Supplementary Information

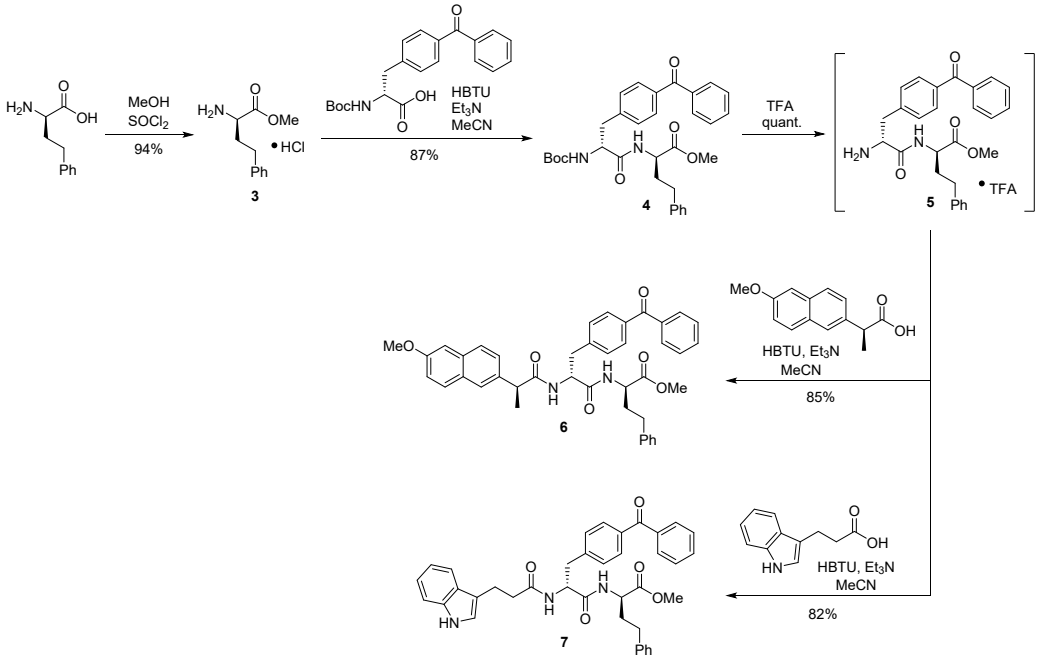
An injectable, naproxen-conjugated, supramolecular hydrogel with ultra-low critical gelation concentration — prepared from a known folate receptor ligand

Carlos B. P. Oliveira,a Sérgio R. S. Veloso,b Pedro R. Figueiredo,c,d Alexandra T. P. Carvalho,c,e Loic Hilliou,f Renato B. Pereira,g David M. Pereira,g José A. Martins,a Paula M. T. Ferreira,a Peter J. Jervis*a

[a.] *Centre of Chemistry (CQUM), University of Minho, Campus de Gualtar, 4710-057 Braga, Portugal.*
[b.] *Centre of Physics (CFUM), University of Minho, Campus de Gualtar, 4710-057 Braga, Portugal.*
[c.] *Institute for Polymers and Composites/I3N, Department of Polymer Engineering, University of Minho, Campus de Azur´em, 4800-058 Guimarães, Portugal.*
[d.] *REQUIMTE/LAQV, Lab. of Pharmacognosy, Dep. of Chemistry, Faculty of Pharmacy, University of Porto, R. Jorge Viterbo Ferreira, 228, 4050-313 Porto, Portugal.*

Contents:

Synthesis of compounds **3**, **4**, **6** and **7**



*H-D-HPhe-OMe.HCl (**3**).* Thionyl chloride (1.21 mL, 16.7 mmol) was slowly added to cooled (0 °C) MeOH (12 mL) over 5 min. D-homophenylalanine (1.00 g, 5.58 mmol) was then added slowly, and the temperature of the mixture was raised to 40 °C and left for 4 h. The solvent was removed under reduced pressure to afford a white solid, which was washed with Et_2O (12 mL). Removal of residual Et_2O under reduced pressure afforded H-D-HPhe-OMe.HCl (**3**) as a white solid (1.20 g, 94%). ^{1}H NMR (300 MHz, DMSO-d_6, δ): 2.04-2.13 (m, 1H, γ-CH_2Ph), 2.56-2.68 (m, 1H, β-CH_AH$_B$), 2.70-2.80 (m, 1H, β-CH$_A$$H_B$), 3.73 (s, 3H, CO_2CH_3), 4.00 (m, 1H, α-C*H*), 7.17-7.23 (m, 3H, Ph*H*), 7.27-7.33 (m, 2H, Ph*H*), 8.65 (br s, NH_3^+). ^{13}C NMR (100.6 MHz, DMSO-d_6: δ): 30.2 (CH_2, β-*C*H$_2$ of HPhe), 31.1 (CH_2, γ-*C*H$_2$ of HPhe), 51.5 (CH, α-*C*H of HPhe), 52.7 (CH_3, CO_2CH_3), 126.2 (CH, Ar), 128.3 (CH, Ar), 128.4 (CH, Ar), 140.2 (C, Ar), 169.7 (C, *C*=O). m/z (TOF ES+) 194.1 ([M + H]$^+$, 100%).

