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# A supramolecular topical gel derived from a non steroidal anti-inflammatory drug-fenoprofen capable of treating skin inflammation in mice

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**Abstract**

A new series of bioconjugates derived from a non-steroidal-anti-inflammatory drug (NSAID) namely fenoprofen has been synthesised by amidation with various biogenic molecules such as β-alanine, aminocaproic acid and tyramine with the aim of converting the NSAID into a supramolecular gelator for plausible biomedical applications. One such bioconjugate (**2**) showed gelation ability with methylsalicylate (MS) and 1 % menthol in methyl salicylate (MMS) solvents. These gels were characterized by table top rheology, high resolution-transmission electron microscopy (HR-TEM) and dynamic rheology. Gelator **2** was found to be biostable both in proteolytic enzymes and blood serum of BALB/c mouse under physiological conditions. It was also found to be biocompatible as revealed by methyl thiazolyldiphenyl tetrazolium bromide (MTT) assay in mouse macrophage RAW 264.7 and mouse myoblast C2C12 cells. The anti-inflammatory response (prostaglandin E2 assay - $PGE_2$ assay) of **2** was comparable to that of the parent drug fenoprofen calcium salt. Finally, a topical gel formulation of **2** displayed *in vivo* self-delivery application in treating imiquimod (IMQ) induced skin inflammation in BALB/c mice.

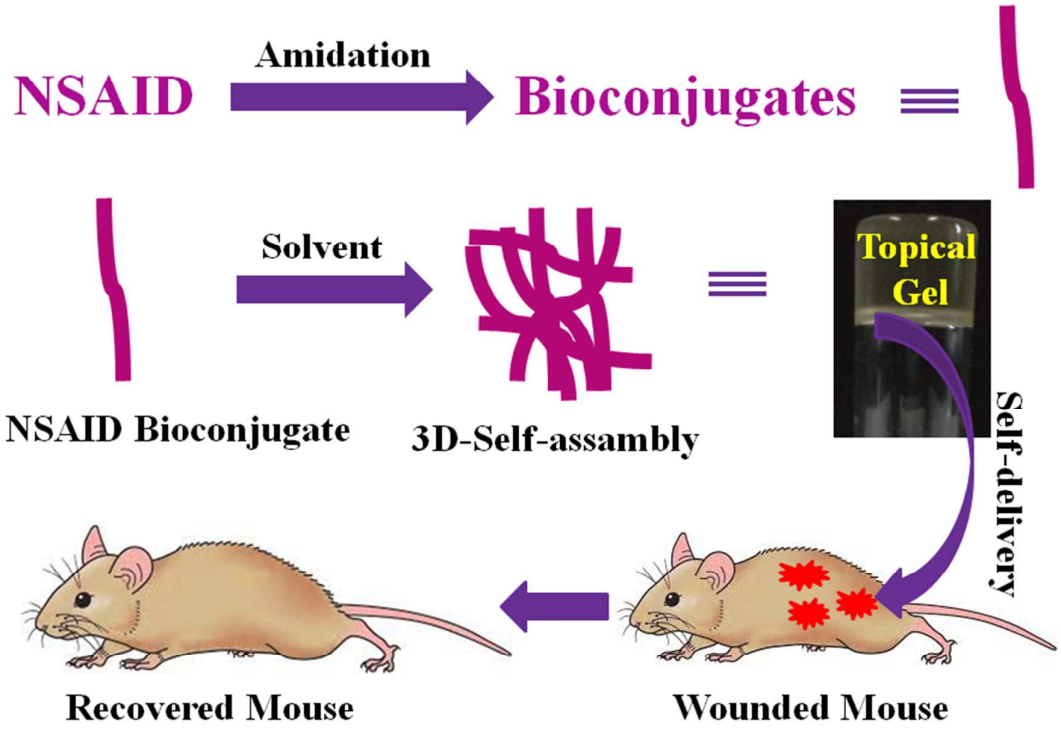
## Introduction

Small molecular weight (MW < 3000) compounds capable of gelling various organic and aqueous solvents are popularly known as low molecular weight gelators (LMWGs).[1] Gelator molecules first self-assemble to form various 1D supramolecular architectures which further entangle through various weak intermolecular forces such as hydrogen bonding, π-π stacking, van der Waals interactions, hydrophobic interactions, charge-transfer interactions etc. to form 3D self-assembled-fibrillar-networks (SAFiNs)[2] within which the solvent molecules are immobilized to form gel. Over the decade, LMWG based supramolecular gels have received much interest due to their wide range of applications such as in developing sensors,[3] electro-optics/photonics,[4] cosmetics,[5] template for crystalization,[6] oxygen storage materials,[7] structure-directing agents,[8] catalysis,[9] conservation of art,[10] tissue engineering,[11] regenerative medicine,[12] biomineralization,[13] wound healing,[14] medical diagnostics,[15] enzyme mimics,[16] inhibitors of cancer cells,[17] 3D cell cultures,[18] immunosuppressive materials[19] etc. Beside these, supramolecular gels are also being explored as controlled-release drug delivery systems[20] and in recent time much attention have been employed to develop various drugs/prodrugs/bioactive agents based supramolecular gels for developing self-delivery systems[21] - wherein the delivery vehicle (usually a polymer) is not required; drug/pro-drug molecules are synthetically modified to supramolecular gelators and the corresponding gel itself can self-deliver the drug thereby getting rid of the carrier molecule.

The objective of the present study is to develop supramolecular gel for self-delivery applications for treating inflammation. For this purpose, we choose to work with NSAIDs which are commonly prescribed as painkillers for the treatment of patients with acute or chronic inflammatory diseases. However, conventional NSAIDs do have considerable side effects. The major problems associated with NSAIDs are their poor water solubility[22] and hence lower bioavailability requiring high dose, which causes various adverse side effects

such as gastrointestinal-irritation, renal problem, cardiovascular risks etc.[23] These side effects could be avoided by converting the NSAID molecule into supramolecular gel based self-delivery systems which can be applied topically to deliver the gelator drug onto the affected site. Such self-delivery not only avoids the gastrointestinal-irritation associated with NSAID's oral administration, but also prevents the metabolism of the drug in the liver thereby reducing renal problems. Commercially available NSAID based topical gels are comprised of a carrier molecule (usually a polymer) and a drug; however, such formulation often faces various problems such as inefficient drug loading into the carrier molecule, its irregular release, synthetic challenges associated with the functionalization of the carrier molecule with the drug, toxicity and biodegradability of the carrier molecule etc.[24] On the other hand, supramolecular gel based self-delivery system overcomes these problems by delivering the gelator drug itself at the target site without using any carrier molecule. Thus, it will be advantageous to convert NSAIDs into supramolecular gels. In fact, much attention have been employed in recent years to develop various NSAIDs based supramolecular gels for topical self-delivery applications. For examples, Kim and co-workers showed enzyme-triggered release of NSAID ibuprofen from its peptide based hydrogel.[25] Stupp et al. converted NSAID-nabumetone into a peptide amphiphile based hydrogel and studied its release at physiological condition.[26] Xu et al. conjugated various D-amino acids with the NSAID-naproxen and prepared a series of supramolecular hydrogels and studied their *in vitro* release profile in PBS buffer.[27] We also reported NSAID-naproxen based supramolecular hydrogelators that displayed remarkable biostability, biocompatibility, and anti-inflammatory properties.[28] In this context, the drug fenoprofen (which is clinically used for symptomatic relief for rheumatoid arthritis, osteoarthritis, and mild to moderate pain) has still not been explored for self-delivery application. In the present study, we report design, synthesise and characterization of a series of β-amino acid linked fenoprofen bioconjugates for topical self-

delivery application as shown in Scheme 1. Thus a series of fenoprofen bioconjugates (**1-4)** has been synthesized (Scheme 2). One of these bioconjugates (**2**), was capable of producing supramolecular gels with methylsalicylate and menthol containing methylsalicylate, which are commonly found in many commercial formulation of topical gels. The boiconjugate **2** showed excellent *in vitro* biostability and biocompatibility. A menthol/methylsalicylate supramolecular gel of **2** displayed *in vivo* topical self-delivery application in treating IMQ-induced skin inflammation in mice. To the best of our knowledge, this is the first report of NSAID-fenoprofen based supramolecular gel for topical self-delivery application.

