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Improved mass spectrometric detection of acidic peptides by variations in the functional group pK_a values of reverse micelle extraction agents

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Abstract

Polymeric reverse micelles can be used to selectively extract peptides from complex mixtures via a two-phase extraction approach. In previous work, we have shown that the charge polarity of the hydrophilic functional group that is in the interior of the reverse micelle dictates the extraction selectivity. To investigate how the extraction is influenced by the inherent pK_a of the functional group, we designed and tested a series of polymeric reverse micelles with variations in the hydrophilic functional group. From this series of polymers, we find that the extraction capability of the reverse micelles in an apolar phase is directly related to the aqueous phase pK_a of the interior functional group, suggesting that the functional groups maintain their inherent chemistry even in the confined environment of the reverse micelle interior. Because these functional groups maintain their inherent pK_a in the reverse micelle interior, they provide predictable extraction selectivity upon changes in aqueous phase pH. We exploit this finding to demonstrate that sulfonate-containing polymers can be used to remove basic peptides from complex mixtures, thereby allowing the improved detection of acidic peptides. Using these new materials, we also demonstrate a new means of isoelectric point (pI) bracketing that allows the mass spectrometric detection of peptides with a defined and narrow range of pI values.

Graphical abstract

Functional groups in reverse micelles maintain their aqueous phase pK_a and allow selective extraction of peptides according to isoelectric point.

INTRODUCTION

Simplification of complex biological mixtures is of great interest, especially for a variety of metabolomics and proteomics measurements that rely on mass spectrometric detection.^{1–12} Due to limitations in the dynamic range of MS based detection platforms, effective protein/peptide sample separation strategies are usually required prior to MS analysis to exploit the full detection capabilities of MS.^{13–20} Detection of acidic peptides and proteins in complex mixtures is particularly challenging because the negatively charged nature of these molecules usually causes them to be suppressed during ionization or detected inefficiently by most techniques.^{21–24}

Over the years, various isoelectric point (pI) based fractionation methods have been developed to improve the detection of acidic proteins/peptides, such as isoelectric focusing mass spectrometry (IEF-MS)^{25–27} and ion exchange chromatography MS (IEC-MS).^{28–31} To improve acidic peptide and protein identifications in mixtures, pI-based separations have also been coupled with techniques like activated ion negative electron-transfer dissociation (AI-NETD) for proteome analysis.^{32–33} Our group has recently demonstrated that self-assembled amphiphilic polymers can selectively simplify peptide mixtures via a biphasic extraction format, thereby allowing sensitive detection of different fractions by MS.^{34–37} By assembling these amphiphilic polymers as reverse micelles in an apolar phase, we have demonstrated the ability of the charged interiors of these materials to selectively sequester peptide biomarkers from serum.³⁸ We have also shown that materials containing positively-charged functional groups or metal-bound functional groups can selectively extract and sensitively detect negatively-charged peptides and phosphorylated peptides, respectively.^{39–40}

In previous work using an amphiphilic homopolymer with carboxylate functionality, we discovered a pH dependency to how efficiently positively charged peptides could be extracted from an aqueous phase, with extraction capacities being minimal at pH values around 5, which is close to the typical pK_a values of carboxylates. From this observation, we suspected that the charge state of the functional group in the reverse micelle might behave in a similar manner to how it behaves in free solution.³⁵ In addition to being fundamentally interesting, this behavior causes carboxylate-based materials to have inherent limitations with respect to extraction efficiency at lower pH values. To better understand the relationship between the inherent pK_a of functional groups and their effective pK_a in the interior of the

reverse micelles, we synthesized new amphiphilic polymers with phosphonate and sulfonate groups, which have much lower pK_a values than carboxylates. In this work, we describe the extraction capabilities of these new materials. Our results indicate that functional groups preserve their small-molecule pK_a in the reverse micellar state. Moreover, we show that materials having functionalities with lower pK_a values allow us to achieve more effective separations of acidic peptides. In addition, we report a “pI bracketing” method that can specifically detect acidic peptides within a small pI range.

