Supplementary Information

Pirfenidone Loaded Spray Dressing Based on Lyotropic Liquid Crystal for Deep Partial Thickness Burn Treatment: Healing Promotion and Scar Prophylaxis

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1 Experimental section

1.1 Optimization of matrix and solvent

GMO and DMAC was uniformly mixed at a ratio of 1:9 to 9:1 and then added to excessive water for observation of gel formation. GMO and DMAC ratio at 7:3, 8:2 and 9:1 (LLC70, LLC80, LLC90) was able to form bulk gel in contact with water and selected for further optimization. In order to investigate the effect of water on the precursor, water was also added in a weight amount of 10% to the precursor (WLLC70, WLLC80, WLLC90). Rheological test was performed to thoroughly optimize the

formulation, including viscosity, yielding stress and viscoelasticity, using Kinexus Lab^{+} Rheometer (Malvern, UK) in accordance to the testing sequence provided by rspace software (Malvern, UK). Specifically, shear rate was set from 0.1to 100 s^{-1} in viscosity test while shear stress ranged from 0 to 100 Pa·s in yielding stress test. For viscoelasticity test, amplitude sweep was adopted with a strain range from 0.1% to 100% and sweep frequency at 1Hz. For complex modulus, the frequency and shear strain was fixed in 1 Hz and 1%. Single frequency test was also conducted with shear strain at 1%, frequency at 1Hz and testing time at 300s.

1.2 HA addition

The matrix ratio of GMO: DMAC: water was chose at 72:18:10 (w/w). HA with various molecular weight (MW) and content was added to the matrix for thorough investigation and optimization through water absorption and rheological property. HA MW varied from 200~400 kD, 800~100 kD, 1300~1500 kD to 1800~2200 kD while HA content range was 0.1%, 0.5%, 1%, 2%, 3% and 5%.

Water absorption was conducted as followed. Around 0.5 g precursor was placed on the bottom of a 5 mL tube and 4 mL water was carefully added to the tube without scouring the precursor. The tube was placed at 37˚C, 100 rpm in a shaker (THZ-82B, Jingda, China) and taken out to remove all the water at predetermined interval by tilting the tubes. Weight of sample and tube were recorded and the water absorption rate (R_{wu}) was calculated according to the following equation:

$$R_{wu} = \frac{w_t - w_{ep} - w_p}{w_p} \qquad \text{Equation (S1)}$$

where w_t is the total weight of tube and gel after water removal, w_{ep} is the weight of tube and w_p is the weight of the added LLC precursor.

1.3 Drug release behavior

The matrix ratio of GMO: DMAC: water was chose at 72:18:10 (w/w) according to previous study. HA with various molecular weight (MW) and content was added to the matrix for thorough drug release behavior. HA MW varied from 200~400 kD, 800~100 kD, 1300~1500 kD to 1800~2200 kD while HA content range was 0.1%, 0.5%, 1%, 2%, 3% and 5%. Different content of drug PFD (0.5%, 1%, 1.5%, 2% and 3%) was also loaded into the precursor and the drug release behavior was investigated. Moreover, complex modulus of HA solution, LLC matrix and HA contained LLC matrix at different HA (800~1000 kD) content was also evaluated.

Drug release was conducted using dialysis bag method. Approximately 0.4 g HLCSD precursor was added to dialysis bag (cut-off MW, 3500 D, Jinsui Bio-Technology Co. Ltd, Shanghai, China) and placed in a tube filled with 10 mL PBS solution as drug release medium. The tube was placed in a shaker at 37˚C, 100 rpm. At predetermined interval 1 mL drug release medium was withdrawn and subjected to HPLC to detect the released drug amount. The tube was supplemented with equivalent medium. HPLC (UlitMate3000, Dionex, Sunnyvale, CA, USA) was performed using a InertSustain C18 column (4.6 inner diameter x150 mm length, GL Sciences, Japan) under the following conditions: wavelength: 317 nm, mobile phase: 0.02 M KH_2PO_4 : acetonitrile (V/V), flow speed: 20 uL/min. Cumulative drug release was calculated and

plot as a function of time.

