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Cationic Supramolecular Hydrogels for Overcoming the Skin Barrier in Drug Delivery

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A cationic bis-imidazolium-based amphiphile was used to form thermoreversible nanostructured supramolecular hydrogels incorporating neutral and cationic drugs for the topical treatment of rosacea. The concentration of the gelator and the type and concentration of the drug incorporated were found to be factors that strongly influenced the gelling temperature, gel-formation period, and overall stability and morphology. The incorporation of brimonidine tartrate resulted in the formation of the most homogeneous material of the three drugs explored, whereas the incorporation of betamethasone resulted in a gel with a completely different morphology comprising linked particles. NMR spectroscopy studies proved that these gels kept the drug not only at the interstitial space but also within the fibers. Due to the design of the gelator, drug release was up to 10 times faster and retention of the drug within the skin was up to 20 times more effective than that observed for commercial products. Experiments in vivo demonstrated the rapid efficacy of these gels in reducing erythema, especially in the case of the gel with brimonidine. The lack of coulombic attraction between the gelator–host and the guest–drug seemed particularly important in highly effective release, and the intermolecular interactions operating between them were found to lie at the root of the excellent properties of the materials for topical delivery and treatment of rosacea.

1. Introduction

Gels, formed by a network of fibers in a solvent, have a particularity in their rheological behavior: they do not flow even though typically over 95% of their composition is liquid.[1–4] Many gels are made out of polymer molecules, and they can be classified depending on the way the molecules attach to each other to form the fibers: they can be covalently bonded throughout the whole system to form a polymer (chemical gels), or they can be composed of smaller subunits that are attached to each other through weaker chemical forces such as hydrogen bonds, coulombic forces, and van der Waals interactions, among others (supramolecular gels).[2,5] The covalent attachment of chemical gels leads to the formation of an irreversible, robust material that can be useful in certain biomedical applications such as contact lenses;[2,6] however, for drug delivery or other biomedical applications, supramolecular gels (also called physical gels) are more suitable because they are softer and non-permanent.[3,7–9] The majority of existing gels for cutaneous applications are supramolecular gels made out of polymeric subunits.[10] There has been recent interest in the development of supramolecular hydrogels for different biomedical purposes, such as vaccine adjuvants, and for the incorporation of nanoparticles for thermochemotherapy;[11–14] nonetheless, they are made out of polymeric subunits, and gel formation needs the presence of either a second substance or an enzymatic reaction to take place.

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In contrast, low-molecular-weight gelators (LMWGs) are smaller molecules that self-assemble to form fibers, and this leads to supramolecular gels that are commonly thermoreversible, more easily degraded than polymeric ones, and therefore optimum for certain biomedical applications such as drug delivery.[2,5] Examples of supramolecular hydrogels can be found in the literature, in which the importance of ionic interactions between the gelator and a host in gel formation/melting was shown.[15] Interesting examples include supramolecular gels for which host–guest interactions induce self-assembly only above a certain temperature and melt below it, as opposed to the majority of thermoreversible gels.[16] Also, the use of bis-imidazolium derivatives as LMWGs has been reported,[7,8] but gel formation can also need the presence of additional solvents such as glycerol and octanol or ionic liquids.[17]

Our group reported that bis-imidazolium salts are cationic surfactants that could interact favorably with anions, including anionic drugs, and possessed a variety of advantages for biomedical applications. For instance, they were shown to form micelles[18] and to deliver anionic drugs when they are stabilizing gold nanoparticles.[19,20] These surfactants can also act as LMWGs and form supramolecular hydrogels for drug delivery.[7,8] However, recently, all cases of drugs incorporated into cationic gels have been anionic, as the electrostatic attraction between the positively charged surfactant and the negatively charged drug was thought to be the interaction responsible for the behavior of the material, and these drugs displayed slow and sustained release. Nevertheless, an extensive part of the therapy of dermatological diseases is based on the administration of non-negatively charged drugs. For instance, rosacea is a chronic, inflammatory skin disease characterized by erythema, telangiectasia, and papulopustules.[21,22] Its etiology is not yet completely known, and pharmaceutical products on the market commonly contain neutral or cationic drugs that only act upon the medical symptoms. Brimonidine tartrate (Figure 1), an α_2 adrenergic agonist, is commercialized as the carbopol-based gel Mirvaso® for topical use in the treatment of rosacea, and it provides vasoconstriction.[10] Also, glucocorticoids are potent anti-inflammatory drugs that prevent vasodilation; their indirect mechanism decreases the expression of inflammatory cytokines and increases the expression of β_2-adrenergic receptors.[23] Triamcinolone acetonide (Figure 1), considered an "intermediate-acting" glucocorticoid because of its plasma half-life, has potency that is five times higher than that of cortisol.[24] It is commercially available in combination with other drugs, for example, as the cream Positon®.[25] Betamethasone 17-valerate (Figure 1) is considered a long-acting glucocorticoid with a potency that is 25 to 40 times higher than that of cortisol.[24] It is available as the cream Celecrem®,[26,27] as well as other brands, for topical application in inflammatory and pruritic dermatoses.

Moreover, there is still a lack of appropriate and fast animal models of rosacea for testing new products in vivo. The new formulations developed with already-marketed ingredients can be tested directly in clinical trials without performing animal tests (e.g. Mirvaso®);[10] nevertheless, a significant improvement in the treatment of a disease might need the exploration of completely new materials such as these supramolecular hydrogels, in which case animal tests must first be performed. Thus, the development of easy and fast animal models is desired for progress in rosacea therapy.

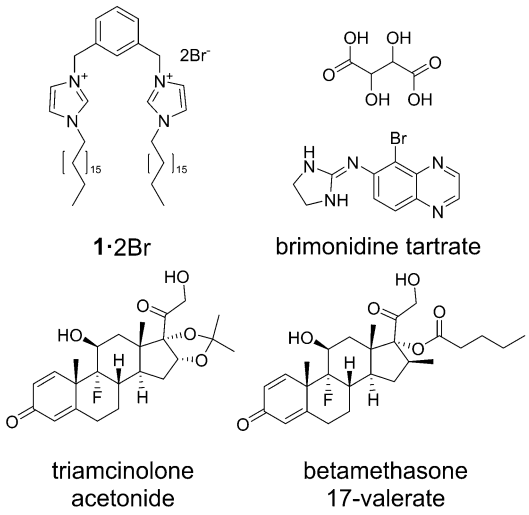

Figure 1. Chemical structures of compound **1·2 Br** and drugs incorporated in gels.

Furthermore, ethanol is used in many dermatological products on the market, as it works as a disinfectant and provides a fresh sensation if applied on skin. Hence, its presence in topical formulations could counteract the local temperature increase in the inflammatory process occurring in rosacea.

For all these reasons, the ability of compound **1·2 Br** (Figure 1) to form gels in the presence of neutral or cationic drugs that could be used in the treatment of rosacea was explored by using only water and ethanol as solvents. It was expected that the chemical interactions between the gelator host network and the guest drugs, lacking ionic interactions, would drive the delivery behavior of the hydrogels, for instance, by promoting very fast and effective release, which would render an excellent material for topical delivery. Here, brimonidine tartrate, triamcinolone acetonide, and betamethasone 17-valerate were incorporated in gels based on **1·2 Br**. Optimum conditions for gel formation were found, and the gels were extensively characterized. The release of drugs from the gels was studied, and drug-permeation experiments in human skin were performed to assess the suitability of this novel material as a possible topical drug-delivery agent.

Finally, a possible rapid animal model of rosacea was also developed on the basis of measuring the decrease in erythema after inducing vasodilation, and in vivo experiments were performed by using this new model to assess the efficacy of these novel supramolecular gels as a topical treatment for this disease.

2. Results and Discussion

2.1. Supramolecular Gel Formation

To study the gelling ability of **1·2 Br**, the gelator was dissolved in ethanol, water was added as an antisolvent, and the mixture

was left to stand without disturbance. The influences of the proportion of solvent, concentration of the gelator, and temperature were screened as an approach to choose suitable gelling conditions.

The proportion of the solvent had a very strong influence on gel formation, as ethanol was needed to dissolve gelator **1·2 Br** completely, whereas water played the role of the antisolvent. Gels could be formed with ethanol contents between 35 and 50%, and the latter resulted in the fastest formation (10 min). The gels were found to be stable for at least 6 months at room temperature. Higher proportions of ethanol prevented the formation of the matrix due to the high solubility of **1·2 Br** in this solvent and only led to partial gels (see Figure S1 in the Supporting Information).

