability and wide applicability. C18-bonded silica is the most common

reversed-phase column packing, but other stationary phases, such as C4, C8 and phenyl groups, can also provide alternative selectivity for optimizing peptide separation.[9,13-15] Though reversed-phase chromatography is commonly used, some peptides can be more efficiently separated by other selective methods such as ion-exchange or size-exclusion. Ion exchange chromatography can be used for peptide separation because the amino acids in the peptide can be either positively charged or negatively charged. Size-exclusion chromatography (SEC) separates molecules based on their molecular size rather than chemical properties such as charge or polarity. It is a low-resolution technique, and the resolution can be affected by the volume and concentration of the sample.[14] SEC is generally not effective for separating peptides of similar size. Due to their limitations, these other separation techniques are sometimes combined with reversed-phase chromatography to create a two-step process for the purification of difficult samples.

While numerous separation techniques are available for different purposes, they are usually require specialized instrumentation, for example, the most commonly used HPLC. Simpler approaches for the isolation of desired peptides are needed. Here, we describe polymeric supramolecular assemblies that can purify peptides based on their chemical characteristics, including attributes such as isoelectric point (pI) or modifications.[15-18] Through selective capture and sequential release, peptides can be isolated by the self-assembling nanoassemblies (Figure 4.1). These nanoassemblies, formed by amphiphilic polymers that consisting of a styrene scaffold and a functional group, can combine the advantages of the separation techniques for good separation efficiency. They can work over a wide pH range, and the self-assembly feature of these materials allows for flexibility of simple optimization for peptides that are difficult to separate.

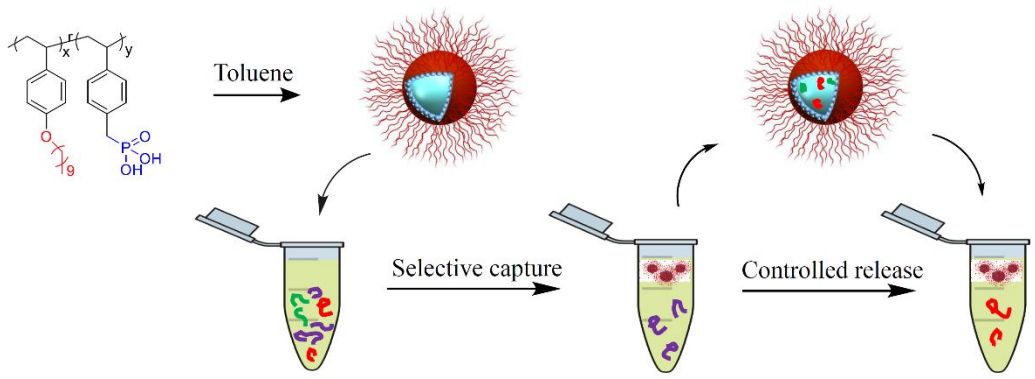


**Figure 4.1.** Selective capture and controlled release of peptides using amphiphilic nanoassemblies.

## Results and Discussion

### Selective Capture and Release of Peptides based on Charge

To develop a means of selectively isolating peptides of interest, we first explored separations based on peptide charge. In previous work, we had demonstrated that peptides could be selectively extracted from an aqueous phase into an organic phase by utilizing the isoelectric point (pI) of peptides and the aqueous phase pH. In this work, we investigated whether peptides extracted into an organic phase could be selectively released back into an aqueous phase having a different pH. A mixture of seven peptides with pI values ranging from 3.37 to 12.4 were chosen to test this possibility (Figure 4.2). After extraction of the peptide mixture at pH 6 using polymer **P1**, the two negatively charged peptides with pI values < 6 are left in aqueous phase because of the charge repulsion they experience with the phosphonate groups of polymer **P1** (Figure 4.2c). In contrast, the five positively-charge peptides with pI values > 6 are extracted into the organic phase because of their complementary charge (Figure 4.2d). This extraction behavior is identical to previous work with reverse micelle-like nanoassemblies containing negatively charged hydrophilic

groups.[20] To test the possibility of selectively releasing some of the peptides from the organic phase, a fresh aqueous phase at pH 9.5 was mixed with the organic phase containing the five peptides in the nanoassemblies of polymer **P1**. We predicted that the two peptides with pI values below 9.5 would be released into the new aqueous phase as the pH of the water pool inside the nanoassemblies equilibrated with the new aqueous phase and changed the charge state of these peptides. The two peptides with pI values of 7.91 and 6.92 would become negatively charged, causing repulsion with the negatively charged phosphonate groups in the interior of the nanoassemblies. Indeed, upon analyzing the new

