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Abstract
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molecular imprinting, micelles, cross-linking, binding, biomimetic, molecular recognition, hydrophobic interactions

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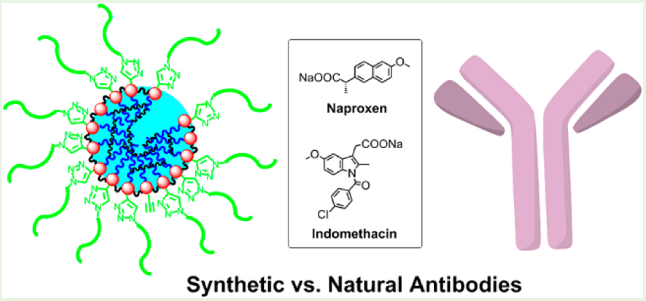
Supporting Information

ABSTRACT: The wide usage and subsequent leakage of nonsteroidal anti-inflammatory drugs (NSAIDs) into the environment present an urgent need to create materials for selective binding of NSAID drugs, which are highly similar to one another in structure and functionality. Surface−core double-cross-linking of cationic micelles containing Naproxen or Indomethacin as the template yielded molecularly imprinted nanoparticles (MINPs) for these drugs. The nanoparticle receptors resembled water-soluble proteins in their hydrophilic exterior and hydrophobic core with guest-tailored binding pockets. Their binding selectivity for their templates over other NSAID analogues rivaled that of antibodies prepared through much lengthier procedures.



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INTRODUCTION

The immune system has remarkable abilities to generate antibodies for virtually any molecule of biological interest. The strong and specific recognition of antigens by their antibodies is at the heart of immune response and vital to the biological host's survival. Their extraordinary molecular recognition also makes antibodies powerful tools in diagnostics, therapeutics, imaging, analysis, and elucidation of biological mechanisms.[1]

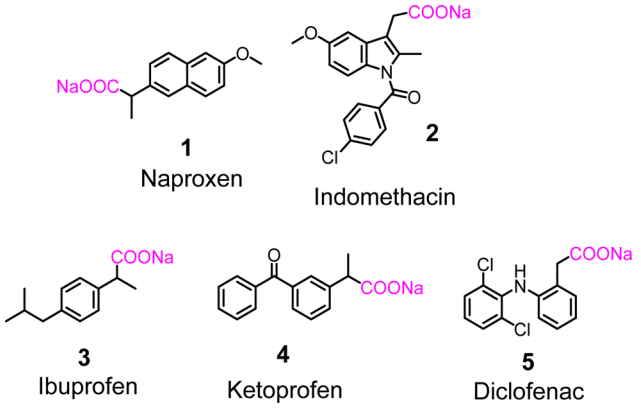
However, antibodies are expensive biomolecules requiring lengthy procedures to produce. Immunization of animals itself often takes weeks of time, even if the molecules readily elicit immune responses. The (polyclonal) antibodies generated then need to be isolated and purified. Monoclonal antibodies are even more cumbersome to prepare, as they need to come from a single cell line. Just like any proteins, antibodies are subject to denaturation, whether by adverse temperatures, adsorption to surfaces, or exposure to organic solvents, surfactants, or other chemical entities.

Chemists for decades have tried to create receptors for molecules of interest,[2,3] in a way similar to what nature does with antibodies. Although remarkable receptors have been made, sometimes with biological kind of affinity and specificity,[4−6] synthetic receptors tend to be limited to specific classes of molecules or ions and a general method to create strong and specific antibody-resembling receptors remains an elusive goal.