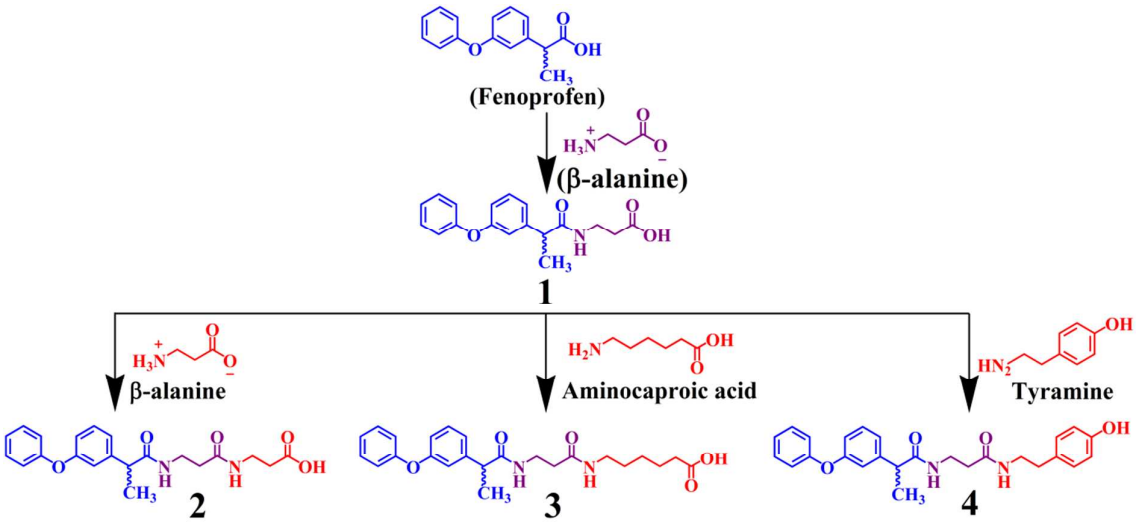



**Scheme 1**. Schematic representation of designing NSAID based supramolecular topical gel for treating skin inflammation in mouse.

**Results and Discussion**

**Design, synthesis and gelation:** In order to design supramolecular gelator from a drug for self-delivery application (e.g. topical gel), the following important criterion should be fulfilled: a) proteolytic degradation of the drug derivative should be prevented, b) it should

have minimum cytotoxicity, c) it should display improved or unchanged durg activity (e.g. anti-inflammatory property in the present case) and finally, d) it should have suitable self-assembling functionalities conducive for gelation. One of the major pathways of degradation of drug in living system is proteolytic cleavage wherein an enzyme e.g. carboxypeptidase Y cleaves the C-terminal peptide bond of L-amino acid in the drug derivative. In order to prevent such degradation, we considered various biogenic molecules such as β-amino acid (β-alanine), amino-caproic acid and tyramine to make peptide conjugate of the parent drug fenoprofen following benzotriazole coupling chemistry,[29] since proteolytic enzymes do not recognise amide/peptide bond made from β-amino acid[30] and are also not expected to cleave the amide bond made from either aminocaproic acid or tyramine. The resulting derivatives **1-4** are expected to display biocompatibility comparable to the parent drug. Among the various noncovalent interactions, hydrogen bonding plays a pivotal role in gelation.[31] Thus, the peptide conjugates (**1-4**) decorated with other hydrogen bonding functionalities (-COOH, -OH) in addition to the amide moiety are expected to display gelation ability.




**Scheme 2.** Various bioconjugates derived from NSAID-fenoprofen.

Next, we checked the gelation ability of these bioconjugates **1-4** with methyl salicylate as it is an important ingredient of commercially available topical gel formulation of various NSAIDs. Among these, only bioconjugate **2** showed excellent gelation capability with methylsalicylate (minimum gelator concentration (MGC) = 3.0 wt %) and it was also able to gel methylsalicylate in presence of 1 % menthol (see Table S1 in supporting information). Gel formation was confirmed by the test tube inversion wherein no gravitational flow of the materials was observed upon inverting the test tube. It appeared that the compound **2** was able to achieve the needed balance between lipophilicity (via amide functionality) and lipophobicity (via bis-β-alanine backbone) in order to self-assemble to form gel. Whereas, compounds **1**, **3** and **4** failed to gel presumably because of their differences in lipophobicity (**3** contains longer alkyl chain length and **4** contains aromatic moiety) and lipophilicity (**1** contains only one amide functionality) compared to the gelator molecule **2**.

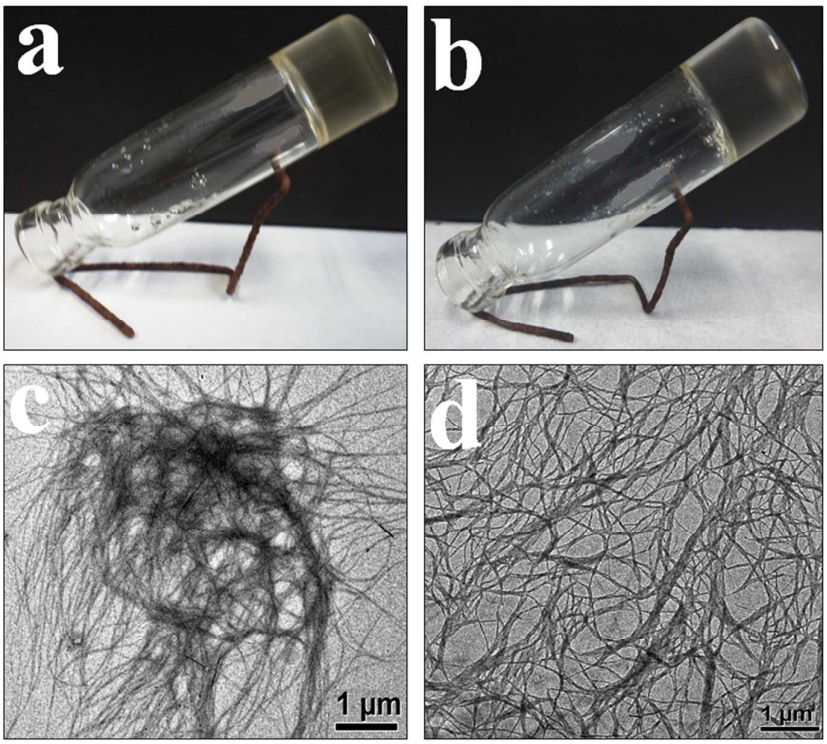



**Figure 1**. (a) and (b) optical images of 3 wt % methylsalicylate gel and 3 wt % menthol/methylsalicylate gel of **2,** respectively; (c) and (d) HR-TEM micrographs of methylsalicylate gel and menthol/methylsalicylate gel of **2,** respectively.

Both the MS and MMS gels were transparent and the morphology of these gels was characterized using HR-TEM. Both the gels displayed highly entangled network of fiber having width of 20-45 nm and 30-50 nm range for MS and MMS gel, respectively. These results suggested that menthol did not seem to have much influence on the gel morphology (Figure 1).

Both these gels showed thermo-reversible gel to sol transition ($T_{gel}$) behaviour as revealed in dropping-ball experiments[32] (see Figure S5 in supporting information). $T_{gel}$ vs. gelator concentration plot for both the gels showed that $T_{gel}$ temperatures increased with the increasing gelator concentration indicating the role of various supramolecular interactions in gel network formation (Figure 2).