The concentration of the gelator was also crucial to gel formation and stability. Gels could be formed at room temperature only if the final concentration of **1·2 Br** in the gels was $\geq$4 mg mL^{-1}. More dilute samples resulted in the formation of only partial gels. The gel with a concentration of 5 mg mL^{-1} was already formed after 10 min. An increase in the gelator concentration decreased the gelling time; the fastest gelling time was 2 min, which was observed in the 7.5 and 10 mg mL^{-1} samples (Figure S2). However, no clear difference in consistency or macroscopic appearance was observed upon increasing the concentration above 5 mg mL^{-1}. All formed gels were stable for at least 6 months at room temperature (below 30 °C). Pictures of the gels with different concentrations of **1·2 Br** were taken at different times and are shown in Figure S3.

The temperature was also an important parameter to consider upon forming gels with LMWGs[28] such as **1·2 Br**. Upon preparing the mixture and leaving to stand at room temperature (≈24 °C) or at 5 °C, gels were formed in only 10 min. Nevertheless, the gels formed at low temperatures had supernatant liquids and appeared to flocculate. At 35 °C, gelation did not occur, and the sample remained as a solution, as it was above the gel-melting temperature. However, cooling down to room temperature resulted in a homogeneous gel. No clear difference in consistency or macroscopic appearance was observed, except for the slight flocculation observed at 4 °C. A picture of the gels at different temperatures can be seen in Figure S4.

The time for complete gelation was influenced by the type and amount of drug incorporated in the mixture. Brimonidine tartrate was first dissolved in water and was then added to a solution of the gelator in ethanol. On the other hand, both triamcinolone acetonide and betamethasone 17-valerate were dissolved in ethanol together with the gelator, and water was added to the solution. In general, the gelling time was not altered in the presence of low amounts of drug relative to the gels without the drug (10 min), but it increased at higher drug concentrations. An exception was observed in the case of **1·triamcinolone** gels, for which, at all drug concentrations tested, the gelation was faster (2 min) than in gels without the drug. The results obtained are displayed in Figure 2 and Table S1. For all cases, no significant differences in consistency or macroscopic appearance were observed.

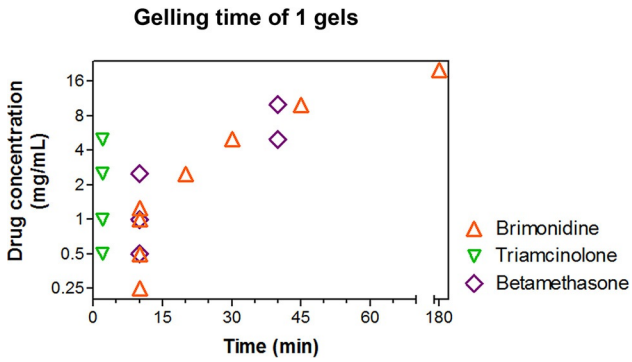

Figure 2. Influence of type and concentration of drug on gelling time.

Unless stated otherwise, the optimum conditions were chosen as 5 mg mL^{-1} of **1·2 Br** with an ethanol/water ratio of 1:1, which allowed both making and storage of the mixture at room temperature. A drug concentration of 5 mg mL^{-1} was selected for comparison purposes, as it was higher than that of the commercially available products, which implied an advantage in terms of reaching the therapeutic dose.

2.2. Rheological Measurements

Rheological studies of gel **1·2 Br** with brimonidine tartrate, triamcinolone acetonide, or betamethasone 17-valerate or without a drug were performed to know their viscoelastic behaviors. Amplitude sweep tests were performed at a constant frequency of 1 Hz to determine their resistance to rupture, as shown by the critical stress value (Table S2). The results showed that the addition of a drug decreased the critical stress values, and consequently, the structure could be broken more easily if anointed. In all cases, if the critical stress was reached, gels showed abrupt rupture rather than slow rupture. This showed the suitability of this material for a topical pharmaceutical form.

Additionally, frequency sweep tests were performed in all gels to determine their resistance to deformation at different frequencies, shown by the storage (G') and loss (G'') moduli, upon applying a constant shear stress of $\tau = 0.5$ Pa to be within the viscoelastic region. As can be observed (see Figure S5), despite the drug contained in the gel and the frequency applied, the storage modulus was higher than the loss modulus ($G' > G''$), with no crossover value ($G' = G''$). This elastic plateau permits these gels to be classified as "solid-like" gels.[3,29] Other examples of elastic plateau behavior can also be found in polymeric gels[30–33] and in gels made out of LMWGs.[29] By comparing the G' values (see Table S2), **1·triamcinolone** did not significantly change its resistance to deformation relative to that observed for gel **1·2 Br**. Instead, the addition of either brimonidine tartrate or betamethasone 17-valerate produced gels that were almost four times softer, which is even more suitable for dermal application.

2.3. Drug Incorporation into Gel Fibers

Supramolecular gels formed with isomeric compound **1·2 Br** can incorporate anionic drugs not only in the interstitial space of the gel but also inside the gel fibers.[8] This was thought to be due to an electrostatic interaction between the positively charged gelator and the negatively charged drug. However, neither the mechanism of the assembly leading to gelation nor that of drug incorporation is yet completely understood. That is why we challenged gel formation with neutral and cationic drugs such as triamcinolone acetonide, betamethasone 17-valerate, and brimonidine tartrate.

Indeed, gels of **1·2 Br** can be formed with these neutral and cationic drugs, and ^{1}H NMR spectroscopy shows that these supramolecular assemblies incorporate non-negatively charged drugs inside their fibers. By analyzing the spectra of gels formed in an NMR tube (see Figures S6–S8), no signals of compound **1·2 Br** can be observed, and consequently, compound **1·2 Br** assembles completely to form a gel. Also, **1·2 Br** can assemble in the presence of cationic and neutral drugs and incorporate them inside its fibers, not only in the interstitial space. This indicates that such incorporation is not mainly driven by an electrostatic attraction. If gel **1·2 Br** is formed in the presence of a twofold amount of the drug, the amount of drug incorporated in the fibers is 58% for brimonidine, 84% for triamcinolone acetonide, and 65% for betamethasone 17-valerate. This high drug-incorporation behavior makes this supramolecular material very interesting as a novel drug-delivery system, as the material can act as a package for the drug by protecting and facilitating its permeation through the skin.

The scanning electron microscopy (SEM) images of the gels studied herein with the incorporated drugs reveal a clear difference in the structures of the fibers depending on the drug incorporated. As observed in Figure 3a,b, the xerogel of **1·2 Br** alone has long fibers, and most of them are longer than 10 μm with a width of approximately 100 nm. Small groups of two to six fibers tend to stick together longitudinally to give a ribbon appearance, for which fibers can be individually seen. These groups of "ribbons" can bend and intertwine without any geometrical pattern to give a densely packed structure with reduced interstitial areas. According to the rheological studies, the gels are soft and can also break abruptly if critical stress is reached. This macroscopic break is indeed due to fracture of the fibers at the microscopic level if anointed. An example of how these fibers break can be observed in Figure 3c,d. This soft and easy-to-break behavior becomes especially important if thinking about rosacea patients, whose inflamed skin demands a comfortable application.

The addition of a drug to gel **1·2 Br** results in changes in the overall microscopic appearance, including the morphology of the fibers. For instance, gel **1·brimonidine** (Figure 3e,f) appears to have a larger number of fibers stuck longitudinally than gel **1·2 Br**, whereas gel **1·triamcinolone** (Figure 3g,h) has reduced interstitial areas due to flocculation induced by the drug.