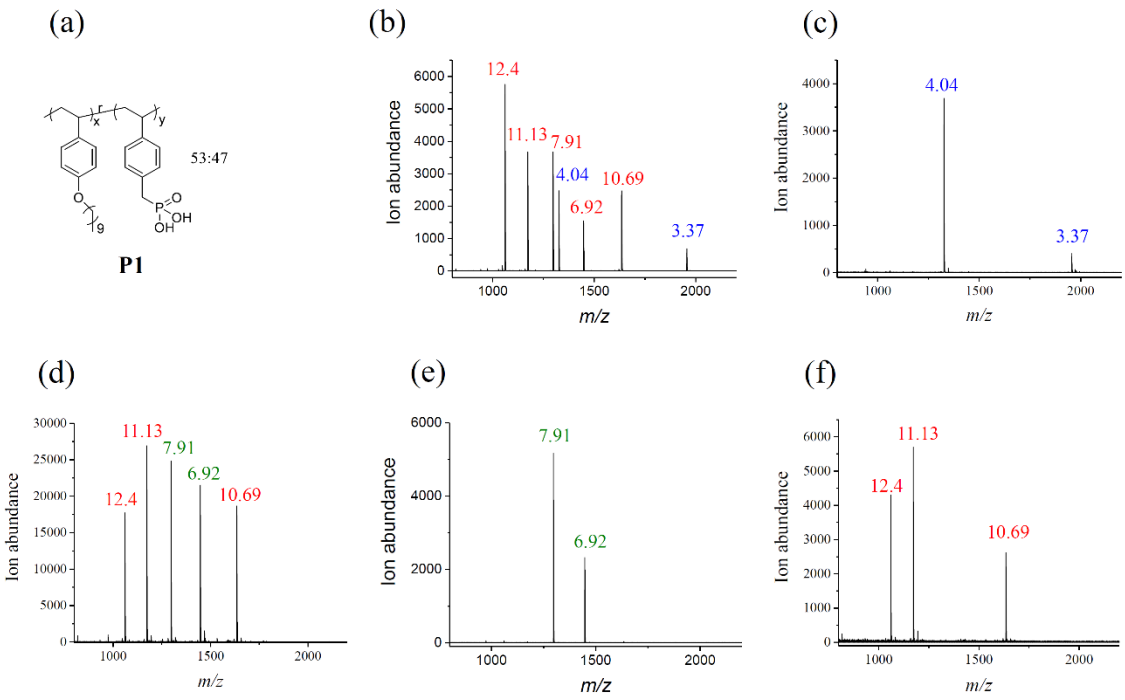


**Figure 4.2.** MS analysis of peptides before and after separation using polymer **P1**. (a) Structural features of polymer **P1**; (b) MALDI mass spectrum of peptide mixture before extraction. The peptides include bradykinin (pI 12.40), kinetensin (pI 11.13), angiotensin I (pI 7.91), β-amyloid 1-11 (pI 4.04), β-amyloid 10-20 (pI 6.92), malantide (pI 10.69) and preproenkephalin (pI 3.37); (c) MALDI mass spectrum of peptides left in aqueous phase after selective extraction by polymer **P1** at pH 6; (d) MALDI mass spectrum of peptides extracted into the organic phase by polymer **P1** at pH 6; (e) MALDI mass spectrum of peptides selectively released from the organic phase containing polymer **P1** into a new aqueous buffer of pH 9.5; (f) MALDI mass spectrum of peptides left in polymer phase after release at pH 9.5.

aqueous phase after peptide release, we detect only the two peptides in this phase (Figure 4.2e). The nanoassemblies in the organic phase remain intact as the other positively charged peptides remain in the organic phase (Figure 4.2f). In effect, two selective capture steps and one controlled release step offer an opportunity to separate the seven peptides into three separate groups according to pI: (1) pI < 6; (2) 6 < pI < 9.5; and (3) pI > 9.5.