The release kinetics curves of different were fitted in models including zero order (y = kt + a), first order (ln(100-y) = kt + a), Higuchi (y = kt^1/2 + a), Hixon Crowell ((100-y)^1/3 = kt + a) and ritger pepper ($y = kt^n$) model, where *y* is the drug release at time *t*, a and n are constants, and *K* is the drug release rate coefficient. The correlation coefficient R^2 between each discrete release point and the fitted curve was determined to infer possible drug release mechanism.

2. Result and discussion

2.1 Optimization of matrix and solvent

GMO and DMAC mixed at a ratio of 1:9 to 9:1 demonstrated different ability for gel formation. When contacting excessive water, GMO and DMAC at a ratio from 1:9 to 4:6 showed no phase transition. When the ratio increase to 5:5 and 6:4, incomplete gel formation can be observed instantly with white floccules or cluster in morphology. GMO and DMAC mixed at ratio from 7:3 to 9:1 showed instant transition in contact with water and formed transparent bulk gel. Hence, these ratios were selected for further optimization.

The further optimization of GMO and DMAC ratio was conducted through rheological assessment. The viscosity of LLC was all shear thinning, which indicated the pseudoplastic property. When exterior force was applied to the precursor and the shear rate rose drastically, the viscosity would sharply descend accordingly and thus facilitated the spray process. Viscosity of LLC90 (Figure S1C) was one order

magnitude higher than that of LLC70 (Figure S1A) and LLC80 (Figure S1B) due to the higher percentage of GMO. Water addition to LLC cause different changes to LLC. For LLC70 and 80, the high content of DMAC ensure a complete mixture of matrix and solvent, which disperse the water uniformly and cause slight change on the shear-thinning viscosity. For LLC90. GMO has composed the majority of matrix hence the introduction of water had no influence on the shear thinning property.

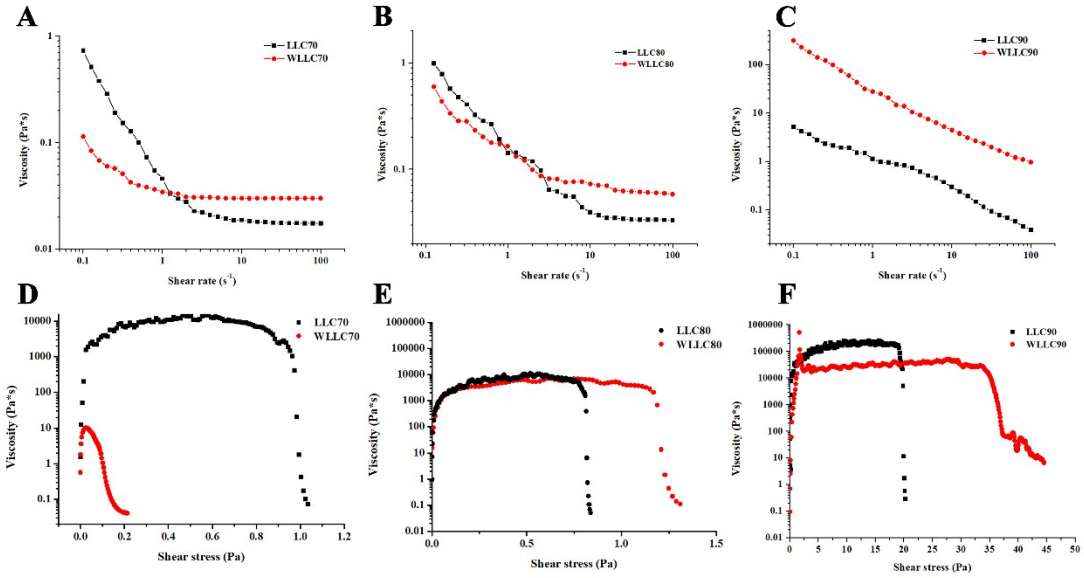

Figure S1. Rheological property of LLC spray dressing precursor with different composition. A,B,C viscosity; D,E,F, yielding stress.

