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Molecular Features Influencing the Release of Peptides from Amphiphilic Polymeric Reverse Micelles

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Abstract

Efficient and controlled release of peptides bound to polymeric reverse micelle assemblies can be achieved through the cooperative effects of disassembly and disruption of charge-charge interactions. Through the examination of various peptides and polymer architectures, we have identified the factors that affect the release efficiency of the electrostatically bound peptides. Peptide guests and polymers with a greater number of complementary charges result in less efficient release than peptides and polymers with lower numbers of charges. Interestingly, we find that the presence of adjacent charged groups on the monomeric unit of the polymer exhibit exceptionally low release efficiency, perhaps due to a chelate-like effect, even when the total polymer charge is lower. Overall, our findings inform the design principles for catch-and-release systems based on polymeric reverse micelles, which offer great versatility and tunability.

TOC Graphic

Keywords

polymeric reverse micelles; host-guest interaction; disassembly; release efficiency

INTRODUCTION

Polymer-based supramolecular assemblies that form organized structures such as micelles, reverse micelles, and vesicles, have attracted attention in recent years for their use in

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ASSOCIATED CONTENT

Supporting Information

Materials and methods, syntheses and characterization of polymers, and supporting figures. This material is available free of charge via the Internet at <http://pubs.acs.org>.

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delivery, separations, catalysis, biomimetics, sensing, and as nanoreactors.^{1–11} Such applications often rely on the polymeric assembly acting as a host to accommodate guest molecules through noncovalent interactions. Remarkably, although these host-guest interactions employ relatively weak noncovalent interactions that render the binding reversible and dynamic, the multivalent nature of the polymeric host affords additional strength and versatility to the binding. The existence of cooperativity among the subunits of a supramolecular assembly imparts properties that are different from its individual components, and this manifests not only in the process of host-guest binding/association but also in guest release/dissociation. Understanding the factors that influence guest release is just as important as knowing the mechanism of host-guest binding, as evidenced by the emergence of stimuli-responsive assemblies that take advantage of diverse strategies for regulated guest release.^{12–16} Depending on the desired application, either a slow, controlled release may be preferred,^{17–19} as in the case of drug delivery, or an immediate, quantitative release may be needed,^{20,22} as in the case of separations and purification. In either case, a deeper understanding of the guest release process is necessary to advance such applications, especially in systems involving the capture and release of complex biomolecules.

Encapsulation of biomolecules, particularly peptides and proteins, is typically intended either to isolate them from complex media, protect them from degradation, increase their biostability and sustain their bioactivity, or offer confinement that could modulate their activity.^{23–27} Because these large biopolymers possess diverse chemistries and functionalities in their amino acid precursors, selective capture and release of peptides and proteins into and out of supramolecular hosts require a means of discriminating a selected set of proteins over others. Our group has been particularly interested in the use of amphiphilic polymers, such as the carboxylate-functionalized homopolymer **P1** (Figure 1) for the selective extraction of peptides and proteins. The amphiphilic nature of these polymers enables them to self-assemble into reverse micelles in nonpolar solvents, such that the charged functional groups form a hydrophilic core via solvophobic effects while the hydrophobic moieties are oriented outside in contact with the nonpolar solvent. These polymeric reverse micelles serve as ideal supramolecular hosts for extracting peptides and proteins into nonpolar solvents due to the inherent stability of the assemblies, tunable selectivity and optimizable capacity.^{28–30} In the past, we have shown how these assemblies can be utilized for the selective enrichment and sensitive detection of peptides and proteins through a simple biphasic extraction.³¹ By virtue of a peptide's isoelectric point (pI), which dictates its charge at a given solution pH, the polymeric reverse micelles bearing a charged hydrophilic interior can selectively sequester peptides having a complementary charge into the organic phase. The polymeric reverse micelles can also control the reactivity of peptides that are encapsulated in their interior, enabling residue-selective bioconjugation.³² However, the factors that control the release of the sequestered biomolecules from the reverse micelles have not been explored. Although there have been numerous studies demonstrating guest release from small molecule surfactant reverse micelles such as AOT (bis(2-ethylhexyl) sulfosuccinate sodium),^{20,33,36} systematic studies of the factors that influence guest release from polymeric reverse micelles have been limited.^{4,37} Moreover, because of the high kinetic stability of polymeric reverse micelle assemblies, most guest release mechanisms are

of the slow type,³⁸ simply relying on the inherently dynamic but gradual exchange of guest molecules between the hydrophilic interior of the reverse micelles and the bulk solvent.