MATERIALS AND METHODS

Polymer Synthesis and Characterization

All reagents were commercially available and used as received unless stated otherwise. To confirm the structures of random copolymers and synthetic small molecules, ^1H -NMR spectra were recorded on a 400 MHz Bruker NMR spectrometer using residual proton resonance of the solvents as internal standards. Chemical shifts are reported in parts per million (ppm). To further validate the monomer structures, mass spectra were obtained using a Bruker AmaZon quadrupole ion trap mass spectrometer coupled with electrospray ionization source. The electrospray needle voltage was kept at ~4 kV, and the capillary temperature was set at 220 °C. Gel permeation chromatography (GPC) was used to estimate the molecular weight and polydispersity index (PDI) of the polymers using THF as eluent, and 1 μL of toluene was added as the internal reference. Polystyrene standards were used for calibration and data analysis. Sizes of the reverse micelle assemblies were measured using dynamic light scattering (DLS) on a Malvern Zetasizer instrument.

Extraction method

Reagents—The following peptides were purchased from the Bachem (Torrance, CA): kinetensin (IARRHPYFL), Angiotensin II (DRVYIHPF), β -amyloid fragment 1-11 (DAEFRHDSGYE). The peptide bradykinin (RPPGFSPFR) and the proteins bovine serum albumin (BSA) and lysozyme were obtained from Sigma-Aldrich (St. Louis, MO). The protein β 2-microglobulin, purified from human urine, was purchased from Lee Biosolutions (Maryland Heights, MO). Immobilized trypsin, immobilized chymotrypsin and triethylamine acetate (pH 8.0, 1 M) were obtained from Princeton Separations (Adelphia, NJ). Trifluoroacetic acid (TFA), and cyano-4-hydroxy-cinnamic acid (CHCA) were purchased from Sigma-Aldrich (St. Louis, MO). Acetonitrile, tetrahydrofuran, toluene, sodium hydroxide, ammonium acetate and HPLC grade water were all purchased from Fisher Scientific (Fair Lawn, NJ).

Proteolytic digestion—The protein sample was dissolved by adding 100 mM triethylamine acetate (pH 8.0). To reduce the disulfide bonds, TCEP in water was added at a protein:TCEP molar ratio of 1:80, and the sample was incubated at 37 °C for 10 min. To alkylate the reduced cysteines, an iodoacetamide stock solution (made in 100 mM triethylamine acetate, pH 8.0) was added at a protein:iodoacetamide molar ratio of 1:80, and the sample was incubated in the dark at room temperature for 30 min. Next, the sample was incubated with 10% (v/v) acetonitrile at 50 °C for 45 min to denature the protein. The protein sample was then applied to the selected enzyme at an enzyme: substrate ratio of

1:10. Digestion of β 2m was performed with immobilized chymotrypsin. Digestion of BSA and lysozyme were performed with immobilized trypsin. After 2 h of digestion at 37 °C, the enzyme was separated from the mixture by centrifugation, and the supernatant was collected. After the digests of each protein were collected individually, they were mixed for subsequent extraction.

Preparation of Polymeric Reverse Micelles—Amphiphilic random copolymers were dispersed in toluene at a final concentration of 1.0×10^{-4} M. 1 μ L of 0.01 M NaOH aqueous solution was added per 1 mL of the reverse micelle solution. Sonication was conducted until the solution became optically clear.

Matrix Solutions—CHCA was prepared at a concentration of 10 mg/mL in a mixture of 50 μ L acetonitrile, 47.5 μ L H₂O, and 2.5 μ L TFA. CHCA in THF was prepared at a concentration of 30 mg/mL in a mixture of 350 μ L THF, 150 μ L H₂O, and 6 μ L TFA.

Peptide Extraction and Sample Preparation—The general extraction procedure is described elsewhere,³⁷ but in short, the following procedure was used. Peptide solutions were prepared in 50 mM Tris buffer where the pH of the buffer was adjusted beforehand. One mL of the buffered peptide solution was mixed vigorously with 200 μ L of the polymeric reverse micelle solution in toluene for 2 hours. After mixing, the solution was centrifuged at 13,000 rpm for 60 minutes to break the emulsion. The aqueous phase was withdrawn and kept in a separate microtube. 10 μ L of the aqueous phase sample was then mixed with 10 μ L of the CHCA matrix solution in acetonitrile. From this sample, 2 μ L was spotted on the MALDI target plate for MALDI-MS analysis. The remaining organic phase was dried by blowing N₂ gas. The dried residue of the mixture of polymer and the extracted peptides were re-dissolved in 10 μ L of THF and then 20 μ L of the THF-containing CHCA matrix solution. From this sample, 1 μ L was spotted on the MALDI target plate for MALDI-MS analysis.

