IOWA STATE UNIVERSITY
Digital Repository

Chemistry Publications | Chemistry

2017

Sequence-Selective Binding of Oligopeptides in Water through Hydrophobic Coding

Joseph K. Awino
Iowa State University, jkhadori@iastate.edu

Roshan W. Gunasekara
Iowa State University

Yan Zhao
Iowa State University, zhaoy@iastate.edu

Follow this and additional works at: https://lib.dr.iastate.edu/chem_pubs

Part of the Materials Chemistry Commons, Nanoscience and Nanotechnology Commons, and the Organic Chemistry Commons

The complete bibliographic information for this item can be found at https://lib.dr.iastate.edu/chem_pubs/1094. For information on how to cite this item, please visit http://lib.dr.iastate.edu/howtocite.html.

This Article is brought to you for free and open access by the Chemistry at Iowa State University Digital Repository. It has been accepted for inclusion in Chemistry Publications by an authorized administrator of Iowa State University Digital Repository. For more information, please contact digirep@iastate.edu.

Sequence-Selective Binding of Oligopeptides in Water through Hydrophobic Coding

Abstract
A general method for sequence-specific binding of peptides remains elusive despite decades of research. By creating an array of "hydrophobically coded dimples" on the surface of surface–core doubly cross-linked micelles, we synthesized water-soluble nanoparticle receptors to recognize peptides by the location, number, and nature of their hydrophobic side chains. Minute differences in the side chains could be distinguished, and affinities up to 20 nM were obtained for biologically active oligopeptides in water.

Disciplines
Materials Chemistry | Nanoscience and Nanotechnology | Organic Chemistry

Comments
This document is the Accepted Manuscript version of a Published Work that appeared in final form in *Journal of the American Chemical Society*, copyright © American Chemical Society after peer review and technical editing by the publisher. To access the final edited and published work see DOI: 10.1021/jacs.6b12949. Posted with permission.

This article is available at Iowa State University Digital Repository: https://lib.dr.iastate.edu/chem_pubs/1094

Author Manuscript
Author Manuscript
Author Manuscript
Author Manuscript

HHS Public Access
Author manuscript
J Am Chem Soc. Author manuscript; available in PMC 2018 February 15.

Published in final edited form as:
J Am Chem Soc. 2017 February 15; 139(6): 2188–2191. doi:10.1021/jacs.6b12949.

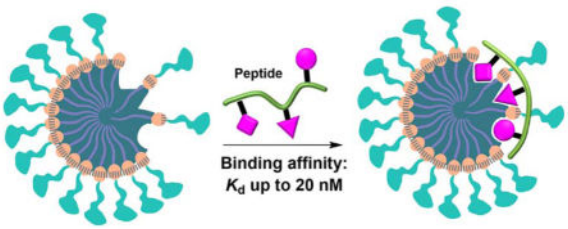
Sequence-Selective Binding of Oligopeptides in Water through Hydrophobic Coding

Joseph K. Awino, **Roshan W. Gunasekara**, and **Yan Zhao***
Department of Chemistry, Iowa State University, Ames, Iowa 50011-3111

Abstract

A general method for sequence-specific binding of peptides remains elusive despite decades of research. By creating an array of "hydrophobically coded dimples" on the surface of a surface–core doubly cross-linked micelle, we synthesized water-soluble nanoparticle receptors to recognize peptides by the location, number, and nature of their hydrophobic side chains. Minute differences in the side chains could be distinguished and affinities up to 20 nM were obtained for biologically active oligopeptides in water.

Graphical Abstract



Chemists and biologists have long been interested in sequence-specific molecular recognition of oligopeptides.[1–12] It represents the first step toward a general method for protein recognition.[13] Also, oligopeptides act as neurotransmitters, neuromodulators, and hormones in many organisms and their interactions with protein receptors influence cell–cell communications, metabolism, and immune response.[14] Peptide-recognizing synthetic materials thus can be used to better understand these interactions, inhibit them when necessary, and isolate or detect these peptides in their natural milieu.

Many materials have been used to construct peptide receptors including macrocycles[1,3] such as cyclodextrin[2,4] and cucurbituril,[8,10,12] amide oligomers[1,6,7,9,11] and self-assembled nanocages.[5] Molecularly imprinted polymers[15–20] have also been employed.[21–26] Despite decades of research, however, a general method for sequence-selective binding of peptides in water remains elusive.[13,27] A notable challenge comes from the recognized difficulty in

Corresponding Author: zhaoy@iastate.edu.