Yield stress is defined as the critical stress applied to non-Newtonian fluid where it tends to flow while still remains the original state. The viscosity is in a relatively high level and the deformation was barely seen. Once the stress passes the critical value, the fluid starts to flow and the viscosity will decrease vigorously. It could be regarded as an indicator for the fluidity of the precursor to assess the spray feasibility. The yield stress of LLC70 (Figure S1D) and LLC80 (Figure S1E) was rather small compared with

LLC90 due to their smaller GMO amount (Figure S1F). Water addition to the matrix would cause mild fluctuation to the yield stress considering the different formulation composition. For LLC70, the large amount of DMAC ensure a sufficient mixture of the aqueous phase and lipid phase once water was added, so the fluidity was improved. As GMO ratio increased in the matrix, it became more difficult for the added water to fully disperse into the matrix and thus the yield stress for the precursor would rise. For LLC80, the yield stress was around 1 Pa·s, which was close to LLC70 and in favor of the precursor spray. Meanwhile, yield stress of LLC90 grew to over 30 Pa·s, significantly hinder the application of precursor as spray dressing. The result was in agreement with that of viscosity test, reflecting the ability to flow in the state of precursor.

Viscoelasticity test was performed to evaluate the solid-liquid state and property of the precursor since it was non-Newton liquid with both feature. The complex modulus under different shear strain was an indicator hardness which could be decomposed into elastic modulus (G') and viscous modulus (G''). When the value of G' surpasses G'' and the phase angle was smaller than 45^{o}, the dominant state of the fluid is regarded to be solid, and liquid versa. Viscosity could be referred as an indicator of the fluid state under different exterior condition.

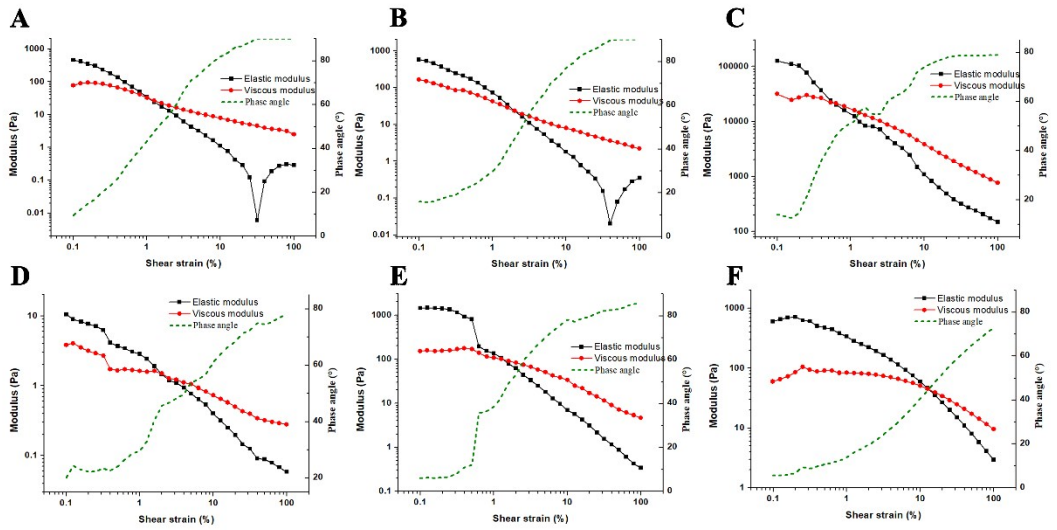

Figure S2. Rheological property of LLC spray dressing precursor with different composition. A,B,C, viscoelasticity test results of LLC70 (A), LLC 80 (B) and LLC90 (C); D,E,F, viscoelasticity test results of of WLLC70 (D), WLLC 80 (E) and WLLC90 (F).

