ELECTRONIC SUPPLEMENTARY INFORMATION

Microscale coiling in bis-imidazolium supramolecular hydrogel fibres induced by release of a cationic serine protease inhibitor

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EXPERIMENTAL SECTION

Materials

All reagents were of analytical grade. 4-(2-aminoethyl)benzenesulfonyl fluoride hydrochloride (**AEBSF·HCl**) was purchased from Fisher Scientific. Phosphate buffered saline and Ethanol HPLC grade were purchased from Sigma-Aldrich. Water was obtained from a MilliQ equipment from Millipore®.

Synthesis

Compound 1,3-bis[(3-octadecyl-1-imidazolio)methyl]benzene dibromide (**1·2Br**) was prepared as reported previously.[1]

Methods

Gel preparation

Gels were always prepared by dissolving compound **1·2Br** in ethanol, adding distilled water as the anti-solvent, mixing gently and storing without disturbance in closed vials to prevent solvent evaporation.

Influence of drug concentration on gel formation

Solubility of **AEBSF·HCl** was previously assessed in water in order to determine the drug concentrations in gel to be assayed. For the gels with **1·2Br** and **AEBSF·HCl** 10 mg of the gelator was dissolved in 1 mL of ethanol. 1 mL of an aqueous solution containing different amounts of drug (1, 2.5, 3.5, 5, 10, 20 and 40 mg) was added and the solution was gently stirred and left to stand at room temperature as described above.

Optimum conditions for gel fabrication

Optimum conditions were chosen for the preparation of gels, which were used in the rest of the experiments either with or without drug, unless stated otherwise. For instance, the final volume was 2 mL, with a final concentration of 1·2Br of 5 mg/mL as the gelator molecule, using a proportion of 50% ethanol and 50% water, and both mixing and storing at room temperature. Concentration of **AEBSF·HCl** was 5 mg/mL unless differently stated for being the highest that presents no problems for gelling, except for SEM images in which three different concentrations were used, and drug incorporation studies (NMR), in which the molar ratio gelator:drug was 1:1. Gelator **1·2Br** was dissolved in ethanol and was mixed with the drug solution in water. Samples were mixed gently, closed for preventing solvent evaporation, and left to stand without disturbance.

Gel characterization

Rheology Experiments

Rheological studies of gels **1·2Br** and **1·AEBSF** were performed in order to know their viscoelastic behaviour. Amplitude sweep tests show their resistance to rupture by the critical stress value.

For rheological studies, gels were formed in 7 cm diameter glass Petri dishes, forming a total volume of 27 mL. Prepared gels were always kept at room temperature overnight before study.

The rheological characterization was performed using a Haake Rheostress1 rheometer (Thermo Fisher Scientific, Karlsruhe, Germany) connected to a temperature control Thermo Haake Phoenix II + Haake C25P and equipped with parallel plate geometry (Haake PP60 Ti, 60 mm diameter, 3 mm gap between plates).

Oscillation amplitude tests: The amplitude in shear stress τ was increased for 0.01 to 100 Pa with constant frequency of 1 Hz for evaluating the gel strength. Oscillation frequency tests were carried out from 0.01 to 10 Hz at a constant shear stress within the linear viscoelastic region, in order to determine the related variation of storage modulus (G')

and loss modulus (G") at 32ºC. Both viscoelastic moduli are defined as follows: $G'=\tau_0/\gamma_0 \cos\delta$ and $G''= \tau_0/\gamma_0 \sin\delta$ (where τ_0 and γ_0 are the amplitudes of stress and strain, and δ is the phase shift between them).

The software Haake RheoWin®Job Manager V.3.3 and RheoWin®Data Manager V.3.3 (Thermo Electron Corporation, Karlsruhe, Germany) were used to carry out the test and analysis of the obtained data, respectively.

Drug incorporation into gel fibres

Incorporation of drug into the gel **1·2Br** was quantified by ^{1}H NMR spectroscopy using a Varian 400 MHz NMR spectrometer from the *Centres Científics I Tecnològics de la Universitat* de Barcelona (CCiT-UB). 32 scans were recorded in every measurement.

For quantifying the incorporation of drug inside the gel fibres, gels from **1·2Br** incorporating drug at a molar ration of 1:1 were formed inside the NMR tube, and the drug signals in spectra were compared to those from a drug solution at the same concentration. Peaks of the aromatic moeity from the drug were taken as the reference signal. For instance, two aliquots containing (8.28 μmol) of **AEBSF·HCl** each were dissolved in 0.75 mL of deuterium oxide and ^{1}H NMR spectra of both (Tubes A and B) were recorded (Record 1). After that, 0.75 mL of deuterated methanol was added to tube A and 7.5 mg (8.28 μmol) of **1·2Br** dissolved in 0.75 mL of deuterated methanol was added to tube B. Both tubes were shaken to promote mixing and the gel formation in tube B was observed, while tube A remained in solution. ^{1}H NMR spectra of both tubes were recorded in the same conditions (Record 2).

Microscopy: SEM/EDX

In all cases, xerogel samples were prepared by completely evaporating the solvent from freshly and two-week old gels.

Scanning Electron Microscopy (SEM) images and EDX analyses were acquired by the Electron Microscopy Service in the Institut de Ciència de Materials de Barcelona – Consejo Superior de Investigaciones Científicas (ICMAB-CSIC) on a QUANTA FEI 200 FEG-ESEM system on samples deposited on carbon tape, dried with N2 .and coated with a layer of gold.

Calorimetric studies

We have used this technique to evaluate the influence that the incorporation of **AEBSF·HCl** in gel **1·AEBSF** has on the temperature and time of gelation, as well as on the changes in thermodynamic parameters such as enthalpy and entropy, as compared to gel **1·2Br**. The strategy consists on introducing the freshly prepared mixture of the gelator, the solvents, and **AEBSF·HCl** if it is the case, into the equipment above the gel's melting temperature. After that, temperature is slowly decreased in order to form the gel inside the equipment, while continuously tracking heat changes. Note that thermograms are plotted in an increasing temperature scale, but experiments are performed by decreasing the temperature, for which plots should be read from right to left.

A Microcal VP-DSC from Mettler-Toledo was used for performing the gelation of compound **1·2Br**. 0.5 mL of a mixture of compound **1·2Br** with both ethanol and water was introduced in the equipment at 35 °C. The sample was then cooled down slowly at 1 °C/min, from 35 °C to 5 °C, in order to form a gel inside the equipment, while monitoring the specific heat capacity (C_p) during the cooling of the sample.