The authors declare no competing financial interests.

Supporting Information
Experimental details, ITC titration curves, and additional figures. This material is available free of charge via the Internet at http://pubs.acs.org.

Author Manuscript Author Manuscript Author Manuscript Author Manuscript

molecular recognition in water.[28,29] Another challenge is derived from the small differences between many amino acids: leucine (L) and isoleucine (I) differ by the position of one methyl group; phenylalanine (F) misses a single hydroxyl from tyrosine (Y); and glutamic acid contains one extra methylene than aspartic acid. Also, when several amino acids exchange positions on a peptide, the overall hydrophobicity and charge characters stay the same but the peptides become completely different from the structural and functional point of view.

Herein, we report water-soluble nanoparticle receptors that can differentiate peptides based on the location, number, and nature of their hydrophobic side chains. Minute differences in the side chains could be distinguished and affinities up to 20 nM were obtained for biologically active oligopeptides in water.

The design of our peptide-binding materials is based on the “hydrophobic coding” of a peptide. Hydrophobic interactions are often considered nonspecific. However, a peptide chain consists of amino acids with varying degrees of hydrophobicity. Even for the conventionally classified hydrophobic amino acids such as leucine, isoleucine, and tryptophan (W), their side chains differ in size, shape, and hydrophobicity. Thus, a “hydrophobic code” exists with each peptide that describes the number, size, shape, and distribution of hydrophobic side chains. As long as a complementary array of hydrophobic indentations or “dimples” can be created on a material to match this code, the material should be able to bind the peptide strongly and selectively.

To create the complementary hydrophobic code for a peptide, we turned to molecular imprinting in micelles, a method recently developed by our laboratory.[30,31] In general, a hydrophobic template molecule is solubilized by the micelle of cross-linkable surfactant **1** in water (Scheme 1). Click-cross-linking using diazide **2** yields an alkyne-functionalized surface-cross-linked micelle (alkynyl-SCM), which is conveniently functionalized by ligand **3** using another round of click reaction.[32] Free radical polymerization is then initiated photochemically using DMPA (the photoinitiator) in the core to cross-link the methacrylate of **1** and DVB solubilized in the micelle. After the tem-plate is removed by repeated solvent washing, a hydrophobic binding site is left on the surface of the resulting molecularly imprinted nanoparticle (MINP), complementary to the template in size and shape.

The method worked well for large hydrophobic molecules, and MINPs have been prepared to distinguish bile salt derivatives[30] and aromatic sulfonates.[33] With appropriate functional monomers, MINP could also be made to bind hydrophilic compounds such as sugars.[34] However, the subtle differences among the hydrophobic side chains of amino acids make their differentiation particularly challenging.

To find out whether peptide receptors could be created through micellar molecular imprinting, we first studied peptides containing tryptophan (Table 1). It large size, strong hydrophobicity, and fluorescence make such peptides excellent candidates to test the concept.

The results were very promising. MINP imprinted against WWGG, for example, bound its template in water with a very impressive binding constant ($K_a = 970 \times 10^4\ M^{-1}$) according

Author Manuscript Author Manuscript Author Manuscript Author Manuscript

to isothermal titration calorimetry (ITC), equivalent to a binding free energy of $\Delta G = -9.52$ kcal/mol (entry 1). ITC also showed an average of 0.92 binding sites (N) per nanoparticle. This feature was achieved by keeping the surfactant/template ratio close to the micelle aggregation number (~50) during MINP preparation. With a higher ratio of template used, we have shown previously that more than more binding site could be obtained on the MINP.[30] The binding affinity obtained by ITC was confirmed by fluorescence titration (Table S1, Figure S40). The 1:1 binding stoichiometry was also verified by the Job plot (Figure S44). In HEPES buffer (pH 7.4), a very similar binding constant ($K_a = 936 \times 10^4$ M^{-1}) was obtained.

Because hydrophobic interactions are the main driving force in typical MINP binding,[30] we expected the positions of the tryptophan groups to play a critical role in the binding selectivity. Because WWGG and GWWG both have the two hydrophobic side chains right next to each other, we had thought it would be difficult for the MINP to distinguish the two. Surprisingly, the binding of GWWG by MINP(WWGG) was nearly 5 times weaker than that of the template (entry 3). Thus, even the glycine, which lacks a hydrophobic side chain, affected the binding. Possibly, the hydrophilic groups such as the amides and the carboxylate of the peptides engaged in hydrogen bonds and electrostatic interactions with the MINP. These interactions might have played secondary roles in the binding and selectivity of the imprinted receptor.

