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D-Amino Acids Boost the Selectivity and Confer Supramolecular Hydrogels of a Nonsteroidal Anti-Inflammatory Drug (NSAID)

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Supporting Information

ABSTRACT: As systemically used therapeutics for treating acute or chronic pains or inflammations, nonsteroidal anti-inflammatory drugs (NSAIDs) also associate with the adverse gastrointestinal and renal effects and cardiovascular risks. Thus, it is beneficial to develop topical gels that selectively inhibit cyclooxygenase-2 (COX-2) for the management of local inflammation. In this work, we demonstrate that the covalent conjugation of D-amino acids to naproxen (i.e., a NSAID) not only affords supramolecular hydrogelators for the topical gels but also unexpectedly and significantly elevates the selectivity toward COX-2 about 20× at little expense of the activity of naproxen. This work illustrates a previously unexplored approach that employs D-amino acids for the development of functional molecules that have dual or multiple roles and exceptional biostability, which offers a new class of molecular hydrogels of therapeutic agents.

We report the design, synthesis, and characterization of hydrogelators made of D-amino acids and a nonsteroidal anti-inflammatory drug (NSAID) for the development of multifunctional supramolecular hydrogelators that have excellent selectivity for inhibiting cyclooxygenase-2 (COX-2). As widely, systemically used drugs for the treatment of acute or chronic pains or inflammations, NSAIDs usually are administered in high dosage, which causes the adverse gastrointestinal and renal effects[1] when they inhibit COX-1,[2] and are associated with cardiovascular risks with COX-2 inhibition.[2,3] Such paradoxical effects demand NSAID selectivity to be modulated according to therapeutic objectives as well as minimize systemic use of NSAIDs for localized acute or chronic pains.[4] Therefore, it is clinically beneficial to explore NSAID gels as a topical agent for treating inflammation and relieving pain, as demonstrated by the use of diclofenac lotion for managing moderate osteoarthritis.[5] Encouraging results of diclofenac lotion indicate that it is worthwhile to develop other NSAID gels for more effective localized treatment of severe chronic pains. Thus, we choose to develop supramolecular NSAID hydrogels because, despite their promising potential, they are less explored,[6] particularly in terms of enhancing biostability and maintaining desired activity—two essential requirements of topical gels for sustained release.

Supramolecular hydrogels, a hydrogel type resulting from self-assembly of small molecules (usually termed as hydrogelators) in water,[7] have become an attractive choice of soft nanomaterials[8] for a variety of applications, such as scaffolds for tissue engineering,[9,10] carriers for drug delivery,[11,12] ultrathin membranes,[13] and new matrices for enzyme assay,[14] antibacterial cell culture,[10] gel electrophoresis,[15] and protein pull-down assay.[16] Since the hydrogelators only associate with each other through noncovalent interactions, supramolecular hydrogels are inherently biocompatible and biodegradable, which also makes them attractive candidates for self-delivery therapeutics, i.e., drug molecules themselves are hydrogelators.[12] Comparing use of biodegradable polymers to encapsulate therapeutic agents for controlled release of drugs by adjusting the pore sizes and functionality of the polymer networks,[17] self-delivery hydrogels based on supramolecular hydrogelators minimize several inherent shortcomings, such as inflammations,[18] limited loading of drug molecules,[19] and the difficulties of functionalizing the polymers with drug molecules, that limit the application of polymeric hydrogels.

