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Esterase-Activated Release of Naproxen from Supramolecular Nanofibres

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Abstract

Nanofibre forming peptide amphiphiles were conjugated to naproxen through an esterase-sensitive linker. The amount of naproxen released, in the presence of enzymes, was influenced by the linker conjugating the drug to the supramolecular assembly. *In vitro* studies showed the anti-inflammatory activity of the released drug was maintained.

Over the past few years various types of nanocarriers have been developed to achieve controlled drug release in response to external stimuli such as pH, temperature, light, or enzymes.^{1–4} These strategies can minimize the limitations of small molecule therapies including metabolism to inactive products and side effects.^{5, 6} Bioactive supramolecular nanostructures based on peptide amphiphiles (PAs) are a versatile platform for the delivery of small molecules. PAs are composed of natural amino acid sequences covalently conjugated to hydrophobic segments. These molecules can self-assemble in aqueous solutions into high-aspect-ratio nanostructures, such as fibers and ribbons, with their hydrophobic segments packed away from the aqueous interface.^{7–9} The peptide domain near the hydrophobic core can be varied to tune the supramolecular cohesion of the nanostructures.¹⁰ PAs can be functionalized to display on the surfaces of the nanostructures bioactive cues for cell signalling and targeting.^{11–13} Recently, PAs have been used as drug delivery systems by covalently attaching small molecules containing a ketone functionality via a pH-labile hydrazone linkage.^{14, 15} In a similar approach, Cui and co-workers conjugated camptothecin (CPT) to a glutathione-responsive disulphylbutyrate linker and attached it to a peptide domain. This approach places the CPT segments as the hydrophobic

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core of the nanostructure. Under tumor-relevant conditions, these nanostructures exhibited *in vitro* efficacy against cancer cell lines.¹⁶

The previous examples, hydrazine-PA and disulphylbutyrate-PA nanofibres, are useful platforms to deliver either ketone- or alcohol-containing therapeutic agents. However, these strategies cannot be extended to the large family of drugs with an acidic functionality, including atorvastatin (Lipitor®), pregabalin (Lyrica®), and Lev ofloxacin (Levaquin®). Naproxen is an acid-bearing nonsteroidal anti-inflammatory drug used to treat osteoarthritis,¹⁷ rheumatoid arthritis,¹⁸ and cancer.¹⁹ We report here the development of a peptide-based supramolecular nanostructure bearing sensitive naproxen-ester linkers for enzyme-triggered release. Enzymatic drug release is attractive because it does not require an external stimulus and can be tailored to diseases expressing specific enzymes.²⁰

Esterases are ubiquitous enzymes found in the body with varying degrees of specificity.²¹ Many esterases, such as human carboxylesterase and porcine liver esterase (PLE),²² present the conserved catalytic triad sequence SHE deep inside the protein,²³ around 19 Å from the surface (Fig. S1). Thus, any nanofibre-drug conjugate must have a linker long enough to access the catalytic site to be hydrolysed by the enzyme. Scheme 1 shows the synthesis of the PA-naproxen conjugate **3** (32.5 Å from ester bond to the PA core, linker in red, naproxen in blue, also Fig. S2). In addition, we prepared PAs **4** and **5**, with linkers of with a 19.4 Å or 11.6 Å extended length, respectively, to study their differences in enzymatic hydrolysis (Scheme 2). Such molecular design allows the presentation of the naproxen-ester derivatives on the surface of the nanofibres, where we expect greater conformational degrees of freedom relative to the hydrophobic core for optimal esterase accessibility.^{24, 25}

We also synthesized C₁₆-V₃A₃E₃ (**E3**)²⁶ PA with the same V₃A₃ β-sheet-forming domain to dilute the drug-conjugated PAs **3–5** within the supramolecular nanofibres. In previous investigations, we have found that reducing the surface density of bioactive PAs on the nanofibres by mixing with diluent PAs promoted enhanced biological responses *in vitro* and *in vivo*.^{27–29} In this work we co-assembled the naproxen-conjugated PAs with PA **E3** at 1:3 ratio (a higher proportion of the drug-conjugated PAs resulted in poor water solubility). After co-assembly, cryogenic transmission electron microscopy micrographs revealed the formation of nanofibres (Fig. 1 and Fig. S3). As previously reported, one-dimensional micron-long nanofibres are typical of the C₁₆-V₃A₃E₃ sequence.^{26, 28} In addition, circular dichroism of the same systems indicated the presence of β-sheets (Fig. S4).