In this paper, we describe investigations into the conditions that affect peptide release efficiency from polymeric reverse micelles and show that efficient guest release requires the disassembly of the reverse micelles and weakening of the non-covalent interactions between polymer and peptide. We further examine the features of the supramolecular host (polymeric reverse micelles) and guest (peptide) that influence the release process.

EXPERIMENTAL

Reagents.

The peptides bradykinin (RPPGFSPFR) and angiotensin I (DRVYIHPFHL) were purchased from Sigma-Aldrich. TAMRA-bradykinin (TAMRA dye conjugated at the N-terminus of the peptide) was obtained from Biopeptek Inc. The following peptides were purchased from the American Peptide Company: kinetensin (IARRHPYFL), malantide (RTKRSGSVYEPLKI), β -amyloid fragment 10-20 (DAEFRHDSGYE). Trifluoroacetic acid (TFA), MOPS buffer and α -cyano-4-hydroxy-cinnamic acid (CHCA) were purchased from Sigma-Aldrich. Tris(hydroxymethyl)-aminomethane (Tris buffer), acetonitrile, formic acid, hydrochloric acid (HCl), sodium hydroxide, ammonium hydroxide (NH₄OH), glacial acetic acid, toluene, tetrahydrofuran (THF) and purified water were obtained from Fisher Scientific. THF was distilled over Na/Ph₂CO before use.

Polymer Synthesis and Characterization.

Seven different polymers (**P1-P7**) were synthesized to study the effect of the supramolecular host on guest release. Refer to Supplementary Information for synthetic and characterization details.

Preparation of Polymeric Reverse Micelle.

Solutions of the amphiphilic polymers were prepared in toluene to form the reverse micelle assemblies. A concentration of 1×10^{-4} M was prepared by weighing the appropriate amount of polymer and dissolving in toluene by sonication until a clear solution was obtained. To serve as the water pool for the reverse micelles, 2 equivalents of water per carboxylate were added to the solution and sonicated again until optically clear.

Extraction and Release of Peptides.

Peptide solutions were prepared in an aqueous buffer of 50 mM MOPS, pH 6.5. Extractions were done by adding 200 μ L of the polymeric reverse micelle solution to 1 mL of the buffered peptide solution (0.2 μ M of peptides bradykinin, angiotensin I, malantide, kinetensin and β -amyloid 10-20; 0.5 μ M of TAMRA-bradykinin), mixing them by vortex for 2 hours, and separating the two phases by centrifugation at 12,500 rpm for 60 minutes. The aqueous phase was carefully drawn out and transferred to a separate tube, leaving the organic phase with the extracted peptides in the original tube. Peptide release was accomplished by the addition of 100 μ L of THF (unless otherwise noted), followed by the addition of the specified stripping aqueous phase (*e.g.* 10% acetic acid, water, buffer, or

NaCl solutions). The mixture was vortexed for 2 hours to allow for peptide release from the organic phase into the stripping aqueous phase. The released peptides in the aqueous phase were analyzed either by MALDI-MS, LC-MS, or fluorescence spectroscopy for quantification (protocols and methods described in Supplementary Information). The release efficiency was calculated as:

$$\text{Release efficiency} = \frac{[\text{Concentration of peptide in stripping aqueous phase}]}{[\text{initial concentration of peptide}] \bullet \text{Concentration Factor}} \times 100$$

$$\text{where Concentration Factor} = \frac{\text{initial volume of peptide}}{\text{final vol of peptide in stripping aqueous phase}}$$

RESULTS AND DISCUSSION

In devising strategies for efficient peptide release, we first considered the simple disruption of the electrostatic interactions between the polymer and the peptide that are essential for selective binding. Our attempts at screening out the electrostatic interaction by increasing the ionic strength was not successful for quantitative recovery. Hence, three strategies (Figure 1) were considered: (1) creation of charge-charge repulsion between the peptide and polymer by deprotonating the peptide through the addition of base; (2) neutralization of the polymer's carboxylate groups via the addition of acid to abolish the charge-charge interaction between the polymer and the peptide; and (3) disassembly of the reverse micelles prior to the addition of the stripping aqueous solution.