MALDI-MS Analysis—A Bruker Autoflex III time-of-flight mass spectrometer was used to acquire mass spectra in reflectron mode with an accelerating voltage of 19 kV. Each spectrum was the average of 500 laser shots at 40% laser power. For the MALDI-MS analysis of pI bracketing of peptide mixtures and protein digests, a Bruker UltrafleXtreme MALDI-TOF/TOF mass spectrometer was used. Acquisition of all mass spectra was done in reflectron mode with an accelerating voltage of 20 kV. Each spectrum was the average of 2000 laser shots at 50% laser power.

Extraction capacity determination—Increasing concentrations of the peptide kinetensin (IARRHPYFL, pI 11.01) were extracted using 100 μ L of 1.0×10^{-4} M of each polymer in toluene, and the left-over peptide in the aqueous phase was mixed on a 1:1 volume ratio with the acetonitrile containing CHCA matrix solution before analysis by MALDI-MS. The extraction capacity is taken as the concentration of peptide at which a measurable amount of peptide remains in the aqueous phase, suggesting that the reverse micelles are saturated and can no longer accommodate more peptides in the organic phase. The capacity is determined by performing a linear regression on a plot of the peptide signal in the aqueous phase after extraction as a function of peptide concentration (e.g. Figure S3). The capacity concentration is chosen by extrapolating the line to zero peptide signal.

RESULTS AND DISCUSSION

Previously, we developed the random copolymer **P1** (Figure 1a) containing carboxylate functional groups that can selectively simplify peptide mixtures via electrostatic interactions.⁴¹ The extraction selectivity depends on (i) peptide pI values and (ii) the pH of the bulk aqueous phase, with positively-charged peptides being selectively extracted by the negatively-charged polymer. It is important to note that the carboxylate groups are charged according to their pK_a, but it is interesting to see if this charging behavior is the same in the confined environment of reverse micelles as it is in free solution. To test this idea, a reverse micelle solution of polymer **P1** was prepared in toluene at a final concentration of 1.0×10^{-4} M. 200 μ L of this solution was then used to perform bi-phasic extractions of peptide mixtures containing angiotensin II (DRVYIHPF, pI= 7.78), kinetensin (IARRHPYFL, pI= 11.01), and β -amyloid (1-11) (DAEFRHDSGYE, pI= 3.93) at three different pHs. Table 1 shows relevant properties of each these peptides.

After extraction, the corresponding spectra of the organic and aqueous phases were obtained via MALDI-MS. We observed that, at pH 9, negatively charged **P1** was able to selectively extract kinetensin, which has a positive net charge, to the organic phase while leaving the negatively charged peptides, angiotensin II and β -amyloid (1-11), in the aqueous phase (Figure 1b). At pH 5.5, both kinetensin and angiotensin II are positively charged and are thus extracted by reverse micelles solution of **P1**, while the negatively-charged β -amyloid (1-11) is not extracted (Figure 1c). However, at pH 3 where all three peptides are positively charged, reverse micelles of **P1** cannot fully extract the peptides despite using the same polymer concentration, as indicated by peptides remaining in the aqueous phase after extraction (Figure 1d). We speculate that this behavior is due to an increased number of carboxylate functional groups being protonated inside the reverse micelles when the aqueous phase pH is 3. In other words, the carboxylate groups in the confined environment reverse micelle interiors are perhaps being protonated in a manner that reflects the inherent pK_a values of these functionality in an aqueous phase. Thus, they have a lower number of negative charges for binding positively-charged peptides, causing a significant fraction of the peptides to remain in the aqueous phase. It should be stated that the organic phase with the amphiphilic copolymers was prepared in an identical fashion for each extraction shown in Figure 1, suggesting that the water pool in the interior of the reverse micelles ‘communicates’ with the aqueous phase upon extraction. We also note that no peptides are extracted at any pH when polymers are absence from the organic phase, which indicates the polymers are essential for an effective extraction.

