A Bioinspired Medical Adhesive Derived from Skin Secretion of *Andrias davidianus* for Wound Healing

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Existing surgical tissue adhesives on the market cannot meet the desired demand for clinical operations due to their limited adhesivity or undesired cytotoxicity. A new bioadhesive is derived from the skin secretion of *Andrias davidianus* (SSAD). This bioinspired SSAD has significantly stronger tissue adhesion than the fibrin glue and improved elasticity and biocompatibility when compared to the cyanoacrylate glue both ex vivo and in vivo. Additionally, the SSAD-based adhesive decreases skin wound healing time and promotes wound regeneration and angiogenesis. The SSAD-based adhesive is completely degradable, strongly adhesive, and easily produced from a renewable source. Based on these favorable properties, the SSAD-based bioadhesive demonstrates potential as a surgical bioadhesive for a broad range of medical applications.

1. Introduction

Wound closure is essential in surgical operations. Clinically, 60% wounds are closed by suture or staples after surgical procedures.\(^{[1]}\) Mechanical closure could cause secondary damage due to the fragility of soft tissue and localized stress, and subsequent removal of non-degradable sutures or staples is required. Therefore, sutureless wound closure, primarily relying on the utilization of medical adhesives, could simplify operation procedures, shorten recovery times, and improve patient care quality.\(^{[2]}\)

To date, the reported adhesive materials can be mainly divided into the following classifications. The first class includes natural protein-derived fibrin,\(^{[3]}\) gelatin,\(^{[4]}\) albumin,\(^{[5]}\) and collagen.\(^{[6]}\) Alternatively, polysaccharides such as chitosan,\(^{[7]}\) alginate,\(^{[8]}\) hyaluronic acid,\(^{[9]}\) and dextran\(^{[10]}\) have been used. Another category includes synthetic polymers such as polycyanoacrylate,\(^{[11]}\) polyurethane,\(^{[12]}\) and polyethylene glycol. More recently, naturally derived adhesives, such as the biomimetic mussel-inspired dopamine, have been reported.\(^{[13]}\) The basic characteristics of an ideal medical adhesive include strong adhesion, good biocompatibility, low cost, and simple production.

Unfortunately, most available options have limitations. For example, the most widely used commercial synthetic adhesive, the cyanoacrylate glue, has excellent adhesive strength,\(^{[11]}\) but its unignorable cytotoxic effect is the major concern. Additionally, cyanoacrylate glue produces excessive heat due to fast redox polymerization during bonding,\(^{[14]}\) and its poor elasticity cannot accommodate the movement of soft tissues such as the skin.

Compared to cyanoacrylate, the fibrin glue has much better biocompatibility. However, because of its unreliable adhesive strength, the fibrin glue is less suitable to be used alone, which

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limits its application. The strong adhesive strength of mussels to rocks under the sea inspired 3,4-dihydroxyphenylalanine (DOPA)-based bioadhesives. Regrettably, DOPA-based bioadhesives require oxidants or base buffers for polymerization, and long polymerization action time and relatively high production cost have also limited their applications. Therefore, although a wide range of medical adhesives is available, the development of new sealants that feature advantageous properties, such as strong adhesion and good biocompatibility, remains imperative.

Interestingly, nature provides a rich source for tissue adhesives. The species *Andrias davidianus* (Chinese giant salamander), which is an endemic amphibian in China, is the longest-living and largest amphibian species in the world. Chinese giant salamanders, which can reach a length of 1.8 m in adulthood, are thought to have originated 200 million years ago (making them the same age as the dinosaurs) and are thus termed a “living fossil”. Over billions of years of evolution, the skin of *Andrias davidianus* has developed mucous glands that are capable of secreting large amounts of mucus following scraping or other external stimulation, which helps the animal escape dangers or heal. The Chinese have used the skin secretion of *Andrias davidianus* (SSAD) for the treatment of burns for approximately 1600 yr, according to the Bencao Jing Ji Zhu, complied by Hongjing Tao in the North-and-South Dynasty. According to incomplete statistics, there were more than 20 million giant salamanders under cultivation in China in 2018 primarily towards the protection purpose, and some of these salamanders are starting to be used commercially such as for the collection of their skin secretions. Recently, Xu et al. applied gel gene ontology, mass spectrometry, and 2D electrophoresis analyses, which showed that SSAD contains 155 polypeptides or proteins that may participate in diverse physiological activities, including respiration, host immune defense response, extracellular matrix (ECM) synthesis, and tissue regeneration. However, as far as we know, SSAD has not been systematically studied in bioadhesive applications. Herein, we demonstrate the potential of SSAD as a bioinspired medical adhesive derived from a simple and environmentally friendly production process. In this study, cyanoacrylate and fibrin glues (i.e., the two most common bioadhesives on the market) were used as controls. Compared with the two controls, our hydrogel derived from SSAD exhibited strong adhesion, elasticity, and flexibility on porcine skins ex vivo, along with excellent biocompatibility, degradability, and minimal inflammatory response in a rat skin incision adhesion model in vivo. We further revealed that SSAD-derived bioadhesive facilitated wound healing in a diabetic Sprague Dawley (SD) rat model with a full-thickness skin defect in vivo, suggesting its potential as a medical bioadhesive for surgical operations.