We tested these strategies with 200 pmol of a model positively charged peptide, angiotensin I (sequence: DRVYIHPFHL, pI 7.9), which can be readily sequestered into the reverse micelles via a biphasic extraction approach described in the experimental section. This peptide was chosen as a model because its pI is predictable, and its diversity of amino acids makes it more representative of biologically active peptides. Peptide release into the stripping aqueous phase was monitored by matrix-assisted laser desorption/ionization mass spectrometry (MALDI-MS) analysis. Using polymer **P1** as an example, we can use the peptide ion signal at m/z 1296.7 as an indicator of how much peptide was released using each of the strategies illustrated in Figure 1. In parallel, the toluene phase was also analyzed after peptide release to detect any peptides that might remain in the reverse micelles in the organic phase. For reference, Figure 2A shows the MALDI-MS signal for an angiotensin I standard that has a concentration of 1 μM (200 pmol in 200 μL), which is the expected concentration if peptide release in the stripping aqueous phase is 100%, while Figure 2B shows the MALDI-MS signal for 200 pmol angiotensin I in the reverse micelles in toluene before any peptide is released (*i.e.* 0% release).

Adding base to deprotonate the peptide (strategy 1) does not yield significant release as indicated by the low MALDI-MS peptide ion signal seen in the stripping aqueous phase (Figure 2C) and an abundant amount still remaining in the toluene phase (Figure 2D). Adding acid to neutralize the charged groups in the polymeric reverse micelles (strategy 2)

did not release much peptide either (Figure 2E), with a significant amount of peptide remaining with the reverse micelles in the toluene phase (Figure 2F).

Results from the first two strategies suggest that simply eliminating the charge-charge interaction between the polymer and peptide through a pH change is not sufficient to cause efficient guest release. We posited that their confinement inside the reverse micelle assemblies in the organic phase may be preventing their release into the aqueous phase. We then considered disruption of the assemblies using tetrahydrofuran (THF), which is considered to be a good solvent for both the hydrophobic and hydrophilic moieties of the polymer, thereby eliminating the solvophobic effects that drive self-assembly. DLS measurements before and after THF addition confirm that the assemblies become unstable (erratic correlograms and unreliable sizes) in a mixture of THF and toluene (Figure S1A and S1B). Interestingly, UV-Vis measurements indicate that the polymer does not partition to the stripping aqueous phase after THF addition (Figure S1C), confirming that the polymer is still in the organic phase but just not as stable assemblies.

Even though THF disassembles the reverse micelles, addition of this solvent is not sufficient to efficiently release the peptides from the organic phase when water (pH 6) is added as the stripping aqueous phase (Figure 2G and Figure 2H). However, when acid (pH 1.7) is used as the stripping aqueous phase after THF addition, almost quantitative release of the peptides into the aqueous phase is seen (Figure 2I). The peptide signal in this case is close to what is expected if the peptide were initially dissolved in an aqueous solution (Figure 2A). Furthermore, almost no peptide remains in the organic phase (Figure 2J).

An illustrative and quantitative indication of guest release can also be observed and measured via the release of the fluorescently labeled guest peptide, TAMRA-bradykinin (sequence: RPPGFSPFR). Figure 3 shows the efficiency of guest release using each strategy as measured by fluorescence spectroscopy, which is consistent with results obtained from the MALDI-MS analysis. Taken as a whole, the results in Figures 2 and 3 indicate that simply neutralizing the electrostatic interaction between polymer and peptide, nor merely disassembling the reverse micelles in the organic phase is sufficient to cause efficient guest release from the reverse micelles. Instead, both disassembly and neutralization are necessary for efficient guest release. Thus, it appears that guest release from the polymeric reverse micelles is subject to a significant degree of cooperativity in that both disassembly of the reverse micelles and disruption of the polymer-peptide interactions are necessary.

We then explored the extent of cooperativity in guest release for three other amphiphilic polymer scaffolds (see Figure 4A) to better understand the structural factors that influence this process. An easily tunable amphiphilic random copolymer (*i.e.* **P2**), which contains a similar styrene-based hydrophobic decyl chain and a hydrophilic carboxylate moiety similar to **P1**, was considered to test the effect of amphiphilic group flexibility as the hydrophobic and hydrophilic groups are not constrained as they are in polymer **P1**. Polymer **P3**, which is also a random copolymer scaffold bearing a hydrophobic decyl chain and a hydrophilic carboxylate group but has an acid-cleavable citraconic acid amide moiety,³⁹ was chosen to study how charge reversal from negative to positive might drive guest release further due to charge-charge repulsion with the positively charged peptides. A carboxylate-functionalized

polyacrylamide-based amphiphilic homopolymer **P4** was also considered to see if a less bulky scaffold would affect guest release. Moreover, several peptides spanning a range of basicity were used as guests in reverse micelles of polymers **P1-P4** to investigate what properties of the host (polymer) and guest (peptide) influence release efficiency.

