2007

Use Of Functionalized Gold Nanoparticles To Efficiently Extract And Concentrate Peptides For Maldi-tof-ms Detection

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USE OF FUNCTIONALIZED GOLD NANOPARTICLES TO EFFICIENTLY EXTRACT AND CONCENTRATE PEPTIDES FOR MALDI-TOF-MS DETECTION

A Thesis Presented

by

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Submitted to the Graduate School of the
University of Massachusetts Amherst in partial fulfillment
of the requirements for the degree of

MASTER OF SCIENCE

May 2007

Chemistry

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DEDICATION

To my son, Eugenius and loving wife, Christiana

ACKNOWLEDGMENTS

I am particularly indebted to Richard W. Vachet, my advisor, for his thoughtful guidance and gentle support throughout this work. I would also like to extend my gratitude to Ricardo B. Metz, for agreeing to serve on my thesis committee.

I wish to express my sincere appreciation to our collaborators (the Rotello group), especially to Gang Han, for his special efforts in providing us with the nanoparticles for our experiments. A special thanks to all the members of the Vachet group and to all those whose friendship helped me to come this far.

ABSTRACT

USE OF FUNCTIONALIZED GOLD NANOPARTICLES TO EFFICIENTLY EXTRACT AND CONCENTRATE PEPTIDES FOR MALDI-TOF-MS DETECTION

MAY 2007

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We have developed a straight forward method that uses monolayer protected clusters (MPCs) and mixed monolayer protected clusters (MMPCs) as selective extraction and concentration probes for peptides. After extraction with these nanoparticles (NPs), the peptides are analyzed by MALDI-TOF-MS to obtain their mass fingerprints. Application of the method to a test library of 146 tryptic peptides showed that cationic MPCs/MMPCs target negatively charged peptides while the anionic MPCs/MMPCs target positively charged peptides. The extraction with these NPs is also accompanied by high concentration factors such they can be used to extract and concentrate microcystin-LR at levels below the WHO guideline of 1μg/L.

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CHAPTER 1

INTRODUCTION

Recent advancements in mass spectrometric instrumentation allow the masses and sequences of peptides to be sensitively determined, and this enables protein identification. Typically, proteins from cell lysates are separated with two-dimensional gel electrophoresis (2DE) and stained spots on the gel are excised and digested to produce peptides of different lengths and sequences. The resulting peptides are then separated by on-line HPLC, and the eluting peptides are ionized by ESI and are further fragmented to obtain sequence information. Spectra from selected peptides are compared with computer generated mass spectra from a sequence database to identify the protein.[1] However, the complexity and the wide dynamic range of the biological matrices from which these biomolecules are derived can limit our ability to detect them efficiently. To take full advantage of powerful mass spectrometric instrumentation so that peptides can be adequately analyzed requires the use of highly efficient and selective extraction and concentration methods.

A number of extraction and concentration techniques have been developed and applied to peptides prior to their analysis, with the two most successful ones being solid-phase extraction (SPE)[2,3] and bioaffinity retention (BA) systems.[4] In SPE, the sample to be extracted is passed through a cartridge, which retains them by adsorption. The adsorbed samples can then be eluted from the cartridge using the appropriate solvent (e.g. polar organic solvents such as methanol), [5] and the analytes of interest can be effectively concentrated by elution with a smaller volume than the initial loading

volume. Alternatively, the extracts can be reduced in volume by evaporation, resulting in increased sample concentration. Even though SPE is suitable for many applications, it has limited extraction capacity because of relatively low analyte specificity and relatively low surface areas. Sol gels have been investigated as materials for SPE because they offer good control over surface recognition sites and provide moderately high surface areas.[6] These materials, however, tend to have recognition sites with a range of specificity and analyte access, and the very high surface area materials (i.e. aerogels) are very fragile.

BA systems, on the other hand, use the natural affinity of the analyte of interest for its natural biological complement (e.g. a ligand immobilized on the surface of the stationary phase).[7] BA systems rely on the reversible formation of complexes, and have been used as a part of the traditional 2DE-MS workflow for protein identification, often to deplete biological matrices of high abundance proteins so that proteins present at low concentrations can be detected. An example is the use of an affinity system fabricated by conjugating β-casein and bovine immunoglobulin G (IgG)-specific IgG with sepharose to remove major proteins including β-casein and IgG from bovine milk, which makes identification of low-abundance milk proteins possible.[8] In general, BA systems use ligands that have long-term storage problems and limited utility under harsh conditions. In addition, BA systems use micron-sized support materials with relatively low surface-area-to-volume ratios, which may limit their extraction capacities due to limited recognition site loading.

Very recently, several researchers have demonstrated the extraction capabilities of nanometer-sized particles.[9-18] As extraction agents, nanoparticles (NPs) have significant advantages over micron-sized materials, which are the standard support materials used in SPE and BA extraction systems. Most notable is a higher surface area-to-volume ratio. As particle radius decreases from 1 μm to 10 nm, for example, the surface area-to-volume ratio increases 100-fold. This increase translates into much greater extraction capacity, and NPs have great potential in this regard. Indeed, several researchers have recently used the inherent extraction efficiency of NPs in conjunction with MALDI-MS analysis. Chen and co-workers have demonstrated that gold NPs coordinated with citrate and chloride ions (i.e. negatively-charged surfaces) can trap and concentrate positively-charged peptides from 100 nM solutions,[9] while TiO_2-coated Fe_3O_4 NPs can be used to selectively concentrate phosphopeptides at 500 pM concentrations.[10] Gold NPs dissolved in toluene have also been used as part of a liquid-liquid microextraction system to concentrate negatively-charged peptides present at μM concentrations in urine samples.[11] Other NP materials such as silica,[12,13] diamond [14], and zeolites [15] have also been used to concentrate peptides from very dilute solutions. Hsiao and co-workers have also employed C18 functionalized magnetic nanoparticles to enrich and identify phosphopeptides from phosphorylated myelin basic protein.[16] Chen et al. demonstrated that nanocomposites of ion oxide magnetic nanoparticles coated with sol-gel alumina can be used to selectively enrich phosphoproteins and phosphopeptides from tryptic digest products of proteins.[17] Yang et al. have also derivatized calcium carbonate nanopaticels with poly(methyl methacrylate) and applied them to enrich peptides mixtures from very dilute solutions.[18]

In addition to high surface area-to-volume ratios, some NPs offer the ability to build materials with "bottom-up" design control. This feature enables the creation of particles with surface functionality that can selectively recognize a particular class of compound.[19-29] In particular, monolayer-protected gold clusters (MPCs), which are core/shell NPs, provide many desirable attributes for the creation of selective extraction agents. MPCs can be formed via a one-pot synthesis.[28] Their core size can be varied from < 1 nm to ~ 8 nm by controlling the thiol (shell)-gold (core) stoichiometry. The functionality at the surface and interior of the monolayer can be controllably varied through the thiol(s) used for capping. Furthermore, the functional diversity of MPCs can be easily extended by forming mixed monolayer protected clusters (MMPCs), which can be formed directly using mixed thiols during NP formation or by a place displacement reaction.[29] Taken together, these features make MPCs and MMPCs attractive systems for developing specifically tailored NP extraction materials.

The goal of this work is to use MPCs and MMPCs in a general methodology that will allow direct, efficient, and selective extraction and concentration of peptides from very dilute solution mixtures. These materials will be used in conjunction with MALDI-MS for efficient detection of these peptides. MALDI-TOF-MS will be used to detect the extracted/concentrated analytes because of its capability in the high-mass range and its sensitivity in the femtomole to picomole range. MALDI-TOF MS also has high sample transmission efficiency, which minimizes sample loss, rapid analysis times and relatively high tolerance for sample contamination.

Microcystins, a family of hepato-toxic cyclic peptides will be another target for our NPs. This family of cyanotoxins is produced in lakes and other fresh water bodies

by bloom-forming cyanobacteria genera that include *Microcystis, Anabaena, Planktothrix* and *Nostoc*.[30-32] To date, there are over 60 variants of microcystins that have been characterized and that differ in their toxicities,[33] with microcystin-LR being the most common in cyanobacteria; however, it is common to find more than one microcystin in a particular strain of cyanobacterium. Microcystins are potent hepato-toxins (LD_{50} 40–800 mg/kg (mice, intraperitoneal injection)),[34-37] and an inhibitory dosage (ID_{50}) of 1-10 mg (per person) by an unspecified route of exposure has also been estimated.[38]The toxins have been implicated in the deaths of livestock and wildlife,[39-42] however, the most serious incident of human intoxication occurred in 1996, when the deaths of about 60 patients at a hemodialysis clinic in Brazil were attributed to microcystins, which were later identified in the clinic's water supply.[43-45] The toxins have also been suggested as potential biological warfare agents.[38]

In structure, microcystins are made of five invariant amino acids namely, D-alanine (position 1), D-methylaspartic acid (position 3), 3-amino-9-methoxy-2, 6, 8-trimethyl-10-phenyl-4, 6-dienoic acid (Adda) (position 5), D-glutamic acid (position 6) and *N*-methyldehydroalanine (position 7) as well as two variant amino acids at positions 2 and 4, which are normally L-amino acids (fig. 1.1).

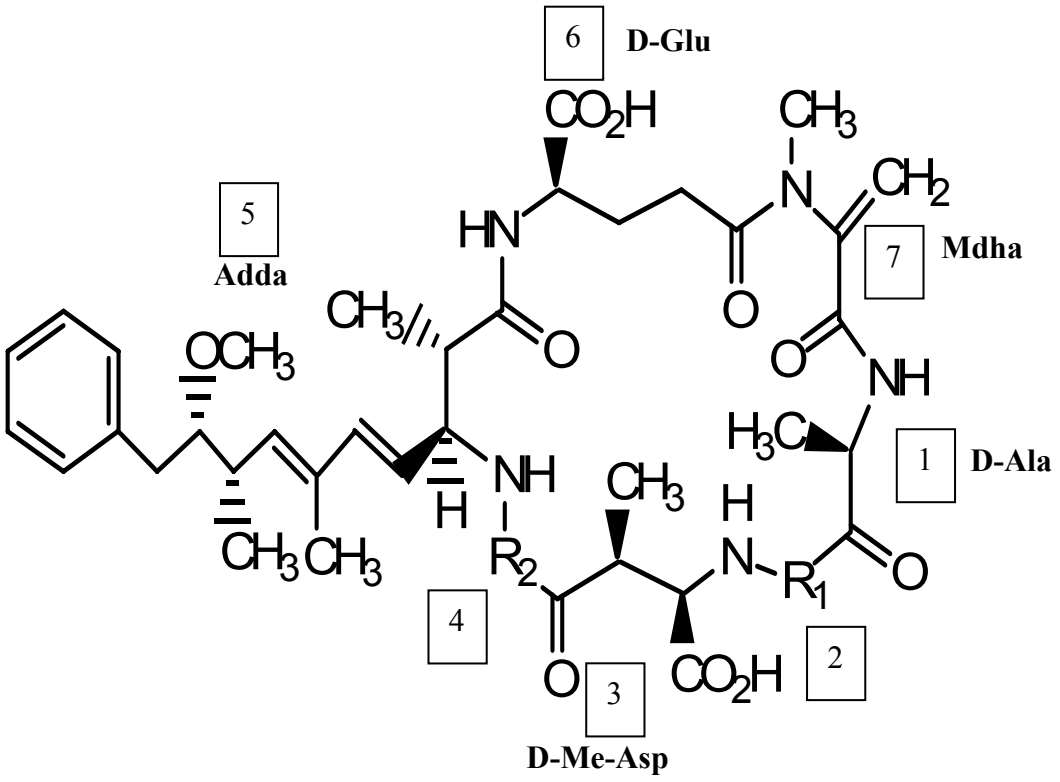


Figure 1.1: General structure of microcystins (MCs), where R_1 and R_2 are variable L-amino acids.

The Adda moiety is a unique β-amino acid and is one of the invariant amino acids.[46,47] The toxins are named according to the two variable L-amino acids at positions 2 and 4 (fig. 1.1). For example, microcystin-LR (MC-LR) contains the amino acids leucine (L) and arginine (R) at these positions. At the molecular level, they are known to bind irreversibly to several serine/threonine protein phosphatases including protein phosphatase 1 (PP1) and protein phophatase 2A (PP2A) and thus inhibit these enzymes' functions.[48, 49]

The extent of human fatalities caused by microcystin toxicity has necessitated the development of fast, sensitive methods to detect these toxins. These methods should

be able to detect the toxin at concentrations below the WHO guideline of 1 μg/L.[50] These concentrations are, however, difficult to analyze and thus very efficient concentration and clean-up methods are needed prior to analysis. We anticipate that the high surface-area-to-volume of MPCs/MMPCs will afford the required extraction capacity to detect the toxins at levels below the WHO provisional guideline.

The body of this thesis is divided into four chapters. Chapter 2 focuses on developing and testing a general protocol for selective extraction and concentration of peptides with MMPCs. Chapter 3 describes the evaluation of several different NPs, in order to understand the extraction selectively of these NPs for the target peptides. The use of different NPs enabled us to further understand the characteristic features of the NPs that allow them to function as highly efficient and selective extraction agents for peptides. In chapter 4, we describe the use MMPCs to extract and concentrate microcystin-LR from very dilute solutions, and finally, chapter 5 outlines the future direction of this work.

CHAPTER 2

GENERAL METHOD DEVELOPMENT

In this chapter, we describe, the use of mixed monolayer protected clusters (MMPCs) to develop a method to selectively extract and concentrate peptides with specified physical characteristics from very dilute solutions. Two types of MMPCs were used, one is positively-charged (TmaNP (I)) and the other is negatively-charged (CxyNP). It is held that these nanoparticles interact with their targets mainly by means of complementary electrostatic interactions.[51] In our experiments, we mixed the NPs with peptides in a specified mole amount, and once extracted, the peptide-bound NP are directly analyzed by MALDI-TOF-MS in a manner that reduces sample handling and thus minimizes any associated sample losses.

2.1 EXPERIMENTAL

2.1.1 Materials and Reagents

Human glucagon (19-29), T7-peptide, human ACTH (1-13) and kinetensin were obtained from Global Peptides Inc. Angiotensin I, bradykinin, α-cyano-4-hydroxycinnamic acid (α-CHCA) and all chemicals for the NP fabrication were obtained from Aldrich. Tris-(hydroxymethyl) aminomethane (Tris) and tris-(hydroxymethyl) aminomethane hydrochloride (Tris-HCl) were purchased from EM science. The water used in all the experiments was obtained from a Milli-Q water purification system (Millipore, Bedford, MA, USA). The Centricon filters and centrifuge tubes were purchased from Millipore Corporation.

2.1.2 Preparation of NP Systems

Fabrication and characterization of the gold NP systems were accomplished via previously reported procedures. [52, 53] For the cationic NPs (TmaNP (I)), the octanethiol-functionalized precursor was place exchanged with 11-trimethylammonium-undecanethiol moieties [54] and for the anionic NPs (CxyNP), the octanethiol precursor was place exchanged with ω-thiol carboxylic acid units. [55] NMR analysis of the end groups of the TmaNP indicated that the trimethylammonium side chain functionality was 71% of the thiol monolayer. A 1:1 ratio of ω-thiol carboxylic acid to octanethiol was obtained for the CxyNP.

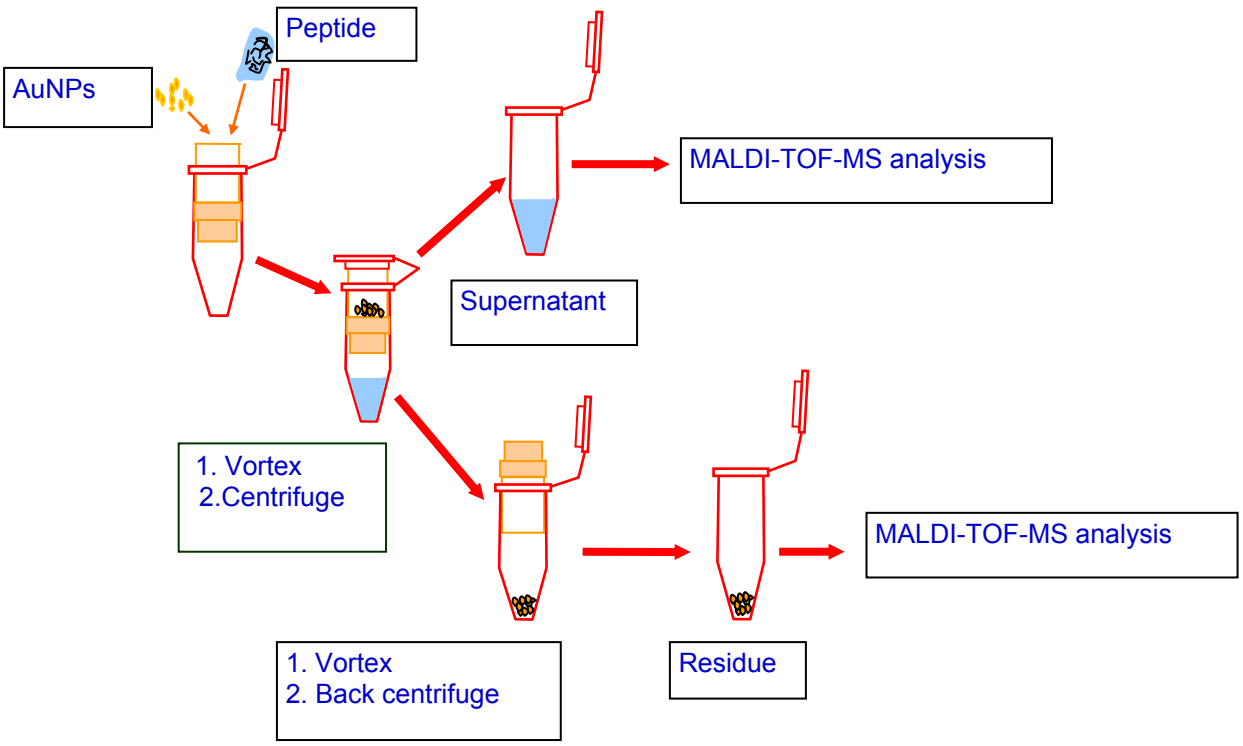


Figure 2.1: Schematic of the extraction process for MALDI-MS analysis with nanoparticle systems.