2. Results and Discussions

2.1. SSAD Collection

As an old amphibian species, the skin of *Andrias davidianus* is different from that of aquatic and terrestrial species. First, due to their not-yet-evolved lungs, the amphibian skin is an important respiratory organ. Moreover, unlike other species, skin glands in amphibians could secret white mucus when irritated. The main ingredients of SSAD are proteins, amino acids, mucopolysaccharides, and antimicrobial peptides. In this study, before SSAD collection, Chinese giant salamanders were rinsed with clean water to remove molting. By gently scratching the back skin, the animals secreted mucus, as shown in Figure 1b and Movie S1 in the Supporting Information. Approximately, 3–5 mL of mucus could be collected from each giant salamander twice per month. After freeze-drying, approximately 200 mg of SSAD powder could be obtained from every 1 mL of mucus collected (Figure 1c). When blended with a small amount of water, SSAD showed good adhesion (Figure 1d and Movie S2, Supporting Information), and a homogeneous SSAD hydrogel could be reconstituted within a short period (within 2 min; Figure 1e and Movie S3, Supporting Information).
2.2. Characterization of SSAD

2.2.1. Formation of SSAD Hydrogel

SSAD powder harvested after lyophilization had the appearance of inhomogeneous lumps under scanning electron microscopy (SEM, Figure 2a) and was insoluble in neither water nor ethanol. However, the polypeptide chains in the lumps could swell upon mixing the SSAD powder with water by hydrating and permeating each other to form a highly water-containing hydrogel with a porous structure (Figure 2a). With the increase of hydration time, the porous structure was more pronounced. Interestingly, as shown in Figure 2b, while the pore sizes of the samples were relatively centralized after 12 h of gelation and the average size was 107.08 ± 9.1 μm, they were more dispersed at 2 h of gelation. Moreover, the walls of the cavities after 12 h of hydration seemed to be denser than those after 2 h, indicating that the hydrogel network might be more stable with improved chain density. In addition, as demonstrated in Figure 2c, the storage modulus ($G'$) of the SSAD hydrogel increased dramatically from 65.29 ± 1.16 Pa at 2 h to $566.86 \pm 2.72$ Pa at 12 h. Meanwhile, the loss modulus ($G''$) increased from $13.03 \pm 0.89$ Pa at 2 h to $88.51 \pm 4.54$ Pa at 12 h, and the $G''/G'$ ratio was reduced from 0.20 to 0.15, which suggested that gelation was more complete with increased hydration time to form the hydrogel. Also, the walls of the cavities after hydration of 12 h seemed to be denser than those of 2 h (Figure 2a), indicating that the hydrogel network might be more stable with improved chain density.

According to a previous report based on proteomics, there are 155 proteins in SSAD associated with the adhesive characteristics of the mucus of the species, such as annexin-B11, apolipoprotein A-IV, galectin-2, galectin-7, and talin-2, and 10 proteins related to wound healing, such as β-actin, cGMP-dependent 3',5'-cyclic phosphodiesterase, and osteonectin.[20] Here, we analyzed the amino acid composition of SSAD. As shown in Table S1 in the Supporting Information, there were 18 amino acid residues identified. The contents of tyrosine, phenylalanine, and tryptophan were 5.59%, 5.08%, and 0.94%, respectively, all of which contain a benzene ring in their respective structures. Fourier-transform infrared spectroscopy (FTIR) spectra were further used to understand the primary structures of SSAD, as shown in Figure 3a. The stretch vibration peak of N–H and the bands of amide I/II/III exhibited redshift after hydration due to hydrogen bond formation between proteins and water molecules, which moderately altered the protein-hydrogen bond network within or between the original protein molecules.[17] Secondary structural analyses of proteins are illustrated in Figure 3b,c, and the composition of α-helix, β-sheet, β-turn, and unordered conformation are listed in Table 1.

Compared to the powder, the proportion of the β-sheet structure in the hydrogel increased to 31.7%, whereas that of the β-turn structure decreased correspondingly. These results suggested that the secondary structure of the proteins in SSAD changed after hydration due to intramolecular and intermolecular hydrogen interactions,[26] which was also confirmed by Raman spectra. An obvious decrease in the tyrosine doublet ratio, $I_{850}/I_{830}$ cm$^{-1}$ (powder: 1.21; hydrogel: 0.97), was revealed upon hydration (Figure 3e,f), indicating that the hydrophobic groups of tyrosine residues were buried in a more hydrophobic environment and served as strong hydrogen bond donors to enhance the intermolecular hydrogen bond reaction.[27] Additionally, compared with the weak S–S characteristic bands in the powder sample, the standout peak of 500–600 cm$^{-1}$ in the hydrogel highlighted the SH–SS exchange reaction between the protein molecules (Figure 3d), which was perhaps the source...
of additional strength to help maintain stability of the 3D porous network.\textsuperscript{[28]} To further confirm the effects of hydrogen bonding and S–S on the hydration of the mucus, we analyzed the reduction of intermolecular/intramolecular hydrogen and S–S bonds in the hydrogels by employing treatments with urea\textsuperscript{[29]} and mercaptoethanol\textsuperscript{[17]} respectively. As Figure 2b shows, compared with the control group (hydrogel–water mixture), there was an obvious loss of viscoelasticity after exposure of hydrogel to urea solution (8 M) and a slight loss of that in mercaptoethanol solution (10%). These results suggested the pivotal roles of hydrogen bonding in hydrogel formation, and S–S bonds enhanced the 3D structure of the hydrogel. Accordingly, the possible mechanistic interpretation of SSAD hydration is proposed in Figure 4a.