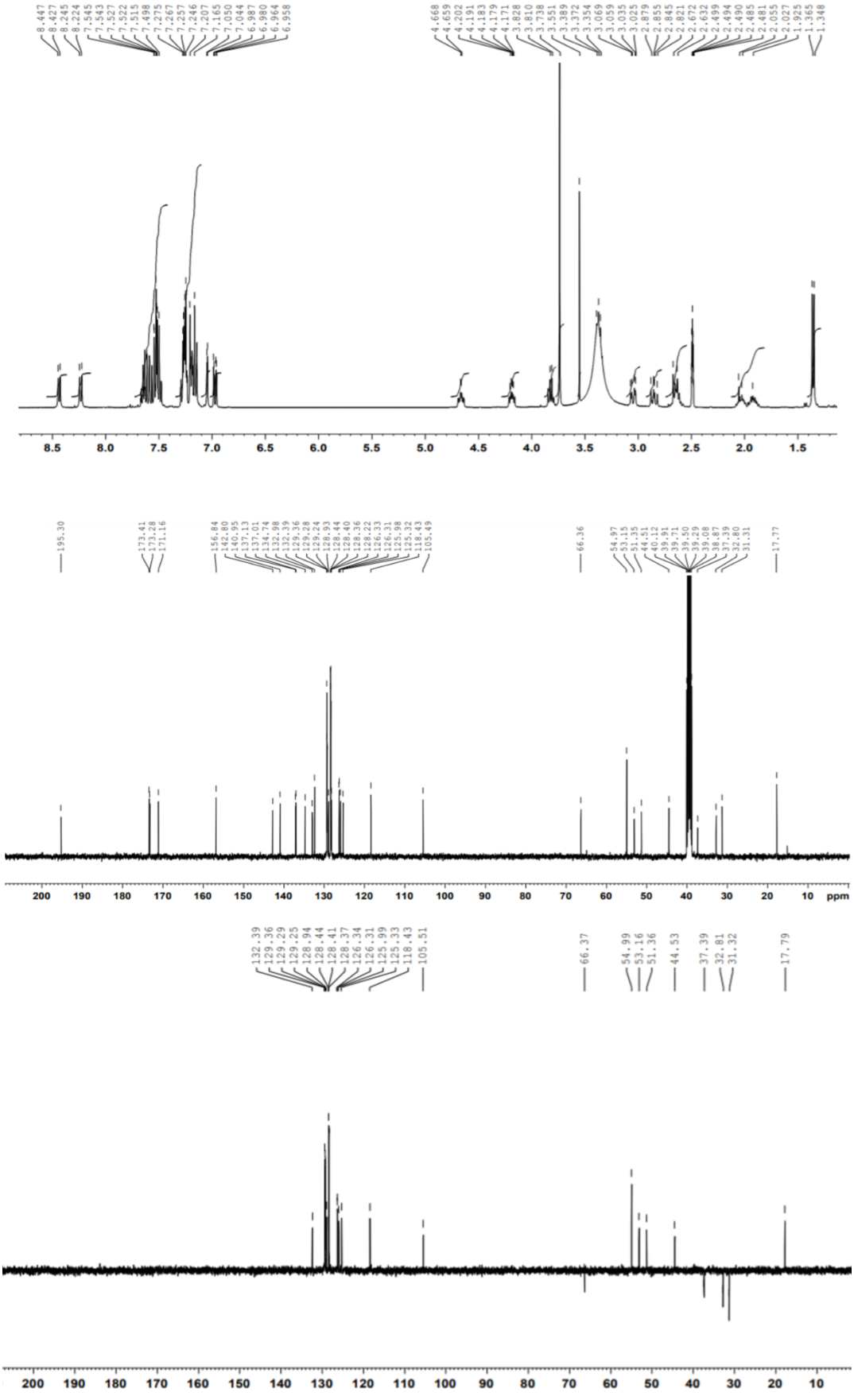
*Boc-D-BPh-D-HPhe-OMe (**4**).* Boc-D-BPhe-OH (200 mg, 0.54 mmol) was dissolved in MeCN (5 mL) and cooled to 0 °C. H-D-HPhe-OMe (**3**) (124 mg, 0.54 mmol), Et_3N (225 uL, 1.62 mmol) and HBTU (225 mg, 0.59 mmol) were added sequentially, with 2 min between each addition, and the mixture was stirred at rt overnight. The solvent was removed under reduced pressure to afford a residue that was partitioned between EtOAc (50 mL) and $KHSO_4$ (50 mL, 1 M). After separation of the phases, the organic phase was thoroughly washed with $KHSO_4$ (1 M, 2 × 50 mL), $NaHCO_3$ (1 M, 3 × 50 mL), and brine (3 × 50 mL) and then dried with $MgSO_4$. Filtration followed by removal of the solvent under reduced pressure afforded compound **4** (248 mg, 87%). ^{1}H-NMR (400 MHz, $CDCl_3$, δ): 1.42 (s, 9H, O(CH_3)$_3$), 1.96-2.05 (m, 1H, β-CH_AH$_B$ of HPhe), 2.14-2.23 (m, 1H, β-CH$_A$$H_B$), 2.61 (app. t, 2H, J = 8.0, γ-CH_2 of HPhe), 3.08 (dd, 1H, J = 13.4 6.8, β-CH_AH$_B$ of BPhe), 3.20 (dd, 1H, J = 13.4, 6.4, β-CH$_A$$H_B$ of BPhe), 3.69 (s, 3H, CO_2CH_3), 4.40-4.42 (m, 1H, α-C*H* of HPhe), 4.60 (app. q, J = 6.6, α-C*H* of BPhe), 5.00 (1H, d, J = 6.6, N*H*), 6.53 (d, 1H, J = 7.6, N*H*), 7.14 (2H, d, J = 6.8, ArH), 7.20 (1H, d, J = 7.2, ArH), 7.23-7.29 (2H, m, Ar*H*), 7.33 (2H, d, J = 8.0, Ar*H*), 7.47 (2H, t, J = 6.4, Ar*H*), 7.57-7.62 (1H, m, Ar*H*), 7.72-7.81 (4H, m, Ar*H*). ^{13}C NMR (100.6 MHz, $CDCl_3$, δ): 28.2 (CH_3, OC(*C*H$_3$)$_3$, 31.4 (CH_2, γ-*C*H$_2$ of HPhe), 33.6 (CH_2, β-*C*H$_2$ of HPhe), 38.0 (CH_2, β-*C*H$_2$ of BPhe), 52.1 (CH, α-*C*H of BPhe), 52.4 (CH_3, CO_2CH_3), 55.5 (CH, α-*C*H of HPhe), 126.2 (CH, Ar), 128.2 (CH, Ar), 128.3 (CH, Ar), 128.5 (CH, Ar), 129.3 (CH, Ar), 129.9 (CH, Ar), 130.4 (CH, Ar), 132.4 (CH, Ar), 136.2 (C, Ar), 137.6 (C, Ar), 140.5 (C, Ar), 141.6 (C, Ar), 155.3 (C, *C*=O), 170.6 (C, *C*=O), 172.0 (C, *C*=O), 196.3 (C, *C*=O). m/z (TOF ES+) 567.3 ([M + Na]$^+$, 100%).