Nonsteroidal anti-inflammatory drugs (NSAIDs) are one of the most used over-the-counter drugs (see Chart 1 for selected structures).[7] Because of their wide usage and subsequent leakage into the environment, there is high interest in monitoring and detecting them in natural settings.[8] In addition to chemical methods, enzyme-linked immunosorbent assays (ELISA), which rely on NSAID-specific antibodies for operation, have been used for NSAID drug analysis.[9,10] The challenge in designing antibody-like receptors for these drugs (**1**−**5**) lies in their structural similarity: all have a carboxylate and a hydrophobic aromatic moiety. Naproxen and Ibuprofen (or Ketoprofen) in particular resemble one another, even in the size and shape of the aromatic group. Needless to say, to recognize these drugs selectively, the receptor needs to have remarkable precision in its binding. Ideally, one also needs the receptors to function in water for drug monitoring or analysis.

Herein, we report that antibody-like polymeric nanoparticle receptors can be created for NSAIDs through molecular

Chart 1. Structures of Common NSAIDs



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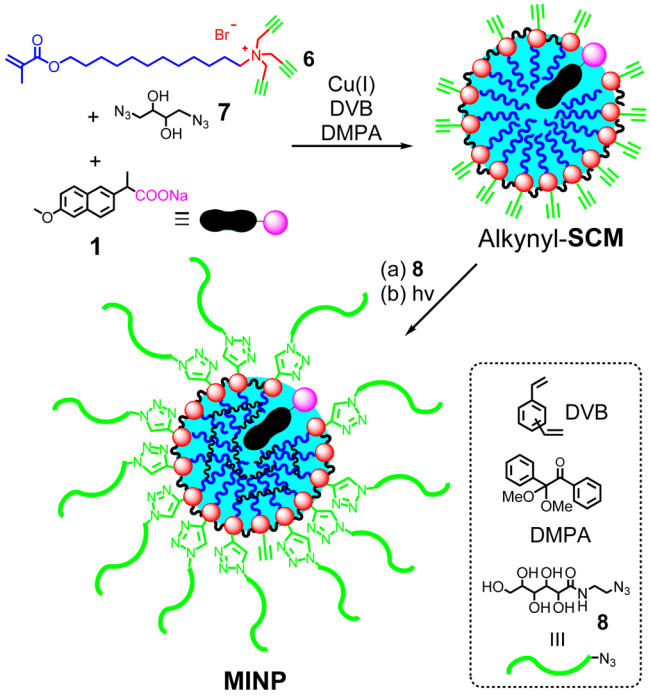
imprinting in cross-linked micelles. The binding selectivity displayed by our "synthetic antibodies" was comparable to that found in biologically generated antibodies. Our materials, however, can be produced in 2−3 days rather than weeks without special techniques, provided that the building blocks (the polymerizable surfactants and cross-linkers) are available.

RESULTS AND DISCUSSION

The synthesis of our nanoparticle receptors starts with micellization of cationic surfactant **6** above its critical micelle concentration (CMC) of 0.55 mM in water (Scheme 1).

Scheme 1. Preparation of MINP with Naproxen as the Template



Because the headgroup of this surfactant is a tripropargylammonium cation, its micellization places a layer of terminal alkynes on the surface of the micelle. In the presence of a diazide cross-linker (**7**) and Cu(I) catalysts, the highly efficient copper-catalyzed alkyne−azide cycloaddition[11] quickly cross-links the surface of the micelles to afford alkynyl-SCMs as water-soluble nanoparticles.

In our previous work, we have shown that water- or organic-soluble azide-functionalized ligands could be easily installed on the SCM surface if a sufficient number of alkyne groups are left on the surface of the cross-linked micelles.[12−15] This feature was achieved by using a ratio of [**6**]/[**7**] = 1:1.2 during the SCM preparation, as an excess of alkynes will be left after cross-linking. The alkynyl-SCMs were functionalized with sugar-derived ligand **8** so that the resulting nanoparticles were highly hydrophilic on the surface and completely soluble in water.

