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'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] and David M. Pereira [2]

[1] Centro de Quimica, Universidade do Minho 4710-057, Braga, Portugal; pmf@quimica.uminho.pt (P.M.T.F.); jmartins@quimica.uminho.pt (J.A.M.)

[2] Faculdade de Farmácia, Universidade do Porto 4099-002, Porto, Portugal; rutemartinsmoreira@hotmail.com (R.M.); dpereira@ff.up.pt (D.M.P.)

* Correspondence: peterjervis@quimica.uminho.pt

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Abstract: Supramolecular hydrogels where the hydrogelator molecule is endowed with intrinsic pharmacological properties can potentially fulfil a dual function in drug delivery systems as nanocariers for incorporated drugs and as active drugs themselves. In this present study, we investigated the pharmacological activities of a panel of naproxen-dehydrodipeptide conjugates, previously studied for their hydrogelation ability and as nanocarriers for drug-delivery applications. A library of dehydrodipeptides, containing N-terminal canonical amino acids (Phe, Tyr, Trp, Ala, Asp, Lys, Met) N-capped with naproxen and linked to a C-terminal dehydroaminoacid (ΔPhe, ΔAbu), were evaluated for their anti-inflammatory and anti-cancer activities, as well as for their cytotoxicity to non-cancer cells, using a variety of enzymatic and cellular assays. All compounds except one were able to significantly inhibit lipoxygenase (LOX) enzyme at a similar level to naproxen. One of the compounds was able to inhibit the cyclooxygenase-2 (COX-2) to a greater extent than naproxen, without inhibiting cyclooxygenase-1 (COX-1), and therefore is a potential lead in the search for selective COX-2 inhibitors. This hydrogelator is a potential candidate for dual COX/LOX inhibition as an optimised strategy for treating inflammatory conditions.

Keywords: hydrogelator; anti-inflammatory; NSAID; peptide; naproxen; cyclooxygenase; lipoxygenase; proteasome; supramolecular; self-assembly

1. Introduction

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 [1]. 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 -stacking interactions. They possess some advantages over other types of hydrogelators, such ease of synthesis, low toxicity, trends in mechanical properties can be readily tuned by the physical–chemical properties of the amino acid side chains [2].

Their 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), indole- 3-acetyl or naphthalene derivatives. The aromatic group provides the -stacking and hydrophobic interactions required for self-assembly [3].

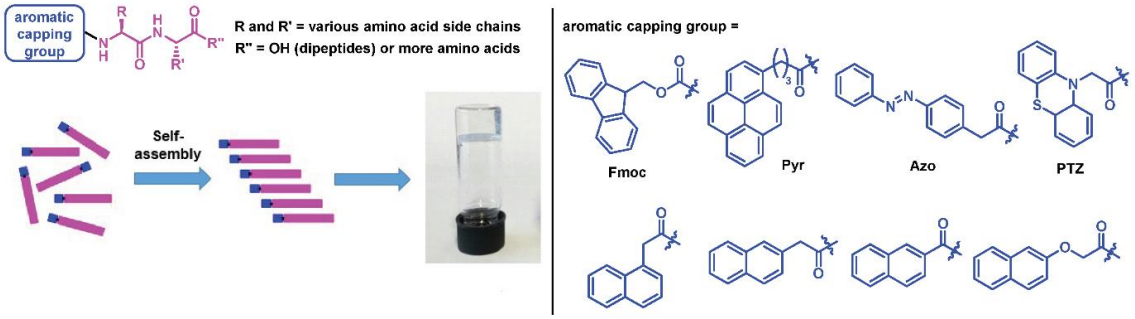


Figure 1. Left: Short peptides attached to an aromatic N-capping groups are often able to undergo self-assembly to form supramolecular hydrogels. Right: Some of the known aromatic capping groups.