When both THF and acid were added (Figure 4B), all guest peptides were released efficiently (90-100%) from the polymers indicating that all the polymers are subject to the same cooperativity noted above. Interestingly, the charge-conversion polymer **P3**, which generates a repulsive interaction upon addition of acid, does not demonstrate a significant advantage over the other three polymers in terms of release efficiency, indicating that disassembly and charge shielding after THF addition is sufficient for effective guest release. However, when only THF and water are used (Figure 4C), subtle differences in release efficiency can be observed. At the pH of water (~5.6-6.5), protonation of all the carboxylate groups in the polymer presumably does not occur, resulting in residual electrostatic binding between the polymers and the positively charged peptides. A close look at these data reveals that release efficiency is influenced by the type of polymer and the peptide guest. For instance, less basic peptides like angiotensin I (pI=7.9) are released more easily than highly basic peptides (*vide infra* for comparisons). Also, the random copolymers (**P2** and **P3**) release the peptides into water better than do the homopolymers (**P1** and **P4**), especially for the more basic peptides. These subtle effects evidently arise when the pH of the stripping aqueous solution is closer to neutral, as this condition gives a discriminating effect between weak and strong interactions. Based on these observations, we hypothesize that the strength of electrostatic interaction between the polymer carboxylate groups and the peptides influences the guest release efficiency. We also considered that peptide hydrophobicity might influence release efficiency, but there appears to be no trend between calculated peptide hydrophobicity and release efficiency (Table S1).

The hypothesis that electrostatic interactions between the peptide and polymer influence guest release is supported upon considering the peptides' net charges as a function of pH (Figure 4D). Using release from polymer **P1** in Figure 4C as an example, we find that guest release efficiency increases as the peptide net charge decreases at pH 6. Release of the more basic peptides, malantide, kinetensin, and bradykinin, is much lower than the release of β -amyloid and angiotensin I, which follows the theoretically calculated net charge on each peptide at pH 6. That is, the higher the net charge, the stronger the peptide's electrostatic interaction with the polymer, and the more difficult it is to be released. Polymers **P2** and **P4** show a similar trend as **P1**, although the trend is less dramatic for the random copolymer **P2**. Overall, under conditions where the carboxylate groups in the polymer are not fully neutralized, the release efficiency depends on the magnitude of the net charge of the peptide at that pH. Polymer **P3**, however, does not clearly show this trend and instead we see better release efficiency for the more basic peptides compared with the other polymers. This is perhaps because some fraction of the acid-cleavable units is being converted to positively charged amine groups at pH 6, thereby giving rise to some repulsive interactions that enable this polymer to more easily release highly positively charged peptides compared to the other polymers without this charge-conversion moiety. To confirm this, we performed the release of malantide, a hard-to-release peptide, at a slightly lower pH where the charge conversion

has been shown to occur,³⁹ and was able to observe a consequent increase in release efficiency as a result of the hydrolysis (Figure S2).

Interestingly, we can also take advantage of this “release by repulsion” to achieve selective peptide release by using a stripping aqueous phase pH that causes a peptide to have a net negative charge. For example, if kinetensin (pI=11) and angiotensin (pI=7.9) are loaded into reverse micelles of **P1** and then released using THF and a stripping aqueous phase at a pH of 9.5, angiotensin I is selectively released (Figure 5A), while kinetensin remains in the organic phase (Figure 5B). Presumably, angiotensin I is released because it becomes negatively charged at the pH above its pI, whereas kinetensin remains because it retains its positive charge at the pH below its pI. This observation provides further proof for the importance of electrostatic interactions in the release process, but it also suggests a new mode by which selective release can be achieved, which could have implications for peptide separations/purifications or selective delivery of multiple cargos.

Further evidence for the importance of electrostatic interactions on guest release is found by varying the number of charges in the polymer via changes in the pH of the stripping aqueous phase used after THF addition. We find that peptides are released from the reverse micelles of **P1** with a titration curve-like dependence (Figure 6). As an example, TAMRA-bradykinin is released quantitatively at low pH, presumably as a result of protonation of the polymer carboxylate groups, while release efficiency decreases as the pH is increased, providing evidence that neutralizing the carboxylate groups weakens the polymer's hold on the peptide upon disassembly by THF. Notably, the pH at which 50% of the peptides are released is around pH 4.7, which may be the effective pK_a of the carboxylic acid groups in the polymer. Interestingly, in previous work we showed that polymer **P1** has the same pH dependency when extracting peptides from an aqueous phase into an organic phase,⁴⁰ with an inflection point at around pH 5, highlighting the reversible behavior of this homopolymer. The titration curve-like dependence and effective pK_a observed in Figure 6 is similar to what is obtained for a carboxylate-functionalized random copolymer (Figure S3), suggesting that this behavior is independent of the polymer scaffold. These observations are important as they indicate that the extent of peptide release can be predicted and controlled based on the identity of the charged functional group.