**Sequential Release Peptides for Peptide Isolation**

We also explored the possibility of sequentially releasing peptides one-by-one via a step-wise increase in the pH of the aqueous buffer that was mixed with the organic phase containing the extracted peptides. This approach is illustrated with a three-peptide mixture (Figure 4.3). From an aqueous solution of pH 3, β-amyloid 1-11, β-amyloid 1-16 and ACTH 1-13 were extracted by polymer **P1** (Figure 4.3a) as the net charge of the peptides is positive (Figure 4.3b). After mixing the polymer phase with an aqueous phase at pH 5, β-amyloid 1-11 was selectively released (Figure 4.3c) since the net charge of this peptide became negative, which is repulsive to the polymer phase. Upon further sequential mixing of the remaining polymer phase with new aqueous phases at pH 9 and pH 11, β-amyloid 1-16 and ACTH 1-13 were selectively released and isolated (Figure 4.e and g). This peptide isolation method is easily operated in the two-phase liquid-liquid separation technique. An interesting observation is that the release behavior of these peptides as a function of pH follows the trend that would be expected for the negatively-charged species of these peptides based on the $pK_a$ values of their different functional groups (Figure 4.3d, f, and h). As the pH of the aqueous phase is increased, the peptides are released as they become negatively-charged, although there is a slight shift to higher pH values, which is likely

attributed to increases in the $pK_a$ of the peptide functional groups upon interacting with the phosphonate groups in the polymer.

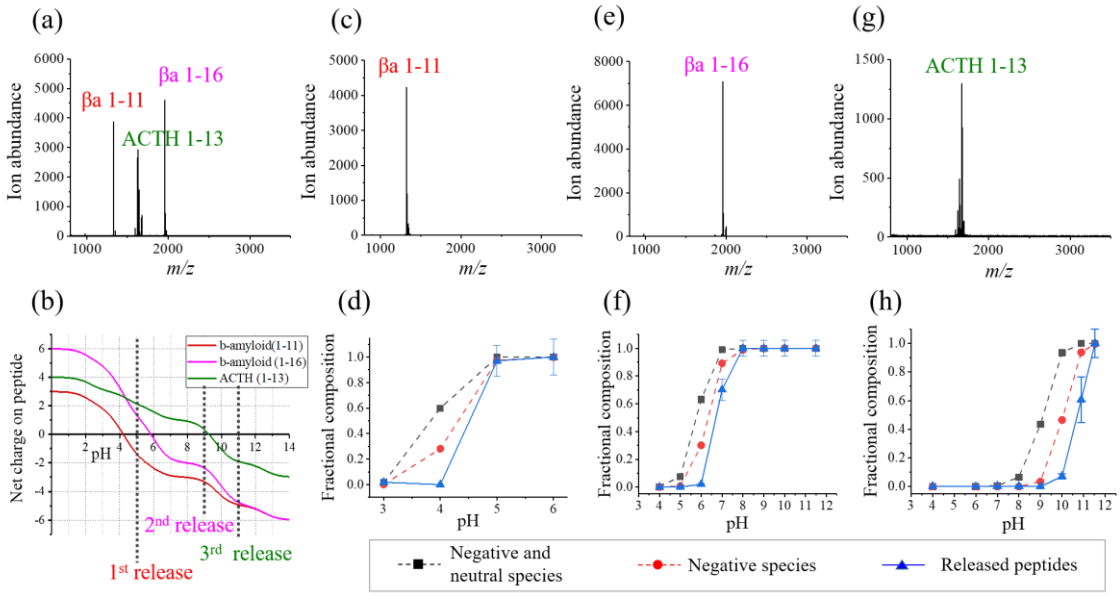


**Figure 4.3.** Sequential release of peptides into new buffer and their release behavior study. (a) MALDI mass spectrum of β-amyloid 1-11, β-amyloid 1-16 and ACTH 1-13; and (b) their net charge distribution over pH. c) MALDI mass spectrum of β-amyloid 1-11 being isolated at the first releasing pH 5 and (d) its release behavior is studied from pH 3 to 6; (e) MALDI mass spectrum of β-amyloid 1-16 isolated at the second releasing pH 9 and (f) its release behavior is studied from pH 4 to 12; and (g) MALDI mass spectrum of the ACTH 1-13 isolated at the third releasing pH 11 and (h) its release is studied from pH 4 to 12.