When one or two glycine residues were inserted into the two tryptophans, the peptide in principle should be able to fold itself to insert its tryptophans into the binding pockets imprinted from WWGG. The binding, however, should be weaker due to the unfavorable conformational change imposed by the binding. The prediction was confirmed by our experiments: K_a for WGWG and WGGW by MINP(WWGG) was ~13 and 23 times weaker, respectively, than that for the template itself (compare entries 4 and 5 with 1).

We also prepared MINPs for the other three peptides and all the MINPs bound their own templating peptides well, with K_a in the range of 400–750 × 10^4 M^{-1} (Table 1, entries 6–8). In general, binding was stronger when the templating peptides had the two aromatic side chains closer to each other. The trend most likely was a result of hydrophobic imprinting. Assuming that the binding is mainly driven by hydrophobic interactions, the overall hydrophobic driving force is largely the same among the four peptides, determined by the (same) size and number of the hydrophobic side chains (W). For peptides with the two tryptophans next to each other, their two glycines could move relatively freely after binding. For the peptides with the tryptophans farther apart, the glycine(s) in between would be restricted by the two hydrophobic anchors (i.e., tryptophans) upon binding, and this decrease of freedom might have weakened the binding.[35]

Encouraged by these results, we turned to peptides with smaller hydrophobic side chains. MINP(FF) bound its own template with a K_a of 92.2 × 10^4 M^{-1} (Table 2, entry 1). The value is an order of magnitude lower than that between WWGG and its MINP (Table 1, entry 1). These results support hydrophobic interactions being chiefly responsible for the binding: because phenylalanine is smaller than tryptophan, placing the hydrophobic groups of FF in a

Author Manuscript Author Manuscript Author Manuscript Author Manuscript

complementary binding pocket buries a smaller hydrophobic surface area than doing so with WWGG.

The most exciting results came from the binding selectivity. Replacing one of the two phenylalanines with other hydrophobic amino acids weakened the binding significantly. Alanine- and isoleucine-replacement lowered K_a by 21- and 40-fold, respectively (entries 2 and 3). Interestingly, leucine was tolerated by MINP(FF) much better than isoleucine (entries 4 and 5), even though these two are constitutional isomers with identical number of carbons.

The large difference between leucine and isoleucine suggests that our MINP has a very strong shapememory of the template. As shown in Figure 1, the benzyl group of phenylalanine has a primary carbon bonded to the α carbon of the amino acid, followed by a secondary carbon on the phenyl ring. This particular pattern also exists in leucine and is probably why the residue was tolerated by the phenylalanine-imprinted binding pocket. Isoleucine, on the other hand, has a secondary carbon bonded to the α carbon and should have difficulty fitting into the same binding site if the site closely resembles benzyl in shape and size.

The shape memory testifies to the success of our micellar cross-linking. In the MINP preparation, we typically use a 1:1 ratio between the cross-linkable surfactant **1** and DVB. With approximately 50 DVB molecules in a doubly cross-linked micelle ~4 nm in diameter, the cross-linking density is very high. As shown by our previous work, a high cross-linking density is essential to the binding selectivity of the MINP.[30]

Another interesting feature of the peptide-binding MINP is that binding sites created after a larger hydrophobic group can bind a smaller group (albeit with a lower affinity) but the reverse is not true. MINP(FF), for example, showed very weak binding for FW (Table 2, entry 5), which has a larger hydrophobic group (tryptophan). In other words, despite its stronger driving force to enter a hydrophobic pocket, FW had difficulty fitting into the pocket custom-designed for FF. Likewise, MINP(FF) showed an extremely weak binding affinity for Boc-protected FF (entry 7), but MINP(Boc-FF) a considerable affinity for FF, with $K_a = 41 \times 10^4\ M^{-1}$ (entry 9).

Hydrophobicity clearly is the most important factor, as the binding for Boc-FF by MINP(Boc-FF) was significantly stronger than FF by MINP(FF). The binding data showed that a single *t*-butyl contributed ~0.65 kcal/mol to the binding (compare entries 1 and 8). Likely for the same reason, insertion of a hydroxyl on the phenyl ring weakened the binding by nearly 1.9 kcal/mol (entry 6).