To evaluate the stability of the ester linkage, we first investigated base-promoted hydrolysis of the PA-ester conjugates **3** diluted with a filler PA **E3** (1:3 molar ratio) at different pH values: 7.4, 9.0 and 12.0. The PA-ester conjugate exhibited chemical stability towards hydrolysis at pH 7.4, with only 2.8 ± 2.3% of Naproxen released after 7 d (Fig. S5). The stability of the ester bond on the PAs at physiological pH was comparable to previously reported polymeric systems containing either ibuprofen or naproxen.^{2, 30} At higher pH, the ester linker was hydrolysed to a much greater extent with increased drug release: 22 ± 4% at pH 9 and 76 ± 5% at pH 12 after 7 d.

In order to evaluate enzymatic hydrolysis, PA **3** was diluted with PA **E3** (1:3 molar ratio) and incubated in PBS (pH 7.4) with porcine liver esterase (PLE) at 20 U mL⁻¹, a physiologically relevant dose.³¹ Fig. 2A shows the HPLC trace of PA **3** before and after the addition of PLE. The peaks were identified by mass spectrometry as naproxen (11.8 min), PA **E3** (14.6 min), hydrolysed PA **3** (15.6 min), and non-hydrolysed PA **3** (17.3 min). Naproxen release from PA **3** was 50 ± 4% after 5 h and 56 ± 2% after 24 h (Fig. 2b) with no substantial release at later time points. This result indicated that PA **3**, which is stable in PBS at physiological pH, can be enzymatically cleaved to release the drug. However, we did not observe complete hydrolysis, as evidenced by the presence of non-hydrolysed PA **3** in the HPLC trace. Interestingly, we found that introducing additional PLE (10 U mL⁻¹) after 24 h only increased naproxen hydrolysis to 61 ± 3% (Fig. S6). In addition, when PA **3**- PA **E3** (1:3 molar ratio) was incubated 24 h with varied amounts of PLE, we did not find a substantial difference in hydrolysis between 5 and 20 U mL⁻¹ of the enzyme (Fig. S7). Since epitope crowding on the nanofibre surface has been shown to decrease interactions with proteins,^{27–29} we postulate that the 25% dilution of the drug-conjugated PA was too high for optimal esterase accessibility. To test our hypothesis, we incubated the PEG3-Naproxen ligand **2** in the presence of PLE (20 U mL⁻¹) with and without PA **E3** at 1:3 molar ratio to keep the same concentrations of the earlier experiments (Fig. S8). In both cases we observed complete hydrolysis of the ester after 1 d, which implies that the PLE activity was not disrupted when ligand **2** is not tethered to the nanostructures. Next, a further dilution of PA **3** from 25% to 10% by PA **E3** exhibited a 15% increase in enzymatic hydrolysis (Fig. S9), which suggests that naproxen crowding was an important factor for incomplete hydrolysis. Furthermore, when naproxen-conjugated PAs are co-assembled with PA **E3**, it is possible that some naproxen is buried in the hydrophobic parts of the nanofibre, which can also diminish the percentage of hydrolysis. Nonetheless, we have observed here that modification of monomer ratio allows the tuning of drug release from these supramolecular nanostructures.