In all cases, some drug precipitates to give particles of different shapes and sizes. This precipitate corresponds to the drug dissolved in the space between the gel fibers that precipitates upon evaporation of the solvent before analysis. In the cases of **1·brimonidine** and **1·triamcinolone**, the precipitates are prismatic crystals, whereas for **1·betamethasone** (Figure 3i,j) they are a spherical amorphous material. Considering that upon application on the skin some drug could precipitate,

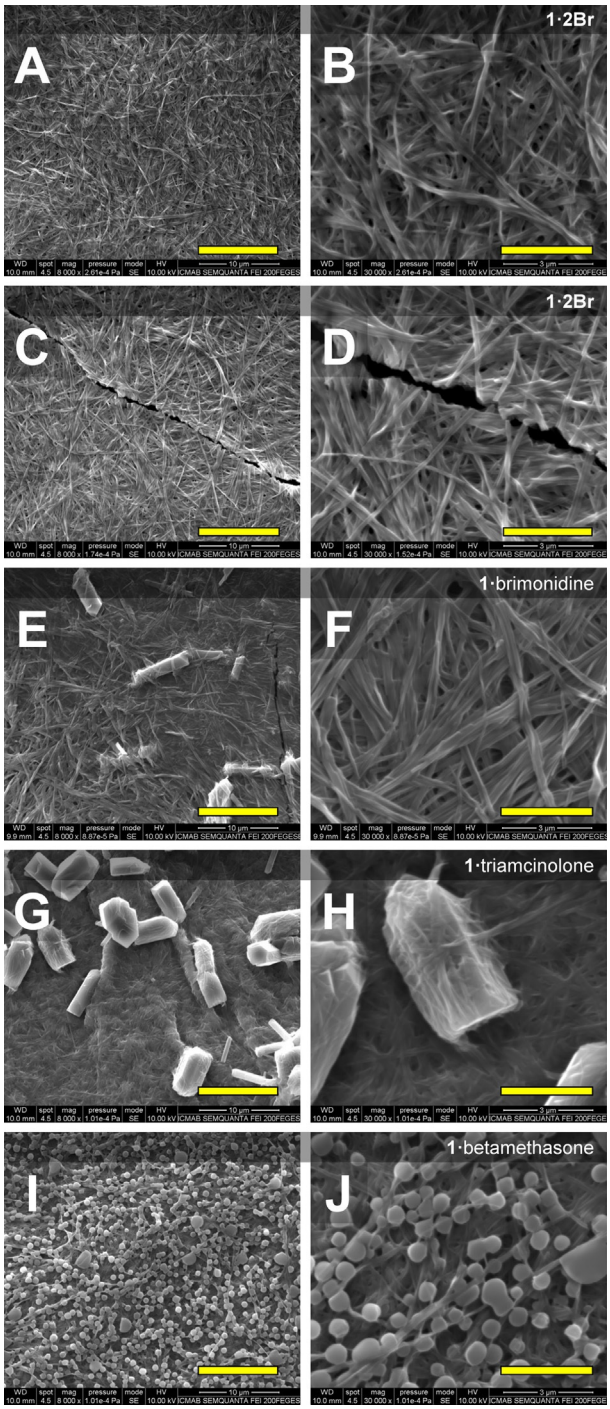

Figure 3. SEM images of gels a–d) **1·2 Br**, e,f) **1·brimonidine**, g,h) **1·triamcinolone**, and i,j) **1·betamethasone**. Rupture pattern of gels **1·2 Br** are shown in panels c and d; the same pattern was observed in all gels with drug. Scale bars represent 10 μm for the images on the left and 3 μm for the images on the right.

a drug in an amorphous material is ideal for topical administration, because it can be redissolved in the lipids and water of the skin more easily than crystals.[34,35]

Differential scanning calorimetry was used to observe energy changes upon gelation of **1·2 Br**. The strategy consisted in placing the freshly prepared mixture of compound **1·2 Br** with water and ethanol inside the equipment cell above the melting temperature to prevent gelation. The sample was then cooled down slowly below the melting temperature to form a gel inside the equipment while measuring the heat capacity (C_p) as a function of temperature. Thereby, if the gelation was an exothermic process, a signal would be observed, and the total enthalpy could be obtained by calculating the area under the curve. The results (Figure S9a) clearly show that the gelation of **1·2 Br** is an exothermic process, and the process is similar to what occurs in a crystallization event with a total enthalpy release of 14400 cal mol^{-1}. Note that the thermograms are plotted on an increasing temperature scale, but the experiments were performed by decreasing the temperature, and this they should be read from right to left. As the cooling speed is known to be 1 °C min^{-1}, the speed of gelation at each moment can also be determined from the heat-capacity values; for instance, the onset temperature (T_{onset}) at 22.1 °C is the point at which gelation starts, and the maximum value (T_{max}) at 20.2 °C is the point at which gelation occurs faster, releasing 5650 cal mol^{-1} min^{-1}. Interestingly, it can be seen that the signal is not symmetrical in shape, but it has a tail on the left side at 18.8 °C. Thus, this technique permits observation that, at this point and down to cooler temperatures, the gelling becomes considerably slower and follows asymptotic behavior. The derivative of C_p with respect to temperature (dC_p/dT) (Figure S9a) represents the change in the speed of gelation. From this value, it can be determined that within this interval of temperatures (22.1 to 18.8 °C) the majority of the gel is formed in only 3.3 min, during which 75% of the total gelling enthalpy (t_{gel75}) is already released, as seen by the area under the curve. This range of temperatures through which gelation occurs faster is especially interesting if thinking about the large-scale production of pharmaceutical formulations, as fabrication times can be importantly decreased.

To assess the thermoreversibility of the material, after cooling the sample down to 5 °C it was heated up again to 35 °C to melt the gel. Subsequent gelling cycles were performed, during which the exothermic signal was also observed but the T_{onset} value was shifted to 24 °C in the second cycle with no further shifts (Figure S9a). These results prove that this material is completely thermoreversible, whereas the shift in T_{onset} suggests that heating the sample up to 35 °C melts the gel but still leaves some gel nucleation points unmelted; it is these nucleation points that facilitate the start of the subsequent gelation. This is also in accordance with the observations of the enthalpy released, which decreases very slightly (3%) from the first cycle to the second cycle but with no further change. Detailed data are given in Table S3.

On the other hand, Figure S9b shows that the volume of the sample does not influence the gelling temperature (T_{onset}) but does influence the gelling speed and, therefore, the gelling time. Upon decreasing the volume of the sample by 50%, the gelling time (t_{gel75}) is also reduced by 50% (1.6 min) and reaches a maximum gelling speed that is three times higher (17190 cal mol^{-1} min^{-1}) than that of the original sample. This suggests that a higher surface area between the container and the mixture facilitates gelation, as adhesion of the liquid to the material helps supramolecular ordering for the formation of fibers. Therefore, the lower the volume of the sample, the higher the surface area per milliliter, and this increases the speed of gelation and reduces the gelling time. This interesting behavior can be very useful in industrial production, as the prepared mixture can be packed directly in single doses if it is still liquid, which makes the process easier and much faster and, furthermore, useful from the patient's point of view, as the dosage becomes more comfortable.

The same calorimetric studies were performed with gels including brimonidine tartrate, triamcinolone acetonide, and betamethasone 17-valerate. Figure S9b shows that the type of drug can influence the gelling time and temperature. Thus, the gelling process of gel **1·betamethasone** is not different from that of gel **1·2 Br**, and the addition of brimonidine tartrate promotes the start of the gelation at a higher T_{onset}. Gel **1·triamcinolone** starts to form at the highest T_{onset}, though the gelling speed is slower, especially at higher temperatures, and thus, the time for complete gelling is twice that of **1·2 Br** (Table S3).

This technique demonstrates that a difference in one single Celsius degree greatly influences the gelling speed and thus the gelling time. For this reason, the differences observed between these experiments and the naked-eye observations (Figure 2) are related to the room temperature, which varies between 18 and 28 °C.

2.4. Drug-Release Studies

Drug-release experiments were performed to prove that the drugs incorporated in the gel could be released from the material so they could have therapeutic activity if applied on the skin and also to demonstrate that this release profile did not limit permeation of the drugs into human skin. The experimental conditions were adjusted to simulate those in the skin-permeation experiments, including the use of Franz cells and matching the stirring speed and temperature (32 °C is the normal temperature of the skin). The solvent in the receptor chamber was always chosen to comply with SINK conditions to discard the possibility that low solubility would compromise the results.[36]

In all cases, the drugs could be released from the gel following a one-phase exponential association model. Two commercial products were tested for comparison purposes: Positon®, containing triamcinolone acetonide, and Celecrem®, containing betamethasone 17-valerate. These formulations also followed the same model. The release profiles are plotted in Figure 4a, and the equation parameters of all the gels studied are available (see Table S4).

