Technical Note

A tissue adhesives evaluated *in vitro* and *in vivo* analysis

Xiumei Mo,[1] Hiroo Iwata,[2] Yoshito Ikada[3]

[1]Biomaterials and Tissue Engineering Laboratory, Donghua University, Shanghai 201620, China
[2]Institute for Frontier Medical Science, Kyoto University, Kyoto, Japan
[3]Department of Indoor Environmental Medicine, Nara Medical University, Nara 634-8522, Japan

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Abstract: In this study, three kinds of two-component adhesive glues were prepared, namely, gel-dext glue made from modified gelatin and dextran, gel-HES glue made from modified gelatin and hydroxyethyl starch (HES), and chit-dext glue made from chitosan and modified dextran. Upon mixing the two-component solution together crosslinking occurred and a gel formed in several seconds, which would seal the wound tissue and stop the bleeding. The adhesive ability of those three prepared glues was evaluated *in vitro* and *in vivo* separately by measuring the bonding strength to two piece of porcine skin and the adhesive strength after sealing the skin incisions on the back of rat. Fibrin glue was used as comparing. Gel-dext glue and gel-HES glue shown higher bonding strength and adhesive strength than chit-dext glue and fibrin glue. Histology test of incision tissues given by both HE and MTC methods, the former shown that gel-dext and gel-HES glues, like fibrin glue, have only normal initial inflammation to skin tissue, which almost disappear from 9 days but chit-dext glue seams have heaver inflammation, which may last to 12 days; the later shown gel-dext and gel-HES glues similar to fibrin glue, can heal the wound fast than that of chit-dext glue. The hemostatic ability for gel-HES glue was also tested on a cut liver of rat, which depend on the gel formation speed when the two-composite solutions were mixed together. J Biomed Mater Res Part A: 94A: 326–332, 2010.

Key Words: tissue adhesive, hemostatic agent, gelatin, chitosan, polysaccharides

INTRODUCTION

Tissue adhesive is a very useful agent for various surgeries, such as skin closure in wound care,[1] brain vascular anastomosis in brain surgery,[2] aorta vascular anastomosis,[3] as well as bone piece adhesive in osteosurgery. Varied requirements cause that a single adhesive could not meet so many needs. Some tissue adhesives have been developed for clinic application.[4] Gelatin-Resorcinal-Fomol glue (GRF) was designed as closure agent for aorta vascular,[5,6] but the closure point is hard, which has the danger to brake the aorta vascular nearby the closure point. Cyanoarylate show strong adhesive to tissue but it can not be used for big amount as its body is nonbiodegradable and toxic to tissue.[7] Fibrin glue is a very useful glue in almost all the surgeries,[8] but it still has its disadvantage like that the danger to distribute disease and week adhesive strength. Therefore, a great deal of effort has been made to prepare synthetic or semisynthetic glues applicable as surgical adhesives and hemostatic agents. Ikada's Lab has reported two kinds of tissue adhesives that consist of gelatin, poly(L-glutamic acid) (PLGA) and water soluble carbodiimide, and gelatin and *N*-hydroxysuccinimide-activated PLGA.[9,10] These can promptly form a gel and firmly bond to soft tissue when mixed. However, urea derivative or *N*-hydroxysuccinimide are released during formation of gels.

This study was conducted to develop new surgical adhesive and hemostatic glue, which does not release any low molecular weight substance. Our new glue is based on the Schiff base reaction between the amino groups in modified gelatin (gel) or chitosan and the aldehyde goups in oxidized dextran (ald-dextran) or oxidized hydroxyethyl starch (ald-HES). A gel is quickly formed when these two aqueous solutions are mixed together, which can stop bleeding and seal the tissue. The preparation and properties of the new adhesives have been reported.[11] In this paper, *in vitro* and *in vivo* tissue adhesive ability was evaluated for preparing three kinds of two-component polymer glues, that is, modified gelatin and dextran glue (gel-dext), modified gelatin and hydroxylethyl starch glue (HES) (gel-HES), chitosan and modified dextran (chit-dext) to compare with clinically used fibrin glue. The hemostatic ability of gel-HES glue has been tested by the method of coating the glue on the cut liver surface of rat and measuring the bleeding amount of cut liver to compare the bleeding amount of cut liver without

Correspondence to: X. Mo; e-mail: xmm@dhu.edu.cn
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coating with gel-HES glue. Gel-dext and gel-HES glues have been certificated the effective adhesive glue and hemostatic agent comparing with fibrin glue.

