Supplementary materials

*In-situ* Gelation of rhEGF-containing Liquid Crystalline Precursor with Good Cargo Stability and System Mechanical Properties: A Novel Delivery System for Chronic Wounds Treatment

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S1 Formulation details and the key physical properties of rhEGF-LLCP<sub>1</sub>–rhEGF-LLCP<sub>8</sub>

The formulation compositions of all prepared formulations (rhEGF-LLCP<sub>1</sub>–rhEGF-LLCP<sub>8</sub>) and the apparent viscosity and gelation time thereof are summarized in Table S1.

Table S1 Formulation compositions of rhEGF-LLCP<sub>1</sub>–rhEGF-LLCP<sub>8</sub>, and their apparent viscosity and gelation time (n = 3).

<table>
<thead>
<tr>
<th>Formulation</th>
<th>GMO (%)</th>
<th>EtOH (%)</th>
<th>DMAC (%)</th>
<th>PEG400 (%)</th>
<th>Apparent viscosity (mPa·s)</th>
<th>Gelation time (s)</th>
</tr>
</thead>
<tbody>
<tr>
<td>rhEGF-LLCP&lt;sub&gt;1&lt;/sub&gt;</td>
<td>58.4</td>
<td>14.6</td>
<td>0</td>
<td>27.0</td>
<td>43.99 ± 0.91</td>
<td>&lt; 1</td>
</tr>
<tr>
<td>rhEGF-LLCP&lt;sub&gt;2&lt;/sub&gt;</td>
<td>51.1</td>
<td>0</td>
<td>21.9</td>
<td>27.0</td>
<td>41.15 ± 2.55</td>
<td>&lt; 1</td>
</tr>
<tr>
<td>rhEGF-LLCP&lt;sub&gt;3&lt;/sub&gt;</td>
<td>80.0</td>
<td>20.0</td>
<td>0</td>
<td>0</td>
<td>28.99 ± 0.87</td>
<td>&lt; 1</td>
</tr>
<tr>
<td>rhEGF-LLCP&lt;sub&gt;4&lt;/sub&gt;</td>
<td>70.0</td>
<td>0</td>
<td>30.0</td>
<td>0</td>
<td>36.77 ± 0.66</td>
<td>&lt; 1</td>
</tr>
<tr>
<td>rhEGF-LLCP&lt;sub&gt;5&lt;/sub&gt;</td>
<td>70.0</td>
<td>30.0</td>
<td>0</td>
<td>0</td>
<td>20.22 ± 0.69</td>
<td>4</td>
</tr>
<tr>
<td>rhEGF-LLCP&lt;sub&gt;6&lt;/sub&gt;</td>
<td>60.0</td>
<td>40.0</td>
<td>0</td>
<td>0</td>
<td>12.12 ± 0.38</td>
<td>6</td>
</tr>
<tr>
<td>rhEGF-LLCP&lt;sub&gt;7&lt;/sub&gt;</td>
<td>80.0</td>
<td>0</td>
<td>20.0</td>
<td>0</td>
<td>48.97 ± 1.13</td>
<td>1</td>
</tr>
<tr>
<td>rhEGF-LLCP&lt;sub&gt;8&lt;/sub&gt;</td>
<td>60.0</td>
<td>0</td>
<td>40.0</td>
<td>0</td>
<td>23.88 ± 0.59</td>
<td>3</td>
</tr>
</tbody>
</table>

S2 Validation of the rhEGF assay by ELISA kit

A parabolic calibration curve for rhEGF quantification was established within 0.82~200.00 pg/mL, and the equation was \( y = -0.1917 x^2 + 0.9662 x - 0.6900 \) (\( R^2 = 0.9997 \)). The accuracy of the assay was within 100.00~100.63%, and the RSD values determined in intra-day precision and inter-day precision was all below 2.14%. The recovery of the assay was within 99.56~100.33%. The rhEGF assay by ELISA kit was validated and used in subsequent studies.
S3 Models for the fitting of *in vitro* release profiles

The *in vitro* release profiles of rhEGF-LLCP₁–rhEGF-LLCP₄ were fitted by zero-order model, first-order model, Higuchi model and Ritger-Peppas model. The formulae of these models are listed in Table S2.