According to the results, our carrier material permitted a high level of drug release and did not limit permeation of the drug into human skin. Gels **1·triamcinolone** and **1·beta-**

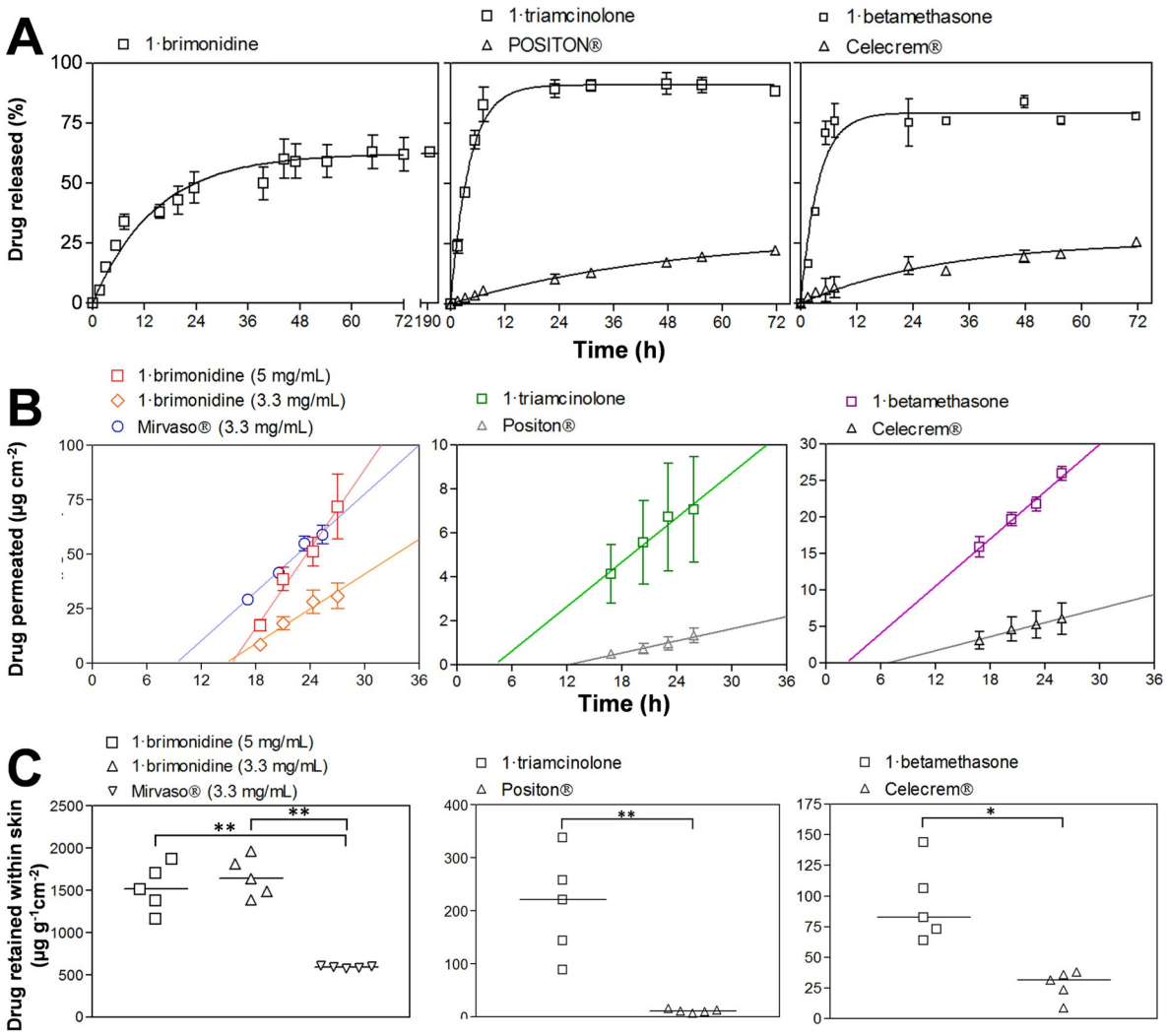

Figure 4. a) Drug released [%] from gel based on **1·2 Br** incorporating brimonidine tartrate (left), triamcinolone acetonide (center), or betamethasone 17-valerate (right). Commercially available formulations containing either triamcinolone acetonide (Positon®) or betamethasone 17-valerate (Celecrem®) were studied for comparison purposes. All values represent the mean of three replicas, and error bars represent one standard deviation. b) Skin permeation of drugs from a gel based on **1·2 Br** or commercial formulations. Gel **1·brimonidine** and Mirvaso® both contain brimonidine tartrate (left), **1·triamcinolone** and Positon® both contain triamcinolone acetonide (center), and **1·betamethasone** and Celecrem® both contain betamethasone 17-valerate (right). Values represent the means, and error bars represent one standard deviation. c) Amount of drug retained within the skin per gram and square centimeter after application either in gel from **1·2 Br** or in commercial formulations [brimonidine tartrate (left), triamcinolone acetonide (center), and betamethasone 17-valerate (right)]. Differences with statistical significance: $*p < 0.05$, $**p < 0.01$.

methasone presented both the highest amount of drug released and the highest speed of release. Moreover, gels derived from **1·2 Br** released up to three times more triamcinolone or betamethasone than commercial products. Also, the speed of release of these drugs was ten times faster if they were incorporated in gel **1·2 Br**. These results show a promising improvement in the dermal bioavailability of such drugs if they are formulated by using this novel material.

2.5. Skin-Permeation Studies

Gels were prepared by incorporating brimonidine tartrate, triamcinolone acetonide, or betamethasone 17-valerate at a drug concentration of 5 mg mL^{-1}, and permeation studies were performed in the same Franz cells as those used for the drug-release studies. Available marketed formulations containing brimonidine tartrate (Mirvaso®), triamcinolone acetonide (Positon®), or betamethasone 17-valerate (Celecrem®) were also studied for comparison purposes. Moreover, to assess the influence of the gel material itself and to discard other factors such as a different concentration of the drug, brimonidine tartrate was taken as a model and was also incorporated into the gel of **1·2 Br** at the same concentration as the commercial product Mirvaso® (3.3 mg mL^{-1}).

As observed in Figure 4b and Table S8 [*J* values], the gel promoted permeation of the drug at a rate up to four times faster than the marketed products, as observed in **1·triamcinolone** and **1·betamethasone**. Gel **1·brimonidine**, in contrast,

showed slightly slower permeation than Mirvaso® if loaded at the same concentration; nonetheless, if loaded at a concentration of 5 mg mL^{-1}, the flux became almost two times higher.

Also, the abscissa intercept in the plots shows the lag time (t_{lag}), which represents the time it takes for the drug to cross the epidermis completely and to cross part of the dermis. These results show that gel **1·2 Br** promotes the entry of the drugthrough the skin four times more rapidly than the commercial formulations, which implies faster action. An exception is the gel **1·brimonidine**, since brimonidine tartrate takes a few less hours if applied in Mirvaso®; nonetheless, the higher flux values of gel **1·brimonidine** provide more drug than Mirvaso® 25 h after the initial application, at which time better efficacy can be expected (see the Supporting Information, brimonidine tartrate flux vs. lag time). However, in the case of rosacea, the lag time becomes a less-important parameter in considering that the chronic nature of the disease implies repeated administration of the dose.

In addition, Figure 4c shows the total amount of drug that can be retained inside the skin after topical application. It is very interesting that in all cases the gel significantly promotes retention of the drug, as also seen in Table S8 (A_s corr.), and relative to the commercial formulations, the gel provides 2 times more drug in the case of betamethasone, 3 times more drug in the case of brimonidine, and up to 20 times more drug in the case of triamcinolone. The observed permeation flux values of triamcinolone are lower than those of bethametasone, and this is related to its high retention inside the skin. In all of the cases, the ability of the gel to promote this retention is highly beneficial for treatment of rosacea, as the therapeutic targets of these drugs are located at the dermis and, in the case of the glucocorticoids, also at the epidermis. Therefore, as the skin dermatomed includes both the epidermis and part of the dermis, high retention of the drug implies that more drug is available at the pharmacological target and, thus, higher efficacy. Furthermore, the skin can also act as a reservoir for the retained drug, which can continuously be redissolved and permeated to provide sustained pharmacological activity and to permit an increase in the dosing intervals. For this reason, the use of gels based on **1·2 Br** in the treatment of rosacea could also help the common problem of missed doses to be overcome.[37,38]

2.6. Pharmacological Efficacy In Vivo

The lack of sufficient knowledge in the etiology of rosacea may be the reason why there are not enough animal models for this disease. A few examples of possible animal models are based on neovascularization, vasodilation, and inflammation upon injection of either the cathelicidin peptide or the enzyme Kallikrein-5 that produces it; however, such models can last up to 4 weeks.[21,39] Thereby, pharmacological efficacy studies for the treatment of rosacea have always been a challenge. Hence, it would be of great interest for research on rosacea to develop new fast animal models of the disease.