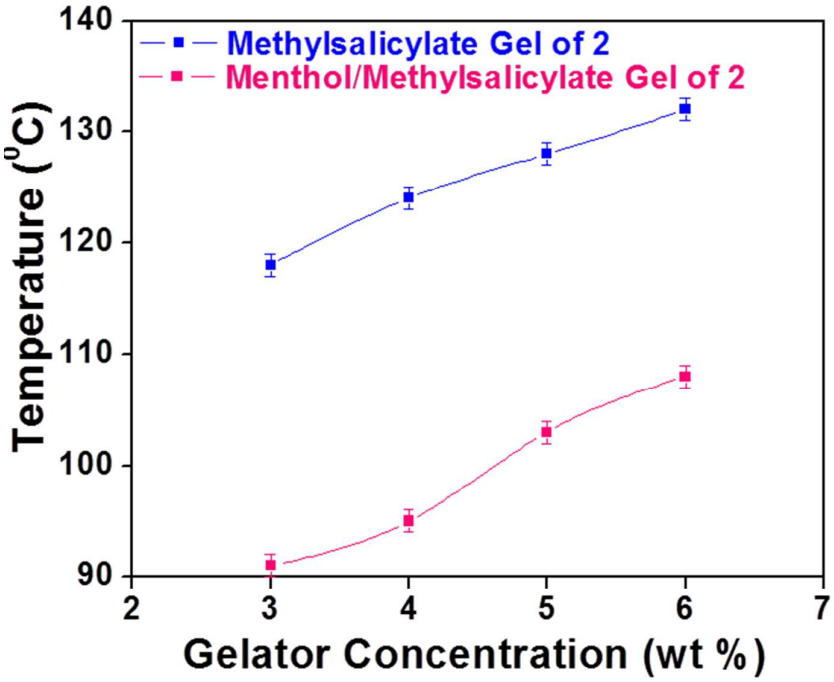



**Figure 2**. $T_{gel}$ vs. gelator concentration plot for methylsalicylate gel and menthol/methylsalicylate gel of **2**.

The visco-elastic (gel) properties of these materials were characterized using various rheological studies. We first performed strain sweep experiments of these gels to find out

their linear visco-elastic region. Figure 3a-b showed the strain sweep behaviour of both the gels. The elastic modulus (G′) of these gels decreased rapidly and fell below the viscous modulus (G″) after the critical strain region (critical strain = 10 % and 4.0 % for MS gel and MMS gel of **2,** respectively), indicating collapse of the gel networks above the critical strain – a typical behaviour of gels.

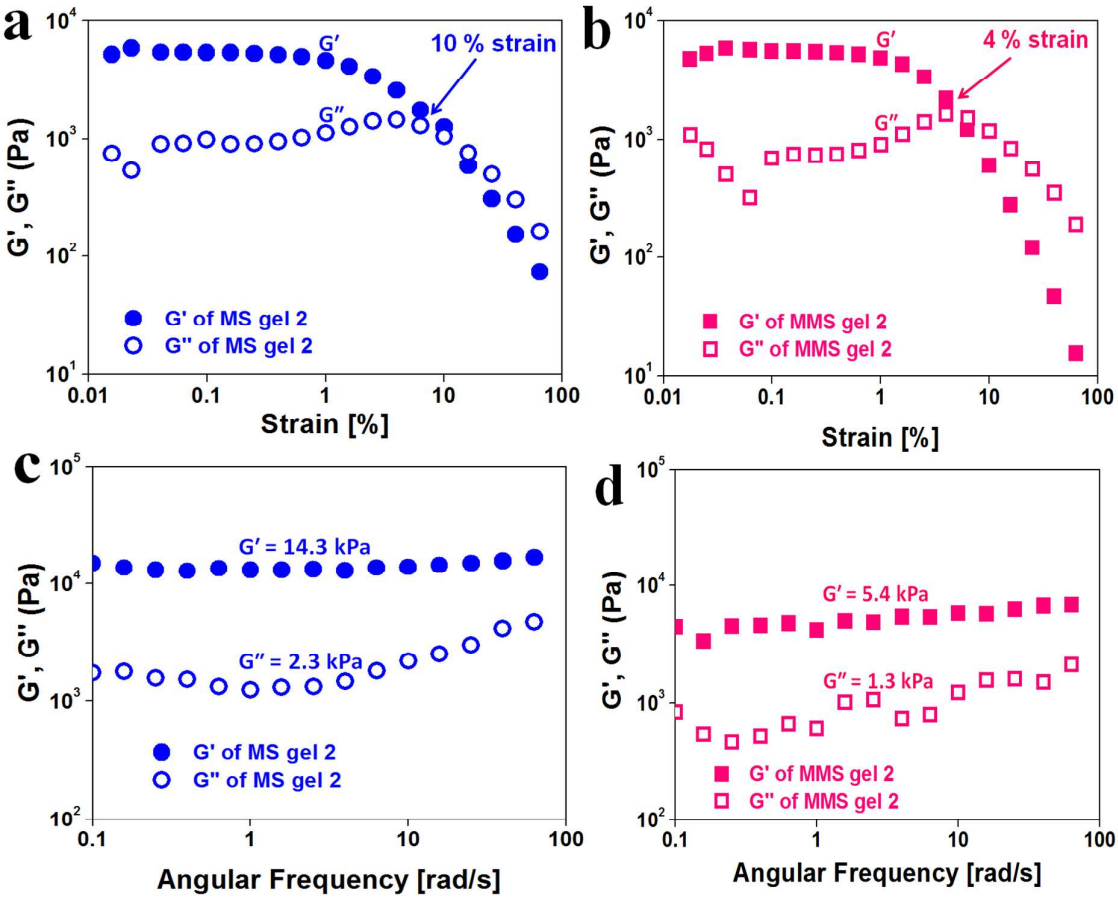



**Figure 3.** Rheological responses of 3 wt % methylsalicylate gel and menthol/methylsalicylate gel of **2**; (a) strain sweep experiment of methylsalicylate gels of **2**; (b) strain sweep experiment of menthol/methylsalicylate gels of **2**; c) and d) frequency sweep rheological behaviour of 3 wt % methylsalicylate gel and menthol/methylsalicylate gel of **2** respectively at constant strain of 0.05 % at 25º C.

The variations of modulus G′ and G″ of these gels against angular frequency ω under constant strain of 0.05 % as suggested by the independent strain sweep experiment are shown in Figure 3c-d. Both the gels displayed visco-elastic response as revealed by the fact that the G′s

were almost frequency invariant and greater than the G″ over the entire range of frequency ω. The rheological stability of the MS gel of **2** was found be better than the corresponding MMS gel ($G'_{MS\ gel}$ = 14.3 kPa > $G'_{MMS\ gel}$ = 5.4 kPa) and the same trend was observed in the thermal stability ($T_{gel}$ studies) of these gels as well (Figure 2).

**Biological studies:** Having successfully converted the NSAID fenoprofen to a supramolecular gelator **2**, it became necessary to evaluate its potential in biological application such as self-delivery. For this purpose, we undertook both *in vitro* and *in vivo* studies. While *in vitro* studies included biostability, MTT and $PGE_2$ assays of the gelator **2**, in *in vivo* experiments, a topical gel of the gelator drug **2** was explored to treat IMQ-induced skin inflammation in mice.

***In vitro* studies:**

**Biostability:** We first checked the stability of gelator bioconjugate **2** under physiological conditions. For this purpose, bioconjugate **2** was incubated in phosphate buffer saline (PBS) of pH 7.4 at 37 °C in a humidified incubator for 72 h. However, no chemical degradation of the bioconjugate **2** was found even after 72 h incubation in PBS as monitored by TLC and HPLC (see Figure S6-7 in supporting information).

Next, we checked the stability of the bioconjugate **2** in presence of proteolytic enzymes. For this purpose, gelator **2** was incubated with carboxypeptidase-Y and aminopeptidase in phosphate buffer saline (PBS) of pH 6.5 and tris-HCl buffer of pH 8.0, respectively at 25 °C in a humidified incubator for 72 h. Again, no chemical degradation of bioconjugate **2** was found after enzymatic incubation as monitored by TLC and HPLC (see Figure S8-9 in supporting information).

Finally, we tested the stability of bioconjugate **2** in blood serum of BALB/c male mouse. We incubated bioconjugate **2** with blood serum of BALB/c mouse at 37 °C in a humidified

incubator for 72 h. Again, no chemical degradation of bioconjugate **2** was found as monitored by TLC and HPLC (see Figure S10 in supporting information). As envisaged, the β-amino acid derived peptide bond in **2** was not recognized by the proteolytic enzymes present in the blood serum. These results indicated that gelator bioconjugate **2** was indeed biostable *in vitro.*

**Biocompatibility:** Biocompatibility of gelator **2** was checked in RAW 264.7 mouse macrophage and C2C12 mouse myoblast cells. Both these cells were treated with various concentrations of calcium salt of fenoprofen and gelator **2** for 72 h at 37 °C, and the cell viability test was performed using MTT assay; while ~ 24% RAW 264.7 cells survived after 72 h incubation at concentration of 1000 μM of calcium salt of fenoprofen, more than twice as much cells (~51 %) survived in presence of the gelator **2** under identical conditions indicating better biocompatibility of **2** compared to the parent drug (Figure 4).