Release studies

Drug release experiments were performed to prove that gel **1·AEBSF** can release **AEBSF·HCl** from the nanocomposite material, and to demonstrate that such profile will not limit the permeation of the drug when applied

on human skin. Conditions such as the Franz cells used and the temperature bath at 32 ºC were adjusted to be similar than those in the skin permeation experiments. PBS was chosen as the receptor medium for complying SINK conditions.

Drug release studies from the gels were performed in a Microette transdermal diffusion system (Microette plus-Hanson Research) following previously reported methodologies.[2,3] Vertically assembled Franz-type diffusion cells (Crown Glass) (2.54 cm^2 diffusion area) were used. Dialysis membranes (Cellu·Sep T3 dialysis membrane, MWCO 12000 – 14000 Da, MFPI, USA), previously hydrated in ethanol:water 7:3, were placed in the Franz-type diffusion cells. Receptor chamber contained 10 mM PBS pH 7.4 for the study of gels with **AEBSF·HCl**, complying with SINK conditions.[4] The dialysis membrane and the donor container were put onto the glass receptor chamber and the assembly was fixed with a joint. The Franz-type cells were connected to a controlled temperature circulating bath set to 32°C. Gels of **1·2Br** were prepared at a drug concentration of 5 mg/mL. Known weights of gel were placed into the donor compartment onto the dialysis membranes and the donor compartment was sealed with plastic paraffin film to prevent solvent evaporation. Samples were taken at given time intervals, and every sample taken was replaced by equal volume of the receptor solution. Release experiments of gels were done in triplicate. Concentrations of samples were determined by HPLC and cumulative amounts of released drug as a function of time were plotted. Kinetic parameters were calculated from the Mean values of three replicas, performing a nonlinear least-squares regression using GraphPad Prism® (version 3.00, GraphPad software, Inc., USA). Different models were tested: Higuchi's square root of time, Korsmeyer-Peppas, One Phase Exponential Assosiation (first-order), Weibull's equation and Zero-order. The best model was chosen accordign to de R^2 value.

Skin permeation studies

The permeation assay was done with human skin from the abdominal region obtained during plastic surgery of a healthy, 40 year-old woman who gave written, informed consent to the use of this material for research purposes. The protocol was similar to that followed in the drug release study, replacing the dialysis membranes with skin previously dermatomed at 0.4 mm thickness, and placed with the stratum corneum facing the donor compartment, according to the guidelines.[5,6] Gel was applied on the donor compartment (408.3±52.7 mg of gel, 2.3±0.3 mg of drug) in contact with the epidermal side of the skin (n=5). The samples were taken at given time intervals for 24 hours. Concentrations were determined using HPLC and cumulative amounts of drug permeated were plotted. Kinetic parameters were calculated from the Median and range values performing a linear least-squares regression in the linear zone of the plot,[7] using GraphPad Prism® (version 3.00, GraphPad software, Inc., USA).

Drug retained inside the skin

Drug extraction from the skin: At the end of the permeation study, extracted drug from the skin was evaluated following a protocol described elsewhere.[2] The skin was removed from the Franz cell, cleaned with gauze soaked in a 0.05% solution of sodium dodecyl sulfate and washed in distilled water accurately. The permeation area of the skin was then excised, punctured with a needle, weighed, and drug contained therein was extracted with 1 mL of the corresponding receptor medium during 20 min of sonication. The resulting solutions were measured by HPLC, yielding the amount of drug extracted from the skin expressed in (μg g^{-1} cm^{-2}). Non-parametric Mann Whitney test statistical analyses were performed to compare drug retention from different formulations. [7]

Drug recovery experiments: A piece of skin from the same patient as in permeation experiments was immersed in 1 mL of a 21 μg/mL AEBSF·HCl solution, using as solvent the receptor medium used in release and skin permeation

studies, and kept at 32°C for 27 hours. The skin piece was cleaned with gauze soaked in a 0.05% solution of sodium dodecyl sulfate and washed in distilled water accurately. Drug concentrations of both solutions "before immersion" and "after immersion" were determined using HPLC in order to know the amount of drug that can be retained within the skin. Skin pieces were punctured with a needle, and drug was extracted with 1 mL of the corresponding receptor solution using sonication, as performed in drug retention experiments. Concentrations of the drug extractions were determined using HPLC. The percentage of drug that can be recovered after being retained within skin was determined as follows:

Drug recovery (%) = drug extracted (μg) / drug retained (μg).

Drug retention inside the skin: The percentage of drug recovery was used for estimating the real amount of drug retained within skin during the skin permeation experiments.

HPLC determination

Concentrations of **AEBSF·HCl** were obtained by HPLC in a Waters 717 plus Autosampler, with a 600 Controller pump, equipped with a 2996 Photodiode Array Detector, using a 4 μm (3.9 mm x 150) Nova-Pack C18 column. The mobile phase consisted of acetonitrile:water (both with 0.07% of trifluoroacetic acid) 45:55, with a flow rate of 0.8 mL min^{-1}, setting a detection wavelength of 226 nm. Each sample had a run time of 4 min. The data were collected using Millennium32 version 4.0.0 software from Waters Corporation.

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[5] Scientific Committee on Consumer Safety, *Eur. Comm.*, 2010, SCCS/1358, 1–14.

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[7] A. C. Williams, P. A. Cornwell and B. W. Barry, *Int. J. Pharm.*, 1992, 86, 69–77.

SUPPORTING RESULTS

Influence of drug concentration on gelation

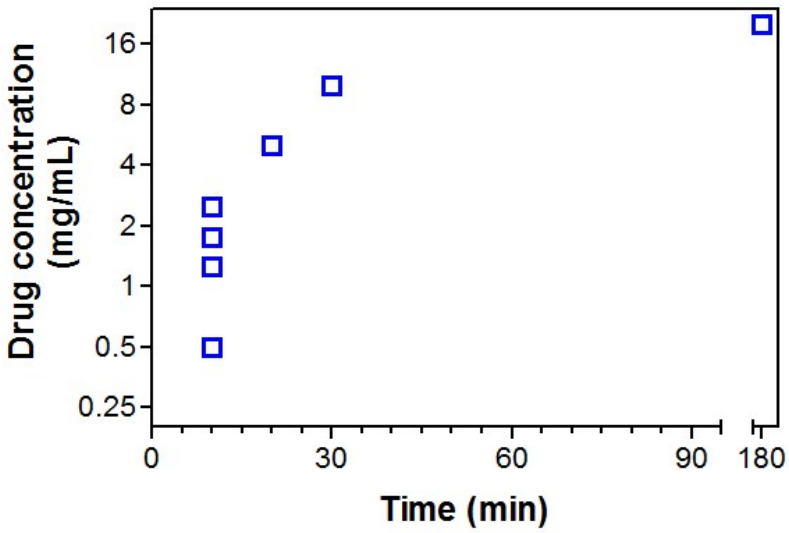

Figure S1. Gel formation time as a function of **AEBSF·HCl** concentration.

