Peptide-Binding Nanoparticle Materials with Tailored Recognition sites for Basic Peptides

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Abstract

Peptides rich in basic residues such as lysine and arginine play important roles in biology such as bacterial defense and cell penetration. Although peptide-binding materials with high sequence-specificity have broad potential applications, the diverse functionalities of peptide side chains make their molecular recognition extremely difficult. By covalently capturing micelles of a doubly cross-linkable surfactant with solubilized peptide templates, we prepared water-soluble molecularly imprinted nanoparticles with high sequence-specificity for basic peptides. The nanoparticles interact with the side chains of lysine and arginine through hydrogen bonds strengthened by the nonpolar environment of the micelle. They have hydrophobic pockets in their core complementary to the hydrophobic side chains in size and shape. These recognition sites allowed the micelles to bind basic biological peptides strongly in water, with tens to hundreds of nanomolar in binding affinity.

Graphical Abstract
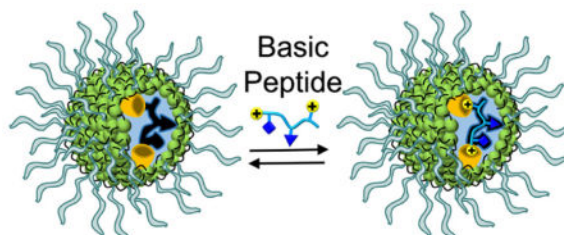
Introduction

Peptides rich in basic residues such as lysine and arginine play important roles in biology. Their cationic charge under physiological conditions help them bind negative groups on cell membranes. Strategically positioned hydrophobic side chains allow the peptides to undergo appropriate conformational changes when they bind to membranes, either lysing the cell as

in antimicrobial peptides[1,2] or crossing the membrane as in cell-penetrating peptides.[3–7] These important features have prompted continuous research effort to understand their structure–activity relationships and develop novel therapeutic and delivery agents. Materials that can selectively bind such peptides in aqueous milieu are expected to be particularly useful in their separation, purification, sensing, and biological study.

Sequence-selective recognition of oligopeptides attracted the attention of chemists and biologists for decades.[8–19] A general method to recognize peptides, however, is elusive due to the many difficult challenges involved.[20,21] One difficulty comes from the highly diverse functional groups on these guest molecules. Even with many tools developed in supramolecular chemistry in the last decades, researchers do not have recognition motifs for the unique hydrophobic, hydrophilic, acidic, and basic side chains of most amino acids. Not only so, the side chains of some amino acids differ minutely: leucine and isoleucine, for example, only have the position of their methyl change by one carbon. Such subtlety demands an extremely high level of precision in an effective peptide receptor.

The second difficulty in peptide recognition is related to size. As a peptide guest gets longer, its receptor, in order to possess a complementary binding interface, necessarily needs to increase in size and complexity. One strand of DNA can bind its complementary strand with matching hydrogen-bonding code, but a concave binding interface is needed to accommodate the different side chains of peptides, implying a much larger host structure is required for peptide recognition. Even if individual recognition motifs or "codes" can be developed for each amino acids, joining the individual motifs on an appropriate scaffold in a preorganized fashion to recognize the matching peptide will take tremendous efforts, especially if the host is a discrete molecule built from the scratch.

For biological applications, the medium represents the third difficulty, as directional intermolecular forces such as hydrogen bonds are compromised severely by competition from water.[22,23] Although hydrophobic interactions can be strong in water, their usage for selective molecular recognition is not as straightforward as in hydrogen-bonded systems with clearly defined donor–acceptor patterns.[24]

Molecular imprinting is a conceptual breakaway from the molecular construction of receptors and relies on "chemical molding"—i.e., binding, polymerization, and cross-linking —around the templates to afford the binding sites directly. Provided that appropriate functional monomers (FMs) are available for the type of templates involved and the imprinting process works well, individual design of the receptor is eliminated.[25–35] This feature makes the method inherently general and potentially highly powerful. Many imprinted materials indeed have been created for peptides, showing promising results for various applications.[36–41]

An ideal peptide receptor should be generally applicable to peptides with varying properties and lengths. For biological applications, water-solubility is important. Their synthesis and purification should be easy, even better if achievable by nonchemists.

We recently reported our first step toward a general method for peptide recognition. Using micellar imprinting, a technique developed in our laboratory,[42] we created molecularly

imprinted nanoparticles (MINPs) with high affinity (as low as 20 nM in water) and exceptional selectivity for peptides 2–12 residues in length.[43] Recognition was based on imprinted "hydrophobic dimples" created on the surface of the cross-linked micelle that complement the hydrophobic side chains of the templating peptide in size and shape. These "dimples" essentially encoded the MINP receptors with molecular recognition information for the peptide guests and were precise enough to distinguish leucine and isoleucine, as well as phenylalanine and tyrosine that differ by one hydroxyl.[43]

The previously reported method, nonetheless, mainly relied on hydrophobic interactions for the imprinting and binding. Thus, highly polar peptides including basic ones are unlikely to work well with the previous materials. Herein, we report methods to target the side chains of lysine and arginine, in addition to hydrophobic amino acids. As mentioned above, the basic and hydrophobic side chains together are critical to the antimicrobial and cell-penetrating properties of this important class of peptides. Materials that can recognize these groups should facilitate the biological study of these peptides. The strategies used in the binding also are useful to the fundamental understanding of molecular recognition in water, an important challenge in supramolecular chemistry.

