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

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Gels formed by a gemini dicationic amphiphile incorporate a serine protease inhibitor, which could be used in a new approach to the treatment of Rosacea, within the fibres as well as in the space between them, affecting a number of gel properties but most importantly inducing remarkable fibre coiling at the microscopic level as a result of drug release from the gel. Drug release and skin permeation experiments show its potential for topical administration.

Low molecular weight gelators (LMWGs) self-assemble to form fibres through non-covalent forces.[1] These supramolecular gels are soft and sometimes thermoreversible, making them suitable for therapeutic applications.[2] Their three-dimensional morphology depends on the nature of the gelator, the self-assembly conditions, and non-covalent interactions established with host molecules incorporated into the gel matrix, like, ion-dipole interactions in metal and anion-binding gels.[3] Also, gel skeletal modification can be made introducing metal ions.[4] However, to the best of our knowledge, no examples are known of changes in the morphology of the gel fibres caused by the release of a previously incorporated host.

Our group has shown that gemini imidazolium salts can deliver anionic drugs,[5] including from hydrogels that are useful for topical applications.[6] The self-assembly of the cationic gelators and the interaction with anionic guests in the supramolecular gels is driven not only by ionic interactions but also hydrogen bonds and hydrophobic forces.[6] Here, we show that the incorporated drug can also be cationic, that this feature makes drug release more effective, leading to a change in the morphology of the cationic gels through coiling of the gel fibres.

We chose the drug 4-(2-aminoethyl)-benzenesulfonyl fluoride hydrochloride (**AEBSF·HCl**), as an irreversible serine protease inhibitor whose activity has shown to be successful, inhibiting Kallikrein-5 (K5),[7] a protein that is overexpressed in ailments such as Rosacea.[8] Its clinical use would imply a new therapeutic strategy that has not been reported, the main drawback being low drugability. A delivery material could overcome this obstacle and make a novel approach in the topical treatment of Rosacea. Furthermore, topical administration helps increase the drug concentration at the target site, lowering the side effects in other tissues.

For all these reasons, the ability of bis-imidazolium **1·2Br** to form gels in presence of **AEBSF·HCl** (Fig. 1) using water and ethanol as solvents was explored and the gelling conditions were optimized. The structure and behaviour of the gels were characterized, and drug release and skin permeation experiments were performed in order to assess their suitability as a possible new topical treatment for Rosacea.

The optimum gelling conditions of compound **1·2Br** are a final concentration of 5 mg/mL in 50:50 ethanol:water, and at room temperature, giving a fast gel formation (*ca.* 10 min.). The influence of **AEBSF·HCl** concentration was assessed using these optimized conditions. Gels **1·AEBSF** are also formed in 10 minutes in the presence of low concentrations of drug, but the gelling time increased significantly at higher concentrations (above 4 mg/mL, See ESI Fig. S1). A final concentration of 5 mg/mL **AEBSF·HCl** was chosen as optimum for being the highest one that permits gelation in 20 minutes or less. This proportion is an approximate 1:4 gelator:drug molar ratio, a much higher loading than that possible using the same gelator and anionic drugs.[6]

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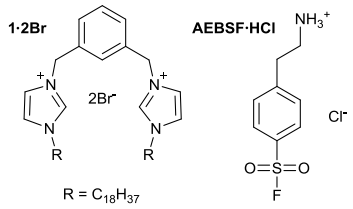

Fig. 1 Chemical structures of the gelator **1·2Br** and the serine protease inhibitor **AEBSF·HCl**.