2.1.3 Sample Extraction with NP's

Stock solutions of all the peptides except for glucagon were prepared in deionized water. Glucagon was prepared in a 70:30 ACN:H_2O (v/v) because of its limited solubility in water. The peptide stock solutions were then diluted to working concentrations in a 25 mM Tris/Tris-HCl buffer at the desired pH. To extract the peptides from solution, a 50 - 250 μL solution of NP's (2.5 – 50 μM) was added to an equal volume of a peptide solution in a 10000 MW Centricon cut-off filter. The mixture was vortexed for 8 – 10 minutes. The peptide-bound NPs were aggregated and separated from the reaction supernatant by centrifugation in an Allegra™ X-22R centrifuge (Beckman Coulter™). The residue (peptide-bound NPs) and supernatant were then analyzed separately by MALDI time-of-flight (TOF) MS (fig. 2.1). For the pH-dependent experiments, 50 μL of a 5 μM NP solution was used to extract a 1 μM solution (50 μL) of angiotensin I. To test the selectivity of the NP systems for peptides with different pI values, 50 μL of a 5 μM NP solution was used to extract an equal volume of a 5 μM solution of each peptide except glucagon and the T7-peptide. These latter peptides were studied at concentrations of 100 μM for glucagon and 60 μM for the T7-peptide. These higher concentrations were used because these two peptides are inefficiently ionized by MALDI.

2.1.4 MALDI TOF-MS

All mass spectra were acquired in positive ion mode using an Omniflex® Reflectron TOF mass spectrometer (Bruker). Each mass spectrum was acquired by accumulation of 100 laser shots at 30% laser power. The matrix solution consisted of α-

CHCA saturated in 70%ACN:30%H_2O, 0.1% TFA (v/v). The dried-droplet method was used for all the samples. Typically, 1 μl of the residue or supernatant was mixed with 5 μl of the matrix solution and an aliquot (0.5 - 1 μl) of this mixture was applied to the stainless steel probe and allowed to air dry before the MALDI-TOF MS measurements.

2.2 RESULTS AND DISCUSSION

2.2.1 Peptide-NP Mole Ratio

We have used both positively-charged (TmaNP (I)) and negatively-charged (CxyNP) NPs to extract and concentrate several different peptides from solution. To study the effect the peptide-NP mole ratio on the resulting MALDI ion signal, angiotensin I and TmaNP (I) were used. A 50 μL solution of 1 μM angiotensin was extracted with the same volume but varying concentrations (5 - 50 μM) of TmaNP (I). Figure 2.2 displays the plot of MALDI ion abundance versus peptide-NP mole ratio. The MALDI ion abundance increases to a maximum at an optimum peptide-NP ratio value of 1:10. Above this ratio, ion abundance drops and then levels off with increasing amount of the NPs. This optimum occurs because of two competing processes that are both dependent on the NP concentration – extraction capacity and MALDI signal suppression. As the NP concentration increases a greater percentage of the peptide is extracted, and thus the MALDI signal associated with the residue increases and the signal associated with the supernatant decreases. At high NP concentrations, though, NP-induced ion suppression is observed. The 1:10 mole ratio represents the optimum trade-off between these competing factors. To overcome the suppression caused by the presence of the NPs during the MALDI process, 5 μL of a 60 μM solution of sodium

cyanide was added to the peptide-NP conjugate after centrifugation to induce dissolution of the gold core of the NP before analysis. The MALDI ion signal is significantly improved (triangles in Fig. 2.2).It is important to note that recent work by Russell and co-workers demonstrated that gold NPs themselves could be used as a MALDI matrix when used at ratios of 1 x 10^7-10^9 analyte molecules to 1 NP.[56] In our experiments, much higher ratios of NPs are necessary for effective extraction and concentration, and at the ratios studied (e.g. 1:10 peptide:NP) the NP's did not act as effective matrices alone.

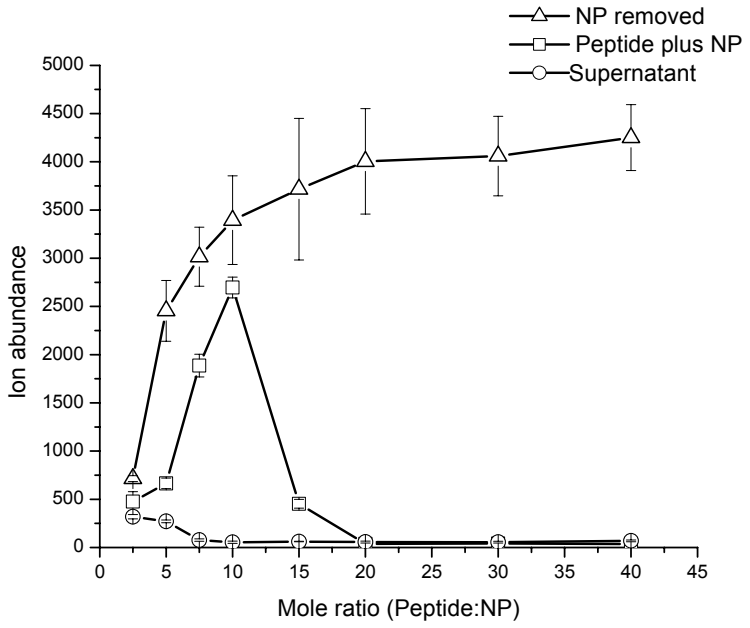


Figure 2.2: MALDI ion abundances of angiotensin I as a function of peptide:NP ratio after extraction with the TmaNP (I). The squares represent the MALDI signal associated with the peptide-NP residue, the circles represent the MALDI signal associated with the supernatant and the triangles represent the MALDI signal associated with residue after removal of the NPs with NaCN. The error bars associated with each measurement represent one standard deviation from the mean.

2.2.2 Extraction Efficiency as a Function of pH

The NPs used in this study were designed to interact with the target peptides through complementary electrostatic interactions. To confirm that the NPs function in this fashion, the MALDI ion abundance of angiotensin was studied as a function of extraction solution pH. Figure 2.3 shows that the residue (peptide-NP conjugate) ion signal increases with increasing pH, whereas the supernatant signal decreases. Because the ionization state of the trimethylammonium functional groups is independent of pH, the solution charging of angiotensin is what gives rise to this pH-dependent behavior. Angiotensin has an isoelectric point (pI) of 7.7, so at pH values below 7.7 the peptide has a net positive charge and is not extracted very efficiently with the positively-charged NPs.

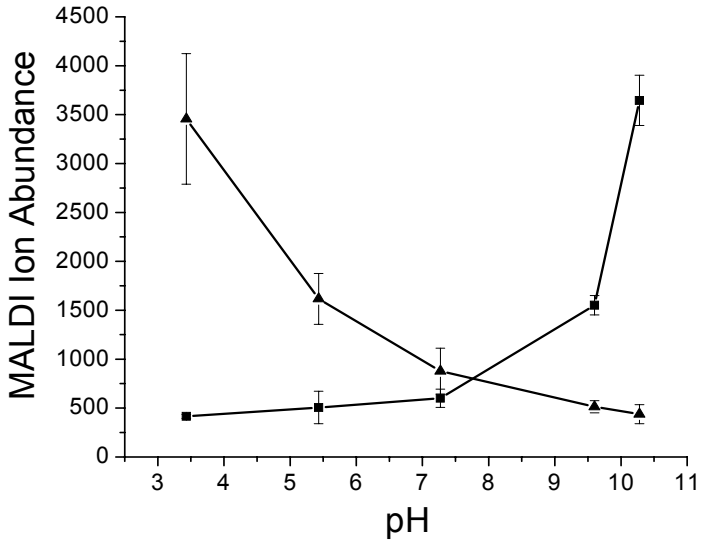


Figure 2.3: MALDI ion abundances of angiotensin I after extraction with TmaNP (I) at different pH values. The squares represent the MALDI signal associated with the peptide-NP residue, and the triangles represent the MALDI signal associated with the supernatant. The error bars associated with each measurement represent one standard deviation from the mean.

At pH values above 7.7, though, angiotensin has a net negative charge and is extracted more efficiently. These observations suggest that complementary electrostatic binding is the main factor controlling extraction of the peptide by TmaNP (I). Even so, angiotensin can still be extracted, albeit inefficiently, at low pH values, which can be attributed to capture of the peptide via hydrophobic interactions between the peptide and the C8 chains that account for about 30% of the NP surface. An interesting, yet not fully understood observation is the relatively low residue and supernatant signals measured when the peptide is extracted at pH values near its pI. If extraction efficiency is poor at pH values around 7.7 as indicated by the low residue signal, then one would expect relatively high MALDI signals for the supernatant, but this is not observed. A temptation is to ascribe this poor supernatant signal to low peptide solubility around its pI, but the low concentrations used in this experiment (i.e. 1 μM) lessen the likelihood of this explanation. This phenomenon, which is observed for several peptides that are extracted near their pIs, needs to be further investigated.

Table 1.1: Peptides used in the nanoparticle extraction experiments.

Peptide	Sequence	pI[a]
ACTH (1-13)	SYSMEHFRWGKPV	9.3
T-7 peptide	MASMTGGQQMG	6.1
Kinetensin	IARRHPYFL	11.2
Glucagon (19-29)	AQDFVQWLMNT	3.8
Bradykinin	RPPGFSPFR	12.5
Angiotensin I	DRVYIHPFHL	7.7

[a] Theoretical values of the peptide pI's were obtained using a software tool located at http://www.bioweb.pasteur.fr/seqanal/tmp/pepstats/

2.2.3 Extraction Capacity as a Function of Peptide pI

Given the observation that electrostatic interactions dominate the extraction efficiency, we tested the selectivity of the NPs for peptides of varying pI values (Table 2.1). Figure 2.4 summarizes the resulting data by plotting extraction capacities as a function of peptide pI. Extraction capacity is defined as the ratio of the MALDI ion abundance of each protonated peptide $(M+H)^+$ in the residue (I_r) to the ion abundance in the reaction supernatant (I_s) ($K=I_r/I_s$). The peptide extraction capacity correlates with peptide pI, and as might be expected, the two NP systems (TmaNP (I) and CxyNP) exhibit opposite selectivity for the peptides. The cationic NPs more efficiently extract peptides with low isoelectric points because these peptides are negatively charged under the solution conditions that were used (i.e. pH 7.4). For example, glucagon, which has a pI of 3.7, is extracted fairly efficiently by TmaNP (I), whereas, bradykinin with a pI of 12.5 is poorly extracted by these positively-charged NPs. In contrast, the anionic NPs more efficiently extract peptides with high pIs because these peptides are positively charged under the solution conditions that were used (i.e. pH 8.6). As an example of this trend, bradykinin is very efficiently extracted, but glucagon is not. The TmaNP (I)'s increase in extraction capacity with decreasing peptide pI (Fig. 2.4a) is explained by the increase in the effective negative charge of the peptides with low pI's, while the CxyNP's increase in extraction capacity with increasing pI (Fig. 2.4b) is due to the increase in the effective positive charge of the peptides with high pI's. Just as was seen with angiotensin in Figure 2.3, both TmaNP (I) and CxyNP can still extract peptides of the same net charge, but they do so with lower efficiency. This result again indicates

that non-electrostatic interactions, possibly hydrophobic and/or hydrogen-bonding interactions, play a minor role in the extraction processes involving these NPs.

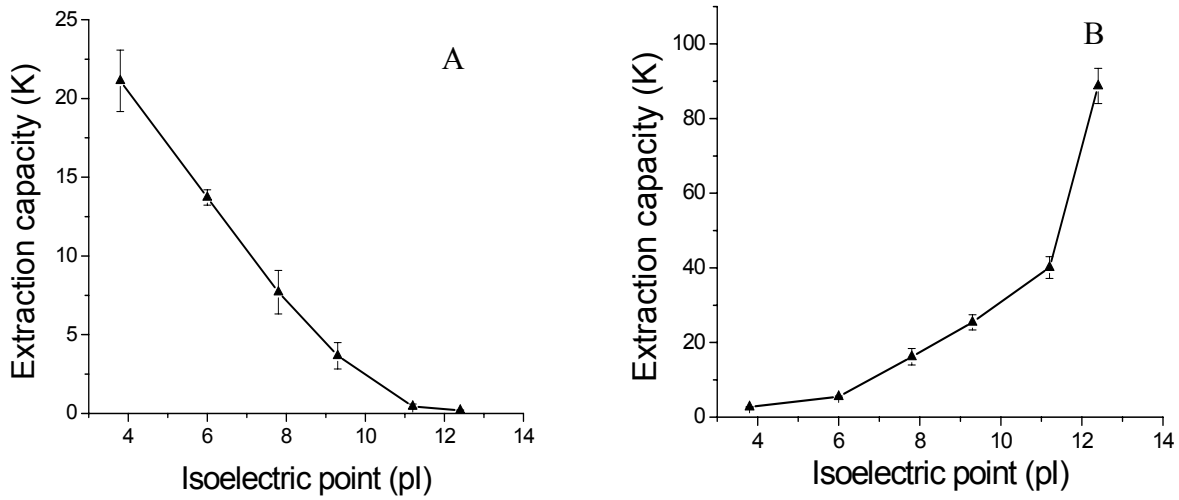


Figure 2.4: Extraction capacity of TmaNP(I) (A) and CxyNP (B) as a function of peptide isoelectric point. The pH of the solution used to extract peptides with the TmaNP(I) was 7.4, while the pH of the solution used to extract peptides with the CxyNP was 8.6. The peptides used in these experiments are shown in Table 1. The error bars associated with each measurement represent one standard deviation from the mean.

2.2.4 Preconcentration

Because the NPs used here can be easily concentrated in solution via centrifugation, their selectivity is accompanied by high concentration factors. Figure 2.5 shows MALDI mass spectra of bradykinin before and after extraction with CxyNP. Virtually no identifiable ion signal is observed for bradykinin when a 1.25 nM solution of the peptide is analyzed directly (Fig. 2.5a). However, upon extraction and concentration of the same sample solution with CxyNP, the protonated species of bradykinin is easily observable in the mass spectrum (Fig. 2.5b). Figure 2.5c displays the results of bradykinin extraction and concentration at a concentration of 500 pM. As

might be expected, no identifiable protonated species is observed for bradykinin before extraction/concentration at 500 pM (data not shown).

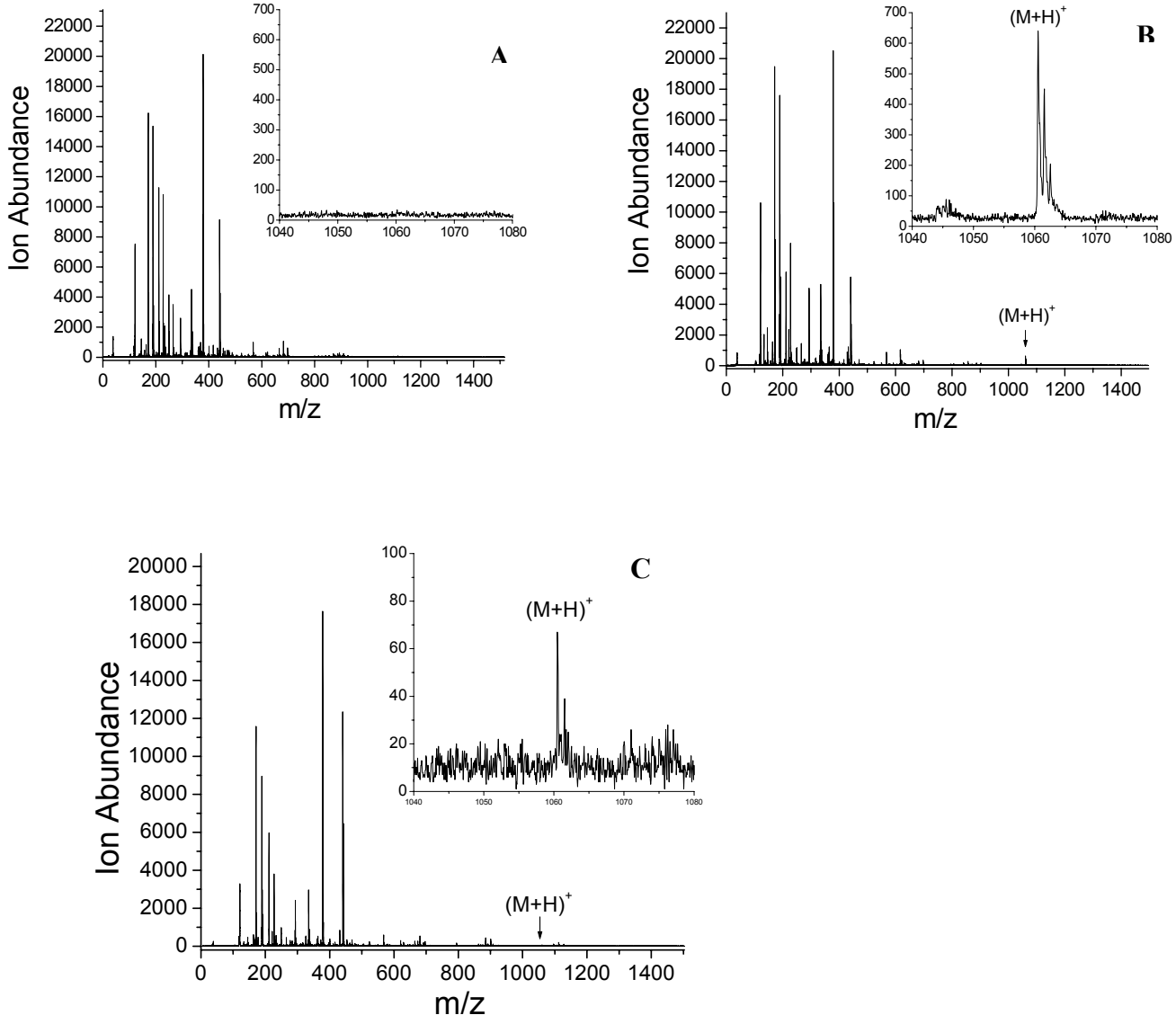


Figure 2.5: MALDI mass spectra of a 1.25 nM solution of bradykinin ($[M+H]^+$ = m/z 1060.5) before (A) and after (B) extraction and concentration with CxyNP. (C) MALDI mass spectrum of a 500 pM solution of bradykinin after extraction and concentration with CxyNP.