When this peptide isolation method was further evaluated with mixtures containing several peptides with high pI values, it was found that the high pI peptides could not be properly released. As an example, when the peptides ACTH 1-13, ACTH 6-24 (pI 12.2), and bradykinin (pI 12.4) are in a mixture, ACTH 6-24 and bradykinin are released at pH values that are too low (Figure 4.4a). This unexpected release was attributed to the instability of polymeric nanoassemblies at high pH.[20] Therefore, polymer **P10** was synthesized (Figure 4.4b), as we previously found that polymers with a lower percentage of the negatively-charged functional group could maintain their stability and ability to bind

positively-charged peptides in the organic phase when exposed to aqueous phases with high pH values.[20] The greater stability of polymers like **P10** at higher pH is thought to be

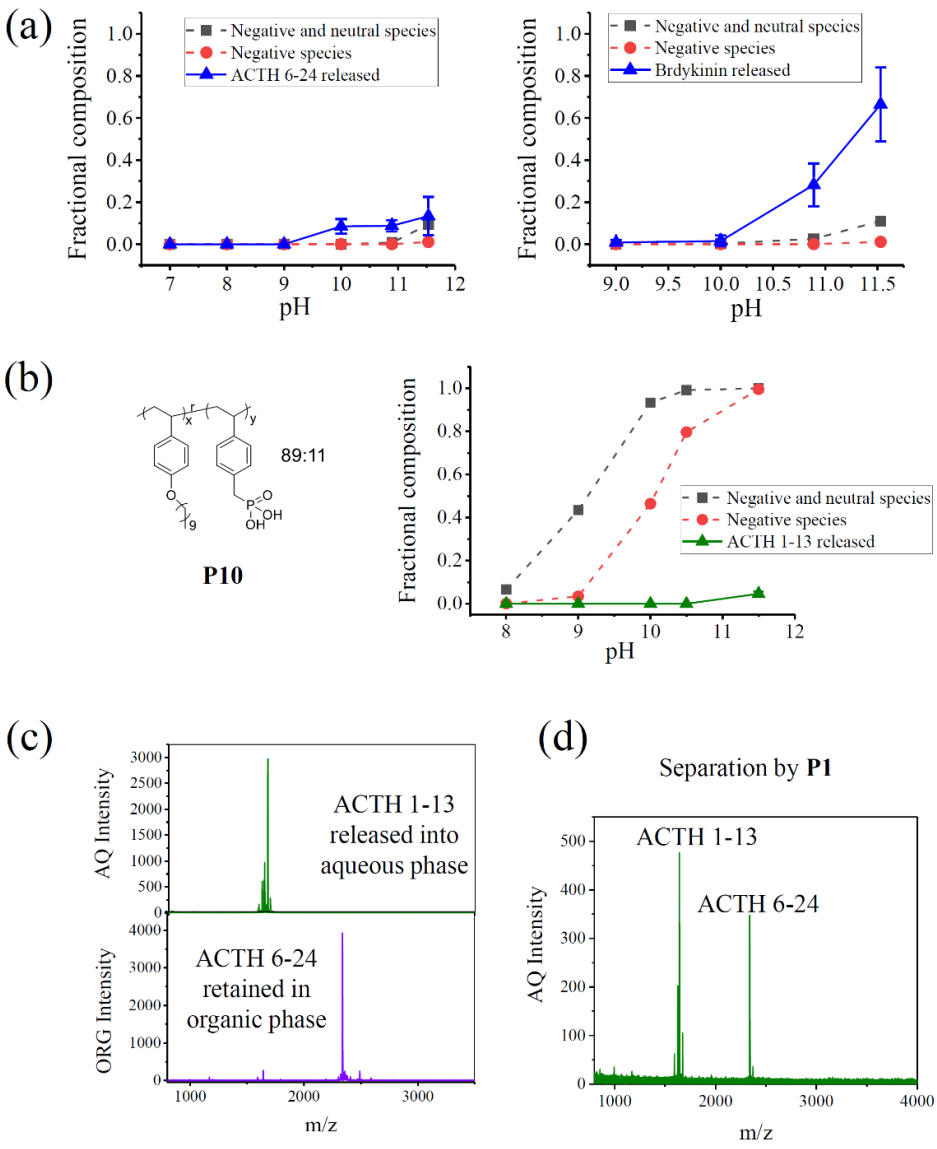


**Figure 4.4.** The capture and release ability of nanoassemblies formed by different amphiphilic polymers. (a) Unexpected release of high pI peptides ACTH 6-24 (pI 12.2) and bradykinin (pI 12.4) from nanoassemblies formed by polymer **P1** at high pH; (b) Structural features of polymer **P10** and ACTH 1-13 (pI 9.3) cannot be properly released by overly stable nanoassemblies formed by polymer **P10** and (c) MALDI spectra of peptides separated using acetonitrile as co-solvent by **P10**, which cannot be separated by polymer **P1** due to the unexpected release (d).