Results and Discussion

Design and Synthesis

The synthesis of receptors for basic peptides was adapted from previous procedures.[42–47] As shown in Scheme 1, the peptide was first solubilized by mixed micelles comprising cross-linkable surfactant **1**, an equivalent amount of divinylbenzene (DVB, a free radical cross-linker), a small amount of 2,2-dimethoxy-2-phenylacetophenone (DMPA, a photoinitiator), and the appropriate FM. Although cationic materials are expected to repel basic peptides that tend to be positively charged under physiological conditions, we envisioned their usage would minimize non-specific adsorption of the peptides and thus enhance selectivity in the binding (vide infra).

The micelles were cross-linked by diazide **2** on surface and then decorated with a layer of hydrophilic groups by sugar-derived azide **3**, both using the Cu(I)-catalyzed click reaction. [48–50] UV irradiation led to free radical polymerization/cross-linking of the core between the methacrylate of **1** and DVB. Repeated solvent washing removed the templates to vacate the binding sites.

The MINPs were characterized by previously reported procedures.[42–47] The surface- and core-cross-linking were monitored by ^{1}H NMR spectroscopy (see Supporting Information for details). The surface-cross-linking has been verified by mass spectrometry after the 1,2-diol in the cross-linked **2** was cleaved.[48,49] Dynamic light scattering (DLS) afforded the size of the nanoparticles and their average molecular weights. The DLS size (~5 nm) was confirmed by transmission electron microscopy (Figure 1).

Creating complementary hydrophobic "dimples" for the hydrophobic side chains of peptides does not require any FMs beyond the cross-linkable surfactant and DVB that polymerize around the template. As long as the double cross-linking of the micelle could "freeze" the

initial peptide-binding state, the resulting MINP could bind the templating peptide afterwards. Given the exceptional selectivity in binding for leucine/isoleucine and phenylalanine/tyrosine, the polymerization and cross-linking in the confined nanospace of micelle must be extremely amenable to imprinting.[43]

For peptides rich in polar amino acids such as lysine and arginine, however, imprinting would not be as easy with the surfactant and DVB. First of all, these polar side chains are expected to stay on the surface of the micelle to be solvated by water. Even if they can migrate into the core of the micelle (e.g., in the amino form for lysine), the hydrophobic driving binding interactions will be quite weak. Thus, to imprint effectively, we need to have appropriate FMs to bind the polar side chains and, importantly, the binding needs to have sufficient strength in an aqueous micellar solution.[51,52]

As shown in Scheme 1, the FM utilized in this work was **4**, prepared from the commercially available azacrown derivative.[53] Its two styrenyl groups enable the FM to be covalently attached to the MINP during the core-cross-linking. Crown ether derivatives (including azacrowns) are known to bind amine,[54] ammonium[55,56] and guanidinium[57–59] groups by various hydrogen-bonding motifs. We envisioned that the sulfonamide groups could provide additional sites for hydrogen-bonding and may be particularly suitable to bind the guanidinium side chain of arginine. Our hope was that the styrenyl groups of **4** would provide enough hydrophobicity for the FM to migrate to the hydrophobic region of the micelle where hydrogen bonds might be sufficiently strong to allow successful imprinting.

Binding Affinities

The first templates studied were tripeptide lysine-tryptophan-tryptophan (KWW) and lysine-lysine-lysine (KKK), with their structures shown below. KWW is very hydrophobic with two large indole side chains and KKK has no hydrophobic side chains. Their comparison thus could help us understand the effectiveness of FM **4** for two very different classes of peptides.
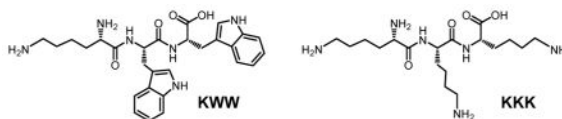

Table 1 summarizes the results of our binding studies. We included the binding data for MINPs prepared with and without FM **4** generally. We also listed the K_{rel} values, which is the binding constant of the MINP with FM **4** relative to that without, and thus is a measure of the effectiveness of the FM in the molecular imprinting.

In the absence of **4**, MINP(KWW), i.e., MINP prepared with KWW as the template, was found to bind its template with a binding constant of $K_a = 20.7 \times 10^5$ M^{-1}, determined by fluorescence titration and nonlinear least squares fitting of the data to a 1:1 binding isotherm. The 1:1 binding stoichiometry was confirmed additionally by the Job plot (Figure S13). The binding constant corresponds to 8.6 kcal/mol of binding free energy ($-\Delta G$).

Addition of **4** in the MINP preparation increased the binding for KWW by <2 fold (entries 2–4). At first appearance, the small K_{rel} seems to suggest that the FM was barely involved and inconsequential. However, when its amount was varied from 1 to 3 equiv to the template, the binding clearly responded and K_{rel} reached the highest value at 2 equiv. Since KWW contains two amino groups, one at the *N* terminus and the other on the lysine side chain, our binding data suggest that a 1:1 ratio between the FM and the amino groups was optimal, a result only possible if the FM was involved in both the imprinting and binding.

The above conclusion was confirmed by MINP(KKK). Once the large hydrophobic side chains of tryptophan were removed, binding (between KKK and its MINP) became much weaker, with $K_a = 0.2 \times 10^5$ M^{-1}. This value was two orders of magnitude lower than that between KWW and MINP(KWW), consistent with the importance of hydrophobic imprinting and binding of KWW. Significantly, up to 13-fold increase in K_a was observed for KKK when the MINP contained FM **4**. Interestingly, K_{rel} reached the highest value at 4 equiv of FM for this peptide and stayed unchanged when additional FM was used (entries 6–10). Because KKK has four amino groups, the optimal ratio of FM to the amino group was thus 1:1 once again, supporting the effectiveness of FM **4** in the imprinting of lysine-containing peptides.