The gelation process is initiated in response to an external trigger, with the most commonly employed being temperature change, pH change, solvent switch or the enzymatic cleavage of a solubilising phosphate group [4]. Other gelation methods include the use of ultrasound, light and magnetic field. The properties closely mimic those of the extracellular matrix (ECM), and as such they have found many medicinal applications, including drug delivery applications, tissue engineering, wound dressings, 3D bioprinting and ECM surrogates for the culture of cancer cells, stem cells and neuronal cells [5].

Our research group is interested in developing hydrogel delivery systems for nonsteroidal anti- inflammatory drugs (NSAIDs) [6–8]. NSAIDs are a class of drug which eases pain, reduces fever, decreases inflammation. They are used in the treatment of inflammatory diseases, such as rheumatoid arthritis, osteoarthritis, tendonitis and bursitis. Commonly used NSAIDs include ibuprofen, naproxen and aspirin [9]. They function by inhibiting 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 [10]. 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, many COX-2 inhibitors were withdrawn from the US and European markets. Possible methods for improving the properties of NSAIDs include the development of alternative formulations, to control the distribution of the drug in vivo. Targeted drug delivery systems may increase efficacy, allow reduced doses and decreased side-effects [11].

The structures of common NSAIDs are shown in Figure 2 (left panel) [9]. They share some key structural features: an aromatic, flat, hydrophobic core and a terminal carboxylic acid head group. The carboxylic acid group allows facile conjugation to other molecules via standard amide coupling chemistry. Therefore, we considered NSAIDs as ideal moieties for replacing the usual aromatic capping group of peptide hydrogelators, and reasoned

that conjugates such as that shown in Figure 2 (right panel) may retain both anti-inflammatory and hydrogelation properties. This may allow the 'self-delivery' of NSAIDs in topical applications [12]. To this end, we synthesised a panel of naproxen- dehydropeptide conjugates and assessed their gelation properties and biological activity [13].

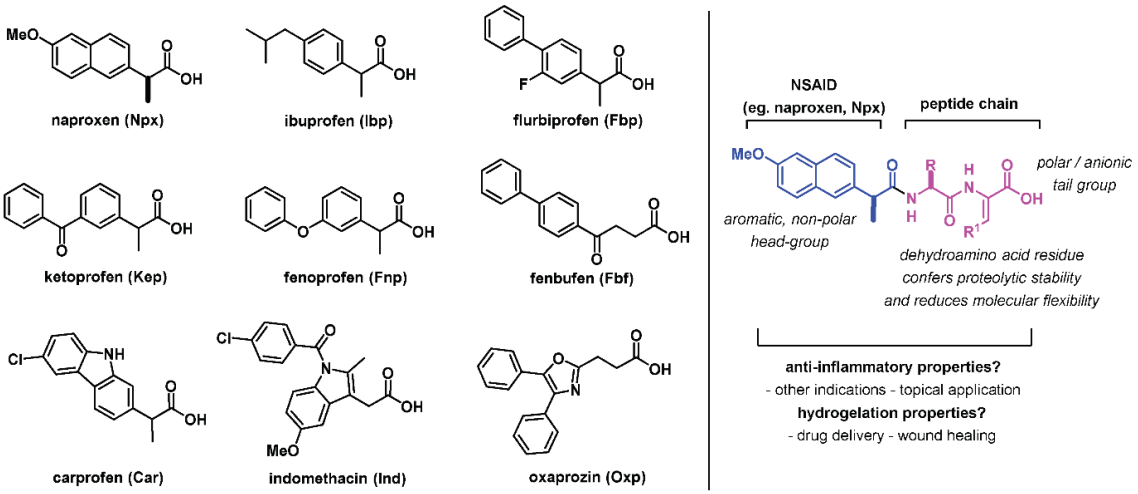


Figure 2. Left: The structures of common NSAIDs. Right: Naproxen-dehydropeptide conjugate, proposed as a potential multifunctional hydrogelator.

2. Methods

Compounds Tested. The chemical reactions required for the synthesis of compound 1-8 have been described previously.[6,7] The partition coefficient between water and n-octanol (Log P) of each compound was estimated using Molinspiration Cheminformatics software (Molinspiration, Slovensky Grob, Slovak Republic, 2017, http://www.molinspiration.com), as a sum of fragment-based contributions and correction factors, and it is used as quantitative descriptor of compound lipophilicity.

