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Rapid Gelation from Lipoic Acid/Trometamol Binary Assembling for Realizing 3D Printing in Poly(Lipoic Acid)-based Hydrogel Bandages Production

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Abstract

Developing a strongly adhesive, easily removable and robust bandage is valuable in trauma emergencies. Poly(lipoic acid) (PLA)-based adhesives have been well-developed, however, the additive manufacturing and depolymerization of which remains a challenge. Herein, LA and trometamol are found to rapidly gel into a supramolecular hydrogel at room temperature with injectability and 3D printing potential. Meanwhile, the synthesized LA-grafted hyaluronic acid (HALA) and cellulose nanocrystals (CNC) are involved not only to optimize 3D printing, but also to be the macromolecular covalent crosslinker and giant physical crosslinker to co-polymerize with LA after printing to effectively promote fidelity and prevent the inverse closed-loop depolymerization of PLA in water. The hydrogel bandage exhibits strong adhesion (the adhesion strength was ~10 times higher than FibrinGlu) and enhanced elastic modulus and toughness, as well as immediate self-healing ability. Meanwhile, the hydrogel bandage can be removed with no residue by water flushing, showing protection to neo-tissue during dressing replacement. The in vivo healing of the incision and full-layer wounds confirms that the application of the hydrogel bandage significantly promoted wound healing by closing the wound, forming a physical barrier and providing an anti-inflammatory effect, showing great potential in future clinical applications.

Keywords

α-lipoic acid; trometamol; binary assembling; 3D printing; hydrogel adhesive
1. Introduction

Skin injuries caused by disasters, wars, etc. are usually open wounds with uncontrolled bleeding, which has always been an important cause of death.\(^1\) As the “gold standard” in clinical surgery, sutures, staples and clips cannot be effectively applied outside the hospital. In addition, they will cause detrimental effects on the surrounding tissue, and show limit in sealing wounds in friable tissues. Bioadhesives have been extensively studied for their ease of use and versatility as an alternative to sutures and staples.\(^2\) For trauma first aid, the bioadhesives should exhibit high adhesive force to effectively close the wound, easy detachment for the subsequent therapies, robust mechanical performance to form a physical barrier.\(^3\) Adhesive hydrogels have received widely attention for their biocompatibility and biomimetic characteristics.\(^4\) As the candidates for surgical sealants, they can form covalent bonding or non-covalent attractions to tissue surfaces.\(^5\) At the same time, the enhanced mechanical performance not only provides a sufficient physical barrier to wounds but also acts as a supplement to interface adhesion to further promote adhesion strength.\(^6\) Thus, developing a strongly adhesive, easily removable, and robust hydrogel bandage is valuable for pre-hospital in trauma emergencies.

\(\alpha\)-Lipoic acid (LA) is a small molecule coenzyme necessary for the aerobic metabolism of mitochondria, which has natural biological safety. It has remarkable electrophilicity and the ability to react with free radicals, can eliminate superoxide and peroxide free radicals.\(^6\) As an effective antioxidant, LA has been applied in clinics. Oxidation, heat, light, and mercaptan can trigger disulfide bond cleavage and exchange reactions in the dithiolane ring to initiate the ring-opening polymerization reaction to prepare poly(lipoic acid) (PLA), which has been reported to possess reliable adhesion.\(^7\) Adhesives preparation, including hydrogel adhesives preparation based on melting polymerization of LA have been widely reported.\(^8\) However, on the one hand, the currently reported LA polymerization methods mainly include thermal and light-stimulated polymerization, solvent volatilization-induced assembly polymerization, etc., which often require multiple operations or long duration. H. Huang et al established deprotonation-promoted ring-opening polymerization of LA as a highly effective and simple method due to the long-range electronic effect and nucleophilic carboxylate, which could be carried out at room temperature but also needed long duration.\(^9\) Among these methods, it is difficult to find the right timing for 3D printing during the
polymerization process. Once LA underwent effective polymerization, it normally has high viscosity and high adhesion to various substrates, which not only affects extrusion but also seriously interferes with path execution. Currently, only a few studies have developed fused deposition modeling (FDM) based on LA melt-polymerization.\cite{21} 3D printing of LA-based hydrogel bioadhesive has not been reported. Considering that 3D printing is important for the molding and processing of biomaterials, developing a new, general but simple approach for realizing 3D printing in the production of PLA-based hydrogel adhesives is significant but challenging.

On the other hand, the as-polymerized PLA-based supramolecular polymers are metastable due to the inverse ring-closing de-polymerization, leading to adhesion failure of PLA-based adhesives. Although PLA-based supramolecular polymers can be stabilized by introducing multiple double-bond monomers, metal ions, ionic liquids, chlorinated organic solvent, phytic acid, etc.,\cite{7,29-32} the unreacted small molecules are difficult to be removed, posing a biosafety concern for biomedical application.\cite{Error! Reference source not found.1} Pioneer attempts have been recently made to stabilize PLA-based hydrogel bioadhesives by introducing polyphenols such as dopamine, tannin, etc., showing better biocompatibility and can be used for wound healing.\cite{Error! Reference source not found.5,33} Nevertheless, the introduction of dopamine, tannin, etc. makes the removal of adhesives more complex.\cite{Error! Reference source not found.} Thus, it is urgent to develop biocompatible methods to effectively stabilize PLA-based adhesives with easily removal feature for biomedical applications.

Herein, LA was found to assemble with trometamol (Tris-base) in water to rapidly form a binary self-assembled hydrogel at room temperature for the first time. Based on this assembling, the controllable 3D printing of the hydrogel adhesive was realized. At the same time, hyaluronic acid grafted with LA (HALA) was synthesized and employed as a macromolecular crosslinker to co-polymerize with LA in the aqueous phase, with the presence of cellulose nanocrystals (CNC) as the giant physical crosslinker. For one thing, HALA and CNC associated with LA-assembled hydrogel could optimize the 3D printing. For another, HALA associated with CNC could prevent PLA chains from depolymerization by hydrogen bonding, leading to a long-term stabilized hydrogel adhesive. Besides, the presence of HALA endowed the hydrogel with higher cohesion and elasticity. Further introduction of cellulose nanocrystals (CNC) formed hydrogen bonding with PLA, endowing the hydrogel with robust features to withstand large deformation. The composite hydrogel bandage
showed strong adhesion to different tissues and enhanced mechanical performances. More importantly, 
the hydrogel bandage could be removed with almost no residue by water flushing, showing protection 
to neo-tissue during dressing replacement (Figure 1).

*Figure 1. Schematic representation of hydrogel bandage preparation and functions.* LA and trometamol 
assembling to realize molding & processing through 3D printing. Stabilization of PLA hydrogel was realized by 
HALA and CNC. The robust hydrogel bandage was used in wound healing to protect the wound and reduce
inflammation.

2. Materials and methods

2.1 Materials

α-Lipoic acid (LA), trometamol, and 4-Dimethylaminopyridine (DMAP) were purchased from Aladdin Biochemical Technology Co., Ltd. Hyaluronic acid (HA, $M_w = 4 \times 10^4$) was purchased from Bloomage BioTechnology Co., Ltd. Cellulose nanocrystals (CNC) was purchased from ScienceK Co., Ltd. N,N'-Carbonyldiimidazole (CDI) was purchased from J&K Scientific Co., Ltd.