<table>
<thead>
<tr>
<th>Model name</th>
<th>Zero-order</th>
<th>First-order</th>
<th>Higuchi</th>
<th>Ritger-Peppas</th>
</tr>
</thead>
<tbody>
<tr>
<td>Formula</td>
<td>$M_t / M_{\infty} = kt$</td>
<td>$M_t / M_{\infty} = 1 - e^{-kt}$</td>
<td>$M_t / M_{\infty} = kt^{0.5}$</td>
<td>$M_t / M_{\infty} = kt^n$</td>
</tr>
</tbody>
</table>

S4 Sterility test

The sterility test was performed under the guidance of Chinese Pharmacopoeia 2015. Briefly, fluid thioglycollate medium and soya-bean casein digest medium were employed to detect the contamination of micro-organisms. The samples were seeded in the media and cultivated for 14 days. Aerobes were incubated under 30~35°C, and anaerobes and fungi were incubated under 20~25°C. The microbial growth was detected by visual or microscopic inspection.

In this study, no microbial growth was found in the samples after electron irradiation sterilization or 3-month storage.

S5 More information of dosing protocols for *in vivo* studies

The procedures used to establish and treat a standardized full-thickness wound (10 mm × 10 mm) is shown in Figure S1.
Figure S1 (A) Protocols of the animal experiments; (B) Typical images taken during the procedures.

S6 Scoring standard for inflammatory recovery and re-epithelialization process

The level of inflammatory recovery and re-epithelialization process was semi-quantitatively scored from the panoramic scan of hematoxylin and eosin (H&E) stained sections which were taken from the center of each wound. Scoring was performed by two observers who were experienced yet blinded in the study, using a visual scoring rubric (Table S3 and S4).
Table S3  Histological scoring standard for the evaluation of inflammation recovery.

<table>
<thead>
<tr>
<th>Score</th>
<th>Histopathologic morphology</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>No inflammatory response</td>
</tr>
<tr>
<td>1</td>
<td>Acute inflammation: formation of fibrin clot and pyogenic membrane, migration of leucocytes and polynuclear neutrophils</td>
</tr>
<tr>
<td>2</td>
<td>Predominance of diffuse acute inflammation: predominance of granulation tissue, vascular neogenesis, and almost presence of the pyogenic membrane</td>
</tr>
<tr>
<td>3</td>
<td>Predominance of chronic inflammation: fibroblast proliferation</td>
</tr>
<tr>
<td>4</td>
<td>Resolution and healing: reduction or disappearance of chronic inflammation, although occasional round cells may persist</td>
</tr>
</tbody>
</table>

Table S4  Histological scoring standard for the evaluation of re-epithelialization process.

<table>
<thead>
<tr>
<th>Score</th>
<th>Re-epithelialization process</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>Re-epithelialization at the edge of the wound</td>
</tr>
<tr>
<td>1</td>
<td>Re-epithelialization covering less than half of the wound</td>
</tr>
<tr>
<td>2</td>
<td>Re-epithelialization covering more than half of the wound</td>
</tr>
<tr>
<td>3</td>
<td>Re-epithelialization covering the entire wound, irregular thickness</td>
</tr>
<tr>
<td>4</td>
<td>Re-epithelialization covering the entire wound, normal thickness</td>
</tr>
</tbody>
</table>

S7 Molecular compactness analysis

The molecular compactness analysis was achieved by molecular dynamics (MD) simulations. Briefly, three-dimensional (3D) structures of GMO and PEG400 molecules were constructed using UCSF Chimera software (University of California, CA) (Pettersen et al., 2004). Structures were optimized with AM1-BCC charges assigned. The initial configuration of the GMO system and the GMO-PEG400 system were established by GROMACS software version 2016.4 (Groningen University, Groningen, Netherlands) (Abraham et al., 2015). GMO and PEG400 molecules were randomly placed
in a cubic box (4 nm × 4 nm × 4 nm). For the former system, 44 GMO molecules were used, while for
the latter system 32 GMO molecules and 29 PEG400 molecules were used. They were surrounded by
water molecules.

MD simulations were performed in GROMACS with AMBER ff99SB-ILDN force field. Each
system was solvated using TIP3P water model (Jorgensen et al., 1983) and SPC216 solvent configuration
(Berendsen et al., 1981). Periodic boundary conditions (PBC) were set to allow free motion along the
3D lattice. Since both systems were electronic neutral, no counter ions were needed. Nonbonded van der
Waals interactions were calculated using the Lennard-Jones 12-6 potentials with a 1.0 nm cutoff, while
long-range electrostatics were treated using the Particle Mesh Ewald (PME) algorithm (Darden et al.,
1993). Dispersion correction was performed to account for energy and pressure cutoffs following the
Verlet cutoff-scheme (Verlet, 1967).