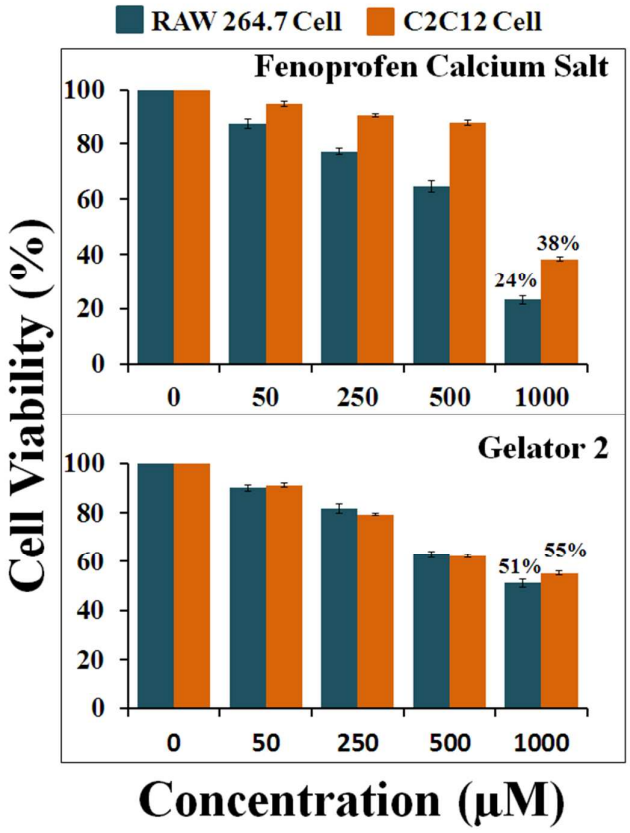



**Figure 4**. MTT assay of parent NSAID fenoprofen calcium salt and its gelator **2** in mouse macrophage RAW 264.7 cells and mouse myoblast C2C12 cells for 72 h at 37 °C.

In the case of C2C12 mouse myoblast cells, we found that ~ 38% cells survived after 72 h incubation at concentration of 1000 μM of calcium salt of fenoprofen, whereas 55% cells survived in presence of the gelator **2** under identical conditions (Figure 4). Thus, the gelator **2** was found to be almost equally biocompatible in both the cell lines.

**Anti-inflammatory Activity ($PGE_2$ assay):** Next, we checked anti-inflammatory activity of gelator **2** by $PGE_2$ assay in RAW 264.7 cell line. In this assay, inflammation inducing agents like lipopolysaccharides (LPS) and interferon gamma (IFN-γ) were added systematically to obtain maximum inflammation response which was monitored by the production of $PGE_2$ in the cell culture medium. In these experiments, we found that 1 μg/mL of LPS and 100 ng/ mL of IFN- γ produced a maximum of 2012 pg/ mL $PGE_2$ in the culture medium of ~ 1 x $10^6$ RAW 264.7 cells after 24 hours. The amount of $PGE_2$ secretion in the cell culture medium was reduced to 277 and 297 pg/mL in presence of 500 μM concentration of fenoprofen calcium salt and gelator **2**, respectively. Thus, these results clearly showed *in vitro* anti-inflammatory activity of gelator **2**, which was comparable to that of the parent drug fenoprofen (Figure 5).

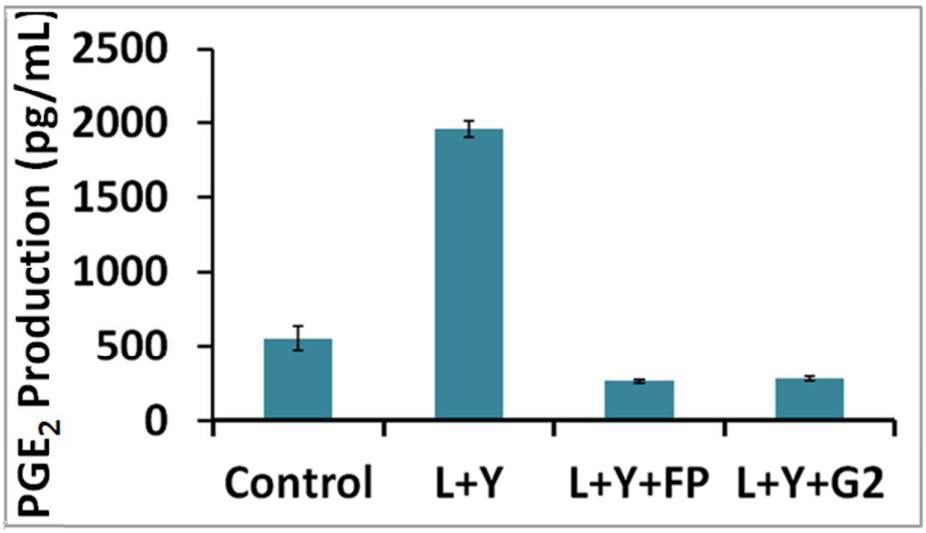



**Figure 5.** $PGE_2$ assay of fenoprofen calcium salt and its gelator **2** using RAW 264.7 cells. Here, Control = Cells grown in DMEM media only, L = Lipopolysaccharide, Y = Interferon-γ., FP= Fenoprofen calcium salt, G2 = Gelator **2**.

***In vivo* study:**

**Topical self-delivery of gelator bioconjugate 2 in treating skin inflammation in mice:** Encouraged by the *in vitro* bioactivity of gelator **2**, we decided to evaluate its *in vivo* bio-activity in mice. For this purpose, we studied the effect of the topical gel formulation of **2** in treating psoriasis-like skin inflammation in BALB/c male mice; such skin inflammation was generated following a published protocol.[33] BALB/c male mice (n=15, aged 16-18 weeks, 1 mouse/cage) were shaved on their dorsal skin by applying hair removing cream veet and they were rested for 48h. The mice were then divided into two groups – group A (3 mice) and B (12 mice). A daily topical dose of 80 mg of the commercially available IMQ cream (5% w/w) was applied on the shaved skin of group B mice whereas group A mice were treated similarly with vaseline as a control group in these experiments. The IMQ application was continued for 6 consecutive days. After 7 days of the first IMQ application, inflammation like symptoms (scaling, erythema and redness) were observed on the dorsal skin of all group B mice while vaseline treated group A control mice showed normal skin condition. Group B mice were further divided into four sub groups (3 mice in each group) – B1, B2, B3 and B4. The skin inflammation of the mice thus generated was treated as detailed in Table 1. Such treatment was continued for 3 conseqetive days. It may be mentioned here that menthol was incorporated in the formulation of the topical gel **2** because of its vasodilation effects[34] allowing easy penetration of the active ingredient through the skin barrier.

**Table 1.** Details of treatment provided in the dorsal skin of BALB/c mice following skin inflammation induction by IMQ.

| **Groups** | **Group A** | **Group B1** | **Group B2** | **Group B3** | **Group B4** |
|---|---|---|---|---|---|
| Treatment provided | Vaseline | No Treatment | 50 µL 1 % menthol in methylsalicylate | 50 µL 1 % menthol containing fenoprofen calcium salt in methylsalicylate (delivering 1 mg of fenoprofen) | 40 mg 1 % menthol containing topical gel of **2** in methylsalicylate (delivering 1 mg of gelator **2**) |

One week after receiving the first dose of the topical gel of **2**, we performed histological analysis of the dorsal skin of the mice of all the groups to see the effects of various treatments (Figure 6). The dorsal skin tissues of the various groups were collected and fixed in 10% formalin and embedded in paraffin wax. The cross sections of these tissues were stained with hematoxylin-eosin (H & E). The histology images showed that well grown hair follicles[35] were present both in the vasline treated normal mice and the topical gel **2** treated group (B4) mice, whereas only hair bulbs were seen in the rest of the control groups i.e. non-treated mice (B1), menthol treated mice (B2) and fenoprofen calcium treated mice (B3). These data clearly indicated that the supramolecular topical gel formulation of **2** was able to cure the IMQ-induced skin inflammation and was more effective than its parent NSAID feneprofen. This *in vivo* data demonstrated that it was possible to self deliver the bioconjugates of **2** in the form of a supramolecular topical gel.

