'Self-Delivery' Using Anti-Inflammatory Hydrogels: Biological Evaluation of NSAID-Dehydrodipeptide Conjugates

Peter J. Jervis[1], Rute Moreira[2], Paula M. T. Ferreira[1], José A. Martins[1], David M. Pereira[2]

[1]Centro de Quimica, Universidade do Minho, Braga, Portugal
[2]Faculdade de Farmácia, Universidade do Porto, Porto, Portugal

Peptide-based supramolecular **hydrogels**

- **Short peptides** (and other small amphoteric molecules) *N*-capped with **aromatic groups** often undergo **self-assembly** in aqueous media to afford **supramolecular hydrogels**, which are highly ordered three-dimensional **molecular networks** consisting of **mainly water** molecules.

- In contrast to the polymer-based chemically cross-linked hydrogels, these physical hydrogels are held together by **non-covalent interactions** such as **hydrogen bonds**, **van der Waals** and **π-stacking interactions**.

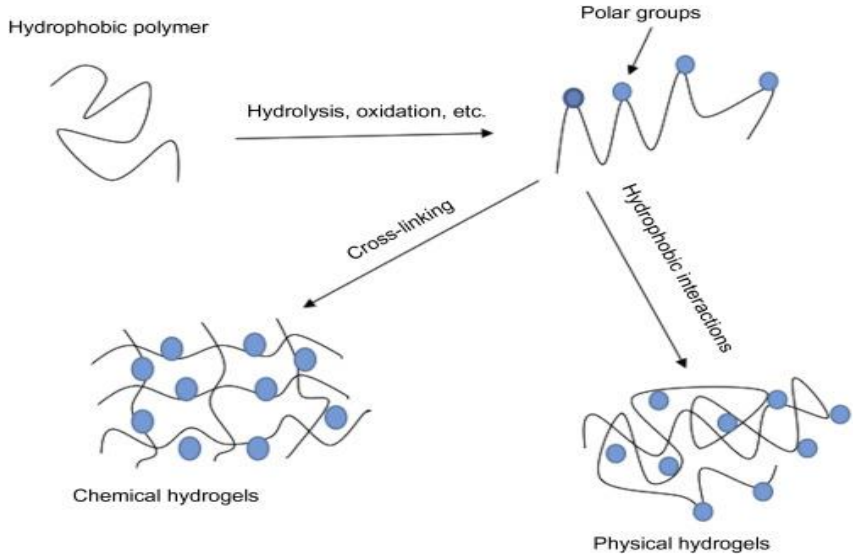
- **Advantages** over other types of hydrogelators: **ease of synthesis**, **low toxicity**, trends in **mechanical properties** can be readily **tuned** by the physical–chemical properties of the **amino acid side chains**.

Peptide-based supramolecular **hydrogels**

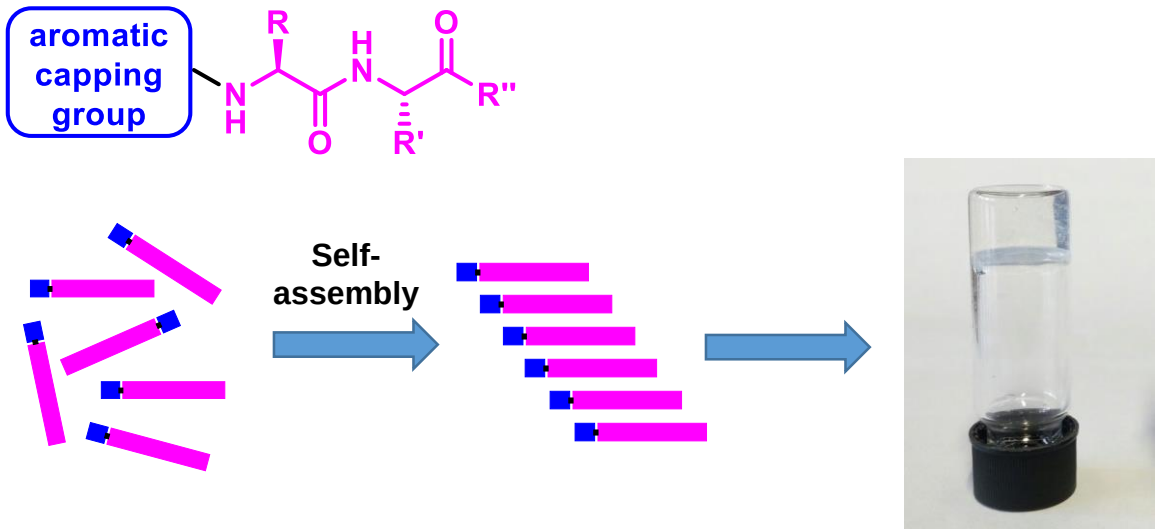
- **Short peptides** (and other small amphoteric molecules) *N*-capped with **aromatic groups** often undergo **self-assembly** in aqueous media to afford **supramolecular hydrogels**, which are highly ordered three-dimensional **molecular networks** consisting of **mainly water** molecules.

- In contrast to the polymer-based chemically cross-linked hydrogels, these physical hydrogels are held together by **non-covalent interactions** such as **hydrogen bonds**, **van der Waals** and **π-stacking interactions**.

- **Advantages** over other types of hydrogelators: **ease of synthesis**, **low toxicity**, trends in **mechanical properties** can be readily **tuned** by the physical–chemical properties of the **amino acid side chains**.



polymer-based hydrogels



peptide-based hydrogels

Biomater. Sci., 2015, **3**, 937–954
Soft Matter, 2019, **15**, 1704–1715

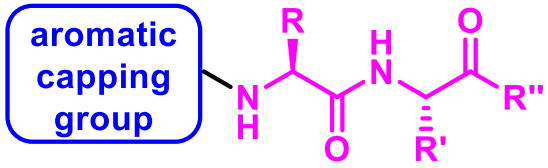
Peptide-based supramolecular **hydrogels**

- Structure consists of a **hydrophilic peptide** chain, ***N*-terminated** with an **aromatic** capping group.

- The peptide chains can associate through **hydrogen bonds** and **ionic** interactions.

- The *N*-capping group is usually a **bulky aromatic moiety**, such as flourenylmethoxycarbonyl (**Fmoc**), i**ndole-3-acetyl** or **naphthalene** derivatives.

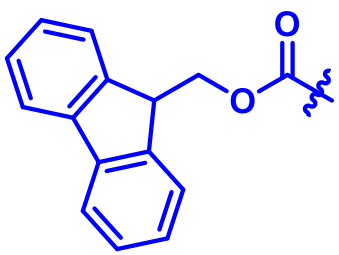
- Aromatic groups provide the **π-stacking** and **hydrophobic interactions** required for **self-assembly**.



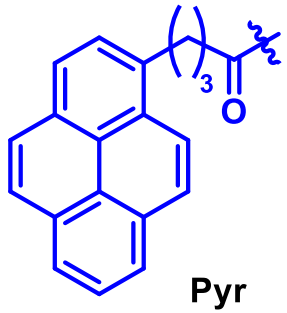
R and R' = various canonical amino acid side chains