The LINCS algorithm was applied to constrain all bonds (Hess, 2008). Firstly, a steepest descent
minimization of 500,000 steps was performed to remove improper atom contacts. Convergence was
reached when the maximum force of no greater than 1000 kJ mol⁻¹ nm⁻¹ for any atom. Subsequently, a
two-step equilibration phase was carried out to simulate both constant volume (NVT) and constant
pressure (NPT) ensembles, respectively. Both phases were simulated for 100 ps maintained at 300 K
through the velocity rescaling (v-rescale) algorithm (Bussi et al., 2007), a modified Berendsen
thermostat. NPT equilibrium was isotropically controlled at 1.0 bar using Parrinello-Rahman barostat
(Parrinello and Rahman, 1981). Following NPT equilibration, MD simulations were conducted for 2 ns
for each system using the same conditions as described. The integration time step applied was 2.0 fs with
the coordinates saved every 1.0 ps. Radial gyration ($R_g$) analysis was carried out to study the compactness
of the system.

The MD simulations results were as follows. The original structure of the systems in the cubic box
is depicted by Figure S2. Two nanoseconds of MD simulations were performed, and root-mean-square
deviation (RMSD) values were recorded during the process (Figure S3). It was shown that the RMSD
values basically reached a pseudo stable state after 1,000 ps, and therefore 2 ns was sufficient for system
equilibration (Kim et al., 2003).
Figure S2 The original structure of GMO-GMO system (left) and GMO-PEG400 (right) system in the cubic box. Large blue balls: GMO molecules; Large purple balls: PEG400 molecules; Small pink balls: water molecules.

Figure S3 RMSD values recorded during the 2-ns process of MD simulations.

The structure after MD simulations is illustrated by Figure S4. In both systems, the aliphatic chains of GMO interacted with each other by hydrophobic force, and were covered by the polarized moieties. The hydroxyl groups and/or carbonyl groups formed hydrogen bonds with each other. Radius of gyration ($R_g$) of these two systems were calculated, and the results are demonstrated by Figure S5. $R_g$ was an indicator of the system volume, and a higher $R_g$ meant a less molecular compactness (Lobanov et al., 2008). GMO-GMO system and GMO-PEG400 system exhibited $R_g$ values of about 1.6 nm and 1.9 nm,
respectively, elucidating that the molecular compactness of GMO-PEG400 system was less than that of GMO-GMO system.

![Figure S4](image)

**Figure S4** The structure of GMO-GMO system (A) and GMO-PEG400 system (B) after MD simulations. The green dashed lines represented hydrogen bonds.

![Figure S5](image)

**Figure S5** The $R_g$ values of GMO-GMO system and GMO-PEG400 system.

### S8 In vitro release profiles after storage

The *in vitro* release profiles before and after 3-month storage of rhEGF-LLCP$_1$ and rhEGF-LLCP$_2$ are illustrated by **Figure S6**. No significant change was observed after the storage, confirming the storage stability of rhEGF-LLCPs.
Figure S6 The *in vitro* release profiles before and after 3-month storage of rhEGF-LLCP₁ (A) and rhEGF-LLCP₂ (B) ($n = 3$).

S9 LVR determination and peripheral discussion about the rheological measurements

The LVR of rhEGF-LLCG₁ and rhEGF-LLCG₂ is determined, and shown in Figure S7. It was demonstrated that $G'$ remained unchanged within 0–0.6% strain, and thus this range was regarded as the LVR.

Figure S7 Results of LVR determination.

Generally, the chronic wounds needed to be bandaged to prevent infection. Therefore, the system mechanical rigidity should be strong enough to resist the bandage pressure to prevent gel movement and breakage. Venous (varicose or stasis) ulcers was a kind of chronic wound, and its main treatment was
ambulatory compression treatment (Gohel et al., 2007). Patients would be treated with multi-layered compression bandaging with 40 mm Hg of pressure (Guest et al., 1999). As shown in Figure 3 A and B, the $G'$ of rhEGF-LLCPs was much higher than 40 mm Hg (5.33 kPa) in the whole frequency range, demonstrating that they could resist the pressure of compression bandage in compression therapy. Therefore, the rhEGF-LLCPs were sufficient for chronic wounds which were treated with conventional bandage.

Also, the gel strength determined by textural measurements were 15.67 ± 0.25 kPa and 16.81 ± 1.15 kPa for rhEGF-LLCG$_1$ and rhEGF-LLCG$_2$, respectively, which were strong enough (> 5.33 kPa) to maintain stable structure and resist the bandage pressure.

**S10 Typical images of cell migration experiments**

Typical images of cell migration at different time intervals in Balb/C 3T3 and HaCaT are shown in Figure S8 and S9, respectively.

![Figure S8](image-url) **Figure S8** Typical images of cell migration at different time intervals in Balb/C 3T3.
Figure S9 Typical images of cell migration at different time intervals in HaCaT.

References