Precursor of both LLC70 (Figure S2A) and LLC80 (Figure S2B) showed large modulus close to 10^2~10^3 Pa at low shear strain area. The dominant solid state ensure stability to cling to the wound surface at a relevant still condition after spraying to the lesion. As the shear strain increased, the modulus gradually decreased to 10^0 Pa and was mainly in liquid state with excellent fluidity. Water addition barely or slightly decreased the modulus (Figure S2D, 2E). LLC90 showed similar trend yet the modulus with or without water was significantly larger than others due to high GMO amount, which might impede the application (Figure S2C, 2F).

Single frequency test was a type of viscoelasticity test in which temperature, sweep frequency and shear strain was fixed. Resulted modulus was recorded as a function of time and the property changes was caused merely due to the inner structure. The result of pure GMO showed constantly increase on both elastic and viscous modulus as high as 10^5 Pa, due to the spontaneous solidification process of unsaturated lipid. The

solidification required heat to restore to liquid, which was of much incompliance. Owing to this property of GMO, LLC 70 (Figure S3A), LLC80 (Figure S3B) and LLC90 (Figure S3C) all demonstrated significant solidification process, featuring growing complex modulus, as well as elastic modulus surpassing viscous modulus. The addition of water could postpone or curb this process to some extent, by drastically decreasing the modulus or maintain the elastic modulus at a higher level than viscous modulus (Figure S3D, 3E, 3F). WLLC80 was finally selected as the optimized formulation (GMO: DMAC: water = 72:18:10) due to the low viscosity and yield stress, dominant liquid state and relatively small amount of organic solvent.

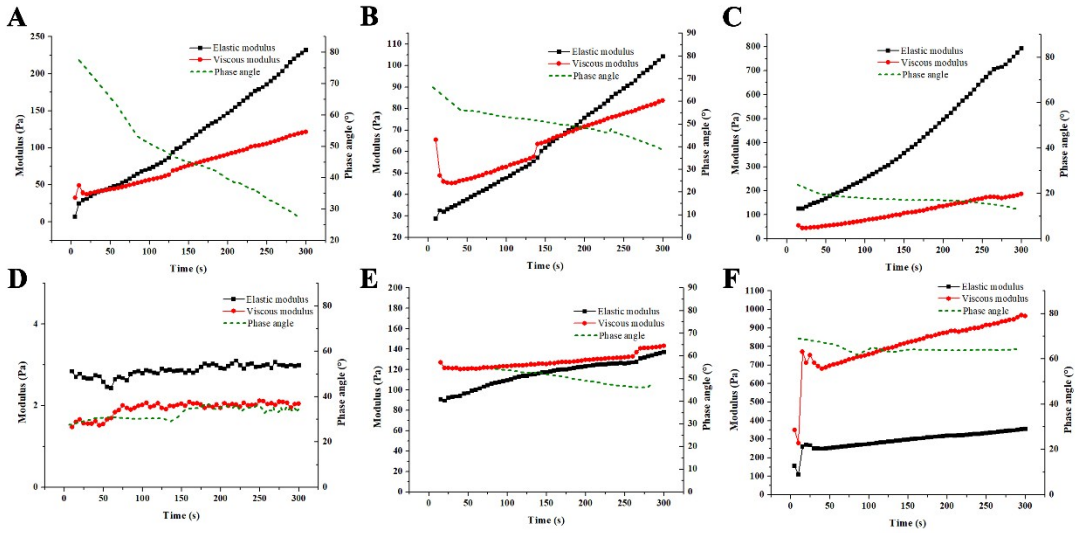

Figure S3. Rheological property of LLC spray dressing precursor with different composition. A,B,C single frequency test results of LLC70 (A), LLC 80 (B) and LLC90 (C); D,E,F, single frequency test results of WLLC70 (D), WLLC 80 (E) and WLLC90 (F).