R'' = OH (for dipeptides) or extra amino acid residues

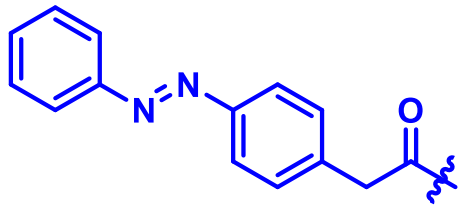
aromatic capping group =



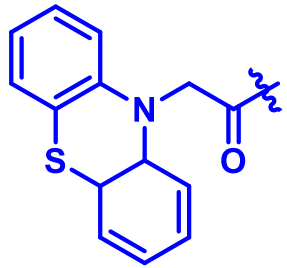
Fmoc



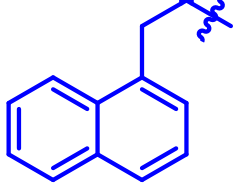
Pyr



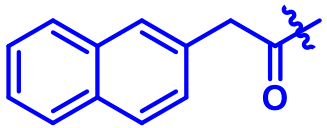
Azo



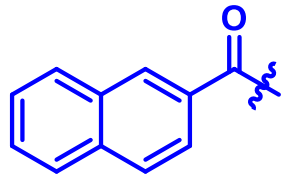
PTZ



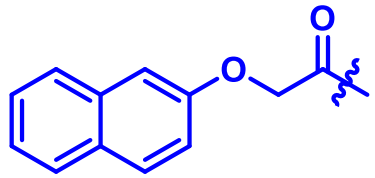
1-naphthalenylacetyl



2-naphthalenylacetyl



2-naphthoyl



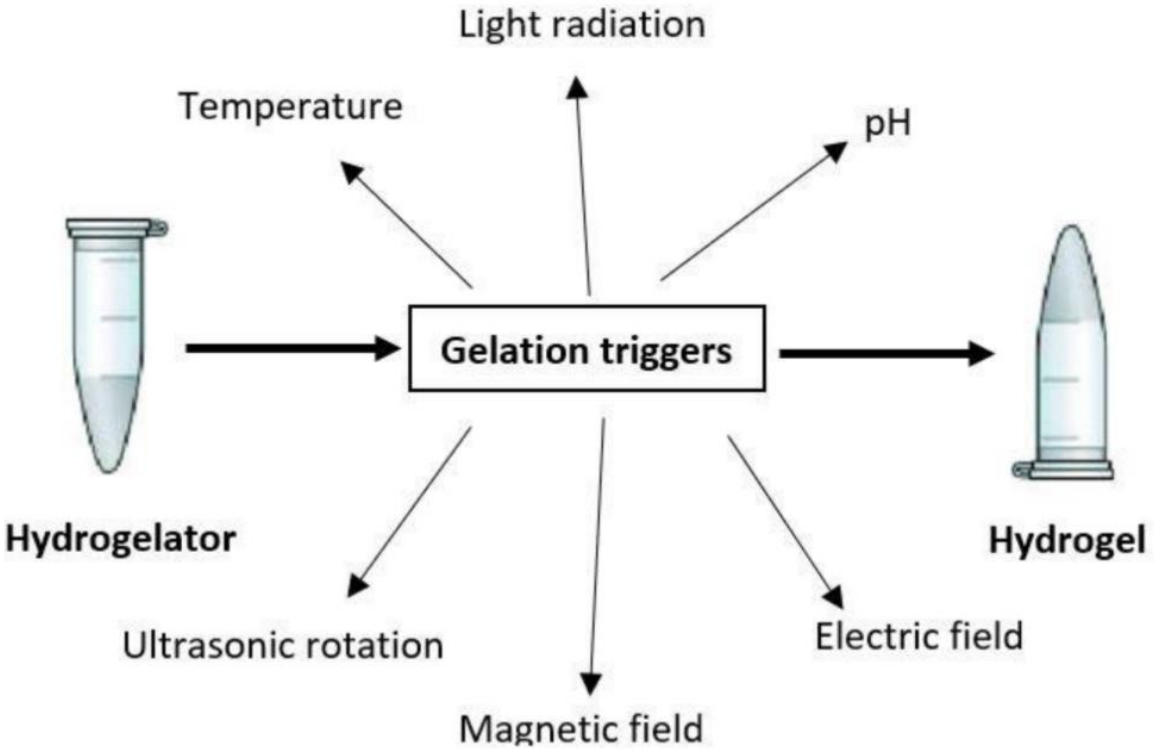
2-naphthalenyloxyacetyl

Langmuir, 2019, **35**, 6506–6521

Peptide-based supramolecular **hydrogels**

- Gelation is **initiated** in response to an **external trigger**.

- Most commonly employed trigger is a **temperature** change, **pH** change, **solvent switch** or **enzymatic cleavage** of a solubilising **phosphate** group.



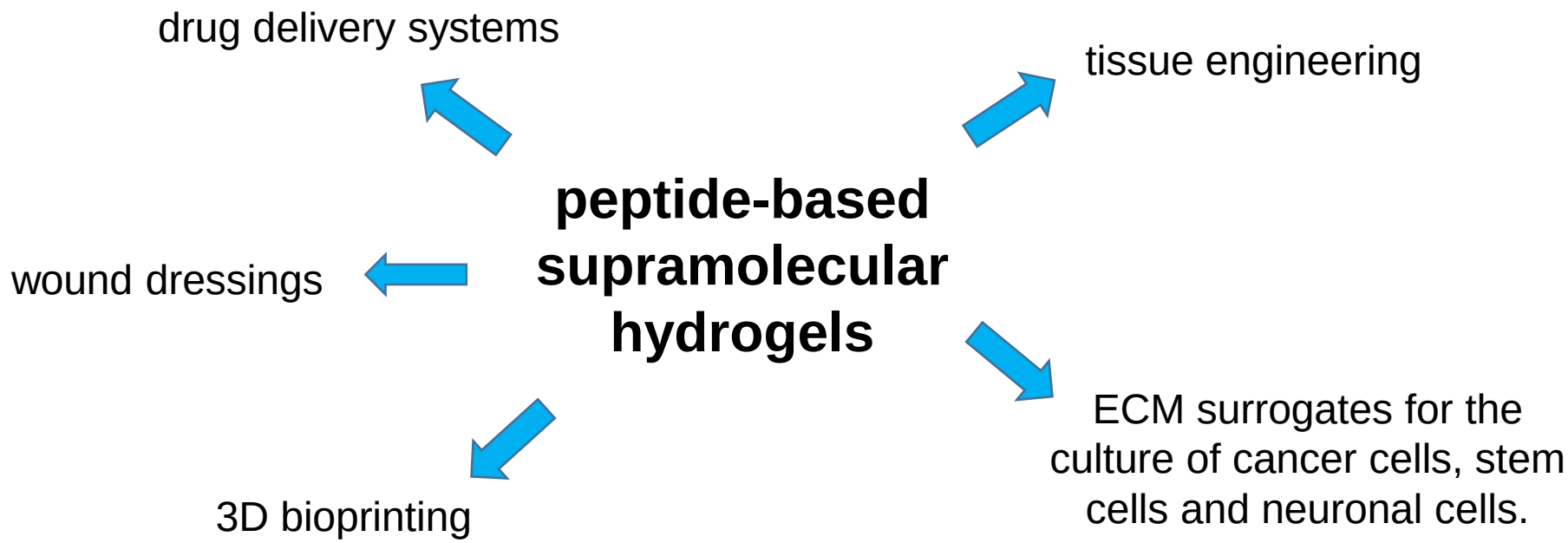
Mater. Today, 2014, **17**, 381–388

Peptide-based supramolecular **hydrogels**

- The properties closely **mimic** those of the **extracellular matrix (ECM)**, and as such they have found many **medicinal applications**:

Peptide-based supramolecular **hydrogels**

- The properties closely **mimic** those of the **extracellular matrix (ECM)**, and as such they have found many **medicinal applications**:



Bioact. Mater., 2019, **4**, 120–131

Nonsteroidal **anti-inflammatory** drugs (NSAIDs)

- Class of drug which eases pain, reduces fever, decreases inflammation

- Treatment of inflammatory diseases, such as **rheumatoid arthritis**, **osteoarthritis**, **tendonitis** and **bursitis**