Gelation Method. Hydrogels were formed using a previously reported method [6]. A typical procedure involves adding NaOH (1 M, 20 L) to a suspension of hydrogelator compound (4.0 mg) in H2O (1.0 mL), in a small tube. When fully dissolved, GdL (4.0 mg) was added and the solution was left to form a gel overnight.

Lipoxygenase Glycine Max (Soybean) Assay. The inhibitory effect on LOX was assessed in 96-well plates, using a modified version of a previously reported procedure from Pereira et al. [14]. The compounds were tested in a reaction mixture of each compound (20 μL), phosphate buffer (200 μL, pH 9.0) and soybean LOX (20 μL, ~100 U). After 5 min pre-incubation at room temperature, the reaction was started by addition of linoleic acid substrate (20 μL of a 4.18 mM solution in ethanol). The reaction was monitored at 234 nm using a multiplate reader (Multiskan Thermo Fisher Scientific Oy, Vantaa, Finland), for 3 min. 2.4.

Cyclooxygenase-1 (COX-1) and Cyclooxygenase-2 (COX-2) Inhibition Assay. The assay was performed using the COX fluorescent inhibitor screening assay kit (Cayman chemical, MI, USA), with some modifications. Briefly, 60 μL of assay buffer (100 mM Tris-HCl, pH 8.0), 5 μL of hemin, 5 μL of enzyme (either COX-1 or COX-2) and 5 μL of compound (25 μM) were added to a black 96-well plate. After 5 min of incubation at room temperature, 5 μL of 10-acetyl-3,7-dihydroxyphenoxazine (ADHP) and 20 μL of a solution containing arachidonic acid (0.5 mM) and KOH (2.5 mM) were added to each well. After a further 2 min at room temperature, the fluorescence of resorufin was monitored with an excitation wavelength between 530–540 nm and an emission wavelength between 585–595 nm, using a multiplate reader (Synergy H1,

Biotek Instruments Winooski, Winooski, VT, USA). SC-560 and DuP-697 inhibitors were used as positive controls to COX-1 and COX-2 assay, respectively.

Cell Culture. Adenocarcinoma gastric cells (AGS; Sigma-Aldrich, St. Louis, MO, USA), murine- macrophage cell line (RAW 264.7; American Type Culture Collection, LGC Standards S.L.U., Barcelona, Spain), and human foetal lung fibroblasts (MRC-5; ECACC, Porton Down Salisbury, UK) were cultured as a monolayer at 37 °C in a humidified incubator with 5% carbon dioxide. AGS and RAW 264.7 cells were grown in DMEM, supplemented with 1% streptomycin/penicillin and 10% FBS (Gibco®). MRC-5 cells were grown in MEM, supplemented with 1% streptomycin/penicillin and 10% FBS.

3-(4,5-Dimethylthiazol-2-yl)-2,5-Diphenyltetrazolium Bromide (MTT) Reduction Assay. Cell viability was evaluated by the MTT reduction assay [28]. Cells were cultured in 96-well plates (15,000 cells/well for AGS, 25,000 cells/well for RAW and 20,000 cells/well for MRC-5) and allowed to attach for 24 h. After incubation with compounds for 24 h, MTT (0.5 mg/mL final concentration) was added to each well and the plate was incubated for 75 min at 37 ◦C. Formazan crystals were dissolved by the addition of a DMSO: isopropanol mixture (3:1) and then quantified spectrophotometrically at 570 nm using a microplate reader (Multiskan Thermo Fisher Scientific Oy, Vantaa, Finland).

3. Results and Discussion

A panel of naproxen-dehydrodipeptide conjugates 1-8 was prepared by solution phase peptide synthesis. A representative example is shown in Figure 3 (top). The panel features a systematic variation of the canonical amino acid residue (Figure 3, bottom).