*Npx-D-BPh-D-HPhe-OMe (**6**).* Boc-D-BPhe-D-HPhe-OMe (**4**) (229 mg, 0.42 mmol) was dissolved in TFA (2.0 mL) and the reaction mixture was stirred at rt for 30 minutes. The mixture was diluted with $CHCl_3$ (10 mL) and concentrated under reduced pressure. Additional $CHCl_3$ (2 × 10 mL) was added and then removed under reduced pressure (to completely remove the residual TFA), to afford H-D-BPh-D-HPhe-OMe•TFA (**5**) as a white

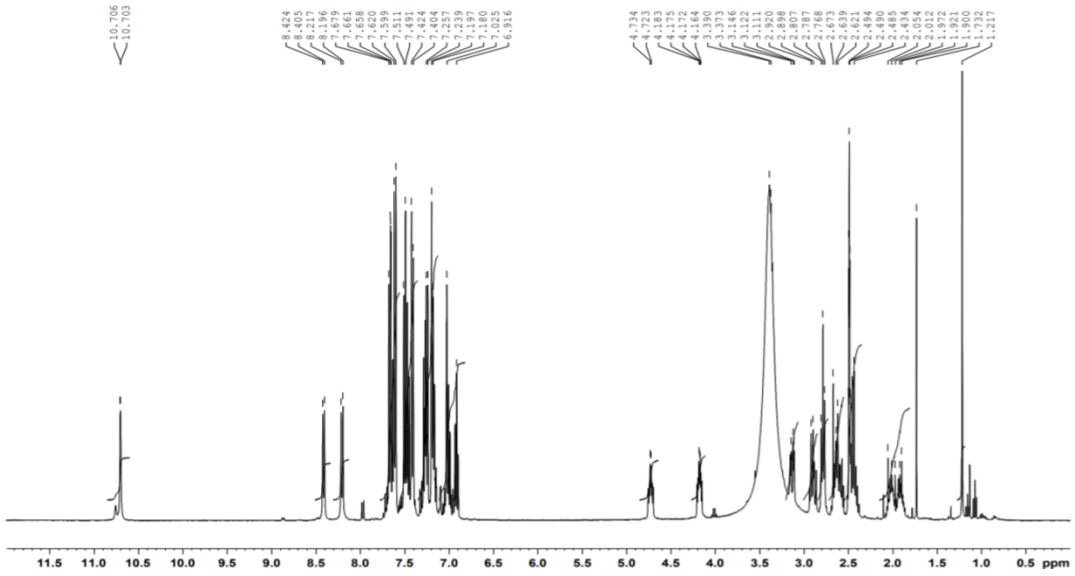
solid, which was dissolved in MeCN (6 mL) and cooled to 0 °C. Naproxen (106 mg, 0.46 mmol), Et_3N (175 uL, 1.26 mmol), and HBTU (175 mg, 0.46 mmol) were added sequentially, with 2 min between each addition, and the mixture was stirred at rt overnight. The solvent was removed under reduced pressure to afford a residue that was partitioned between EtOAc (50 mL) and $KHSO_4$ (1M, 50 mL). After separation of the phases, the organic phase was thoroughly washed with $KHSO_4$ (1 M, 3 × 50 mL), $NaHCO_3$ (1 M, 3 × 50 mL), and brine (3 × 50 mL) and then dried with $MgSO_4$. Filtration followed by removal of the solvent under reduced pressure afforded Npx-D-BPhe-D-HPhe-OMe (**6**) as a white solid (234 mg, 85%). ^{1}H NMR (400 MHz, $CDCl_3$, δ): 1.55 (d, 3H, J = 7.2, C*H*$_3$ of Npx), 1.94-2.01 (m, 1H, β-C*H*$_A$H$_B$ of HPhe), 2.12-2.22 (m, 1H, β-CH$_A$*H*$_B$), 2.56 (app. t, 2H, J = 8.0, γ-C*H*$_2$ of HPhe), 2.99 (dd, 2H, m, β-C*H*$_2$ of BPhe), 3.70 (s, 3H, CO_2CH_3), 3.84 (s, 3H, OC*H*$_3$ of Npx), 4.55 (app. dt, 1H, J = 7.6, 5.2, α-C*H* of HPhe), 4.77 (app. dt, 1H, J = 14.0, 7.2, α-C*H* of BPhe), 6.02 (br d, 1H, J = 8.0, N*H*), 6.75 (1H, br d, J = 8.0, N*H*), 6.94 (d, 2H, J = 8.0, Ar*H*), 7.01-7.13 (m, 4H, Ar*H*), 7.13-7.29 (m, 4H, Ar*H*), 7.40-7.48 (m, 4H, Ar*H*), 7.56-7.65 (m, 4H, Ar*H*), 7.68-7.72 (m, 2H, Ar*H*). ^{13}C NMR (100.6 MHz, $CDCl_3$, δ): 17.9 (CH_3, CH*C*H$_3$ of Npx), 31.4 (CH_2, γ-*C*H$_2$ of HPhe), 33.6 (CH_2, β-*C*H$_2$ of HPhe), 37.4 (CH_2, β-*C*H$_2$ of BPhe), 46.8 (CH_2, CH*C*H$_3$ of Npx), 52.1 (CH, α-*C*H of HPhe), 52.4 (CH_3, CO_2CH_3), 53.6 (CH, α-*C*H of BPhe), 55.2 (CH_3, O*C*H$_3$ of Npx), 105.6 (CH, Ar), 119.3 (CH, Ar), 125.7 (CH, Ar), 126.1 (CH, Ar), 126.2 (CH, Ar), 127.7 (CH, Ar), 128.2 (CH, Ar), 128.3 (CH, Ar), 128.5 (CH, Ar), 128.8 (C, Ar), 129.1 (CH, Ar), 129.9 (CH, Ar), 130.2 (CH, Ar), 132.3 (CH, Ar), 133.7 (C, Ar), 135.7 (C, Ar), 135.9 (C, Ar), 137.5 (C, Ar), 140.4 (C, Ar), 140.9 (C, Ar), 157.7 (C, Ar), 170.3 (C, *C*=O), 172.1 (C, *C*=O), 174.4 (C, *C*=O), 196.1 (C, *C*=O). 1 x (CH, Ar) is missing due to resonance overlap. m/z (TOF ES+) 657.3 ([M + H]$^+$, 100%).