The above results suggest that the effectiveness of FM **4** in the imprinting of basic peptides is inversely related to the overall hydrophobicity of the template. For a peptide rich in hydrophobic side chains such as KWW, hydrophobic interactions provide sufficient driving force in binding with its MINP receptor, evident from the 8.6 kcal/mol binding free energy mentioned above. Under such conditions, additional polar interactions such as the hydrogen bonding interactions between the azacrown and the amino/ammonium groups would play a secondary role. For a peptide relatively hydrophilic, however, the polar interactions become more important, enhancing the binding by up to 13-fold in the case of KKK. It is worth noting that a 5.56 kcal/mol binding energy for its parent MINP, i.e., the MINP prepared without FM **4**, was still fairly impressive, suggesting the "background" imprinting/binding was significant. In our previous work, even glycine was found to influence the imprinting and binding of peptides, despite its lack of a side chain.[43] MINPs contain multiple functional groups including numerous triazole, hydroxyl, ammonium, and amide. These groups can certainly interact with the bound peptide through hydrogen bonds, van der Waals interactions, cationic–π interactions, and electrostatic interactions. Since these are less well-defined than the specific interactions between the hydrophobic "dimples" and the hydrophobic side chains or those between the azacrown and the amine/ammonium, we group them together as the background interactions.

We also studied the effects of pH on the binding of KWW and KKK, with and without FM **4** (Table 1, entries 1, 3, 5, 8). Even though the overall pH effect was relatively small, our data show that lowering pH from 7.0 to 4.7 caused a decrease of binding in the absence of the FM (entries 1 and 5) but an increase in the presence (entries 3 and 8). The results support our imprinting model. In the absence of the FM, imprinting mostly relies on the hydrophobic interactions between the peptide and the micelles. Imprinting of the lysine probably occurs in the nonpolar region of the micelle, with the side chain in the deprotonated (i.e., amino) state. During rebinding, a lower pH leads to more complete protonation of the lysine and

reduces its hydrophobic interactions with the MINP. In the presence of FM **4**, protonated lysine could be imprinted directly through ammonium–azacrown interactions, which are stronger than amine–azacrown intercations.[54] During rebinding, more complete protonation of lysine still occurs at a lower pH but the protonated form can interact with the azacrown more strongly than the amino form. Overall, since the pH effect was rather small and the binding constants were quite similar in buffer and in water, we performed the majority of the binding in Millipore water.

Entries 11–22 of Table 1 show the effects of FM **4** on other peptides. Several interesting trends are obvious. First, the K_{rel} value (at 1:1 FM/basic group) generally increased with the number of basic groups: up to 2.5 for two basic groups, 4.0 for three, and 13 for four. This trend supports the involvement of the azacrown in the imprinting and binding.

Second, for the peptides containing the same number of basic groups, K_{rel} was higher when the amino groups were farther apart, e.g., 1.77 for KWW but 2.51 for WKW. For the peptides containing two lysines and one tryptophan, the same trend persisted: K_{rel} was 2.20 for KKW and 1.75 for WKK, but 4.04 for KWK. It is not exactly clear to us why the binding depends on the distance between the basic groups. Nonetheless, to maximize the binding with its basic peptide guest, the azacrown-functionalized MINP needs to have the appropriate hydrophobic pocket to accommodate the indole ring of tryptophan. Meanwhile, the amine/ammonium group of the peptide needs to be in close proximity to the azacrown to engage in hydrogen bonds. When both tryptophan and FM **4** are quite bulky, the steric requirement might be quite demanding. When the basic groups are too close to each other, it is possible that their binding with FM **4** may cause the hydrophobic binding pocket to be less well-formed during the imprinting. It is also possible that the azacrowns may have difficulty achieving the optimal binding geometry under this situation.

Third, FM **4** was more effective for arginine (R) than for lysine (K). The binding data show that K_{rel} was 2.93 and 7.43 with one and two arginines, but only 1.77 and 2.20 with one and two lysines, respectively. The result supports our hypothesis that the multiple hydrogen-bond acceptors (sulfonamide oxygens, ethereal oxygens, and nitrogen) of FM **4** might make it bind arginine particularly well, which has an abundance of hydrogen-bond donors.

Binding Selectivity

Having confirmed the effectiveness of FM **4** for lysine- and arginine-containing peptides, we examined its impact on the selectivity of binding, using KWW as the templating peptide. Figure 2 shows the binding constants of different peptides to MINP(KWW), normalized to K_a of KWW. The red bars show the selectivity for the MINP prepared without FM and blue bars the MINP with 2 equiv **4**.

In the absence of the azacrown FM, MINP(KWW) easily distinguished KSW, KKW, and KWK. Thus, replacing any one of the two tryptophans with a smaller, polar side chain (of serine and lysine) drastically weakened the binding. However, replacing the lysine of KWW with serine (S), aspartic acid (D), histidine (H), glutamine (Q), and arginine (R) did not weaken the binding as much, affording 26–65% of the binding constant. The parent MINP,

therefore, was excellent at distinguishing the hydrophobic side chains but not very good at polar ones.

For the MINP(KWW) prepared with 2 equiv FM **4**, selectivity generally improved. Its ability to recognize the hydrophobic side chains, to our delight, was maintained, as KSW, KKW, and KWK continued to show low binding. Meanwhile, the selectivity for SWW, DWW, HWW (with histidine also as a basic amino acid), and QWW all improved in comparison to the parent MINP, sometimes dramatically. The improved binding selectivity for these polar amino acids supports lysine being engaged in specific interactions with the aza-crown-functionalized MINP, in line with our design hypothesis and the binding affinities discussed in the previous section.