The details of the molecular imprinting procedure has been reported previously.[16] Briefly, the anionic and hydrophobic nature of the NSAID (e.g., **1** or **2**) allowed it to be readily incorporated into the cationic micelle and the resulting SCM. After the surface cross-linking and functionalization, we initiated core cross-linking of the methacrylate of **6** around the template. This step is the key to the molecular imprinting to form the final binding pocket. To facilitate this process, we solubilized 1 equiv. of divinylbenzene (DVB) and 5 mol % of 2,2-dimethoxy-2-phenylacetophenone (DMPA, a photolytic radical initiator) in the very beginning of the preparation and irradiated the surface-functionalized SCMs with UV light. UV irradiation initiated free radical polymerization between the methacrylate and DVB. Because the template molecule (**1** or **2**) had neither alkyne nor methacrylate to participate in any cross-linking, it acted as a place holder throughout the preparation while surface- and core cross-linking took place around the template to afford the binding pocket.

Molecularly imprinted polymers (MIPs)[17−25] have been reported in the literature for NSAID drugs.[26−29] They are typically prepared by polymerization of a functionalized monomer such as 4-vinylpyridine that binds the carboxylic acid of the NSAID and a large amount of a vinyl cross-linker. The bulk polymerization normally yields insoluble cross-linked polymers that need to be ground into smaller particles, sieved, and washed. In contrast, because the core-cross-linking was confined with each SCM in our case, the final molecularly imprinted nanoparticles (MINPs) were water-soluble nanoparticles similar to water-soluble proteins in size, hydrophilic exterior, hydrophobic interior, and a specific binding pocket in the hydrophobic core.

The MINP synthesis was monitored by ^{1}H NMR spectroscopy and dynamic light scattering (DLS). The SCMs had been characterized earlier additionally by mass spectrometry (after cleaving the 1,2-diol cross-linkages) and TEM.[12] Upon surface-cross-linking, all the ^{1}H NMR signals of surfactant **6** showed characteristic broadening (Figure S1). The alkenic and aromatic protons of both **6** and DVB remained clearly visible in the SCMs but disappeared after the core-cross-linking. The disappearance of the alkenic protons should be caused by their consumption by the polymerization. The aromatic protons disappeared most likely because the high cross-linking density of the core restrained their movements in the core. DLS showed that the alkynyl-SCM, the surface-functionalized SCM, and the final MINP-**1** (i.e., MINP prepared with **1** as the template) had an average size of 4.0, 6.3, and 5.0 nm, respectively (Figure S2). The size change was consistent with our previous results,[16] suggesting that the nanoparticles became larger upon surface decoration and shrank during core-cross-linking.

The MINPs were purified by precipitation from acetone and repeated washing with water/acetone and methanol/acetic acid. The yield of the final MINPs was typically >80%. The fluorescence of Naproxen allowed us to monitor its removal from MINP-**1** by the disappearance of its characteristic emission peak at 360 nm. The carboxylate of the template served two strategic purposes in our imprinting: first, its anionic charge enabled it to interact with the cationic micelle and, ultimately, the MINP, by electrostatic interactions in addition to hydrophobic effect; second, its hydrophilic nature ensured its location on the micelle surface and thus prevented permanent trapping of the template inside the MINP during polymerization. According to DLS, the final MINP-**1** averaged 5.0 nm in diameter, which translated to a molecular weight of 51 000 Da (Figure S3). In the calculation of the molecular weight, the DLS program assumed the power law for the dependency of the MW on the size of the particle. Typical parameters for proteins were used for the MINPs since both proteins and MINPs were compact in the interior (see the Supporting Information for details).[30] Following the same procedures, we

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also prepared MINP-**2** against Indomethacin and characterized the materials similarly (Figures S4–S6).

In traditional MIPs, the insolubility of the receptors means that binding needs to be determined indirectly, often by measuring the amounts of guest absorbed by different amounts of polymer beads.[17−25] Neither the number of binding sites on a polymer bead nor their binding affinity can be controlled, as the polymer beads are obtained by grinding and sieving of insoluble macroporous polymers. A heterogeneous population of binding sites is typically obtained from such imprinting.