Rheological studies of gels **1·2Br** and **1·AEBSF** show their resistance to rupture by the critical stress value: the addition of **AEBSF·HCl** makes the gel three times more elastic as compared to the gel alone (ESI Table S1 and Fig. S2), as opposed to the observations with other drugs.[6b] However, when the critical stress is reached, gels show an abrupt rupture rather than a slow one, making it suitable for a topical pharmaceutical form. Frequency sweep tests showed the gel resistance to deformation at different frequencies, shown by the Storage (G') and Loss (G'') moduli, at a constant shear stress of $\tau = 0.5$ Pa for being within the viscoelastic region. In both the gel **1·AEBSF** and the gel **1·2Br**, independently of the frequency applied, an elastic plateau was observed, where the Storage modulus is higher than the Loss modulus (G' > G''), meaning that gels present a predominant elastic, solid-like behaviour, for which they can be classified as "solid-like" gels.[1a,9] The addition of **AEBSF·HCl** decreases the gel resistance to deformation, making it softer than gel **1·2Br** which is also useful for topical application (ESI Fig. S2).

^{1}H NMR spectroscopy experiments show (ESI Fig. S3) that at a 1:1 molar ratio of **1·2Br** and **AEBSF·HCl** (5:1 mg/mL) the totality of **1·2Br** assembles forming the gel **1·AEBSF**, leaving no remaining compound in solution, as no peaks from compound **1·2Br** can be observed. *Ca*. 76% of the **AEBSF·HCl** present in the mixture is incorporated in the gel fibres, the remainder left in the interstitial space. The versatility of the gelator **1·2Br** to incorporate both anionic and cationic drugs confirms its promise for drug delivery.

Xerogel **1·2Br** has fibres longer than 20 µm and around 100 nm width, that stick together forming ribbons and do not show signs of ageing (Fig. 2a and Fig. S4a in ESI). Contrastingly, the morphology of the gels **1·AEBSF** changes with time, a phenomenon which is also dependent on the concentration of **AEBSF·HCl** used (ranging from 1-5 mg/mL). **AEBSF** precipitates might be expected after complete evaporation of the solvent in the gel, because even at 1 mg/mL 24% of the material is in the interstitial space (as shown by NMR); pure **AEBSF·HCl** precipitates in rod-shaped crystals (Fig. 2b). In all gels with **1·AEBSF**, no clear drug precipitates were found on freshly prepared gels, when gelation takes place in the presence of either 1, 3 or 5 mg/mL of **AEBSF·HCl**, as shown by SEM images (Fig. 2c and ESI Fig. S5). It is interesting that fibres in **1·AEBSF** are densely twisted much more than pure gel **1·2Br**, which could be the reason of their subsequent coiling in order to reduce the tension created.