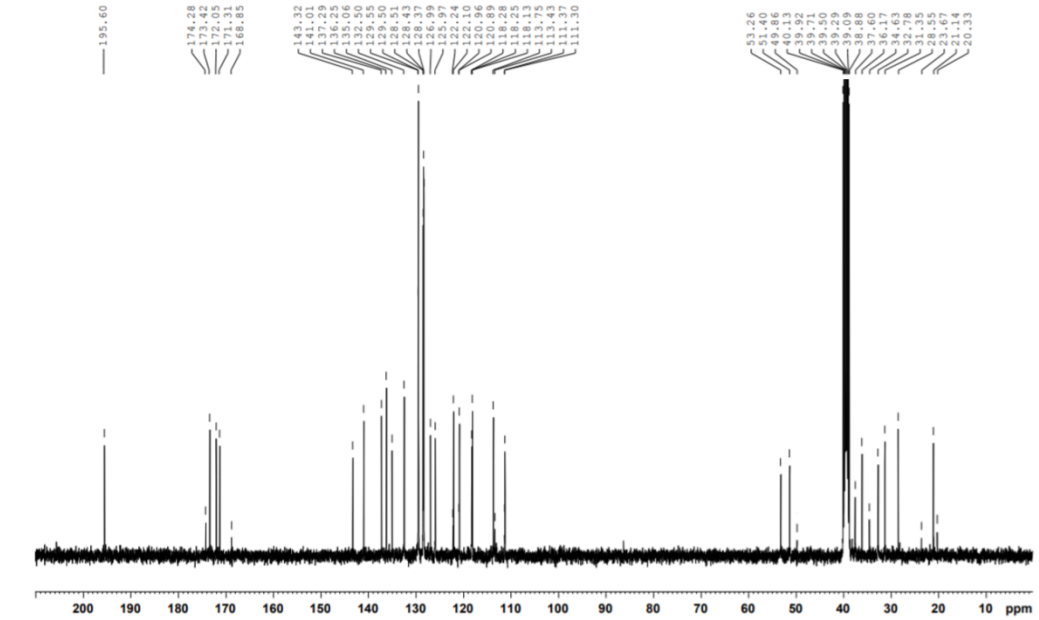
*Ind-D-BPh-D-HPhe-OMe (**7**).* Boc-D-BPhe-D-HPhe-OMe (**4**) (218 mg, 0.40 mmol) was dissolved in TFA (2 mL) and the reaction mixture was stirred at rt for 30 minutes. The mixture was diluted with $CHCl_3$ (10 mL) and concentrated under reduced pressure. Additional $CHCl_3$ (2 × 10 mL) was added and then removed under reduced pressure (to completely remove the residual TFA), to afford H-D-BPh-D-HPhe-OMe•TFA (**5**) as a white solid, which was dissolved in MeCN (6 mL) and cooled to 0 °C. 3-Indolepropionic acid (83 mg, 0.44 mmol), Et_3N (167 uL, 1.20 mmol), and HBTU (167 mg, 0.44 mmol) were added sequentially, with 2 min between each addition, and the mixture was stirred at rt overnight. The solvent was removed under reduced pressure to afford a residue that was partitioned between EtOAc (50 mL) and $KHSO_4$ (1M, 50 mL). After separation of the phases, the organic phase was thoroughly washed with $KHSO_4$ (1 M, 3 × 50 mL), $NaHCO_3$ (1 M, 3 × 50 mL), and brine (3 × 50 mL) and then dried with $MgSO_4$. Filtration followed by removal of the solvent under reduced pressure afforded Ind-D-BPhe-D-HPhe-OMe (**7**) as a white solid (202 mg, 82%). ^{1}H NMR (400 MHz, $CDCl_3$, δ): 1.94-2.01 (m, 1H, β-C*H*$_A$H$_B$ of HPhe), 2.10-2.21 (m, 1H, β-CH$_A$*H*$_B$ of HPhe), 2.50-2.67 (m, 4H, γ-C*H*$_2$ of HPhe and C*H*$_2$ of Ind), 2.90-3.13 (m, 4H, β-CH$_2$ of BPhe and C*H*$_2$ of Ind), 3.70 (s, 3H, CO_2CH_3), 4.54 (app. dt, 1H, J = 7.6, 5.2, α-C*H* of HPhe), 4.76 (app. q, J = 7.2 α-C*H* of BPhe, 5.99 (br d, 1H, J = 8.4, N*H*), 6.57 (br d, 1H, J = 8.0, N*H*), 7.07-7.26 (10H, m, Ar*H*), 7.33 (1H, d, J = 8.0, Ar*H*), 7.48 (2H, t, J = 8.0, Ar*H*), 7.54-7.65 (4H, m, Ar*H*), 7.68-7.72 (2H, m, ArH), 8.22 (br s, 1H, N*H*). ^{13}C NMR (100.6 MHz, $CDCl_3$, δ): 21.1 (CH_2, 1 x *C*H$_2$ of Ind), 31.2 (CH_2, γ-*C*H$_2$ of HPhe), 32.6 (CH_2, β-*C*H$_2$ of HPhe), 36.1 (CH_2, 1 x *C*H$_2$ of Ind), 37.6 (CH_2, , β-*C*H$_2$ of BPhe), 51.4 (CH, α-*C*H of HPhe), 51.9 (CH_3, CO_2CH_3), 53.2 (CH_2, α-*C*H of BPhe), 111.3 (CH, Ar), 113.7 (C, Ar), 118.1 (CH, Ar), 118.2 (CH, Ar), 120.8 (CH, Ar), 122.1 (CH, Ar), 126.0 (CH, Ar), 127.0 (C, Ar), 128.3 (CH, Ar), 128.4 (CH, Ar), 128.5 (CH, Ar), 129.46 (CH, Ar), 129.5 (CH, Ar), 132.5 (CH, Ar), 135.1 (C, Ar), 136.2 (C, Ar), 137.2 (C, Ar), 140.8 (C, Ar), 143.2 (C, Ar), 171.4 (C, *C*=O), 172.0 (C, *C*=O), 172.3 (C, *C*=O), 195.5 (C, *C*=O). m/z (TOF ES+) 638.3 ([M + Na]$^+$, 100%).