Since the formation of supramolecular hydrogels relies on small molecules (i.e., hydrogelators) that self-assemble in water via noncovalent interactions and sustained drug-release depends on hydrogels biostability, the essential requirements for hydrogelators made of NSAIDs are (i) enabling self-assembly of NSAIDs without compromising the NSAIDs activity; and (ii) resisting premature degradation due to proteolytic hydrolysis. To satisfy these two requirements and to demonstrate the design concept, we use one of the prescription NSAIDs, naproxen (denoted as Npx), to generate new hydrogelators that are composed of naproxen and small peptides made of D-amino acids. Based on the ability of diphenylalanine (Phe-Phe)[20] motif to enable functional molecules to self-assemble in water,[21] we conjugate D-Phe-D-Phe with Npx to afford NSAID-containing hydrogelators. The use of D-amino acids for the conjugates not only confers proteolytic resistance to the hydrogelators but also unexpectedly and significantly enhances the hydrogelators selectivity as the Npx derivatives for inhibiting COX-2. As the first example of D-amino acids improving selectivity and maintaining desired activity of a therapeutic agent, this work demonstrates a new approach for improving the selectivity of clinically used therapeutics and for developing multifunctional small molecules that self-assemble in water, resulting in new supramolecular hydrogels of therapeutic agents that are biostable, target specific, and potent.

We design the hydrogelators based on COX-2 crystal structure,[22] which suggests that the conjugation of amino acids to Npx hardly disrupts the Npx to bind COX-2. Figure 1

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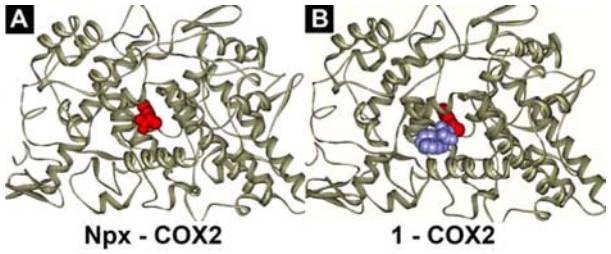
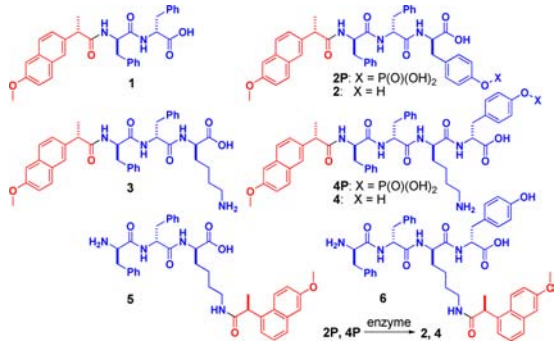


Figure 1. Binding of (A) Npx and (B) **1** with COX-2 enzyme (ligands as CPK model and COX-2 as ribbons).

shows an example of the design. According to the binding of Npx (red) with COX-2 enzyme (gray) (Figure 1A), the Npx carboxylate end is available for modification after Npx binds to COX-2 due to the large open space in the COX-2 structure. Figure 1B shows the predicted binding model of hydrogelator Npx-D-Phe-D-Phe (**1**, Npx-ff, blue spheres represent D-Phe-D-Phe) and COX-2: the bulky D-Phe-D-Phe dipeptide connected to Npx still allows Npx to bind to the COX-2 active site. To generate the hydrogels in situ (or in vivo) via enzymatic reactions,[23] we introduce D-tyrosine phosphate to **1**. Additionally, we connect Npx to the side chain of small peptides of D-amino acids to evaluate the correlation between structure and activity of NSAID hydrogelators. Scheme 1 shows the

Scheme 1. Hydrogelators Structures Consisting of D-Amino Acids and Npx



molecular structures of designed derivatives of Npx in this work. The connection of D-Phe-D-Phe, D-Phe-D-Phe-D-Tyr, D-Phe-D-Phe-D-Lys, or D-Phe-D-Phe-D-Lys-D-Tyr to Npx results in molecules Npx-ff (**1**), Npx-ffy (**2**), Npx-ffk (**3**), or Npx-ffky (**4**), respectively, that contains Npx at the backbone of the small peptide. The conjugation of Npx to the D-Phe-D-Phe-D-Lys or D-Phe-D-Phe-D-Lys-D-Tyr side chain via the D-Lys residue ε-amino group produces molecules ffk(Npx) (**5**) and ffk(Npx)y (**6**). Addition of a phosphate group on the tyrosine residue of **2** and **4** affords the precursors (**2P** and **4P**) that would convert to molecules **2** and **4** followed by the dephosphorylation catalyzed by phosphatases.[23,24]