Therefore, as two of the most important signs of rosacea are erythema and telangiectasia,[22,40] a model of the disease was developed in rabbits. For instance, upon application of *m*-xylene in the dorsal side of the rabbit ears, severe vasodilation was observed, especially at the capillary vessels, which also led to erythema. Both conditions are visible to the naked eye and last for at least 15 min. Moreover, to obtain quantitative and precise results, the decrease in erythema was evaluated by measuring the skin color by using a skin colorimetric probe that can detect much smaller differences in color than the naked eye. Hence, this model was used to assess the pharmacological efficacy of gels **1·brimonidine**, Mirvaso®, **1·triamcinolone**, and **1·betamethasone** in the topical treatment of the disease by inducing vasodilation and by applying the gel while measuring the skin color with time. Pictures of rabbit ears treated with **1·brimonidine** are shown in Figure 5, and those from the rest of the treatments as well as the skin color measurements in RGB code can be found in the Supporting Information (Figures S12–S14, Tables S9 and S10).

The evolution of erythema can be seen by reproducing the skin color as a sequence through the different steps: basal color; induction of vasodilation; and 5, 10, and 15 min after

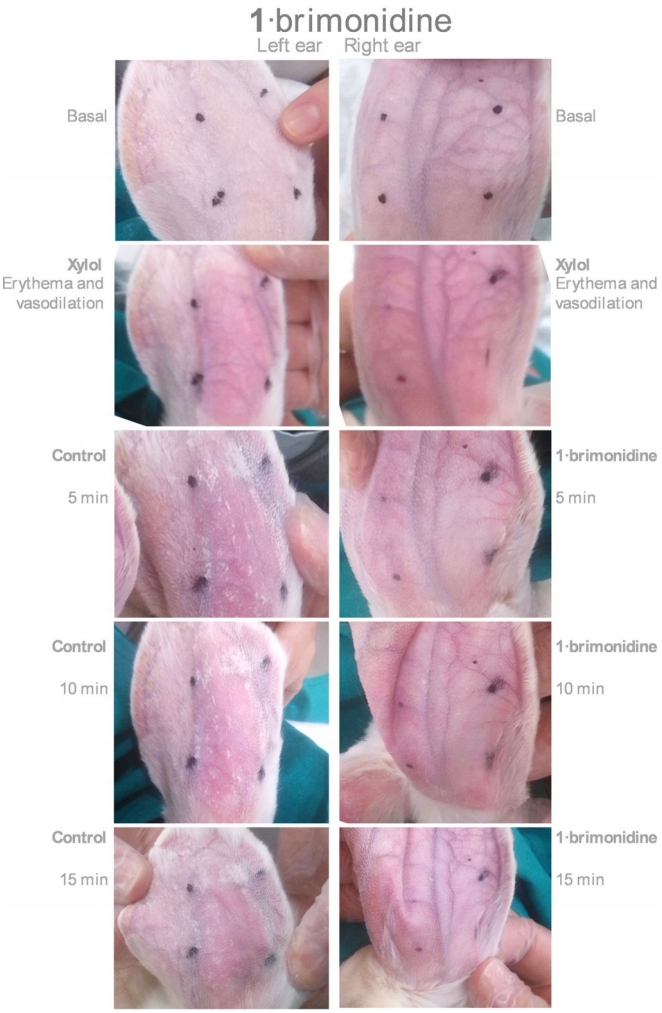

Figure 5. Pictures showing the evolution of erythema at different times after treatment with **1·brimonidine**. Treatment was applied on the dorsal side of the rabbit's right ear, whereas the left ear was not treated as a control.

treatment with either gel (right ear) or without applying any treatment (left ear) (see Figure S11). The results show that the application of *m*-xylene leads to acute erythema, which appears immediately and slightly diminishes after 5 min (left ear); however, there is no evolution afterwards, and the color of the skin does not return to the basal value. On the other hand, by applying a treatment, the erythema decreases even more quickly to give a lighter color. Gel **1·brimonidine** already decreases the erythema from minute 5 and keeps decreasing it until minute 15. In contrast, Mirvaso® shows a clear reduction only at minute 10, which does not evolve. The difference in efficacy between these two treatments can also be observed at the vasoconstrictive level, as gel **1·brimonidine** provides clear vasoconstriction of the capillary vessels (Figure 5 and Figure S12). In contrast, the application of **1·triamcinolone** or **1·betamethasone** as treatment apparently does not reduce erythema after 15 min, as the color of the skin remains similar to that of the control.

To obtain a quantitative comparison between treatments, the colors must be transformed into single numerical values. This cannot be done directly for the nature of the color codes; in this case, colors are given in the RGB code, which is a triple-number value. The transformation into single numerical values can only be done indirectly by calculating the differences between the colors. For instance, the triple code (RGB) is treated as *xyz* Cartesian coordinates in a 3D Euclidean space; thus, a single color is treated as a point and plotted in the *xyz* scatter chart. Consequently, the difference between two colors is obtained by measuring the linear distance between the two respective points. This procedure was used to calculate the difference between the basal color and the color at each stage (vasodilation; 5, 10, and 15 min from treatment). The difference between the basal color and the vasodilation color (vasodilation difference) was considered 100% erythema. Therefore, relative erythema [%] values were calculated with respect to the vasodilation difference.

Figure 6 clearly shows the evolution of erythema either with or without the application of treatment, and it is expressed as relative erythema. This plot confirms that erythema can decrease within 5 min without the application of a treatment (controls). However, this decrease is partial, as the erythema values do not return to the basal state (0%); instead, the majority of the time the values remain around 50%. In contrast, the application of a treatment can reduce the erythema beyond the control value, and such a reduction can appear sooner or later (lag time) depending on the drug it contains.

For instance, **1·brimonidine** had the fastest response, and it significantly reduced erythema only 5 min after application. Moreover, at the 10 min mark, the redness decreased even below the basal value and down to −107% after 15 min; both values are significantly lower than the control ($p < 0.001$). As opposed to **1·brimonidine**, treatment with Mirvaso® showed a lag time of 10 min and provided a relative erythema of 15% with no significant evolution afterwards. Statistical analysis did not show Mirvaso® to be significantly more effective than its control; however, the median relative erythema values were lower. Upon comparing **1·brimonidine** with Mirvaso®, no statistically significant differences were obtained, though it appeared that the former was slightly more effective than the latter at 5 min and was considerably more effective at 10 min.

This higher efficacy is in accordance with the results obtained for the skin-permeation studies, for which the amount