2.2 HA addition

The water absorption result was shown in Figure S4. Pure LLC showed weak water absorption ability. Addition of HA to LLC matrix (HLCSD) significantly

158 increase water absorption. The addition of HA improved the water absorption capacity,
159 indicating a superior exudates absorption ability than pure LLC to create a favorable
160 environment for wound healing. In terms of the molecular weight, low molecular
161 weight HA (LMWHA, 200~400 kD) showed weak improvement in water absorption,
162 with bare increase only when the content exceeded 1% due to the week hydroscopicity
163 and short chain of LMWHA. Medium molecular weight HA (MMWHA, 800~1000 kD,
164 1300~1500 kD) and high molecular weight HA (HMWHA, 1800~2200 kD)
165 significantly enhanced the absorption capacity from 100% to 260%.

166 Pure LLC matrix showed weak capacity in water absorption. The contact area of
167 precursor and medium was controlled to better mimic the physiological contact of
168 dressing and wound during application, which limited a comprehensive contact. The
169 erosion by medium scouring would cause loss of gel, simulating the possible exterior
170 abrasion after administration. The improvement with HA addition may result from
171 strong hydroscopicity and long molecular chain of HA. HA molecule itself possessed
172 great capacity to absorb water and swell with large volume, while the long molecular
173 chain of HA could maintain the integrity of the lattice structure by binding adjacent unit
174 and reduce the gel loss by resisting the medium scouring. Together the water absorption
175 ability was remarkably enhanced. Compared with 800~1000 kD, MW of 1300~1500
176 kD and 1800~2200 kD did not significantly affected the water absorption, indicating a
177 saturation of MW effect on water absorption.

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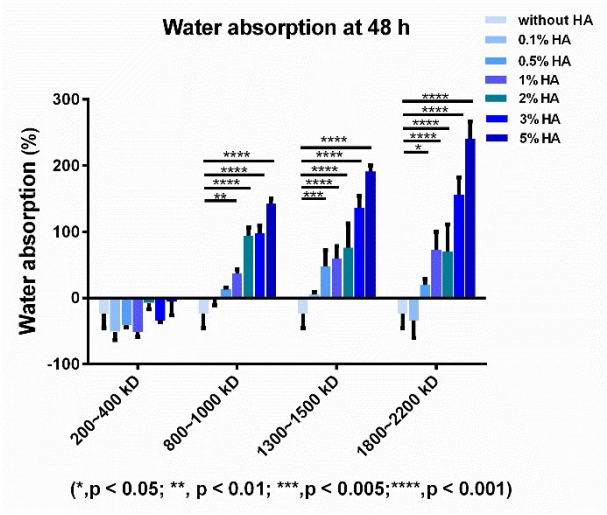

Figure S4. HLCSD water absorption at 48 h with HA of various MW and concentration.

Rheological assessment was conducted to investigate the influence of HA addition to LLC matrix. Ideal spray dressing for wound management should be equipped with low viscosity as precursor and robust modulus as gel. Low viscosity could provide convenience during administration and facilitate the spray process while moderate modulus of gel could serve as a protection for wound from stress and shock.

HA addition of different MW raised the viscosity by 1~5 magnitudes, yet the shear thinning property was still maintained. During spray the stress applied to the precursor may cause a shear rate as high as 10^2 to 10^4 s^{-1}, which could drastically decrease the viscosity of the precursor according to the viscosity/shear rate curves. As the result showed, formulation with HA MW at 1300~1500 kD (Figure S5C) or 1800~2200 kD (Figure S5D) demonstrated a viscosity higher than that of the HA free formulation due to the addition of large MW HA. The high shear rate viscosity of formulation with HA MW at 800~1000 kD restored closely to the HA free formulation, indicating a potential for further study as the precursor of LLC spray dressing (Figure S5B).