Entries 10–13 of Table 2 show the binding selectivity of the MINP created from tripeptide FGL. The peptide has two hydrophobic residues (phenylalanine and leucine) and the small size of leucine makes the imprinting even more challenging. Excellent selectivity, nonetheless, was observed once again. MINP(FGL) easily detected a change from leucine to isoleucine (entry 11), an exchange of positions between leucine and glycine (entry 12), and an insertion of a glycine in between the two hydrophobic residues (entry 13). Remarkably, all changes lowered the K_a value by at least one and sometimes two orders of magnitude.

Author Manuscript Author Manuscript Author Manuscript Author Manuscript

Being confident that our molecular imprinting is able to detect minute changes in hydrophobicity in peptides, we decided to create receptors for several biologically active peptides consisting of seven to over a dozen amino acids (**4**–**8**). All the peptides contain some hydrophobic amino acids but are fully soluble in water.

Gratifyingly, all the MINPs showed excellent binding properties toward their templating peptides (Table 2, entries 14–18). Due to a larger number of hydrophobic residues in the peptide chain, the binding affinities were substantially higher than those listed in Table 1, up to $K_a = 4520 \times 10^4\ M^{-1}$ or a dissociation constant of $K_d \approx 20$ nM.

The selectivity of the MINPs for the biological peptides was remarkable. Figure 2a shows a cross reactivity study, with the five different peptides titrated into the MINP(**5**) solution. ITC titration showed that only the templating peptide was able to bind and all the other peptides were completely silent, despite the presence of similar hydrophobic residues. A reverse cross reactivity study was also performed, using peptide **6** as the example. When the peptide was titrated into different MINP solutions, only MINP**(6)** showed a response (Figure 2b). These experiments not only demonstrated the specificity of the MINP receptors but also ruled out nonspecific binding—which is always a concern for hydrophobically driven molecular recognition—playing any significant roles in our peptide-binding nanoparticles.

A general method for sequence-selective binding of peptides is an important goal in supramolecular and bioorganic chemistry.[13] Without the method of molecular imprinting, it would be difficult to imagine bottom-up construction of synthetic hosts for guests as complex as peptides **4**–**8**. These long and complex peptides contain much more information (on hydrophobicity) than a short peptide. It seems that the information-richer “hydrophobic codes” of these peptides made their distinction by the MINP receptors even easier than the shorter peptides—a pleasant outcome of our strategy. Essentially, because every hydrophobic side chain (and, to a lesser degree, hydrophilic groups that interact with MINP through hydrogen bonds and electrostatic interactions) con-tributes to both the binding affinity and selectivity, the longer the peptide, the stronger the binding and the more selective the MINP receptor will be, as long as the peptide contains a sufficient number of hydrophobic residues.

What is significant in our peptide-binding MINPs is that minute changes in hydrophobic side chains can be differentiated. Another important feature is the generality of the method. Complementary arrays of hydrophobic “dimples” were simply created by “chemical molding” around the interested peptides, without any need for individual design. The same method worked for small and large peptides. Once all the staring materials are available, the entire synthesis and purification of the receptors can be done in 2–3 days, without any special technique. The materials tolerate high temperatures and organic solvents.[30,31] These features should help their adoption by chemists and nonchemists when strong and selective “synthetic antibodies” are needed to bind peptides.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Author Manuscript Author Manuscript Author Manuscript Author Manuscript

Acknowledgments

We thank the National Institute of General Medical Sciences of the National Institutes of Health (R01GM113883) for financial support of this research.