Another way to explore the influence of polymer charge density on release efficiency is to vary the percentage of carboxylate groups on the polymer. This can be easily achieved through our random copolymer scaffold by varying the ratio of the hydrophilic and hydrophobic moieties during polymerization.³⁰ In addition to polymer **P2** which has 50% carboxylate groups, we synthesized random copolymers **P5** and **P6**, containing 20% and 30% carboxylate groups, respectively (Figure 7A). As observed before, quantitative peptide release is achieved when THF and acid are used (Figure 7B), regardless of the percentage of carboxylate groups in the polymer suggesting that the carboxylate groups are effectively protonated at a low pH. However, when using THF and pure water, peptide release efficiency decreases as the percentage of carboxylate groups in the polymer increases (*i.e.* **P5** < **P6** < **P2** < **P1**), suggesting that a higher carboxylate number in the polymer leads to stronger peptide binding and therefore less efficient peptide release. These results have important implications because while an optimal percentage of carboxylate groups is

required for high binding capacity of the reverse micelles,³⁰ guest release efficiency is reduced, especially at near neutral conditions.

A notable exception to the trend in Figure 7B is the result for **P7**, which contains a 17% ratio of a di-carboxylate moiety (or 34% total carboxylate per monomer). With this percentage of carboxylate groups, one would expect peptide release efficiency to be about the same as **P6**, which has 30% carboxylate groups. However, we find that polymer **P7** has an even lower release efficiency than the homopolymer **P1**, which has 100% carboxylate. This observation suggests that the presence of two adjacent carboxylate groups imparts additional avidity through a chelate-like effect that allows the polymer to retain peptides more strongly than what would be expected based on just the global or overall charge density.

The results above demonstrate that peptide release is exquisitely sensitive to pH and polymer charge density, but we also find that peptide release is influenced by ionic strength after disassembly. Moreover, the effect of ionic strength is polymer-dependent (Figure 8). For polymers **P6** and **P2**, quantitative release occurs at NaCl concentrations as low as 0.01 M, suggesting that minimal charge shielding is needed to disrupt the electrostatic interaction between polymer and peptide. In contrast, polymers **P1** and **P7** require salt concentrations as high as 1 M to achieve efficient peptide release. The behavior of polymer **P7** is again remarkable, even though it has a lower overall percentage of carboxylate groups than **P1** or **P2**. The bidentate nature of the carboxylate groups in **P7** presumably confers additional strength to its binding with the peptides such that a greater electrostatic shielding is required to release its peptide guests. Perhaps more intriguing is the fact that the forward extraction capacities of polymers **P6** and **P7** were found in our recent work to be very similar.³⁰ Evidently, the presence of higher local charge density for **P7** does not influence the forward extraction process like it does guest release. This disparity may be attributed to the fact that peptide extraction from the aqueous phase occurs while the polymer exists as intact reverse micelle assemblies, whereas during peptide release the addition of THF causes them to disassemble, leaving **P7**'s local charge density to dominate polymer-guest interactions.

SUMMARY AND CONCLUSIONS

In summary, we have evaluated different strategies for releasing guest peptides from polymeric reverse micelles and investigated the features of the guest and the host that affect the release efficiency. We have shown that: (i) both disassembly of the reverse micelles and neutralization of electrostatic interactions between polymer and peptide are required to efficiently release encapsulated guest peptides, indicating a high degree of cooperativity in the release process; (ii) under conditions where full neutralization of the polymer charged groups is not achieved, guest release efficiency is dependent on the strength of interaction between polymer and peptide, which in turn is dictated by the peptide charge and the nature and amount of charge on the polymer; (iii) peptides with a higher magnitude of complementary charge are harder to release but can be rescued with the use of a charge-conversion polymer; (iv) peptides are selectively released if they bear the same charge as the polymer at a given pH of the stripping aqueous phase; (v) release efficiency at different pHs is governed by the effective pK_a of the charged functional groups in the polymer; (vi) higher charge densities in the polymer typically lead to lower release efficiencies; and (vii)

polymers bearing two adjacent charged groups in their monomeric unit lead to lower release efficiencies due to a chelate-like effect brought about by their multidentate binding ability.