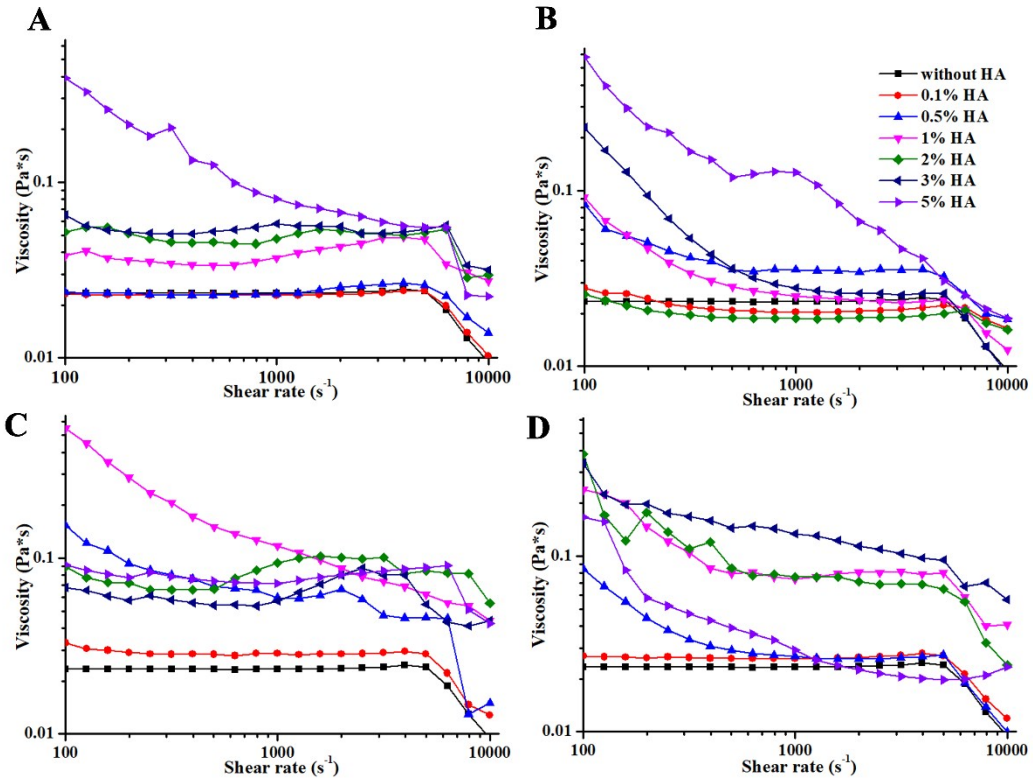

Figure S5. Precursor viscosity with shear rate ranging from 10^2 ~10^4 s^{-1} with HA MW at 200~400 kD (A), 800~100 kD (B), 1300~1500 kD (C) and 1800~2200 kD (D).

Another rheological property of HLCSD worth concerning was the gel modulus which measured the stiffness or resistance to elastic deformation under load. Ideal dressing should possess appropriate modulus (skin modulus 10^0~10^3 kPa) to protect the skin from damage of exterior abrasion and force yet not to cause discomfort to patients. HLCSD gel modulus with different HA content was shown is Figure S6A. Pure LLC without HA showed low modulus at round 1kPa and addition of HA significantly increased the modulus value. The effect of 200~400 kD was limited. For 800~1000 kD HA, the modulus tended to increase as the HA concentration rose. However, some modulus decreased in groups with HA MW over 1300 kD, possibly due to the reason that the excessive swelling of HA loosened and broke the integral structure of LLC gel. Addition of 800~1000 kD HA ensured a stable enhancement to modulus which was

similar to that of skin. It could not only enable the LLC gel to resist exterior force but also bring compliance to patients after administration.