2.3 CONCLUSION

In summary, we have developed a straightforward method that employs functionalized NP systems as selective and efficient extraction agents for peptides from dilute solutions. By designing the NPs with cationic or anionic functional groups, we have demonstrated that these NPs can selectively extract negatively- or positively-charged peptides, respectively, with efficiencies that are dependent upon peptide pI and solution pH. The extraction selectivity is coupled with a significant preconcentration effect such that 500 pM solutions of some peptides can be analyzed when treated with the appropriate NPs. The high extraction capacity is attributed to the very high surface area-to-volume ratios associated with the small size (~ 2 nm diameter) of the NP's. The small dimensions of the NPs also allow them to be used directly in the MALDI analysis without substantial losses to sensitivity or mass spectral resolution as long as the appropriate NP concentration is chosen. Direct analysis of the NPs has practical advantages too as it minimizes sample manipulation and in doing so avoids sample losses and contamination while reducing analysis time. Given the positive attributes of these NPs and the ease with which they can be combined with MALDI-MS, the approach described here has several potential applications. For example, cationic NPs might be used to selectivity concentrate proteolytic peptides with low pIs, and the fact that the extracted peptides have low pI values could be used as a constraint during database searches for protein identification. Furthermore, because the MMPCs can be readily fabricated with a wide range of surface functionality, the selectivity of the NPs can be tuned for even more selective fractionation of complex mixtures. This feature could make these NPs suitable for targeted protein extraction from cell lysates.

CHAPTER 3

FACTORS AFFECTING THE EXTRACTION SELECTIVITY OF THE NANOPARTICLES

Biological matrices, for example, cell lysates are complex in composition and possess wide dynamic range. Highly selective and effective extraction agents are thus needed to successfully fractionate them. To obtain the most efficient extractions for selected targets, the factors that influence the selectivity of our NPs have to be well understood so that they can be correctly tuned for efficient fractionation.

While our preliminary results [chapter 2] indicate that peptide charge appears to be the dominant factor controlling the extraction, it is clearly not the only factor. Other factors might control the extraction efficiency, and these factors are not adequately accounted for by this limited set of peptides. In this chapter therefore, we describe our studies of the factors that affect the extraction selectivity of the NPs for the peptides. Five different types of NPs were used to extract and concentrate several peptides from a test library. The test library was created from tryptic digests of several known proteins. The different NPs were separately used to extract and concentrate the peptides from dilute solutions of the different digest samples (at the same solution concentrations), and both the residue and the reaction supernatant were separately analyzed by MALDI-TOF MS after removal of the NPs from the mixture for comparison.

3.1 EXPERIMENTAL

3.1.1 Materials and Reagents

All proteins, α-cyano-4-hydroxycinnamic acid (α-CHCA), Tris (2-carboxyethyl) phosphine hydrochloride (TCEP) and all chemicals for the NP fabrication were

purchased from Aldrich. Tris-(hydroxymethyl) aminomethane (Tris) and tris-(hydroxymethyl) aminomethane hydrochloride (Tris-HCl) were purchased from EM Science. Sequencing grade modified trypsin (Promega, Madison, WI) was used for all the digestions. The water used in all the experiments was obtained from a Milli-Q water purification system (Millipore, Bedford, MA, USA). The Centricon filters and centrifuge tubes were purchased from Millipore Corporation.

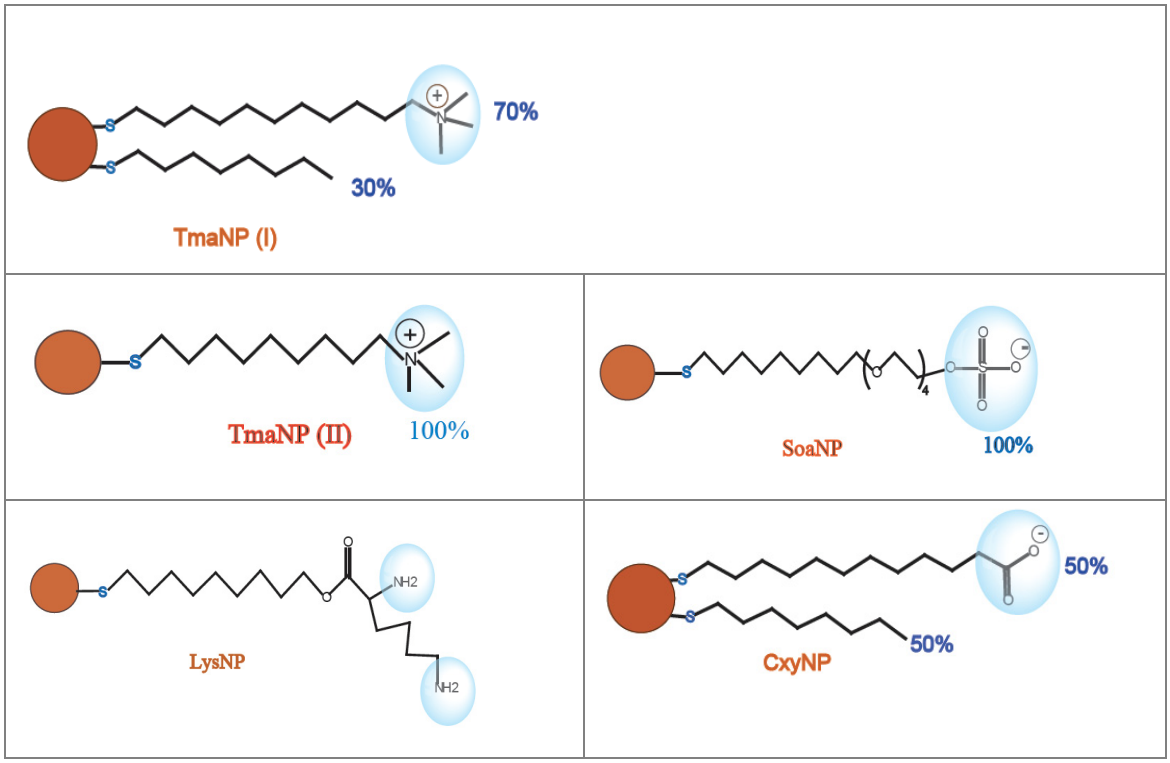
3.1.2 Protein Digestion

Tryptic digestions were carried out on myoglobin, cytochrome C, hemoglobin, bovine serum albumin (BSA), lysozyme and ovalbumin to produce peptides of different lengths and sequences for the extraction experiments. Each of the proteins was digested with trypsin at a concentration ratio of 50:1 (protein/trypsin). To facilitate the digestion, 15 μL of acetonitrile was first added to a 50 μL solution of the protein (625 μM), and the mixture was heated at 60° C for 30 min before adding trypsin. After cooling the protein solution to room temperature, trypsin was added to the appropriate concentration ratio in a 50 mM Tris-Tris/HCl buffer at pH 7.6. The protein/enzyme mixture was then incubated at 37 ºC for 18 h. Each protein digest was analyzed separately by MALDI-TOF MS to confirm the proteins identity, and then it was extracted with the NPs. Proteins that contain disulfide bridges were reduced with TCEP before incubation with acetonitrile and subsequent digestion with trypsin. Six proteins were digested in all, and the experiments resulted in a data set of 145 peptides.

3.1.3 Preparation of NP systems

Fabrication and characterization of the gold NP systems (see Table 3.1) were accomplished via previously reported procedures (see chapter 2). For the positively charged NPs, TmaNP (I) is capped with 30% free alkanethiol and 70% trimethylammonium groups, and TmaNP (II) is capped with 100% trimethylammonium groups.

Table 3.1: Functionalized nanoparticles used for the extraction experiments.



The trimethylammonium ions in both types of NPs are counter-balanced in solution by bromide ions. LysNP is functionalized with 100% alkanethiols terminated with lysine groups. For the negatively charged NPs, CxyNP is functionalized with both free alkanethiols and carboxylate-capped alkanethiol groups, which are counter-balanced in solution by sodium ions. SoaNP is functionalized with 100% alkanethiols that are

capped with sulfonate groups via tetraethyleneglycol (TEG) linkers. The sulfonate ions are counter-balanced in solution by sodium ions.

3.1.4 Sample Extraction with NP's

Stock solutions of all the digests were prepared in deionized water. The digest stock solutions were diluted to working concentrations (2 μM) in a 25 mM Tris/Tris-HCl buffer at the desired pH. To extract the peptides from the digest solutions, a 150 μL solution of NP's (20 μM) was added to an equal volume of a peptide (2 μM) solution in a 10000 MW Centricon cut-off filter, and the mixture was vortexed for 8 – 10 minutes. The peptide-bound NPs were aggregated and separated from the reaction supernatant by centrifugation in an Allegra™ X-22R centrifuge (Beckman Coulter^{TM}). MALDI-TOF MS analysis is then performed on the residue and the supernatant after the gold NPs have been removed from the residue. A mixture of 5% trifluoroacetic acid (TFA), 75%H_2O, and 20% actonitrile (ACN) was used as the releasing agent for the extraction experiments involving TmaNP (II), SoaNP and CxyNP. Aqueous sodium cyanide (NaCN) was used as the releasing agent for the extractions involving TmaNP (I), while a mixture of 5%NH_3/65%H_2O/30%ACN was used for the LysNP experiments.

3.1.5 MALDI TOF-MS

All mass spectra were acquired in positive ion mode using an REFLEX III (Bruker Daltonics) in reflectron mode. The laser fluence was adjusted for optimum resolution and peak intensity in the 600 – 4000 m/z range. Each mass spectrum was acquired by accumulation of 60 laser shots. The matrix solution consisted of α-CHCA

saturated in 70%ACN:30%H_2O with 0.1% TFA(v/v). The dried-droplet method was used for all the samples. Typically, 1 μl of the residue or supernatant was mixed with 5 μl of the matrix solution and an aliquot (1 μl) of this mixture was applied to the stainless steel probe and allowed to air dry before the MALDI-TOF-MS measurements.

3.2 RESULTS AND DISCUSSION

Figure 3.1 displays plots of extraction capacity (K) vs. peptide isoelectric point (pI) for extractions with the five different NPs. K is a measure of how well the NPs extracted the peptides and is expressed as a ratio of the MALDI ion abundance of each protonated peptide in the residue to the MALDI ion abundance of the same protonated peptide in the reaction supernatant (see chapter 1). The peptide pI values were computed using the PEPSTATS software.[57] In all, five different types of NPs, namely TmaNP (I), CxyNP, TmaNP (II), LysNP and SoaNP (table 3.1), were screened against a test library of 146 peptides (table 3.2) to learn about the factors that affect their extraction selectivity.

For the extraction experiments involving LysNP, SoaNP and the two trimethylammonium functionalized NPs (i.e. TmaNP (I) and TmaNP (II)); the pH of the extraction mixture was fixed at 7.4 using a Tris/Tris-HCl buffer. At this solution pH, 82 of the 146 peptides are negatively-charged (i.e. have pI values below the solution pH), 61 are positively-charged (i.e. have pI values above the solution pH), and the remaining 3 peptides are neutral (i.e have pI values equal to the solution pH). CxyNP aggregates at acidic and even neutral pH, and for this reason, the extractions involving this NP were

Figure 3.1: Extraction capacity of TmaNP (I), LysNP, SoaNP, CxyNP and TmaNP (II) as a function of peptide isoelectric point.

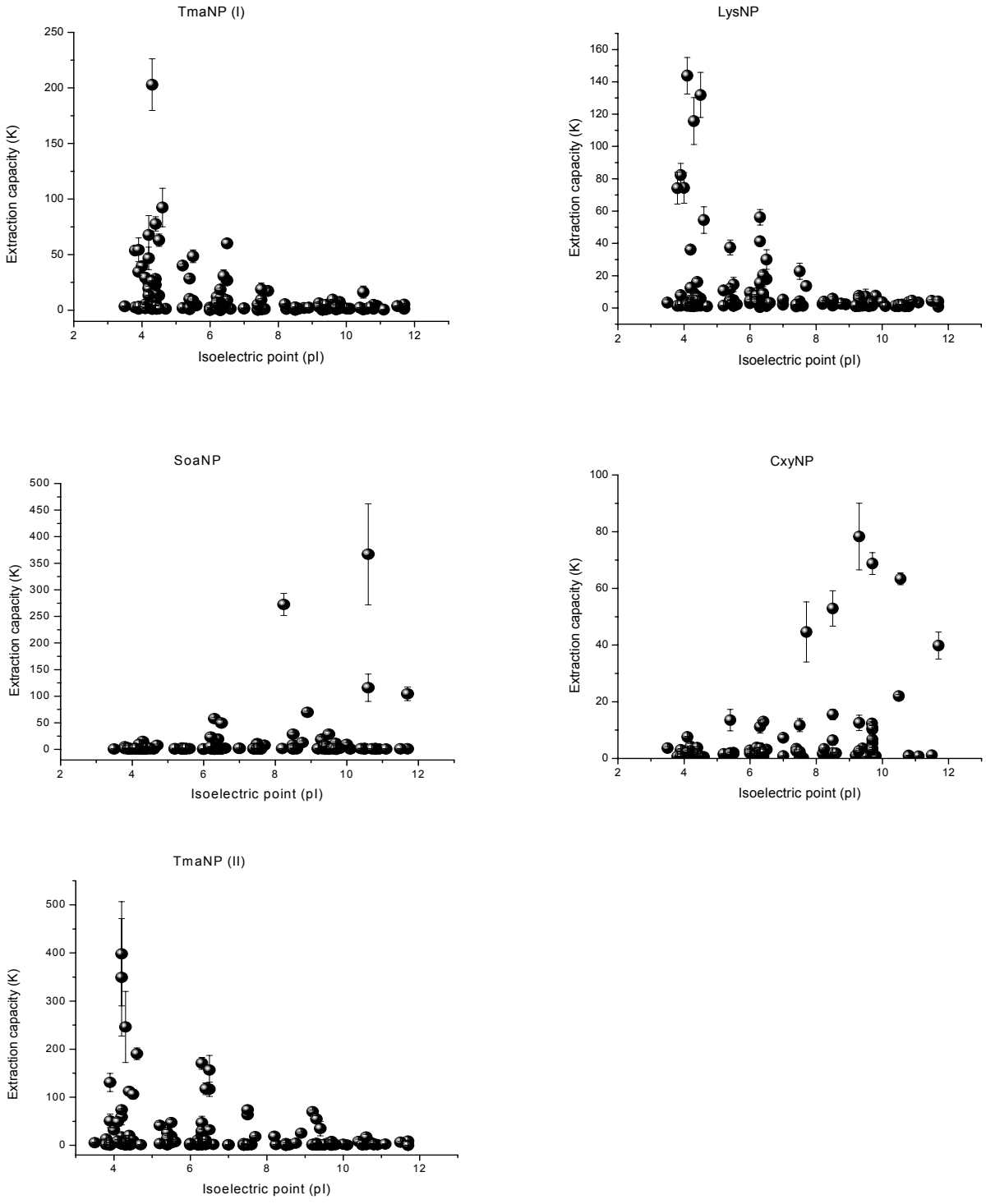


Table 3.2 a: Tryptic peptides used in the extraction experiments (cationic NPs).