The water-solubility of MINP-**1** and the fluorescence of Naproxen enabled us to directly study the binding of MINP-**1** as a receptor by fluorescence titration. Direct characterization by spectroscopic methods is a highly desired feature for molecular imprinted materials and is often difficult in traditional insoluble MIPs.[31] Good solubility and spectroscopic characterization could make our MINPs useful sensors for the drugs to be studied. As shown by Figure 1, upon titration of

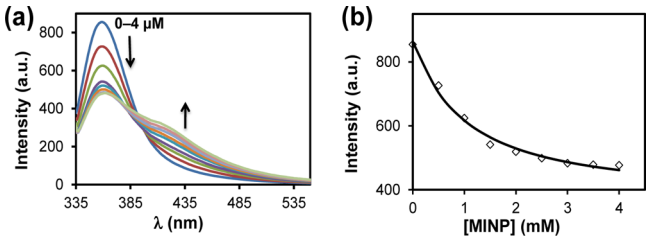

Figure 1. (a) Fluorescence emission spectra of **1** (λ_{ex} = 310 nm) upon addition of different concentrations of MINP-**1**. (b) Nonlinear least-squares curve fitting of the emission intensity of **1** at λ = 358 nm as a function of MINP-**1** concentration. [**1**] = 0.25 μM.

Naproxen by different concentrations of MINP-**1** in an aqueous Tris buffer (50 mM Tris, pH 7.4),[32] the emission peak of the guest at 358 nm gradually decreased and a new peak at 430 nm emerged. The data fit nearly perfectly to a 1:1 binding isotherm to afford a binding constant (K_a) of $(1.1 \pm 0.2) \times 10^6$ M^{-1}. The binding constant translates to a binding free energy of $-\Delta G$ = 8.2 kcal/mol.

As mentioned earlier, a successful receptor for NSAIDs not only needs to have strong binding but also low cross-reactivity with its structural analogues. Because Naproxen is the only fluorescent molecule among the NSAIDs chosen, we turned to isothermal titration calorimetry (ITC) to study the binding of the other drugs by MINP-**1**. As shown by Figure 2a, the titration data for Naproxen and MINP-**1** yielded a K_a value of $(0.91 \pm 0.04) \times 10^6$ M^{-1}, experimentally the same as the value obtained from the fluorescence titration.

Having confirmed the good agreement between the fluorescence and ITC binding data, we proceeded with the ITC binding studies of the other NSAIDs (**2**–**5**) by MINP-**1**. Recognizing the structural and functional-group similarity between the template (Naproxen) and Ibuprofen or Ketoprofen, we were apprehensive whether the MINP was able to distinguish these structural analogues.

The K_a values for **2**, **3**, **4**, and **5** were determined to be 8.0×10^3, 8.7×10^4, 2.9×10^4, and 3.7×10^4 M^{-1}, respectively (Table 1, entries 3–6). Thus, the binding constants for these other NSAIDs by MINP-**1** were at least an order of magnitude weaker than that for the Naproxen template. In the literature, the specificity of an antibody is represented by its cross-reactivity with ligands analogous to its antigen; the cross-

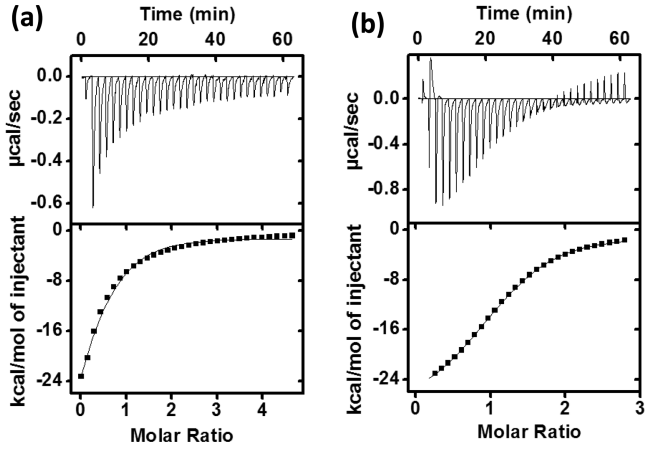

Figure 2. ITC titration curves obtained at 298 K for the binding of (a) **1** by MINP-**1** and (b) (a) **2** by MINP-**2**. The data correspond to entries 1 and 7 in Table 1. Additional ITC titration curves can be found in the Figures S7 and S8.