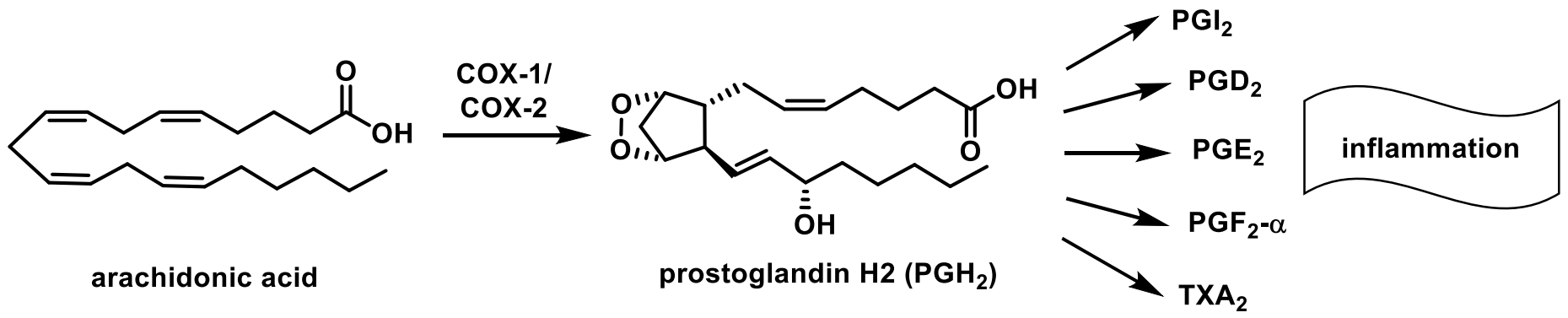
- Include ibuprofen, naproxen and aspirin

- Inhibit cyclooxygenase (**COX**) enzymes, which synthesise the **prostaglandins** responsible for inflammation, from arachidonic acid

J. Pain Res., 2015, **8**, 105–118

Nonsteroidal **anti-inflammatory** drugs (NSAIDs)

- Class of drug which eases pain, reduces fever, decreases inflammation
- Treatment of inflammatory diseases, such as **rheumatoid arthritis**, **osteoarthritis**, **tendonitis** and **bursitis**
- Include ibuprofen, naproxen and aspirin
- Inhibit cyclooxygenase (**COX**) enzymes, which synthesise the **prostaglandins** responsible for inflammation, from arachidonic acid



J. Pain Res., 2015, **8**, 105–118

Nonsteroidal **anti-inflammatory** drugs (NSAIDs)

- Class of drug which eases pain, reduces fever, decreases inflammation

- Treatment of inflammatory diseases, such as **rheumatoid arthritis**, **osteoarthritis**, **tendonitis** and **bursitis**

- Include ibuprofen, naproxen and aspirin

- They inhibit cyclooxygenase (**COX**) enzymes, which synthesise the **prostaglandins** responsible for inflammation, from arachidonic acid

- Two COX isozymes, COX-1 and COX-2. The **COX-1** isozyme serves a **maintenance** function in healthy cells, whilst the **COX-2** isozyme is produced in **response to injury** and is involved in the inflammatory response to tissue damage.

- Inhibition of **COX-2** is responsible for the **anti-inflammatory effect**. However, these **NSAIDs** also inhibit the constitutive **COX-1 isozyme**, which regulates platelet aggregation, gastrointestinal protection and kidney function.

- Unwanted **COX-1 inhibition** can result in **gastric toxicity** and therefore COX inhibitors which **selectively target COX-2** are sought.

J. Pain Res., 2015, **8**, 105–118

Nonsteroidal **anti-inflammatory** drugs (NSAIDs)

The discovery of the COX-2 isozyme led to the development of many COX-2 selective inhibitors, for example rofecoxib, valdecoxib and celecoxib, which exhibited a safer gastric toxicity profile

- **However**, many of the launched COX-2 drugs produced an **increased risk** of **heart attack** and **stroke**. As a result, rofecoxib was withdrawn worldwide in 2004, and valdecoxib was withdrawn from the US and European markets in 2005.

- Celecoxib remains available in the United States and Europe, but carries a boxed warning. Thus, the **search** for **COX inhibitors without side effects** remains active.

Curr. Med. Chem., 2010, **17**, 1563-

Nonsteroidal **anti-inflammatory** drugs (NSAIDs)

The discovery of the COX-2 isozyme led to the development of many COX-2 selective inhibitors, for example rofecoxib, valdecoxib and celecoxib, which exhibited a safer gastric toxicity profile

- **However**, many of the launched COX-2 drugs produced an **increased risk** of **heart attack** and **stroke**. As a result, rofecoxib was withdrawn worldwide in 2004, and valdecoxib was withdrawn from the US and European markets in 2005.

- Celecoxib remains available in the United States and Europe, but carries a boxed warning. Thus, the **search** for **COX inhibitors without side effects** remains active.

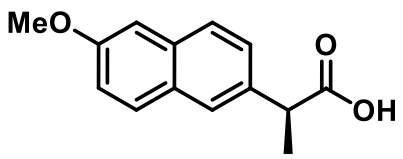
Possible solution:

- **Alternative formulations** of NSAIDs, to **control** the **distribution** of the drug *in vivo*

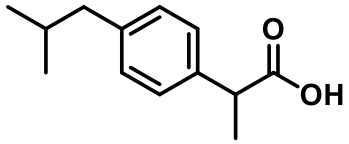
- Develop **targeted drug delivery systems** to **increase efficacy, reduce doses** and **decrease side-effects**

Curr. Med. Chem., 2010, **17**, 1563-

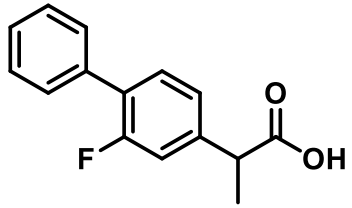
Nonsteroidal **anti-inflammatory** drugs (NSAIDs)



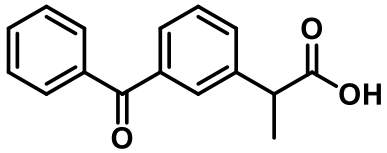
naproxen (Npx)



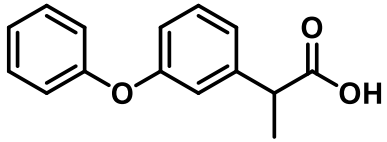
ibuprofen (Ibp)



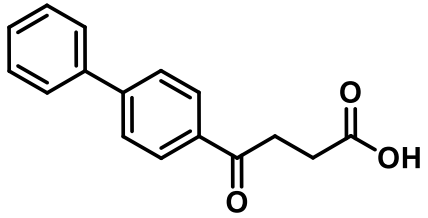
flurbiprofen (Fbp)



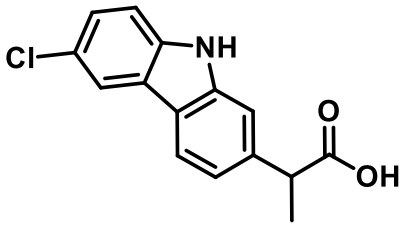
ketoprofen (Kep)



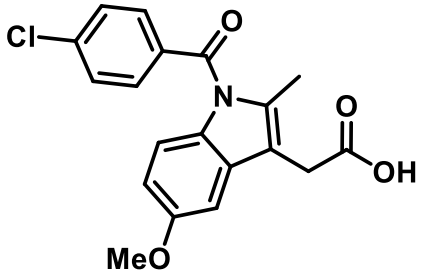
fenoprofen (Fnp)



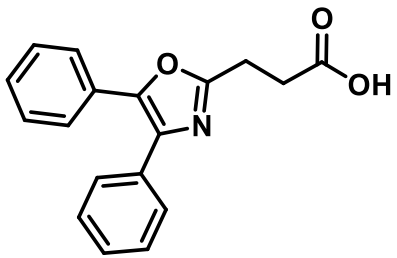
fenbufen (Fbf)



carprofen (Car)



indomethacin (Ind)



oxaprozin (Oxp)

Key structural features:

- **Aromatic**, flat, hydrophobic core
- Terminal **carboxylic acid** head group – allows facile conjugation to other molecules