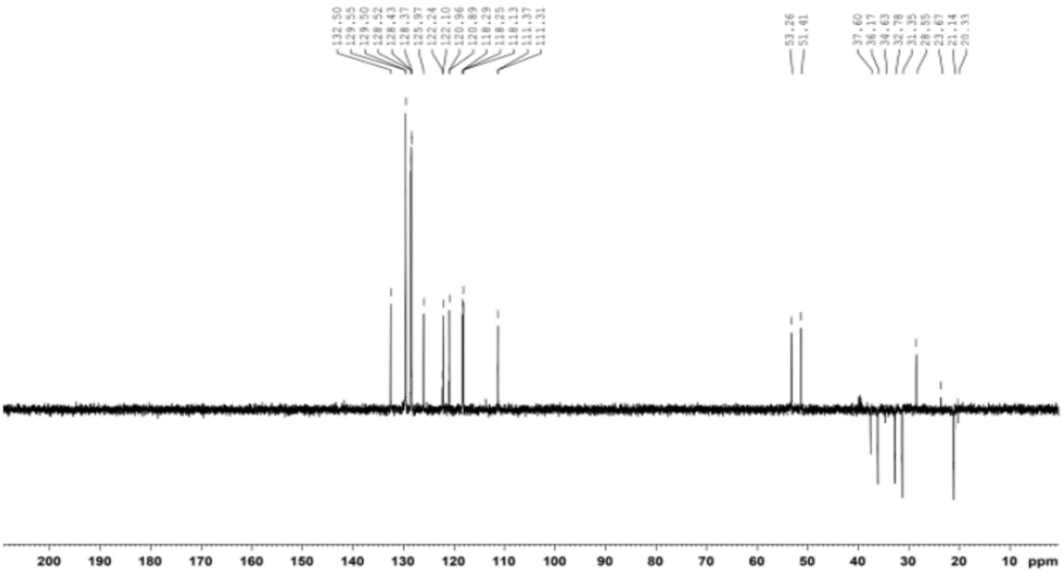
^{1}H and ^{13}C NMR (standard and DEPT) spectra for compound **1**