Table 1. Secondary structure composition of SSAD powder and hydrogel. The bolded numbers indicate significant changes in values between the SSAD powder and hydrogel samples.

<table>
<thead>
<tr>
<th>Secondary structure fractions</th>
<th>Powder</th>
<th>Hydrogel</th>
</tr>
</thead>
<tbody>
<tr>
<td>Peak position [cm\textsuperscript{-1}]</td>
<td>Area [%]</td>
<td>Peak position [cm\textsuperscript{-1}]</td>
</tr>
<tr>
<td>α-helix</td>
<td>1649.3,1656.8</td>
<td>25.6</td>
</tr>
<tr>
<td>β-sheet</td>
<td>1618.6,1626.2,1633.9</td>
<td>26.8</td>
</tr>
<tr>
<td>β-turn</td>
<td>1664.1,1670.6,1676.8,1683.5</td>
<td>29.9</td>
</tr>
<tr>
<td>Random coil</td>
<td>1641.5</td>
<td>13.6</td>
</tr>
<tr>
<td>Indetermination</td>
<td>1691.1</td>
<td>4.1</td>
</tr>
</tbody>
</table>
2.2.2. Adhesion of SSAD

Upon verifying the presence of tyrosine and phenylalanine in the SSAD identified by Raman spectra (Figure 3d),\textsuperscript{[28,30]} the phenolic hydroxyl group, amino acids, and benzene ring were considered to be the functional groups showing the adhesive effect.\textsuperscript{[31]} When dry SSAD powders were blended with water, the polypeptide network swelled rather than dissolved, and the entangled protein network expanded due to the permeation of water molecules and the formation of new hydrogen bonds. Therefore, during swelling, the amino acid residues of polypeptide chains undergo conformation transformation to form a hydrogel adhesive, where the phenolic hydroxyl groups and amino acids act as hydrogen bond donors to promote biological adhesion by hydrogen bonds and van der Waals force.\textsuperscript{[32]} Alternatively, the benzene rings close to the interface build strong interactions with the substrates through \(\pi-\pi\) electron or cation-\(\pi\) interaction upon contact with a hydrophobic surface.\textsuperscript{[33]} Since there are abundant proteins and other ECM molecules at the wound sites, it is easy to form strong adhesion forces between SSAD and the wound through hydrogen bonds, van der Waals force, and/or \(\pi-\pi\) interactions during the hydration of the adhesive powder in the presence of interstitial fluid (Figure 4b).

2.3. Adhesion Evaluations Ex Vivo and In Vivo

2.3.1. Pig Skin Adhesion Assessment Ex Vivo

American Society for Testing and Materials (ASTM) standard tests were performed to evaluate the adhesion properties of SSAD ex vivo. Two commercially available medical adhesives, i.e., cyanoacrylate synthetic glue and fibrin glue, were used as controls. According to ASTM F2255-05, the shear strength of SSAD was measured by the lap shear test (Figure 5a). For edge-to-edge adhesion (Figure 5a.i), the shear adhesion of cyanoacrylate could reach 40.71 ± 3.71 kPa, whereas in the SSAD group, it was 26.66 ± 8.22 kPa, and there was no significant difference between the adhesive strengths of cyanoacrylate and SSAD (\(p > 0.05\)). Although both SSAD and fibrin glue are derived from biological sources, the adhesive strength of fibrin glue was only 3.76 ± 0.16 kPa (Figure 5a.ii,a.iii), which was significantly lower than those of SSAD (\(p < 0.01\)) and cyanoacrylate (\(p < 0.001\)).

The low bond strength of fibrin glue is in accordance with those of other studies, which are typically lower than 5 kPa,\textsuperscript{[34]} and is consistent with its usage instructions, suggesting application as a supplement to sutures.\textsuperscript{[35]} In addition to fibrin glue, the adhesive strength of another natural protein-derived adhesive, i.e., the mussel-inspired adhesive based on DOPA, is improved with a reported bonding strength of 10–30 kPa instantly. When given modifications and more curing time, such as 1 d, the DOPA-based adhesive could achieve even better bonding strength.\textsuperscript{[35,36]}