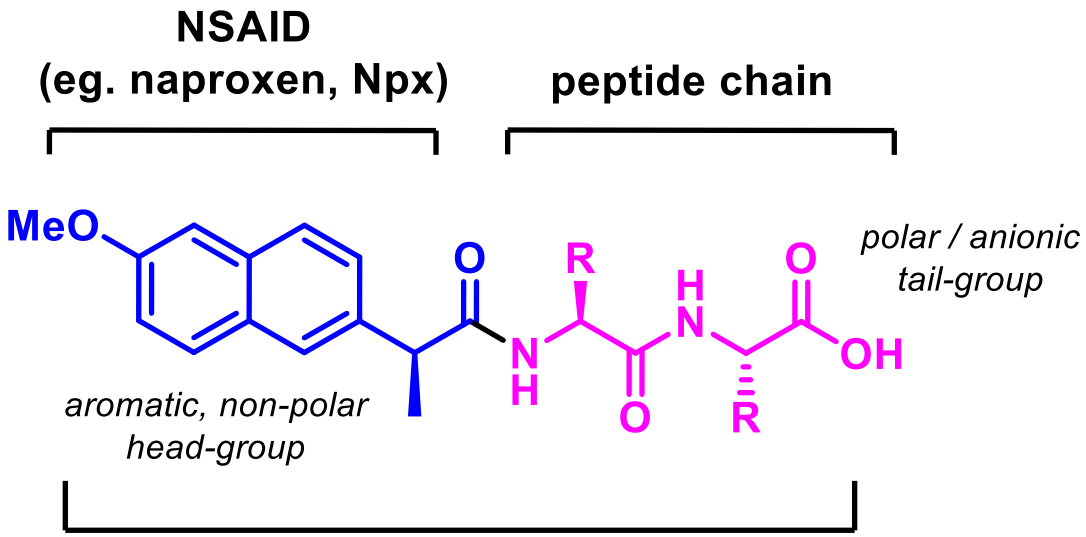
Therefore:

Ideal moieties for replacing the usual **aromatic capping group** of **peptide** hydrogelators

- Retention of **anti-inflammatory** and **hydrogelation** properties**?**
- **‘Self-Delivery’** of **NSAIDs?**

J. Pain Res., 2015, **8**, 105–118

Nonsteroidal **anti-inflammatory** drugs (NSAIDs)



anti-inflammatory properties?
- other indications - topical application

hydrogelation properties?
- drug delivery - wound healing

Key structural features:

- **Aromatic**, flat, hydrophobic core
- Terminal **carboxylic acid** head group – allows facile conjugation to other molecules

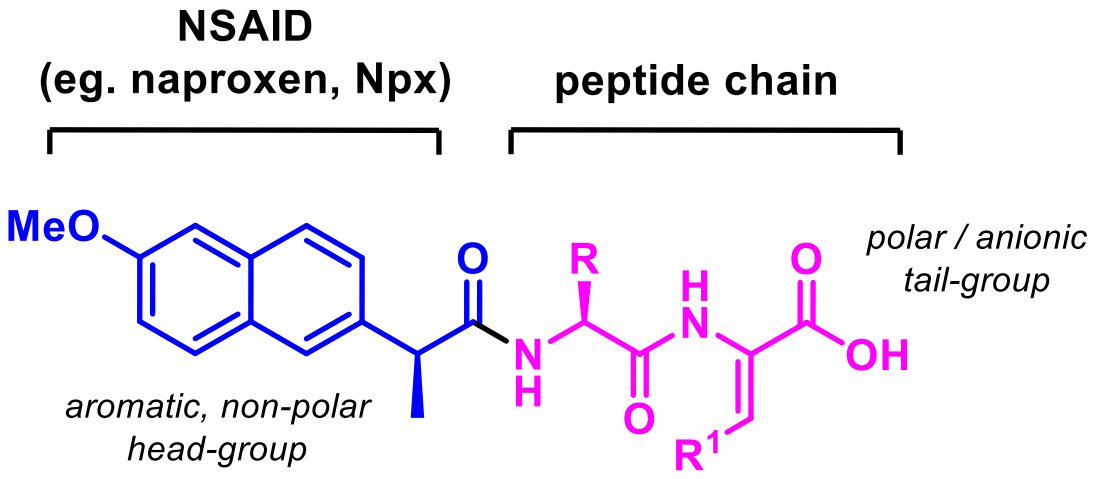
Therefore:

Ideal moieties for replacing the usual **aromatic capping group** of **peptide** hydrogelators

- Retention of **anti-inflammatory** and **hydrogelation** properties**?**
- **'Self-Delivery'** of **NSAIDs?**

J. Pain Res., 2015, **8**, 105–118

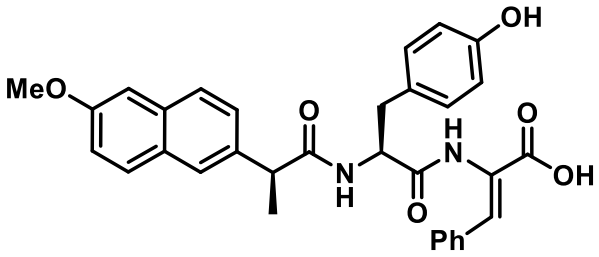
Designing **NSAID-dehydropeptide** conjugates as **hydrogelators**



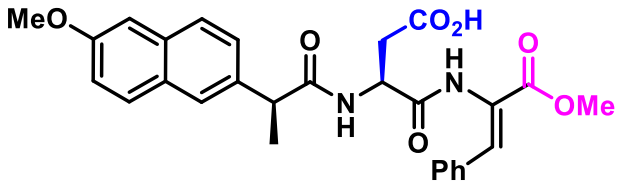
dehydroamino acid *residue gives proteolytic stability and decreases molecular flexibility*

Combined **structural** and **pharmaceutical** function

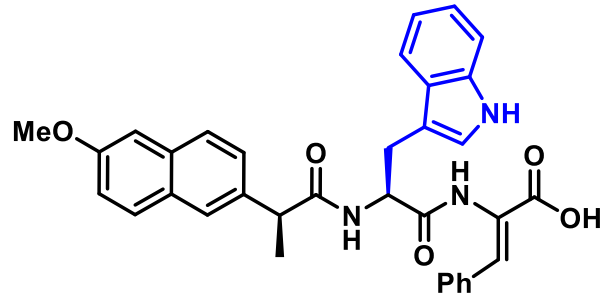
Target Molecules



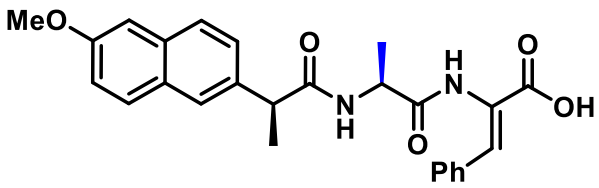
Npx-L-Tyr-Z-ΔPhe-OH (1)
MW = 538.60, cLogP = 5.36



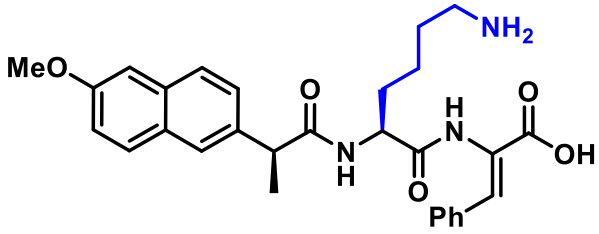
Npx-L-Asp-Z-ΔPhe-OMe (2)
MW = 504.53, cLogP = 4.17



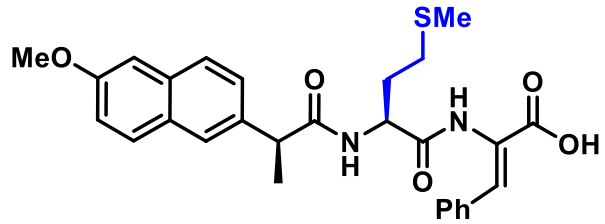
Npx-L-Trp-Z-ΔPhe-OH (3)
MW = 561.63, cLogP = 5.99



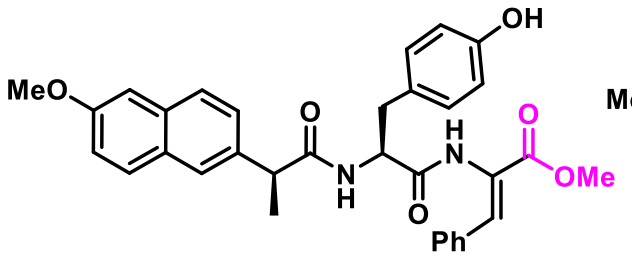
Npx-L-Ala-Z-ΔPhe-OH (4)
MW= 446.50, cLogP = 4.38