References

1. Hong JI, Namgoong SK, Bernardi A, Still WC. J Am Chem Soc. 1991; 113:5111.
2. Breslow R, Yang Z, Ching R, Trojandt G, Odobel F. J Am Chem Soc. 1998; 120:3536.
3. Hossain MA, Schneider HJ. J Am Chem Soc. 1998; 120:11208.
4. Yamamura H, Rekharsky MV, Ishihara Y, Kawai M, Inoue Y. J Am Chem Soc. 2004; 126:14224. [PubMed: 15506789]
5. Tashiro S, Tominaga M, Kawano M, Therrien B, Ozeki T, Fujita M. J Am Chem Soc. 2005; 127:4546. [PubMed: 15796499]
6. Wright AT, Anslyn EV, McDevitt JT. J Am Chem Soc. 2005; 127:17405. [PubMed: 16332090]
7. Schmuck C, Wich P. Angew Chem Int Ed. 2006; 45:4277.
8. Reczek JJ, Kennedy AA, Halbert BT, Urbach AR. J Am Chem Soc. 2009; 131:2408. [PubMed: 19199617]
9. Niebling S, Kuchelmeister HY, Schmuck C, Schlucker S. Chem Sci. 2012; 3:3371.
10. Smith LC, Leach DG, Blaylock BE, Ali OA, Urbach AR. J Am Chem Soc. 2015; 137:3663. [PubMed: 25710854]
11. Faggi E, Vicent C, Luis SV, Alfonso I. Org Biomol Chem. 2015; 13:11721. [PubMed: 26481115]
12. Sonzini S, Marcozzi A, Gubeli RJ, van der Walle CF, Ravn P, Herrmann A, Scherman OA. Angew Chem Int Ed. 2016; 55:14000.
13. Peczuh MW, Hamilton AD. Chem Rev. 2000; 100:2479. [PubMed: 11749292]
14. Sewald, N., Jakubke, H-D. Peptides: chemistry and biology. Wiley-VCH; Weinheim: 2002.
15. Wulff G. Angew Chem Int Ed Engl. 1995; 34:1812.
16. Zimmerman SC, Lemcoff NG. Chem Commun. 2004:5.
17. Haupt K, Mosbach K. Chem Rev. 2000; 100:2495. [PubMed: 11749293]
18. Alexander C, Andersson HS, Andersson LI, Ansell RJ, Kirsch N, Nicholls IA, O'Mahony J, Whitcombe MJ. J Mol Recognit. 2006; 19:106. [PubMed: 16395662]
19. Ye L, Mosbach K. Chem Mater. 2008; 20:859.
20. Sellergren, B., Hall, AJ. Supramol Chem. John Wiley & Sons, Ltd; 2012.
21. Klein JU, Whitcombe MJ, Mulholland F, Vulfson EN. Angew Chem Int Ed. 1999; 38:2057.
22. Nishino H, Huang CS, Shea KJ. Angew Chem Int Ed. 2006; 45:2392.
23. Hoshino Y, Kodama T, Okahata Y, Shea KJ. J Am Chem Soc. 2008; 130:15242. [PubMed: 18942788]
24. Hoshino Y, Koide H, Urakami T, Kanazawa H, Kodama T, Oku N, Shea KJ. J Am Chem Soc. 2010; 132:6644. [PubMed: 20420394]
25. Banerjee S, König B. J Am Chem Soc. 2013; 135:2967. [PubMed: 23398303]
26. Zhang Y, Deng C, Liu S, Wu J, Chen Z, Li C, Lu W. Angew Chem Int Ed. 2015; 54:5157.
27. Maity, D., Schmuck, C. Synthetic Receptors for Biomolecules: Design Principles and Applications. The Royal Society of Chemistry; 2015. p. 326
28. Oshovsky GV, Reinhoudt DN, Verboom W. Angew Chem Int Ed. 2007; 46:2366.
29. Kataev EA, Müller C. Tetrahedron. 2014; 70:137.
30. Awino JK, Zhao Y. J Am Chem Soc. 2013; 135:12552. [PubMed: 23931721]
31. Awino JK, Zhao Y. Chem-Eur J. 2015; 21:655. [PubMed: 25382073]
32. Zhang S, Zhao Y. Macromolecules. 2010; 43:4020.
33. Awino JK, Zhao Y. Chem Commun. 2014; 50:5752.
34. (a) Awino JK, Gunasekara RW, Zhao Y. J Am Chem Soc. 2016; 829:9759.(b) Gunasekara RW, Zhao Y. J Am Chem Soc. 2017; 139:9759.

Author Manuscript Author Manuscript Author Manuscript Author Manuscript

35. The explanantion mostly considers the conformational entropy of the peptide being bound. Because the binding enthalpy and entropy determined by ITC also included the contributions from released water molecules during binding, we could not identify the exact cause of the trend from the ITC binding data.