The effect of linker length on enzymatic hydrolysis was evaluated using PAs **4–5** (Fig. 2). These PAs were diluted with PA **E3** (1:3 molar ratio) and treated with PLE at 20 U mL⁻¹. After 24 h, naproxen release from PA **4** was 36 ± 4%, approximately 20% lower than that from PA **3**. PA **5** exhibited the least amount of drug release after 24 h (6 ± 1 %). Even after 48 h incubation, there was minimal release from PA **5** (7 ± 2). Since esterases require entry of the substrate into their catalytic pockets, it is possible that the longer linker diminished the steric hindrance of nanofibres to enzyme action, allowing easier access of the naproxen conjugate to the PLE-catalytic site. This is in agreement with previous findings that showed bioactive cues were more functional when they were separated from the bulk scaffold via molecular linkages.³² Moreover, D'Emanuele and co-workers have reported that the enzymatic hydrolysis of non-assembling dendrimers containing ester-naproxen conjugates increases with longer linkages.³³

The anti-inflammatory activity of the hydrolysed naproxen was evaluated using a cyclooxygenase (COX)-2 inhibitor assay.³⁴ COX enzymes promote the synthesis of prostaglandins, which are key mediators in inflammation, pain, and other diseases.³⁵ For COX-2 inhibition, it is important that the drug is hydrolysed to a carboxylic acid to promote

electrostatic interactions with the residues on the active site.³⁶ PA **3**, which exhibited the fastest hydrolysis, was diluted with PA **E3** (1:3 molar ratio) and incubated with or without 20 U mL⁻¹ of PLE for 24 h at 37 °C. The collected supernatant was indeed able to inhibit COX-2-activity by 70% (Fig. S10). In addition, we evaluated biocompatibility of this system and did not observe cytotoxicity in mouse mesenchymal stem cells (Fig. S11).

We have shown here that drugs containing carboxylic acid functionalities can be conjugated to PAs through an ester linkage for controlled drug release. The PA-naproxen conjugate nanofibre exhibited stability at physiological pH and temperature, which will be advantageous for long term storage. Enzymatic hydrolysis of PAs shows improved release kinetics with increased spacing between the drug and the PA backbone, likely due to increase enzyme access to the ester linkage. The acid functionality of naproxen is unaltered upon enzymatic action and its bioactivity is preserved as shown by the COX-2 inhibitory assay. The biocompatible nature of the supramolecular assemblies make them ideal platforms for drug delivery applications. In clinical settings, we propose that the naproxen-conjugated nanofibres may be systemically delivered for cancer therapy, or locally injected to form a gel network *in situ* to treat osteoarthritis or rheumatoid arthritis. Furthermore, the chemistry reported here can be applied to many other drugs containing carboxylic acids. Since the lipid tail and β -sheet regions of the PA are unaltered, it is expected that the self-assembled scaffold will be conserved upon release. Therefore, this supramolecular design makes it possible to encapsulate drugs³⁷ or integrate different types of PA monomers into the same nanostructure, allowing sequential release of drugs.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Figure 1. Representative cryogenic transmission electron micrograph of 1:3 molar mixtures of PA **3** with **E3** PA (a). Molecular graphics representation of a nanofibre assembled from PA **3** with **E3** PA in 1:3 molar ratio (b).

Figure 2.

(a) Overlap of PA 3- PA E3 (1:3 molar ratio, 1 mM final concentration) before (black) and after (red), addition of PLE (20 U/mL) at pH 7.4, 60 rpm and 37 °C, 1 day. (b) Naproxen release as a function of time from PAs 3, 4 and 5 with PA E3 (1:3 molar ratio) in the presence of PLE (20 U/mL) at pH 7.4, 60 rpm and 37 °C.

Scheme 1.

Synthesis of PA- naproxen conjugate **3**. i. PyBOP, DIPEA, DME, DCM; ii. TFA, TIPS, H₂O

4, PEG1-NAP-PA

5, C4-NAP-PA

Scheme 2.
Structures of PA-naproxen conjugates **4** and **5**