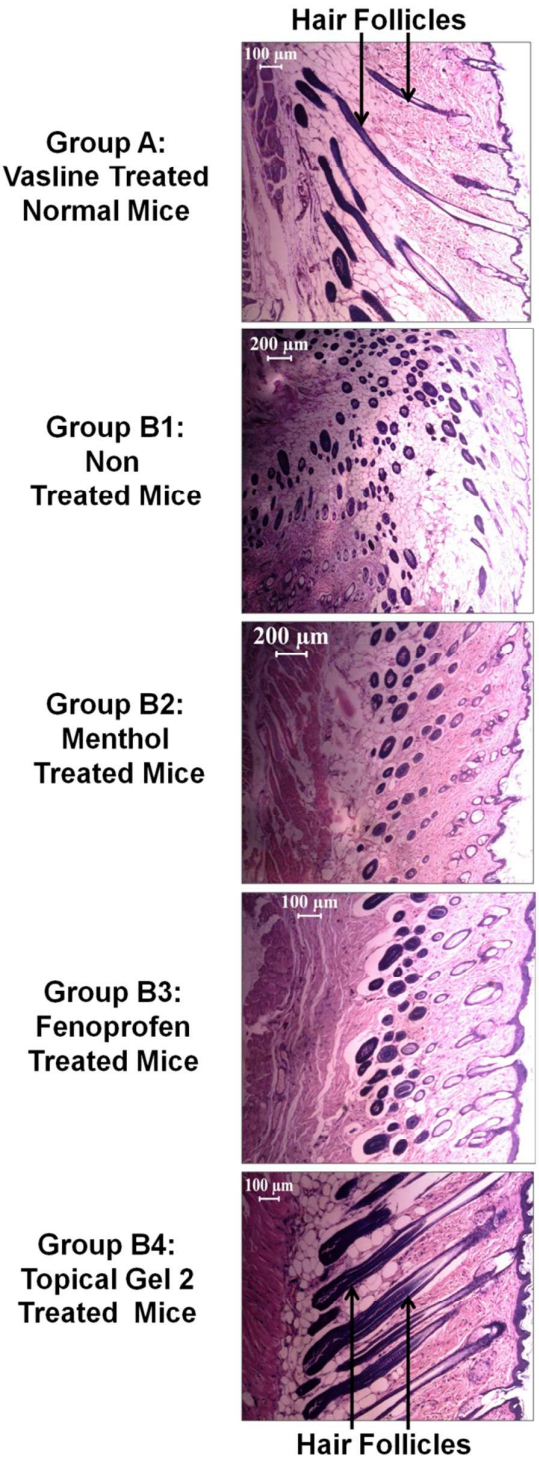



**Figure 6.** Histological evaluation of the dorsal skin tissue sections of mice after one week post treatment. Group A: no skin inflammation induced by IMQ (control mice); Group B1: no treatment after skin inflammation induced by IMQ; Group B2: menthol solution application after skin inflammation induced by IMQ; Group B3: parent drug fenoprofen calcium salt solution application after skin inflammation induced by IMQ; and Group B4:

menthol/methylsalicylate topical gel of **2** application after skin inflammation induced by IMQ; (H & E stain).

## Conclusion

Herein, we reported design, synthesise and characterization of a new series of bioconjugates derived from NSAID-fenoprofen by conjugating it with a small β–amino acid and various small biomolecules such as β–alanine, aminocaproic acid and tyramine. One of the bioconjugates (**2**), thus synthesised was capable of gelling both methylsalicylate and menthol containing methylsalicylate solvent. Microscopic studies of these gels revealed their fibrillar morphology in the gel networks and rheological studies probed their visco-elastic (gel) nature. The gelator bioconjugate **2** showed *in vitro* biostability not only in PBS buffer and in presence of proteolytic enzyme (cayboxypeptidase-Y and aminopeptidase), but also in blood serum of BALB/c mouse. Biocompatibility of the bioconjugates **2** was evaluated by MTT assay in mouse macrophase RAW 264.7 cells and mouse myoblast C2C12 cells. The $PGE_2$ assay of the gelator bioconjugate revealed its anti-inflammatory response, which was comparable to that of the parent drug fenoprofen. A menthol/methylsalicylate supramolecular topical gel made from this bioconjugate was applied in treating IMQ-induced skin inflammation in mice. Histology studies indicated the effect of the gelator bioconjugate **2** on the growth of hair folicles. The supramolecular topical gel formulated in the present study did not require any additional gel matrix (commonly used in commercial topical gel formulation) thereby demonstrating the potential of small β-peptide based NSAID topical gel in self-delivery application.

## Experimental Section

**Materials:** All the chemicals including fenoprofen calcium salt, benzotriazole, thionyl chloride, β-alanine, tyramine hydrochloride, aminocaproic acid, carboxypeptidase-Y,

aminopeptidase etc. were purchased from Sigma Aldrich and used without further purification. Analytical reagent (AR) grade solvents were purchased from local market and used without further distillation. Hair removal cream veet and skin inflammation inducing imiquimod (IMQ) cream used in the *in vivo* experiment were purchased from the local market. BALB/c male mice were purchased from National Center for Laboratory Animal Sciences (NCLAS), Hyderabad, India.

**Methods:** FT-IR spectra of the compounds were obtained using Shimadzu FT-IR 8300 instrument. The HR-MS spectra were recorded on Q-TOF Micro YA263 mass spectrometer. All the NMR spectra were recorded by using Bruker Ultrasheild Plus-500 MHz spectrometer. TEM images were recorded using a JEOL JEM instrument with a 300 mesh copper TEM grid. Rheological experiments were carried out using Anton Paar Modular Compact Rheometer (MCR 102). MTT and $PGE_2$ assays were performed using a multiplate ELISA reader (Varioskan Flash Elisa Reader, Thermo Fisher). Histology images were recorded using a Leica MZ 16 microscope.

**Synthesis:**

**Procedure for the synthesis of benzotriazole activated fenoprofen**

Thionyl chloride (0.20 mL, 3.6 mmol) was added to a solution of benzotriazole (1.071g, 9 mmol) in dry DCM (30 mL) at room temperature, and the reaction mixture was stirred for 10 min. Then fenoprofen free acid (3.0 mmol) was added to it and the mixture was stirred for 12 h at room temperature. The white precipitate obtained was filtered off, and the filtrate was concentrated under reduced pressure to get benzotriazole activated fenoprofen compound.

**Procedure for the synthesis of compound 1**

The benzotriazole activated fenoprofen compound (3 mmol) was added to a solution of amino acid β-alanine (267 mg, 3 mmol) dissolved in 30 ml of $MeCN/H_2O$ (2:1) mixture in the presence of $Et_3N$ (1.26 ml,9 mmol) and allowed to stir for 24 h at room temperature.

Then, the mixture was evaporated under reduced pressure to remove MeCN, acidified with 1N HCl and extracted with ethyl acetate (3×30 ml). The ethyl acetate fractions were then evaporated to dryness under reduced pressure to get the crude residue which was purified through column chromatography using pet ether/ethyl acetate in 1:1 ratio as mobile phase to give pure semi-solid compound **1** (Yield: 80%).

**General procedure for the synthesis of compounds 2 and 3**

Thionyl chloride (0.18 ml, 2.5mmol) was added to a solution of benzotriazole (714mg, 6 mmol) in dry DCM (50 mL) at room temperature and stirred for 10 min. Then compound **1** (2 mmol) was added to it and allowed to stir for 12 h at room temperature. The white precipitate that was filtered off and the filtrate were concentrated under reduced pressure to get the crude benzotriazolide product of **1**, which was then used without any further purification. The benotriazole activated compound (1.5 mmol) thus obtained from **1,** was added to separate solutions of β-alanine (1.5 mmol) and aminocaproic acid (1.5 mmol) dissolved in 30 ml of MeCN/$H_2O$ (2:1) mixture in the presence of $Et_3N$ (0.63 ml,4.5 mmol) and allowed to stir for 24 h at room temperature. The mixtures were evaporated under reduced pressure to remove MeCN, acidified with 1N HCL and extracted with ethyl acetate and the ethyl acetate fractions were then evaporated to dryness under reduced pressure to get the crude residues. The crude residues were then purified through column chromatography using 5 % MeOH in DCM as mobile phase to give pure semi-solid compounds **2** and **3** (Yield: 70 and 72% for **2** and **3,** respectively).