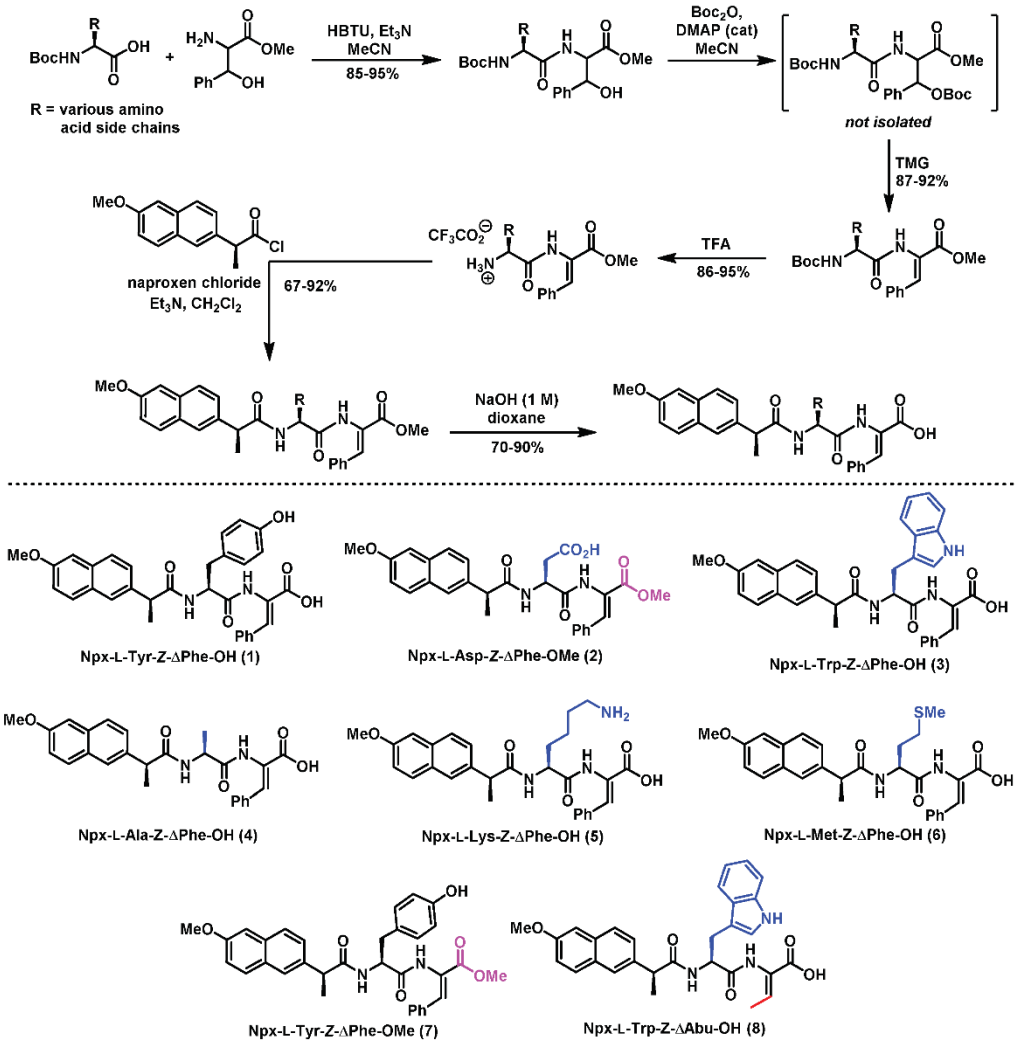