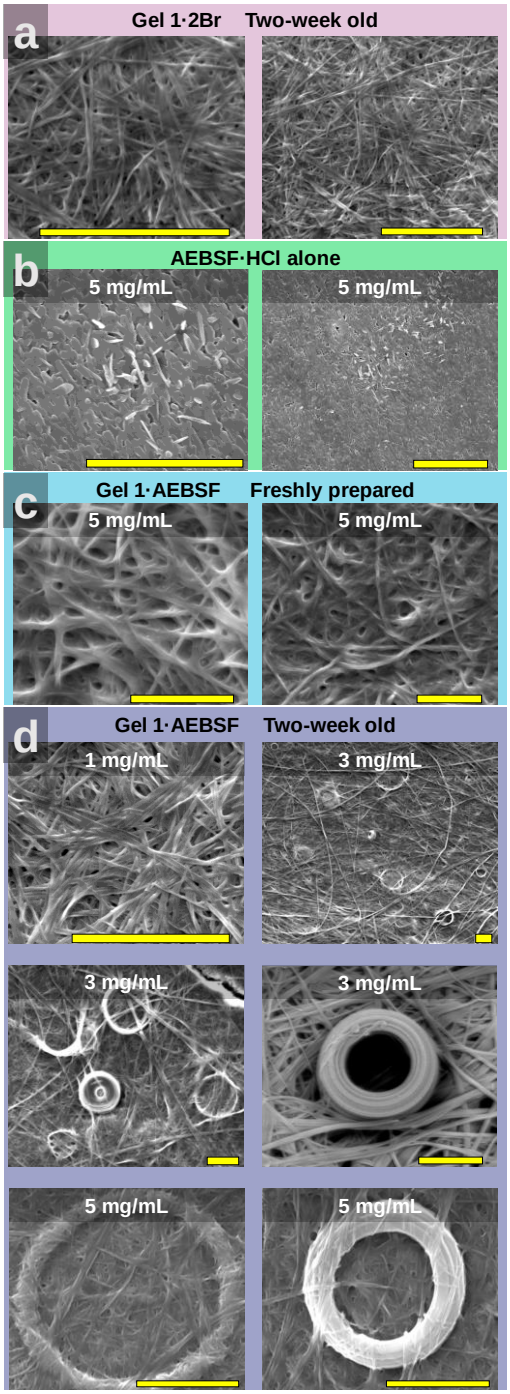

Fig. 2 SEM images showing the influence of drug concentration and age of the gel on the morphology of gel fibres. a) Gel **1·2Br**. b) Precipitates of **AEBSF·HCl**, from a 5 mg/mL solution. c) Freshly prepared **1·AEBSF** gel. d) Influence of the drug concentration in a two-week old gel. Yellow scale bar represents 8 µm in all images.

When gels **1·AEBSF** are left for two weeks in a sealed vial the morphology of the xerogels exhibits changes depending of the amount of **AEBSF·HCl** present in the gelation process. Thus, when 1mg/mL of **AEBSF·HCl** was used, the fibres in **1·AEBSF** retain the same morphology as when freshly prepared, as seen in Fig. 2d (see also ESI Fig. S5a). In contrast, in the two-week old gels formed at a concentration above 3 mg/mL of **AEBSF·HCl**, the bending of the fibres in a circular way, resembling "coiled

ropes" can be observed (Fig. 2d, ESI Fig. S4b, Fig. S5a). These rolls range from 5 to 15 µm in diameter, and the thickness of the ring varies widely due to the number of times the "rope" is coiled. As can be seen, the concentration of **AEBSF·HCl** influences the structure of fibres in two-week old gels **1·AEBSF**. Coils were formed both at 3 and 5 mg/mL gels, the coils being thinner at 3 mg/mL, as fibres are coiled less times. Also, at this concentration some long straight fibres are still starting to bend, suggesting the subsequent coil formation.

Just a few examples of differences in morphology of nanostructured materials have been reported before,[10] but mainly as the consequence of induced self-assembly after evaporation, and very rarely as a result of doping gels with metal ions.[4] The coiling observed on the fibres of **1·AEBSF** appears as an unprecedented example of ordering rearrangement induced by intermolecular interactions. Thus, the cationic drug **AEBSF·HCl** seems to be kinetically entrapped within the gel nanostructure, due to the fast self-assembly in the gelation process, generating a metastable state where the drug is incorporated in the lamellar gel. However, its presence disturbs the interlayer packing of the gelator **1·2Br** that can experiment alterations upon changes in external experimental conditions.

The chemical composition of the fibres was measured by Energy Dispersive X-ray spectroscopy (EDX) on different areas of two-week old **1·AEBSF** xerogels assembled in the presence of 3 mg/mL (ESI Fig. S6) or 5 mg/mL (Fig. 3) of **AEBSF·HCl**. The spectra show the presence of sulphur in the straight fibres in gels for both concentrations, confirming the presence of the drug after two weeks. However, in the coiled fibres the absence or diminution of both the sulphur and fluorine peaks suggests that there is release of drug from the fibres over time, presumably into the interstitial liquid. The release could trigger the disruption in the interlayer packing within the fibres, prompting their coiling.