Rheology experiments

Table S1. Critical shear stress and G' values of gels **1·2Br** and **1·AEBSF**

Gel	G' at τ = 0.5 Pa (Pa) Frequency sweep tests[a]	Critical stress (Pa) [b]
1·2Br	1336	8.1
1·AEBSF	741	24.1

[a] Frequency sweep tests were performed at τ = 0.5 Pa for being within the viscoelastic region. [b] Shear stress experiments were performed at 1 Hz frequency.

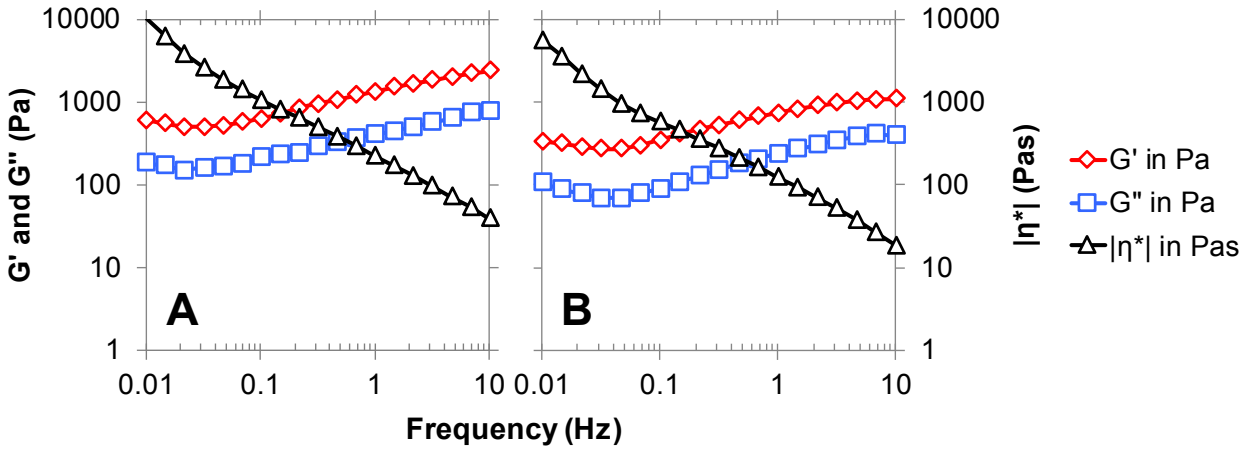

Figure S2. Rheograms of the oscillation sweep frequency test obtained for gels: A) **1·2Br**, B) **1·AEBSF**.

Drug incorporation studies

Identical amounts of drug (8.28 µmol) were dissolved in deuterium oxide (0.75 mL) and put in two NMR tubes (A and B), and a spectrum of both was recorded (Record 1). Later on, a solution of gelator compound **1·2Br** (8.28 µmol) in deuterated methanol (0.75 mL) was added to tube B while the same volume of solvent without compound was added to tube A. Tubes were shaken, resulting in gel formation in tube B but not in tube A. The final concentration of **1·2Br** in tube B was 5 mg/mL. A spectrum of both tubes was recorded again (Record 2) and the intensity of the drug signals between Records 1 and 2 was compared. In both tubes, a decrease in the intensity of drug signals is expected in Record 2 when compared to Record 1 mainly due to the dilution generated by the addition of solvent. However, in tube B the decrease is higher because of some drug incorporation inside the fibres.

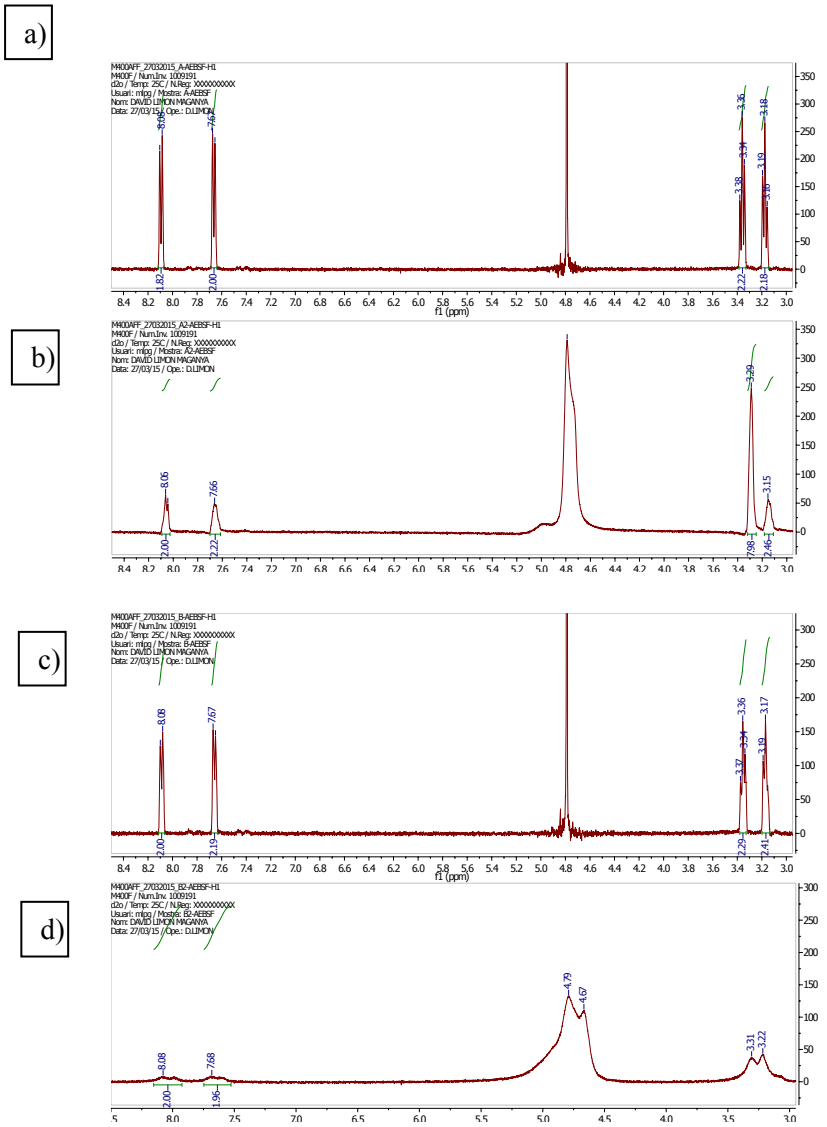

Figure S3. Incorporation of **AEBSF·HCl** in gel **1·2Br**.

a) Tube A, Record 1: **AEBSF·HCl** (8.28 µmol) in 0.75 mL of deuterium oxide.

b) Tube A, Record 2: after addition of 0.75 mL of deuterated methanol.

c) Tube B, Record 1: **AEBSF·HCl** (8.28 µmol) in 0.75mL of deuterium oxide.

d) Tube B, Record 2: after addition of **1·2Br** (8.28 µmol) in 0.75 mL of deuterated methanol.