To test the idea that the functional groups in the reverse micelle interiors have an effective pK_a like they would have in an aqueous phase, we designed and synthesized random copolymers **P2** and **P3** with phosphonate (pK_a $\approx$ 2 & 7) and sulfonate (pK_a $\approx$ -0.5) functional groups respectively (Figures 2a and 2c). Reverse micelles solutions of polymers **P2** and **P3** were used to extract the same peptide mixture at pH 3, the pH at which polymer **P1** could not perform the extraction efficiently. Upon extraction with these polymers, we observe that all the positively charged peptides are extracted to the organic phase and no peptides remain in the aqueous phase (Figures 2b and 2d), which suggest that the functional groups in **P2** and **P3** retain their negative charges at the low extraction pH likely due to the

relatively lower pK_a values. Upon extraction at even lower pHs (i.e. pHs 2.1 and lower), we find that **P2** can efficiently extract peptides at a pH as low as 2 (Figure S1), while **P3** can extract peptides even at a pH as low as 1 (Figure S2). Note that incomplete peptide extraction at pH 1 for polymer **P2** is likely because the phosphonate pK_a is about 2, meaning that many of these functional groups in the reverse micelle interior are protonated in a manner similar to what was observed for polymer **P1** (Figure 1d). In contrast, the very low pK_a of the sulfonate group results in reverse micelles of **P3** maintaining a sufficiently charged interior even at pH 1.

If the extraction efficiency of the reverse micelles is reflecting the inherent pK_a of their functional groups, we then predicted that the polymer extraction capacities would follow a titration-curve like behavior as the extraction pH is varied. To test this idea, we measured the pH-dependent extraction capacity of polymers **P1**, **P2**, and **P3** (Figure 3) using the peptide kinetensin, which remains positively-charged up to a pH of about 11. We find that the capacities of **P1** and **P2** increase from pH 1 to pH 6.5 and have midpoints of about 5.5 and 2.5, respectively (Figure 3a and b), which are close to the pK_a values of typical carboxylates and phosphonates. Despite the relatively high error bars, the capacity of **P3** appears to be somewhat constant over the entire pH range, suggesting that the functional groups in these reverse micelles have an effective pK_a that is like sulfonates in free aqueous solution. Together, these observations further suggest that the functional groups in the interior of the reverse micelle behave like their bulk solution counterparts with regard to their pK_a values.

While the extraction behavior of polymers **P1**, **P2**, and **P3** follow the pK_a values of their functional groups at pH values below 7, there is a clear deviation at pH values above 7 where the capacities decrease (Figure 3); this is especially apparent for **P1** and **P2**. There are at least two possibilities that could explain this capacity decrease. First, at higher pH values more functional groups are deprotonated, resulting in more hydrophilic random copolymers. This increased hydrophilic character could weaken the stability of the assembled reverse micelle structures or cause the assemblies to translocate into the aqueous phase, both of which would result in a lower extraction capacity. Second, the net positive charge of kinetensin decreases at higher pH, thereby weakening the electrostatic driving force that allows peptide extraction into the reverse micelles.

To test if the copolymers remain stably trapped in the organic phase, UV-Vis absorption spectrometry measurements were conducted for all three reverse micelle solutions before and after equilibration with aqueous phase at the pH where the largest capacity decrease occurred, as seen in Figure 3. No measurable polymer is found in the aqueous phase after equilibration (Figure S4), which indicated that **P1**, **P2**, and **P3** remain in the organic phase. Even though the polymers remain in the organic phase, it is possible that the highly charged reverse micelles do not have the proper hydrophilic-lipophilic balance (HLB) and thus do not assemble in an appropriate manner to accommodate peptides effectively.⁴² Therefore, decreasing the charge density and increasing the hydrophobicity might recover the proper HLB at the higher pH values and thus recover the extraction capacity. To test this idea, extractions were performed from pH 5.4 to 8.7 using a random copolymer **P4** (Figure 4a) with a lower carboxylate percentage (i.e. 29% for **P4** vs. 51% for **P1**). Upon using **P4** for the extraction, only a relatively small decrease in extraction capacity, from 1.0 ± 0.2 to 0.8 ± 0.2 ,

is observed for kinetensin in going from pH 7.6 to 8.7 (Figure 4b). This contrasts with **P1** for which the capacity decreased from 1.00 ± 0.03 to 0.5 ± 0.1 over the same pH range (Figure 3a). Similarly, extractions with **P5**, which has 11% phosphonate groups (Figure 4c), show an increase in capacity at even pH values close of 9, which is unlike **P2** that has its capacity decrease dramatically at pH values above 6.5 (Figure 3b). Overall, these findings suggest that an underlying reason for the decreased capacities for **P1** and **P2** at higher pH values is the improper HLB for the polymer assemblies, which evidently must be optimized to maintain extraction capacity at higher pHs.