2.2 Preparation of LA self-assembled supramolecular hydrogel

Trometamol was dissolved in distilled water at room temperature to prepare a 20% (w/v) solution (the trometamol solution at this concentration was used as the solvent in the present study). LA was dissolved in trometamol solution with the concentration of 50% (w/v) and stood for 5 minutes to yield a bulk hydrogel.

The rheological properties of the gelled LA solution (LA 50% w/v, trometamol 20% w/v) were tested using a rheometer (DHR-3, TA, USA) with a 12 mm flat steel plate fixture at room temperature. The gelled LA solution was placed onto the rheometer fixture and tested for their modulus-frequency relationship with a frequency sweep range of 0.01-100 Hz and a strain of 0.1 %. Strain sweep measurements were carried out at a constant frequency of 1 Hz with strain ranging from 0.01 to 1000%. The viscosity change with the shear rate was continuously increased from 0.001 to 100 s$^{-1}$ to evaluate their shear-thinning behavior.

The structure and binding energy of LA and trometamol forming complexes were studied based on density functional theory. All calculations were performed using the ORCA (version 5.0.4) program at the B3LYP-D3 and def2-SVP basis set levels for configuration optimization, while single point energy calculations were performed using the more accurate wB97M-V functional at the def2-TZVP level. All calculations were carried out under implicit water solvents (The Conductor like Continuum Polarization Model CPCM Solvation Models (SMD) have been implemented for water dissolution). The binding energy of a compound could be calculated by the following formula: $E_{\text{binding}} = E_{t-l} - E_l - E_t$. Among them, $E_{\text{binding}}$ was the single point energy of the complex formed by LA and trometamol, while $E_l$ and $E_t$ were the individual single point energies of compounds LA and trometamol. In order to find
the most stable conformations of LA and trometamol, software xTB 6.3 and GFN0-xTB method were used to conduct molecular dynamics (MD) simulation calculations on the possible conformations. The dynamic simulation was carried out using the NVT ensemble at a temperature of 298.15 K and 1 atmospheric pressure, with a step size of 1 fs and a total duration of 10 ps. One conformation was output every 10 steps, resulting in a total of 2000 conformations. Then, the software Molclus was used to screen it and obtain 200 low-energy conformations. Further configuration optimization was performed using the GFN2 xTB method, and the top 10 lowest energy conformations were screened using Molclus. Finally, the structure was optimized using r2SCAN-3C density functional theory to obtain the lowest energy and most stable conformation. The weak interactions between molecules were calculated using Multiwfn software (3.8 (dev)) based on the IGMH method, and the results were analyzed using software VMD.

A self-built and designed 3D bioprinting system was used for 3D printing. LA (50% w/v), HALA and CNC were mixed in trometamol solution (20% w/v) in a 5 mL syringe and placed at room temperature for 10 min to yield a bulk hydrogel. The stepper motor was used to drive the syringe plunger. The bulk hydrogel was printed from nozzles (from 18 G to 21 G) at room temperature. The pneumatic pressure was 40 kPa. The printing speed was 10 mm/s, while the print path was generated by the numerical control system. After printing, the printed hydrogel was heated at 70 °C for 3 h for the polymerization to yield a printed hydrogel.

2.3 PLA adhesive patch preparation

Trometamol was dissolved in distilled water at room temperature to prepare a 20% (w/v) solution (the trometamol solution at this concentration was used as the solvent in the present study). LA was added and fully dissolved in trometamol solution to form a yellow and transparent solution, which was heated at 70 °C for 3 h and cooled to room temperature.

LA was dissolved in trometamol solution to prepare solutions with different concentrations of 0.001, 0.01, 0.05, 0.1, 0.3, 0.5, 0.75, and 1 g/ml. After reacting at 70 °C for 6 h, the reactions were diluted to 0.001 g/mL. A UV spectrophotometer was employed to test the UV absorption spectra of LA at different concentrations.

SmartLab intelligent Diffractometer (XRD, Rigaku, Japan) was used to analyze LA and PLA. An
appropriate amount of LA powder sample and a freeze-dried PLA solid sample was placed on a slotted flat glass sheet with gentle pressing. The crystal structure of the samples was measured. The scanning range of 2θ was 10 °-90 °, and the scanning speed was 10 °/min.

The temperature-dependent rheological behavior of LA was tested at different concentrations (30%, 40%, and 50% (w/v)). The three samples were subjected to temperature scanning using the rotary Rheometer (DHR-3, TA, USA) with a 12 mm clamp. The strain was set to 1%, and the scanning frequency was maintained at 1 rad/s. The temperature scanning test was carried out at a heating rate of 1 °/min between 10 °C and 90 °C.

2.4 PLA-HA-CNC hydrogel bandage preparation

HALA was synthesized by grafting LA to HA via the esterification reaction according to our previous work. HALA and LA were dissolved in trometamol solution in sequence, followed by the addition of CNC to disperse evenly and heated at 70 °C for 3 h to prepare hydrogels, which were cooled to room temperature for study. The feeding conditions for the preparation of hydrogel were shown in the Table 1.

<table>
<thead>
<tr>
<th>Sample</th>
<th>Component (mg)</th>
<th>Solvent (mL)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>trometamol</td>
<td>HALA</td>
</tr>
<tr>
<td>PLA-HA1</td>
<td>200</td>
<td>10</td>
</tr>
<tr>
<td>PLA-HA2</td>
<td>200</td>
<td>20</td>
</tr>
<tr>
<td>PLA-HA-CNC3</td>
<td>200</td>
<td>20</td>
</tr>
<tr>
<td>PLA-HA-CNC5</td>
<td>200</td>
<td>20</td>
</tr>
<tr>
<td>PLA-HA-CNC7</td>
<td>200</td>
<td>20</td>
</tr>
</tbody>
</table>

TEM (JEM-200cx, JEOL, Japan) and DLS (ZS90, Marlven, British) were used to characterize the morphology, size and zeta potential of CNC. FT-IR (NicoletiS10, Thermofischer, Germany) and DLS were used to characterize the interactions in hydrogels. PLA-HA1, PLA-HA2, PLA-HA-CNC3, PLA-HA-CNC5 and PLA-HA-CNC7 precursors were prepared according to the feeding conditions in Table 1. The pH values of these precursors were measured by a pH meter (PHS-3TC, Shanghai). Then, the PLA-HA2, PLA-HA-CNC3, PLA-HA-CNC5 and PLA-HA-CNC7 hydrogels after gelling under reaction conditions were immersed in distilled water for 4 h to record the pH value.
2.5 Lap-shear test

The surface of two glass slides was coated with 5% (w/v) gelatin solution with a coating size of 2.5 cm × 2.5 cm after air drying for use. For the test of PLA adhesives, the PLA adhesives prepared from 30%, 40%, and 50% (w/v) LA solution were evenly spread on the gelatin-coated glass surface with an area size of 2.5 cm × 2.5 cm. For the test of PLA-HA1, PLA-HA2, PLA-HA-CNC3, PLA-HA-CNC5 and PLA-HA-CNC7 hydrogels, the hydrogels with the size of 2.0 cm × 1.0 cm × 0.5 cm were adhered between the two gelatin-coated glasses. For the test of PLA-HA-CNC5 during removal, the PLA-HA-CNC hydrogels with the size of 2.0 cm × 1.0 cm × 0.5 cm were adhered between the two gelatin-coated glasses, which were then immersed in water. After different duration, the glasses were subjected to the lap-shear test. The stretching rate in all lap-shear tests in the present study was set as 50 mm/min.