We synthesize the molecules in Scheme 1 according to synthetic procedures that combine solid-phase synthesis and *N*-hydroxysuccinimide (NHS)-assisted coupling reaction.[25] After the designed hydrogelators synthesis, the gelation test indicates that all hydrogelators in Scheme 1 can form stable hydrogels at the concentration of 0.8 wt % (Figure 2), but hydrogels exhibit a slightly different appearance. For example, the aid of sonication and heating affords the aqueous solution of **1** at pH 9.0, which turns into an opaque hydrogel upon pH

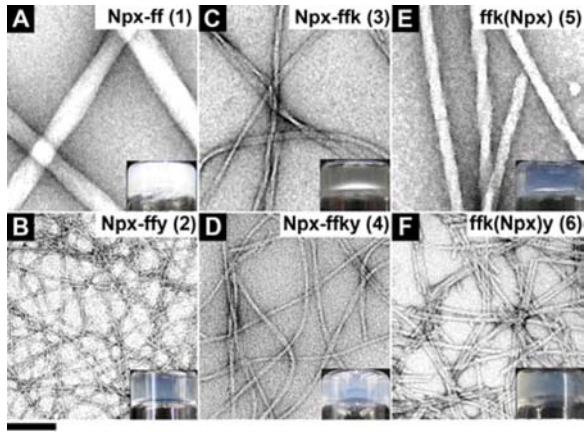


Figure 2. TEM images of hydrogels of (A) **1** (pH 4.0); (B) **2** (pH 7.6); (C) **3** (pH 7.6); (D) **4** (pH 7.6); (E) **5** (pH 7.0); (F) **6** (pH 7.0) (inset: optical images). Hydrogels of **2** and **4** made by adding 0.2 U/mL alkaline phosphatase to the **2P** and **4P** solutions. All hydrogels are at 0.8 wt % (f = D-Phe, k = D-Lys, y = D-Try); scale bar is 100 nm.

adjustment to 4.0 at rt . Unlike **1**, the addition of 0.2 U/mL of alkaline phosphatase to the **2P** solution results in a transparent hydrogel of **2** at pH 7.6. By changing pH and temperature, we obtain hydrogels **3**, **5**, and **6**, respectively.[25] By adding 0.2 U/mL of alkaline phosphatase to the **4P** solution, we obtain the hydrogel of **4**.

We use TEM to examine the Npx-containing hydrogels for evaluating the characteristics of the molecular assemblies. As shown in Figure 2A, hydrogelator **1** self-assembles to afford large and rigid nanofibers with average width of 54 ± 7 nm, while hydrogelator **2** gives long, thin, and flexible nanofibers with average width of 7 ± 2 nm (Figure 2B). Figure 2C shows the nanofibers with helical structure formed in hydrogel of **3**, with average width of 16 ± 3 nm. The enzymatically formed hydrogel of **4** comprises of long and flexible nanofibers with average width of 10 ± 2 nm (Figure 2D). Hydrogel of **5** exhibits helical, rigid, and long nanofibers with average widths of 26 ± 3 nm (Figure 2E), meanwhile hydrogelator **6** self-assembles to give rigid but short nanofibers with average widths of 7 ± 2 nm, which tend to form bundles (Figure 2F). As shown by the images at the bottom row of Figure 2, the hydrogels containing D-Tyr (i.e., hydrogels **2**, **4**, and **6**) exhibit smaller diameter nanofibers that entangle to form network with higher density than their corresponding hydrogels (i.e., hydrogels **1**, **3**, and **5**) without D-tyrosine. Incorporation of D-Lys in hydrogelator **3** also forms more flexible and narrow nanofibers than the nanofibers of hydrogelator **1**. Hydrogel of **4** contains nanofibers that have similar morphologies to those in hydrogel of **2**. The hydrogels **5** and **6**, with Npx connected at the side chain, contain rigid and straight nanofibers, which differ from those flexible and long nanofibers in the hydrogels **3** and **4**. These morphological differences of the hydrogels indicate the Npx position and the Tyr presence at the C-terminals of the hydrogelators likely play a role in their self-assembly in water.