						TmaNP (I)		TmaNP (II)		LysNP	
m/z	**Sequence #**	**Amino acid sequence**	**pI**	**Hb**	**KD**	**K**	**Sd (±)**	**K**	**Sd (±)**	**K**	**Sd (±)**
604.00	56-60	GITWK (5)	9.7	15.88	-1.40	0.90	0.20	1.00	0.10	2.40	0.20
634.00	9-13	IFVQK (5)	9.7	14.61	4.10	1.30	0.30	0.90	0.20	1.80	0.00
678.00	74-79	YIPGTK (6)	9.3	10.36	-3.39	0.30	0.10	4.30	0.80	1.30	0.30
779.34	80-86	MIFAGIK (7)	9.69	27.42	11.20	1.30	0.10	0.50	0.10	2.90	0.70
861.00	1-8	GDVEKGKK (8)	9.4	4.53	-15.30	0.70	0.30	0.80	0.10	1.50	0.40
907.00	80-87	MIFAGIKK (8)	10.1	26.89	7.29	1.40	0.80	0.60	0.10	1.20	0.20
964.00	92-99	EDLIAYLK (8)	4.2	27.29	1.69	24.40	6.00	59.10	16.60	36.10	2.40
1169.00	28-38	TGPNLHGLFGR (11)	10.6	25.01	-4.30	1.30	0.10	1.50	0.20	1.50	0.20
1350.00	89-99	TEREDLIAYLK (11)	4.4	26.99	-6.99	9.70	2.40	1.60	0.20	1.90	0.40
1470.00	40-53	TGQAPGFTYTDANK (14)	6.2	18.89	-13.80	12.00	3.30	0.70	0.10	3.80	2.20
1479.19	89-100	TEREDLIAYLKK (12)	6.6	25.42	-10.89	1.30	0.20	2.00	0.10	3.10	0.40
1495.00	61-72	EETLMEYLENPK (12)	3.8	32.15	-19.40	53.70	4.10	13.20	1.50	74.20	9.80
1508.95	92-104	EDLIAYLKKATNE (13)	4.43	30.3	0.30	1.20	0.10	1.20	0.30	4.10	0.60
1598.00	39-53	KTGQAPGFTYTDANK (15)	9.3	17.4	-17.70	1.30	0.30	1.70	0.40	2.50	0.90
1607.00	87-99	KKTEREDLIAYLK (13)	9.2	25.75	-14.79	3.40	1.00	1.00	0.10	1.40	0.20
1623.00	61-73	EETLMEYLENPKK (13)	4.2	31.06	-15.50	20.10	3.70	2.80	0.80	2.20	0.40
1634.85	9-22	IFVQKCAQCHTVEK (14)	8.24	20.23	-8.10	1.10	0.10	1.80	0.30	3.90	0.70
748.00	133-138	ALELFR (6)	6	26.75	4.19	2.20	0.10	4.00	0.50	9.50	1.10
941.00	145-153	YKELGFQG (8)	6	20.86	-6.40	0.90	0.30	2.90	0.30	3.00	0.60
1062.00	43-50	FDKFKHLK (8)	9.7	23.65	-8.99	0.80	0.10	1.90	0.40	1.60	0.20
1272.00	32-42	LFTGHPETLEK (11)	5.4	23.1	-7.10	1.00	0.20	1.20	0.50	11.90	1.10
1361.00	46-56	FKHLKTEAEMK (11)	8.5	19.05	-12.30	2.40	0.70	3.80	1.30	1.70	0.10
1378.80	64-77	HGTVVLTALGGILK (14)	8.76	40.29	16.40	2.00	0.20	4.60	1.70	2.70	0.60
1502.00	118-132	HPGDFGADAQGAMTK (15)	5.2	20.18	-11.00	2.00	0.50	3.70	1.30	10.90	2.50
1507.00	63-77	KHGTVVLTALGGILK (15)	10.8	38.79	12.49	1.30	0.30	1.60	0.20	1.40	0.30
1554.00	140-153	NDIAAKYKELGFQG (14)	7	27.43	-9.20	1.50	0.10	1.50	0.20	2.10	0.30
1607.00	17-31	VEADIAGHGQEVLIR (15)	4.6	27.29	2.29	92.40	17.30	190.40	12.10	54.40	8.20
1816.00	1-16	GLSDGEWQQVLNVWGK (16)	5.2	41.59	-9.20	40.10	1.40	41.40	6.30	1.50	0.10
1853.00	79-95	GHHEAELKPLAQSHATK (17)	7	18.04	-18.39	1.70	0.30	1.10	0.30	5.10	0.40
1885.02	103-118	YLEFISDAIIHVLHSK (16)	6.5	45.92	9.70	60.20	3.60	32.40	6.30	1.30	0.10
1982.00	78-95	KGHHEAELKPLAQSHATK (18)	8.5	16.14	-22.29	0.60	0.10	0.90	0.20	4.20	0.40
2861.36	17-42	VEADIAGHGQEVLIRLFTGHPETLEK (26)	4.7	37.29	-4.80	1.20	0.20	1.40	0.20	1.00	0.30
3405.33	1-31	GLSDGEWQQVLNVWGKVEADIAGHGQEVLIR (31)	4.2	48.53	-6.89	67.70	17.50	74.00	2.20	1.50	0.20
648.09	280-284	VYLPR (5)	9.3	15.64	0.60	4.16	0.84	0.67	0.09	2.60	0.60
780.16	105-110	LYAEER (6)	4.3	8.41	-7.20	1.68	0.35	2.77	1.21	5.20	0.30
823.14	219-226	VASMASEK (8)	6	6.87	0.70	0.14	0.03	0.71	0.15	4.40	1.00
887.21	278-284	IKVYLPR (7)	10	22.45	1.20	1.47	0.20	2.44	0.20	3.60	0.30
906.09	181-187	VYLPRMK (7)	10.5	21.4	-1.40	0.90	0.10	1.28	0.46	1.80	0.50
976.16	182-189	GLWEKAFK (8)	9.5	26.9	-4.20	1.80	0.37	1.66	1.24	1.70	0.10
1015.37	277-284	KIKVYLPR (8)	10.9	24.01	-2.70	3.85	0.39	1.26	0.16	4.50	0.80
1062.58	47-55	DSTRTQINK (9)	9.7	6.45	-16.60	2.64	0.28	0.31	0.20	2.60	0.80
1080.99	219-228	VASMASEKMK (10)	9.5	13.8	-1.30	2.58	0.39	0.75	0.15	2.00	0.10
1190.23	360-369	ADHPFLFCIK (10)	7.4	33.56	6.00	0.90	0.00	0.64	0.29	2.00	0.20
1209.39	190-199	DEDTQAMPFR (10)	3.9	19.14	-14.30	1.19	0.33	0.55	0.06	7.90	1.70
1345.57	370-381	HIATNAVLFFGR (12)	10.6	36.49	9.40	7.43	1.01	17.36	5.26	2.10	0.20
1467.19	111-122	YPILPEYLQCVK (12)	6.2	34.8	2.10	2.57	0.49	1.29	0.64	7.60	0.80
1555.53	187-199	AFKDEDTQAMPFR (13)	4.4	25.23	-13.60	28.08	2.16	112.51	7.95	16.10	2.00
1581.52	264-276	LTEWTSSNVMEER (13)	4	26.63	-12.50	39.56	3.35	32.22	4.05	74.40	9.50
1687.71	127-142	GGLEPINFQTAADQAR (16)	4.2	29.68	-8.60	19.30	2.35	349.29	122.14	12.50	3.10
1709.85	264-277	LTEWTSSNVMEERK (14)	4.5	26.04	-16.40	13.20	1.65	9.92	2.13	5.90	2.00

Table 3.2 a (continued)

						TmaNP (I)		TmaNP (II)		LysNP	
m/z	**Sequence #**	**Amino acid sequence**	**pI**	**Hb**	**KD**	**K**	**Sd (±)**	**K**	**Sd (±)**	**K**	**Sd (±)**
1774.80	323-339	ISQAVHAAHAEINEAGR (17)	6.5	20.99	-3.90	26.85	2.61	156.42	30.50	30.00	6.00
1859.91	143-158	ELINSWVESQTNGIIR (16)	4.3	37.43	-4.10	202.94	23.33	246.30	73.85	115.70	14.50
2250.14	123-142	ELYRGGLEPINFQTAADQAR (20)	4.4	35.58	-14.10	15.39	2.13	3.81	3.31	6.50	2.60
2281.23	85-104	DILNQITKPNDVYSFSLASR (20)	6.4	39.65	-6.50	10.12	2.03	2.53	1.04	8.90	4.30
2461.24	159-181	NVLQPSSVDSQTAMVLVNAIVFK (23)	6.3	46.23	15.30	6.42	0.84	1.00	0.00	1.70	0.40
729.59	1-7	VLSPADK (7)	6.3	10.1	0.00	0.22	0.03	1.58	0.09	3.00	1.00
766.93	201-207	VKAHGKK (7)	11.1	2.1	-9.30	0.43	0.08	2.67	0.17	3.40	1.00
819.03	93-99	VDPVNFK (7)	6.3	17.65	-1.30	0.08	0.01	2.22	0.32	2.90	1.80
931.89	150-158	SAVTALWGK (9)	9.7	23.96	4.90	1.16	0.12	1.05	0.14	3.80	1.80
951.99	142-149	VHLTPEEK (8)	5.4	13.28	-8.40	1.39	0.40	2.07	0.36	5.10	0.30
973.93	8-16	TNVKAAWGK (9)	10.8	16.62	-5.50	2.80	0.49	1.66	0.40	3.50	1.30
1071.03	32-40	MFLSFPTTK (9)	9.7	32.47	3.60	1.33	0.21	3.07	0.71	5.50	0.70
1087.13	91-99	LRVDPVNFK (9)	9.7	23.68	-2.00	1.10	0.26	1.36	0.04	3.50	0.20
1126.01	237-245	LHVDPENFR (9)	5.4	21.86	-9.00	28.41	1.73	26.73	6.04	37.50	4.60
1150.02	274-285	VVAGVANALAHK (12)	9.7	22.62	12.60	4.59	0.57	1.02	0.23	4.20	2.80
1170.99	1-11	VLSPADKTNVK (11)	9.5	13.39	-3.90	1.70	0.31	1.60	0.12	7.10	4.60
1252.13	128-139	FLASVSTVLTSK (12)	9.7	29.07	12.90	1.25	0.37	6.47	2.84	2.30	0.90
1269.04	140-149	YRVHLTPEEK (10)	7.5	17.65	-14.20	0.65	0.31	1.90	0.36	1.60	0.10
1274.12	172-181	LLVVYPWTQR (10)	**9.3**	33.32	3.50	3.64	0.94	54.81	6.74	7.50	0.40
1314.10	159-171	VNVDEVGGEALGR (13)	3.9	21.19	-1.50	34.62	3.52	51.05	14.14	82.20	7.30
1378.12	262-273	EFTPPVQAAYQK (12)	6.4	21.68	-9.00	3.68	1.35	2.95	0.70	7.20	4.00
1422.00	224-236	GTFATLSELHCDK (13)	5.4	28.82	-2.00	1.50	0.50	2.07	0.60	4.90	1.90
1449.15	274-287	VVAGVANALAHKYH (14)	9.3	24.67	8.10	1.46	0.34	1.46	0.24	2.00	0.20
1529.17	17-31	VGAHAGEYGAEALER (15)	4.5	20.9	-5.50	63.02	5.51	106.27	9.65	131.90	14.00
1669.37	208-223	VLGAFSDGLAHLDNLK (16)	5.4	36.74	6.60	10.14	2.28	14.37	3.08	3.80	0.70
1719.77	246-261	LLGNVLVCVLAHHFGK (16)	8.6	39.24	20.30	1.33	0.09	1.52	0.04	2.70	1.00
1798.36	207-223	KVLGAFSDGLAHLDNLK (17)	7.5	38.19	2.70	2.13	0.87	1.65	0.24	2.40	0.10
1834.44	41-56	TYFPHFDLSHGSAQVK (16)	**7.7**	31.7	-7.50	17.37	2.54	18.37	1.74	13.60	2.90
2060.05	182-200	FFESFGDLSTPDAVMGNPK (19)	3.9	38.05	-4.10	1.40	0.40	6.72	1.95	4.50	0.50
606.08	1-5	KVFGR (5)	11.7	9.6	-1.80	1.17	0.39	0.12	0.02	4.00	1.00
874.26	15-21	HGLDNYR (7)	7.5	11.54	-12.60	1.15	0.27	0.73	0.08	22.70	5.00
936.17	62-68	WWCNDGR (7)	6.2	19.77	-11.20	1.43	0.32	4.55	1.88	6.80	1.50
1030.53	14-21	RHGLDNYR (8)	9.3	12.35	-17.10	1.25	0.09	1.01	0.17	6.00	2.90
1045.44	117-125	GTDVQAWIR (9)	6.3	22.94	-2.99	10.42	4.89	20.41	0.21	56.20	4.80
1268.47	22-33	GYSLGNWVCAAK (12)	8.5	29.56	2.90	0.33	0.21	0.19	0.01	5.70	1.30
1428.56	34-45	FESNFNTQATNR (12)	6.4	16.15	-16.80	2.50	2.01	10.26	2.06	20.50	1.80
1450.48	2-14	VFGRCELAAAMKR (13)	9.8	25.08	3.80	1.64	0.33	1.26	0.36	7.60	2.20
1675.90	98-112	IVSDGNGMNAWVAWR (15)	6.3	32.93	0.00	5.42	2.35	29.54	3.84	15.60	3.30
1753.95	46-61	NTDGSTDYGILQINSR (16)	4.1	26.96	-14.30	29.30	4.06	49.13	7.02	143.80	11.30
609.73	500-504	AFDEK (5)	**4.2**	4.92	-6.30	4.60	0.40	5.15	1.18	1.30	0.20
649.09	199-204	CASIQK (6)	**8.5**	4.44	0.60	1.20	0.20	0.84	0.10	4.10	4.50
658.03	94-98	QEPER (5)	**4.3**	1.65	-16.60	5.90	1.60	4.58	1.28	1.60	0.10
688.29	212-217	AWSVAR (6)	**10.5**	13.09	1.60	0.80	0.10	5.03	0.46	1.20	0.10
701.08	174-180	GACLLPK (7)	**8.5**	17.65	6.00	2.50	0.50	1.38	0.50	1.60	0.40
712.15	5-10	SEIAHR (6)	**7.6**	4.99	-5.70	1.10	0.30	1.25	0.24	1.40	0.10
733.06	188-194	VLTSSAR (7)	**10.5**	7.88	3.00	1.10	0.20	1.11	0.23	1.20	0.10
752.19	317-322	NYQEAK (6)	**6.4**	3.1	-13.90	2.70	0.80	1.65	0.11	1.80	0.40
787.91	233-239	LVTDLTK (7)	**6.3**	17.02	2.99	3.30	0.20	3.31	0.56	0.50	0.10
818.03	538-544	ATEEQLK (7)	**4.3**	6.4	-9.50	1.10	0.30	1.18	0.29	1.90	0.20
840.89	459-465	LCVLHEK (7)	**7.4**	15.76	3.70	0.30	0.10	0.32	0.10	0.90	0.20

Table 3.2 a (continued)

						TmaNP (I)		TmaNP (II)		LysNP	
m/z	Sequence #	Amino acid sequence	pI	Hb	KD	K	Sd (±)	K	Sd (±)	K	Sd (±)
846.90	218-224	LSQKFPK (7)	**10.8**	15.69	-7.10	4.70	0.30	3.27	0.24	1.10	0.20
886.30	107-114	DDSPDLPK (8)	**3.8**	12.43	-14.60	2.60	0.10	1.66	0.39	1.40	0.50
922.05	225-232	AEFVEVTK (8)	**4.3**	20.05	1.40	2.70	0.30	0.78	0.12	1.20	0.30
927.36	137-143	YLYEIAR (7)	**6.5**	24.44	-0.50	8.90	1.19	116.57	14.66	17.80	0.90
974.34	13-20	DLGEEHFK (8)	**4.4**	16.23	-11.40	22.80	2.80	11.20	3.03	7.20	2.10
977.36	99-106	NECFLSHK (8)	**7.4**	16.6	-5.80	5.10	1.00	3.61	0.22	2.10	0.10
1011.11	389-396	QNCDQFEK (8)	**4.2**	7.55	-16.09	4.20	0.30	2.76	0.35	3.30	0.30
1014.57	525-533	QTALVELLK (9)	**6.3**	32.23	5.80	4.00	1.20	2.43	1.10	2.10	0.10
1050.39	564-573	EACFAVEGPK (10)	**4.4**	19.69	0.20	6.10	2.00	6.54	2.46	1.80	0.30
1084.59	137-144	YLYEIARR (8)	**9.2**	24.29	-5.00	6.23	0.21	69.89	3.26	1.10	0.20
1144.68	212-221	AWSVARLSQK (10)	**11.7**	21.64	-2.80	5.00	1.00	8.70	0.88	0.80	0.30
1163.47	42-51	LVNELTEFAK (10)	**4.3**	28.48	1.30	26.20	0.40	10.72	5.33	9.00	1.10
1197.56	313-322	DVCKNYQEAK (10)	**6.3**	6.24	-14.60	6.90	0.40	47.16	13.36	1.10	0.10
1202.63	195-204	QRLRCASIQK (10)	**11.5**	11.9	-8.10	3.80	0.30	6.45	2.47	4.40	0.80
1249.39	11-20	FKDLGEEHFK (10)	**5.5**	22.54	-12.50	5.40	0.60	7.74	4.98	4.80	0.50
1283.67	337-347	HPEYAVSVLLR (11)	**7.5**	32.31	2.89	18.86	5.59	63.66	8.64	4.10	0.20
1293.68	222-232	FPKAEFVEVTK (11)	**6.4**	27.48	-1.30	30.70	5.60	117.64	12.30	1.70	0.30
1305.61	378-388	HLVDEPQNLIK (11)	**5.5**	25.69	-6.40	48.50	5.70	19.08	7.40	14.50	4.40
1345.51	174-185	GACLLPKIETMR (12)	**6.2**	28.89	3.70	6.90	0.20	5.37	2.62	9.20	1.60
1361.09	65-76	SLHTLFGDELCK (12)	**5.5**	30.18	0.70	7.50	0.20	4.88	4.44	1.40	0.20
1386.31	262-273	YICDNQDTISSK (12)	**4.1**	14.82	-10.00	5.10	0.30	5.96	1.02	3.80	0.60
1399.63	545-556	TVMENFVAFVDK (12)	**4.2**	37.53	6.80	46.67	10.07	17.45	1.98	7.30	0.50
1439.83	336-347	RHPEYAVSVLLR (12)	**9.4**	32.16	-1.60	4.70	0.50	34.92	15.19	3.60	0.30
1450.60	199-211	CASIQKFGERALK (13)	**9.6**	23.36	-3.30	9.50	0.10	4.68	1.21	1.10	0.20
1464.73	432-444	VGTRCCTKPESER (13)	**8.2**	5.56	-14.89	5.40	0.40	18.85	7.83	2.50	0.70
1479.85	397-409	LGEYGFQNALIVR (13)	**6.3**	35.04	3.80	18.64	1.22	170.71	12.44	41.20	2.60
1496.69	363-375	DDPHACYTSVFDK (13)	**4.3**	21.87	-10.70	4.10	0.20	6.99	2.39	1.10	0.20
1503.09	525-537	QTALVELLKHKPK (13)	**10.5**	31.59	-6.80	16.30	5.50	6.84	3.38	2.00	0.30
1511.91	521-533	VPQVSTPTLVEVSR (14)	**6.3**	27.05	2.90	3.40	0.40	10.36	2.54	2.90	0.00
1519.58	115-127	LKPDPNTLCDEFK (13)	**4.4**	25.03	-12.80	4.40	0.20	20.55	0.11	3.40	0.80
1546.05	376-388	LKHLVDEPQNLIK (13)	**7.5**	27.83	-6.50	9.40	1.00	73.85	9.06	2.10	0.20
1567.81	323-335	DAFLGSFLYEYSR (13)	**4.2**	39.68	-1.10	13.40	0.10	398.24	108.41	3.10	0.40
1578.07	243-256	ECCHGDLLECADDR (14)	**3.9**	20.09	-8.70	3.10	0.10	5.22	0.57	2.10	0.30
1616.27	94-106	QEPERNECFLSHK (13)	**5.6**	17.93	-22.40	4.30	0.30	7.40	1.26	2.20	0.90
1630.00	524-537	YNGVFQECCQAEDK (14)	**3.9**	19.98	-12.80	54.30	10.70	130.77	19.07	1.50	0.30
1640.11	413-427	KVPQVSTPTLVEVSR (15)	**9.7**	26.99	-1.00	2.30	0.20	8.25	3.68	2.60	0.10
1741.07	363-377	DDPHACYTSVFDKLK (15)	**5.5**	27.43	-10.80	8.60	1.50	47.23	6.27	1.40	0.30
1755.88	557-573	CCAADDKEACFAVEGPK (17)	**4.1**	20.98	-2.10	2.70	0.30	7.40	3.62	2.30	0.40
1824.10	484-499	RPCFSALTPDETYVPK (16)	**6.2**	32.51	-8.60	6.30	0.20	11.16	3.60	3.40	1.00
1851.03	505-520	LFTFHADICTLPDTEK (16)	**4.4**	35.93	0.70	2.70	0.00	10.08	1.15	1.40	0.30
1956.36	295-312	DAIPEDLPPLTADFAEDK (18)	**3.5**	36.35	-10.10	3.50	0.40	5.65	0.85	3.30	1.70
2025.48	413-431	KVPQVSTPTLVEVSRSLGK (19)	**10.7**	32.29	-2.30	2.50	0.20	6.68	1.70	1.00	0.20
2046.53	144-159	RHPYFYAPELLYYANK (16)	**8.9**	37.08	-13.00	2.30	0.10	25.36	2.56	2.30	0.50
2060.42	410-427	YTRKVPQVSTPTLVEVSR (18)	**10.4**	29.03	-7.50	2.20	0.20	8.07	4.64	1.20	0.40
2199.68	538-556	ATEEQLKTVMENFVAFVDK (19)	**4.1**	40.96	-2.70	1.70	0.10	8.84	3.68	1.40	0.30
2414.16	484-504	RPCFSALTPDETYVPKAFDEK (21)	**4.5**	37.02	-14.90	1.50	0.00	4.30	0.61	1.50	0.40