Having shown that functional group pK_a affects a reverse micelles' pH-dependent extraction capacity and that polymers such as **P2** and **P3** can efficiently extract positively-charged peptides at low pH, we were interested in testing the ability of these polymers to simplify protein digest mixtures. The idea that we decided to explore is whether these materials can separate out high pI peptides and leave behind low pI peptides in the aqueous phase that could then be more efficiently detected. Generally speaking, acidic peptides can be difficult to detect due to their inherently lower ionization efficiency and the fact that their ionization is often suppressed by high pI peptides. Selectively removing high pI peptides would benefit the detection of acidic peptides. To test this idea, a protein digest mixture consisting of BSA, Lysozyme (Lyz) and β -2-microglobulin (β 2m) was prepared and extracted at a pH of 4.5 using reverse micelles of **P1**, **P2**, and **P3**. At this pH, peptides with pI values higher than 4.5 are expected to be extracted as they carry a net positive charge. Upon extraction, we observe that **P1** fails to remove positively charged peptides as efficiently as **P2** and **P3** (Figure 5), likely due to the relatively high pK_a of its carboxylates and its correspondingly low capacity at pH 4.5. While **P2** simplifies the peptide mixture more effectively than **P1**, 11 peptides with pI values > 4.5 remain in the aqueous phase. Reverse micelles of **P3** provide the best removal of peptides with pI values > 4.5 (detailed peptide information can be found in Tables S1-S4), presumably due to the low pK_a of the sulfonate functional group. A nice advantage of this approach over other liquid separation techniques (e.g. ion-exchange chromatography) is that the peptides that remain in the aqueous phase do not undergo any dilution and can be readily subjected to further analyses.

The ability of **P3** to effectively extract peptides at low pH values enables a pI bracketing scheme that can selectively purify and detect peptides with a defined range of pI values, as is illustrated in Figure 6. The same protein digest mixture of BSA, Lyz, and β 2m was prepared and first extracted at pH 6.0 using a reverse micelle solution of **P3**. During the first extraction, peptides with pI values higher than 6 are extracted into the organic phase (Figure S5a and Table S5) while those with lower pI values remain in the aqueous phase (Figure S5b and Table S6). Upon lowering the pH of the remaining aqueous phase to 4.5, during which peptides having pIs between 4.5 and 6 become positively charged, an extraction with a fresh solution of **P3** reverse micelles results in the successful extraction and detection of only peptides having pIs between 4.5 and 6 (Figure 7a). Moreover, more acidic peptides with pIs lower than 4.5 still remain in the corresponding aqueous phase (Figure 7b). It should be noted that some peptides having pI values between 4.5 and 6 remain in the aqueous phase, but this might be due to the **P3** reverse micelles having a slightly lower capacity at pH 4.5. Interestingly, 15 low pI peptides (see Tables S5-S8), which were not detectable in the

original digests mixtures, were detected only after sequential extractions, emphasizing how signal suppression can be alleviated by simplifying the mixtures during this pI bracketing approach. Separating and selectively detecting peptides within a pI range of 1–2 units could be useful for improving protein identifications via database searching,^{43–45} especially for acidic proteins.

Conclusion

In summary, we have synthesized random copolymers with carboxylate, phosphonate, sulfonate functional groups that can form reverse micelle type assemblies and simplify peptide mixtures through a biphasic extraction method. Using pH dependent extraction capacities of standard peptides as indicators, we show that functional group pK_a values in the confined reverse micelle state are consistent with the expected values in free aqueous solution. In addition to the pK_a -dependence, a decrease in capacity was also observed at high pHs, which is attributed to the change in the HLB of the polymer. By optimizing the HLB through variations in the polymer structure, the decreased capacity can be recovered. Using polymers having functional groups with low pK_a values, we have shown that complex peptide mixtures can be efficiently simplified at acidic pH. Furthermore, extraction and bracketing of peptides within a narrow pI range enables selective and sensitive detection of acidic peptides. Overall, our study here explores variations in functional group pK_a values in the context of their interactions with peptides inside the confined environment of supramolecular assemblies. The fundamental understanding developed here could have broad implications in a variety of applications involving separation of peptides. Taking the advantage of low pK_a polymers to realize specific isolation and detection of acidic peptides could potentially benefit studies in proteomics and detection of disease biomarkers in complex mixtures. Compared to other techniques used for acidic peptide detection, such as isoelectric focusing (IEF)-MS and ion-exchange chromatography (IEC)-MS, our method provides predictable separations with a variable and definable pI range and is readily compatible with detection by MS without a need for significant sample pre-treatment before analysis by MS.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Figure 1.