^{1}H and ^{13}C NMR (standard and DEPT) spectra for compound **2**







Figures S1 and S2 related to Molecular Dynamics of compounds **1**

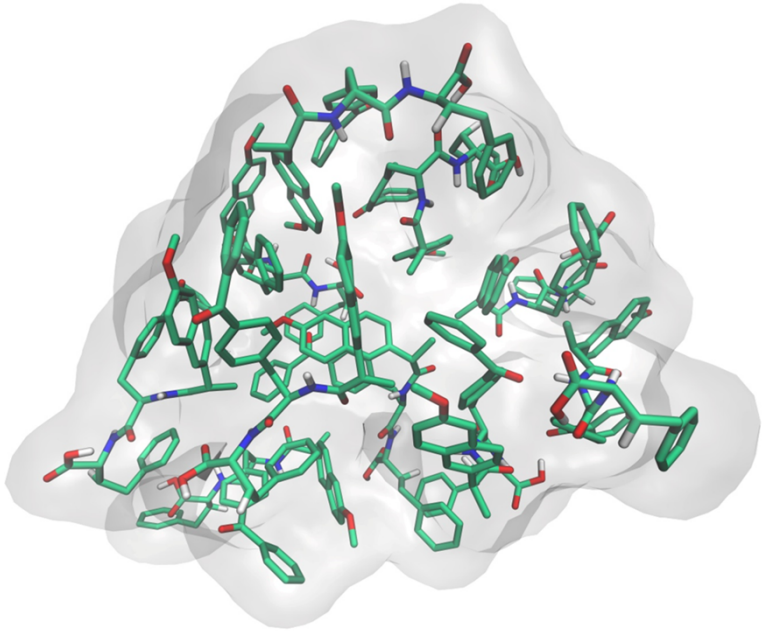


Figure S1. MD simulation snapshot from the self-assembly of dipeptide **1** after equilibration. For simplicity, water molecules, non-polar hydrogens, and periodic boundary conditions are not represented.

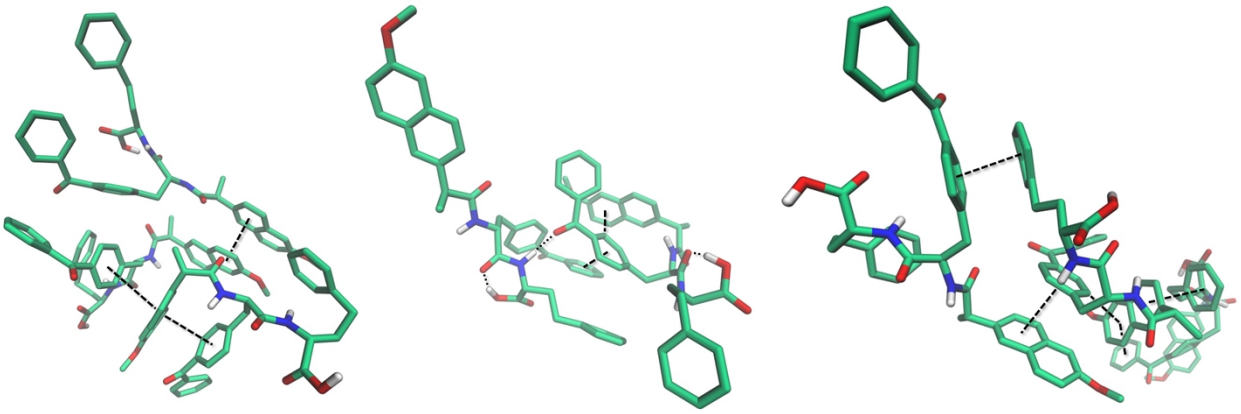


Figure S2. Molecular interactions at the simulation endpoint (500 ns) for the dipeptide **1**. Dashed lines represent the π-interactions (sandwich and T-shape) and dotted lines the hydrogen bonds.

Fitting the Drug Release Data to the Korsmeyer-Peppa Model of Release Kinetics

To describe the release of the cargo from the hydrogel network, the Korsmeyer-Peppa's model was used. This mathematical model includes both diffusion and erosion of polymer (**Figure S3**).

The following equation describes the Korsmeyer-Peppa's model:

$$\frac{M_t}{M} = kt^n$$

M_t: amount of cargo released at time t;

M: Total amount of cargo used for the release study;

k: release rate constant incorporating structural and geometric characteristics of drug dosage form;

n: release exponent.

In this model, the n value is associated with the diffusion mechanism of the drug as described in **Table S1**.[1,2]

Table S1: Interpretation of diffusional release mechanisms.

Release exponent (n)	**Drug transport mechanism**
0.5	Fickian diffusion
0.45 < n = 0.89	Non – Fickian transport
0.89	Case II transport
Higher than 0.89	Super case II transport