Table 3.2 b: Tryptic peptides used in the extraction experiments (anionic NPs)

						CxyNP		SoaNP	
m/z	**Sequence #**	**Amino acid sequence**	**pI**	**Hb**	**KD**	**K**	**Sd (±)**	**K**	**Sd (±)**
604.00	56-60	GITWK (5)	9.7	15.88	-1.40	28.84	7.33	4.80	1.10
634.00	9-13	IFVQK (5)	9.7	14.61	4.10	5.84	2.53	2.70	0.40
678.00	74-79	YIPGTK (6)	9.3	10.36	-3.39	5.62	0.93	1.50	0.10
779.34	80-86	MIFAGIK (7)	9.69	27.42	11.20	9.50	3.20	11.20	3.00
861.00	1-8	GDVEKGKK (8)	9.4	4.53	-15.30	1.55	0.51	0.10	0.00
907.00	80-87	MIFAGIKK (8)	10.1	26.89	7.29	4.12	1.01	0.70	0.10
964.00	92-99	EDLIAYLK (8)	4.2	27.29	1.69	1.62	0.41	2.50	0.40
1169.00	28-38	TGPNLHGLFGR (11)	10.6	25.01	-4.30	63.38	2.10	115.90	25.80
1350.00	89-99	TEREDLIAYLK (11)	4.4	26.99	-6.99	1.94	0.34	1.80	0.20
1470.00	40-53	TGQAPGFTYTDANK (14)	6.2	18.89	-13.80	3.53	2.00	2.10	0.40
1479.19	89-100	TEREDLIAYLKK (12)	6.6	25.42	-10.89	1.84	0.06	2.10	0.30
1495.00	61-72	EETLMEYLENPK (12)	3.8	32.15	-19.40	1.21	0.61	4.80	1.70
1508.95	92-104	EDLIAYLKKATNE (13)	4.43	30.3	0.30	2.36	0.60	3.20	0.40
1598.00	39-53	KTGQAPGFTYTDANK (15)	9.3	17.4	-17.70	14.05	5.02	4.10	0.20
1607.00	87-99	KKTEREDLIAYLK (13)	9.2	25.75	-14.79	1.38	0.21	1.70	0.30
1623.00	61-73	EETLMEYLENPKK (13)	4.2	31.06	-15.50	1.46	0.60	2.90	0.10
1634.85	9-22	IFVQKCAQCHTVEK (14)	8.24	20.23	-8.10	6.41	0.54	272.60	20.80
748.00	133-138	ALELFR (6)	6	26.75	4.19	1.15	0.34	0.40	0.20
941.00	145-153	YKELGFQG (8)	6	20.86	-6.40	2.71	0.53	0.90	0.30
1062.00	43-50	FDKFKHLK (8)	9.7	23.65	-8.99	0.93	0.16	1.20	0.20
1272.00	32-42	LFTGHPETLEK (11)	5.4	23.1	-7.10	2.78	0.59	0.60	0.10
1361.00	46-56	FKHLKTEAEMK (11)	8.5	19.05	-12.30	6.41	1.70	10.30	0.50
1378.80	64-77	HGTVVLTALGGILK (14)	8.76	40.29	16.40	1.30	0.12	12.90	3.00
1502.00	118-132	HPGDFGADAQGAMTK (15)	5.2	20.18	-11.00	2.02	0.16	0.80	0.40
1507.00	63-77	KHGTVVLTALGGILK (15)	10.8	38.79	12.49	1.30	0.06	1.50	0.40
1554.00	140-153	NDIAAKYKELGFQG (14)	7	27.43	-9.20	0.77	0.67	1.50	0.00
1607.00	17-31	VEADIAGHGQEVLIR (15)	4.6	27.29	2.29	0.49	0.10	0.60	0.50
1816.00	1-16	GLSDGEWQQVLNVWGK (16)	5.2	41.59	-9.20	1.99	0.66	1.40	0.90
1853.00	79-95	GHHEAELKPLAQSHATK (17)	7	18.04	-18.39	4.86	1.70	2.20	1.10
1885.02	103-118	YLEFISDAIIHVLHSK (16)	6.5	45.92	9.70	1.44	0.14	49.40	5.50
1982.00	78-95	KGHHEAELKPLAQSHATK (18)	8.5	16.14	-22.29	15.49	1.80	2.60	0.30
2861.36	17-42	VEADIAGHGQEVLIRLFTGHPETLEK (26)	4.7	37.29	-4.80	1.47	0.14	7.50	2.20
3405.33	1-31	GLSDGEWQQVLNVWGKVEADIAGHGQEVLIR (31)	4.2	48.53	-6.89	3.00	0.54	4.90	1.00
648.09	280-284	VYLPR (5)	9.3	15.64	0.60	3.60	0.30	18.98	5.74
780.16	105-110	LYAEER (6)	4.3	8.41	-7.20	0.70	0.20	1.26	0.42
823.14	219-226	VASMASEK (8)	6	6.87	0.70	0.90	0.10	0.10	0.03
887.21	278-284	IKVYLPR (7)	10	22.45	1.20	3.10	0.90	9.33	3.43
906.09	181-187	VYLPRMK (7)	10.5	21.4	-1.40	3.80	0.40	1.47	0.56
976.16	182-189	GLWEKAFK (8)	9.5	26.9	-4.20	1.60	0.30	0.68	0.29
1015.37	277-284	KIKVYLPR (8)	10.9	24.01	-2.70	1.20	0.10	0.80	0.33
1062.58	47-55	DSTRTQINK (9)	9.7	6.45	-16.60	1.00	0.10	29.27	7.82
1080.99	219-228	VASMASEKMK (10)	9.5	13.8	-1.30	3.40	1.80	28.50	6.19
1190.23	360-369	ADHPFLFCIK (10)	7.4	33.56	6.00	1.80	0.60	1.66	0.49
1209.39	190-199	DEDTQAMPFR (10)	3.9	19.14	-14.30	1.80	0.40	1.12	0.29
1345.57	370-381	HIATNAVLFFGR (12)	10.6	36.49	9.40	20.50	4.00	367.00	95.02
1467.19	111-122	YPILPEYLQCVK (12)	6.2	34.8	2.10	1.70	0.40	3.44	1.90
1555.53	187-199	AFKDEDTQAMPFR (13)	4.4	25.23	-13.60	5.30	0.40	3.52	2.92
1581.52	264-276	LTEWTSSNVMEER (13)	4	26.63	-12.50	2.50	0.10	0.81	0.17
1687.71	127-142	GGLEPINFQTAADQAR (16)	4.2	29.68	-8.60	3.40	0.20	2.63	2.22
1709.85	264-277	LTEWTSSNVMEERK (14)	4.5	26.04	-16.40	2.40	0.50	1.40	0.36

Table 3.2 b (continued)

						CxyNP		SoaNP	
m/z	**Sequence #**	**Amino acid sequence**	**pI**	**Hb**	**KD**	**K**	**Sd (±)**	**K**	**Sd (±)**
1774.80	323-339	ISQAVHAAHAEINEAGR (17)	6.5	20.99	-3.90	9.50	1.80	1.50	1.02
1859.91	143-158	ELINSWVESQTNGIIR (16)	4.3	37.43	-4.10	3.90	0.40	14.85	2.94
2250.14	123-142	ELYRGGLEPINFQTAADQAR (20)	4.4	35.58	-14.10	6.70	0.80	3.41	0.73
2281.23	85-104	DILNQITKPNDVYSFSLASR (20)	6.4	39.65	-6.50	2.10	1.00	19.25	8.06
2461.24	159-181	NVLQPSSVDSQTAMVLVNAIVFK (23)	6.3	46.23	15.30	2.40	0.80	12.10	1.80
729.59	1-7	VLSPADK (7)	6.3	10.1	0.00	1.44	0.17	0.13	0.04
766.93	201-207	VKAHGKK (7)	11.1	2.1	-9.30	0.73	0.20	0.72	0.13
819.03	93-99	VDPVNFK (7)	6.3	17.65	-1.30	1.09	0.04	0.54	0.18
931.89	150-158	SAVTALWGK (9)	9.7	23.96	4.90	3.55	0.34	0.50	0.11
951.99	142-149	VHLTPEEK (8)	5.4	13.28	-8.40	1.12	0.11	0.27	0.04
973.93	8-16	TNVKAAWGK (9)	10.8	16.62	-5.50	0.79	0.03	0.65	0.17
1071.03	32-40	MFLSFPTTK (9)	9.7	32.47	3.60	10.24	1.36	0.38	0.13
1087.13	91-99	LRVDPVNFK (9)	9.7	23.68	-2.00	10.78	3.16	0.47	0.04
1126.01	237-245	LHVDPENFR (9)	5.4	21.86	-9.00	1.31	0.18	1.38	0.35
1150.02	274-285	VVAGVANALAHK (12)	9.7	22.62	12.60	6.70	1.36	0.17	0.03
1170.99	1-11	VLSPADKTNVK (11)	9.5	13.39	-3.90	0.65	0.09	0.08	0.00
1252.13	128-139	FLASVSTVLTSK (12)	9.7	29.07	12.90	3.07	0.06	0.92	0.07
1269.04	140-149	YRVHLTPEEK (10)	7.5	17.65	-14.20	1.88	0.33	10.80	8.10
1274.12	172-181	LLVVYPWTQR (10)	**9.3**	33.32	3.50	78.30	11.74	18.48	3.81
1314.10	159-171	VNVDEVGGEALGR (13)	3.9	21.19	-1.50	0.70	0.11	0.40	0.10
1378.12	262-273	EFTPPVQAAYQK (12)	6.4	21.68	-9.00	1.98	0.21	0.57	0.03
1422.00	224-236	GTFATLSELHCDK (13)	5.4	28.82	-2.00	1.69	0.36	2.39	0.17
1449.15	274-287	VVAGVANALAHKYH (14)	9.3	24.67	8.10	2.60	0.34	1.50	0.12
1529.17	17-31	VGAHAGEYGAEALER (15)	4.5	20.9	-5.50	0.61	0.12	1.31	0.12
1669.37	208-223	VLGAFSDGLAHLDNLK (16)	5.4	36.74	6.60	13.50	3.76	0.49	0.13
1719.77	246-261	LLGNVLVCVLAHHFGK (16)	8.6	39.24	20.30	1.83	0.41	1.08	0.22
1798.36	207-223	KVLGAFSDGLAHLDNLK (17)	7.5	38.19	2.70	2.34	0.04	2.20	0.38
1834.44	41-56	TYFPHFDLSHGSAQVK (16)	**7.7**	31.7	-7.50	44.63	10.59	7.68	1.44
2060.05	182-200	FFESFGDLSTPDAVMGNPK (19)	3.9	38.05	-4.10	4.74	1.17	1.50	0.40
606.08	1-5	KVFGR (5)	11.7	9.6	-1.80	39.83	4.75	104.45	12.64
874.26	15-21	HGLDNYR (7)	7.5	11.54	-12.60	0.33	0.05	8.89	3.01
936.17	62-68	WWCNDGR (7)	6.2	19.77	-11.20	3.87	1.42	23.08	5.08
1030.53	14-21	RHGLDNYR (8)	9.3	12.35	-17.10	2.19	1.33	3.04	0.62
1045.44	117-125	GTDVQAWIR (9)	6.3	22.94	-2.99	3.62	1.75	2.04	0.93
1268.47	22-33	GYSLGNWVCAAK (12)	8.5	29.56	2.90	52.90	6.22	28.39	3.69
1428.56	34-45	FESNFNTQATNR (12)	6.4	16.15	-16.80	12.99	0.93	1.14	0.69
1450.48	2-14	VFGRCELAAAMKR (13)	9.8	25.08	3.80	0.78	0.35	1.73	0.48
1675.90	98-112	IVSDGNGMNAWVAWR (15)	6.3	32.93	0.00	2.16	0.18	57.84	2.70
1753.95	46-61	NTDGSTDYGILQINSR (16)	4.1	26.96	-14.30	1.95	0.44	1.74	1.30
609.73	500-504	AFDEK (5)	**4.2**	4.92	-6.30	0.90	0.20	1.40	0.50
649.09	199-204	CASIQK (6)	**8.5**	4.44	0.60	2.30	0.50	0.70	0.40
658.03	94-98	QEPER (5)	**4.3**	1.65	-16.60	1.00	0.40	0.80	0.30
688.29	212-217	AWSVAR (6)	**10.5**	13.09	1.60	8.10	2.90	0.60	0.10
701.08	174-180	GACLLPK (7)	**8.5**	17.65	6.00	2.20	0.30	1.10	0.20
712.15	5-10	SEIAHR (6)	**7.6**	4.99	-5.70	0.60	0.20	0.40	0.10
733.06	188-194	VLTSSAR (7)	**10.5**	7.88	3.00	1.20	0.30	0.70	0.10
752.19	317-322	NYQEAK (6)	**6.4**	3.1	-13.90	1.10	0.30	1.00	0.30
787.91	233-239	LVTDLTK (7)	**6.3**	17.02	2.99	1.20	0.40	1.20	0.40
818.03	538-544	ATEEQLK (7)	**4.3**	6.4	-9.50	1.80	0.40	0.90	0.20
840.89	459-465	LCVLHEK (7)	**7.4**	15.76	3.70	2.50	0.60	1.10	0.50

Table 3.2 b (continued)