2.6 Self-healing test

The self-healing behavior of the hydrogel was evaluated by cyclic measurement of its storage modulus and loss modulus at 1% and 500% strain. Each test was carried out at a frequency of 1 Hz and a scanning time of 200 s.

2.7 Bursting pressure test

The burst pressure test was carried out according to the reported method. Gelatin solution (5% (w/v)) was prepared and applied evenly to the rubber tube that connected to the syringe. After air drying at room temperature, a hole with the diameter of 1 cm was created in the position where was coated with gelatin. Then, the hydrogel with the thickness of 2 mm was adhered to cover the hole for 5 min for the measurement of the burst pressure. The peak pressure before pressure loss was considered as the burst pressure. After the hydrogel was blasted, it was kept for 5 min, and then experienced the measurement for the second burst pressure. All measurements were repeated three times.

2.8 In vitro biocompatibility test

Rat fibroblasts were purchased from Bluebio Biology (Shanghai, China) and cultured in Dulbecco’s Modified Eagle’s medium (DMEM) with 10% fetal bovine serum (FBS) at 37 °C with 5% CO₂. Cells were seeded in the 96-well plates and after 24 h. The hydrogel particles with the same weight were
placed into wells (one per well) to co-cultured with cells. A cell counting kit-8 (CCK-8, Dojindo, Japan) assay was used at 1, 3, 5, and 7 days after co-culture. The hydrogel particles and culture medium were removed and the wells were washed 3 times with PBS. Then 10 μL of CCK-8 reagent was added into each well and incubated for 90 min. The absorbance was measured at 450 nm with a microplate reader (Tecan, Switzerland).

At the same time, the cell viability was observed using a LIVE/DEAD viability/cytotoxicity kit (Beyotime, China). The hydrogel particles and culture medium were removed and the wells were washed 3 times with PBS. The staining solution containing 1 μM calcein AM and 1 μM propidium iodide was added into the well and the cells were incubated for 30 min at 37 ℃ in the dark. Live (green stain) and dead (red stain) cells were imaged using an inverted fluorescence microscope (Carl Zeiss Meditec, Germany).

2.9 Mechanical tests

The mechanical properties of the hydrogel were characterized by the rheological test. Cylindrical hydrogel samples were prepared with a diameter of 12 mm and a thickness of 5 mm, which were placed on the center of the sample table of the rotary Rheometer with a 12 mm clamp. The test was carried out at 25 ℃ using frequency scanning mode (1% strain, 0.01-100 rad/s).

A dynamic thermo mechanical analyzer (DMA 500) was employed for the compression tests. The cylindrical hydrogels with a diameter of 9 mm and a thickness of 5 mm were subjected to the compression test with a strain range of 0-100% to get the stress-strain curves. The compressive strength of the hydrogel sample referred to the stress of the sample at the fracture strain, which was recorded as δ\text{max}. Compression moduli were calculated according to the following formula:

\[ E = \frac{\delta_{10} - \delta_5}{\varepsilon_{10} - \varepsilon_5} \]

δ5 and δ10 were the stress values of the hydrogel samples at the strain of 5% and 10%, respectively.

Hydrogel samples were then subjected to the cyclic compression test at room temperature. The cylindrical hydrogels were placed between the compression plate clamps of the dynamic thermomechanical analyzer. The compression rate and recovery rate were both 10%/min. After 20 cycles, the residual stress percentage was calculated according to the following formula:
Residual Stress Percentage (\(\%\)) = \(\frac{\varepsilon_{\text{max}} - \varepsilon_0}{\varepsilon_{\text{max}} - 1}\) \(\times 100\%\)

Tensile tests were performed on hydrogel samples with the size of 20 mm \(\times\) 10 mm \(\times\) 2 mm with a stretching rate of 10 mm/min on a universal testing machine (Instron-5943). The toughness was calculated from the area under the stress-strain curve. The universal testing machine (Instron-5943) was also used to carry out cyclic tensile tests on hydrogel samples with the cyclic tensile rate of 10 mm/min. The samples were restored to the original position at the same rate. The energy dissipation of the hydrogel and the recoverable performance were recorded and calculated.

2.10 Wound healing in vivo

All of the animal care, housing, and study procedures for rats complied with the ARRIVE guidelines, and were performed in accordance with the National Research Council's Guide for the Care and Use of Laboratory Animals. The animal experiment was approved by The First Affiliated Hospital of Zhejiang University Ethics Committee (ZJ1H-2022-R-687). SD rats (male) were anesthetized by isoflurane gas, shaved and washed with betadine.

For incision repair, two incisions with a length of 2 cm were created on the back of each rat. The incision treated with hydrogel bandage was employed as the Bandage group. The hydrogel bandage was changed on day 3, 7 and 10 post-surgery. The incision treated with hemostasis only was employed as the Blank group. Rats were sacrificed on day 7 and 14 for skin tissues collection. The middle parts of the scar section were subject to H&E and Masson staining. A total of 10 rats were used in this model.

For full-thickness wound repair, two circular full-layer wounds with a diameter of 10 mm were established on the dorsum of each rat. The metal rings were covered on the wounds and sutured with the surrounding skin to fix the wounds. The wound treated with hydrogel bandage was employed as the Bandage group. The hydrogel bandage was changed on day 3, 5, 7, 9, 11 and 13 post-surgery. The wounds were washed with physiological saline to easily remove the old hydrogel bandages. The wound treated with hemostasis only was employed as the Blank group. A Simulated diagram of wounds was drawn by PowerPoint for area quantification. The proportion of wound area was obtained according to the following formula:
Proportion of wound area = \( \frac{S_t}{S_0} \times 100\% \)

S₀ represented the initial wound area on day 0, Sₜ represented the wound area on day t.

Rats were sacrificed on day 7 and 14 for skin tissues collection (5 samples in each group). Samples were fixed with 4 wt% paraformaldehyde, embedded in paraffin and sliced into pieces of 5 μm thickness. Then, the sections were histologically stained with H&E, Masson and CD31 immunohistochemical staining. Meanwhile, another 5 rats were sacrificed on day 3 for skin tissues collection. Immunofluorescence staining was performed on samples on day 3 to detect the expression of IL-1β, TNF-α, IL-4 and TGF-β1, as well as the expression of iNOS and CD206. Fluorescence images were analyzed by Image J. A total of 15 rats were used in this model.

2.11 Statistical analysis

Each measurement reported was based on a duplicate analysis of at least three independent experiments. The quantitative results were presented as mean ± SD. One-way analysis of variance (ANOVA) was used to reveal statistical differences, and p < 0.05 was considered statistically significant.