Author Manuscript Author Manuscript Author Manuscript Author Manuscript

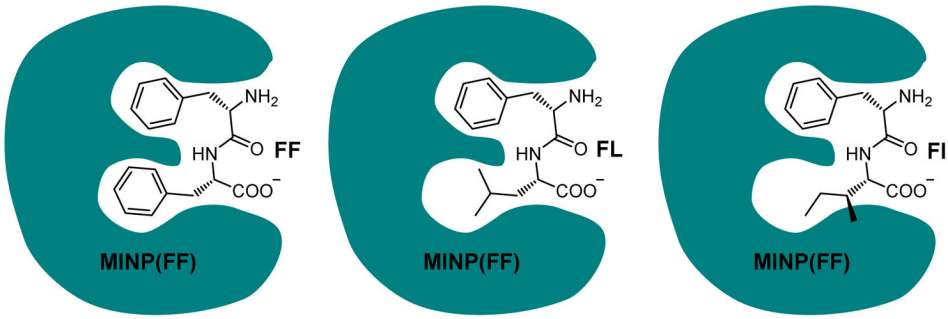


Figure 1.
Comparison of FF, FI, and FL in the binding site of MINP(FF).

Author Manuscript Author Manuscript Author Manuscript Author Manuscript

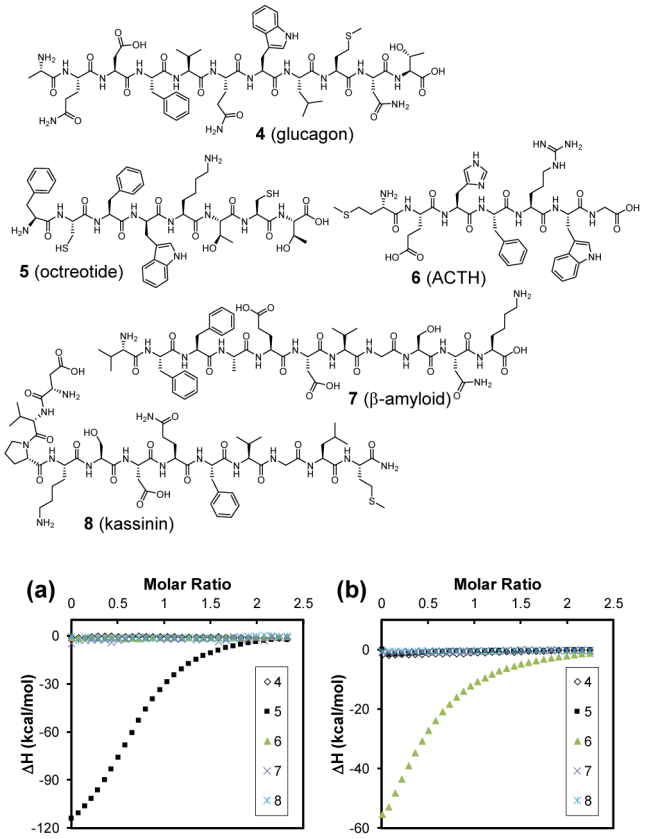
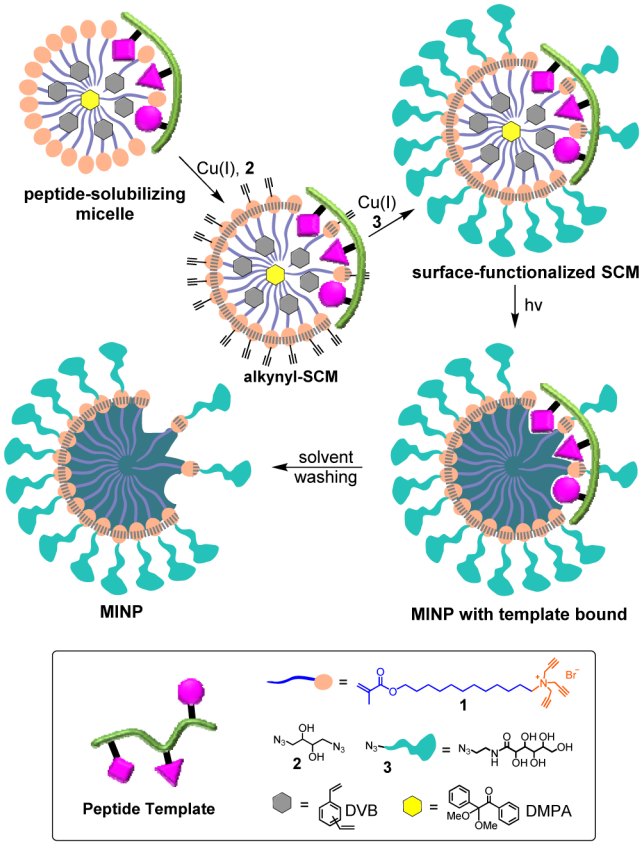


Figure 2.