						CxyNP		SoaNP	
m/z	**Sequence #**	**Amino acid sequence**	**pI**	**Hb**	**KD**	**K**	**Sd (±)**	**K**	**Sd (±)**
846.90	218-224	LSQKFPK (7)	**10.8**	15.69	-7.10	1.30	0.10	1.10	0.50
886.30	107-114	DDSPDLPK (8)	**3.8**	12.43	-14.60	1.10	0.10	1.30	0.30
922.05	225-232	AEFVEVTK (8)	**4.3**	20.05	1.40	1.90	0.20	1.20	0.20
927.36	137-143	YLYEIAR (7)	**6.5**	24.44	-0.50	16.30	0.60	0.30	0.10
974.34	13-20	DLGEEHFK (8)	**4.4**	16.23	-11.40	1.30	0.20	0.60	0.00
977.36	99-106	NECFLSHK (8)	**7.4**	16.6	-5.80	2.20	0.40	1.00	0.00
1011.11	389-396	QNCDQFEK (8)	**4.2**	7.55	-16.09	2.50	0.50	1.60	0.20
1014.57	525-533	QTALVELLK (9)	**6.3**	32.23	5.80	3.40	1.20	0.80	0.20
1050.39	564-573	EACFAVEGPK (10)	**4.4**	19.69	0.20	4.60	1.60	1.60	0.50
1084.59	137-144	YLYEIARR (8)	**9.2**	24.29	-5.00	1.30	0.30	1.60	0.40
1144.68	212-221	AWSVARLSQK (10)	**11.7**	21.64	-2.80	1.40	0.10	1.10	0.20
1163.47	42-51	LVNELTEFAK (10)	**4.3**	28.48	1.30	7.40	1.70	1.00	0.10
1197.56	313-322	DVCKNYQEAK (10)	**6.3**	6.24	-14.60	1.70	0.20	1.00	0.10
1202.63	195-204	QRLRCASIQK (10)	**11.5**	11.9	-8.10	2.00	0.30	1.00	0.10
1249.39	11-20	FKDLGEEHFK (10)	**5.5**	22.54	-12.50	3.20	1.00	1.30	0.40
1283.67	337-347	HPEYAVSVLLR (11)	**7.5**	32.31	2.89	9.80	1.00	2.20	0.50
1293.68	222-232	FPKAEFVEVTK (11)	**6.4**	27.48	-1.30	2.00	0.20	1.50	0.20
1305.61	378-388	HLVDEPQNLIK (11)	**5.5**	25.69	-6.40	9.00	2.10	1.10	0.30
1345.51	174-185	GACLLPKIETMR (12)	**6.2**	28.89	3.70	2.00	0.30	0.90	0.10
1361.09	65-76	SLHTLFGDELCK (12)	**5.5**	30.18	0.70	2.00	0.40	1.10	0.20
1386.31	262-273	YICDNQDTISSK (12)	**4.1**	14.82	-10.00	4.00	1.10	1.00	0.10
1399.63	545-556	TVMENFVAFVDK (12)	**4.2**	37.53	6.80	5.20	1.30	0.60	0.10
1439.83	336-347	RHPEYAVSVLLR (12)	**9.4**	32.16	-1.60	8.00	0.60	7.50	2.50
1450.60	199-211	CASIQKFGERALK (13)	**9.6**	23.36	-3.30	1.50	0.30	1.20	0.10
1464.73	432-444	VGTRCCTKPESER (13)	**8.2**	5.56	-14.89	2.00	0.30	1.50	0.20
1479.85	397-409	LGEYGFQNALIVR (13)	**6.3**	35.04	3.80	25.80	4.00	2.40	0.50
1496.69	363-375	DDPHACYTSVFDK (13)	**4.3**	21.87	-10.70	2.80	0.60	1.10	0.10
1503.09	525-537	QTALVELLKHKPK (13)	**10.5**	31.59	-6.80	2.30	0.30	1.40	0.40
1511.91	521-533	VPQVSTPTLVEVSR (14)	**6.3**	27.05	2.90	14.20	5.90	0.60	0.10
1519.58	115-127	LKPDPNTLCDEFK (13)	**4.4**	25.03	-12.80	2.90	0.60	1.30	0.40
1546.05	376-388	LKHLVDEPQNLIK (13)	**7.5**	27.83	-6.50	1.50	0.30	0.80	0.20
1567.81	323-335	DAFLGSFLYEYSR (13)	**4.2**	39.68	-1.10	7.00	1.20	9.32	1.09
1578.07	243-256	ECCHGDLLECADDR (14)	**3.9**	20.09	-8.70	2.20	0.50	2.60	0.10
1616.27	94-106	QEPERNECFLSHK (13)	**5.6**	17.93	-22.40	2.60	0.70	1.20	0.10
1630.00	524-537	YNGVFQECCQAEDK (14)	**3.9**	19.98	-12.80	3.40	0.50	1.10	0.20
1640.11	413-427	KVPQVSTPTLVEVSR (15)	**9.7**	26.99	-1.00	85.20	10.10	0.60	0.10
1741.07	363-377	DDPHACYTSVFDKLK (15)	**5.5**	27.43	-10.80	1.40	0.20	1.20	0.20
1755.88	557-573	CCAADDKEACFAVEGPK (17)	**4.1**	20.98	-2.10	3.20	0.20	1.80	0.20
1824.10	484-499	RPCFSALTPDETYVPK (16)	**6.2**	32.51	-8.60	3.70	0.50	4.40	0.30
1851.03	505-520	LFTFHADICTLPDTEK (16)	**4.4**	35.93	0.70	1.80	0.40	1.30	0.30
1956.36	295-312	DAIPEDLPPLTADFAEDK (18)	**3.5**	36.35	-10.10	8.50	1.60	0.80	0.10
2025.48	413-431	KVPQVSTPTLVEVSRSLGK (19)	**10.7**	32.29	-2.30	2.20	0.30	1.10	0.10
2046.53	144-159	RHPYFYAPELLYYANK (16)	**8.9**	37.08	-13.00	4.60	1.40	69.57	2.25
2060.42	410-427	YTRKVPQVSTPTLVEVSR (18)	**10.4**	29.03	-7.50	2.50	0.60	1.30	0.60
2199.68	538-556	ATEEQLKTVMENFVAFVDK (19)	**4.1**	40.96	-2.70	2.30	0.50	1.10	0.20
2414.16	484-504	RPCFSALTPDETYVPKAFDEK (21)	**4.5**	37.02	-14.90	3.30	0.60	1.50	0.20

Hb is hydrophobicity calculated using the SSR software and KD is hydrophobicity calculated using the Kyte-Doolittle scale (peptides that have Hb values above zero are hydrophobic while peptides that have Hb values below zero are hydrophilic).

Table 3.3: **Summary (peptides used)**

NPs	# peptides	pI<pH	pI>pH	pI = pH
TmaNP (I)	146	82	61	3
TmaNP(II)	146	82	61	3
LysNP	146	82	61	3
SoaNP	146	82	61	3
CxyNP	146	99	46	1

carried out at a solution pH of 8.6. As a result, 99 of the peptides are negatively-charged, 46 are positively charged, and one peptide is neutral (table 3.3).

Due to the many potential factors (e.g. charge, hydrophobicity, H-bonding capability, peptide amino acid content, etc.) that can contribute to the extractions process, we have used multiple least squares regressions (MLSR) to develop a model to predict the K values using peptide pI and hydrophobicity (Hb). Two scales were used to calculate the hydrophobicity values of the peptides. The Hb values used in the model were obtained using the Shaun D. Black (SDB) scale, [58] which is based on a modification of hydrophobic fragmental constant approach of Rekker. [59] Rekker studied the phase partition behavior of a very large database of compounds and by means of multiple regression analysis he obtained polarity constants for a large number of molecular fragments.

Chemically, these hydrophobic fragmental constants can be combined to assess with good accuracy the polarity of essentially any compound. The Kyte-Doolitle (KD) scale [61] was employed to assist the general data interpretation. This scale used the water-vapor transfer free energies plus interior-exterior distribution of amino acid side chains in assessing the hydropathy values of the amino acid residues. In table 3.2, peptides that have negative KD values are hydrophilic while those that have positive

values are hydrophobic.

As indicated above, for a typical extraction experiment, 1 μM of digest is extracted with 10 μM NP in 300 μL of solution. The digest-NP mixture is then concentrated to 10 μL (residue). This is followed by removal of the trapped peptides from the NPs with 5 μL of the releasing agent, so that the extracted peptides are in a final volume of 15 μL. The residue is thus 20 times concentrated assuming complete extraction. 5 μL of the MALDI matrix is then added to 1 μL of both the residue and the supernatant for the MALDI-TOF MS analysis. Given the concentration factor, we have arbitrarily chosen a K value of 20 as the cutoff for describing whether a peptide is extracted or not. In effect, this cutoff value corresponds to a peptide equally partitioning between the NP and the solution. Based on this threshold value, TmaNP (I), TmaNP (II) and LysNP extracted only 14.38%, 20.55% and 9.59% of the peptides, respectively, while CxyNP and SoaNP extracted only 6.16 % and 7.53% of the peptides, respectively (Table 3.4). As a result, peptides with K values below the cutoff value ($K< 20$) were assigned a value of $K = 0$ and those with $20\geq K<30$ were assigned $K=1$ for the MLSR model.

Table 3.4: Extracted peptides

NPs	# peptides	K>20	% (K>20)
TmaNP (I)	146	21	14.38
TmaNP(II)	146	30	20.55
LysNP	146	14	9.59
SoaNP	146	11	7.53
CxyNP	146	9	6.16

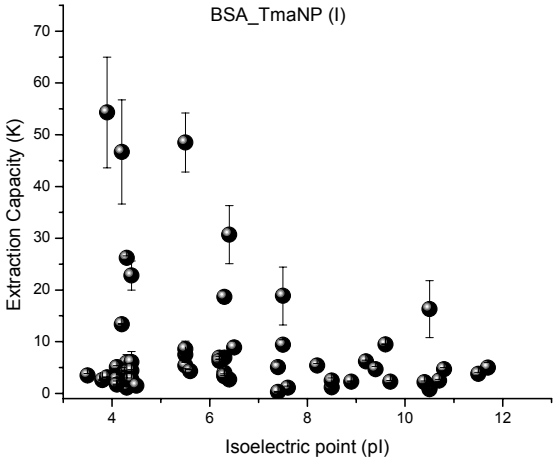
K> 20 is the threshold value for whether a peptide is extracted or not.

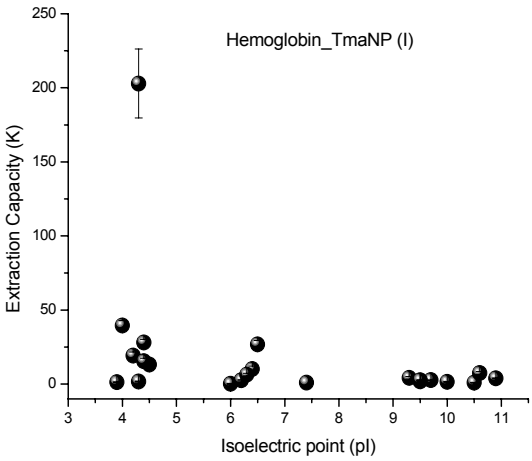
To explain the large number of peptides that were not extracted (K<20) by the

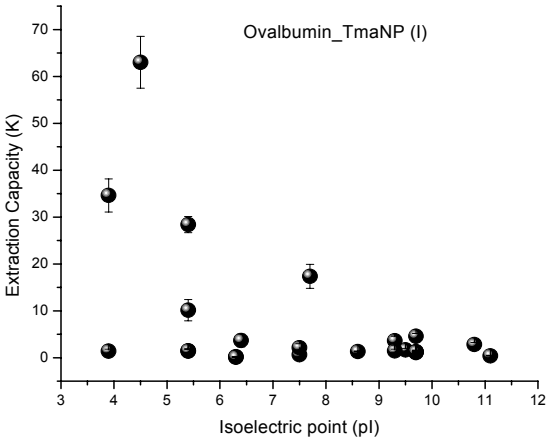
NPs, we estimated the number of total binding sites present on a collection of NPs in the following way. We estimate that one NP can bind and accommodate up to 5 peptide molecules on its surface (i.e. a nanoparticle with a diameter of 3 nm has a total surface area of 28.26 nm^2. An average peptide with 13 amino acids has a surface area of 22.13 nm^2, assuming an average amino acid surface area of 1.70 nm^2. [62] By further assuming that about one quarter of the total surface area of a given peptide will be utilized in binding to the NP, we estimate that a single NP can bind a maximum of five peptides). Thus, the effective concentration of NP binding sites is approximately 50 μM when the NP solution concentration is 10 μM. After digestion the total concentration of peptides is between 10 and 50 μM, depending on the number of peptide fragments produced from a protein whose concentration began at 1 μM. Upon considering the relative concentrations of the peptides and the NP binding sites, it becomes clear why the overall number of peptides that are extracted is relatively low. Significant competition for the binding sites exists, and only those with high affinities or significantly higher concentrations were efficiently extracted.

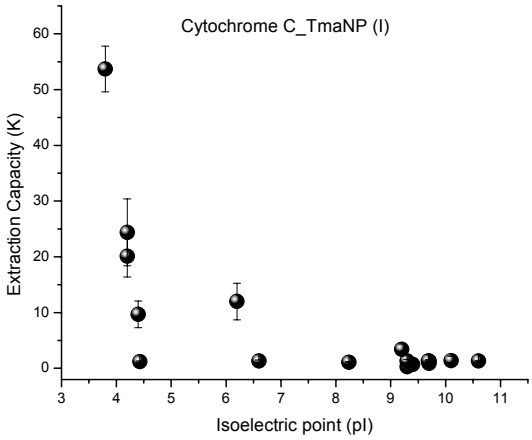
Also, from figure 3.2 – 3.6, almost all of the peptides that were extracted with K>20 have at least one aromatic amino acid residue in its sequence, and it is likely that after binding to the NPs, the bulky side chains of these peptides might have hindered the other peptides from binding to the NPS. It is also possible that some of the peptides were lost when they were being removed from the NPs. Also, the high concentration of aqueous NaCN (65 mM) required for the dissolution of the core of TmaNP (I) might have acted to suppress the MALDI ion signals for the peptides extracted with this NP.

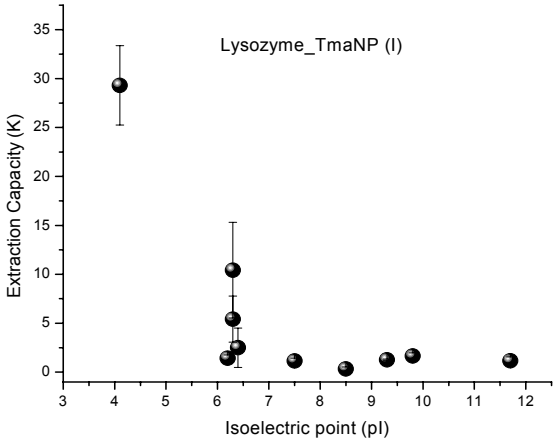
Figure 3.2: Extractions with TmaNP (I)











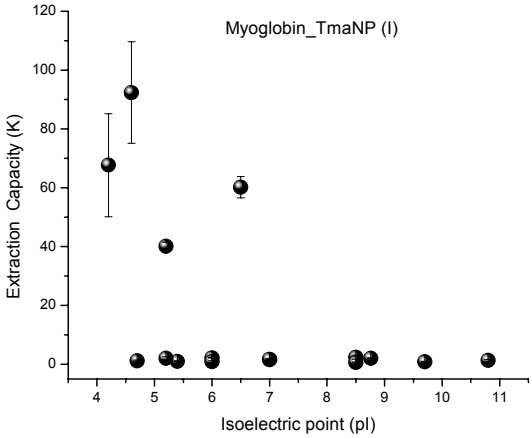
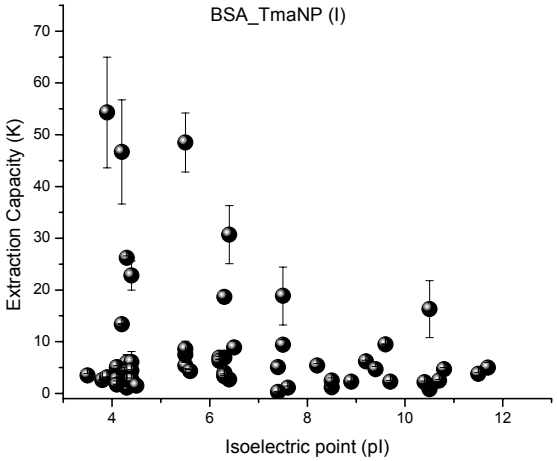
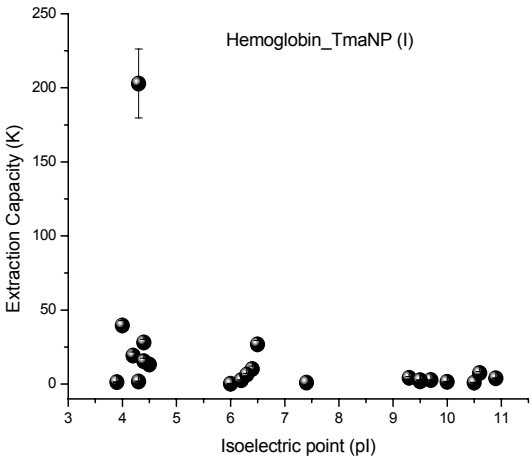
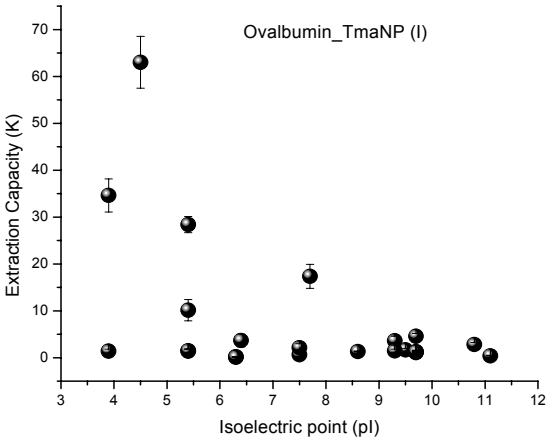
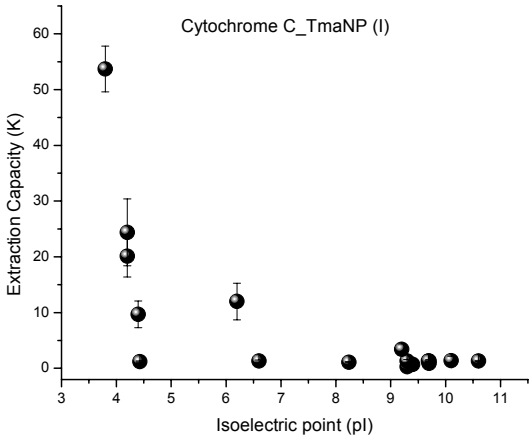


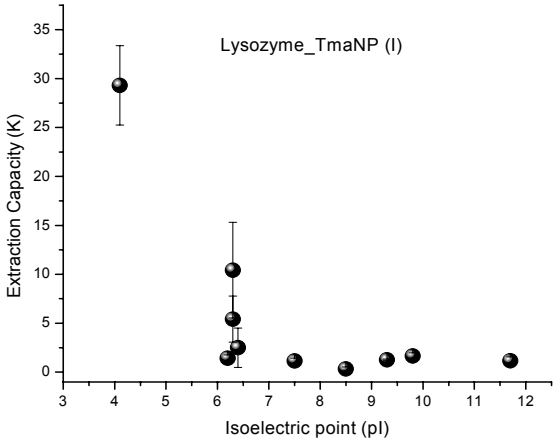
Figure 3.3: Extractions with TmaNP (II)