due to the decreased charge density in the interiors of the nanoassemblies when the high pH causes almost complete deprotonation of the phosphonate groups. While nanoassemblies of **P10** are more capable of binding peptides at high pH, release from **P10** is not efficient (Figure 4.4b) as it barely releases any ACTH 1-13 (pI 9.3) even at pH 11.5, as compared to the release of ACTH 1-13 starting at pH 10 using polymer **P1** (Figure 4.3h). However, by modifying the release solution with 10% acetonitrile, which has a polarity between toluene and water, we are able to disrupt the nanoassembly of polymer **P10** to a greater extent and release peptides more effectively, while maintaining release selectivity. With the addition of 10% acetonitrile, ACTH 1-13 can now be separated from ACTH 6-24 by selective release at pH 10.89 (Figure 4.4c), while the same separation could not be achieved by polymer **P1** under the same release conditions, due to the unexpected release of ACTH 6-24 (Figure 4.4d). These results highlight the flexibility of this method for simple optimization.

The performance of the method on separation of a complex mixture was evaluated using BSA digests (Figure 4.5). After forward extraction of a BSA digest (Figure 4.5a) at pH 4 using polymer **P1**, primarily peptides with pI values < 4 remained in the aqueous phase (Figure 4.5b), while peptides with pI > 4 were extracted and enriched (Figure 4.5c). The organic phase containing the reverse micelles and extracted peptides was then mixed with a new aqueous solution at pH 6, and primarily peptides with pI values between 4 and 6 were released (Figure 4.5d). Because BSA has a pI of 5, a large number of peptides were released. Mixing the remaining polymer phase with an aqueous solution at pH 8, further releases more peptides, including ones that were ineffectively released at the lower pH (Figure 4.5e and f). After these extraction and releases, the peptides in the BSA digest were

in effect sorted according to their pI values. In addition, because the mixtures were simplified along the way, overall more peptides (34 vs. 29) are detected as compared to the direct analysis by MALDI (Figure 4.5a). From the data in Figure 4.5, it is clear that the pI-based separation is not perfect because other factors, such as peptide hydrophobicity, have not been taken into account yet, but 76% of the peptides are sorted according to their pI values. Future work will investigate the peptide-dependent release behavior for more complex mixtures to determine if selective release from even more complicated digests is possible.