Differential Scanning Calorimetry (DSC) showed that the time needed for gel formation of **1·AEBSF** and the thermodyna

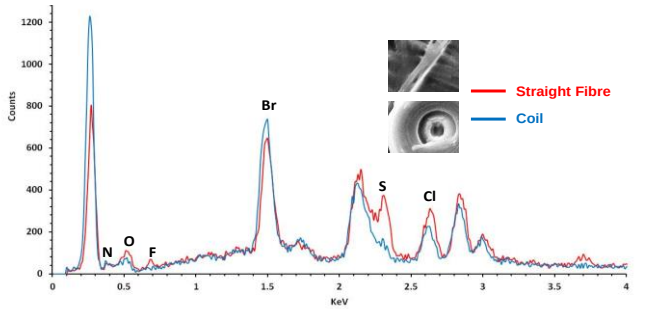

Fig. 3 EDX spectra from straight fibre and coiled fibre of 5 mg/mL **1·AEBSF** gel.

mic parameters associated with the phase transition are very different to gel **1·2Br**. The addition of **AEBSF·HCl** to gelator **1·2Br** influences greatly the gelling temperature (Fig. S7 in ESI), time of gelation, and associated enthalpy change. Gel **1·2Br** spontaneously starts forming at around 21 °C, while gel **1·AEBSF** starts forming at *ca.* 30 °C. This shows that adding the drug makes the gel more stable at higher temperatures. Conversely, the whole width of the peak indicates the total time for gel formation, which increases considerably from around 5 min to 20 min by adding **AEBSF·HCl**, similar to the observations with the naked eye, and suggesting that the interaction between the drug and the gelator lengthens the gelling period, presumably because of slower gel fibre assembly. The heat capacity (C_p) in the plot also represents the speed of gelation, where the onset temperature is the point at which gelation starts, and the maximum value is when the gelation occurs fastest. For instance, the gelation of **1·AEBSF** is 20 times slower than that of gel **1·2Br**, which is in accordance to the increase in the gelling period.

The most noticeable change observed is in the thermodynamic parameters of the process. The gelation of **1·2Br** is exothermic, and is related to the decrease of entropy upon the formation of fibres. Very differently, the gelling of **1·AEBSF** shows both an exothermic event in the beginning, and an endothermic one at lower temperatures, giving an overall enthalpy close to zero. These results indicate that upon the mixture of **1·2Br** with **AEBSF·HCl** and the solvents, not only the gelation occurs, but at least a second process is happening at the same time, which is endothermic, and therefore, necessarily entropic. This event could be an adsorption of the drug in the interstitial space of the gel to the fibres, and might be related to an increase in the surface tension of the solvent. The thermoreversibility of **1·AEBSF** was proven by subsequent heating-cooling cycles, in order to melt the gel and form it again, and similar peaks were observed. However, a slight decrease in C_p values, and a slight increase in the gelling temperature, occur in each cycle, which suggests that heating up the sample to 35 °C melts the gel but still leaves some gel nucleation points intact, not seen macroscopically, which facilitate the subsequent gelation on cooling (ESI, Table S2 and Fig. S8).

Drug release experiments from the nanocomposite material using PBS as the receptor medium for complying SINK conditions,[11] to prove of the drug when applied on human skin is not limited, showed that gel **1·AEBSF** releases almost 92% of the drug during the first 15 hours (fitting a one phase exponential association model). Afterwards, drug degradation occurs in the receptor chamber,[12] following a one phase exponential decay model (Fig. 4 and Table S3). This degradation would not compromise the therapeutical efficacy of the gel when applied on the skin, as the speed of release is ten times higher than the speed of degradation, whose half-life (55 h) is much longer than the usual administration intervals of topical formulations. Moreover, at the normal pH of the skin (5.5), degradation would barely occur. Permeation studies on human skin show that **1·AEBSF** promotes the complete permeation of **AEBSF** through the skin in 6 hours (lag-time, see Fig. 5 and ESI Table S4).

As K5 is located mainly at the epidermis, specifically at the cornified and granular layers,[13,14] the amount of drug retained inside the skin becomes an even more important parameter to be considered. The total amount of **AEBSF** retained (A_s corr.) is estimated by considering both the amount of drug extracted from the skin after the experiment [A_s] and the percentage of drug that can actually be extracted out of the total drug

retained (recovery experiments). After topical application, around 3484 μg/g·cm², is retained in the skin, where it has its therapeutic activity, equivalent to 69% of the total dose applied (Fig. 5).