3. Results and discussion

3.1 Self-assembling of LA and trometamol

Due to the poor solubility of LA in water, trometamol, as a weakly alkaline buffer component, is commonly used to achieve the dissolution of LA. In clinics, trometamol is usually used as an adjunct for LA-injections. The trometamol salt of LA that has been shown in clinical studies to be neuroprotective.\(^{[36]}\) Trometamol is a commonly used buffer, which is usually named as Tris-base. Although trometamol is commonly used as a reagent for dissolving LA, its effect on assembly of LA has not been reported. Interestingly, it was found in the present study that when the molar ratio of LA to trometamol was in the range of 1.2~1.6:1, the viscosity of LA solution increased significantly and rapidly at room temperature. Especially when the molar ratio of LA to trometamol was 1.4~1.5:1, LA could rapidly gel within 5 minutes after dissolution at room temperature (Figure 2A). This effect of trometamol on the rapid self-gelation of LA aqueous solution has not been reported. Reducing the
dosage of trometamol would make it difficult for LA to dissolve. Increasing the dosage of trometamol
would make LA more soluble, but gelation would not occur at room temperature (Figure 2B). At the
same time, LA concentration had to be higher than 30% w/v to realize a rapid and clear gelation. In
fact, the dissolution process of LA was a slow dissolution process with a small amount of multi-step
addition. When all LA was added, it had already shown an approximate solid state. Thus, as shown in
Figure 2C, after LA total dissolution, the G’ had been already higher than G”. In addition, there was
a significant increase in G’ at room temperature along with duration, confirming that the solution
underwent a quick gelation during and after dissolution. Through the evaluation of the viscosity of LA
solution (50% w/v) with different trometamol content, it was found in Figure 2D that within the range
of gelation molar ratio, LA solution quickly self-gelled to exhibit a viscosity significantly higher than
that of LA solution (trometamol concentration = 25% w/v).
Figure 2. Self-assembling of LA and trometamol. (A,B) The effect of trometamol concentration on gelation of LA solution at room temperature. (C) The change of G' and G” with time after LA dissolution (strain 1%, frequency 1 Hz). (D) Rheological testing of viscosity. (E) FT-IR spectrums. (F) Molecular dynamics simulation. (G) The influence of triethanolamine and triethylamine on the assembly of LA. (H) Diagram of LA assembling mechanization.

The mechanization for rapid gelation was further studied. The polymerization of LA was a possible reason. Accordingly, high concentrations may trigger the polymerization of LA. However, at the same LA concentration (50% w/v), the concentration of LA in 30% w/v trometamol aqueous solution did
not trigger effective polymerization, and the solution flowability was very good. Effective LA polymerization typically requires high temperature conditions to promote the reaction towards ring opening polymerization. As shown in Figure S1, solution containing 50% w/v of LA and 30% w/v of trometamol exhibited a significant increase of $G'$ only when it underwent long duration of heating. Therefore, it was speculated that the rapid self-gelation of LA at room temperature was not dominated by LA polymerization, but the assembly of LA. FT-IR was used to study the possible mechanism leading to this phenomenon. As shown in Figure 2E, for the LA solution with the concentration of 50% w/v, when trometamol concentration was 30% w/v, due to the deprotonation of carboxyl group, the stretching vibration of the carboxyl group shifted to $\sim1560 \text{ cm}^{-1}$. The absorption peak near $\sim1700 \text{ cm}^{-1}$ corresponding to the stretching vibration of the carboxyl group of LA powder had almost completely disappeared (Figure S2). More importantly, the characteristic vibration absorption peak at $\sim1590 \text{ cm}^{-1}$ belonging to the amino group of trometamol (Figure S2) significantly shifted to $\sim1631 \text{ cm}^{-1}$, indicating that the characteristic absorption of the amino group changed after proton abstraction. However, when trometamol concentration was 20% w/v, the characteristic vibration absorption peak of the amino group shifted to $1617 \text{ cm}^{-1}$ indicating that the amino group was affected by new interaction.

Molecular dynamics simulation (MD) was performed to illustrate the stable configuration of LA and trometamol. As shown in Figure 2F, Table S1, two kinds of stable combination were obtained from the results of MD. One was that the deprotonated carboxyl group of LA interacted simultaneously with the protonated amino group and adjacent hydroxyl groups of amino group of trometamol, with the $\Delta E_{\text{binding}} = -106.24 \text{ kJ/mol}$; The other was the deprotonation of carboxyl groups in LA interacted with one hydroxyl group of trometamol, with the $\Delta E_{\text{binding}} = -98.67 \text{ kJ/mol}$. The former had a lower $\Delta E$, indicating it was more stable. The carboxylate group tended to interact with the protonated amino group and hydroxyl groups of trometamol. We thus speculated that trometamol, which has multiple hydrogen bond donor functional groups, was used as a small molecule crosslinker to link with multiple LA molecules. To verify this hypothesis, this study selected two organic bases, triethanolamine and triethylamine, to evaluate their assembly of LA (Figure 2G). The molecule structures of triethanolamine and triethylamine are similar, with the difference being that triethanolamine has three hydroxyl groups. As we speculated, at the same molar ratio, LA and triethanolamine could rapidly gel
at room temperature, but LA did not solidify after dissolution in triethanolamine. This result indicated that amine containing polyhydroxy groups could be assembled with LA to form hydrogel. In addition to the effect of polyhydroxy groups on LA assembling, the content of trometamol was also another important factor that significantly dominated the effective gelation of LA. From the molecular dynamics simulation results, it was worth noting that hydrophobic disulfide bonds also formed weak interactions with the amino groups of trometamol, which was conducive to the full dissolution of LA in water. Therefore, when the content of trometamol was high, it could achieve the dispersion of LA in water by interacting with multiple sites of LA. However, when the content of trometamol was low, its amino and hydroxyl groups would preferentially bind to the deprotonated carboxyl groups of LA, forming a form of trometamol molecule binding to multiple deprotonated carboxyl groups of LA. The dithiolane rings in the LA molecules were associated through hydrophobic interactions, thereby synergistically constructing a three-dimensional network structure with trometamol (Figure 2H).

Therefore, it was speculated that the rapid gelation of LA was dominated by the self-assembly of LA and trometamol at a certain molar ratio. Meanwhile, it was also difficult to deny that there was no ring opening reaction of dithiolane rings in this process. Because at higher concentrations and hydrophobic aggregation of the dithiolane rings, the exchange reaction of disulfide bonds was possible. However, this reaction obviously did not dominate rapid gelation at room temperature.

3.2 3D printing protocol

Although PLA-based adhesives and PLA-based hydrogel adhesives have been widely reported recently, the molding method of them is limited, which cannot meet the requirements of personalized, precise, fast, and high repeatability manufacturing. At present, 3D printing of PLA-based hydrogels is rarely reported. This is closely related to the high viscosity of LA after polymerization. While, the LA solution without polymerization cannot be used for 3D printing due to its fluidity. The rapid self-gelation of LA exhibited significant weaker adhesion before heating when compared to the polymerized hydrogel. Therefore, the present study proposed a method based on the self-gelation of LA to realize 3D printing.
Figure 3. 3D printing protocol of PLA-based hydrogels. (A) Frequency scanning to evaluate the G' and G''. (B) Strain scanning. (C) Shear viscosity with increasing shear rates. (D) Images to show the extrusion of different inks. (E) G' of PLA polymerized form LA solution with the concentration of 50% w/v during heating. (F) The appearance of PLA hydrogel with HALA/CNC, PLA hydrogel and PLA hydrogel with CNC. PLA-CNC hydrogel also was paste-like (Red line indicated the bottom end of the lifting rod). (G) G' and G'' of the hydrogels after heating for
polymerization. (H) 3D printing protocol demo.