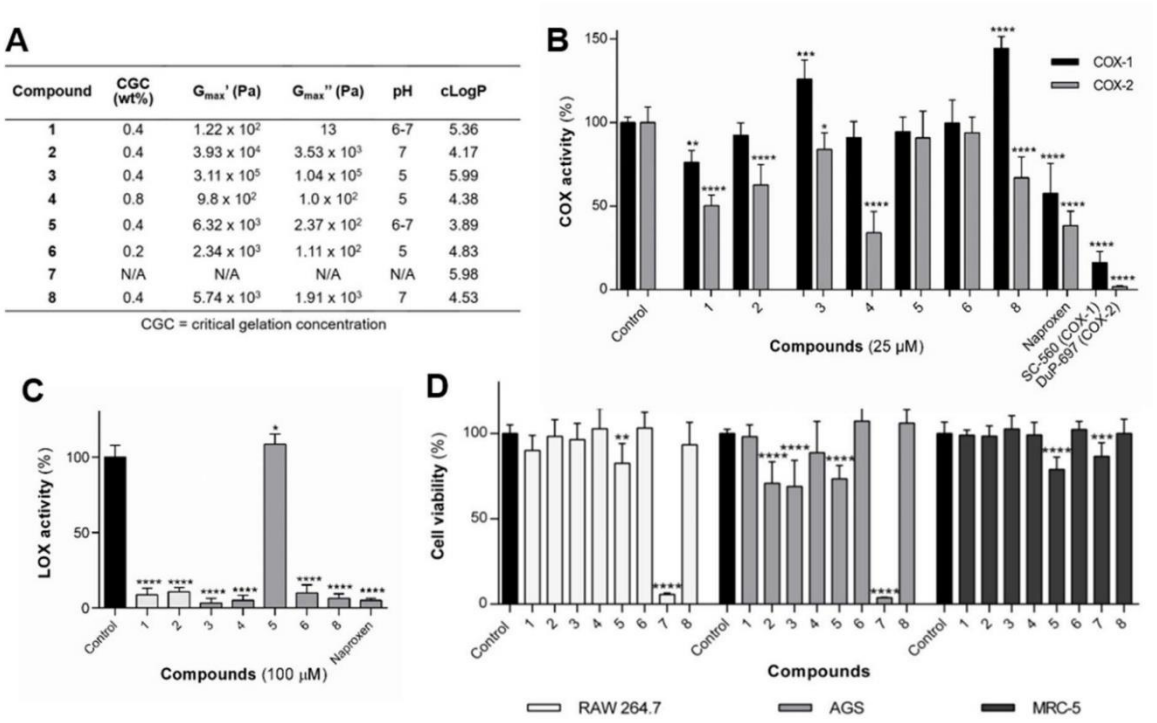