**Separate Peptides of Protein Digests**

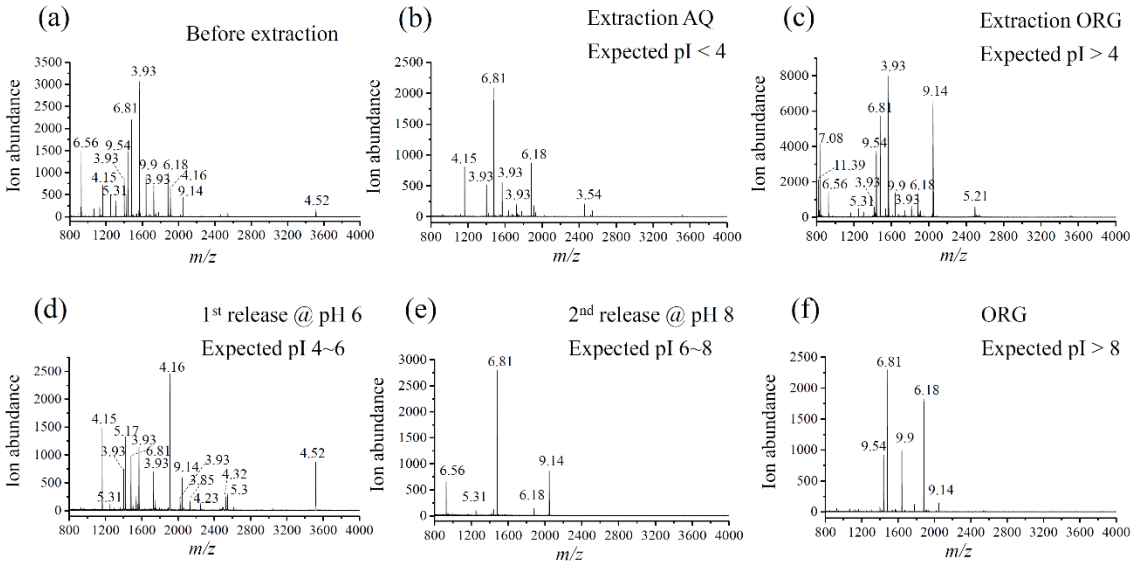


**Figure 4.5.** MS analysis of protein (BSA) digest separation by selective extraction and controlled release. (a) MALDI mass spectrum of direct analysis of BSA digests; (b) MALDI mass spectrum of peptides left in aqueous phase after extraction at pH 4 using polymer **P1**; (c) MALDI mass spectrum of peptides extracted by polymer **P1** at pH 4; (d) MALDI mass spectrum of peptides first released at pH 6 and further released at pH 8 from organic phase and (f) MALDI mass spectrum of peptides left in organic phase after sequential release.

## Conclusions

We have developed a simple method that can selectively capture and controllably release peptides from peptide mixtures. This new method relies on anionic polymeric nanoassemblies that bind and release peptides based on charge complementarity. The materials can isolate peptides through sequential release of a mixture of extracted peptides, and the release pattern follows closely to the calculated fractional composition of the negatively-charged species. Release of high pI peptides and high pH values requires optimization of the polymer architecture and the release buffer to ensure efficient release of high pI peptides. Future work will apply these methods for peptide purification after solid-phase peptide synthesis.

## Experimental Section

### Materials and Reagents

Peptides bradykinin (RPPGFSPFR, MW 1060), kinetensin (IARRHPYFL, MW 1172), angiotensin I (DRVYIHPFHL, MW 1296), β-amyloid 1-11 (DAEFRHDSGYE, MW 1325), β-amyloid 10-20 (YEVHHQKLVFF, MW 1446), malantide (RTKRSGSVYEPLKI, MW 1633), preproenkephalin (SSEVAGEGDGDSMGHEDLY, MW 1954) and ACTH 6-24 (HFRWGKPVGKKRRPVKVYP, MW 2336) were acquired from the American Peptide Company. Peptides β-amyloid 1-16 (DAEFRHDSGYEVHHQK, MW 1955) and ACTH 1-13 (SYSMEHFRWGKPV, MW 1624) were purchased from Bachem Company. Bovine serum albumin (BSA), DL-dithiothreitol (DTT), iodoacetamide (IAM), trifluoracetic acid (TFA) and potassium acetate were obtained from Sigma-Aldrich. BSA digests were obtained according to our

previously reported procedure.[21] Trypsin was purchased from Promega. Sodium periodate, sodium phosphate, 2, 5-dihydroxybenzoic acid (DHB), acetic acid, toluene, tetrahydrofuran (THF) and acetonitrile (ACN) were obtained from Fisher Scientific. Urea was purchased from MP Biomedicals. Ammonium bicarbonate ($NH_4HCO_3$) was obtained from Fluka. Water was purified using a Milli-Q water purification system. THF was distilled before use. All other chemicals were used as received from commercial sources.

**Polymer Synthesis and Nanoassembly Formation in Toluene**

Polymers **P1** and **P10** were synthesized as previously described.[20,21] The nanoassembly solution of polymer was prepared by dissolving the polymer in toluene at the concentration of 1 mg/mL. The solution was sonicated until clear before being used for the two-phase liquid-liquid extraction.

**Peptide Extraction and Selective Release**

Before extraction with the nanoassemblies, the peptide mixture or protein digests were diluted to the concentration of 1 μM and to the desired pH. 200 μL of the nanoassembly solution was added to 1 mL of the peptide solution and vortex mixed vigorously for 1.5 h. After extraction, centrifugation at 13000 rpm for 20 min was used to separate the two phases. The aqueous phase was removed for MS analysis, and 1 mL of fresh buffer with desired pH was added to the left organic phase and shaken for 1 h for selective release. Centrifugation and peptide release were repeated as needed to accomplish sequential release of different peptides.

**Mass Spectrometry Analysis**

The peptide solutions resulting from the nanoassembly separations were analyzed by MALDI-MS. 0.5 µL of peptide solution was mixed with 0.5 µL of DHB matrix solution (25 mg/mL in 70% ACN, 29% $H_2O$ and 1% TFA) and spotted on MALDI target for analysis. MALDI analyses were performed on a Bruker Autoflex III time-of-flight mass spectrometer. All mass spectra were obtained in positive reflectron mode and represent an average of 300 shots acquired at 45% laser power with an accelerating voltage of 19kV.