Microscopy (SEM)

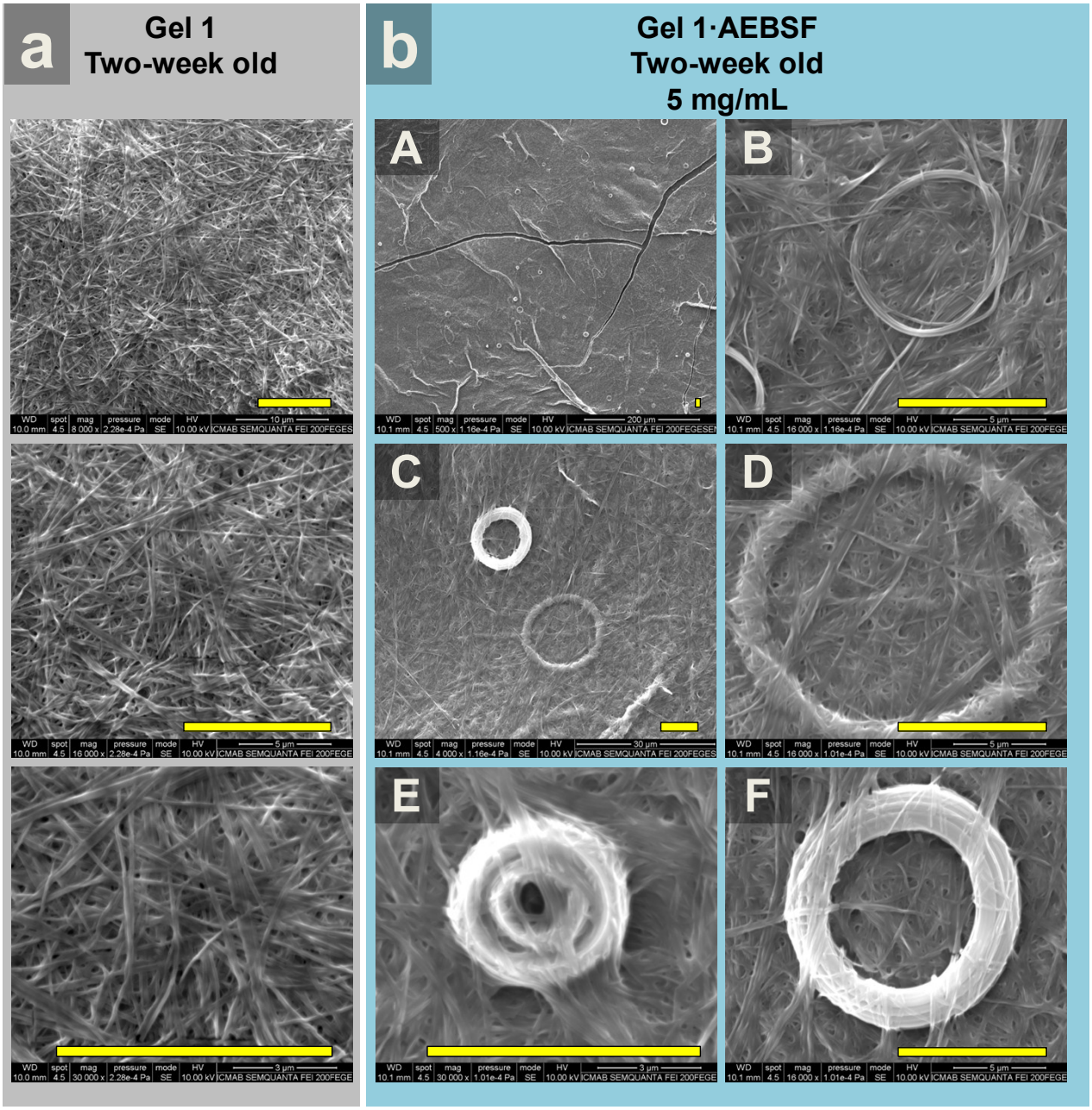

Figure S4. SEM micrographs of two-week old gels. a) Gel **1·2Br**. b) Gel **1·AEBSF** at a drug concentration of 5 mg/mL. Yellow scale bar represents 8 μm in all images.