The rheological properties of the self-gelled system were further evaluated by rheological tests. In the frequency scanning mode, the rheological test showed that the G’ of the rapidly gelled LA was greater than G’’, confirming that it had the elasticity of bulk hydrogel (Figure 3A). Most importantly, due to the reversibility of supramolecular interactions, the strain scanning mode test showed that the G’ of the gelled LA became lower than G’’ under a larger strain, indicating that it changed from solid state to fluid state (Figure 3B). At the same time, with the increase of shear rate, the viscosity of the gelled LA decreased, which indicated that it had the characteristic of shear-thinning (Figure 3C). Such rheological properties made it possible for the gelled LA to be extruded. As shown in Figure 3D, the newly dissolved LA solution could be injected, but did not have continuous extrudability due to its low viscosity, and there was no immediate plasticity after extrusion. After being placed at room temperature for 5 minutes, the extrudability of the gelled LA was significantly improved, enabling continuous extrusion and better plasticity after extrusion. However, the phenomenon of extrusion swelling was obvious.

To further optimize the printability, HALA and CNC were involved. The preparation and characterization of HALA and CNC were shown and discussed in Figure S3, S4, S5 and Table S2. The introduction of HALA and CNC into LA solution (LA 50% w/v, trometamol 20% w/v) would also lead to the same rapid gelation phenomenon. The rheological behaviors of the gelled LA/HALA/CNC were similar to the gelled LA. However, the G’ and viscosity of the gelled LA/HALA/CNC were higher than those of the gelled LA (Figure 3A,B,C). As shown in Figure 3D, after being placed at room temperature for 5 minutes, the extrudability of the gelled LA/HALA/CNC was significantly improved, enabling continuous extrusion without significant extrusion swelling. At the same time, the extruded filament possessed better plasticity.

However, due to the limited interaction in the LA-assembled hydrogel, its mechanical strength was both weak, leading to another printing issue, fidelity. Previous studies have shown that ring opening polymerization (ROP) of LA could be triggered by heating.\[^{37}\] Similarly, in the aqueous phase, dissolved LA with the presence of HALA and CNC experienced ROP, with a significant increase in storage modulus (Figure 3E). The effect of LA concentration on the polymerization process was studied and showed in Figure S6. The polymerized hydrogel after heating possessed significantly
enhanced self-support (Figure 3E). Of note, the presence of HALA and CNC not only optimized the extrusion of the ink but also ensured the stable plasticity and fidelity after polymerization. As shown in Figure 3F, given that there was no chemical crosslinking in PLA hydrogel, as well as PLA-CNC hydrogel, the weak hydrogen bonding made these two hydrogels like paste without formability after polymerization, which was not benefit to the fidelity. However, the simultaneous participation of HALA and CNC endowed the hydrogel with improved fidelity. The G’ of PLA-HALA-CNC hydrogel was significantly higher than that of PLA-CNC hydrogel and PLA hydrogel (Figure 3G). Based on these characteristics, the present study proposed a protocol for realizing 3D printing in the production of PLA-based hydrogel. As shown in Figure 3H, a LA/HALA/CNC solution was prepared in the printing material tank, followed by being placed at room temperature for 5-10 minutes, and then subjected for 3D printing. After printing, it was heated at 70 °C for 3 hours for LA-polymerization and stabilization to yield a more stable 3D printed hydrogel. Thus, the influence of trometamol on LA assembling and the positive influence of HALA and CNC on the rheological properties and extrudability of the ink realized and optimized the molding and processing of PLA-based hydrogels through 3D printing.

3.3 The effect of HALA/CNC on PLA stability

After the molding of PLA hydrogel, the stability of PLA hydrogel was another important issue that needed to be focused. The ROP of LA led to the alternative existence of disulfide bonds in the main chain, thus forming a linear polymer. However, because of the presence of thiol radicals, PLA could not remain stable after falling to room temperature and would depolymerize into oligomers, resulting in a decrease in viscosity and exhibiting fluidity. The de-polymerization of PLA would significantly affect its performance. In order to study the influence of the introduction of HA, HALA and CNC on the stability of PLA, hydrogels with different compositions, including PLA, PLA-HA, PLA-CNC, PLA-HALA, PLA-HALA-CNC were prepared by heating. The stability of hydrogels was observed by inversion experiment at room temperature. As shown in Figure 4A, the bulk hydrogel (polymerized from 40% LA) formed by heating polymerization could stably exist at the bottom of the centrifuge tube when it was inverted. However, after 3 days at room temperature, due to de-polymerization, PLA became a flowable liquid and flowed down under gravity. But other hydrogels remained stable at 3 days. After 7 days, PLA hydrogel and PLA-HA hydrogel underwent obvious phase
transformation, revealing the significant de-polymerization. The other hydrogels did not undergo obvious phase transformation. PLA-CNC showed significant fluidity after 9 days, while PLA-HALA showed significant fluidity after 14 days. Both CNC and HALA showed significant effect on stabilizing PLA. The present study continued to extend the observation time, and finally found that PLA-HALA-CNC hydrogel was the most stable and did not undergo obvious de-polymerization during the 4-weeks observation duration. Further statistics were carried out on the stability of hydrogels with different compositions (Figure 4B). For PLA hydrogel fabricated from 50% LA, the effect of CNC on stabilizing PLA was more significant. The contribution of CNC and HALA to the stability of PLA was similar. The results confirmed that the introduction of either CNC or HALA could significantly improve the stability of PLA, so that the polymerized hydrogel could maintain solid state for a long time and show elasticity. Collaboration between CNC and HALA could better stabilize PLA. HA could also improve the stability of PLA, but this effect seemed weak.

Accordingly, the terminal sulfur radicals of PLA initiate the reverse ring-closing de-polymerization and revert to monomers after cooling, attesting to a more thermodynamically stable LA monomer. Thus, strategies such as the introduction of 1,3-diisopropenylbenzene (DIB) and chlorinated solvents, etc. were used to stabilize PLA by quenching the terminal sulfur radicals. At the same time, 1-ethyl-3-methylimidazolium ethyl sulfate ([EMI][ES]) and the deprotonated LA monomer, etc. were also used to form hydrogen bonds with PLA to lower the potential energy of PLA, thus stabilizing PLA. In the present study, FT-IR was used to illustrate the possible mechanisms. As shown in Figure 4C, the sharp absorption peak at 1702 cm\(^{-1}\) corresponding to the stretching vibration of the carboxyl group shifted to 1545 cm\(^{-1}\), indicating that carboxyl groups in PLA were deprotonated in trometamol aqueous solution. For PLA-CNC hydrogel, the O-H stretching vibration peak at 3400 cm\(^{-1}\) underwent a significant red shift, and the C-O stretching vibration peaks in the 1000-1300 cm\(^{-1}\) region also changed, confirming the formation of H-bonding between the hydroxyl groups of CNC as H donors and the -COO\(^{-}\) of PLA (Figure 4D). In addition, the zeta potential of CNC was -57.53 ± 1.67 mV, which dropped to -11.22 ± 1.68 mV after the interaction with LA in solution. The reduction of negative charges indicated the hydroxyl groups of CNC were shielded due to their interaction with the -COO\(^{-}\) (Figure S7). At the same time, HALA acted as the macromolecular crosslinking agent that could achieve network
formation of PLA, reduce the efficiency of sulfur radicals reaction, significantly improving PLA stability (Figure 4E).

Figure 4. The effect of HALA and CNC on PLA stability. (A) Representative images to show the stability of PLA hydrogels. (B) Stability duration statistics. (C) FT-IR spectrums of LA powder and PLA in solution. (D) FT-IR spectrums of PLA and PLA-CNC. (E) Schematic representation of PLA-HA-CNC hydrogel network.