We use oscillatory rheology to examine hydrogel viscoelastic properties. All Npx-containing hydrogels exhibit viscoelastic properties of a solid-like material because their storage moduli (G') are significantly higher than their loss moduli (G'') and are independent to frequency (Figure S14). Table 1 summarizes the critical strains and the moduli of hydrogels measured from strain and dynamic frequency sweeps. The relatively large

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Table 1. Hydrogels Rheological Properties and TEM Characteristics of D-Amino Acids and Npx Conjugates

	dynamic strain sweep	dynamic frequency sweep	TEM images
	critical strain (%)	G', G'' [a] (Pa)	fiber width (nm)
1	1.0	5.3×10^4, 2.3×10^3	54 ± 7
2	1.6	6.2×10^2, 6.7×10	7 ± 2
3	5.2	3.9×10^2, 4.9×10	16 ± 3
4	5.5	1.5×10^2, 3.0×10	10 ± 2
5	0.41	3.8×10^3, 4.2×10^2	26 ± 3
6	0.40	1.4×10^3, 4.4×10^2	7 ± 2

[a]Value is taken at frequency = 6.28 rad/s.

critical strains of **3** and **4** suggest that the ε-amino group from the lysine residue makes the networks of the hydrogels to be resilient. The low critical strains of hydrogels **1**, **5**, and **6** apparently agree with the rigidity of the nanofibers in those hydrogels, which also confers relatively high storage moduli (G'). These results provide insights on the correlation between molecular structures of the hydrogelators and the viscoelasticity of the supramolecular hydrogels. Moreover, the critical strains of the hydrogels are comparable or higher than that of commercial topical gels (e.g., Gelrite with the critical strain of 1%[26]), suggesting that the hydrogels are rheologically suitable for topical use.

We perform in vitro inhibition assays[27] for both COX-1 and COX-2 enzymes to evaluate the efficacies of the NSAID-containing hydrogelators. In Figure 3A, IC_{50} values of

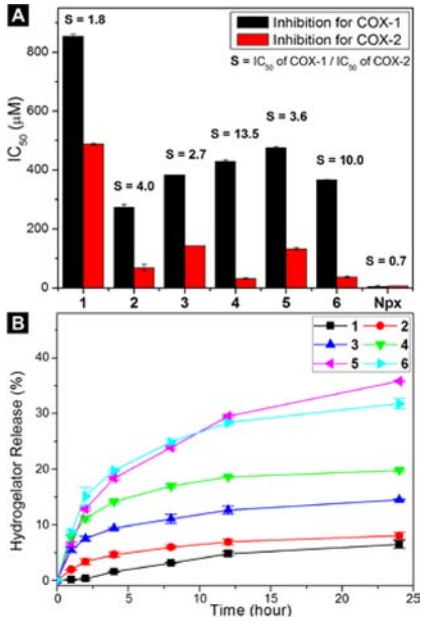

Figure 3. (A) IC_{50} values of Npx-based hydrogelators for inhibiting COX enzyme (selectivity, defined as the ratio of the IC_{50} values towards COX-1 and COX-2, is labeled on top of bars). (B) Release profiles of Npx-based hydrogelators from hydrogels **1**–**6**.