For the azacrown-functionalized MINP, the notable exception to the improved selectivity was RWW. Whereas the parent MINP(KWW) was able to distinguish RWW with a modest selectivity (ca. 1:0.5 or 2:1), the azacrown-functionalized MINP(KWW) showed essentially the same binding for KWW and RWW.

This pattern of lysine/arginine selectivity was confirmed by additional studies (Table 2). The binding selectivity in this table is shown by K_{rel}, the binding constant of a nontemplating peptide over that of the template for the same MINP receptor. The smaller the K_{rel}, the more selective the peptide is. In the absence of FM **4**, MINP(KKW) containing two lysines once again afforded reasonable selectivity for RRW (with $K_{rel} = 0.44$ or ~2.3:1 selectivity). Meanwhile, MINP(RRW) could not distinguish KKW at all: if anything, the K_{rel} value (1.09) suggested reversed selectivity, meaning that the receptor bound the nontemplating peptide KKW slightly more strongly than its own template.

In the presence of FM **4**, the completely opposite trend was observed. MINP(RRW) distinguished KKW fairly easily, with $K_{rel} = 0.38$ or ~2.6:1 selectivity. MINP(KKW), however, bound KKW and RRW both very well. The slightly reversed selectivity ($K_{rel} = 1.12$) was observed once again.

These results indicate that, for the two basic amino acids, there is a preference for lysine by the parent MINPs and a preference for arginine by the azacrown-functionalized MINPs. The preferences can be understood from the different types of interactions involved in the two types of imprinted receptors.

In the absence of the azacrown, imprinting relies strongly on hydrophobic interactions. Lysine can deprotonate easily on the side chain due to weaker basicity of the amino group in comparison to guanidine. Once deprotonated, the side chain can insert itself into the micelle, which is beneficial to the hydrophobic imprinting. Arginine, however, has to stay on the surface of the micelle due to its charged nature, not contributing to the hydrophobic imprinting as much. The explanation is supported by the binding affinity data: MINP(RRW) bound its template with $K_a = 1.18 \times 10^5$ M^{-1} but MINP(KKW) bound its template more strongly, with $K_a = 1.86 \times 10^5$ M^{-1} (Table 2, entries 1 and 3). During rebinding, RRW has difficulty fitting in the binding site inside MINP(KKW) generated for KKW, due to the highly charged nature of the arginine side chain. KKW, on the other hand, could *in its*

protonated form "pretend" to be RRW and stay on the surface of MINP(RRW), exposed to water, just like arginine does.

Once the micelle contains FM **4**, the azacrown can interact with arginine better than lysine, because of its stronger and more numerous hydrogen-bond donors. Indeed, MINP(RRW) now has better binding affinity for its template than MINP(KKW) for its own (Table 2, entries 5 and 7). The stronger guanidinium–azacrown interactions not only can explain the selectivity of MINP(RRW) for KKW (entry 8) but also the reversed selectivity of RMINP(KKW) for RRW (entry 6). This is because, when KKW is used as the template, the lysine– azacrown interactions were strong enough to place the azacrown at the right place— also evident from our binding data in Table 1. Since arginine is quite close to lysine in length, the guanidinium side chain should be able to reach the azacrown binding site. The stronger arginine–azacrown interactions apparently can more than compensate for any imperfection in binding geometry and make the nontemplating RRW bind to MINP(KKW) more strongly than the templating KKW.

The binding selectivity so far was determined from the different binding constants of various peptides for the same MINP. To further confirm the selectivity, we added MINP(KWW) to an equimolar mixture of four peptides (KWW, RWW, SWW, and KKW). The peptide mixtures contained the template and three other representative peptides from Figure 2. The amounts of peptides adsorbed by the MINPs were determined by LCMS after ultracentrifugation to precipitate the MINPs and washing the MINPs with methanol (see the Experimental Section for details). The peptide binding assay essentially represents a solid-phase extraction study. The percent yields of recovery were then plotted against the amounts of MINP used in the extraction (Figures 3 and 4).

In general, the extraction yields were in line with the binding constants of the different peptides. For example, KKW showed the weakest binding for MINP(KWW) among the four peptides, whether the MINP contained FM **4** or not (Figure 2). During the extractions, its adsorption by the MINP was also the lowest, whether the MINP contained FM **4** or not (Figures 3 and 4). MINP(KWW) without the azacrown FM were able to distinguish SWW and RWW from its template in Figure 2. The extraction yields of SWW and RWW by MINP(KWW) were also lower than that of KWW in Figure 3.

On the other hand, MINP(KWW) with FM **4** had difficulty distinguishing RWW but distinguished SWW quite well (Figure 2). The extraction yields showed exactly the same trend (Figure 4), suggesting the binding selectivity was maintained in mixtures of peptides. These results, obtained from a single extraction, bode well for the usage of MINPs in chromatographic separation of peptides.

Binding of Biological Basic Peptides

Figure 5 shows the binding data for several biological peptides, all containing a significant number of lysine/arginine. PKKKRKV is a nuclear localization signal peptide very useful in delivery.[60] Without FM **4**, its MINP receptor gave a dissociation constant (K_d) of 5.5 μM and the azacrown FM was able to lower the K_d to 0.96 μM. The other three peptides are KKALRRQETVDAL (a selective substrate for Ca^{2+}/calmodulin-dependent protein kinase),

RVLSFIKGTK (an immune epitope for Influenza A virus), LRRASLG (a phosphate receptor peptide). With slightly more hydrophobic residues and/or a longer chain length, they could be bound by the azacrown-functionalized MINP receptor with affinities in the range of 100–200 nM in water.