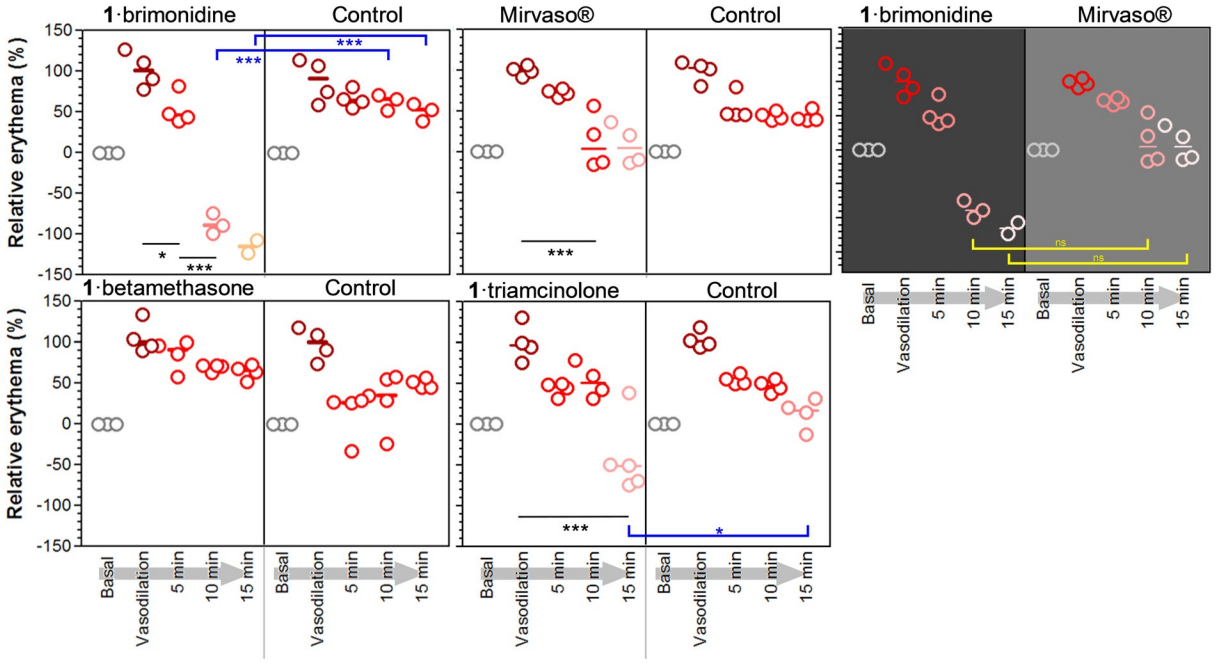

Figure 6. Evolution of erythema at different times as expressed as relative erythema [%] with respect to the vasodilation stage. Comparison between treatment with **1·brimonidine**, Mirvaso®, **1·betamethasone**, or **1·triamcinolone** and their respective controls (no treatment). A comparison between **1·brimonidine** and Mirvaso® is shown (right). Horizontal bars represent the median value. Statistical significance: $*p < 0.05$, $**p < 0.01$, $***p < 0.001$, ns = nonsignificant.

of drug retained inside the skin was almost three times higher upon applying **1·brimonidine** than upon applying Mirvaso®. This shows the effect provided by gel **1·2 Br** both in facilitating entry of the drug in the skin and in promoting its retention within the skin, where it has its therapeutic effect as a vasoconstrictor and decreases erythema. Also, this retention explains why **1·brimonidine** requires a longer time than Mirvaso® to cross the skin completely (permeation lag time). All of these results show that gel **1·brimonidine** is a promising formulation for the topical treatment of rosacea.

Likewise, treatment with **1·triamcinolone** shows a significant reduction in erythema after 15 min, also below the basal value (−53 %), and this gel is thus significantly more effective than the control. In contrast, relative to that observed for the control, treatment with **1·betamethasone** does not show a significant reduction in erythema.

Upon considering that erythema can decrease without the application of any treatment, the efficacy of the different gels can be estimated more accurately by calculating the decrease provided by the treatment itself and subtracting the control value from the treatment value (treatment contribution). Table 1 shows that the highest treatment contribution is provided by gel **1·brimonidine** (160 %), being over six times higher than that of Mirvaso (25 %). Similarly, gel **1·triamcinolone** decreases erythema very effectively (69 %). In contrast, **1·betamethasone** shows only a slight increase in erythema.

Table 1. Lag time, relative erythema values, and treatment contribution after 15 min.

Gel	t_{lag}[a] [min]	Relative erythema [%] Treatment[b]	Control[c]	Treatment contribution[d] [%]
1·brimonidine	5	−107	53	160
Mirvaso®	10	14	39	25
1·triamcinolone	15	−53	16	69
1·betamethasone	n.o.[e]	66	49	−17

[a] Time before a significant reduction in erythema was observed (lag time). [b] Relative erythema 15 min after application of treatment. [c] Relative erythema without the application of treatment (15 min). [d] Reduction in erythema provided by the treatment itself after 15 min, considering the relative erythema of both treatment and control. n.o.: No significant reduction in erythema was observed.

However, the differences in the efficacies of the treatments are not only related to permeation parameters such as flux values or the amount retained within the skin but are also related to the intrinsic therapeutic activity of the drugs. For instance, in the cases of **1·brimonidine** and Mirvaso®, the fast efficacy of both treatments may be due to the mechanism of action of brimonidine tartrate, which occurs immediately upon arriving at the α_2-adrenergic receptors in the endothelial cells. In contrast, both **1·triamcinolone** and **1·betamethasone** incorporate glucocorticoid drugs, the mechanism of action of which is indirect and occurs by regulating the transcription of genes encoding inflammatory cytokines or adrenergic receptors, which may be much slower. Even though the skin-permeation experiments show that the flux and retention within the skin provided by these gels are higher than those provided by the commercial formulations, the animal model employed evaluates the efficacy only on the short-term (15 min). Hence, the lack of activity with **1·betamethasone** could be due to this indirect, slower mechanism of action, and accurate efficacy would be more easily observed over a longer term. Despite this, gel **1·triamcinolone** does show a reduction in erythema 15 min after treatment. A possible explanation could be the intrinsic pharmacokinetics of this drug, which are faster than those of betamethasone; that is why triamcinolone is considered an "intermediate-acting" glucocorticoid, whereas betamethasone is considered a "long-acting" glucocorticoid.[24] In any case, improved efficacy could be observed over a longer term.

3. Conclusions

The supramolecular hydrogels formed upon mixing bis-imidazolium amphiphile **1·2 Br** with water and ethanol were strongly influenced by both neutral and cationic drugs, whereas previous work involved studying the incorporation of anionic drugs and very recently the incorporation of a cationic drug was studied;[41] these hydrogels were studied for the treatment of rosacea. The type of drug strongly influenced the nanometric structure of the gel fibers, and importantly, NMR spectroscopy indicated that the drugs not only remained dissolved at the interstitial space of the gel (as indicated by SEM) but that up to 0.84 moles of drug per mole of gelator were incorporated within the fibers. Such incorporation did not seem to be driven by the charge of the drug, which makes this novel material a versatile nanocarrier as a drug-delivery agent. The lack of a positive coulombic interaction between the gelator host network and the drug seemed particularly important for effective release.

Calorimetry showed that the speed of the supramolecular assembly was highly influenced by the temperature and the total amount of gel, and assembly was faster at smaller volumes and at 20 °C. Also, the type of drug added influenced this gelling temperature and speed. The soft structure of the gel observed in the rheological study showed its suitability for dermal application. This gel could release more drug than the commercial formulations available, and it did it with a faster profile. Upon application to human skin, the gel permitted faster drug permeation than commercial products. Also, independent of the permeation rate, it was very interesting that a gel based on **1·2 Br** promoted retention of the drug within the skin, which is optimum for targeting rosacea and other skin diseases. This drug retention could also act as a drug depot and could provide sustained activity for long periods of time, which would permit an increase in the dosing intervals and would overcome the problem of missed doses. Furthermore, gels from **1·2 Br** could be formed at drug concentrations higher than those of the existing products, which permits a smaller application of the gel to obtain the same therapeutic efficacy.

According to the rosacea model developed, the in vivo studies demonstrated the efficacy of gel **1·triamcinolone** 15 min after treatment and especially that of gel **1·brimonidine** after

5 min of treatment, and thus, these gels are more efficient than the commercial product Mirvaso® and reduced erythema even below the basal value.

Finally, the easy and fast preparation of this gel under simple conditions, that is, room temperature, soft mixing, and single-dose packing if it is still liquid, makes it a great option for the industrial-scale production of dermatological formulations in the treatment of rosacea and other skin diseases of a chronic nature.

Experimental Section

Materials

All reagents were of analytical grade. Brimonidine tartrate, betamethasone 17-valerate, and triamcinolone acetonide were purchased from Acofarma (Spain). Phosphate-buffered saline and *m*-xylene were purchased from Sigma–Aldrich. Compound 1,3-bis[(3-octadecyl-1-imidazolio)methyl]benzene dibromide (**1·2 Br**) was prepared as reported previously.[42]

Methods

Gel Preparation

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

Optimization of Gel Formation

Gelling time and overall macroscopic appearance was evaluated as a function of different factors such as solvent proportion, concentration of gelator, temperature, drug type, and drug concentration.

Influence of Solvent Proportions on Gel Formation

Gel **1·2 Br** (10 mg) was weighed into different vials. A different volume of ethanol was added to each vial (0.7, 0.8, 0.9, 1, and 1.1 mL), and the compound was dissolved by sonication. Water was added to complete a final volume of 2 mL, and samples were mixed gently with a micropipette. The vials were then left to stand at room temperature (24 °C) without disturbance. The final concentration of **1·2 Br** in all gels was 5 mg mL^{-1}, and the final proportions of ethanol in the gels were 35, 40, 45, 50, and 55%. Gel formation and stability were confirmed by the titling method and finally by inverting the vials after 2, 10, and 30 min; 1, 3, and 6 h; 4 days; and 6 months.