Thus, HALA and CNC synergistically improved the stability of PLA, allowing it to maintain long-term stability even in a low-temperature storage environment of 4 °C. The improvement of PLA stability is significant for the maintenance of its function.

3.4 Adhesion and self-healing performance of hydrogel bandage
According to the above research results, the PLA-based hydrogel in this study could be produced by 3D printing, and had reliable stability. Then, its adhesion as a bioadhesive needed to be evaluated next. In order to study the adhesion of the present PLA-based hydrogel, PLA-based hydrogel patches without complex structure were prepared. Due to a large number of carboxyl groups, PLA has been reported to have adhesive ability. As shown in Figure 5A,B,C, PLA-CNC hydrogel also showed adhesion performance. It was capable of tightly bonding two glass sheets coated with gelatin. Two glass sheets bonded by PLA-CNC hydrogel could withstand a weight of 100 g. When the two glass sheets were pulled apart, the adhesive still firmly adhered to them, but the adhesive itself was damaged, confirming that the adhesive had strong interfacial adhesion. According to lap-shear tests, the adhesion strength increased with the increase in LA concentration. In addition, the presence of CNC formed hydrogen bonding with PLA, promoting the cohesion, leading to the promotion of adhesion strength. The failure of adhesion mainly lied in the destruction of hydrogel adhesive, which was obviously related to the low cohesion of the adhesive. Because of the absence of chemical crosslinking in PLA hydrogel adhesive and PLA-CNC hydrogel adhesive, these two groups of hydrogel adhesives were paste-like without formability.

The presence of HALA could make up for this deficiency. The presence of HALA could not only significantly improve the stability of PLA but also improve the cohesion and strength of hydrogel bandages through the formation of covalent crosslinking. As shown in Figure 5D and Figure S8, the PLA-HA-CNC hydrogel could be molded to form a patch or a bandage. The PLA-HA-CNC hydrogel could firmly adhere to the skin surface, and could twist with the skin twisting without falling off (Figure 5E). The lap-shear test showed that the adhesion strength of PLA-HA1 (PLA: 50% w/v; HALA: 1% w/v), PLA-HA2 (PLA: 50% w/v; HALA: 2% w/v), PLA-HA-CNC3 (PLA: 50% w/v; HALA: 2% w/v; CNC: 3%), PLHA-HA-CNC5 (PLA: 50% w/v; HALA: 2% w/v; CNC: 5%) and PLA-HA-CNC7 (PLA: 50% w/v; HALA: 2% w/v; CNC: 7%) hydrogel bandages was ~102 kPa, ~111 kPa, ~122 kPa, ~134 kPa and ~83 kPa, respectively (Figure 5F,G). The adhesion strength increased with the increase of the HALA content and the CNC content. However, excessive CNC content significantly reduced the adhesion strength of the PLA-HA-CNC bandage. The presence of CNC formed hydrogen bonding with the carboxyl groups of PLA, which consumed the carboxyl content in the hydrogel network, thus reducing the effective groups interacting with the tissues. FibrinGlue (Porcine Fibrin...
Sealant Kit, BIOSEAL BIOTECH CO., LTD.) was used as the control adhesive and underwent the same lap-shear test. The adhesion strength of FibrinGlue was ~12 kPa. Thus, the hydrogel bandages showed significantly higher adhesion than the clinic used FibrinGlue.

**Figure 5. Adhesion and self-healing of the bandage.** (A) Images to show PLA-CNC adhered to two pieces of glasses coated with gelatin. (B) Lap-shear curves to show adhesion strength of PLA and PLA-CNC hydrogel adhesives. (C) Adhesion strength of PLA and PLA-CNC hydrogel adhesives. (D) Image to show the plastic PLA-HA-CNC hydrogel patch/bandage. (E) Images to show bandage PLA-HA-CNC adhered skin. (F) Lap-shear curves. (G) Adhesion strength. (H) Images to show the healing of the bandage PLA-HA-CNC. (I) Evaluation of self-healing of the bandage under alternating strains of 1% and 500%. (J) Images to show the burst pressure test, and the burst pressure value (defined as the peak pressure before pressure loss). (K) Images to show the removal of the hydrogel bandage by water flushing. (L) Adhesion strength monitoring by lap-shear test.
Due to the strong adhesion, the two cut hydrogel bandages could instantly adhere and heal after contact immediately. And the healing was firm, which could withstand large tensile deformation without fracture (Figure 5H). High and low cyclic strain tests had also confirmed that the hydrogel bandage had excellent self-healing performance (Figure 5I). After two cycles of alternating strain, the storage modulus and loss modulus of hydrogel bandage could be almost completely recovered. Based on the strong adhesion and self-healing properties, the performance of the hydrogel bandage in the burst pressure test was impressive. As shown in Figure 5J, a long cut of 1 cm was made on the rubber tube plugged at one end. The cut was blocked with the hydrogel bandage. It was found that the hydrogel bandage was closely attached to the rubber tube. With the injection of air, the hydrogel bandage expanded like a balloon due to pressure until it broke. The average bursting pressure of PLA-HA2, PLA-HA-CNC3, PLA-HA-CNC5 and PLA-HA-CNC7 hydrogel bandages were ~21.67 kPa, ~36 kPa, ~35 kPa and ~47 kPa, respectively. After the fracture, the hydrogel bandage still adhered tightly to the rubber tube. Because of the excellent self-healing performance of the hydrogel bandage, the second test was carried out immediately within 5s after the fracture, and the bursting pressure of the hydrogel bandage still reached 80%-95% of the original bursting pressure.

3.5 Bandage removal and re-adhesion

Usually, in clinical applications, dressings need to be replaced regularly. Excellent adhesion performance may also lead to another challenge, which is the difficulty of separating and removing from the adhesive site, leading to secondary damage to the wound. How to achieve simple and residue-free removal is crucial for wound healing in clinic. There are different methods for removing adhesives based on different adhesion mechanisms, such as rapidly degrading adhesives and reducing the interfacial adhesion between adhesives and tissues through competitive reactions. However, small molecules or ions are usually involved, which may bring potential risks.

In the present study, although the hydrogel bandage possessed excellent adhesion, it could be removed without residue. As shown in Figure 5K, the adhesion between hydrogel bandage (PLA-HA-CNC5) and substrate could be significantly reduced by using water washing, thus realizing the detachment of the hydrogel bandage with almost no residual. According to the monitoring of adhesion strength, it was found that the adhesion strength reduced to ~0 kPa within 60 s (Figure 5L). Because
a large number of dense carboxyl groups and HA in hydrogel bandage could absorb water molecules
to form a lubricating hydration layer at the interface after combining with hydrogel, which would
destroy the binding force between hydrogel and substrate. Water would affect the adhesion of the
hydrogel bandage, but according to the monitoring of adhesion strength in Figure 5L, a small amount
of water would not affect the stable adhesion of the hydrogel bandage on the substrates. The complete
desorption process required a considerable amount of water. Besides, after drying at 37 °C for 30 min,
the removed hydrogel bandage could restore its adhesion. However, the adhesion strength decreased
with the increase in adhesion-detachment times (Figure S9).

3.6 Mechanical performance of the hydrogel bandage

Generally, hydrogels exhibit soft and weak, or hard and brittle mechanical properties. As a wound
bandage for first aid of trauma, hydrogel should exhibit mechanical performance similar to skin to
adapt to activities at different parts. Thus, the mechanical performance of the hydrogel bandages was
evaluated.