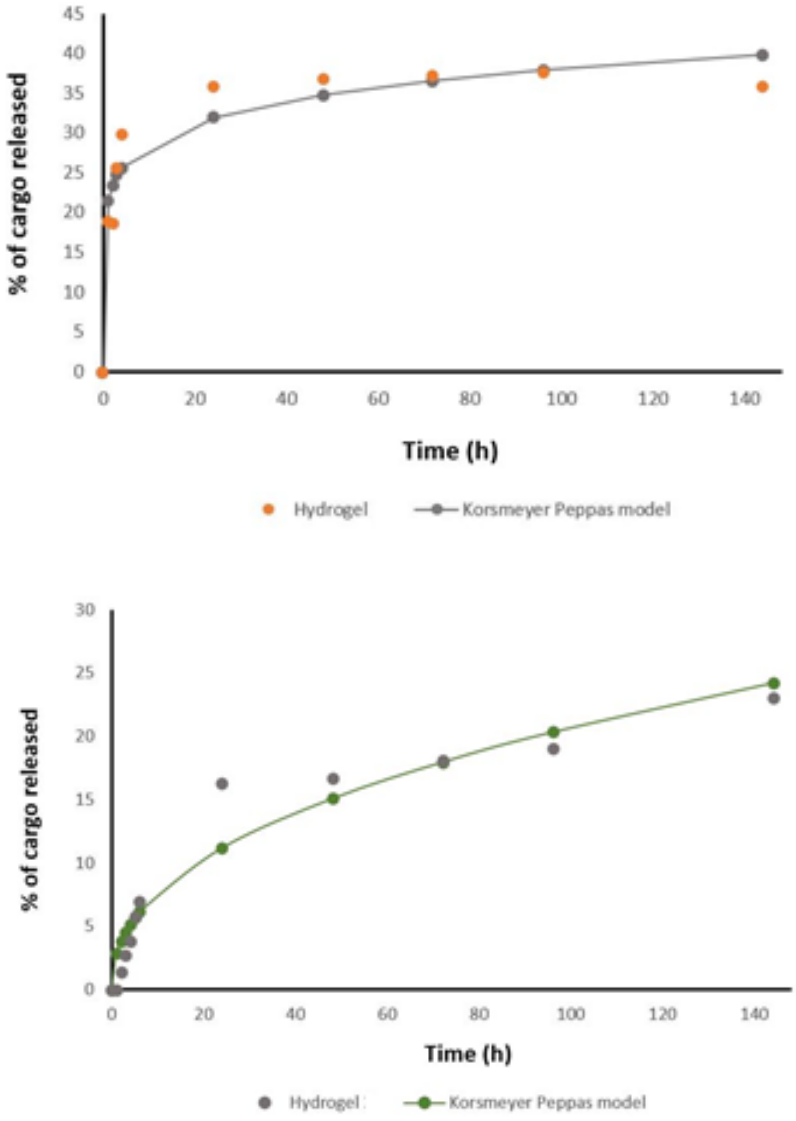


Figure S3: Data to Korsmeyer-Peppas Model to describe the release kinetics of methyl orange (top) and ciprofloxacin (bottom) from hydrogel **1**.

The determined parameters of this model (k and n) and the value of R^2 are presented in **Table S2**. The data show that release of ciprofloxacin from hydrogel **1** is faster (higher k value) than methyl orange and in both cases is associated with a diffusion-controlled release mechanism (n value).

Table S2: Release coefficients of the Korsmeyer-Peppas model obtained for methyl orange and ciprofloxacin release profiles in hydrogels from **1**.

Cargo	k	*n*	R^2
Methyl Orange	21.5687	0.1235	0.9090
Ciprofloxacin	29.9747	0.4309	0.9679

Rheology figures:

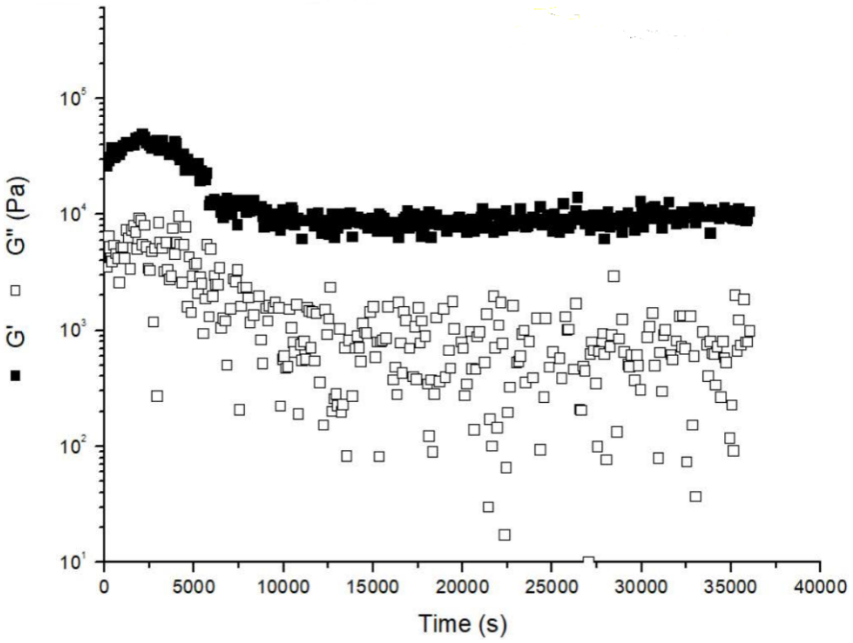


Figure S4: Elastic and viscous modulus during the kinetic process of gelation for compound **1**.