Influence of Concentration of 1·2 Br on Gel Formation

Gels of **1·2 Br** (2 mL) were prepared by weighing different amounts of **1·2 Br** (6, 8, 10, 12, 15, and 20 mg) into separate vials. The compound in each vial was dissolved in ethanol (1 mL) with sonication, after which water (1 mL) was added, and the sample was mixed gently. The final concentrations of **1·2 Br** in the gels were 3, 4, 5, 6, 7.5, 8, and 10 mg mL^{-1}. Gel formation and stability were confirmed as described above.

Influence of Temperature on Gel Formation

Gel **1·2 Br** (10 mg) was weighed into three different vials. The compound in each vial was dissolved in ethanol (1 mL) with sonication, and water (1 mL) was added. The mixtures were stirred gently, and the vials were closed to prevent solvent evaporation. Immediately, the vials were left to stand at three different temperatures: 5, 24, and 40 °C. Gel formation and stability were confirmed as described above.

Influence of Drug Concentration on Gel Formation

The solubilities of the tested drugs were previously determined in ethanol and water to determine the drug concentrations in the gel to be assayed. For the gels with **1·2 Br** and brimonidine tartrate, the gelator (10 mg) was dissolved in ethanol (1 mL). An aqueous solution (1 mL) 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. For the gel with **1·2 Br** and triamcinolone acetonide or betamethasone 17-valerate, ethanol (1 mL) containing different amounts of drug (1, 2.5, 5, and 10 mg; and up to 20 mg in the case of betamethasone 17-valerate) was added to **1·2 Br** (10 mg), and the mixture was sonicated until a homogeneous solution was obtained. Water (1 mL) was added, and the mixture was gently stirred. The vial was closed to prevent solvent evaporation and was left to stand at room temperature. Gel formation and stability were confirmed as described above.

Optimum Conditions for Gel Fabrication

Optimum conditions were chosen for the preparation of gels that 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 5 mg mL^{-1} of **1·2 Br** as the gelator using 50% ethanol and 50% water with both mixing and storing at room temperature. The drug concentration was always 5 mg mL^{-1} so there were no problems for gelling, and this concentration, in all cases, was higher than the concentrations of the commercially available products, which implied therapeutic doses would be reached. In the case of the brimonidine tartrate gel (**1·brimonidine**), gelator **1·2 Br** was dissolved in ethanol and was mixed with the drug dissolved in water. In the cases of the betamethasone 17-valerate (**1·betamethasone**) and triamcinolone acetonide (**1·triamcinolone**) gels, the drug was dissolved in ethanol, gelator was dissolved in the same ethanol solution, and water was added. Samples were mixed gently, closed to prevent solvent evaporation, and left to stand without disturbance.

Rheological Measurements

For rheological studies, gels with or without drug were formed in 7 cm diameter glass Petri dishes to form a total volume of 27 mL. Prepared gels were always kept at room temperature overnight before study. The rheological characterization of each formulation was performed by using a Haake Rheostress1 rheometer (Thermo Fisher Scientific, Karlsruhe, Germany) connected to a temperature controlled Thermo Haake Phoenix II + Haake C25P and equipped with a 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 from 0.01 to 100 Pa with a constant frequency of 1 Hz with the purpose of evaluating gel strength. Oscillation frequency tests were performed from 0.01 to 10 Hz at a constant shear stress of 0.5 Pa to be within the linear viscoelastic region to determine the related variation in storage modulus (G') and loss modulus (G'') at 32 °C. Both viscoelastic moduli are defined as follows [Eqs. (1) and (2)]:

$$G' = \frac{\tau_0}{\gamma_0 \cos\delta} \quad (1)$$

$$G'' = \frac{\tau_0}{\gamma_0 \sin\delta} \quad (2)$$

in which τ_0 and γ_0 are the amplitudes of stress and strain, respectively, 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 perform the test and analysis of the obtained data, respectively.

Drug Incorporation into Gel Fibers

Incorporation of drug into the fibers of gel **1·2 Br** was quantified by ^{1}H NMR spectroscopy[8] by using a Varian 400 MHz NMR spectrometer from the *Centres Científics i Tecnològics de la Universitat de Barcelona* (CCiTUB). A total of 32 scans were recorded in every measurement. Gels from **1·2 Br** incorporating the drug in a molar ratio of 1:1 were formed inside the NMR tube, and the drug signals in the spectra were compared to those from a drug solution at the same concentration. Aromatic signals from the drug were taken as the reference signals. To quantify the incorporation of brimonidine tartrate, two aliquots containing the drug (8.28 μmol, 3.66 mg) were each dissolved in deuterium oxide (0.75 mL), and the ^{1}H NMR spectra of both (tubes A and B) were recorded (record 1). After that, deuterated methanol (0.75 mL) was added to tube A and **1·2 Br** (7.5 mg, 8.28 μmol) dissolved in deuterated methanol (0.75 mL) was added to tube B. Both tubes were shaken to promote mixing, and gel formation in tube B was observed, whereas tube A remained in solution. The ^{1}H NMR spectra of both tubes were recorded under the same conditions (record 2). To quantify incorporation of either betamethasone 17-valerate or triamcinolone acetonide, two aliquots containing the drug (8.28 μmol; 3.94 mg of betamethasone 17-valerate, 3.59 mg of triamcinolone acetonide) were each dissolved in deuterated methanol (0.75 mL), and the ^{1}H NMR spectra of both (tubes A and B) were recorded (record 1). After that, **1·2 Br** (7.5 mg, 8.28 μmol) was dissolved in tube B with sonication, and deuterium oxide (0.75 mL) was added to each tube. Both tubes were shaken to promote mixing, and gel formation was observed in tube B, whereas tube A remained in solution. The ^{1}H NMR spectra of both tubes were recorded under the same conditions (record 2). The intensities of the drug signals in records 1 and 2 in the aromatic region were compared to obtain the amount of drug incorporated inside the gel fibers.

Microscopy

Scanning electron microscopy (SEM) images were acquired by the Electron Microscopy Service in the *Institut de Ciència de Materials de Barcelona—Consejo Superior de Investigaciones Científicas* (ICMAB-CSIC) with a QUANTA FEI 200 FEG-ESEM system for samples deposited on gold on mica from the gel. Contacts were made between the gold surface and the sample holder with graphite paste to avoid charging of the unstained xerogels. In all cases, gels were 2 weeks old, and the xerogels were observed after complete evaporation of the solvent.

Calorimetric Studies

The gelation of compound **1·2 Br** was performed in a Microcal VP-DSC. A mixture (1 mL) of compound **1·2 Br** with both ethanol and water was introduced in the equipment at 35 °C. The sample was then slowly cooled from 35 to 5 °C at a rate of 1 °C min^{-1} to form a gel inside the equipment while monitoring the specific heat capacity (C_p) during cooling of the sample. The thermoreversibility was assayed by performing subsequent heating–cooling cycles. The experiment was repeated with another sample (0.5 mL) to assess the influence of the sample volume. Finally, experiments were performed with gels incorporating either drug (0.5 mL samples). Data were treated and plotted by using Origin software and Microsoft Excel software.

Drug-Release Studies

Drug-release studies for the gels were performed in a Microette transdermal diffusion system (Microette plus-Hanson Research) following a protocol similar to those previously reported.[43,44] 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. The receptor chamber contained 10 mM phosphate-buffered saline (PBS) (pH 7.4) to study gels with brimonidine tartrate and ethanol/water (7:3) for gels with either betamethasone 17-valerate or triamcinolone acetonide, all of which complied with SINK conditions.[36] 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 **1·2 Br** were prepared at a drug concentration of 5 mg mL^{-1}. Known weights of gel **1·2 Br** with either drug or commercial formulations (≈1.6 mg drug) 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 an equal volume of the receptor solution. Release experiments of gels were done in triplicate. The concentrations of the 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 in each type of gel, and nonlinear least-squares regression was performed by 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 according to the R^2 value obtained.