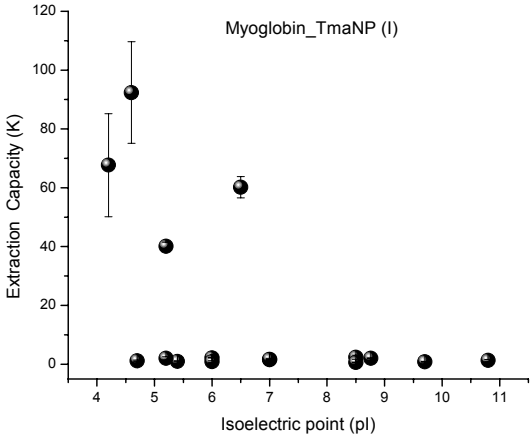


Figure 3.4: Extractions with LysNP

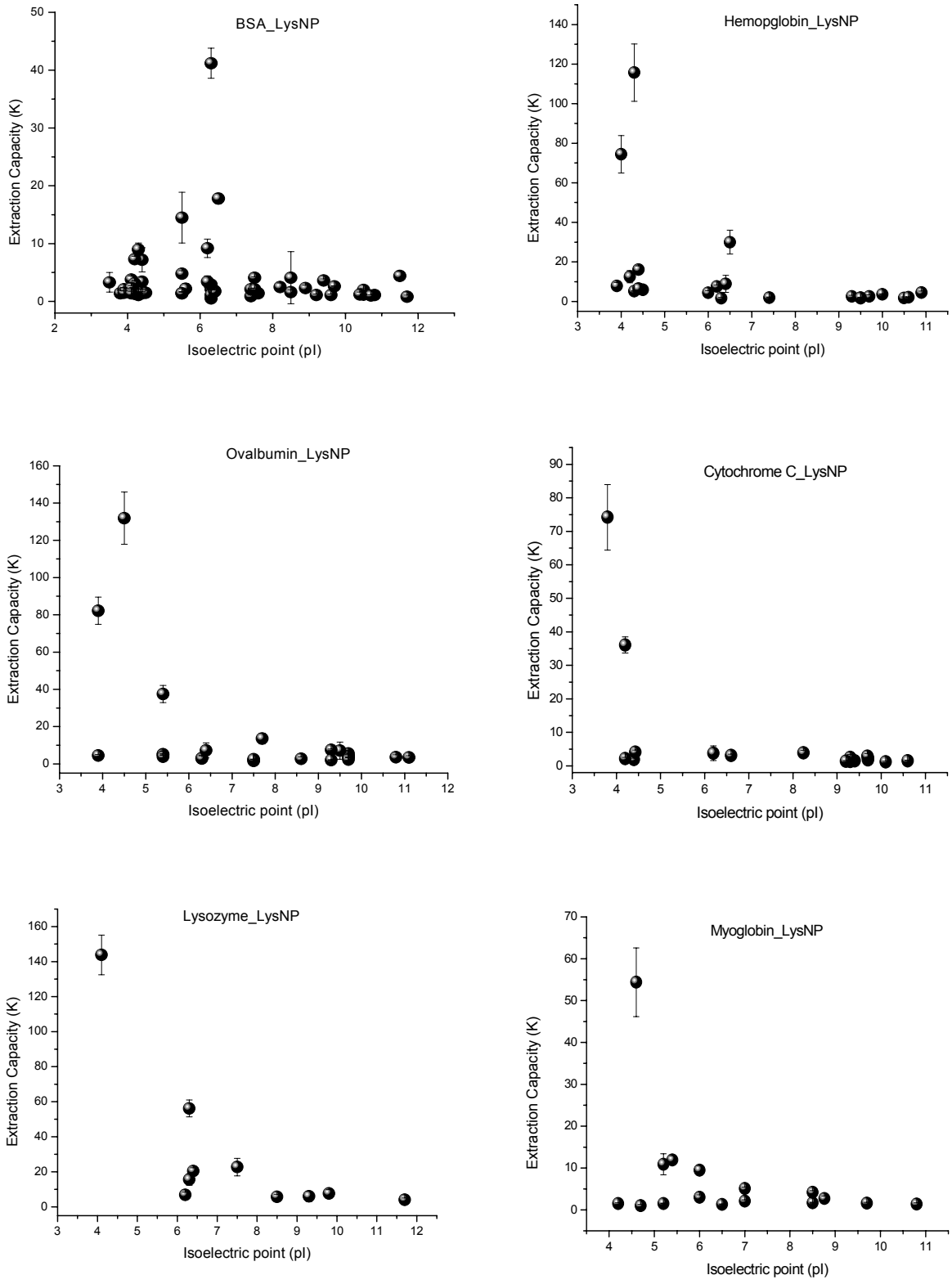


Figure 3.5: Extractions with CxyNP

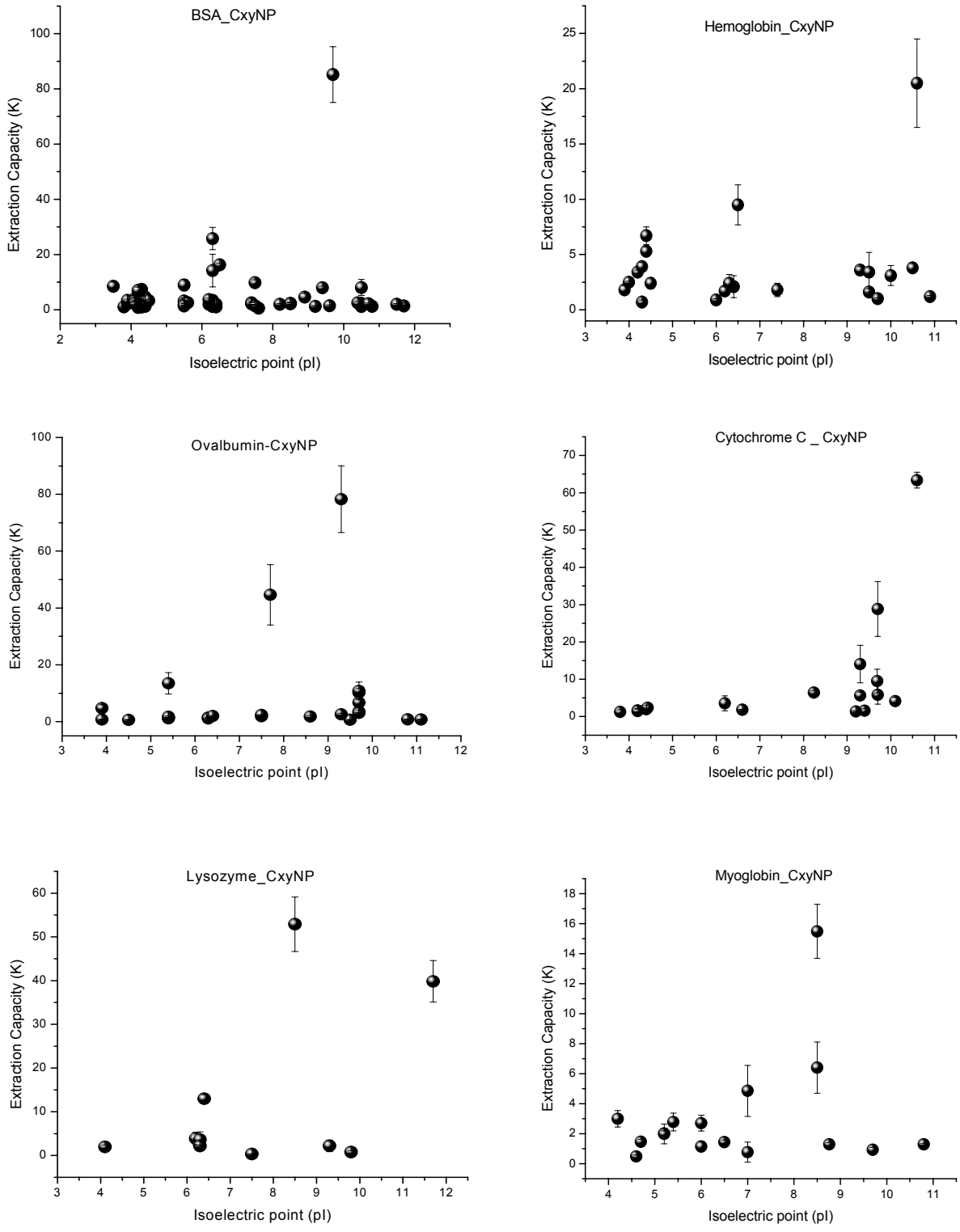
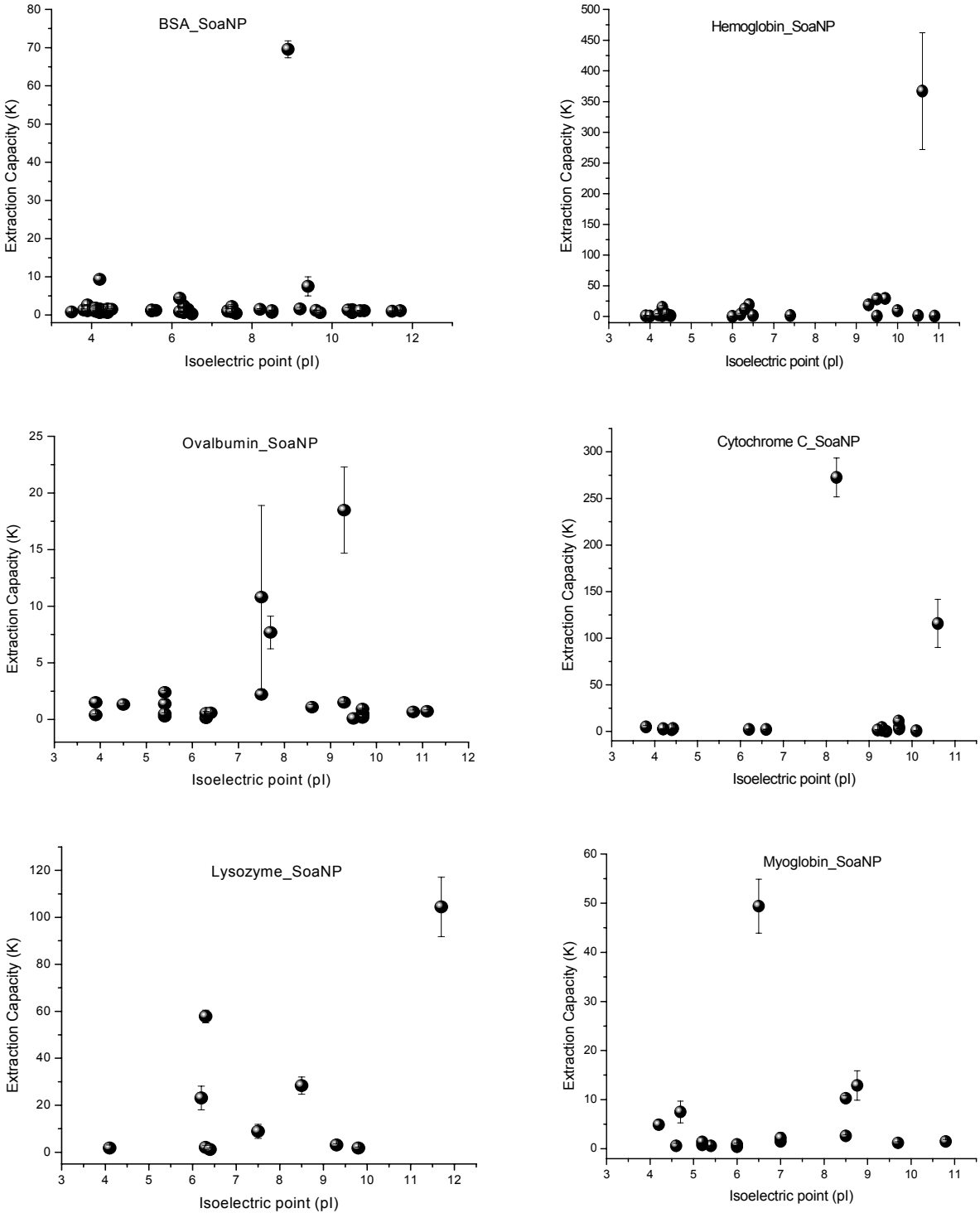


Figure 3.6: Extractions with SoaNP



After performing the MLSR analysis using a K value of 20 as a cutoff, it becomes clear that pI is the most important factor affecting extraction efficiency. This is further supported by the observation that less than 50% of the total peptides extracted by the NPs are hydeophobic (i.e. have positive KD values), table 3.5. A summary of the MLSR analysis, displaying the regression coefficients, the intercepts and the coefficients of the model equation for the various NPs is shown in table 3.6.

Table 3.5: Percent hydrophobicity (total)

NPs	K>20	Hb	%Hb	K>100	%Hb(K>100
TmaNP (I)	21	5	23.81	1	0
TmaNP(II)	30	7	23.33	11	18.2
LysNP	14	3	21.43	3	0
SoaNP	11	4	36.36	0	0
CxyNP	9	4	44.44	4	25

The Hb values used in this tablel were computed from the KD scale

Table 3.6: Summary of model* parameters

NP	**a**	**b_1**	**b_2**	**R^2**
TmaNP (I)	13.905	-2.355	1.418	0.095
TmaNP (II)	39.793	-5.823	3.421	0.085
LysNP	16.215	-2.611	1.366	0.097
SoaNP	-26.564	3.473	1.589	0.046
CxyNP	-13.12	1.288	1.109	0.093

* The model is based on the equation $K = b_1(pI) + b_2(Hb) + a$; where K is the extraction capacity, a is the intercept and b_1 and b_2 are the coefficients of the peptide isoelectric point (pI) and hydrophobicity respectively. The Hb values used in the model was computed from the SDB scale

The model revealed that for all the extractions involving the three positively-charged NPs, peptide pI correlates negatively with K, while for the extractions involving the negatively-charged NPs peptide pI correlates positively with K.

This observation is in agreement with the K vs. pI plots shown in figure 1 and indicates that the cationic and anionic NP systems exhibit opposite selectivity towards the peptides, which was also observed in chapter 1 for a more limited set of peptides. In other words, the cationic NPs showed a high preference for the peptides that have low pI values while the anionic NPs showed a preference for the peptides that have high pI values. For example, TmaNP (I) extracted 25.61% (21 out of 82) of the peptides that have low pI (i.e. below 7.4) values. Likewise, TmaNP (II) and LysNP respectively extracted 29.27% (24 out of 82) and 15.85% (13 out of 82) of the low pI peptides. Conversely, SoaNP and CxyNP respectively extracted 13.11% (8 out of 61) and 13.04% (6 out of 46) of the peptides that have high pI values (table 3.7). The cationic NPs extracted the peptides that have low pI values because these peptides were negatively charged at the solution pH for the extraction. In other words, the low pI (negatively-charged) peptides experienced strong electrostatic interactions with the cationic NPs. On the contrary, the peptides that have high pI values were extracted by the anionic NPs because they were positively charged at the solution pH for the extraction.

Table 3.7: Percent extractions (high and low pI peptides)

NPs	pI<pH	pI>pH	K>20	LpI (K>20)	%LpI (K>20)	HpI (K>20)	%HpI (K>20)
TmaNP (I)	82	61	21	21	25.61	0	0
TmaNP(II)	82	61	30	24	29.27	6	9.84
LysNP	82	61	14	14	15.85	1	1.64
SoaNP	82	61	11	3	3.66	8	13.11
CxyNP	99	46	9	3	3.03	6	13.04

Where %HpI and %LpI are respectively the percentages of the peptides extracted, that have high or low pI values.

The extraction of the peptides was accompanied by high concentration factors such that some of the NPs extracted peptides with K values greater than100 (that is more than five times the expected concentration factor). For example, TmaNP (II) gave K values greater than 100 for 11 negatively-charged peptides, and SoaNPs gave K values greater than 100 for 4 positively-charged peptides (table 3.5). One common thing about the peptides that were extracted with K>100 is that all of them are polar in nature, for instance, the KD scale predicted that only 18.8% (2 out of 11) of the negatively charged (pI < pH) peptides that were extracted by TmaNP (II), and only 25% (1 out of 4) of positively charged (pI > pH) peptides extracted by SoaNP are hydrophobic in nature (table 3.5), which suggests that the extraction of these peptides might have been driven mainly by complementary electrostatic interactions.

It is also important to note that both the cationic and anionic NPs extracted peptides of the same polarity. In other words, the cationic NPs extracted peptides that are positively-charged and the anionic NPs extracted peptides that are negatively-charged under the solution conditions for the extraction. For example, CxyNP and SoaNP respectively extracted 3.03% (3 out of 99) and 3.66% (3 out of 82) negatively-charged peptides, and while TmaNP (I) did not extract any of the positively-charged peptides, TmaNP (II) and LysNP respectively extracted 9.84% (6 out of 61) and 1.64% (1 out of 61) positively-charged peptides. Again, using the KD scale, we found that 66.67% (2 out of 3) of the negatively charged peptides that were extracted by CxyNP and SoaNP, 33.33% (2 out of 6) of the positively-charged peptides extracted by TmaNP (II), and none (one out of one) of the positively charged peptide that was extracted by LysNP are hydrophobic in nature (table 3.8).

Table 3.8: Percent hydrophobicity* of the extracted peptides.

NPs	# peptides K>20	# peptides pI<pH	# peptides pI>pH	% Hb (pI<pH)	% Hb (pI>pH)
TmaNP (I)	21	21	0	23.81	0.00
TmaNP(II)	30	24	6	20.83	33.33
LysNP	14	13	1	23.08	0.00
SoaNP	11	3	8	66.67	25.00
CxyNP	9	3	6	66.67	33.33

*Estimations were done using the KD scale

Thus, even though this involves a small set of peptides, it seems again, that complementary electrostatic interactions dominated their extraction.

The MLSR data further predicted that hydrophobicity correlates positively with K for all the NP systems. This correlation is consistent with the presence of alkane groups in the shells of all the NPs (table 3.1). Thus, it is not surprising that peptide hydrophobicity is an important factor that controls extraction efficiency in addition to complementary electrostatic interactions. However, complementary electrostatic interactions tend to dominate the extraction process because the peptides that were extracted by the NP systems appear to have greater effective polar character (table 3.8).

Also besides their abilities to interact with the peptides via hydrophobic and electrostatic interactions, the NP systems, especially LysNP, SoaNP and CxyNP have hydrogen bonding capabilities too, and can thus hydrogen bond with the peptides. Hydrogen bonding is not accounted for in the model, however, which in part may account for the low R^2 values yielded by the model. For the K values obtained for TmaNP (I), for example, the model gave an R^2 value 0.095 (table 3.6), which means that complementary electrostatic and hydrophobic interactions could account for only

9.5% of the variances in the K values, for the extractions with this NP. Another factor that may account for the low R^2 value is the method we employed to remove the trapped peptides from the NPs before the MALDI-TOF analysis. We suspect that the releasing agents used did not remove all the trapped peptides with the same efficiency, and this could bias the K values for some NPs.