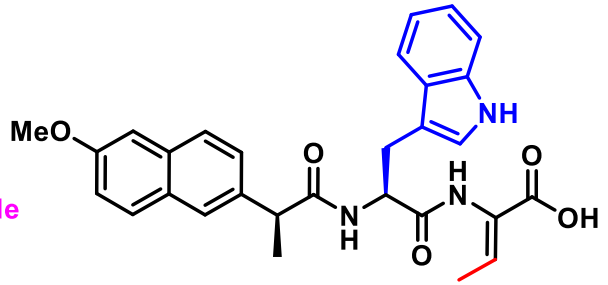
Npx-L-Lys-Z-ΔPhe-OH (5)
MW = 503.59, cLogP = 3.89



Npx-L-Met-Z-ΔPhe-OH (6)
MW = 506.61, cLogP = 4.83

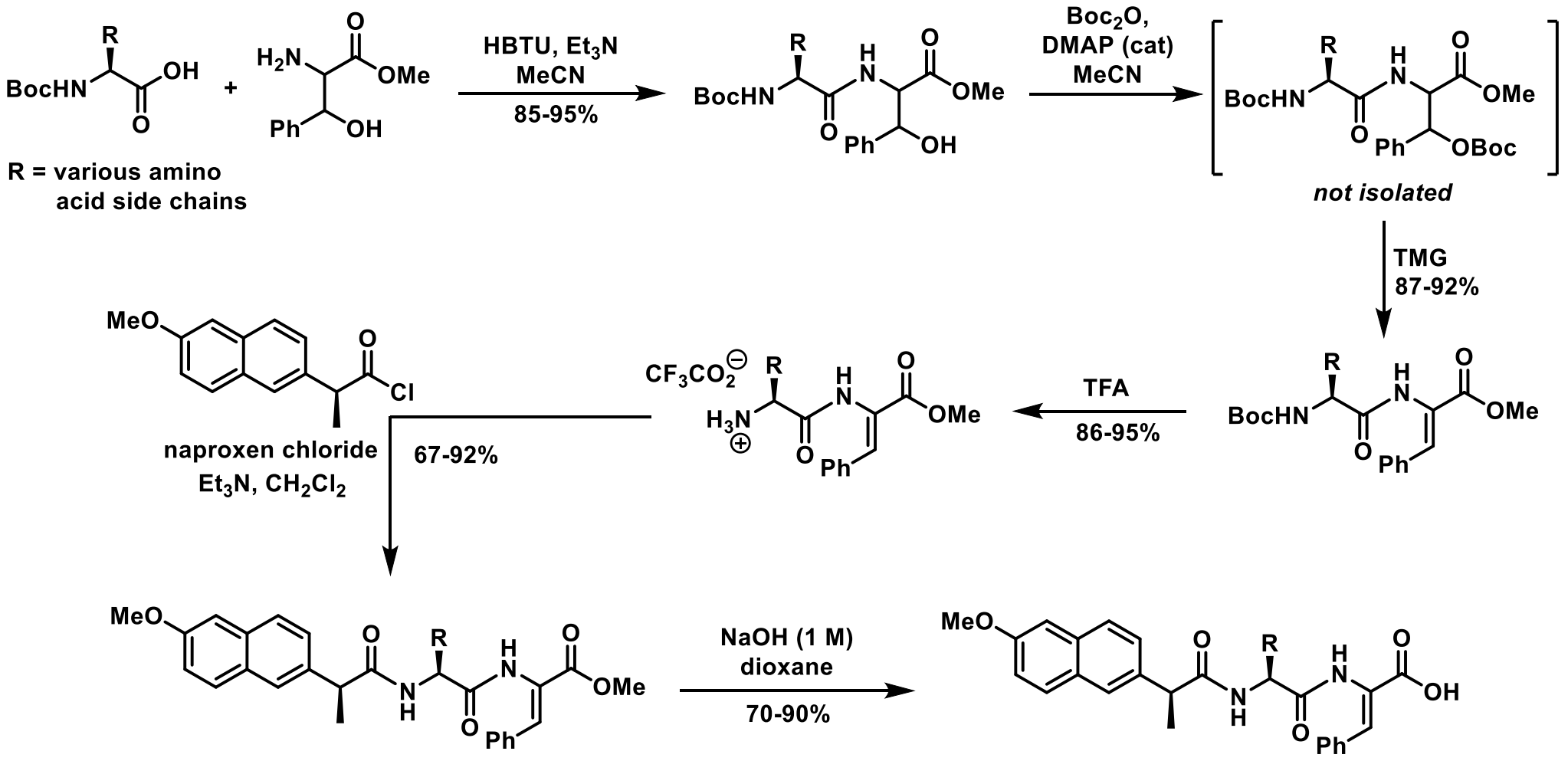


Npx-L-Tyr-Z-ΔPhe-OMe (7)
MW = 552.62, cLogP = 5.98



Npx-L-Trp-Z-ΔAbu-OH (8)
MW = 499.56, cLogP = 4.53

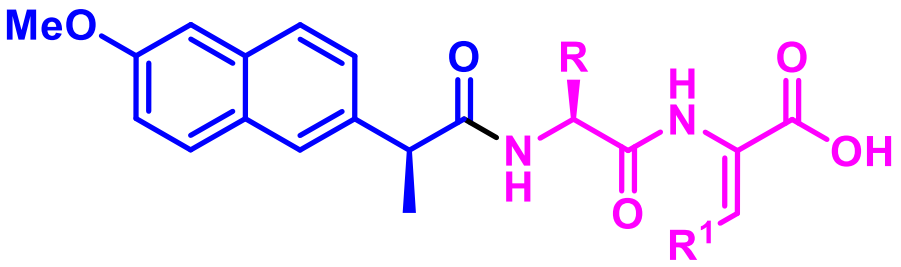
Synthesis of **NSAID-dehydropeptide** conjugates



Facile synthesis by **solution phase** peptide synthesis

Nanomaterials, 2019, **9**, 541
J. Mater. Chem. B, 2015, **3**, 6355–6367
Biomacromolecules, 2015, **16**, 3562–3573

Gelation properties of **NSAID-dehydropeptide** conjugates



Typical gelation procedure:

Conjugate (1.0-8.0 mg) suspension in water (1.0 mL)

1 M NaOH (20 μL)

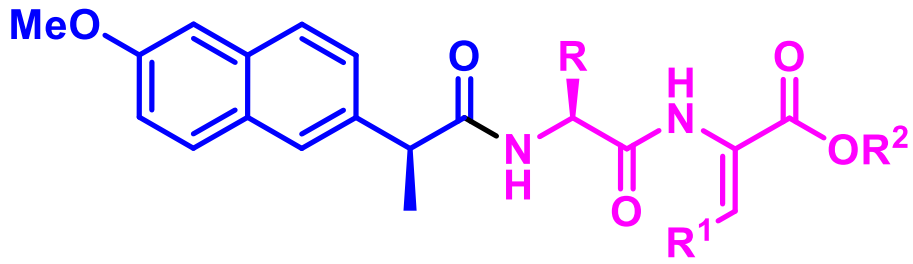
Conjugate in solution

GdL (4.0 mg) (slow release of H^+)