Conclusions

A general method to recognize oligopeptides in a sequence-selective manner has been an unsolved problem in supramolecular and bioorganic chemistry for decades. With the micellar imprinting technique, we can prepare and purify artificial peptide receptors in 2 days if all the starting surfactants, FMs, and cross-linkers are available. The receptors can distinguish peptides based on their hydrophobic side chains[43] and, as shown in this work, recognize basic amino acids (lysine and arginine) when appropriate functional monomers are included.

Importantly, the azacrown-derived FM **4** generally improved both the binding affinity and selectivity of the imprinted micelles (Table 1, Figure 2). Although two reversed selectivities were observed in our binding studies (Table 2), it is fortunate that the parent MNP was more selective for lysine and the azacrown-functionalized MINP more selective for arginine. Thus, selectivity for these two particular amino acids could be controlled by whether or not to include the azacrown in the formation. According to our previous study, all residues contribute to the binding affinity and selectivity, either by primary or secondary (i.e., background) interactions.[43] As long as two peptide differ in multiple places including in chain length, their distinction is quite likely because every mismatch contributes to selectivity.

Experimental Section

Syntheses of compound **1**–**3** have been reported.[42]

Compound 4

To a solution of diaza-18-crown-6 (131 mg, 0.5 mmol) in DCM (6 mL), 4-vinylbenzenesulfonyl chloride (405 mg, 2 mmol) in DCM (4 mL) and TEA (278 μL, 2 mmol) were added dropwise at 0 °C gradually. The mixture was allowed to warm to room temperature and stirred overnight. The organic solution was extracted with water (3×10 mL), dried over anhydrous sodium sulfate and concentrated by rotary evaporation. The residue was purified by column chromatography over silica gel using 1: 200 methanol/DCM as eluent to afford compound 4 as white powder (201 mg, 67 %). ^{1}H NMR (600 MHz, DMSO-d$_6$) δ 7.77 (d, $J = 8.0$ Hz, 2H), 7.68 (d, $J = 8.0$ Hz, 2H), 6.82 (dd, $J_1 = 17.6$ Hz, $J_2 = 10.9$ Hz, 1H), 6.01 (d, $J = 17.6$ Hz, 1H), 5.75 (d, $J = 1.3$ Hz, 1H), 5.45 (d, $J = 10.9$ Hz, 1H), 3.52 (t, $J = 5.9$ Hz, 4H), 3.45 (s, 4H), 3.30 (t, $J = 6.0$ Hz, 4H) ppm. ^{13}C NMR (100 MHz, d6-DMSO) δ 141.7, 138.42 135.7, 127.7, 127.3, 118.3, 70.3, 70.1, 49.0. ESI-HRMS calcd for $C_{28}H_{38}N_2O_8S_2$ (*m/z*): [M + H]$^+$, 595.2142; found, 595.2147.

Typical procedure for the synthesis of MINPs.[42]

To a micellar solution of compound **1** (9.3 mg, 0.02 mmol) in H_2O (2.0 mL), divinylbenzene (DVB, 2.8 μL, 0.02 mmol), compound **4** in H_2O (10 μL of a 14.1 mg/mL in H_2O, 0.0004 mmol), and 2,2-dimethoxy-2-phenylacetophenone (DMPA,10 μL of a 12.8 mg/mL solution in DMSO, 0.0005 mmol) were added. The mixture was subjected to ultrasonication for 10 min before compound **2** (4.1 mg, 0.024 mmol), $CuCl_2$ (10 μL of a 6.7 mg/mL solution in H_2O, 0.0005 mmol), and sodium ascorbate (10 μL of a 99 mg/mL solution in H_2O, 0.005 mmol) were added. After the reaction mixture was stirred slowly at room temperature for 12 h, compound **3** (10.6 mg, 0.04 mmol), $CuCl_2$ (10 μL of a 6.7 mg/mL solution in H_2O, 0.0005 mmol), and sodium ascorbate (10 μL of a 99 mg/mL solution in H_2O, 0.005 mmol) were added. After being stirred for another 6 h at room temperature, the reaction mixture was transferred to a glass vial, purged with nitrogen for 15 min, sealed with a rubber stopper, and irradiated in a Rayonet reactor for 12 h. The reaction mixture was poured into acetone (8 mL). The precipitate was collected by centrifugation and washed with a mixture of acetone/water (5 mL/1 mL) three times, followed by methanol/acetic acid (5 mL/0.1 mL) three times. The product was dried in air to afford MINP(**4**). Yields generally were >80%.

ITC Titration

ITC was performed using a MicroCal VP-ITC Microcalorimeter with Origin 7 software and VPViewer2000 (GE Healthcare, Northampton, MA). The determination of binding constants by ITC followed standard procedures.[61–63] In general, a solution of an appropriate guest in Millipore water was injected in equal steps into 1.43 mL of the corresponding MINP in the same solution. The top panel shows the raw calorimetric data. The area under each peak represents the amount of heat generated at each ejection and is plotted against the molar ratio of the MINP to the guest. The smooth solid line is the best fit of the experimental data to the sequential binding of N binding site on the MINP. The heat of dilution for the guest, obtained by titration carried out beyond the saturation point, was subtracted from the heat released during the binding. Binding parameters were auto-generated after curve fitting using Microcal Origin 7.