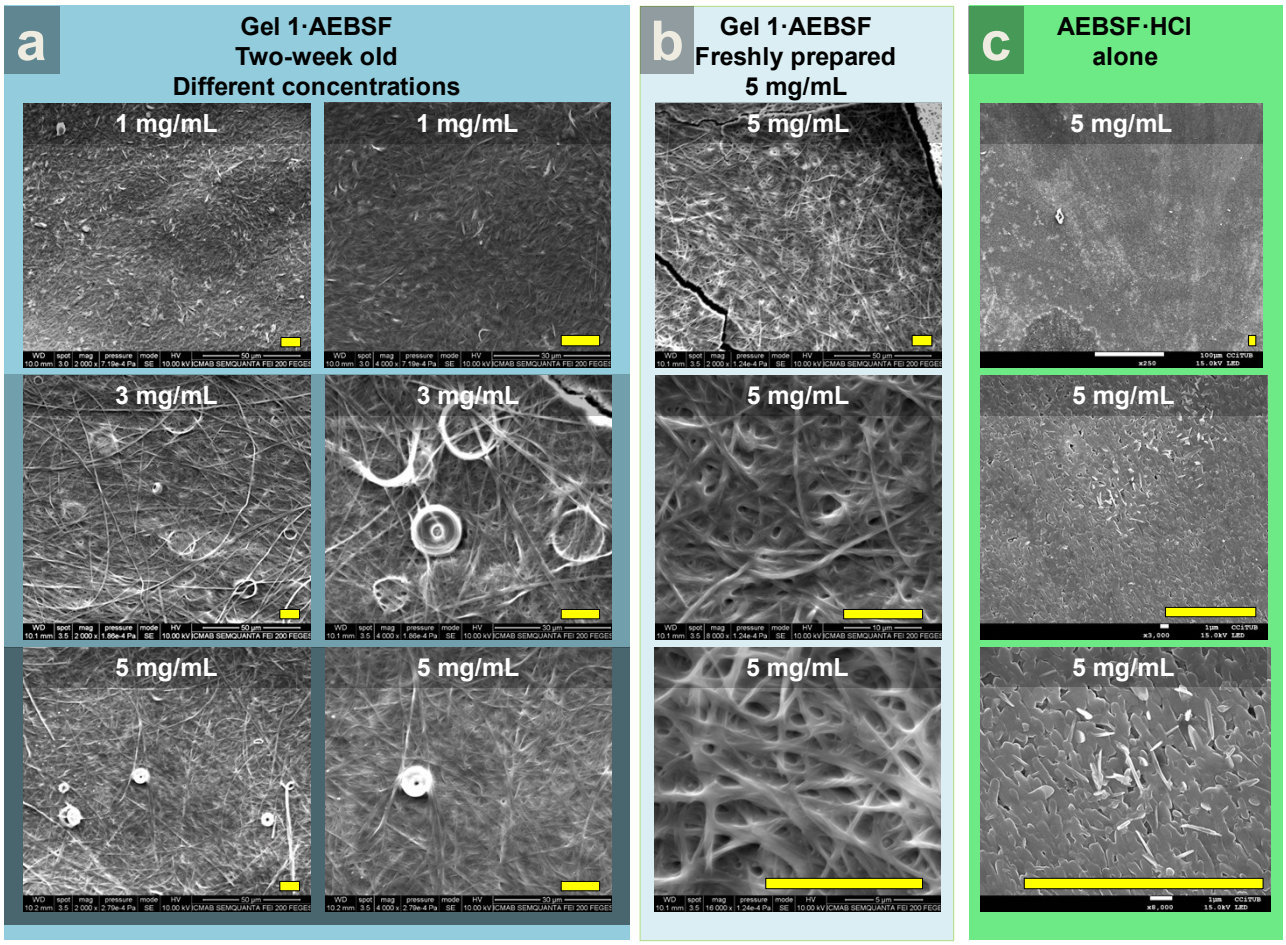

Figure S5. SEM images showing the influence of drug concentration and age of the gel on the structure of gel **1·AEBSF** fibres. a) Influence of the drug concentration in a two-week old gel. The increase in drug concentration induces the formation of coils. No drug precipitates are observed. b) Influence of the age in a 5 mg/mL drug concentration gel. In a freshly prepared gel, coils are still not formed, but fibres are twisted. No drug precipitates are observed. c) Precipitates of drug **AEBSF·HCl** alone, previously in a 5 mg/mL solution. Yellow scale bar represents 10 μm in all images.

Microscopy (EDX)

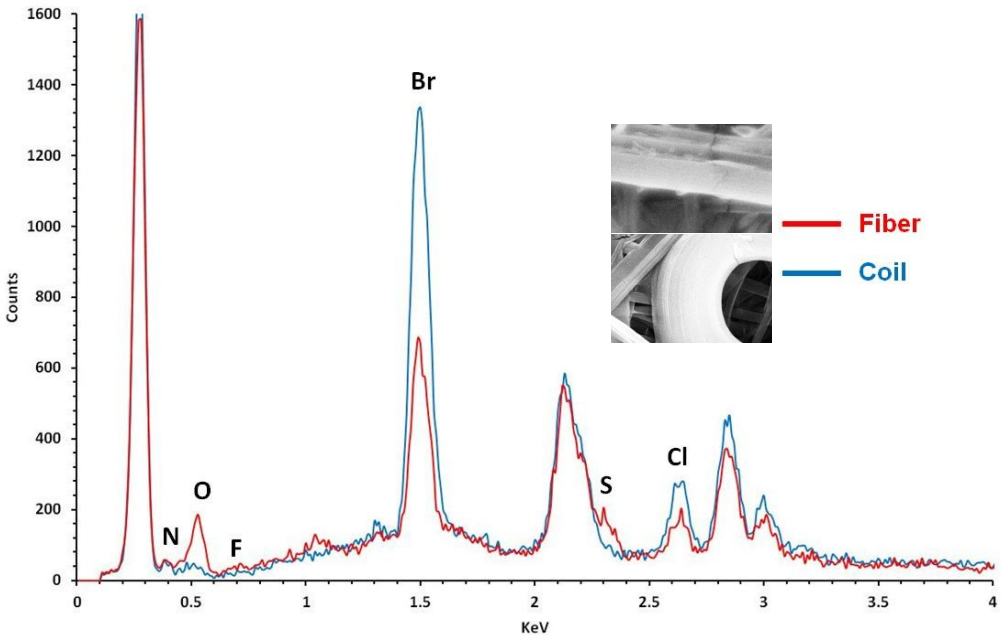

Figure S6. EDX spectra from a straight fibre and a coiled fibre of 3 mg/mL **1·AEBSF** gel. No sulphur or oxygen are observed in the coiled fibre, suggesting a loss of drug.

Calorimetric studies

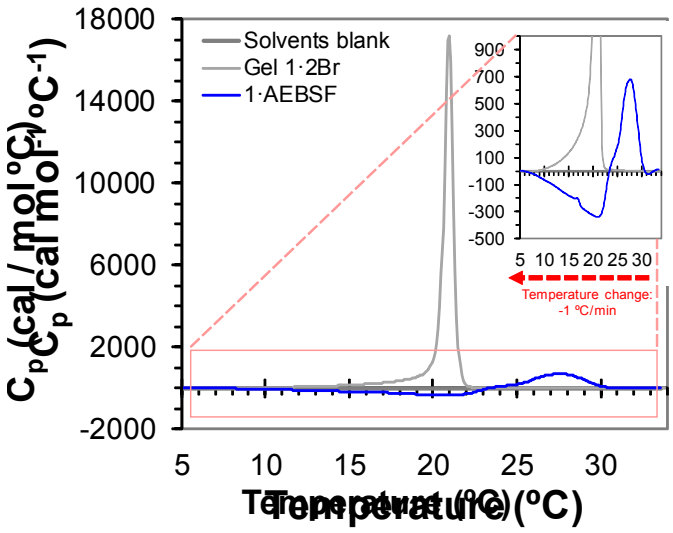

Figure S7. DSC thermogram plotting the heat capacity (C_p) of gel formation, which represents the speed of gelation, both from gel **1·2Br** and gel **1·AEBSF**. A magnification is shown in the inset. Temperature was decreased at a speed of 1 °C min^{-1}.

Table S2. Influence of **AEBSF·HCl** addition on gel formation time and temperature.