The strong adhesion of cyanoacrylate is its greatest advantage. In other studies, the adhesive strength of cyanoacrylate varied from 55\textsuperscript{[39]} to 170 kPa.\textsuperscript{[40]} However, in this research, the adhesive strength of cyanoacrylate was not remarkably higher than that of SSAD (Figure 5a.iii). The differences in adhesion were possibly due to the differences of the adhesive substrates.\textsuperscript{[17]} Indeed, as Figure S2 in the Supporting Information shows, the adhesive strength of cyanoacrylate on subcutaneous fat was very weak. This observation perhaps explains the relatively low adhesive strength of the glue illustrated in Figure 5a, which had to be calculated against the entire cross-sectional area of the skin, including the epidermal and fat layers. In comparison, the adhesive strength of SSAD on fat tissues seemed to be fairly good (Figure S2, Supporting Information). Therefore, although cyanoacrylate had the strongest adhesion of the three adhesives in apposition bonding, only the epidermis (not the subcutaneous fat layer) could be cemented, indicating its application limitations.

The superiority of SSAD in joining subcutaneous fat tissues together was further demonstrated in the lap shear test (Figure 5b.i). Quantitative results in Figure 5b.ii,b.iii revealed that, when adhering subcutaneous fat tissues, the shear adhesion capacity of SSAD reached a remarkable value of 26.11 ± 7.72 kPa, which was similar to that in the edge-to-edge adhesion mode; however, the shear bonding strengths of the...
other two glues were lower than 7 kPa. The excellent adhesion ability of SSAD to fat tissues makes it a potential adhesive for abdominal operations such as cesarean sections, where large amounts of fat might be present at the incision site, which usually affects wound closure and delays healing.\(^{[37]}\) The adhesion mechanisms of SSAD to subcutaneous fat tissue could possibly be attributed to the hydrophobic interactions between the hydrophobic residues of some amino acids in SSAD and the alkyl groups in the subcutaneous fat tissue. Additionally, the cationic amine residues of lysine groups can displace hydrated cations from the tissue surface in a humid environment allowing benzene group of phenylalanine and tyrosine to adhere to the subcutaneous tissue via cation–π interaction.\(^{[38]}\) Therefore, combined with hydrophobic interactions, cation–π interaction, as well as the inherent hydrogen bonding between the hydroxyl/amidogen groups of SSAD hydrogels and the carboxyl groups of fatty acids would potentially have contributed to the strong adhesion observed for the fat tissues.\(^{[39]}\)

In addition to adhesivity, elasticity is another requirement of a bioadhesive,\(^{[40]}\) especially for soft tissues such as skin in tension zones, lungs, and cardiac vesicles. A three-point bonding test was conducted on pig skin with a 2-cm incision in the middle (Figure 5c.i). To achieve the same displacement, SSAD needed only 7.84 ± 1.17 N, which was in a similar range with that of the fibrin glue (7.00 ± 0.99 N) and significantly lower than the force needed in the cyanoacrylate group (12.33 ± 1.53 N, Figure 5c.ii,c.iii). This observation indicated that cyanoacrylate provided rigid bonding to tissues while SSAD could provide flexible bonding. Therefore, the SSAD hydrogel may be more suitable for use as a surgical adhesive than cyanoacrylate.

### 2.3.2. Skin Incision Adhesion and Wound Healing in Normal SD Rats In Vivo

The wound healing performance of SSAD in vivo was evaluated using a rat skin incision model, where 5 mg of SSAD powder was applied to each 2-cm incision, and then 20 µL of PBS was dropped on the incision, followed by gentle pressing of the two
wound edges into contact for approximately 30 s (Movie S4, Supporting Information). It was observed that, through this simple procedure, the site of the wound was reattached. In other groups, incision sites underwent regular suturing, cyanoacrylate synthetic glue, fibrin glue, or hemostasis treatments. During the operations, infrared thermal images were taken to record temperature changes (Figure 6a). It was observed that cyanoacrylate produced heat, increasing the temperature from $34.2 \pm 1.6$ to $43.2 \pm 0.8 \, ^\circ C$, which was much higher than the temperature elevations in other groups (Figure S3, Supporting Information). Additionally, consistent with the ex vivo tests, cyanoacrylate showed rigid bonding on the skin (Movie S5, Supporting Information) compared with the skin bonded by SSAD, which was as flexible as the normal skin (Movie S4, Supporting Information). Although the adhesion zone treated by the fibrin glue was also flexible, the bonded incision site easily ruptured during movement due to the relatively low adhesion ability of the glue (Movie S6, Supporting Information).

The five wound sites were all examined daily to observe the healing process (Figure 6b). At 5 d post-operation, wounds treated with cyanoacrylate and sutures recovered, unlike those of the fibrin glue group and the control group of hemostasis treatment. Interestingly, SSAD treatment resulted in almost no scar formation, showing the best healing effect among all groups. Around the wound site in the SSAD group, no signs of infection or inflammation were observed, and no closed dehiscence was observed in the wounds in the blank group (Figure 6b). In addition, hair regeneration was observed through the incision without obvious scarring, indicating that SSAD promoted the overall healing of the wound.