Solid-Phase Extraction of Peptides

Four 1.0 mL solutions of peptide mixtures were prepared in Millipore water containing KWW, RWW, SWW and KKW each at 100 μM. MINP(KWW) was added to the four solutions, at 100, 200, 300, and 400 μM, respectively. The solutions were diluted to 100 mL by Millipore water and stirred at room temperature overnight.[64] For the determination of the amounts of peptides adsorbed by the MINPs, the solutions were centrifuged at 42,000 g for 2 min to precipitate the MINPs. The MINP precipitates were washed with methanol (3×10 mL) and the combined organic phase was concentrated under vacuum to dryness for each sample. The residue in each sample was dissolved in 0.95 mL of Millipore water, followed by the addition of 50 μL of 1.0 mM *L*-Tryptophan aqueous solution as the internal standard. The amounts of the peptides in the solutions were analyzed by an Agilent 1200 Series Binary VWD HPLC system coupled to an Agilent 6540 UHD Accurate Mass Q-TOF mass spectrometry detector.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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64. The extractions were performed at micromolar concentrations because of the strong binding affinities of the MINP for the peptides.
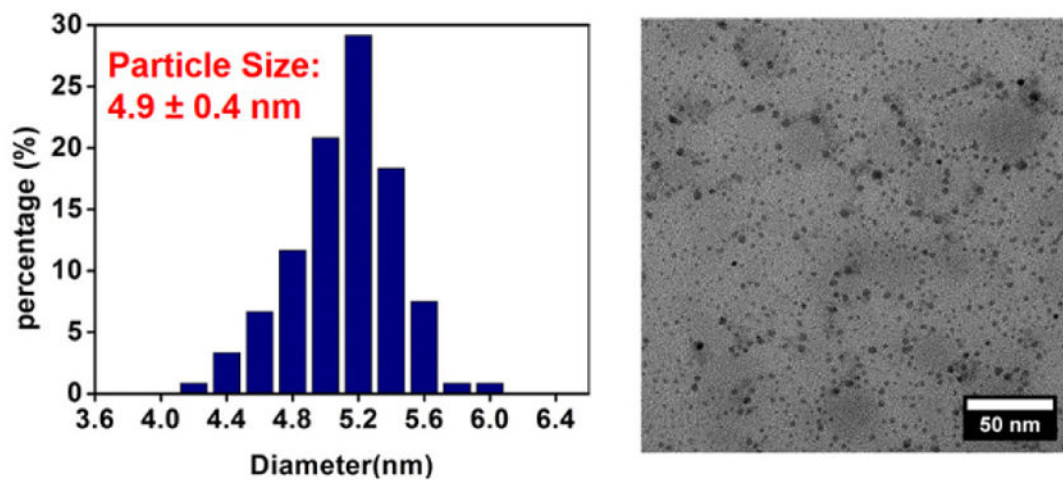
FIGURE 1.
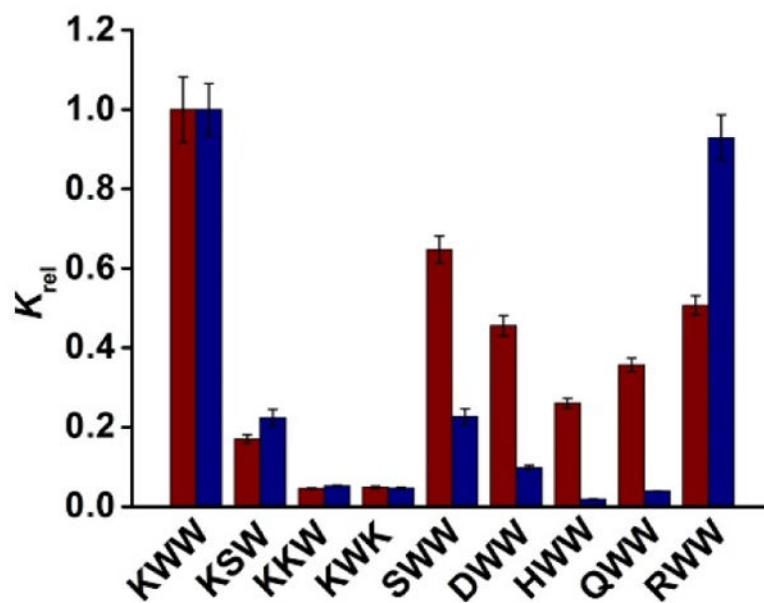Particle size distribution from TEM imaging of MINPs.

FIGURE 2.
Binding selectivity of MINP(KWW) prepared without (red bars) and with FM **4** (blue bars). The binding data are reported in Table S1 in the Supporting Information.
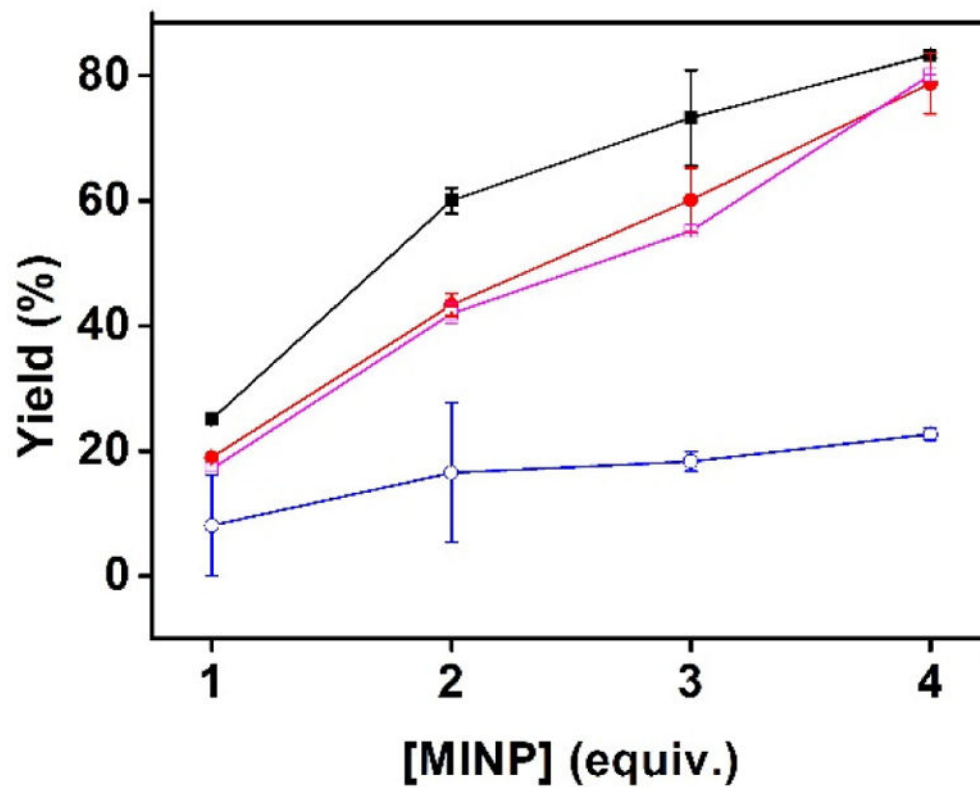
FIGURE 3.
Extraction yields of different peptides (■: KWW; ●: RWW; □: SWW; ○: KKW) by
MINP(KWW) prepared without FM **4**. [All peptides in the mixture] = 1.0 μM. [MINP used
for extraction] = 1–4 μM.