EXPERIMENTAL

Materials

Modified gelatin (Amino-gelatin), modified HES and modified dextran (ald-HES and ald-dextran) were prepared from acid gelatin (Nitta Gelatin Co. Osaka, Japan), dextran (M_w = 200,000, Wako Pure Chemical industries) and HES (Sigma-Aldrich Japan, Tokyo) in our laboratory. Chitosan (deacetylated degree 100%, viscosity 20 cps, from Koyo Chemical Co. Osaka, Japan) was directly used as a component of the adhesive. Fibrin glue was purchased from Chemo-Sero-Therapeutic Research Institute, Kumamoto, Japan. SD female rats of 11 weeks older were punched from Shimizu laboratory supply, Kyoto, Japan.

Preparation of modified polysaccharide and gelatin. Polysaccharides were oxidized by sodium metaperiodate into dialdehyde groups and modified gelatin was also prepared by introducing more amino group in the gelatin with the method described in the previous article.[11]

Gelation time measurement

A glass tube with a l cm magnetic bar was incubated at 37°C and the stirring speed was fixed at three and 0.5 mL of one component solution of adhesive glue was dropped into the tube and mixed with 0.5 mL of another component solution. The time period required for the magnetic bar to stop was recorded as gelation time of the adhesive glue.

Bonding strength measurement

The fatty layer of porcine skin was removed using a scalpel and the fatty-layer-free porcine skin was sliced into 1 × 3 cm^2. Twenty five microliter of one component solution of adhesive glue was put on the dermal side of each skin slice, and then the same volume of another component solution was mixed together on the skin slice. Two skins were then overlapped to a bonding area of 1 × 1 cm^2. After loading a weight of 50 g for 10 min, unless otherwise indicated, the bonding strength was measured at 25°C using a tensile machine, Autograph (Shimadzu, Kyoto, Japan), at a testing rate of 10 mm/min.

Animal experiment for skin closure and *in vivo* degradation test of the adhesive glue

The rats were anesthetized by inhaling with diethyl ether. Four incisions of 2 cm long and as deep as skin thickness were made on both side of the rat back. Each incision was separately coated with different two-component glue of gel-HES, gel-dext, chit-dext glue, and fibrin glue, then the incision was quickly close to let the cut skins adhered tightly. Finally, relief suture was used to the incision to avoid it being broken by force. At 3, 6, 9, and 12 days after implantation the closure skin were cut in 2 × 2 cm^2 size on each wound, half of the skin was used for the measurement of adhesive strength and the remain was fixed in aldehyde solution for the histological test by Masson's trichrome method and Hematoxylin and eosin (HE) method. The *in vivo* degradation of the glue and the skin closure could be observed directly from HE histology image.

Adhesive strength measurement

The freshly cut skin with 2 cm in length and 1 cm in width was used to measure the adhesive strength of the wound immediately with the tensile force perpendicular to the wounded line, in the sample the wounded line is 1 cm long. Same machine and testing conditions as that used for bonding strength measurement were adopted to measure the adhesive strength of the healed wound.

Animal experiments for stopping bleeding

The rats were anesthetized with diethyl ether. Open the chest of the rat to let the rat liver released and can be operated easily. Take a leaf of the liver and cut a piece of it with a intersection about 1 cm length, quickly coat the adhesive glue on the cut surface, at same time collect the bleeding with a filter paper within 3 min. Bleeding amount was measured with hemoglobin testing method.

A controlled bleeding amount also be collected and measured by the same method as above without coating anything on the cut liver surface.