Table 1. Binding Data for MINPs Obtained by ITC[a]

entry	guest	host	K_a ($\times 10^4$ M^{-1})	-ΔG (kcal/mol)	CRR[b]	N
1	**1**	MINP-**1**	112 ± 20	8.2	[c]	[c]
2	**1**	MINP-**1**	91 ± 4	8.1	1	0.6 ± 0.1
3	**2**	MINP-**1**	0.8 ± 0.1	5.6	0.01	1.1 ± 0.1
4	**3**	MINP-**1**	8.7 ± 0.5	6.7	0.1	1.0 ± 0.1
5	**4**	MINP-**1**	2.9 ± 0.4	6.1	0.03	0.9 ± 0.1
6	**5**	MINP-**1**	3.7 ± 0.2	6.2	0.04	1.1 ± 0.1
7	**2**	MINP-**2**	98 ± 5	8.2	1	1.1 ± 0.1
8	**1**	MINP-**2**	5.2 ± 1.2	−6.4	0.05	0.7 ± 0.1
9	**3**	MINP-**2**	8.0 ± 0.1	−6.7	0.08	0.6 ± 0.1
10	**4**	MINP-**2**	0.8 ± 0.1	−5.3	0.01	0.4 ± 0.1
11	**5**	MINP-**2**	9.4 ± 1.0	−6.8	0.10	0.4 ± 0.1

[a]The titrations were generally performed in duplicates in 50 mM Tris buffer (pH 7.4) and the errors between the runs were <15%. [b]CRR = cross-reactivity = binding constant of a given compound by a MINP receptor normalized to that of the template by the same MINP. [c]The binding constant was obtained from fluorescence titration and thus was not compared with the ITC binding data.

reactivity may be obtained from either ITC binding data[33] or antibody-based assays such as ELISA.[9] The binding constants in our studies translate to cross-reactivity of 0.01, 0.1, 0.03, and 0.04 for **2** (Indomethacin), **3** (Ibuprofen), **4** (Ketoprofen), and **5** (Diclofenac), respectively.

In the literature, polyclonal antibodies have been prepared for Naproxen.[9] The immunization (of rabbits) was reported to take 25 weeks. Competitive ELISA was then used to determine the specificity of the antibody for both NSAID and other analogues. The cross-reativity for Ibuprofen and Diclofenac was 0.09 and 0.04, respectively.[9] Thus, the specificity displayed by our plastic antibodies were essentially identical to that exhibited by the antibodies generated through much lengthier and more expensive procedures.

Because all the NSAIDs studied carry the identical carboxylate, the selectivity of our MINP should derive from the size/shape of the binding pocket. In other words, hydrophobic matching between the host and the guest was responsibile for the selectivity. We were delighted to see that Ibuprofen and Ketoprofen, two extremely similar analogues of Naproxen could be distinguished nicely by MINP-**1**.

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Apparently, the binding pocket was so well formed that even an insertion of a single ketone in the middle of the aromatic moiety (in Ketoprofen) was not tolerated by the binding pocket.

Another feature of our MINP is its controllable number of binding sites. The ITC titration revealed that the number of binding site per particle averaged 0.6−1.1. The number agreed well with our prepration: since the micelle aggreation number was ca. 50 and the [template]/[surfactant] ratio was 1:50 in our MINP prepration,[9] we anticipated a single binding site per particle on average. The number of binding sites obtained also compared favorably with typical numbers of protein/antibody receptors, as inactive receptors, impurities, and inaccuracies in the molecular weight of the materials frequently cause diviation of the binding site from unity, even when the orignial bioreceptor contains a single binding site.[33] It should be mentioned that the average number of binding sites per particle could be easily tuned by the [template]/[surfactant] ratio, as demonstrated by our previous work.[16a]