At 5 d post-operation, histological analyses were additionally carried out to evaluate wound healing and possible side effects in the treatment groups (Figure 6c). After SSAD treatment, the incision fused with the basement membrane continuously, and there was no deep opening in the tissue. The incision treated only by hemostasis had granulation tissue, with obvious scabbing and an ulcerous surface. In the sutured wound site, there was a gap present between the incision edges, while the incision site treated by SSAD was full of nuclear-stained neonate cells. For the incision treated with cyanoacrylate, apparent cavitation was found at the bottom of the incision site, possibly consisting of cyanoacrylate residuals. Moreover, some necrotic cells were present around this region, and no obvious nuclear blue-stained cells could be seen in the basal area. The fact that fewer cells could be found around the cyanoacrylate glue might be attributed to the toxicity exhibited by acrylic derivatives, which are present prior to polymerization and are the degradation products of the glues. The incision treated with fibrin glue showed an ulcerous surface, and a small portion of fibrin glue that had not yet degraded was left. This result was consistent with that of Marcus et al., where it took approximately 25 d for fibrin glue, and much longer for cyanoacrylate, to degrade.

Combining ex vivo and in vivo results, the cyanoacrylate glue exhibited the highest adhesive strength to the epidermis. In addition to the weak adhesive strength of cyanoacrylate to fat tissues, the bonding interface provided by cyanoacrylate was rigid. Fibrin-based adhesives have similar properties to soft tissues, but they have low adhesive strength and cannot be used alone. The wound recovery rate following SSAD treatment was significantly higher than that after suture treatment, and there was no obvious wound infection or inflammation. It should also be noted that cytotoxicity of the cyanoacrylate glue was pronounced, in comparison to the relatively toxicity...
of SSAD at the concentration evaluated; as expected, the fibrin glue was supportive of cell growth (Figure S4, Supporting Information).

2.4. Wound Healing Effect in Diabetic SD Rats In Vivo

Angiogenesis is required to restore normal physiologic conditions in wound healing processes, which is essential for transporting oxygen and nutrients to cells at wound sites.\(^{[43]}\) The delayed wound healing in diabetes is well-documented and multifactorial, and impaired angiogenesis is considered one of the primary contributing factors.\(^{[44]}\) Dysfunctional angiogenesis is characterized by hyperglycemia-related microvascular changes and deficiencies in both endothelial progenitor and endothelial cells, leading to diabetic wounds that do not heal.\(^{[43–45]}\)

To further prove the potency of SSAD on wound healing in vivo, full-thickness round skin defects (diameter = 1 cm) were made on the dorsal side of diabetic SD rats by punching; the defects were covered with cotton gauze (negative control) or SSAD powder (30 mg per defect). According to the time interval shown in Figure 7a, wound healing was recorded by photography.

Figure 7. Assessment of full-thickness skin wound healing rate and quality in vivo. a) Macroscopic observation of wounds covered with cotton gauze (control) or treated with the SSAD powder at 0, 3, 7, 14, and 21 d (diameter of blue scale = 10 mm). b) Wound closure percentages (calculated by equation (2) of untreated (control) or treated with the SSAD powder at 3, 7, 14, and 21 d). c) Histologic observation for epidermal healing of wounded tissues. The black line represents the test line for skin thickness. d) The skin thickness ratios (STRs) of control and SSAD treatment groups compared with normal skin. e) Masson’s trichrome stain of a skin defect on day 21. HF, SG, and BV (*p < 0.05 compared to control, and **p < 0.01 compared to control). f) The number of BV, HF, and SG in each visual field. g) Immunofluorescence stain: i) on day 7, only a few endothelial cells (CD31, red) and mural cells (α-SMA, green) could be seen (white arrow) in the i) empty control and iii) normal groups; (ii) many more endothelial cells and mural cells could be seen in the SSAD group. On day 14, both the iv) empty control and v) SSAD groups showed endothelial cells and mural cells stains. iv) Histograms representing the quantitation of percentages of CD-31 and α-SMA stain-positive cells. (***p < 0.001 compared to control).
The wound closure rate for 21 d was measured (Figure 7b). On day 0, no noticeable visual difference was recognized between the two groups (except for the white SSAD powder covering the defect area). At the 3rd day after wound dressing, an obviously higher wound closure rate was observed in the SSAD-treated group (30.9 ± 8.2%) versus that in the non-treatment group (10.4 ± 1.5%) (Figure 7a,b). On the 7th day, the wound closure rate of the control group was 24.4 ± 5.5%, whereas the SSAD-treated group demonstrated wound closures of 54.5 ± 12.4%. On the 14th day after operation, the wound treated with SSAD increased to 80.9 ± 7.5%, while the wound healing percentage was 58.2 ± 11.4% for the control group. On the 21st day, the wound almost healed completely (98.1 ± 2.6%) for the SSAD-treated group and hairs started to regrow at the initial injury site, whereas only 71.9 ± 6.4% wound healing was observed for the control group. It could be concluded that SSAD promoted wound closure rate and improved the appearance of wounds, while untreated scars were significantly larger and lasted longer. This observation could be attributed to the growth factors in SSAD that facilitate accelerated wound healing.[20]

Proper wound healing requires initial re-epithelialization and connective tissue contraction, collagen production, and angiogenesis of the newly generated dermis.[43] To observe the effect of initial re-epithelialization and contraction of connective tissue, histology analyses were performed 21 d after wound dressing (Figure 7c). Hematoxylin and eosin (H&E) staining revealed that the SSAD-treated group led to thicker skin regeneration (similar to that of normal skin) and a smaller ulcerous area when compared with the non-treatment (control) group (Figure 7d).