Hemoglobin testing. A composite solution of 17 m*M* Tris-HCl water solution and 0.75% NH_4Cl solution was prepared and adjusted to pH 7.6. The filter paper soaked with blood was dipped into 10 mL above composite solution in a plastic tube. Shake the tube in a water bath shaker for 24 h at 37°C. Freeze the blood solution for the following measurement.

A staining solution (Potassium cyanide 7.8 mmol/L and Potassium ferricyanide 6.1 mmol/L) was diluted nine times with distilled water. Draw 200 μL blood solution into a glass tube and then add 5 mL diluted staining solution, the optical density of the blood solution was measured at 540 nm using a UV-VI spectrometer. The bleeding amount was calculated from the absorbance. The hemostatic ability of the glues was evaluated by hemostatic ratio.

$$\text{Haemostatic Ratio} = \{(W_C - W_H)/W_C\} \times 100\%$$

W_C: Controlled bleeding amount.

W_H: Bleeding amount with adhesive agent coating on the bleeding surface.

RESULTS AND DISCUSSION

Modified gelatin and polysaccharides

The detail description of modified gelatin and polysaccharides has been published.[8] In the molecular chain of gelatin there are amino group and carboxyl group. By reacting with ethylenediamine, the carboxyl group can be changed into amino group, thus, gelatin was modified to introduce more amino group. Amino group introduced in gelatin can be varied by the addition of the amount of ethylenediamine in the reaction. The neighboring hydroxyl groups in the glucose

TABLE I. Gelation Time and Bonding Strength of Different Glues

Adhesive Glue	Condition	Gelation Time (s)	Bonding Strength (gf/cm^2)
[a]Gel-dext	20% amino-gelatin + 10% ald-dextran	8	210
[b]Gel-HES-1	20% amino-gelatin + 10% ald-HES-1	8	183
[c]Gel-HES-2	20% amino-gelatin + 10% ald-HES-2	2	227
[d]Chit-dext	3% chitosan + 10% ald-dextran	4	130
[e]Fibrin glue	Fibrinogen + thrombin	5	120

[a] 20% amino-gelatin aqueous solution with 55% introduce amino group content and 10% ald-dextran aqueous solution with 70% dialdehyde content.
[b] 20% amino-gelatin aqueous solution with 55% introduce amino group content and 10% ald-HES aqueous solution with 43% dialdehyde content.
[c] 20% amino-gelatin aqueous solution with 55% introduce amino group content and 10% ald-HES aqueous solution with 92% dialdehyde content.
[d] 3% chitosan in 0.2*N* acetic acid solution and 10% ald-dextran aqueous solution with 70% dialdehyde content.
[e] Fibrinogen solution and thrombin solution prepared as the instruction of the product purchased.

ring of polysaccharides, such as dextran and hydroxyethylene starch, can be oxidized by sodium metaperiodate into dialdehyde group to introduce aldehyde group in polysaccharides. Introduced aldehyde group amount can be controlled by the amount of sodium metaperiodate.

Gelation time and bonding strength

Gelatin time was used to measure how fast the two-composite glue can form a gel when was mixed together, which can be used as an evolution of the hemostatic efficiency, hence to stop bleeding, a quick formed gel will be needed to cover the bleeding surface. Binding strength was given from the tensile strength for two adhered skin to be broken, which can be used as an evaluation of *in vitro* adhesive ability of the adhesive agent.

Table I shows the gelation time and bonding strength of different adhesive glues. The gelation time of fibrin glue is 5 s. Usually 5 s gelation time is fast enough to be as a hemostatic agent or adhesive agent. Gelation time of gel-dext and gel-HES can be easily adjusted as near 5 s and even shorter than 5 s by changing the aldehyde content in ald-dext or ald-HES, as shown in Table I, that the gelatin time of gel-HES-2 is shorter than that of gel-HES-1 as the dialdehyde content in ald-HES-2 (92%) is higher than that in ald-HES-1 (43%). The bonding strength is higher for gel-HES-2 than for gel-HES-1, that means the higher aldehyde content in HES benefit the adhesion properties. The bonding strength of gel-dext and gel-HES are obviously higher than that of chit-dext and fibrin glue, that is because of the different bonding mechanism caused by the materials difference.