**Procedure for the synthesis of compound 4**

The benzotriazolide product (1 mmol) obtained from **1,** was added to 50mL THF solution containing free tyramine (1 mmol) and allowed to stir for 24 h at room temperature. After 24 hrs, the mixture was evaporated under reduced pressure to get the crude residue. The crude

residue was then purified through column chromatography using 5 % MeOH in DCM as mobile phase to give pure semi-solid compound **4** (Yield: 70%).

**FT-IR Sample Preparation:**

Each of the semi-solid bioconjugate was dissolved in chloroform (spectroscopy grade) and 5-10 µL of the resulting solution was drop-casted on KBr pellet and subjected to FT-IR after evaporation of the solvent.

**Characterization Data:**

**Compound 1**

**FT- IR (NEAT):** 3301 (s, amide N-H stretch), 1726 (m, acid C = O stretch), 1642 (s, amide C=O stretch) $cm^{-1}$. **HR-MS, ESI (MeOH) m/z (100%):** calculated for $[C_{18}H_{19}NO_4 + Na]^+$ is 336.1212, found 336.1212. **$^1$HNMR (400MHz, MeOD, 25$^0$C):** δ = 7.36-7.33 (t, *J*= 8 Hz, 2H), 7.30-7.27 (t, *J* = 7.5 Hz, 1H), 7.12-7.07 (q, *J* = 7.5 Hz, 2H), 6.99- 6.97 (d, *J* = 8.5 Hz, 3H), 6.85-6.83 (d, *J* = 8 Hz, 1H), 3.62- 3.58(q, *J* = 8 Hz, 1H), 3.40-3.38 (t, *J* = 6.5 Hz, 2H), 2.46-2.39 (q, *J* = 12 Hz, 2H), 1.42-1.40 (d, *J* = 7 Hz, 3H) ppm. **$^{13}$CNMR (400 MHz, MeOD, 25$^0$C):** δ = 176.68, 158.90, 158.64, 145.11, 130.87, 124.39, 123.38, 119.90, 118.86, 118.21, 47.33, 36.86, 35.53, 18.77 ppm (see Figure S1 in supporting information).

**Compound 2**

**FT-IR (NEAT):** 3305 (s, amide N-H stretch), 1705 (m, acid C=O stretch), 1647 (s, amide C=O stretch) $cm^{-1}$. **HR-MS, ESI (MeOH) m/z (100%):** calculated for $[C_{21}H_{24}N_2O_5+Na]^+$ is 407.1583, found 407.1583. **$^1$H-NMR (400 MHz, MeOD, 25ºC):** δ = 7.34 - 7.24 (m, 3H ), 7.10 - 7.05 (q, *J* = 9 Hz, 2H), 6.98 - 6.95 (m, 3H), 6.83 - 6.80 (q, *J* = 2.5 Hz, 1H), 3.61 - 3.56 (q, *J* = 8.5 Hz, 1H), 3.39 - 3.34 (m, 4H), 2.44 - 2.41 (t, *J* = 8.5 Hz, 2H ), 2.33 - 2.30 (t, *J* = 8.5 Hz, 2H ), 1.40-1.38 (d, *J* = 9 Hz, 3H) ppm. **$^{13}$C-NMR (400 MHz, MeOH, 25ºC):** δ = 176.75,

173.60, 158.87, 158.60, 145.11, 130.87, 124.40, 123.37, 119.90, 118.92, 118.18, 47.31, 37.07, 36.62, 36.52, 35.26, 18.74 ppm (see Figure S2 in supporting information).

**Compound 3**

**FT-IR (NEAT):** 3328 (m, amide N-H stretch), 1710 (s, acid C=O stretch), 1646 (s, amide C=O stretch) $cm^{-1}$. **HR-MS, ESI (MeOH) m/z (100%):** calculated for $[C_{24}H_{30}N_2O_5+Na]^+$ is 449.2052, found 449.2052. **$^1$H-NMR (400 MHz, MeOD, 25ºC):** δ = 7.34 - 7.23 (m, 3H ), 7.09 - 7.05 (m, 2H), 6.98 - 6.94 (m, 3H), 6.82 - 6.80 (m, 1H), 3.61 - 3.55 (q, *J* = 7.2 Hz, 1H), 3.40 - 3.36 (m, 2H), 3.12-3.07 (m, 2H), 2.34 - 2.24 (m, 4H ), 1.60-1.56 (t, *J* = 7.5 Hz, 2H ), 1.46-1.43 (t, *J* = 7.5 Hz, 1H), 1.40-1.38 (d, *J* = 7.2 Hz, 3H), 1.32-1.31 (d, *J* = 6.5 Hz, 2H) ppm. **$^{13}$C-NMR (400 MHz, MeOH, 25ºC):** δ = 177.49, 176.74, 173.45, 158.80, 158.53, 145.03, 130.87, 124.40, 123.35, 119.85, 118.93, 118.17, 47.29, 40.20, 37.13, 36.51, 34.78, 29.96, 27.43, 25.65, 18.78 ppm (see Figure S3 in supporting information).

**Compound 4**

**FT-IR (NEAT):** 3298 (brs, O-H stretch), 1656 (m, amide C=O stretch), 1645 (s, amide C=O stretch) $cm^{-1}$. **HR-MS, ESI (MeOH) m/z (100%):** calculated for $[C_{26}H_{28}N_2O_4+Na]^+$ is 455.1947, found 455.1947. **$^1$H-NMR (400 MHz, MeOD, 25$^0$C):** δ = 7.32 - 7.25 (m, 3H ), 7.10 - 7.07 (m, 2H), 7.01 - 6.94 (m, 5H), 6.72 - 6.70 (d, *J* = 6 Hz, 2H), 6.82 - 6.81 (d, *J* = 6 Hz, 1H), 3.60 - 3.56 (q, *J* = 7 Hz, 1H), 3.40 - 3.36 (q, *J* = 6.5 Hz, 2H), 3.29 - 3.27 (q, *J* = 5 Hz, 2H), 2.64-2.61 (t, *J* = 7 Hz, 2H), 2.33-2.30 (t, *J* = 7 Hz, 2H), 1.99 (s, 1 O-H), 1.41 - 1.39 (d, *J* = 6.5 Hz, 3H) ppm. **$^{13}$C-NMR (400 MHz, MeOH, 25$^0$C):** δ = 176.73, 173.43, 158.86, 158.52, 156.88, 145.06, 131.19, 130.90, 130.86, 1$^{30}$.69, 124.40, 123.32, 119.91, 118.91, 118.13, 116.25, 116.07, 47.31, 42.27, 37.13, 36.52, 35.66, 18.76 ppm (see Figure S4 in supporting information).

**Gelation Experiment:** 20 mg of each bioconjugate was taken in a culture vial and dissolved in 0.5 mL of target solvents (water, methylsalicylate and 1% menthol containing methylsalicylate) by heating until a clear solution was obtained. The solution was then allowed to cool to room temperature. After 8-10 h stable gel formation was observed only for the bioconjugate **2**. Gel formation was confirmed by test tube inversion method.

**$T_{gel}$ Measurement:** $T_{gel}$ (gel-sol transition temperature) of these gels were determined by the dropping ball method at different gelator concentrations. In this experiment, a glass ball weighing 468.91 mg was placed on a 0.5 ml gel in a test tube. The test tube was then immersed in an oil bath. The temperature at which the ball touches the bottom of the test tube was noted. (see Figure S5 in supporting information).

**Microscopy:** Each of the TEM sample was prepared by painting a small amount of gel on a carbon coated Cu (300 mesh) TEM grid. The grid was dried at room temperature for 24 h and then under vacuum for one hour and used for recording HR-TEM images using an accelerating voltage of 100 kV without staining.

**Rheology:** All the rheological studies such as strain sweep, frequency sweep experiments etc. were done at room temperature (25 °C) on parallel-plate geometry (25 mm diameter, 1 mm gap). In these experiments, 3 wt % of methylsalicylate and menthol in methylsalicylate gels of **2** were used.

**Biostability Experiment:**

**PBS incubation of the bioconjugate 2**

The stability of the gelator **2** was assessed by incubating 1 mg of it in 1 ml PBS of pH 7.4 at 37 $^{0}$C for 72 h. After incubation, the mixture was acidified with 1M HCl and extracted with ethyl acetate and analyzed by HPLC (see Figure S6-7 in supporting information).