Indomethacin (**2**) was the largest NSAID drug in our study. Because it is generally easier to fit a smaller molecule in a larger binding pocket than vice versa, we were especially curious about the specificity of MINP-**2** for these NSAIDs. As shown in Table 1 (entry 7, see Figure 2b for the titration curve), the binding constant of MINP-**2** for the template itself was $(0.98 \pm 0.05) \times 10^6$ M^{-1}, very similar to the value for Naproxen by MINP-**1**. Because Indomethacin is signficantly larger in size than Naproxen, one would anticipate that the binding of the larger ligand by its correspoinding MINP receptor should be stronger, as a major driving force in the binding should be the expulsion of the water molecules in the binding pocket by the corresponding ligand and a larger guest should expel more water molecules from the (larger) binding pocket.

There could be several possible reasons why the larger Indomethacin did not display stronger binding than the smaller Naproxen. First, Indomethacin contained an amide group in the structure. The amide carbonyl oxygen is an excellent hydrogen-bond acceptor and is expected to be solvated quite well by water prior to its entrance into the binding pocket. Desolvation of the guest costs free energy and is expected to negatively impact the binding affinity. Second, if the amide group was hydrophilic enough to stay near the surface of the micelle during molecular imprinting, the binding pocket obtained from Indomethacin in MINP-**2** could be shallower than that obtained from Naproxen in MINP-**1**. If this is indeed the case, the shallower binding pocket would reduce the hydrophobic driving force for the binding, as part of the guest molecule might still be exposed to water after the binding. In contrast, a more deeply embedded binding pocket should bury the hydrophobic guest more completely; thus, the smaller but overall more hydrophobic aromatic group in Naproxen might be better shielded from water by its binding pocket. Third, a rigid aromatic group (of Naproxen and the top protion of Indomethacin) has little conformational freedom, whereas the tertiary amide bond of Indomethacin could adopt either trans or cis configuration prior to binding. Because binding will fix the conformation of the guest (by fitting the guest into the preformed binding pocket), the loss of conformaitonal entropy will also lower the potential driving force for the binding.

Although the binding for Indomethacin was not signfiicantly stronger than for Naproxen by their corresponding MINPs, it is encouraging to see that MINP-**2** remained highly selective. The cross-reactivity of this MINP was 0.05, 0.08, 0.01, and 0.1 for **1** (Naproxen), **3** (Ibuprofen), **4** (Ketoprofen), and **5** (Diclofenac), respectively. Thus, similar to MINP-**1**, the highest cross-reactivity for the nontemplated NSAIDs was 0.1, similar to what was observed for the natural antibodies.

A close examination of our binding data suggests that a smaller guest indeed can fit into a larger pocket more easily than a larger guest does a smaller pocket. For example, the cross-reactivity of Indomethacin relative to Naproxen in MINP-**1** was 0.01, indicating that the large guest had difficulty fitting into the binding pocket generated by the small guest. The cross-reactivity of Naproxen to Indomethacin in MINP-**2**, however, was 0.05. Thus, although the pocket was quite selective for the NSAIDs studied, relatively speaking, a small guest indeed fitted better to a larger pocket than vice versa.

CONCLUSIONS

Traditionally, chemists use molecular synthesis to create discrete, well-defined molecular receptors for molecules of interest.[2,3] However, building concave receptors with guest-complementary binding surfaces require ingenious molecular design, lengthy synthesis, and many times is simply impossible for complex shaped/functionalized molecules or simple molecules lacking proper functional-group handles. As shown in this study, the great similarity and subtle differences among the NSAIDs make it extremely challenging to create specific molecular receptors for them. Although one could turn to biological methods to generate antibodies for the drugs, the procedures involve lengthy immunization and cumbersome purification and the resulting biomolecules are unstable under many conditions. In contrast, our molecular imprinting in cross-linked micelles readily yielded "synthetic antibodies" with antibody-like specificity. Although higher binding affinities (than the current micromolar affinities) would be even better, the ease of the synthesis, the diversity of the MINP receptors that can be created, the strong tolerance of the materials for organic solvent and high temperatures because of their high cross-linking density,[16] and the excellent molecular recognition of the materials suggest that MINPs could become attractive substitutes for antibodies in appropriate applications.