Gelation occurs

Langmuir, 2019, **35**, 6506–6521

Gelation properties of **NSAID-dehydropeptide** conjugates

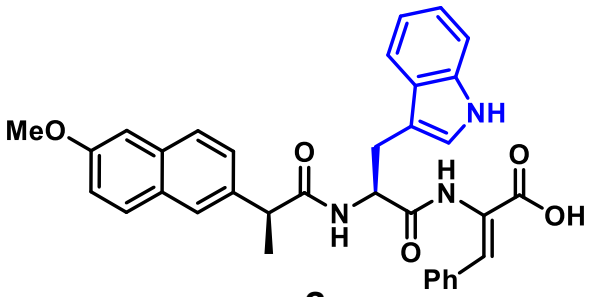


Compound		Critical Gelation Concentration (wt%)	G_{max}' (Pa)	G_{max}'' (Pa)	pH
Npx-L-Tyr-*Z*-ΔPhe-OH	**1**	0.4	1.22×10^2	13	6-7
Npx-L-Asp-*Z*-ΔPhe-OMe	**2**	0.4	3.93×10^4	3.53×10^3	7
Npx-L-Trp-*Z*-ΔPhe-OH	**3**	0.4	3.11×10^5	1.04×10^5	5
Npx-L-Ala-*Z*-ΔPhe-OH	**4**	0.8	9.8×10^2	1.0×10^2	5
Npx-L-Lys-*Z*-ΔPhe-OH	**5**	0.4	6.32×10^3	2.37×10^2	6-7
Npx-L-Met-*Z*-ΔPhe-OH	**6**	0.2	2.34×10^3	1.11×10^2	5
Npx-L-Tyr-*Z*-ΔPhe-OMe	**7**	N/A	N/A	N/A	N/A
Npx-L-Trp-*Z*-ΔAbu-OH	**8**	0.4	5.74×10^3	1.91×10^3	7

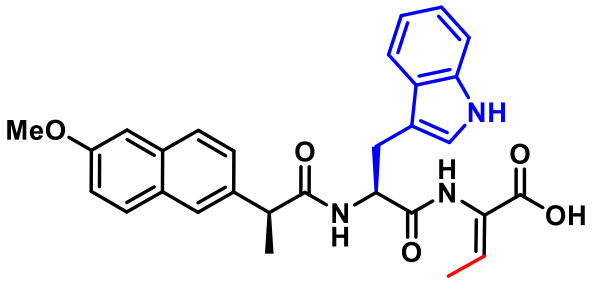
Summary of structural properties of hydrogels of the naproxen-dehydropeptide conjugates **1-8**

Nanomaterials, 2019, **9**, 541
J. Mater. Chem. B, 2015, **3**, 6355–6367
Biomacromolecules, 2015, **16**, 3562–3573

Potential for **drug delivery** applications



3

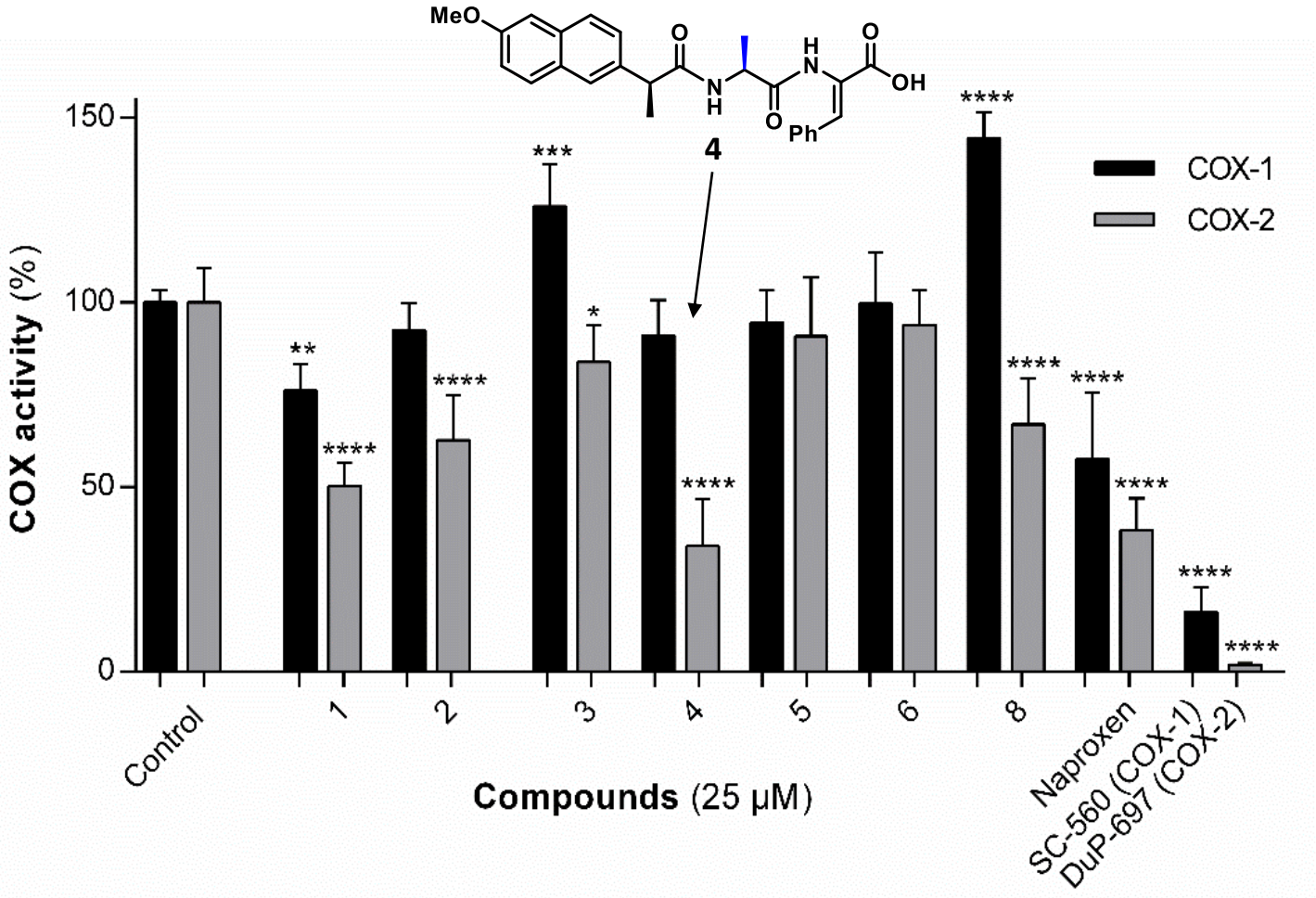


8

- Compounds **3** and **8** were chosen as examples to investigate the drug deliver properties.
- A potential **anti-tumour** thieno[3,2-b]pyridine derivative could be **non-covalently incorporated** into the hydrogel structure – therefore potential as drug nanocarriers.
- **Förster** resonance energy transfer experiments revealed that the loaded drug was located in a **hydrophobic environment** within the **hydrogel matrix**, associated with the peptide fibers.
- In a different study using a longer naproxen-dehydropeptide conjugate, **FRET** studies showed **curcumin** is incorporated into the hydrogel structure and interacts non-covalently with the hydrogel fibrils. **Curcumin** could be **delivered** from the hydrogel into **model membranes** (SUVs) loaded with Nile red.

Biomacromolecules, 2015, **16**, 3562–3573
Mater. Chem. B, 2017, **5**, 8607–8617

Cycloogenoxygenase enzymatic assays (**COX-1** and **COX-2**)



COX-1 and COX-2 activities in the presence of compounds **1**-**6** and **8** at 25 µM. Values are shown with mean ± SD. * $p \leq 0.05$; ** $p \leq 0.01$; *** $p \leq 0.001$, **** $p \leq 0.0001$.