Compression tests showed that the cylindrical hydrogel could be largely deformed to withstand
extreme compression without being destroyed (Figure 6A). Consistent with rheology tests, the
compression modulus significantly improved with the addition of CNC (Figure 6B,C). At the same
time, the anti-fatigue properties of the hydrogels were evaluated by compression cycling test. As shown
in Figure 6D, after the first cycle, the stress of the hydrogels showed a downward trend, but with the
increase in cycle times, the mechanical properties of the hydrogels tended to be stable. The average
residual stress percentages of the hydrogel PLA-HA2, PLA-HA-CNC3, PLA-HA-CNC5 and PLA-
HA-CNC7 after 20 cycles of compression were ~70%, ~70%, ~86% and ~80%, respectively (Figure
S10).
Figure 6. Mechanical performance of hydrogel bandages. (A) Images to show the cylindrical hydrogel under compression. (B) Compressive stress-strain curves. (C) Compression modulus of hydrogels. (D) Stress-time curves of hydrogels in the cyclic compression test. (E) Images to show hydrogel bandage deformation in the tensile test. (F) Images to show hydrogel bandage adhered on two pig skins with loading and unloading of external force. (G) Tensile stress-strain curves. (H) Toughness of hydrogels. (I) Young’s moduli of hydrogels. (J) Tensile stress-strain curves in the cyclic tensile test. (K) Yield stress and yield strain of hydrogel in the cyclic tensile test. (L) Hysteresis energy of hydrogels.
Tensile tests further showed that the hydrogel bandage could be stretched to a high deformation (Figure 6E). As shown in Figure 6F, two separated pig skin pieces were adhered by hydrogel PLA-HA-CNC3. A 100 g weight was hung on one skin piece to exert a loading stress. It was found that the hydrogel tightly adhered to the pig skins and stretched to large deformation under the action of external force. After removing the external force, the hydrogel shrank rapidly with most of the deformation restored. The fracture strain of hydrogel PLA-HA2, PLA-HA-CNC3, PLA-HA-CNC5 and PLA-HA-CNC7 was ~348%, ~476%, ~565% and ~396%, respectively (Figure 6G). Through calculation, the toughness of hydrogel PLA-HA2, PLA-HA-CNC3, PLA-HA-CNC5 and PLA-HA-CNC7 was 153.75±5.66 kJ/m$^3$, 220.79±5.38 kJ/m$^3$, 406.04±4.63 kJ/m$^3$ and 347.05±9.86 kJ/m$^3$, respectively (Figure 6H). The Young’s modulus of hydrogel PLA-HA2, PLA-HA-CNC3, PLA-HA-CNC5 and PLA-HA-CNC7 was 35.96±1 kPa, 38.43±3.52 kPa, 65.72±4.13 kPa and 81.71±1.46 kPa, respectively (Figure 6I). Of note, the presence of CNC significantly improved the modulus and the fracture strain of hydrogel. This was mainly attributed to the extensive hydrogen bonding between CNC and PLA. For one thing, nanoparticles filling and the hydrogen bonding promoted the modulus of hydrogels. For another, under the action of external force, these hydrogen bonds were destroyed, which dissipated the external force and enabled the hydrogel to withstand large deformation. However, the fracture strain of the hydrogel PLA-HA-CNC7 decreased when compared with hydrogel PLA-HA-CNC5, which might be related to the uneven dispersion of CNC at high concentration.

The test of loading and unloading stress was further carried out to study the deformation recovery of hydrogel bandages under tensile mode. The cyclic tensile testing of the hydrogel showed that the hydrogels had obvious hysteresis after the loading and unloading cycle at 200% strain (Figure 6J). The yield stress of hydrogel PLA-HA2, PLA-HA-CNC3, PLA-HA-CNC5 and PLA-HA-CNC7 was 45.12±2.25 kPa, 49.25±2.35 kPa, 52.64±3.72 kPa and 62.4±2.89 kPa, respectively. The yield strain of hydrogel PLA-HA2, PLA-HA-CNC3, PLA-HA-CNC5 and PLA-HA-CNC7 was 158.24±2.7%, 158.16±3.15%, 162.43±2.82% and 131.89±2.52%, respectively (Figure 6K). Through calculation, the hysteresis energy of hydrogel PLA-HA2, PLA-HA-CNC3, PLA-HA-CNC5 and PLA-HA-CNC7 was 48.57±3.79 kPa, 45.38±2.76 kPa, 49.83±2.05 kPa and 67.06±2.73 kPa, respectively (Figure 6L).

The mechanical tests thus illustrated that the hydrogel bandages were robust to withstand and adapt to
3.7 Application of hydrogel bandage in wound incisions

Before being used in vivo, the biosafety of the hydrogel without any post-treatment was first evaluated. Of note, the prepared hydrogel bandages without post-processing possessed a pH value close to neutral (Figure S11). Moreover, the hydrogel bandage, PLA-HA-CNC5, co-cultured with fibroblasts showed no significant cytotoxicity. Almost no dead cells were observed during the 7 days culture (Figure S12). Cells kept proliferation during the in vitro co-culture and showed no significant difference with the control group, revealing the safety and biocompatibility of the hydrogel bandage. Normally, to ensure biocompatibility, it is necessary to remove small molecules and crosslinking agents that have potential cytotoxicity in the preparation process of adhesive hydrogels. However, LA is a coenzyme involved in acyl transfer in the metabolism of substances in the body, which can eliminate free radicals that lead to accelerated aging and disease. Low concentrations do not cause toxicity. At the same time, HA and CNC also have excellent biocompatibility. Therefore, the hydrogel formed by the polymerization of LA as the monomer could be used without post-treatment. This reduced the technical and equipment threshold of hydrogel bandage preparation and greatly improved the generalization of hydrogel bandage preparation.

Considering its well-performed adhesion, robust feature and biocompatibility, hydrogel PLA-HA-CNC5 was used to treat wounds to evaluate its potential as a sutureless material for wound closure. As shown in Figure 7A, the untreated incision in the Blank group did not achieve healing on day 7 post-surgery with the presence of the scab. After 14 days, the incision still did not fully heal. Different from the incision in the Blank group, the incision treated with hydrogel bandage showed significant closure on day 7 post-surgery, and achieved healing on day 14 with inconspicuous healing marks. Because the hydrogel bandage was easy to remove, no secondary damage was caused during hydrogel bandage replacement. Histological staining, including H&E staining and Masson staining showed that incision tissue in the Blank group was discontinuous on day 7 (Figure 7B). Normal skin tissues were filled with neo-fibrous tissue in the middle. The neo-tissue showed poor epidermal continuity and was thinner than normal skin. At the same time, a large amount of vascular infiltration confirms that the tissue was in the repair stage. After 14 days, the neo-tissue showed increased thickness. However, the
structure remained incomplete and was significantly different from the surrounding normal tissue.

On the contrary, the incision treated with the hydrogel bandage on day 7 showed that the skins were well-integrated by the neo-tissue that possessed continuous and complete epidermal continuity, as well as similar thickness with normal skin. Appendages were also observed. After 14 days, the incision further shrunk, leading to a well-integration of skins. Although the arrangement and density of collagen fibers were different from normal skin tissue, the appearance of the continuous epidermis and appendages indicated that the application of the hydrogel bandage significantly promoted the healing of the skin incision.