EXPERIMENTAL SECTION

General. Methanol, methylene chloride, and ethyl acetate were of HPLC grade and were purchased from Fisher Scientific. All other reagents and solvents were of ACS-certified grade or higher, and were used as received from commercial suppliers. Routine ^1H and ^{13}C NMR spectra were recorded on a Bruker DRX-400 or on a Varian VXR-400 spectrometer. ESI-MS mass was recorded on Shimadzu LCMS-2010 mass spectrometer. Fluorescence spectra were recorded at ambient temperature on a Varian Cary Eclipse Fluorescence spectrophotometer. ITC was performed using a MicroCal VP-ITC Microcalorimeter with Origin 7 software and VPViewer2000 (GE Healthcare, Northampton, MA). Dynamic light scattering (DLS) was performed on a PD2000DLS+ dynamic light scattering detector.

Typical MINP Synthesis. To a micellar solution of surfactant **6** (9.3 mg, 0.02 mmol) in D_2O (2.0 mL), divinylbenzene (DVB, 2.8 μL, 0.02 mmol), **1** in D_2O (10 μL of a solution of 10.1 mg/mL, 0.0004 mmol), and 2,2-dimethoxy-2-phenylacetophenone (DMPA) in DMSO (10 μL of a 12.8 mg/mL, 0.0005 mmol) were added.[34] The mixture was ultrasonicated for 10 min. Compound **6** (4.1 mg, 0.024 mmol), $CuCl_2$ in D_2O (10 μL of 6.7 mg/mL, 0.0005 mmol), and sodium ascorbate in D_2O (10 μL of 99 mg/mL, 0.005 mmol) were then added and the reaction mixture was stirred slowly at room temperature for 12 h. Compound **8** (10.6 mg, 0.04 mmol), $CuCl_2$ (10 μL of a 6.7 mg/mL solution in D_2O, 0.0005 mmol), and sodium ascorbate (10 μL of a 99 mg/mL solution in D_2O, 0.005 mmol) were then added and the

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solution 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. ^{1}H NMR spectroscopy was used to monitor the progress of reaction. 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. The crude product was washed with methanol/acetic acid (5 mL/0.1 mL) five times, and then with methanol (2 mL), followed by excess acetone. The off-white product was dried in air to afford the final MINPs (17 mg, 85%).

Determination of Binding Constants. For fluorescence titration, a stock solution of MINP-**1** (4.0×10^{-4} M) in Tris buffer (50 mM Tris, pH 7.4) was added to a solution of Naproxen in the same buffer (0.25 μM). The excitation wavelength was 310 nm. The association constant (K_a) was obtained by nonlinear least-squares curve fitting of the emission intensity of Naproxen at 358 nm following standard procedures.[35] The determination of binding constants by ITC also followed standard procedures.[36−38] Specifically, an aqueous solution of compound **1** (1.20 mM) in Tris buffer (50 mM Tris, pH 7.4) was injected in equal steps of 10 μL into 1.43 mL of a solution of MINP-**1** (0.10 mM) in the same buffer at intervals of 120 s for a total of 30 injections. The heat of dilution for compound **1**, obtained by injecting an aqueous solution of compound **1** (1.20 mM) in Tris buffer (50 mM Tris, pH 7.4) into the buffer, was subtracted from the heat released during the binding. Curve fitting was achieved using Microcal's Origin 7 software (MicroCal VP-ITC, Northampton, MA) that autogenerates the binding parameters.

■ ASSOCIATED CONTENT

Supporting Information

Characterization of MINPs and additional figures. The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acsbiomaterials.5b00042.

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Notes

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