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 for 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 guidelines.[45,46] Skin was used for every different formulation ($n=5$). The temperature was adjusted to 32 °C, the normal skin temperature in humans, and experiments were performed for only 27 h to prevent damage of the biological material. The receptor medium was always elected to comply with SINK conditions to discard the possibility that a slow permeation profile would be limited by the low solubility of the drug in the receptor medium.[36] For instance, 10 mM PBS buffer pH 7.4 was used for the skin permeation of brimonidine tartrate, and a mixture of ethanol/water (7:3) was used for the permeation of triamcinolone acetonide and betamethasone 17-valerate. Available marketed formulations containing brimonidine tartrate (Mirvaso®), triamcinolone acetonide (Positon®), or betamethasone 17-valerate (Celecrem®) were also studied for comparison purposes. Moreover, to assess the influence of the gel material itself and to discard other factors such as different concentrations of the drug, brimonidine tartrate was taken as a model and was also incorporated in gel **1·2 Br** at the same concentration as the commercial product Mirvaso® (3.3 mg mL^{-1}). The gel was applied on the donor compartment in contact with the epidermal side of the skin (≈1.6 mg drug). The samples were taken at given time intervals for 24 h. Concentrations were determined by using HPLC, and cumulative amounts of drug permeated were plotted. Kinetic parameters were calculated from the median and range values by performing a linear least-squares regression in the linear zone of the plot[47] by using GraphPad Prism (version 3.00, GraphPad Software, Inc., USA).

Drug Retention on Skin

At the end of the permeation study, the amount of retained drug inside the skin was evaluated.

Drug Extracted from the Skin

At the end of the permeation study, retained drug inside the skin was evaluated by following a protocol described elsewhere.[43] 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, and weighed, and the drug contained therein was extracted with the corresponding receptor medium (1 mL) during 20 min of sonication. The resulting solutions were measured by HPLC, which yielded the amount of drug extracted from the skin expressed in μg g^{-1} cm^{-2}.

Drug-Recovery Experiments

Drug solutions at known concentrations were prepared by using the receptor medium used in the release and skin-permeation studies as the solvent. For instance, concentrations were 133 μg mL^{-1} for brimonidine tartrate and 5000 μg mL^{-1} for both triamcinolone acetonide and betamethasone 17-valerate. An aliquot (1 mL) of each drug was taken, and pieces of skin from the same patient as in the permeation experiments were immersed in each drug solution and kept at 32 °C for 27 h. The skin pieces were cleaned with gauze soaked in a 0.05% solution of sodium dodecyl sulfate and were washed in distilled water accurately. Drug concentrations of both solutions "before immersion" and "after immersion" were determined by using HPLC to know the amount of drug that could be retained within the skin. Skin pieces were punctured with a needle, and drug was extracted with the corresponding receptor solution (1 mL) by using sonication, as performed in the drug-retention experiments. Concentrations of the drug extractions were determined by using HPLC. The percentage of drug that could be recovered after being retained within skin was determined as follows [Eq. (3)]:

$$\text{Drug recovery}\,[\%] = \frac{(\text{drug extracted}\,[\mu g])}{(\text{drug retained}\,[\mu g])} \quad (3)$$

Drug Retention Inside the Skin

The percentage of drug recovery was used to estimate the real amount of drug retained within skin during the skin-permeation experiments. Nonparametric Mann Whitney test statistical analyses were performed to compare the drug retention values from different formulations.[47]

HPLC Determination

Concentrations of brimonidine tartrate in the samples were obtained by HPLC with a Waters LC Module I by using a Waters Spherisorb 5 μm ODS-2 (4.6 mm×150 mm) column. Concentrations of triamcinolone acetonide and betamethasone 17-valerate in the samples were obtained by using an HPLC with a Waters 717 plus autosampler and a 600 controller pump equipped with a 2996 photodiode array detector by using a 4 μm (3.9 mm×150) Nova-Pack C18 column. To quantify brimonidine tartrate, the mobile phase consisted of acetonitrile/water (50:50, acidified to pH 2.4 with acetic acid) with a flow rate of 1 mL min^{-1} setting a detection wavelength of 316 nm. To quantify betamethasone 17-valerate, the mobile phase consisted of acetonitrile/water following a gradient that consisted of 35:65 at $t=0$ min, 100:0 at $t=10$ min, 35:65 at $t=11$ min, and 35:65 at $t=16$ min with a flow rate of 1 mL min^{-1}. The detection wavelength was set to 239 nm. Each sample had a run time of 16 min. To quantify triamcinolone acetonide, the mobile phase consisted of acetonitrile/water following a gradient that consisted of 40:60 at $t=0$ min, 85:15 at $t=8$ min, 40:60 at $t=9$ min, and 40:60 at $t=14$ min with a flow rate of 1 mL min^{-1}. The detection wavelength was set to 239 nm. Each sample had a run time of 14 min. The data were collected by using Millennium32 version 4.0.0 software from Waters Corporation.

Pharmacological Efficacy In Vivo

All experiments were performed in compliance with the Universitat de Barcelona Committee of Ethics at the Animal Experimentation Unit of Hospital de Bellvitge. The pharmacological efficacies of gels **1·brimonidine**, Mirvaso®, **1·triamcinolone**, and **1·betamethasone** were tested in four groups of rabbits, each group corresponding to one kind of gel ($n=4$). Gel **1·brimonidine** was used at a drug concentration of 3.3 mg mL^{-1}, the same as that of Mirvaso, for comparison purposes. Gels **1·triamcinolone** and **1·betamethasone** were used at a drug concentration of 5 mg mL^{-1}. The skin color of the rabbit was determined in both ears by using a MPA5 Multiprobe adapter from Courage+Khazaka electronic GmbH, equipped with a CL400 skin colorimeter probe from Courage+Khazaka electronic GmbH, Germany. The device emitted a white LED light that homogeneously illuminated a circular part of the skin. The light scattered by the skin was detected by the colorimeter probe, and it is expressed as the intensity of light of the three basic light components, R, G, and B (red, green, and blue), on the scale of 0 to

255 each. After measuring the basal skin color, vasodilation and erythema were induced in both ears by applying *m*-xylene in a limited region of the dorsal side of the ears with the help of sterile gauze, and the skin color of both ears was determined again. Immediately after, an aliquot (400 μL) of **1·brimonidine**, Mirvaso®, **1·triamcinolone**, or **1·betamethasone** was applied to the limited region in the right ear as treatment, leaving the left ear without treatment as control. Skin color determinations were performed after 5, 10, and 15 min of treatment. Pictures of the rabbit ears from the first rabbit of each group were taken immediately before each color measurement. Colors were reproduced by using Microsoft Excel software from the RGB codes and were plotted as a sequence. To determine the difference between the colors, RGB codes were treated as *xyz* Cartesian coordinates in the Euclidean space, (red as *x*, green as *y*, and blue as *z*) and were plotted in a 3D scatter plot. For any pair of colors *A* (R_1, G_1, B_1) and *B* (R_2, G_2, B_2), the difference was obtained first by calculating the linear distance in the space between these two points and then by assessing the sense of the distance. The linear distance was obtained by using the analytical geometry equation [Eq. (4)]:

$$\left|\overrightarrow{AB}\right| = \sqrt{(R_2 - R_1)^2 + (G_2 - G_1)^2 + (B_2 - B_1)^2} \quad (4)$$

The linear distance does not permit determination of whether a color is darker or lighter than the other, but only how different it is. Therefore, the sense of the distance was obtained by calculating the overall difference in light intensity [Eq. (5)]:

$$\Delta_{\text{intensity}} = (R_2 - R_1) + (G_2 - G_1) + (B_2 - B_1) \quad (5)$$

For this equation, a negative result corresponds to a darker color, which was the case of erythema and to which a sense value of (+1) was assigned, and a positive result corresponds to a lighter color, which implies less erythema and to which a sense value of (−1) was assigned. Finally, the difference between the two colors was obtained by multiplying the linear distance by the sense of the distance. Differences were calculated between the basal median value and each measurement. The difference obtained between the basal color and the one after inducing vasodilation (vasodilation difference) was considered 100% erythema. Hence, relative erythema [%] values were calculated with respect to the vasodilation difference and were plotted as a sequence of the different stages to see the evolution of erythema. One-way analysis of variance (ANOVA) with Tukey's multiple comparison tests was performed to assess the statistical significance of both the evolution of erythema and the comparison between treatment and control. Nonparametric Kruskal–Wallis tests were performed to assess the statistical significance of the comparison between different treatments (**1·brimonidine** vs. Mirvaso®). All statistical analyses were performed by using GraphPad Prism software version 3.0.

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Conflict of Interest

The authors declare no conflict of interest.

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