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# CHAPTER V

## CONCLUSIONS AND FUTURE DIRECTIONS

In this dissertation, amphiphilic nanoassemblies were utilized to selectively enrich peptides of interest from complex mixtures through modification-specific and complementary charge-based interactions. In chapter II, the selectivity of the polymeric nanoassemblies was easily changed from pI-based interactions into phosphonate-metal(IV) chelation-based extraction by loading metal ions into the supramolecular nanoassemblies. This means that this material has a broad application in biomarker study as a simple optimization of the material allows for the study of a new type of biomarkers. Besides the flexibility of changing its affinity to peptides, the extraction selectivity and efficiency of the material can also be easily optimized at the molecular level. By evaluating the performance of the Zr loaded nanoassemblies on the phosphopeptide extraction under different conditions such as varying the amount of metal ion loading or changing the polymer structure, we found that an optimal chemical interactions between polymer functional groups, metal ion and phosphopeptides can provide the best extraction result.

Though it has been commonly agreed that metal (IV) ions offer the best selectivity targeting phosphopeptides, how the metal ions are loaded to nanomaterials vary greatly. Previous work has mostly accomplished this by loading metal ions to polymers having phosphate group on the surface of the magnetic beads, which is easy for the phase separation after the extraction of phosphopeptides, while loading and maintaining the metal ions need harsh conditions, like very low pH to minimize hydrolysis of the metal ions.[1,2] However, loading metals ions into the amphiphilic nanoassemblies with the phosphonate

groups inside of the material provides a new possibility of keeping the metal ions balanced between being protected from hydrolysis and becoming accessible for phosphopeptide binding. However, comparing to the materials that have been applied to centrifuge filters or HPLC columns,[2] metal-loaded nanoassemblies still require more systemic studies for wider application in mass spectrometry experiment.

A promising direction following the work in Chapter II would be to develop ways to enrich phosphorylated proteins since some phosphorylation happens only on a specific proteoform.[3,4] Specific detection of phosphorylated proteins would provide a potentially powerful way to measure biomarkers that cannot be unambiguously detected at peptide level. Our method has shown good extraction performance over a broad pH range from acidic to physiological pH, which then has the potential to study a broad range of biomarkers from gastric acid (pH = 1.0-2.0) to urine (pH = 5.0-7.5), and to human milk (pH = 6.9-7.0 ) and blood (pH = 7.35-7.45). More importantly, these extractions could be done without disturbing the structure of the biomarker proteins. We are also encouraged by a recent study by our collaborator who has shown that these supramolecular nanoassemblies can transport proteins from aqueous phase to organic phase through electrostatics and ligand-protein interactions and maintain the tertiary structure and function of the transported proteins.[5] Therefore, extraction of the intact phosphorylated protein, coupled with top-down MS approach has a very good potential in providing information of a specific proteoform biomarker.

Another interesting direction would be applying the metal-loaded nanoassemblies to on-line separation strategies, which would allow us to quantitatively analyze biomarkers more efficiently and with better reproducibility, especially for analyzing large numbers of

samples for reliable comparison between diseased and normal patient groups. A monolithic HPLC column can provide both the nanoassembly structure and its function in targeting phosphopeptides. Also, our new understanding about the chemistry between metal, phosphopeptides, and the phosphonate/PEG groups would allow currently available online phosphopeptide enrichment strategies work in a broader pH range and in milder conditions.

To further explore the possibility of using this material in targeting types of biomarkers with heterogeneity like glycopeptides, we utilized a covalent binding-based interaction in the supramolecular system for higher selectivity and a non-covalent interaction for higher extraction efficiency (i.e. chapter III). Both the covalent and non-covalent interactions have been explored in the previous glycopeptide enrichment studies.[6-10] Non-covalent interactions, such as hydrophilic, lectin and chelation-based interactions are widely used as they can be easily incorporated into column-based separation methods.[6,7] Covalent binding using hydrazide chemistry or boronic acid recognition are also used because they can offer better selectivity than non-covalent interactions.[8,9] We then provided a strategy that combines these two interactions in a way different from previous work. Most previous work has used non-covalent and covalent interactions in sequence, which limits how much the chemical interactions in peptide binding can improve enrichment efficiency. For example, Song, et al used lectin affinity chromatography and hydrazide chemistry separately and so be able to determine and confirm glycopeptide/ glycoprotein biomarkers in human blood serum.[10] However, using non-covalent and covalent interactions at the same time provides the opportunity to improve enrichment efficiency in a way that is not just additive. As our work is in the early development stage, it may require more studies to obtain new scientific insight for its further development.