To evaluate collagen deposition in the defects, the healed tissues were analyzed by Masson’s trichrome staining. Collagen, myofibril, and nuclei were stained blue, red, and dark blue, respectively, by trichrome staining (Figure 7e). During wound healing, collagen fibers play a leading role in the repair structure and function.[46] On the 21st day, the untreated skin tissue was poorly reconstructed. The number of collagen fibers in the untreated group was much lower than that in the SSAD-treated group, which was similar to that of normal skin. Staining further revealed that the SSAD-treated group induced the formation of more mature granulation tissues with a higher number of blood vessels (BV) than normal skin (Figure 7e), since 21 d is still in the granulation tissue proliferation and remodeling stage. In addition to epidermal healing, a mature dermal structure was formed in the SSAD treatment group on the basis of normal tissue components (including hair follicles, HF and sebaceous glands, SG), which was not found in the control group (Figure 7f).

To analyze angiogenesis, endothelial (CD31) and mural (α-smooth muscle actin, α-SMA) cell-associated biomarkers were analyzed to measure newly formed BV.[47] BV structures could be clearly visualized within the repaired tissue areas by CD31-expressing structures and were used to determine the percentage of coverage in neodermis (Figure 7). BV density was significantly higher (p < 0.001) in wounds treated with SSAD adhesive (4.42 ± 0.55%) than in the blank control group (1.48 ± 0.39%) on day 7, and on day 14, more BV appeared in the regenerated skin tissues of the wounds treated with SSAD (13.03 ± 1.03%) than in those of the control group (8.30 ± 1.59%, Figure 7g,i,v).

In addition, α-SMA-positive staining was quantified to indicate maturation of the newly formed BV.[47] On day 7, the control group revealed 0.05% ± 0.004% α-SMA expression, whereas the SSAD-treated group demonstrated 0.12 ± 0.05% expression. On day 14, the SSAD-treated group exhibited a very high density of α-SMA-positive cells (1.96 ± 0.41%) compared to the control group (0.53 ± 0.09%, Figure 7g,vi). Furthermore, colocalization of CD31/α-SMA stains (yellow) could be observed in the SSAD-treated group on day 14 (Figure 7g,v), which was probably a sign of newly formed, potentially mature vessels, indicating good angiogenesis.

Serious skin defects, especially full-thickness wounds, including damages to the epidermis and dermis, skin appendages, and underlying subcutaneous tissues, usually lead to the formation of nonfunctional scars.[48] Scar formation mainly occurs due to disordered collagen and elastic fiber network and results in a lack of functional skin appendages (such as HF, sweat glands, and SG).[49] Wound healing includes a series of steps such as cell proliferation and migration and ECM formation and re-establishment of the natural barrier between the body and the external environment. A main challenge in wound healing, especially with diabetes, is to support adequate vascularization and avoid wound site infections. As mentioned previously, SSAD contains 10 proteins related to wound healing. In addition, 50 antimicrobial peptides of 26 different categories have been identified in skin mucus of three types of amphibians, one of which possesses an obvious inhibitory effect on the development of bacteria.[50] Moreover, compared with the hard and dry surface of an untreated defect, SSAD can keep the wound moist and possibly preserve bioactive cytokines produced at the initial stage of wound healing.[51]

2.5. Degradation of SSAD Hydrogel in Normal SD Rats In Vivo

SSAD hydrogel was subcutaneously implanted into SD rats to evaluate its degradation, host immune response, and interaction with local tissues. SSAD samples were harvested on days 3, 7, 14, and 21 according to gradually reduced macroscopic sizes of the implanting sites (Figure S4, Supporting Information). H&E staining (Figure 8a) and Masson’s trichrome staining (Figure 8b) both confirmed the good degradability of the SSAD hydrogel in vivo. After the SSAD hydrogel was implanted, it induced acute inflammation cell gathering, hydrogel compression, swelling, breakdown, and disappearance during a 21-d period (Figure 8a,b). Three days after implantation, a moderate inflammatory response was found in the outermost layer of the implanted SSAD hydrogel. The representative inflammatory cells were stained dark blue (Figure 8a,i,b,i). At 7 d after implantation, the structure of the SSAD hydrogel started to dissolve and was nearly filled with invasive inflammatory cells without obvious fibrous capsules, indicating that the host response against the SSAD hydrogel was weak. At 14 d after implantation, almost no SSAD and only a few inflammatory cells were left in the implant site (Figure 8b,iii). At day 21, SSAD hydrogel and inflammatory cells could no longer be found (Figure 8a,iv,b,iv), which revealed that SSAD hydrogel could be thoroughly degraded in vivo. This timeline is also consistent with that needed for skin healing phases I–II (2–3 weeks).[52]
Corroborating with histological observations, immunofluorescence staining against surface markers of inflammatory cells indicated that, at day 3, lymphocyte infiltration (CD3) accumulated around the SSAD hydrogel (0.23 ± 0.06%, Figure 8c.i,d) and a slight macrophage invasion into the interface between the sample and the host tissue (CD68) was found (Figure 8c.i,e). Macrophage infiltration reached a maximum (3.21 ± 0.87%) on day 7 (Figure 8c.ii,e). Over time, the numbers of lymphocytes and macrophages both decreased, and almost completely disappeared by day 21 (Figure 8c–e). This observation is aligned with many commercial tissue sealants, especially those produce from biological protein sources,[36c,53] caution is still necessary when applying the adhesive to the human body. Further systematic analyses should be conducted to analyze its full spectrum of immunogenicity.