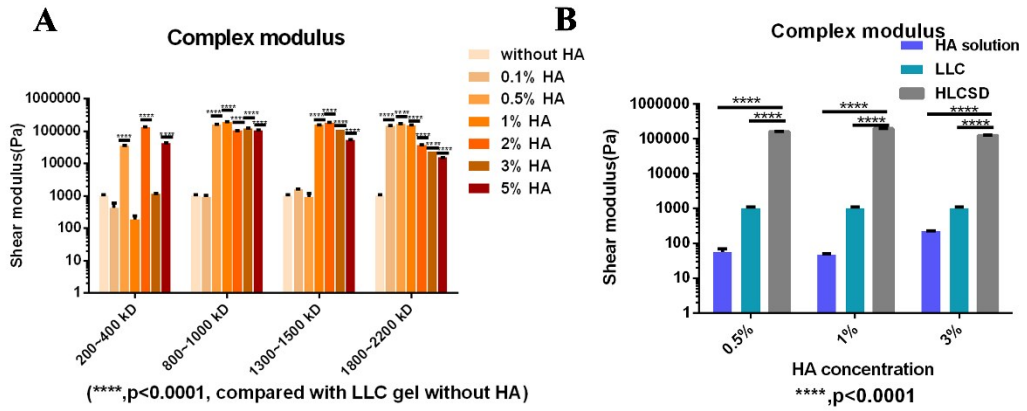

Figure S6. A. Complex modulus of HLCSD gel with different HA; B, Modulus of HA solution, LLC dressing and HLCSD dressing at different HA (MW 800~1000 kD) content.

Complex modulus of HA solution, LLC and HLCSD at different HA (800~1000 kD) content was evaluated to briefly compare their individual and combined modulus. The results showed that both individual HA and LLC matrix possessed low modulus at approximately 0.1-1 kPa. However, after HA addition into LLC matrix (HLCSD), the combination of HA with LLC in HLCSD demonstrated modulus 2-3 magnitude higher than pure HA solution or LLC matrix without HA (Figure S6B), which indicated a robust crystal structure with modulus similar to normal skin. It could be inferred that the addition of HA strengthen the lattice structure formed by LLC self-assemble and increase gel modulus. The molecular chain of HA may insert into the bicontinuous lipid layer of LLC and bind the adjacent unit to maintain the integrity of lattice structure. Hence the modulus was strengthened and enhanced with the combination of HA and LLC. The result further necessitated the integration of HA into LLC precursor in this

study.

2.3 Drug release behavior

The drug release behavior of formulation with different HA content was shown in Figure S7A. PFD was released from HLCSD mainly through diffusion. The size of water channels in HLCSD allowed a quick and complete diffusion of the water-soluble PFD.[1] Of note, the diffusion of PFD could be significantly affected by the medium exchange rate.[2] Therefore, the dialysis bag method with 10 mL release medium at 100 rpm was adopted to restrict the medium exchange rate in the *in vitro* drug release investigation for the simulation of in vivo conditions.

It was worth mentioning that the tissue fluid exchange on the wound was much slower. Therefore, the rapid drug release in the early stage in the *in vitro* drug release investigation probably resulted in a prolonged drug release with a longer retention on the wound *in vivo*. This in turn provided a long-acting regulation of collagen synthesis and further control of scar formation, with a consecutive dressing replacement every two days. Further study will be conducted to optimize the method of the *in vitro* drug release.

The effect of HLCSD composition on drug release was well investigated. The addition of HA to LLC precursor appeared to have insignificant effect on drug release rate and extent. Instant release of drug was achieved in 48 h. The enhanced water absorption in phase transition could enlarge water channel and thus facilitate drug

diffusion through the water channel of LLC. Moreover, HA molecular inserted into the LLC lipid bilayer may bond the adjacent lattice and make the structure more compact, which is against the drug release from the system. It may be the neutralization of these two effect that make the drug release behavior barely affected by HA addition. Different drug loading from 0.5% to 3% (w/w) also showed no significantly difference on drug release indicating that drug content at this range was far from saturation and hardly had an impact on drug release behavior (**Figure S7B**).