(a) Chemical structure of carboxylate random copolymer **P1**. (b) MALDI mass spectrum of the aqueous (AQ) and organic phases (ORG) after extraction using reverse micelles of polymer **P1** at pH 9. (c) MALDI mass spectrum of the aqueous (AQ) and organic phases (ORG) after extraction using reverse micelles of polymer **P1** at pH 5.5. (d) MALDI mass spectrum of the aqueous (AQ) and organic phases (ORG) after extraction using reverse micelles of polymer **P1** at pH 3.

Figure 2.

(a) Chemical structure of phosphonate random copolymer **P2**. (b) MALDI mass spectrum of aqueous phase (AQ) and organic phase (ORG) after extraction using reverse micelles of polymer **P2** at pH 3. (c) Chemical structure of sulfonate random copolymer **P3**. (d) MALDI mass spectrum of aqueous phase (AQ) and organic phase (ORG) after extraction using reverse micelles of polymer **P3** at pH 3.

Figure 3.

(a) The normalized capacity of reverse micelles of **P1** over aqueous solution pH 1.0 to pH 8.7. (b) The normalized capacity of reverse micelles of **P2** over aqueous solution pH 1.0 to pH 8.7. (c) The normalized capacity of reverse micelles of **P3** over aqueous solution pH 1.0 to pH 9.8. The peptide capacities were determined as described in the materials and method and as illustrated in Figure S3. Error bars indicate the standard deviations of three experimental replicates. The more significant errors for some measurements, particularly for the sulfonate polymer (c), arise from variations in the fits used to determine the capacity values. For each polymer, capacities were normalized to the highest capacity over the tested pH range.

Figure 4.

(a) Chemical structures of random copolymers **P1** and **P4**. (b) The normalized capacity of reverse micelles **P1** and **P4** for kinetensin at aqueous solution pH values of 5.4 to 8.7. (c) Chemical structures of random copolymers **P2** and **P5**. (d) The normalized capacity of reverse micelles **P2** and **P5** for kinetensin at aqueous solution pH values of 5.4 to 9.8.

Figure 5. MALDI mass spectrum of (a) a protein digest mixture of BSA, Lyz and $\beta 2m$ before extraction, with each protein at $0.1 \mu M$; (b) the remaining aqueous phase after extraction using $200 \mu L$ of $1.0 \times 10^{-4} M$ of **P1** at pH 4.5; (c) the remaining aqueous phase after extraction using $200 \mu L$ of $1.0 \times 10^{-4} M$ of **P2** at pH 4.5; and (d) the remaining aqueous phase after extraction using $200 \mu L$ of $1.0 \times 10^{-4} M$ of **P3** at pH 4.5. (The number above each peak corresponds to the calculated peptide pI.)

Figure 6. Schematic representation of the pI bracketing scheme based on sequential extractions with polymeric reverse micelles.

Figure 7.

The MALDI mass spectrum of (a) the organic phase after the sequential extraction using **P3** at pH 4.5. (b) the remaining aqueous phase after the sequential extraction using 400 μ L of 2.0×10^{-5} M of **P3** at pH 4.5. (The number above the peaks correspond to the calculated peptide pI values.)

Table 1

Chemical and physical characteristics of peptides used in the extraction experiments associated with Figures 1 and 2.

	Peptide	m/z	Sequence	pI ^a	Concentration in aqueous phase (μM)	Net charge at pH 3	Net charge at pH 5.5	Net charge at pH 9
1	Angiotensin II	1046.2	DRVYIHPF	7.78	0.10	+	+	-
2	Kinetensin	1172.4	IARRHPYFL	11.01	0.10	+	+	+
3	B-Amyloid (1-11)	1325.3	DAEFRHDSGYE	3.93	0.15	+	-	-

^aCalculated using the program available at <http://pepcalc.com>