FIGURE 4.
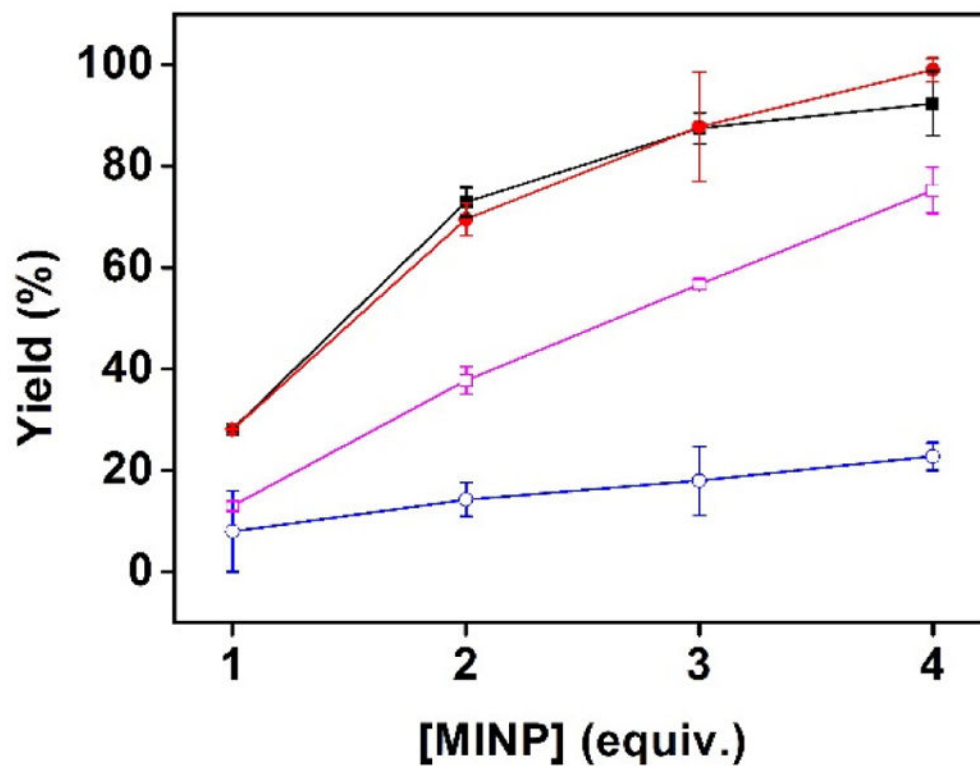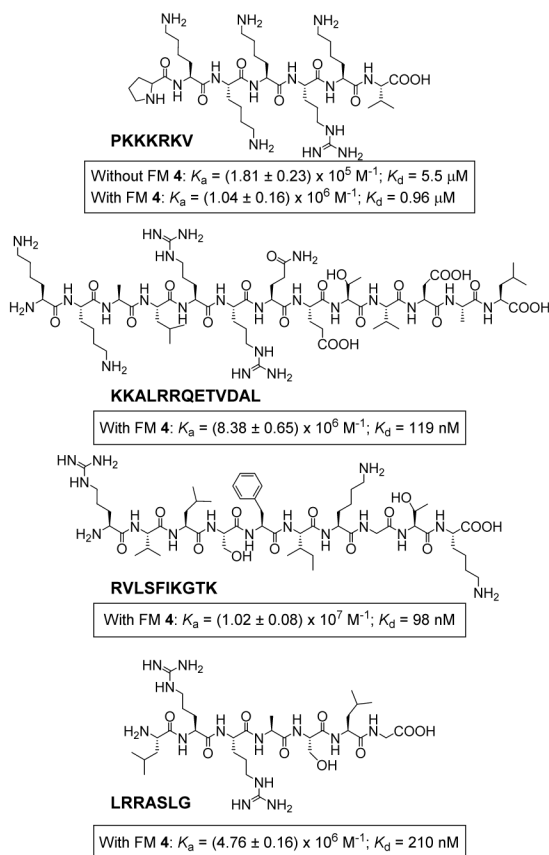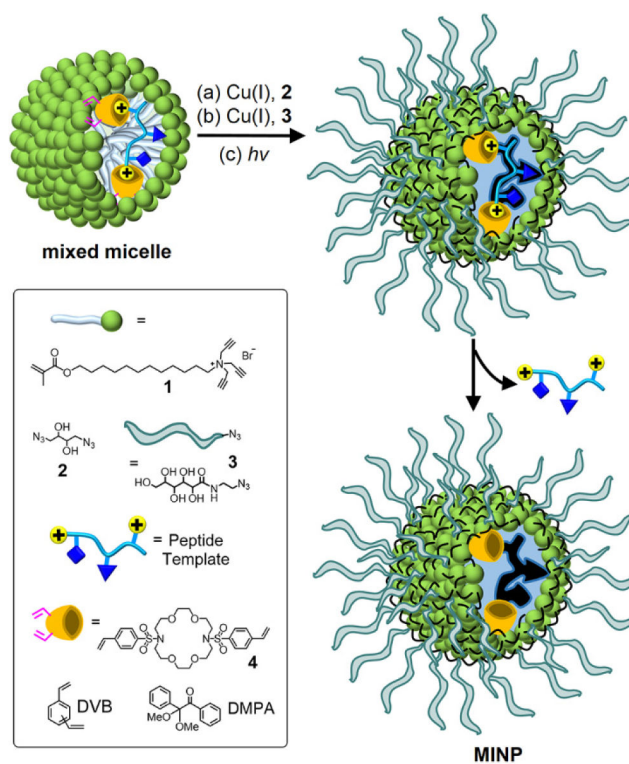Extraction yields of different peptides (■: KWW; ●: RWW; □: SWW; ○: KKW) by
MINP(KWW) prepared with 2 equiv FM **4**. [All peptides in the mixture] = 1.0 μM. [MINP
used for extraction] = 1–4 μM.