Another interesting observation is that, in general, the cationic NPs extracted a lower percentage of peptides than the anionic NPs. The reasons for this are not clear. Interestingly, though, is the fact that SoaNP, while extracting only only 8% of the peptides, together with TmaNP (II) gave the highest K values. These results suggest that SoaNP may be highly selective, and future experiments will attempt to identify the nature of this selectivity.

3.3 CONCLUSION

In summary, we have created and screened five different NP systems with cationic or anionic functional groups against a test library of 146 peptides to learn about the factors that affect the extraction selectivity of the NPs. MLSR shows that even though complementary electrostatic interactions dominate the extraction process, hydrophobic-hydrophobic interactions are also important and that the two parameters together account for an average of 8.3% of the variance in K for all the NPs.

The cationic or anionic NPs can selectively extract negatively- or positively-charged peptides, respectively, with efficiencies that depend upon the peptides' pI values and the solution pH. That is, as we have suggested before [chapter 1], these NPs can still be used to fractionate digests of new proteins for example, and their ability to

discriminate between peptides with low- and high-pI can be used as a constraint during database searches for protein identification.

CHAPTER 4

USE OF FUNCTIONALIZED NANOPARTICLES AS SELECTIVE EXTRACTION AND CONCENTRATION AGENTS FOR MICROCYSTINS

Due to their small size dimensions, nanoparticles (NPs) possess large surface area-to-volume ratio and have been used by many researchers, including our group to trap and concentrate biological molecules from dilute solutions [chapters 1] prior to analysis. Despite their positive attributes, NPs have not been used to extract and concentrate MC-LR from dilute solutions prior to their detection. Microcystins are relatively polar molecules due to the presence of free carboxylic acids in their structures and the frequent presence of arginines at positions 2 and 4 [chapter 1]. Thus, NPs terminated with cationic or anionic ligands can be designed to target the D –MeAsp and D –Glu groups at positions 3 and 6 or the side chain of arginine, respectively. In this chapter, we examined the feasibility of using cationic mixed monolayer protected gold NPs as efficient extraction and concentration agents in conjunction MALDI-TOF MS to detect MC-LR from dilute solutions.

4.1 EXPERIMENTAL

4.1.1 Materials and Reagents

Microcystin LR (MC-LR), α-cyano-4-hydroxycinnamic acid (α-CHCA) and all chemicals for the NP fabrication were purchased from Aldrich. Tris-(hydroxymethyl) aminomethane (Tris) and tris-(hydroxymethyl) aminomethane hydrochloride (Tris-HCl) were purchased from EM science. The water used in all the experiments was obtained from a Milli-Q water purification system (Millipore, Bedford, MA, USA). The

Centricon filters and centrifuge tubes were purchased from Millipore Corporation.

4.1.2 Sample Extraction with NP's

The fabrication and characterization of the gold NP (TmaNP (I)) system used in this work have been described elsewhere [chapter 1]. A stock solution of MC-LR was prepared in 50/50 deionized water/methanol. The stock solution was diluted to working concentrations in a 25 mM Tris/Tris-HCl buffer at the desired pH (8.6). To extract the toxin from solutions, different amounts of NP solutions were added to different amounts of the toxin in a 10000 MW Centricon cut-off filter and the mixture was vortexed for 10 minutes. The toxin-bound NPs were aggregated and separated from the reaction supernatant by centrifugation in an Allegra™ X-22R centrifuge (Beckman Coulter^{TM}). MALDI-TOF MS analysis was then performed on the residue and the supernatant after the gold NPs had been removed from the residue. A solution mixture of trifluoroacetic acid, acetonitrile and water (5%TFA/20%ACN /75%H_2O) was used as the releasing agent.

4.1.3 MALDI-TOF-MS

All mass spectra were acquired in positive ion mode using an REFLEX III (Bruker Daltonics) in reflectron mode. The laser fluence was adjusted for optimum resolution and peak intensity in the 900 - 1200 m/z range. each mass spectrum was acquired by accumulation of 100 laser shots. The matrix solution consisted of α-CHCA saturated in 70%ACN:30%H_2O, 0.1% TFA (v/v). The dried-droplet method was used for all the samples. Typically, 1 μl of the residue or supernatant was mixed with 5 μl of

the matrix solution, and an aliquot (1 μl) of this mixture was applied to the stainless steel probe and allowed to air dry before the MALDI-TOF-MS measurements.

4.2 RESULTS AND DISCUSSION

In chapter 1 we found that cationic and anionic mixed monolayer protected gold clusters (MMPCs) were remarkably good extracting agents for peptides. We attributed the high extraction capacities of the NPs for the peptides to the high surface area-to-volume ratio of the NPs, which allows for efficient capture of the target peptides into a limited analysis volume, thereby effecting sample preconcentration.

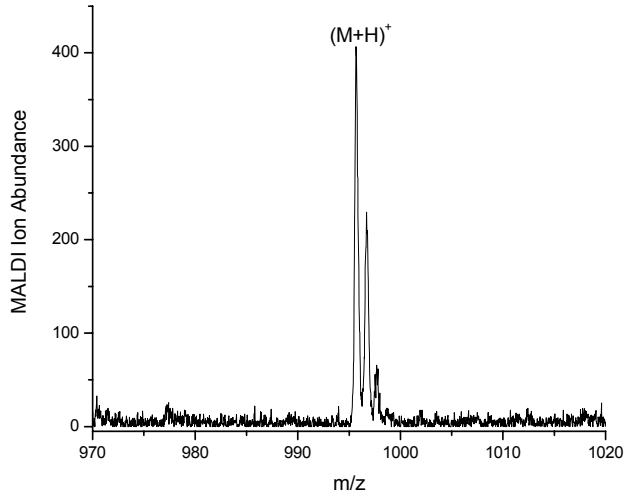


Figure 4.1: MALDI mass spectrum of a 80 nM solution of microcystins-LR ($[M+H]^+$ = m/z 995.17) after extraction and concentration with TmaNP (I). The extraction was carried out at a pH of 8.6

Figure 4.1 shows the results of the analysis of a solution of 80 nM MC-LR after extraction with a 5 μM solution of TmaNP (I) in 500 μL at a pH of 8.6. Virtually no identifiable ion signal is observed for MC-LR when solution of the peptide is analyzed directly (not shown). However, upon extraction and concentration of the same sample

solution with TmaNP (I), the protonated species of MC-LR is easily observable in the mass spectrum (Fig 4.1). After extraction, the mixture was concentrated to a final volume of 5 μL, and 5 μL of the releasing agent was added to remove the gold NP prior to MALDI-TOF MS analysis, resulting in an expected concentration factor of 33.

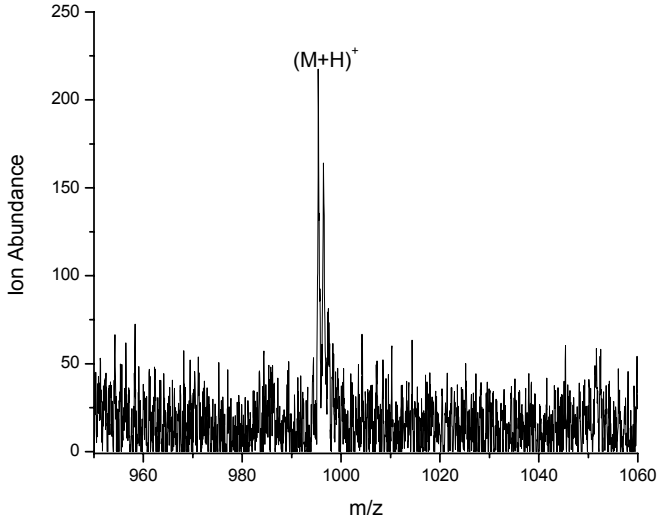


Figure 4.2: MALDI mass spectra of a 0.8 nM solution of microcystin-LR ($[M+H]^+$ = m/z 995.17) after extraction and concentration with TmaNP (I). The extraction was carried out at a pH of 8.6

Figure 4.2 further displays the results of extraction and concentration of 0.8 nM of MC-LR using a 2.5 μM solution of TmaNP (I) in 2 mL at pH 8.6. The mixture was concentrated to a final volume of ~ 1 μL. 10 μL of the releasing agent was used to rinse the peptides off the NPs before the MALDI-TOF MS analysis, yielding a concentration factor of ~ 182. As might be expected, no identifiable protonated species is observed for the toxin before extraction/concentration (data not shown). The protonated species can, however, be observed after treatment with the gold NP. In this experiment, the starting concentration (0.796 μg/L) is below the WHO provisional guideline of 1 μg/L. Thus,

even though this experiment was performed by spiking deionized water with the toxin, we suspect that this NP could be used to assist the detection of relevant concentrations of the toxin in more complex environments, like from sea water and lake water samples.

4.3 CONCLUSION

Following the method described in chapter 1, we have used a cationic MMPC (TmaNP (I)) in combination with MALDI-TOF MS to detect MC-LR from very dilute solutions. Even though this NP may not be really specific for the toxin, we have taken advantage of its high extraction capacity to extract and concentrate MC-LR at a concentration (0.796 μg/L) below the WHO provisional guideline of 1 μg/L. This MMPC, therefore, has the potential of serving as extraction and concentrating agents for microcystins from complex environmental sample like lake and sea water, prior to their detections by other techniques.

CHAPTER 5

FUTURE DIRECTIONS

So far, we have developed and tested a general peptides extraction protocol using functionalized gold NPs, and we have also screen several NPs against a test library of 146 peptides in order to understand the factors that dictate their extraction selectivity. Yet much more remains to be done. For example, we do not completely understand the factors that govern the selectivity of the NPs for the peptides as about 80% of the peptides were not extracted. We therefore recommend that the peptide-NP ratio for the extraction of digest should be further investigated. For example, the current peptide concentration of 1μL can be halved to see if at that concentration, the extraction capacity of the NPs can be improved for the digest. The size of the test peptide library should also be increased, as this will add more features to the pI vs. K plots and thus enrich our understanding of the extraction selectivity of the NPs. Also, future experiments should be designed such that only one batch of NPs and protein digests are used for all the extractions. It is expected that this would minimize the variance in K for peptides from the same protein.

We have also exploited the large extraction capacities associated with extractions with these NPs to extract and concentrate microcystin-LR at levels below the WHO provisional guideline of 1μg/L. Even though the NP used for this experiment is not really specific for the target, we recommend that the selectivity of this NP for MC-LR should be tested by applying it to MC-LR in more complex environments like lake water and sea water.

5.1 COVALENT CAPTURE OF MICROCYSTINS

Also, as indicated earlier, microcystins are known to bind covalently to several serine/threonine protein phosphatases including protein phosphatase 1 (PP1) and protein phophatase 2A (PP2A) via a Michael addition reaction and thus inhibit their functions [chapter 1]. We will exploit this very reaction to capture the toxins covalently from very dilute solutions. A Diels-Alder reaction will also be investigated as an alternative means to capture and extract the toxin covalently from very dilute solutions.

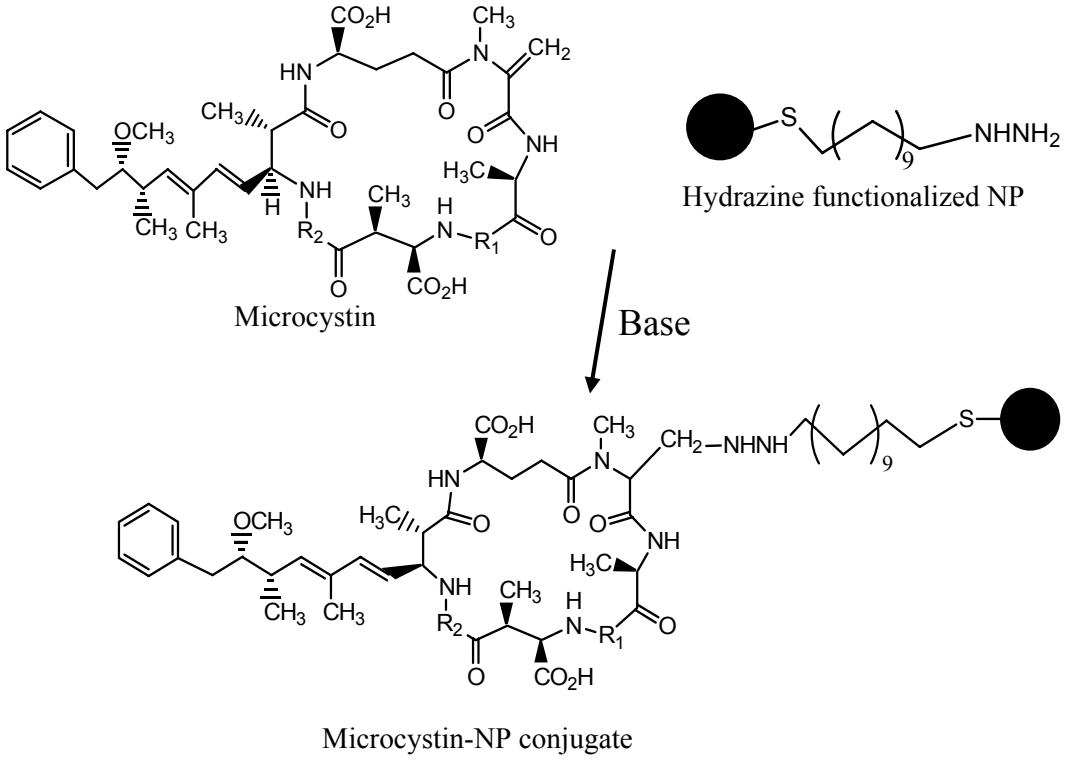


Figure 5.1: Reaction scheme for microcystin and the hydrazine functionalized NP

For the covalent capture via a nucleophilic Michael addition reaction, tap water and sea water will be spiked with the toxins, and NPs that are functionalized with nucleophilic Michael donors (i.e. hydrazine) will be used to extract and concentrate the

toxins covalently. These NP systems are expected to bind covalently to the α–β unsaturated groups of the N-methyldehydroalanine (Mdha) group of the toxins (fig. 5.1) in a 1, 4-conjugated addition reaction, which is similar to the way that the toxins inhibit protein phosphatases. It is expected that the ionic functional groups on the NPs will guide microcystins to the reaction site. Aggregation and separation of the toxin-NP conjugate will be done following the general protocol described above, and the extract will be detected using MALDI-TOF MS.

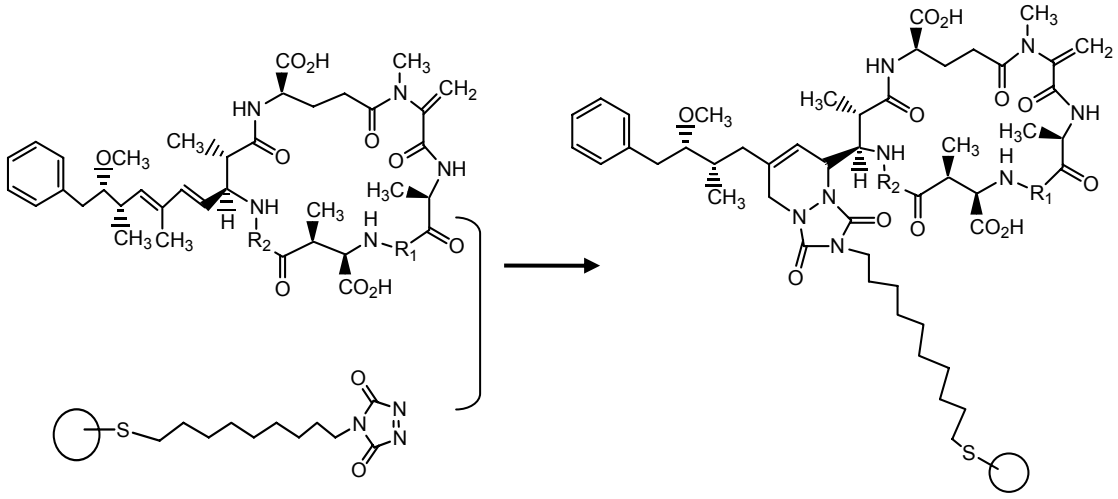


Figure 5.2: Reaction scheme for microcystin and the 1, 2, 4-triazoline-3, 5-dione functionalized NP

For the covalent capture via the Diels-Alder reaction, NPs bearing ligands that are terminated with 1, 2, 4-triazoline-3, 5-dione will be created and applied to the toxins in a manner similar to the Michael extraction. This NP system is expected to bind covalently with the conjugated diene in the side group of the "Adda" amino acid in a [2 + 4] Diels-Alder reaction (fig. 5.2). The 1, 2, 4-triazoline-3, 5-dione moiety is one of the most reactive dienophiles and has recently been attached to a fluorescent dye to

successfully detect microcystins [63]. In our approach, however, the target-NP conjugate will be detected with MALDI-TOF-MS, after extraction and concentration. Unlike the non-covalent approach, the binding between the NPs and the toxins will not be lost during the aggregation and separation protocol. Furthermore, covalent capture is more specific and is thus expected to give even more efficient and highly selective extraction.

Despite the advantages associated with covalent capture, the approach is also expected to pose problems when it comes to releasing the captured toxins for MALDI-TOF analysis. To overcome this problem, aqueous sodium cyanide will be used to decompose the gold core of the NP, and the captured toxin will be detected as an dduct. Another approach to overcome this problem is the use of acid-labile linkers. In this case, the hydrazine and the 1, 2, 4-triazoline-3, 5-dione functionalities of the two NPs will be attached to the gold clusters of the NP through an acid cleavable ligand. Such a linker will contain an ester unit, which can be easily cleaved at acidic pH prior to the MS analysis. In the case of very complex mixtures, the MS analysis could be simplified by using linkers that are synthesized with isotope labels (e.g. ^{79}Br and ^{81}Br) that provide a distinct signature during MS analysis. Because most MALDI matrices are acidic, it is expected that the linker will be cleaved upon mixing with the MALDI matrix.

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