2.6. In Vivo Evaluation of Systemic Toxicity

Assessment of the long-term toxicity of the SSAD hydrogel in vivo is another essential factor of its biological applications. During the treatment period and subsequent observations (up to 35 d post-injury), no effects on the general health or behaviors of the mice were observed. Furthermore, histological examination of the hearts, livers, spleens, lungs, and kidneys with H&E staining did not reveal any noticeable systemic damages (Figure S6a, Supporting Information). The toxic effects of
SSAD on the kidneys (BUN) and liver (AST and ALT) were also evaluated, as well as general damage (LDH). After implantation of SSAD hydrogel (400 mg in 800 µL) for 30 d, these parameters fell within the normal reference ranges of the control groups (Figure S6b, Supporting Information), suggesting no significant damage to the major organs after treatment with SSAD.

3. Conclusions

In summary, we have reported, for the first time, an unmodified SSAD-based tissue adhesive for wound healing applications. SSAD could rapidly (<30 s) close a bleeding skin incision in rats and effectively heal a full-skin defect in diabetic SD rats. Moreover, SSAD could be thoroughly degraded in vivo within 3 weeks with mild inflammation. We anticipate that the low cost, environmentally friendly production, healing-promotion ability, and good biocompatibility of SSAD provide a promising and practical option for sutureless wound closure, as shown by the current research. SSAD will likely overcome some limitations associated with currently available surgical glues and can perhaps be used to heal wounds on other delicate internal organs and tissues.

4. Experimental Section

Andrias davidianus Care and Sample Collection: Healthy adult Andrias davidianus (5–7 yr old, 70–120 cm in length, and 3.2 ± 0.8 kg in weight) were provided by HaoGuan Aquaculture Co. LTD, Chongqing, China (Figure 1a). Before collection, the animals were washed with clean water (Movie S1, Supporting Information). Mucus secretion was induced from freshly prepared SSAD could form an adhesive hydrogel by swelling in double deionized water (Figure 1d,e). The SSAD powder was stored at −20 °C until further use. During this study, no Chinese giant salamanders were sacrificed.

Characterizations of SSAD Powder and Hydrogel: Freeze-dried powder from freshly prepared SSAD could form an adhesive hydrogel by swelling in double deionized water (Figure 1d,e). The SSAD powders and hydrogels were examined via SEM, FTIR, and Raman spectroscopy. FTIR spectra (4000–400 cm⁻¹) were recorded using a Shimadzu FTIR spectrometer (Shimadzu Spectrum 400 FTIR, Japan) in transmission mode, and spectral data were recorded as absorbance units. Pesakfit 4.12 software was applied to analyze the secondary structure, where the raw spectra (1600–1700 cm⁻¹) of amide I were deconvoluted, peak-split, and curve-fitted by Gaussian functions. For Raman analysis, samples were measured in triplicate by a micro Raman spectrometer (785-nm IDRaman micro, Ocean, USA) with a 40 x objective (11.5 mW, 10-s acquisition time) and a spectral coverage of 400–1800 cm⁻¹. A Savitsky–Golay filter (third order, 15 points) was used to smooth the spectra. SEM characterizations were performed using an S-3400N II SEM (Hitachi, Japan).

SEM Analysis: SSAD powder and freeze-dried SSAD hydrogel at different hydration times (2 and 12 h) were lyophilized and mounted on silicon holders. After gold sputter-coating, all samples were imaged by an SEM (XL30 FEG, FEI/Philips, USA) at 15 kV. Pore size and porosity were calculated by ImageJ software (National Institutes of Health, NIH, USA). Samples were imaged in triplicates.

Test of Tissue Adhesive Strength Ex Vivo: Lap shear experiments were carried out to evaluate the bonding properties of SSAD using a universal testing machine (MTS Criterion, Model 43, USA). Porcine skins were used as the bonding matrix. The skins were cut into 1 x 8-cm² rectangles and bonded by cyanoacrylate glue (Baiyun Medical Adhesive; China), fibrin glue (FIBINGLURAS, China), or SSAD adhesive in two ways, i.e., apposition adhesion and side adhesion. For the SSAD adhesive, SSAD powder was spread at the site of interest on the porcine skin, a small amount of PBS was added, and the skin was joined with the other piece to achieve bonding within 30 s. Cyanoacrylate and fibrin glue were used following the manufacturer’s instructions. At 2 h post-adhesion, the adhesivity and flexibility of the adhesive zone were evaluated using the universal testing machine under a 100-N load and at a speed of 1 mm min⁻¹.