- Most compounds tested show little COX-2 selectivity.
- Compound **4**, which contains the smallest canonical amino acid residue (alanine) tested, **inhibits COX-2** to a **greater level** than **naproxen**, whilst providing **no COX-1 inhibition**.
- Therefore, compound **4** is a **selective COX-2 inhibitor.**

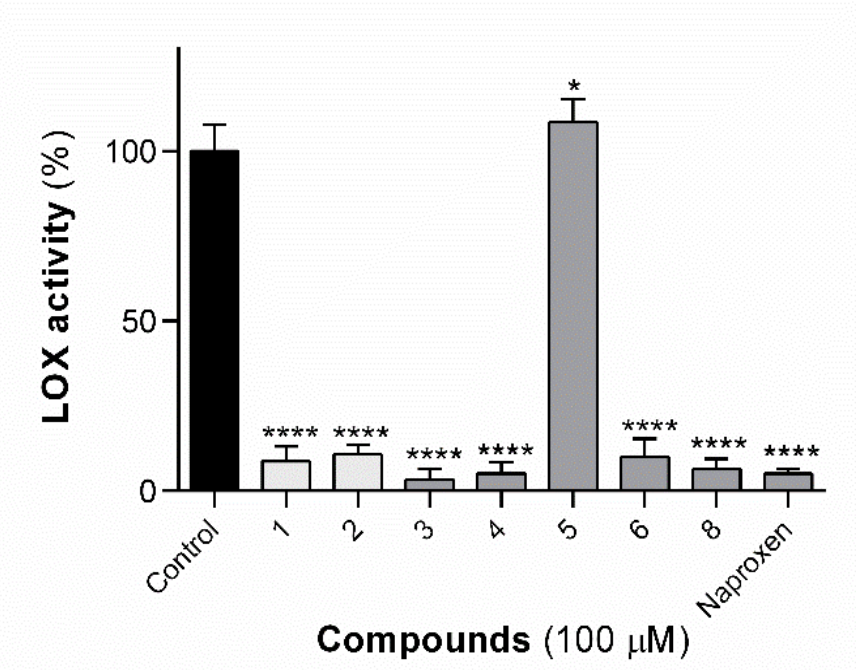
Pharmaceutics, 2020, **12**, 122

Lipoxygenase enzymatic assays (**5-LOX**)

- **LOX** enzyme is responsible for the **production of inflammatory leukotrienes**, which are a major **cause of inflammation** in asthma, allergic rhinitis and osteoarthritis.

- **All** of the compounds showed a **strong ability to inhibit LOX** enzyme at 100 μM, **except** compound **5** (the **most polar example**), which was inactive.

- In a dose-response assay, **IC_{50}** values **slightly higher** than observed for the parent molecule, **naproxen**.



LOX activity in the presence of compounds **1**-**6** and **8** at 100 μM. Values are shown with mean ± SD. **** $p \leq 0.0001$.

IC_{50} of the compounds for LOX activity.

Compound	IC50 (μM)
1	54.1
2	67.4
3	55.9
4	55.7
6	60.3
8	48.9
Naproxen	22.0

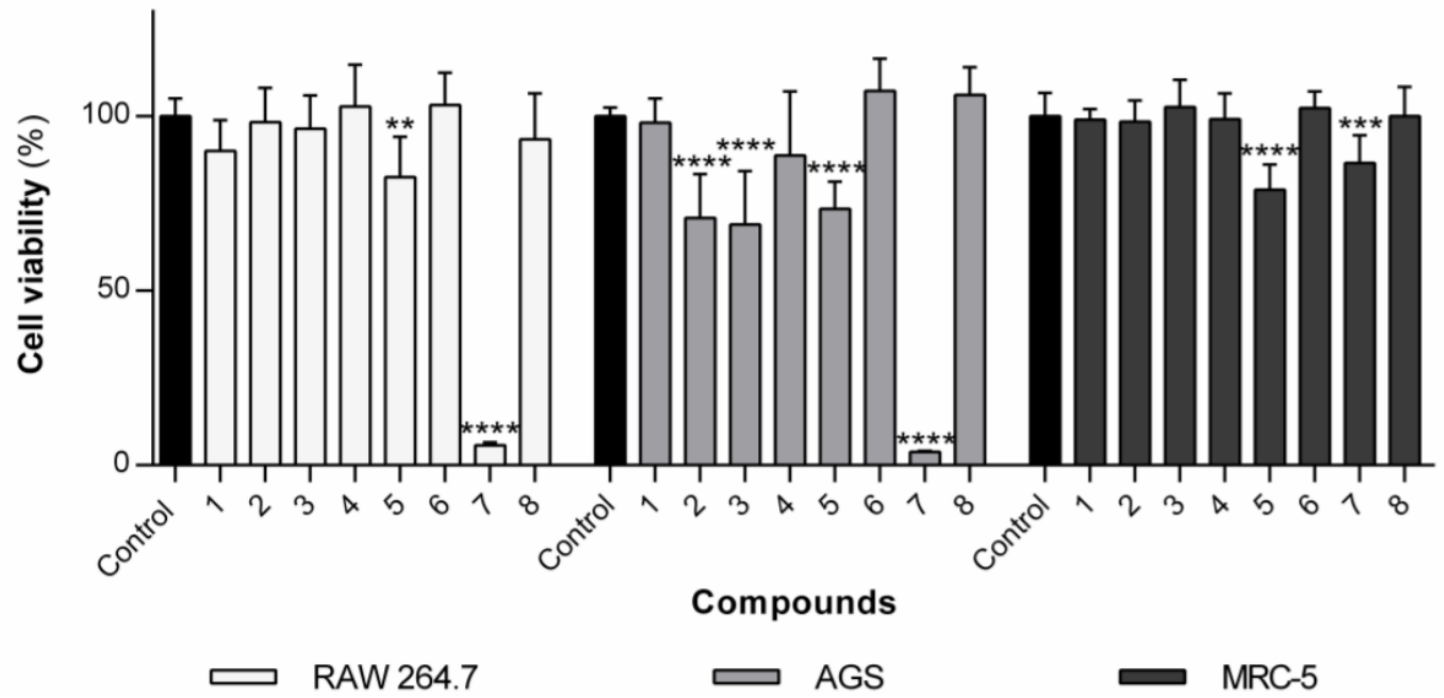
Pharmaceutics, 2020, **12**, 122

Cytotoxicity – **viability** assays

- **Toxicity** of the compounds to **RAW 264.7** (macrophages involved in **inflammation**), **AGS** (human **cancer** cell-line) and **MRC-5** (**human** fibroblast cell-line) was tested.

- In general, the compounds showed **little toxicity** to **RAW 264.7** and **AGS**. An **exception** is **compound 7**. Seems due to the presence of the ester group and the higher hydrophobicity of **7** (comparison with **1**)

- The compounds show **little toxicity** to **human** fibroblast cell-line, **MRC-5**.



Cell viability of RAW 264.7, AGS and MRC-5 in the presence of compounds **1**-**8** at 100 µM for 24h. Values are shown with mean ± SD. ** p ≤ 0.01, **** p ≤ 0.0001.

Pharmaceutics, 2020, **12**, 122

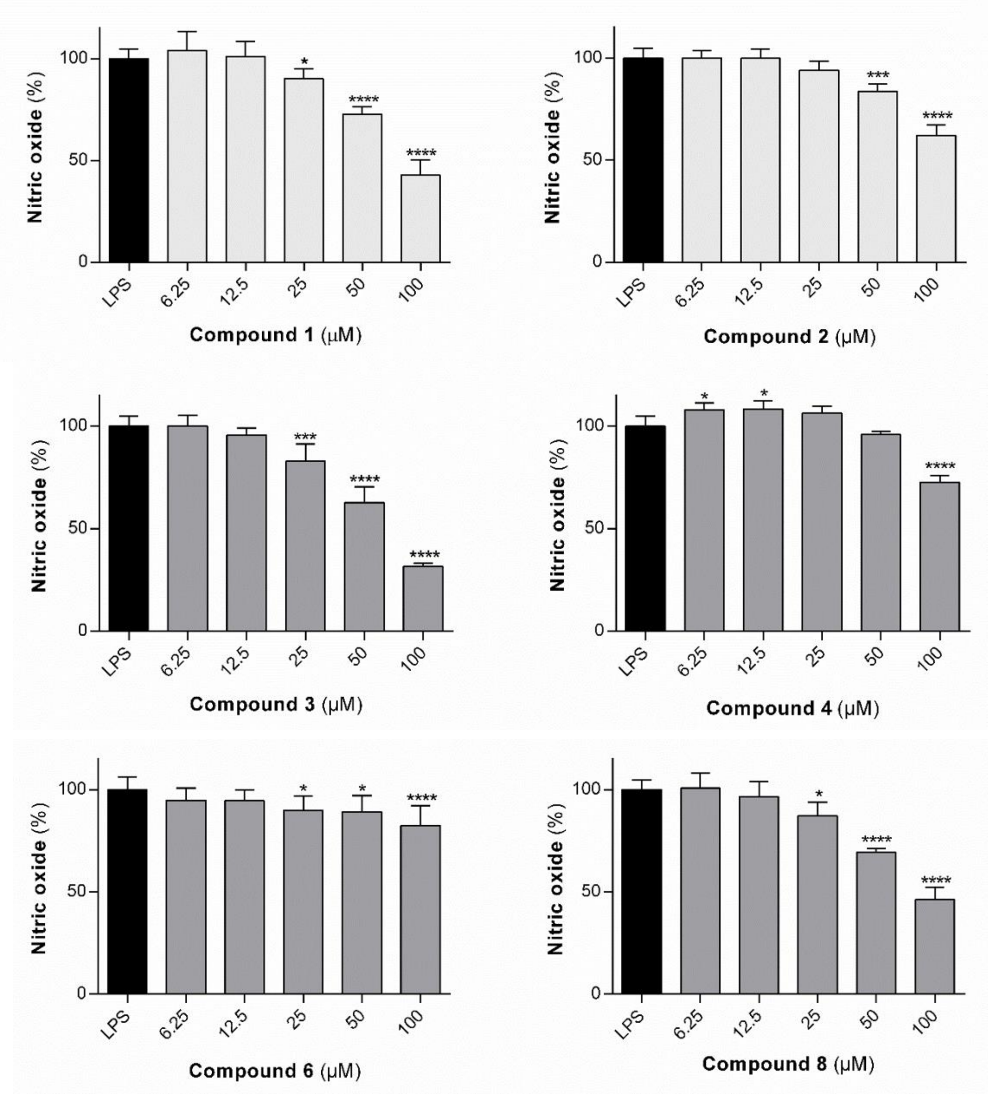
Effect of the compounds on the production of •NO in RAW 264.7