The bonding mechanism for gel-dext and gel-HES is based on the Schiff base reaction in between amino groups in modified gelatin and aldehyde group in modified polysaccharides. The bonding mechanism for chit-dext is based on the Schiff base reaction in between amino groups in chitosan and aldehyde group in modified dextran. Chitosan has stiff molecular chain comparing with gelatin, the formed gel from chitosan and dextran are too stiff to give higher binding strength. The mechanism of gel formation for fibrin glue is based on a polymerization of peptide. The two composite of fibrinogen and thrombin when was mixed together, thrombin as an enzyme will break the fibrinogen into active fibrinopeptide A and fibrinopeptide B, which then polymerized into fibrin gel in the presence of activated factor XIII, thus the wound tissue was sealed by the polymerized fibrin gel. During the polymerized fibrin gel formation some defects may occur inside the adhesive interface, like air bubble, which may be one of the reasons to give week binding strength.

Adhesive strength

To get good closure for the cut skin on the incision one composite glue was first dropped with a syringe. After dropped the other component the incision were closed quickly to avoid too much crosslinked gel leave on the incision. Four different glues shown in Table I gel-dext, gel-HES-2, chit-dext, and fibrin glue was chosen for the skin closure test. Four kind glues all can sealed the incision quickly and tightly and no obvious inflammation on the rat skin can be seen during 12 days observation. At 3, 6, 9, and 12 days after the incision was closed the adhesive strength was measured as in Figure 1.

Figure 1 shows that the incisions closed by different adhesive glue all have increased adhesive strength with increasing the healing time. Gel-dext [Fig. 1(a)] and gel-HES-2 [Fig. 1(b)] glues give higher adhesive strength than that of chit-dext [Fig. 1(c), $p < 0.05$] and fibrin glue [Fig. 1(d)]. The adhesive strength of fibrin glue is not stable. During the experiments, it has been found that some incision sealed by fibrin glue have bubble, same result has also been reported by Sekine et al.[12] That may be one of the reasons why fibrin glue could not have strong adhesive ability. Those *in vivo* rat skin adhesive results are also coincide with the *in vitro* bonding strength measurement result.

Modified gelatin and modified polysaccharides adhesive glue, gel-dext, and gel-HES, has shown their special higher adhesive ability to tissue both *in vitro* and *in vivo* evaluation. As we know gelatin, dextran, and hydroxyethyl starch are all non toxic and biodegradable materials and have been widely used for biomedical application.[13–15] Although aldehyde group introduced in dextran and HES has a certain cytotoxicicity, but during adhesive glue application most aldehyde groups have been changed by the Schiff base

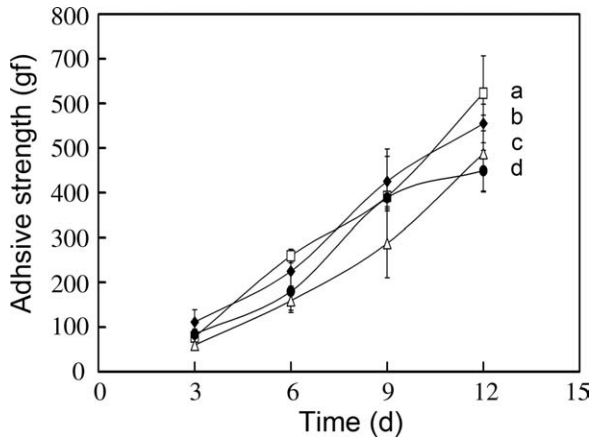

FIGURE 1. Healing time and adhesive strength of wounded skins sealed by the adhesive glues: (a) gel-dext, (b) gel-HES, (c) chit-dext, and (d) fibrin glue. Error bars represent stand deviation (SE). Date represents mean + SE statistically analyzed by Fisher's PLSD ANOVA.

reaction with amino group in the gel formation, so gel-dext and gel-HES can be considered as nontoxic glue.