Gel	T_{onset}[a] (ºC)	T_{max}[b] (ºC)	T_{offset}[c] (ºC)	t_{gel}[d] (min)
1·2Br (without drug)	21.7	20.8	19.4	3.3
1·AEBSF (exo)	30.5	27.8	23.2	7.3
1·AEBSF (endo)[e]	23.2	21.2	5.9	17.3

[a]Temperature at which the gelling starts. [b]Temperature at which the gelling occurs at the highest speed. [c]Temperature at which gelling speed stops changing significantly. [d]Time required for gel formation. [e]Gel **1·AEBSF** showed both and exothermic and an endothermic peak.

The thermoreversibility of gel **1·AEBSF** is proven by performing various heating-cooling cycles, showing a decrease in C_p values, and suggesting that the heating of the gel to 35 ºC macroscopically melts the gel, but leaves some gel nucleation points unmelted, which facilitates the subsequent gelation.

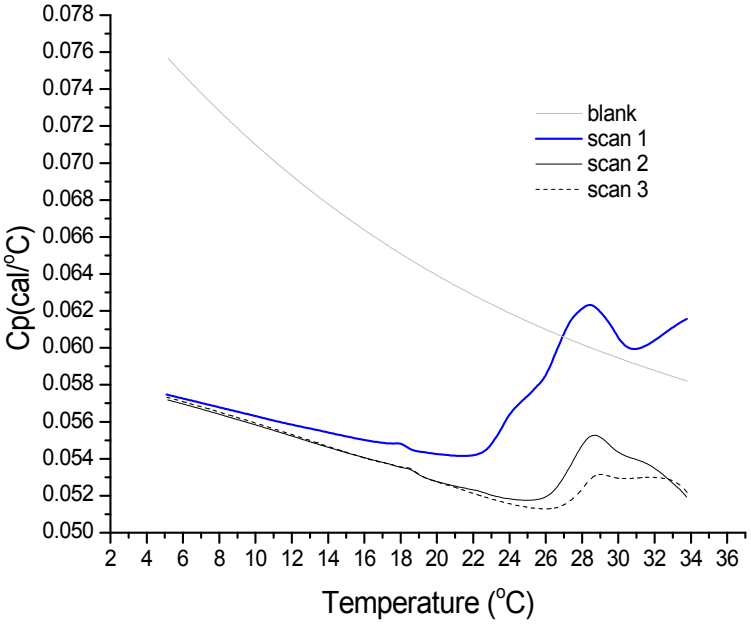

Figure S8. Thermograms of gel **1·AEBSF** formation. Different cycles were performed to prove thermoreversibility.

Drug release

In Figure 4 in the main article. Drug release is shown to follow a *One phase exponential association model* [$Y = Y_{max} (1 - e^{-K X})$], where Y represents the cumulative percentage of drug released at a certain time, Y_{max} is the maximum amount of drug that can be released, K is the rate of release (h^{-1}) and X is time (h). Degradation follows a *One phase exponential decay* model, described by the equation: [$Y = Span \cdot e^{-KX} + Plateau$], where Y is the amount of drug present in the receptor chamber at a certain time (%), (Span+Plateau) is the theoretical amount of drug at time zero (%), K is the speed of degradation (h^{-1}), and Plateau is the amount of drug remaining at an infinite time (%).

Table S3. Drug release and degradation parameters of **AEBSF** when released from gel **1·2Br**. Values represent the Means ± one standard deviation (n=3).

	Drug release	Drug degradation
Y_{max} (%)[a]	91.92 ± 3.23	
K (h^{-1})[b]	0.146 ± 0.013	0.013 ± 0.002
Span (%)[c]		91.43 ± 2.51
Plateau (%)[e]		7.28 ± 3.42
Half-life (h)[f]		55.57
R^2	0.9967	0.9970

[a]Maximum drug release (%). [b]Release/degradation speed rate (h^{-1}). [c]Theoretical amount of drug at time zero in degradation model (%) [e]Drug remaining at infinite time [f]Time for the degradation of 50% of drug remaining in receptor chamber.

Skin permeation

Table S4. Skin permeation parameters of **AEBSF** from gel **1·2Br**.

Gel	**1·AEBSF** (5 mg/mL)
A_{18} (μg/cm^2)[a]	40.64 (40.62 - 72.63)
A_{21} (μg/cm^2)[a]	45.88 (40.40 - 79.43)
A_{24} (μg/cm^2)[a]	72.17 (49.20 - 95.59)
A_{27} (μg/cm^2)[a]	80.72 (66.40 - 122.05)
J (μg/h·cm^2) [b]	5.27 (3.01 – 5.71)
T_{lag} (h)[c]	6.59 (6.44 – 11.47)
$K_p \cdot 10^3$ (cm/h)[d]	1.05 (0.60 – 1.14)
A_s (μg/g·cm^2)[e]	90.59 (40.31 - 107.17)
Percentage of recove	2.6%
A_s corr. (μg/g·cm^2)[g]	3484.20 (1550.38 – 4121.92)

[a]A18, A21, A24, A27 is the cumulative amount of drug permeated after 18, 21, 24 and 27 hours, respectively. [b]J represents the permeation flux of drug through the skin (μg/h·cm^2). [c]T_{lag} represents the time the drug takes to completely cross the skin to the receptor chamber. [d]K_p is the Permeability coefficient (cm/h) [e]A_s is the amount of drug extracted after the experiment per gram and square centimeter of skin (μg/g·cm^2). [f]Percentage of drug that can be extracted out of all the drug retained inside the skin. [g]Total estimated amount of drug retained inside the skin according to the percentage of recovery. Values represent the Median and range.