FIGURE 5.

Structures of biological peptides examined in this study and the binding constants (K_a) and dissociation constants (K_d) obtained.

SCHEME 1.
Preparation of peptide-binding MINP.

Table 1

Comparison of binding for peptides by MINPs prepared with and without FM **4**.[a]

Entry	template	FM	– G (kcal/mol)	K_a (×10⁵ M⁻¹)	K_{rel}
1	KWW	none	8.62	20.7 ± 1.7	1
	KWW[b]	none	8.58	19.5 ± 2.2	--
	KWW[c]	none	8.47	16.3 ± 1.8	--
2	KWW	1 equiv **4**	8.78	27.5 ± 1.2	1.33
3	KWW	2 equiv **4**	8.96	36.7 ± 2.4	1.77
	KWW[b]	2 equiv **4**	8.89	33.1 ± 1.9	--
	KWW[c]	2 equiv **4**	8.99	39.2 ± 3.0	--
4	KWW	3 equiv **4**	8.94	35.9 ± 2.2	1.73
5	KKK	none	5.56	0.12 ± 0.02	1
	KKK[b]	none	5.70	0.15 ± 0.01	--
	KKK[c]	none	5.32	0.08 ± 0.01	--
6	KKK	2 equiv **4**	5.74	0.16 ± 0.02	1.3
7	KKK	3 equiv **4**	6.58	0.67 ± 0.02	5.6
8	KKK	4 equiv **4**	7.10	1.59 ± 0.02	13
	KKK[b]	4 equiv **4**	7.04	1.45 ± 0.12	--
	KKK[c]	4 equiv **4**	7.24	2.04 ± 0.10	--
9	KKK	5 equiv **4**	7.08	1.54 ± 0.08	13
10	KKK	6 equiv **4**	7.08	1.55 ± 0.04	13
11	WKW	none	8.33	12.8 ± 0.6	1
12	WKW	2 equiv **4**	8.88	32.1 ± 2.3	2.51
13	KKW	none	7.19	1.86 ± 0.05	1
14	KKW	3 equiv **4**	7.65	4.08 ± 0.24	2.20
15	WKK	none	7.26	2.11 ± 0.07	1
16	WKK	3 equiv **4**	7.60	3.70 ± 0.09	1.75
17	KWK	none	6.98	1.31 ± 0.05	1
18	KWK	3 equiv **4**	7.81	5.29 ± 0.52	4.04
19	RWW	none	8.41	14.7 ± 1.0	1

Entry	template	FM	− G (kcal/mol)	K_a (×10^5 M^{-1})	K_{rel}
20	RWW	2 equiv 4	9.05	43.1 ± 3.8	2.93
21	RRW	none	6.92	1.18 ± 0.04	1
22	RRW	3 equiv 4	8.11	8.77 ± 0.52	7.43

[a]Binding constants were determined by fluorescence titrations in Millipore water for tryptophan-containing peptides and by ITC for KKK. K_{rel} is the binding constant of the MINP with FM 4 relative to that without in Millipore water.

[b]The binding was measured by ITC in HEPES buffer (pH 7.0).

[c]The binding was measured by ITC in HEPES buffer (pH 4.7).

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Table 2

Differentiation of lysine (K) and arginine acid (R).[a]

Entry	Host	Guest	FM 4	K_a ($\times 10^5\,M^{-1}$)	K_{rel}
1	MINP(KKW)	KKW	none	1.86 ± 0.05	
2	MINP(KKW)	RRW	none	0.81 ± 0.02	0.44
3	MINP(RRW)	RRW	none	1.18 ± 0.04	
4	MINP(RRW)	KKW	none	1.27 ± 0.02	1.09
5	MINP(KKW)	KKW	3 equiv	4.08 ± 0.24	
6	MINP(KKW)	RRW	3 equiv	4.57 ± 0.13	1.12
7	MINP(RRW)	RRW	3 equiv	8.77 ± 0.52	
8	MINP(RRW)	KKW	3 equiv	3.32 ± 0.17	0.38

[a] The K_a values were determined by fluorescence titrations in Millipore water. K_{rel} is the binding constant of a guest relative to that of the templating peptide for a particular MINP.