**Enzymatic incubation of the bioconjugate 2**

1 mg of the gelator **2** was taken in 1mL PBS buffer of pH 6.5. Then 20 µL enzyme carboxypeptidase-Y solution (from a stock solution of 1mg/mL in water) was added to it and incubated for 72 h at 25 $^0$C. After incubation, the above mixture was acidified with 1M HCl and extracted with ethyl acetate and analyzed by HPLC (see Figure S8 in supporting information).

1 mg of the gelator **2** was taken in 1mL tris-HCl buffer of pH 8.0. Then 20 µL enzyme aminopeptidase solution (from a stock solution of 1mg/mL in water) was added to it and incubated for 72 h at 25 $^0$C. After incubation, the above mixture was acidified with 1M HCl and extracted with ethyl acetate and analyzed by HPLC (see Figure S9 in supporting information).

**Serum incubation of the bioconjugate 2**

1 mg of the gelator **2** was taken in 1mL blood serum of BALB/c mouse and the mixture was incubated for 72 h at 37 $^0$C. After incubation, the above mixture was acidified with 1M HCl and extracted with ethyl acetate and analyzed by HPLC (see Figure S10 in supporting information).

**HPLC Experiment**

Instrument: Agilent HPLC System with 1100 series UV/VIS-detector; Column Specification: Thermo ODS hypersil, 5µm, 4.6 x 250 mm; Elution: Isocratic; Mobile Phase: $CH_3OH$: $H_2O$ (8:2); Flow Rate: 1.0 mL/ min; Column Compartment Temperature: 25 ºC; Injection Volume: 5 µL; UV detector: Variable wavelength detector 272 nm.

**MTT Assay**

Mouse macrophage RAW 264.7 and mouse myoblast C2C12 cells were purchased from American Type Culture Collection (ATCC) and cultured following their guidelines. The cells were grown in high glucose Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 10% fetal bovine serum (FBS) and 1% penicillin and streptomycin in a humidified

incubator at 37 °C and 5% $CO_2$. Approximately $1 \times 10^4$ cells/well were taken in 96-well plates. The cells were seeded for 24 h and treated with various concentrations (up to 1000 μM) of fenoprofen calcium salt, the gelator **2** or DMEM alone for 72 h at 37 °C and 5% $CO_2$. Next, the culture medium was replaced with 100 μg of MTT dye per well and kept at 37 °C for 4 h. 100 μL DMSO was added to each well to dissolve the formazan produced by mitochondrial reductase from live cells and the plate was further incubated for 30 minutes at 37 °C. The absorbance of the purple formazan solution was recorded at 570 nm using a multiplate ELISA reader (Varioskan Flash Elisa Reader, Thermo Fisher). The percentage of live cells in fenoprofen calcium salt and the gelator **2** treated samples was calculated by considering the DMEM-treated sample as 100%.

**$PGE_2$ Assay**

The $PGE_2$ assay of the gelator molecule **2** was performed following a published protocol. Approximately $1 \times 10^6$ RAW 264.7 cells/well (using 6-well plates) were seeded for 24 h. One of the wells was treated with 1 mL of DMEM (control), the rests were treated with 1 μg/mL LPS and 100 ng/mL IFN-γ; out of these LPS/IFN-γ treated wells, one well was treated with 500 μM fenoprofen calcium salt and one well was treated with 500 μM gelator **2** in such a way that the total media (DMEM) volume in all the wells was 1 mL and further incubated for 24 h. The amount of $PGE_2$ produced in the culture medium was measured using a Prostaglandin E2 EIA kit − monoclonal (Cayman Chemicals, MI).

**Animals Experiment:** All the BALB/c male mice (weighing 20-25gm) of aged 16-18 weeks were maintained in the animal house facility of Department of Biological Chemistry, Indian Association for the Cultivation of Science, Kolkata as per guidelines of Institutional Animal Ethics Committee (IAEC). Both animal care and the experiment protocol were approved by a local animal ethics committee. Experimental animals were placed in separate cages at a temperature between 22 and 25℃ with a 12 hour light and dark cycle. Animals were given an

unlimited amount of water and food throughout the experiment. Blood serum used in the *in vitro* experiment was collected from heart of the normal and healthy BALB/c male mouse.

**Histology Experiment:** After 7 days treatment, dorsal skin tissue samples were collected from one mouse of each group. The skin tissues were fixed in 10% formalin and embedded in paraffin wax and each section was stained with hematoxylin-eosin (H & E) and examined by LEICA MZ 16 optical microscope.

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**Supporting Information**

$^{1}$H-NMR and $^{13}$C-NMR spectra of the compounds, gelation data, and HPLC traces of gelator **2**.

**References**

1. (a) P. Terech and R. G. Weiss, *Chem. Rev.,* 1997, **97**, 3133-3159; (b) L. A. Estroff and A. D. Hamilton, *Chem. Rev.,* 2004, **104**, 1201-1217; (c) M. Suzuki and K. Hanabusa, *Chem. Soc. Rev.,* 2009, **38**, 967-975; (d) R. G. Weiss and P. Terech, Eds. *Molecular Gels: Materials with Self- Assembled Fibrillar Networks*, Springer: Dordrecht, The Netherlands, 2005; (e) M.-O. M. Piepenbrock, G. O. Lloyd, N. Clarke and J. W. Steed, *Chem. Rev.,* 2010, **110***,* 1960-2004; (f) C. Tomasini and N. Castellucci, *Chem. Soc. Rev.,* 2013, **42**, 156-172; (g) P.

Dastidar, *Chem. Soc. Rev.,* 2008, **37**, 2699-2715; (h) S. Dong, B. Zheng, D. Xu, X. Yan, M. Zhang and F. Huang, *Adv. Mater.,* 2012, **24**, 3191-3195; (i) G. Yu, X. Yan, C. Han and F. Huang, *Chem. Soc. Rev.,* 2013, **42**, 6697-6722.

2. M. George and R. G. Weiss, *Acc. Chem. Res*., 2006, **39**, 489-497.

3. (a) M. Ikeda, K. Fukuda, T. Tanida, T. Yoshii and I. Hamachi, *Chem. Commun.,* 2012, **48**, 2716-2718, (b) X. Zhang, S. Lee, Y. Liu, M. Lee, J. Yin, J. L. Sessler and J. Yoon, *Sci. Rep.,* 2014, **4**, 4593; (c) J. Chen, W. Wu and A. J. McNeil, *Chem. Commun.,* 2012, **48**, 7310-7312.

4. (a) V. K. Praveen, C. Ranjith and N. Armaroli, *Angew. Chem., Int. Ed.,* 2014, **53**, 365-368; (b) S. S. Babu, V. K. Praveen and A. Ajayaghosh, *Chem. Rev.,* 2014, **114**, 1973-2129; (c) E. Krieg, E. Shirman, H. Weissman, E. Shimoni, S. G. Wolf, I. Pinkas and B. Rybtchinski, *J. Am. Chem. Soc.* 2009, **131**, 14365-14373; (d) X. Cao, A. Gao, H. Lv, Y. Wu, X. Wang and Y. Fan, *Org. Biomol. Chem.*, 2013, **11**, 7931-7937; (e) T. Haino, Y. Hirai, T. Ikeda and H. Saito, *Org. Biomol. Chem.*, 2013, **11**, 4164-4170.

5. A. Wynne, M. Whitefield, A. J. Dixon and S. Anderson, *J. Dermatol. Treat*., 2002, **13**, 61-66.

6. J. A. Foster, M-O. M. Piepenbrock, G. O. Lloyd, N. Clarke, J. A. K. Howard and J. W. Steed, *Nat. Chem.,* 2010, **2**, 1037-1043.

7. A. P. Piccionello, A. Guarcello, A. Calabrese, I. Pibiri, A. Pace and S. Buscemi, *Org. Biomol. Chem.*, 2012, **10**, 3044-3052.

8. H. Basit, A. Pal, S. Sen and S. Bhattacharya, *Chem. Eur. J.,* 2008, **14,** 6534-6545.

9. (a) B. Escuder, F. Rodrguez-Llansola and J. F. Miravet, *New J. Chem*., 2010, **34**, 1044-1054; (b) J. Potier, S. Menuel, M. H. Chambrier, L. Burylo, J. F. Blach, P. Woisel, E. Monflier and F. Hapiot, *ACS Catal.,* 2013, **3**, 1618-1621.