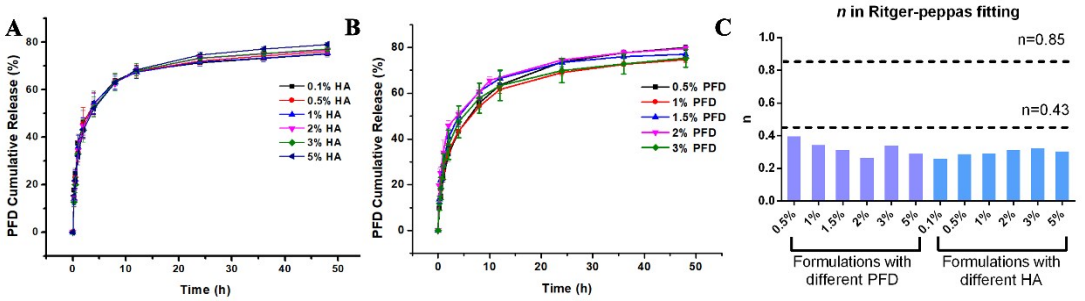

Figure S7. A. PFD release behavior from HLCSD with various HA content (MW 800~1000 kD); B, PFD release behavior from HLCSD with various drug loading. C, *n* value of the fitted Ritger-peppas model from different curves.

As shown by the results, different drug loading from 0.5% to 3% (w/w) had no significantly difference on drug release. According to the results of the cell experiment in manuscript, PFD showed obvious proliferation inhibitory effect over 500 μg/mL (for HSFb) or 1000 μg/mL (for HaCat). For the sake of safety, the PFD content was determined at a lower level. Considering the volume of exudation on wound,[3] 10-fold dilution of PFD was reasonably anticipated during the administration of 100 μL HLCSD precursor. For HLCSD with 0.5% PFD (5000 μg/mL), the exposure of PFD to wound could be controlled to be less than 500 μg/mL, which was regarded biocompatible. For higher PFD content, the exposure concentration was hard to control.

Biocompatible experiment indicated that the administration of HLCSD with 0.5% PFD showed no inhibitory effect to in the model rat. This dosage of administration was also proved efficient by VSS score and immunohistochemical analysis for scar prophylaxis in the pharmacodynamics study. Therefore, PFD content at 0.5% was appropriate and chosen for scar prophylaxis.

The fitting results of drug release curves showed that PFD release from HLCSD could be best fitted in Ritger-peppas model. Further investigation of index *n* demonstrated that the loading of PFD or addition of HA did not significantly change the release behavior. *n* value ($n < 0.43$) indicated that PFD was released from lyotropic liquid crystal structure through Fick diffusion (Figure S7C).

Table S1. PFD release curve fitting in formulation with different PFD

R^2	0.5% PFD	1% PFD	1.5% PFD	2% PFD	3% PFD	5% PFD
Zero-order	0.7331	0.7138	0.6593	0.6867	0.6543	0.6184
First-order	0.8689	0.8328	0.7881	0.829	0.7837	0.7192
Higuchi	0.9045	0.8904	0.8496	0.8668	0.8436	0.8103
Hixson crowell	0.8265	0.7948	0.7465	0.784	0.7414	0.6859
Ritger-peppas	0.9562	0.9541	0.9361	0.9554	0.9288	0.9046

Table S2. PFD release curve fitting in formulation with different HA

R^2	0.1% HA	0.5% HA	1% HA	2% HA	3% HA	5% HA
Zero-order	0.5861	0.5946	0.5858	0.6023	0.609	0.6469
First-order	0.7087	0.7254	0.7152	0.7406	0.745	0.788

Higuchi	0.7852	0.7931	0.7847	0.7994	0.8066	0.8386
Hixson crowell	0.6681	0.6819	0.6724	0.6953	0.7003	0.742
Ritger-peppas	0.905	0.9033	0.888	0.8894	0.8997	0.9328

Reference

1. R. Dorati, J. L. Medina, P. P. DeLuca and K. P. Leung, *AAPS PharmSciTech*, 2018, **19**, 2264-2275.
2. J. Siepmann and F. Siepmann, *Journal of Controlled Release*, 2012, **161**, 351-362.
3. K. Vowden and P. Vowden, *British journal of community nursing*, 2003, **8**, 4-13.