Figure 3. Top: Synthesis of naproxen-dehydrodipeptide hydrogelators. Bottom: Panel of compounds studied (1-8).

The compounds were tested for their hydrogelation properties (Figure 4A). All compounds were able to form hydrogels, except for compound 7, which contains no ionisable carboxylic group. In a separate study, compound 3 and a related compound showed promising results for sustained release in drug delivery applications [15]. The compounds were then assessed for their anti-inflammatory activities in enzymatic assays. In a cyclooxygenase assay, the ability of the compounds to inhibit both the COX-1 and COX-2 isozymes was studied. Most compounds tested showed 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 (Figure 4B). The compounds were then assessed for their ability to inhibit lipoxygenase (LOX) enzyme. 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 M, except compound 5 (the most polar example), which was inactive (Figure 4C).

The 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 MTT viability assays (Figure 4D). In general, the compounds showed little toxicity to RAW 264.7 and AGS, the exception being compound 7. Compound 7 is the methyl esterified analogue of compound 1, which is shown to be non-toxic in the same assays. The toxicity of 7 is therefore presumably due to presence of the ester group and the higher hydrophobicity of 7 compared with 1. The compounds tested displayed little toxicity to the human fibroblast cell-line, MRC-5.



Compound	CGC (wt%)	G_{max}' (Pa)	G_{max}'' (Pa)	pH	cLogP
1	0.4	1.22×10^2	13	6-7	5.36
2	0.4	3.93×10^4	3.53×10^3	7	4.17
3	0.4	3.11×10^5	1.04×10^5	5	5.99
4	0.8	9.8×10^2	1.0×10^2	5	4.38
5	0.4	6.32×10^3	2.37×10^2	6-7	3.89
6	0.2	2.34×10^3	1.11×10^2	5	4.83
7	N/A	N/A	N/A	N/A	5.98
8	0.4	5.74×10^3	1.91×10^3	7	4.53

CGC = critical gelation concentration

Figure 4. (**A**) Table of gelation properties of compounds 1-8; (**B**) COX-1 and COX-2 activities in the presence of compounds 1-6 and 8 at 25 μM; (**C**) LOX activity in the presence of compounds 1-6 and 8 at 100 μM; (**D**) Cell viability of RAW 264.7, AGS and MRC-5 in the presence of compounds 1-8 at 100 μM for 24 h. Values are shown with mean ± SD. * $p \leq 0.05$; ** $p \leq 0.01$; *** $p \leq 0.001$, **** $p \leq 0.0001$.

4. Conclusions

In conclusion, naproxen-dehydropeptide conjugates 1-8 have been synthesised, and the gelation properties and biological activity have been studied. All compounds except compound 7 (containing no ionisable acid group) are effective hydrogelators. The hydrogelators showed 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). Compound 4 will be investigated further in the context of the self-delivery of NSAIDs.

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References

1. Li, J.; Xing, R.; Bai, S.; Yan, X. *Soft Matter* **2019**, *15*, 1704–1715.
2. Dong, R.; Pang, Y.; Su, Y.; Zhu, X. *Biomater. Sci.* **2015**, *3*, 937–954.
3. Draper, E.R.; Adams, D.J. *Langmuir* **2019**, *35*, 6506–6521.
4. Seow, W.Y.; Hauser, C.A.E. *Mater. Today* **2014**, *17*, 381–388.
5. Martin, D.; Thordarson, P.J. *Mater. Chem. B* **2020**, *8*, 863–877.
6. Carvalho, J.; Gallo, D.; Pereira, M.; Valentão, P.; Andrade, P.B.; Hilliou, L.; Ferreira, P.M.T. Bañobre-López, M.; Martins, J.A. *Nanomaterials* **2019**, *9*, 541.
7. Vilaça, H.; Pereira, G.; Castro, T.G.; Hermenegildo, B.F.; Shi, J.; Faria, T.Q.; Micaêlo, N.; Brito, R.M.M.; Xu, B.; Castanheira, E.M.S.; et al. *Mater. Chem. B* **2015**, *3*, 6355–6367.
8. Vilaça, H.; Hortelão, A.C.L.; Castanheira, E.M.S.; Queiroz, M.-J. R. P.; Hilliou, L.; Hamley, I.W.; Martins, J.A.; Ferreira, P.M.T. *Biomacromolecules* **2015**, *16*, 3562–3573.
9. Brune, K.; Patrignani, P.J. *Pain Res.* **2015**, *8*, 105–118.
10. Chakraborti, K.; Garg, S.K.; Kumar, R.; Motiwala, H.F.; Jadhavar, P.S. *Curr. Med. Chem*. **2010**, *17*, 1563–1593.
11. Li, J.; Mooney, D.J. *Nat. Rev. Mater*. **2016**, *1*, 16071.
12. Jervis, P.J.; Amorim, C.; Pereira, T.; Martins, J.A.; Ferreira, P.M.T. *Soft Matter*. 2020, doi:10.1039/d0sm01198c.
13. Moreira, R.; Jervis, P.J.; Carvalho, A.; Ferreira, P.M.T.; Martins, J.A.; Valentão, P.; Andrade, P.B.; Pereira, D.M. *Pharmaceutics* **2020**, *12*, 122.
14. Pereira, R.B.; Taveira, M.; Valentão, P.; Sousa, C.; Andrade, P.B. *RSC Adv*. **2015**, *5*, 8981–8987.
15. Vilaça, H.; Castro, T.; Costa, F.M.G. Melle-Franco, M.; Hilliou, L.; Hamley, I.W.; Castanheira, E.M.S.; Martins, J.A.; Ferreira, P.M.T.J. *Mater. Chem. B* **2017**, *5*, 8607–8617.
16. Werz, O. *Curr. Drug Target Inflamm. Allergy* **2002**, *1*, 23–44.