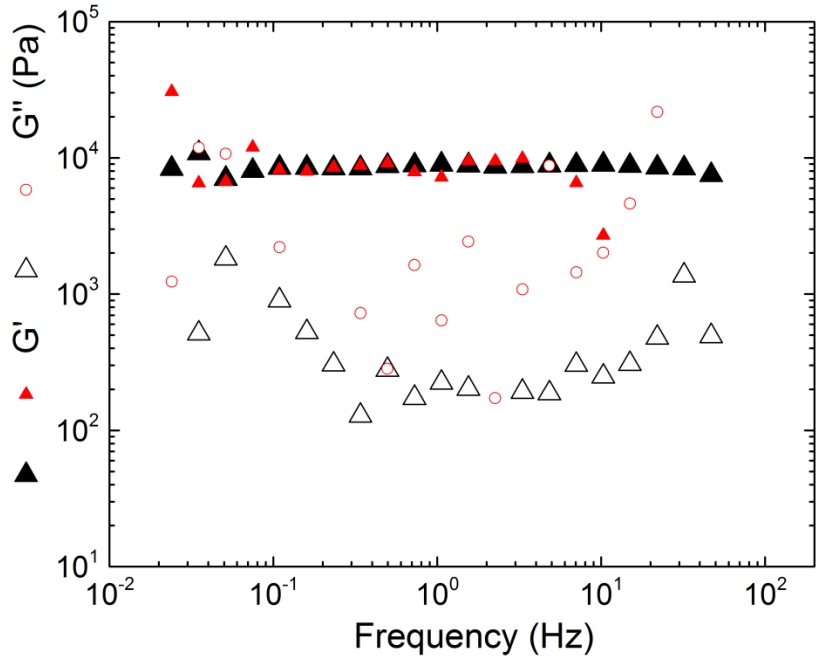


Figure S5: Mechanical spectra of a gel formed from compound **1** after sample loading in the Couette cell and completed gel kinetics (larger black triangles), and of a gel reformed after break-up achieved with a strain sweep (smaller red symbols). The G' and G'' data for the reformed gel are vertically shifted by a factor of 30 to overlay the G' and G'' data of the original gel (larger black symbols).

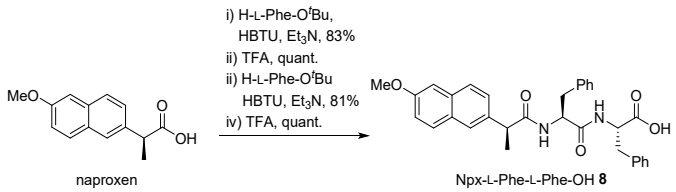


Figure S6: Synthesis of Npx-L-Phe-L-Phe-OH **8** (see Reference 3).

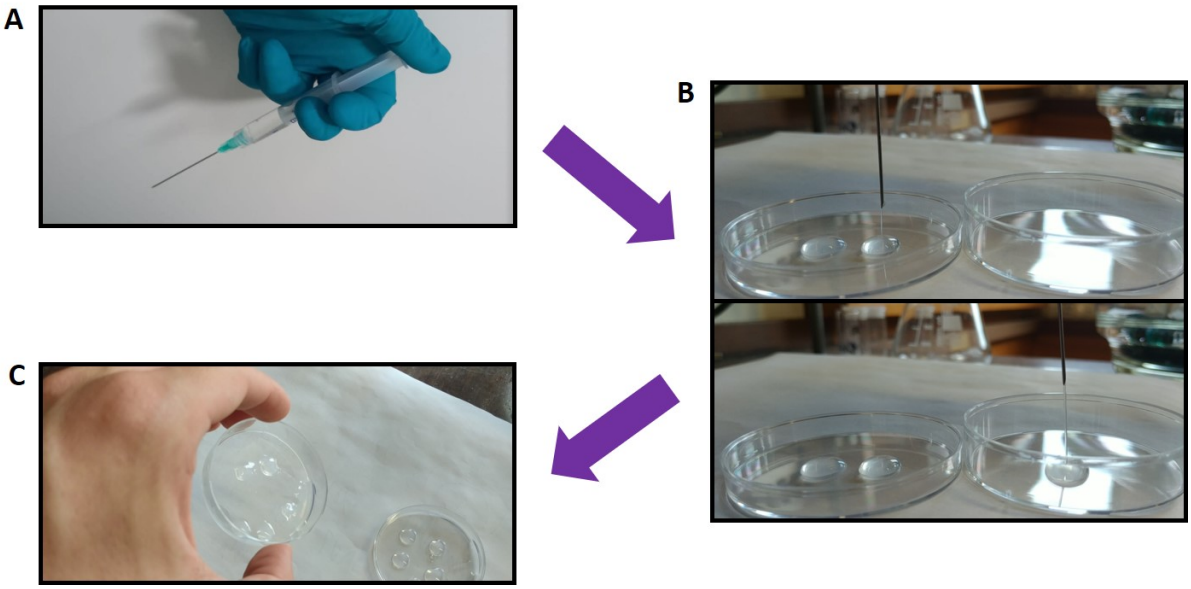


Figure S7: Gelation of compound after being expelled from a syringe: A) A hydrogel is formed inside the syringe, using the conditions described in Section 2.2 (pH change method). B) The pressure of expelling the gel through the needle causes a gel to liquid transition. C) Rapid (<1 min) reforming of the gel after leaving the syringe needle. This strain softening followed by gel structural recovery was predicted by the rheological mechanical spectra (Section 3.3).

References

1. B. L. Abraham, E. S. Toriki, N. J. Tucker, B. L. Nilsson, Electrostatic interactions regulate the release of small molecules from supramolecular hydrogels. *J. Mater. Chem. B.,* 2020, **8**, 6366–6377.
2. S. Dash, P. N. Murthy, L. Nath, P. Chowdhury, Kinetic modeling on drug release from controlled drug delivery systems. *Acta Pol. Pharm.*, 2010, **67**, 217–223.
3. H. Vilaça, G. Pereira, T. G. Castro, B. F. Hermenegildo, J. Shi, T. Q. Faria, N. Micaêlo, R. M. M. Brito, B. Xu, E. M. S. Castanheira, J. A. Martins and P. M. T. Ferreira, J. Mater. Chem. B, 2015, 3, 6355-6367.