10. (a) E. Carretti, M. Bonini, L. Dei, B. H. Berrie, L. V. Angelova, P. Baglioni and R. G. Weiss, *Acc. Chem. Res.,* 2010, **43**, 751-760; (b) E. Carretti, E. Fratini, D. Berti, L. Dei and P. Baglioni, *Angew. Chem., Int. Ed.,* 2009, **48**, 8966-8969.

11. (a) K. Y. Lee and D. J. Mooney, *Chem. Rev.,* 2001, **101**, 1869-1879; (b) A. Khademhosseini and R. Langer, *Biomaterials,* 2007, **28**, 5087-5092.

12. R. N. Shah, N. A. Shah, M. M. D. R. Lim, C. Hsieh, G. Nuber and S. I. Stupp, *Proc. Natl. Acad. Sci.,* 2010, **107**, 3293-3298.

13. Z. A. C. Schnepp, R. Gonzalez-McQuire and S. Mann, *Adv. Mater.,* 2006, **18**, 1869-1872.

14. Z. Yang, G. Liang, M. Ma, A. S. Abbah, W. W. Lu and B. Xu, *Chem. Commun.,* 2007, 843-845.

15. S. Kiyonaka, K. Sada, I. Yoshimura, S. Shinkai, N. Kato and I. Hamachi, *Nat. Mater.,* 2004, **3**, 58-64.

16. Q. Wang, Z. Yang, X. Zhang, X. Xiao, C. K. Chang and B. Xu, *Angew. Chem., Int. Ed.,* 2007, **46**, 4285-4289.

17. (a) S. M. Standley, D. J. Toft, H. Cheng, S. Soukasene, J. Chen, S. M. Raja, V. Band, H. Band, V. L. Cryns1 and S. I. Stupp, *Cancer Res*., 2010, **70**, 3020-3026; (b) C. Yang, D. Li, Q. F. Zhao, L. Wang, L. Wang and Z. Yang, *Org. Biomol. Chem.*, 2013, **11**, 6946-6951

18. (a) V. Jayawarna, M. Ali, T. A. Jowitt, A. F. Miller, A. Saiani, J. E. Gough and R. V. Ulijn, *Adv. Mater.,* 2006, **18**, 611-614; (b) X. Q. Dou, P. Li, D. Zhang and C. L. Feng, *J. Mater. Chem. B,* 2013, **1**, 3562-3568.

19. F. Zhao, B. A. Heesters, I. Chiu, Y. Gao, J. Shi, N. Zhou, M. C. Carroll and B. Xu, *Org. Biomol. Chem.*, 2014, **12**, 6816-6819.

20. (a) A. Friggeri, B. L. Feringa and J. van Esch, *J. Controlled Release,* 2004, **97,** 241-248.

(b) K. J. C. van Bommel, M. C. A. Stuart, B. L. Feringa and J. van Esch, *Org. Biomol. Chem.,* 2005, **3**, 2917-2920.

21. (a) P. K. Vemula, J. Li and G. John, *J. Am. Chem. Soc.,* 2006, **128**, 8932-8938; (b) M. J. Webber, J. B. Matson, V. K. Tamboli and S. I. Stupp, *Biomaterials,* 2012, **33**, 6823-6832; (c) J. Majumder, J. Deb, M. R. Das, S. S. Jana and P. Dastidar, *Chem. Commun.,* 2014*,* **50***,* 1671-1674; (d) X. M. Li, J. Y. Li, Y. A. Gao, Y. Kuang, J. F. Shi and B. Xu, *J. Am. Chem. Soc.,* 2010, **132**, 17707-17709; (e) H. Wang, J. Wei, C. Yang, H. Zhao, D. Li, Z. Yin and Z. Yang, *Biomaterials,* 2012, **33***,* 5848-5853.

22. (a) B. J. Lee and J. R. Lee, *Arch. Pharm. Res.,* 1995, **18**, 22−26; (b) V. K. Tammara, M. M. Narurkar, A. M. Crider and M. A. Khan, *Pharm. Res.,* 1993, **10**, 1191−1199.

23. (a) F. E. Silverstein, G. Faich, J. L. Goldstein, L. S. Simon, T. Pincus, A. Whelton, R. Makuch, G. Eisen, N. M. Agarwal, W. F. Stenson, A. M. Burr, W. W. Zhao, J. D. Kent, J. B. Lefkowith, K. M. Verburg and G. S. Geis, *J. Am. Med. Assoc.,* 2000, **284**, 1247−1255; (b) C. Bombardier, L. Laine, A. Reicin, D. Shapiro, R. Burgos- Vargas, B. Davis, R. Day, M. B. Ferraz, C. J. Hawkey, M. C. Hochberg, T. K. Kvien and T. J. Schnitzer, *N. Engl. J. Med.,* 2000, **343**, 1520−1528.

24. (a) K. E. Uhrich, S. M. Cannizzaro, R. S. Langer and K. M. Shakesheff, *Chem. Rev.,* 1999, **99**, 3181-3198; (b) G. Vilar, J. Tulla-Puche and F. Albericio, *Curr. Drug Delivery,* 2012, **9**, 367-394.

25. S. Bhuniya, Y. J. Seo and B. H. Kim, *Tetrahedron Lett.,* 2006, **47**, 7153−7156.

26. J. B. Matson and S. I. Stupp, *Chem. Commun.,* 2011, **47**, 7962−7964.

27. J. Li, Y. Kuang, Y. Gao, X. Du, J. Shi and B. Xu, *J. Am. Chem. Soc.,* 2013, **135**, 542-545.

28. J. Majumder, M. R. Das, J. Deb, S. S. Jana and P. Dastidar, *Langmuir,* 2013, **29**, 10254-10263.

29. A. R. Katritzky, S. K. Singh, C. Cai and S. Bobrov, *J. Org. Chem.* 2006, **71**, 3364−3374.

30. D. F. Hook, F. Gessier, C. Noti, P. Kast and D. Seebach, *Chem. Biol. Chem.,* 2004, **5**, 691-706.

31. (a) A. Ajayaghosh and S. J. George, *J. Am. Chem. Soc.,* 2001**, 123***,* 5148-5149; (b) K. Yabuuchi, E. Marfo-Owusu and T. Kato, *Org. Biomol. Chem.*, 2003, **1**, 3464-3469; (c) S. E. Paramonov, H-W. Jun, and J. D. Hartgerink, *J. Am. Chem. Soc.,* 2006, **128**, 7291-7298; (d) A. Pal, S. Abraham, M. A. Rogers, J. Dey, and R. G. Weiss, *Langmuir*, 2013, **29**, 6467−6475.

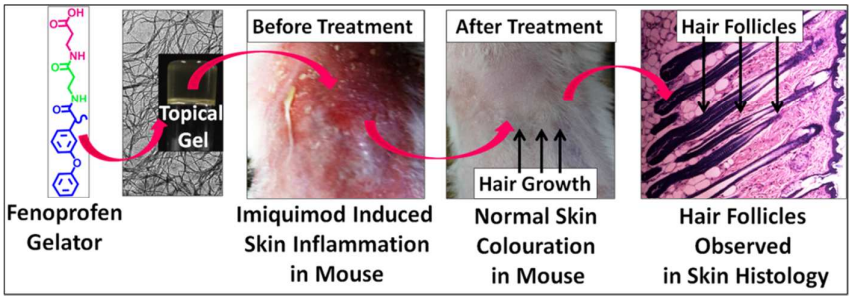
32. A. Takahashi, M. Sakai and T. Kato, *Polym. J.*, 1980, **12**, 335-341.

33. J. O. Baek, D. Byamba, W. H. Wu, T. G. Kim and M. G. Lee, *Arch. Dermatol. Res.*, 2012, **304**, 699-706.

34. R. Eccles, *J. Pharm. Pharmacol.,* 1994, **46**, 618-30.

35. B. Young and J. H. Heath, *Wheater's Functional Histology - A Text and Colour Atlas;* 4th Edition, Harcourt Publishers Limited: London, 2000, pp157-171.

**Table of Contents Graphic:**




A small β-dipeptide functionalized non steroidal anti-inflammatory drug-fenoprofen bioconjugate forms supramolecular gel with menthol containing methylsalicylate solvent. The resulting gel shows topical self-delivery application in treating imiquimod induced skin inflammation in mice.