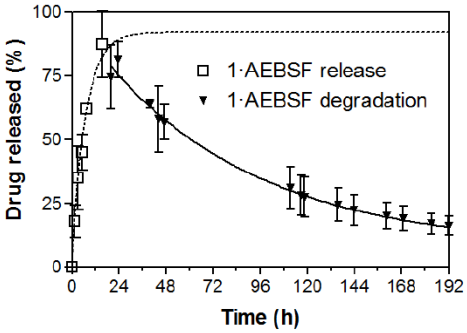

Fig. 4. Cumulative amount of drug released of from gel **1·AEBSF** and degradation in receptor medium. Values are means and error bars represent one standard deviation (n=3). Release and decay both follow one phase exponentials (see ESI and equation parameters in **Table S3**).

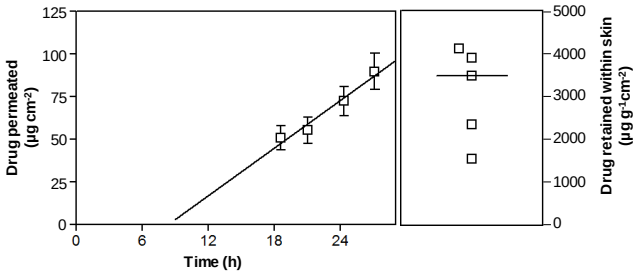

Fig. 5. Cumulative amount of **AEBSF** permeated (left) and retained in human skin (right) after application of gel **1·AEBSF.** Values in permeation experiments represent the Means ± one standard deviation. The bar in retention experiments represents the Median value (n=5).

Changes in the morphology of the gels were scrutinized by SEM after being subjected to release conditions, for a maximum of 16 h, the maximum period permitting almost a total release with no detectable hydrolysis of the drug. In all the samples, some lumpy material arises from the buffer used under those conditions. No variation was observed for the pure gel **1·2Br**, for which only straight fibres were seen (Fig. S9 in ESI). The release of the drug from **1·AEBSF** under these release conditions for 6 and 16 hours is also accompanied by the formation of fibre coils (see SEM images in ESI Fig S9 and S10). The images clearly show the formation of coils and a more structured and curved nature to the fibres of the gelator. Direct quantification is not possible, but the number of coiled fibres seems similar to those on aged gels under storage conditions over a longer period of time. While the morphological change is clear, powder X-ray diffraction of gels before and after release shows no significant structural rearrangement (Fig. S11 in ESI). A model such that in Fig. S12 might explain this observation.

In summary, **AEBSF·HCl** strongly influences the self-assembly of **1·2Br** and the behaviour of the resulting gel, which is soft and thus suitable for dermal application. **AEBSF** is released from the gel, triggering its morphological change evidenced by the twisting of fibres and the subsequent formation of coils, although not all fibres are able to coil, presumably because their length and being trapped physically by other fibres. Almost all the drug incorporated in **1·AEBSF** is released and penetrates human skin, where it is retained. By the nature of the drug incorporated, this gel would imply a novel therapeutic approach in the topical treatment of ailments like Rosacea.

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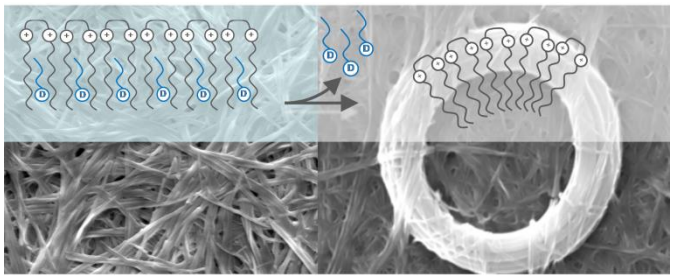

A gemini dicationic amphiphile forms gels with a serine protease inhibitor, which could be used in a new approach to the treatment of Rosacea, and the release of the drug causes remarkable gel fibre coiling.