In chapter III, nanoassembly containing hydrazide functional groups was designed, which showed selective covalent interactions to peptides modified with glycans. Co-assembled with the hydrazide polymer, acidic polymers in the micro extraction environment were found to be able to facilitate the reaction between hydrazide polymer and the oxidized glycans on the glycopeptides. These co-assembled polymers provide adjacent proton donors and conjugate base functionality that are needed in the coupling reaction. These encouraging results show that not only can we improve the selectivity by utilizing different chemical interactions in the supramolecular system, but also, we can catalyze the binding reaction once nanoassemblies interact with low abundant biomarkers, which could significantly improve the biomarker recovery. Although nanoreactors have been well studied mainly due to high reaction efficiency,[11] they are not used in peptide enrichment, while our work shows the possibility of utilizing that strategy in high specific binding of biomarkers using supramolecular materials in the future.

Another significant difference of our method comparing to most currently available glycopeptide enrichment methods is that after extraction of the glycopeptides to their nanomaterials, an enzyme has to be added to the aqueous phase so that peptides, free of glycans, can be collected for MS analysis. Though, by our method, glycopeptides are released from polymer by acid and so that the glycans can be analyzed on the peptide sequence. This means of analysis is important for peptide biomarkers when we need the information about what specific sugar composition on a specific amino acid sequence, but we found that removing glycans from peptides after releasing them with acid allows more sensitive MS detection. Therefore, it would be interesting and more efficient if we could release the peptides from glycans in the reverse micelles after extraction. Perhaps this

release could even be more efficient in the confined environment of the reverse micelles. A future work following the studies described in Chapter III could be to load the enzyme peptide-N(4)-(N-acetyl-beta-D-glucosaminyl)asparagine amidase F (PNGase F) into separate reverse micelles and then merge these loaded reverse micelles with those reverse micelles having the extracted glycopeptides. If the two sets of reverse micelles could merge effectively, PNGaseF could be transferred into the micelles with the glycopeptides, allowing them to be deglycosylated. Subsequent analysis of these deglycosylated peptides could then benefit from the enhanced MALDI-MS analysis that occurs due to 'hot spot' formation.[12] The optimal working pH of the PNGaseF is pH 7.4, and the pI of the enzyme is 8.3, so it is positively-charged at the working pH. Therfore, a negativley-charged polymers like the phosphonate polymer, which also improves the reaction efficiecy of hydrazide and glycan, can be used for the extraction of PNGase F. Another option could be modifiying PNGase F with a tag like glutathione-S-transferase (GST) by expressing it as a GST fusion protein and then glytathione-containg nanoassemblies that have affinity for GST could be used for the extraction of PNGase F.[13]

Besides selective extraction, the possibility of controlled release was studied with amphiphilic nanoassemblies in Chapter IV, which could be very useful in sorting biomarkers into different categories and even isolating one specific biomarker for more careful study. This method was investigated with complementary charge-based interactions as peptides are either positively charged or negatively charged and their net charges are affected by the pH of environment. By taking advantage of that feature, we can change the charge state of the peptides and let them be extracted and sequentially released. This idea was proved successful in peptide isolation from relatively simple mixture and show a good

potential for peptide sorting from complex mixtures like protein digests. In addition, this method has shown great potential in peptide isolation after solid-phase synthesis, which could be a follow up application in the future.

In summary, these studies demonstrate that supramolecular materials formed by amphiphilic polymers can be readily designed to selectively target a broad range of biomarkers. The performance of these materials has been preliminarily evaluated in peptide mixture, and their performance in enriching protein biomarkers are expected to be evaluated in more complex samples like serum and cell lysates in the future. Especially, more enzyme loading work needs to be studied as it will open more possibilities, besides the reaction with extracted glycopeptides, other modifications, such as ubiquitination, which requires more specific ubiquitin receptors with tertiary structure can also be investigated by this method. In addition to exploring new science using these supramolecular nanoassemblies, they also show good potential as separation materials. Investigations of the relationship between polymer structure and their resulting performance has provided us novel insight so we can develop more efficient biomarker enrichment strategies that could one day allow us to diagnose some diseases at an earlier stage.

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