hydrogelators **1**–**6** against COX-1 enzyme are 853.8, 273.7, 383.5, 428.9, 476.3, and 367.3 μM, respectively. All values are almost 2 orders of magnitude higher than the literature IC_{50} values (0.6–4.8 μM)[28] of Npx. Undoubtedly, the attachment of the small D-peptides to Npx greatly reduces its binding to COX-1, which should reduce the associated adverse gastrointestinal and renal effects. For COX-2 enzyme, an inducible enzyme at inflammation sites, the IC_{50} values of hydrogelators **1**–**6** are 487.7, 68.8, 143.2, 31.7, 132.2, and 36.7 μM, respectively. Since the reported IC_{50} of Npx against COX-2 is 2.0–28.4 μM,[28] hydrogelators **4** and **6**, obviously, afford reasonable IC_{50} values for the inhibition of COX-2. Thus, hydrogelators **4** and **6** exhibit excellent selectivity, S = 13.5 and 10.0, respectively, toward COX-2. These results not only validate **4** and **6** as potential candidates for topical NSAID gels but also suggest that the D-Tyr on the D-peptides is beneficial for the activity and selectivity regardless the Npx position on either the side or the main chain of the D-peptide. In the control compound, the use of L-amino acids (L-Phe, L-Lys, and L-Tyr) to replace the D-amino acid residues in hydrogelators **1**–**4** results in the hydrogelators that exhibit higher IC_{50} values and poor selectivity toward COX-2 (Figure S12). For example, L-**4** (Npx-FFKY) exhibits IC_{50} values of 38.0 and 114.8 μM for COX-2 and COX-1, respectively, which affords the selectivity for COX-2 inhibition to be ~3. These results indicate the advantages of using D-peptide for generating Npx-containing hydrogelators to achieve high selectivity.

After studying their drug efficacies, we evaluate the sustained release of Npx-containing hydrogelators from the hydrogels (0.8 wt %). We incubate 100 μL of hydrogels at 37 °C for 24 h with 100 μL of PBS buffer solution (pH 7.4), which is refreshed and monitored at 1, 2, 4, 8, 12, and 24 h. Figure 3B shows the release profile of these hydrogelators. After 24 h, hydrogel **1** slowly and steadily releases 6.5% of gelators. With increased solubility contributed from hydrophilic amino acid residues (i.e., Tyr and Lys), hydrogels **2**–**4** release gelators of 8.0, 14.5, and 19.8%, respectively. Hydrogels **5** and **6** release 35.8 and 31.7% of gelators after 24 h, likely due to the good solubility of the hydrogelators and the brittleness of the nanofiber networks (indicated by hydrogels **5** and **6** low critical strains). Dynamic light scattering of the solution of the released hydrogelator (**1** or **6**) solutions exhibits little difference with that of the PBS buffer (Figure S15), suggesting that the hydrogelators unlikely exist as self-assembled nanofibers after being released from the hydrogels. These results suggest that these Npx-containing hydrogels may serve as topical gels for sustained drug delivery.

We also examined the biocompatibility of the Npx-containing hydrogelators by incubating them with HeLa cells for 72 h at 37 °C. As shown in Figure S13, all of these hydrogelators have IC_{50} values >500 μM, except **1** with IC_{50} value of 357 μM. The hydrogels high IC_{50} values indicate that they are cell compatible. Although the formazan absorption in the MTT assay indicates the promotion of the cell growth when the cells are incubated with hydrogelators **2**, **5**, or **6**, we find no promotion of the cell proliferation based on the change in HeLa cell numbers. Though the exact mechanism remains to be determined, these increased absorptions likely originate from the metabolic activity increase in those HeLa cells treated by **2**, **5**, or **6**, without increasing cell viability.[29]

In conclusion, we have developed multifunctional supramolecular hydrogelators made of D-amino acids and a NSAID, which is a new approach for delivering therapeutic agents by biostable, target specific, and potent hydrogels. In addition, incorporation of D-amino acids makes Npx a highly selective NSAID for inhibiting COX-2 and minimizing its adverse effect. This approach may provide useful strategy for reducing the adverse drug reactions of other therapeutic agents or candidates, a subject being investigated further. Although the phosphate trigger in **2P** or **4P** appears less relevant to NSAID

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topical gel application as a pain reliever, the enzyme triggered gelation may find new applications in other settings when the presence of phosphatases accompanies the change of physiological conditions.

■ ASSOCIATED CONTENT

Ⓢ Supporting Information

Experimental details and characterization data. This material is available free of charge via the Internet at http://pubs.acs.org.

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Notes

The authors declare no competing financial interest.

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