**Figure 7. Healing of incision.** (A) Images to show incisions on day 0, 7 and 14 post-surgery with no treatment in the Blank group, and incisions on day 0, 7 and 14 post-surgery treated with hydrogel bandage, PLA-HA-CNC5 in the Bandage group. (B) H&E staining and Masson staining of incisions on day 7 and 14 post-surgery in the Blank group
3.8 Application of 3D printed porous hydrogel bandage in full-thickness skin wounds

Whether the hydrogel bandage could promote the healing of skin defects in full-thickness skin wounds was further studied. The porous hydrogel bandage was prepared by 3D printing, possessing a filament diameter of 300 μm and a filament gap of 400 μm for providing better breathability to larger wounds. As shown in Figure 8A,B,C, compared with the Blank group, the healing rate of skin wounds in the Bandage group was significantly faster on day 7. The wound residual area in the Bandage group reduced to ~20% on day 7, which was much lower than that in the Blank group (~55%). After 14 days, the wounds treated by hydrogel bandage PLA-HA-CNC5 were completely healed, exhibiting similar color and texture to normal skin. However, more than 20% of the wounds had not been healed in the Blank group on day 14.

H&E staining and Masson staining were performed to further study the effect of the hydrogel bandage on wound healing. As shown in Figure 8D,E, neo-tissues were observed both in the Blank group and Bandage group. The epidermis in the regenerated area of the Bandage group was found to be continuous on day 7. Complete squamous epithelial tissue could be observed. However, the epidermis in the Blank group was not observed. According to Masson staining, collagen deposition could be observed in the two groups. While collagen in the Bandage group showed regular arrangement. After 14 days, the epidermis could be observed in the Blank group, which, however, was still discontinuous. In the Bandage group, the wounds further contracted and healed. The collagen fibers in the Bandage group were thicker and denser, with a more mature phenotype and regular orientation. The Blank group had the least deposition and loose arrangement of collagen fibers. The histological results were consistent with the general observation. In addition, angiogenesis was further monitored. Angiogenesis can continuously provide sufficient oxygen and nutrients to the wound. Error! Reference source not found. According to the staining of CD31, a common indicator to characterize vascular endothelial cells, the positive expression of CD31 in the wound site in the Bandage group was higher than that in the Blank group on day 7, revealing that the hydrogel bandage performed a positive effect on angiogenesis. After 14 days, the expression of CD31 in the Bandage group decreased significantly, indicating that the wound entered the period of vascular inhibition and remodeling. However, the strong expression of CD31 in the Blank group indicated that the wound was in the repair
Figure 8. Healing of full-thickness skin wounds. (A) Images to show wounds on day 0, 7 and 14 post-surgery in the Blank group and the Bandage group. (B) Simulated diagram of wound healing. (C) Quantification of wound residual area rate (**p<0.01). (D) H&E staining of the wounds on day 7 and 14 post-surgery in the Blank group and the Bandage group (Bar scale: 200 μm). (E) Masson staining of the wounds on day 7 and 14 post-surgery in the Blank group and the Bandage group (Bar scale: 200 μm). (F) CD31 immunofluorescence staining of the wounds on day 7 and 14 post-surgery in the Blank group and the Bandage group (Bar scale: 100 μm). n=5.

In addition, to study the related factors of hydrogel bandage promoting wound healing, the
inflammatory microenvironment of the early stage of wounds on day 3 was evaluated. According to interleukin-1β (IL-1β), tumor necrosis factor-α (TNF-α), interleukin-4 (IL-4) and transforming growth factor-β1 (TGF-β1) immunofluorescence staining (Figure 9A,B,C), the lowest fluorescence intensity of IL-1β and TNF-α in the Bandage group illustrated that these pro-inflammatory factors were significantly inhibited. While anti-inflammatory factor (IL-4) expression in the Bandage group was significantly higher than that in the Blank group, revealing that at the early stage of wound healing, the hydrogel bandage promoted the secretion of anti-inflammatory factors and inhibited local inflammatory response to accelerate wound healing. At the same time, TGF-β1 was up-regulated in the Bandage group. TGF-β1 plays an important role in mediating M1/M2 subtype transformation and the subsequent tissue repair and angiogenesis. The up-regulation of TGF-β1 promoted angiogenesis, which had been confirmed with the above CD31 immunofluorescence staining results. Meanwhile, to verify the transformation effect of hydrogel bandage on macrophages in vivo, iNOS (M1 type macrophage marker) staining and CD206 (M2 type macrophage marker) immunofluorescence staining were performed. According to Figure 9D,E, it was found that in the Blank group, iNOS expression was higher, while in the Bandage group, CD206 expression was higher, indicating that the hydrogel bandage significantly affected the macrophage polarization, leading to more M2 macrophages. M2 macrophages mainly secrete high levels of anti-inflammatory cytokines and repair growth factors to promote tissue regeneration. Thus, the CD31 immunofluorescence staining results, the pro-inflammatory and anti-inflammatory factors immunofluorescence staining results, as well as the macrophage marker immunofluorescence staining results illustrated that the application of hydrogel bandage provided an immune microenvironment conducive to tissue repair, thereby promoting wound healing.
Figure 9. Immune microenvironment of wounds at the early stage. (A) Immunofluorescence staining of IL-1β, TNF-α, IL-4 and TGF-β1 on the wound samples on day 3 (Bar scale: 100 μm). (B) Relative staining intensity of IL-1β and TNF-α (*p<0.05, **p<0.01). (C) Relative staining intensity of IL-4 and TGF-β1 (*p<0.05). (D) Immunofluorescence staining of iNOS and CD206 (Bar scale: 100 μm). (E) Relative staining intensity of iNOS and CD206 (*p<0.05). n=5.

4. Conclusion

In summary, the present study reported a rapid gelation phenomenon of LA in water with the assistance of trometamol. The binary system of LA and tromtamol could quickly assemble in water to form injectable supramolecular hydrogel within 5 min. With the help of HALA and CNC, this hydrogel could realize 3D printing for producing PLA-based hydrogel bandages. HALA and CNC were found to exhibit significant effect on stabilizing PLA by preventing PLA de-polymerization. At the same time, HALA and CNC were employed as the macromolecular covalent crosslinker and the giant physical crosslinker to synergistically endow the hydrogel with higher cohesion, significantly improving the modulus and the toughness of the hydrogel. The adhesion strength of the hydrogel bandage PLA-HA-CNC5 reached to ~134 kPa, which was ~10 times higher than that of the clinically used FibrinGlue.

The hydrogel bandage possessed quick self-healing ability. After blasting, the bursting pressure of the
healed hydrogel bandage reached 80%-95% of the original bursting pressure. Although the hydrogel bandage had strong adhesion, it could be removed without residue within 60 seconds by washing with water flowing. The in vivo healing of the incision and full-thickness wounds confirmed that the application of the bioadhesive hydrogel bandage significantly promoted wound healing by closing the wound, forming a physical barrier to the wound and providing an anti-inflammatory effect. The preparation process of the hydrogel bandage did not need complex equipment, as well as tedious and time-consuming post-treatment to ensure the reliable biocompatibility. Therefore, the present hydrogel bandage and its preparation methods had potential for future clinical applications.

Supplementary Information

The online version contains supplementary material available at...

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Data Availability Statement

The data are available from the corresponding author upon reasonable request.

Conflict of Interest

The authors declare no conflict of interest.

References


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