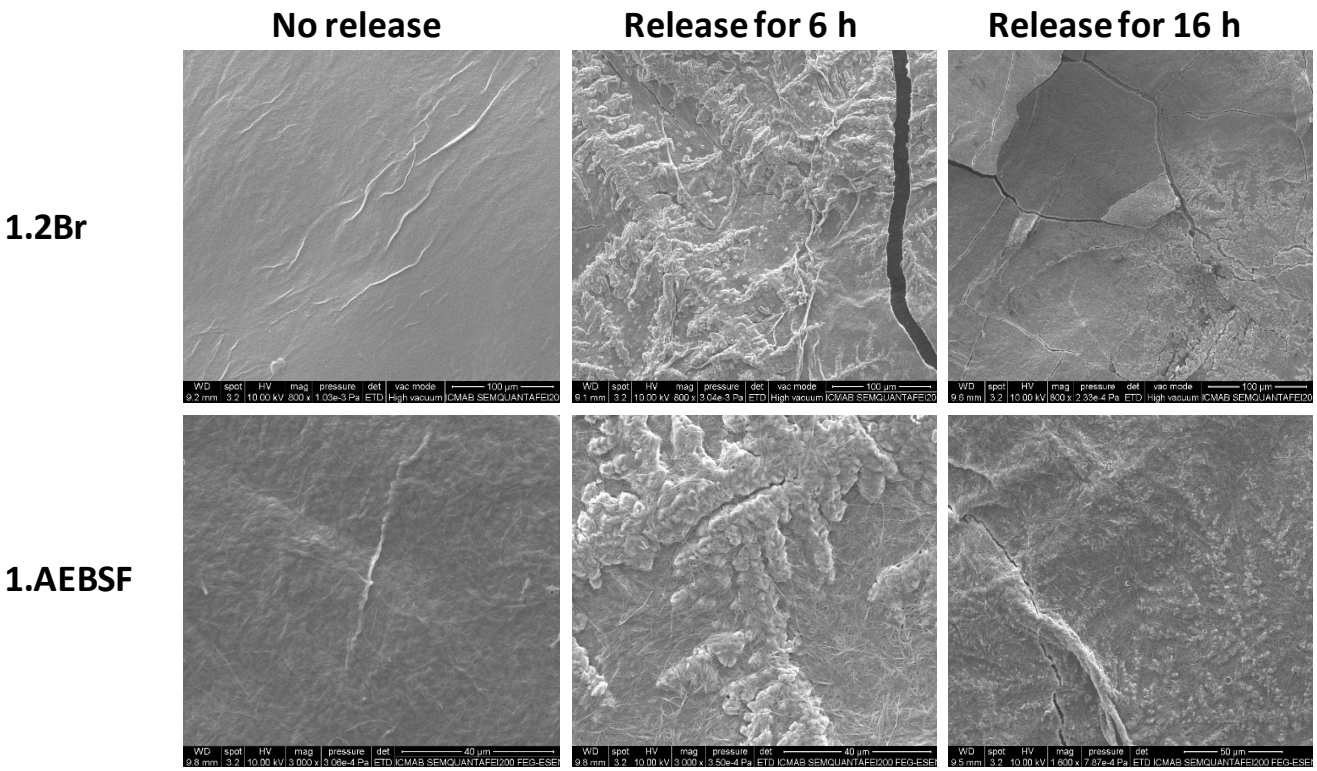

Figure S9. SEM images showing the influence of drug release on the morphology of the fibres. Image are shown for the fresh gels **1·2Br** and **1·AEBSF**, at 6 hours under release conditions and at 16 hours under release conditions. The lumpy material for the samples held under release conditions arises from the buffer used under those conditions.

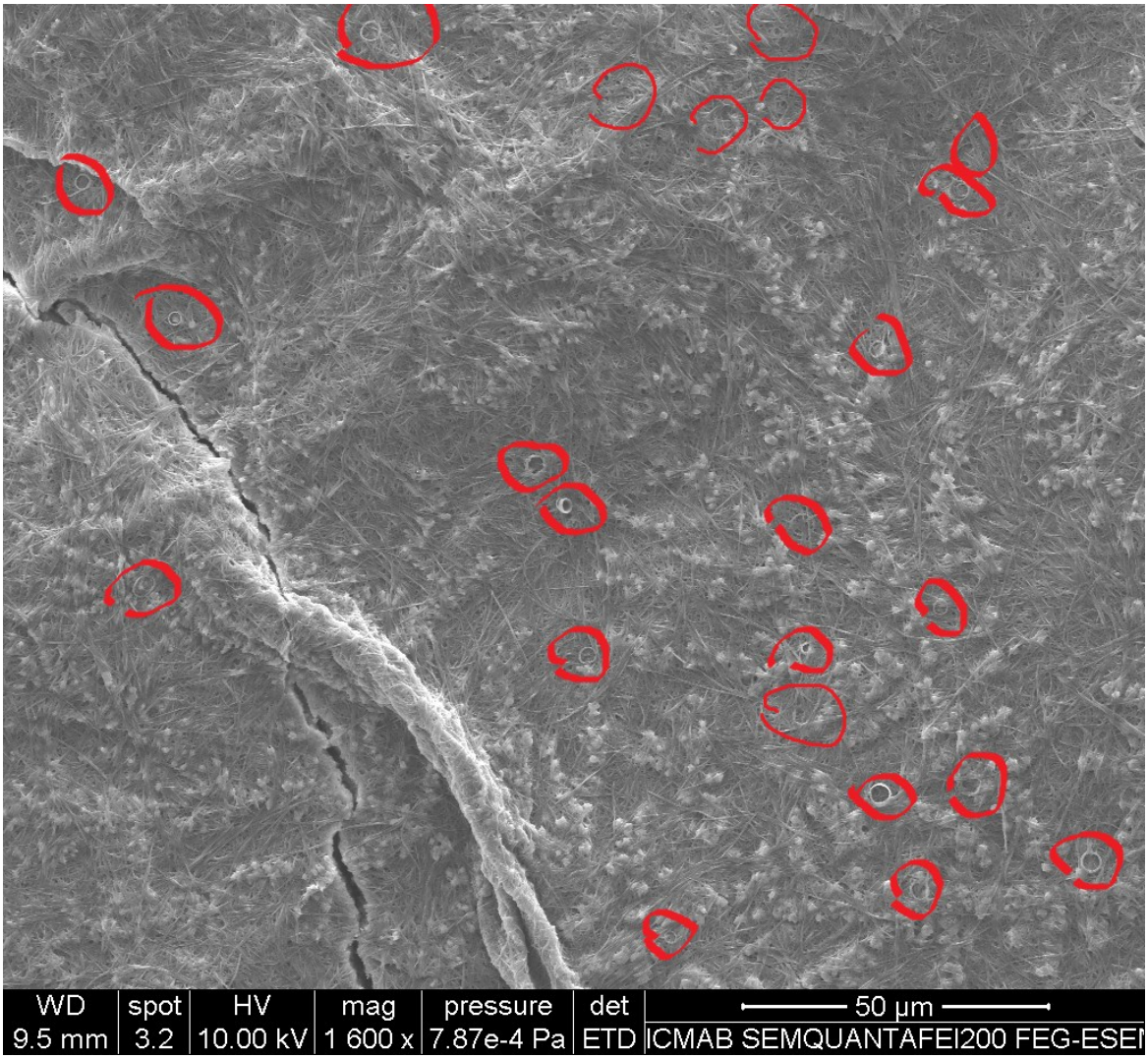

Figure S10. SEM image of **1·AEBSF** after 16 hours under release conditions with the coil structures circled.