Adhesion Assessment In Vivo: All animal studies were carried out according to the National Institutes of Health Laboratory Animal Care and Use Guidelines (NIH Publication No. 85-23 Rev. 1985), and approval was granted by the Dental School of Chongqing Medical University Animal Care and Use Committee (CQHS-REC-2018-01). All experimental animals were purchased from the Experimental Animal Center of Chongqing Medical University.

Male SD rats (6–8 weeks old; 200 ± 20 g) were used to assess the wound adhesive effect of SSAD in vivo. After anesthesia, the backs of the rats were shaved and disinfected by iodine and ethanol, and four incisions (2 cm) were made for each rat. The following four treatments were used: suture closure (4–0 nonresorbable suture), SSAD adhesive, cyanoacrylate glue, and hemostasis only (negative control). All SSAD powders were sterilized under ethylene oxide sterilization. At 5 d post-operation, the rats were sacrificed, and the skins (3 x 3-cm²) were collected for histological analyses.

Wound Healing Assessment In Vivo: The wound healing ability of SSAD in vivo was evaluated using full-thickness skin defects in a streptozotocin (STZ)-induced diabetic SD rat skin model. After the diabetic rats were successfully generated according to our previously published protocol,[26] they were anesthetized with 1% sodium pentobarbital (intraperitoneal injection). Then, their dorsal surfaces were shaved and disinfected. A disposable biopsy punch was used to create a full-thickness round skin defect (diameter = 10 mm) on the back, which was then covered with gauze (negative control) or SSAD powder (30 mg per defect). At 21 d post-injury, the rats were sacrificed for histological analyses. The wound closure percentage, STR, average normal skin thickness, and numbers of cutaneous appendages were statistically analyzed.

The wound closure percentage was calculated according to equation (1) using ImageJ:

\[
\text{Wound closure percentage} = \frac{S_{\text{initial}} - S_{\text{current}}}{S_{\text{initial}}} \times 100\% \quad (1)
\]

where \(S_{\text{initial}}\) is the initial wound size and \(S_{\text{current}}\) is the current wound size. Each test was performed in triplicate.

STR was calculated according to equation (2):

\[
\text{Skin thickness ratio} = \frac{T_{\text{Scar}}}{T_{\text{Normal}}} \times 100\% \quad (2)
\]

where \(T_{\text{Scar}}\) is the average skin thickness of scar tissue and \(T_{\text{Normal}}\) is the average normal skin thickness. Each test was performed in triplicate.

In Vivo Degradation Assay: After general anesthesia, skin incisions (3-cm in length) were created on the back and subcutaneous tissues were separated bluntly. SSAD hydrogels (100-mg SSAD powder in 200-µL PBS) were implanted into the underlying subcutaneous pockets. At 3, 7, and 14 d post-operation, the hydrogels with the surrounding tissues and the associated skin were harvested for histological analyses.

In Vivo Biocompatibility Assay: After treatment with SSAD for 35 d, the hearts, livers, spleens, lungs, and kidneys of the mice were harvested for histological analyses to evaluate the biocompatibility of SSAD. Blood samples were also collected for blood biochemistry analyses.

Histological Analysis: The samples were collected with surrounding tissue, H&E (Solarbio, China), Masson’s trichome (Solarbio, China), and immunofluorescence stains were used for histological analyses.

To assess angiogenesis in the wound healing model, rabbit polyclonal anti-CD31 primary antibody and mouse monoclonal anti-α-SMA primary antibody (Abcam, USA) were used to stain the wound tissues around the skin. Then, goat anti-rabbit IgG-Alexa Fluor 647 secondary antibody and...
goat anti-mouse IgG-Alexa were used. To evaluate the inflammation effect of degradation, anti-CD68 and anti-CD3 primary antibodies (Abcam) and then Alexa fluorescent secondary antibodies (Abcam) were applied. Samples were further counterstained with 4′,6-diamidino-2-phenylindole (Vector Laboratories, UK). Positively stained cells were counted. The values were divided by the areas of the fields by ImageJ software. All illustrations were assembled and processed digitally. Histological images (n = 4 sections per sample) were checked (n = 5 field-of-views per section) with an Axios observation microscope (Zeiss, Germany).

Statistical Analyses: All data were analyzed using SPSS 19.0 software (IBM, USA) and expressed as the mean ± SD. One-way analysis of variance (ANOVA) and student Newman-Kells tests were used to analyze the differences among groups. At P < 0.05, the difference was deemed to be statistically significant. All graphs were drawn by Origin V10.5.30 software (Origin Lab, USA).

Supporting Information
Supporting Information is available from the Wiley Online Library or from the author.

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Conflict of Interest
The authors declare no conflict of interest.

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