(a) Cross reactivity study for the binding of peptides **4**–**8** to MINP(**5**), showing only **5** bound during the ITC titrations. [MINP] = 6.0 μM. [Peptide] = 60 μM. (b) Cross reactivity study for the binding of peptide **6** to MINP(**4**)–MINP(**8**), showing only MINP(**6**) binding during the ITC titrations. [MINP] = 10.0 μM. [Peptide] = 90 μM.

Author Manuscript Author Manuscript Author Manuscript Author Manuscript



Scheme 1.

Preparation of peptide-binding MINP from molecular imprinting in a cross-linked micelle.

Author Manuscript Author Manuscript Author Manuscript Author Manuscript

Table 1

Binding data for MINP obtained by ITC.[a]

Entry	Host	Guest	K_a(10^4 M^{-1})	ΔG (kcal/mol)	N[b]
1	MINP(WWGG)	WWGG	970 ± 77	−9.52	0.92 ± 0.01
2	MINP(WWGG)	WWGG[c]	936 ± 19	−9.50	0.92 ± 0.01
3	MINP(WWGG)	GWWG	199 ± 15	−8.59	1.08 ± 0.01
4	MINP(WWGG)	WGWG	72 ± 51	−7.99	1.07 ± 0.01
5	MINP(WWGG)	WGGW	43 ± 4	−7.68	1.17 ± 0.02
6	MINP(GWWG)	GWWG	742 ± 85	−9.37	1.02 ± 0.01
7	MINP(WGWG)	WGWG	562 ± 39	−9.20	1.00 ± 0.01
8	MINP(WGGW)	WGGW	429 ± 32	−9.04	0.99 ± 0.01

[a]The titrations were generally performed in duplicates in Millipore water and the errors between the runs were <10%. The titration curves are reported in the Supporting Information (Figures S37–S39) including the binding enthalpy and entropy.

[b]N is the number of binding site per nanoparticle measured by ITC.

[c]The titration was performed in HEPES buffer (pH 7.4).

Author Manuscript Author Manuscript Author Manuscript Author Manuscript

Table 2

Binding data for MINP obtained by ITC.[a]

Entry	Host	Guest	K_a(10^4 M^{-1})	ΔG (kcal/mol)	N^b
1	MINP(FF)	FF	92.2 ± 3.8	−8.13	0.84 ± 0.04
2	MINP(FF)	FA	4.40 ± 0.26	−6.33	0.59 ± 0.07
3	MINP(FF)	FI	2.28 ± 0.15	−5.94	0.63 ± 0.08
4	MINP(FF)	FL	16.5 ± 5.2	−7.11	0.62 ± 0.06
5	MINP(FF)	FW	0.63 ± 0.05	−5.18	0.91 ± 0.04
6	MINP(FF)	FY	3.85 ± 0.24	−6.25	0.91 ± 0.04
7	MINP(FF)	Boc-FF	0.026 ± 0.01	−3.29	1.31 ± 0.08
8	MINP(Boc-FF)	Boc-FF	274 ± 19	−8.78	0.77 ± 0.02
9	MINP(Boc-FF)	FF	41.2 ± 4.5	−7.65	1.03 ± 0.05
10	MINP(FGL)	FGL	91.4 ± 5.4	−8.13	0.61 ± 0.09
11	MINP(FGL)	FGI	8.39 ± 0.48	−6.71	0.47 ± 0.09
12	MINP(FGL)	FLG	0.72 ± 0.09	−5.26	1.29 ± 0.33
13	MINP(FGL)	FGGL	7.24 ± 0.38	−6.62	0.55 ± 0.12
14	MINP(**4**)	**4**	4520 ± 100	−10.4	0.96 ± 0.02
15	MINP(**5**)	**5**	2060 ± 100	−9.97	0.85 ± 0.06
16	MINP(**6**)	**6**	480 ± 4	−9.11	0.68 ± 0.03
17	MINP(**7**)	**7**	2850 ± 80	−10.2	0.74 ± 0.06
18	MINP(**8**)	**8**	860 ± 4	−9.45	0.75 ± 0.03

[a]The titrations were generally performed in duplicates in Millipore water and the errors between the runs were <10%. The titration curves are reported in the Supporting Information (Figures S45 –S49) including the binding enthalpy and entropy.

[b]*N* is the number of binding site per nanoparticle measured by ITC.