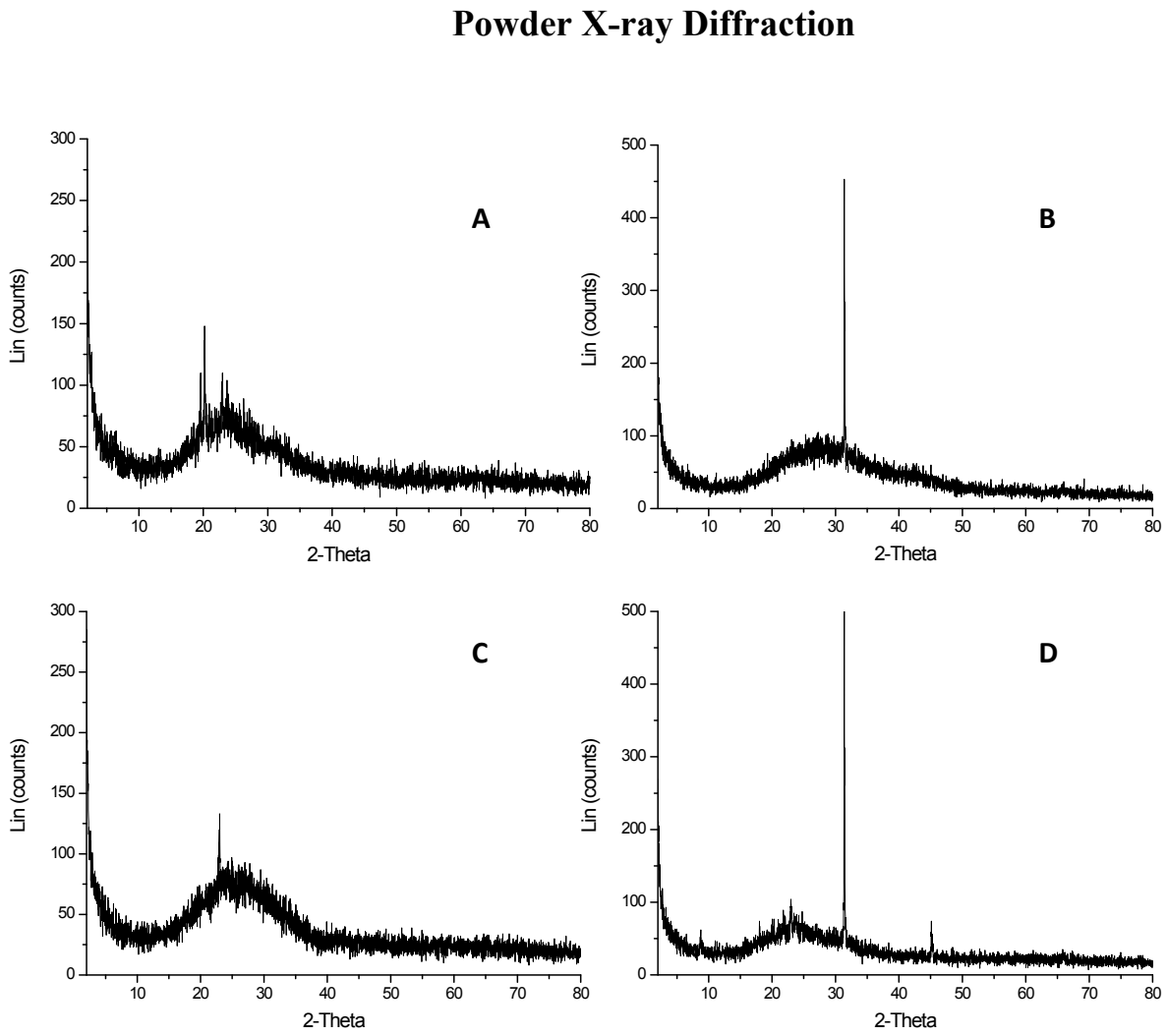

Figure S11. Powder X-ray diffractograms of a fresh gel with drug **1·AEBSF** (A) and after 16h of release (B); an aged gel (2 weeks) with drug (C) and after 16h of release

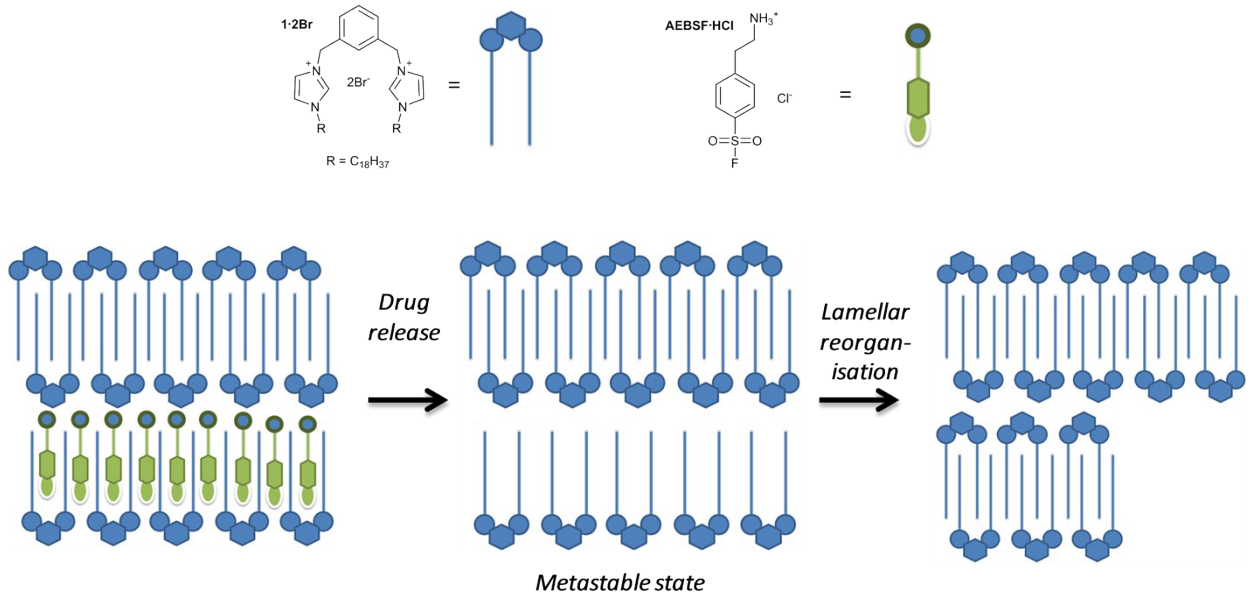

Figure S12. A possible explanation for the generation of stress - and resulting curvature - upon release of the drug from the composite gel. Counter-ions (the anions to both gelator and drug) are not shown in the cartoon but are believed to be located between the lamellae in contact with the cations.