- The compounds **non-toxic** to rat macrophages were **tested** for their **ability** to **inhibit lipopolysaccharide** (LPS)-dependent •NO production in rat macrophages.

- **•NO** is an **important mediator** of the **inflammatory response**, which is synthesised by inducible nitric oxide synthase (iNOS) from oxygen and L-arginine.

- Its **excessive production** is **associated** with **inflammatory diseases**. Thus, the ability of these molecules to decrease the •NO production was assessed.

- The compounds generally elicited **only a modest effect**, with the **most active** compounds being **1**, **3** and **8**, which possessed IC50 values of 79.3 µM, 64.7 µM and 84.4 µM



LPS-induced •NO production in rat macrophages in the presence of the compounds **1**-**4**, **6** and 8 for 24h. Values are shown with mean ± SD. * $p \leq 0.05$; *** $p \leq 0.001$; **** $p \leq 0.0001$.

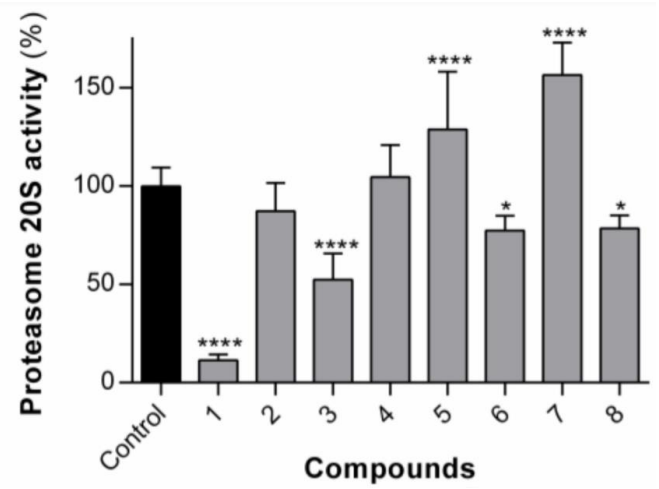
Effect of the compounds on **proteasome** activity

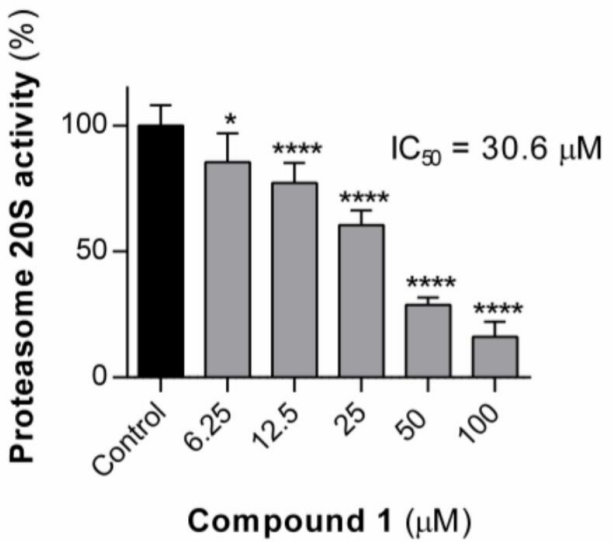
- Compounds tested for their **ability** to **inhibit proteasome** enzymes.

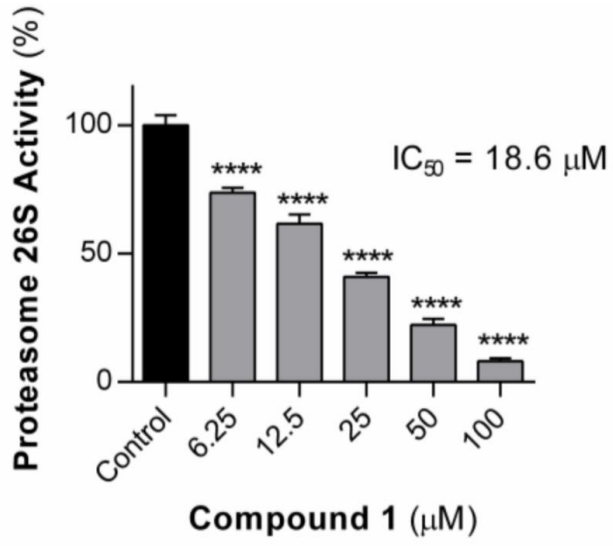
- Proteasomes play an **important regulatory role**, catalysing the **degradation** of **misfolded proteins**. Misfolded proteins are first **polyubiquitinated**, and then proceed through a complex cascade of reactions before being **hydrolysed by the proteasome**.

- The proteasome system is of interest for cancer therapy because **cancer cells** have a faster rate of metabolism that is **more sensitive to problems with proteasome function**, and will die more quickly if the degradation system is interrupted.

- **Compound 1** was the **only compound** able to **significantly inhibit proteasome 20S** (IC50 = 30.6 mM). Compound 1 was also found to inhibit the **proteasome 26S** (IC50 = 18.6 mM).







LPS-induced •NO production in rat macrophages in the presence of the compounds **1**-**4**, **6** and 8 for 24h. Values are shown with mean ± SD. * $p \leq 0.05$; *** $p \leq 0.001$; **** $p \leq 0.0001$.

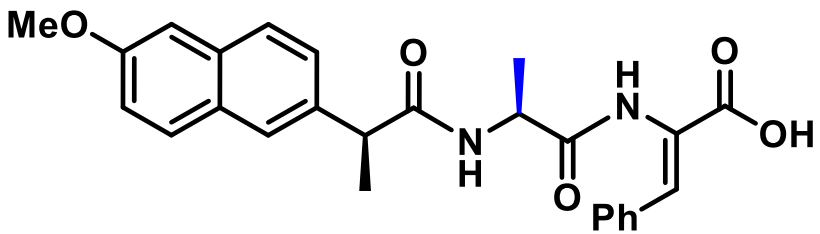
Conclusion

- Naproxen-dehydropeptide conjugates **1**-**8** have been synthesised, and the gelation properties and biological activity studied.

- **All compounds** except compound **7** (contains no ionisable acid group) are **effective hydrogelators**.

- The hydrogelators show potential for sustained release in **drug delivery applications**.

- Compound **4** is the most **promising** compound for **biological applications**, being both a **selective COX-2 inhibitor** and a **LOX inhibitor**, whilst being **non-toxic** to human fibroblasts (MRC-5)



4

Potential candidate for **dual COX-2/LOX inhibitors** as an **optimised strategy** for treating **inflammatory conditions**

- Compound **1** is the only compound which **inhibits proteasome** 20S and 26S.

Acknowledgments