Overall, our findings suggest that much like the forward encapsulation process, guest release efficiency is optimizable depending on the aforementioned features of the guest and polymer host. It should be noted that understanding the pH dependent release of peptides is especially important as changes in pH occur in biological systems such as that between the cytoplasmic (pH 7.0) and the endosomal or lysosomal pH (about 6.5 to 4.5) or in the extracellular acidic environment of tumor tissues. Deeper insights into the underlying features that affect the forward and the reverse processes of encapsulation and guest release thus provides a more complete view for advancing catch-and-release applications involving polymeric reverse micelle assemblies.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1. Different strategies for guest release from carboxylate-functionalized polymeric reverse micelle assemblies.

Figure 2.

Representative MALDI mass spectra for (A) 1 μM Angiotensin I standard (* m/z 1296.7) and (B) 200 pmol angiotensin I fully extracted into the organic phase without any release.

Representative MALDI spectra of the resulting aqueous and organic phases after release of 200 pmol angiotensin I from reverse micelles of **P1** using 200 mM Tris base at pH 10 (C and D), using 10% acetic acid at pH 1.7 (E and F), by the addition of THF then water (G and H), and by the addition of THF then 10% acetic acid (I and J). All stripping aqueous phases are 200 μL in volume.

Figure 3.

TAMRA-bradykinin before (**A**) and after (**B**) extraction into reverse micelles of **P1** in toluene. Release of TAMRA-bradykinin under different conditions (**C**), and quantitative determination of the release efficiency by fluorescence measurement of the stripping aqueous phase (**D**). Initial TAMRA-bradykinin concentration is 0.5 μM in 1 mL of buffer, released to a final volume of 200 μL stripping aqueous phase. Each bar is the average $\pm$ standard deviation obtained for three replicate samples for each condition.

Figure 4.

(A) Chemical structures of amphiphilic polymers **P1**, **P2**, **P3** and **P4**. Efficiency of peptide release from each polymer by addition of THF then 10% acetic acid (**B**), or by addition of THF then water (**C**), as quantified by LC-MS (ND=not detected). Initial concentration of each peptide is 0.2 μ M in 1 mL of buffer, released to a final volume of 50 μ L stripping aqueous phase. Each bar is the average $\pm$ standard deviation of three experimental replicates. Statistical analysis of variance of release efficiency obtained among polymers and among peptides can be found in the supplementary information. (**D**) Theoretically calculated net charge[‡] on the peptide as a function of solution pH (shaded gray region corresponds to pH range of 5.6-6.5 for water). [‡]Values obtained from from *InCharge* software by *EpiQuest* Protein Analysis Software Suite (<https://www.epiquest.co.uk/incarge>). Peptides studied: malantide (RTKRSGSVYEPKLI, pI=10.3), kinetensin (IARRHPYFL, pI=11), bradykinin (RPPGFSPFR, pI=12), β -amyloid 10-20 fragment (YEVHHQKLVFF, pI=7.6), angiotensin I (DRVYIHPFHL, pI=7.9).

Figure 5. MALDI mass spectra of the stripping aqueous phase (**A**) and the organic phase (**B**) after release at pH 9.5 (using THF + 50 mM Tris, pH 9.5), illustrating that angiotensin I can be selectively released.

Figure 6. Release efficiency (blue squares) of TAMRA-bradykinin peptide from reverse micelles of **P1** at various pH superimposed with the theoretically calculated percentage of protonated carboxylate groups in the polymer for each pH (red circles). Error bars represent the standard deviation from three experimental replicates done for each pH. Best fit curves are drawn to guide the eye.

Figure 7.

(A) Structures of polymers **P5**, **P6** and **P7**. (B) Release efficiency for bradykinin, quantified by LC-MS, from reverse micelles of polymers with different percentages of carboxylate functional groups. Each bar is the average $\pm$ standard deviation of three experimental replicates. Statistical ANOVA and significance testing of release efficiency obtained between polymers can be found in the supplementary information.

Figure 8. Release efficiency of TAMRA-bradykinin from reverse micelles of **P7**, **P6**, **P2** and **P1** using THF and a stripping aqueous phase with increasing electrostatic shielding. Error bars represent the standard deviation obtained from three experimental replicates done for each condition.