Histology of healed skin

HE histology of the incision section. Hematoxylin and eosin method (HE) is a normal used histological method. Figure 2 showed the histology of skin cross section sealed by gel-dext glue, similar result has also been found for the gel-HES glue. The blue point around the incision was coursed by the inflammation in tissue, which indicated the nucleuse of microphages. At 3 days obvious inflammation occurred in the incision, it then disappeared quickly, and in 12 days no more inflammation can be observed.

Inflammation in wound healing can be qualitatively separated into acute and chronic inflammation. Acute inflammation is initial foreign body reaction to the implant, generally of short duration and lasting about one week. It is characterized by an increased capillary permeability, which results in the exudation of plasma proteins and emigration of polymorphonuclear neutrophilic leukocytes (PMN) to the site of inflammation. While, chronic inflammation is longer duration and histologicaly associated with macrophages, lymphocytes, proliferation of connective tissues, deposition of matrix proteins, and capillary neogenesis.[16] In Figure 2 all the inflammation is belong to acute inflammation, that is to say, the modified gelatin and modified polysaccharides two-component adhesive glue is safe material to skin closure.

Figure 3 shows the HE histology of chit-dext. More serious inflammation was found in Figure 3 than in Figure 2, and a certain amount of inflammation still can been seen in 12 days after implantation. The serious inflammation may be caused by chitosan solution. As 100% deacetylated chitosan can not dissolve in pure water, a dilute acetic acid solution was used to prepare the chitosan solution which shown the pH of 4.4.

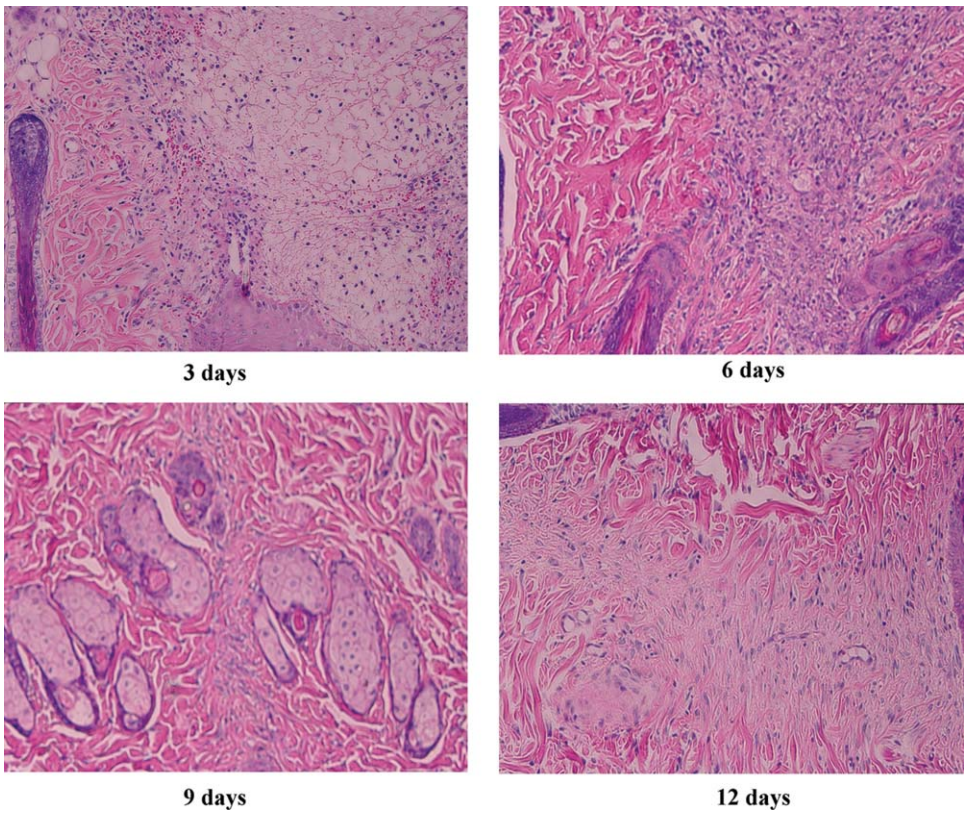

FIGURE 2. HE histological examination for gel-dext at different healing time. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

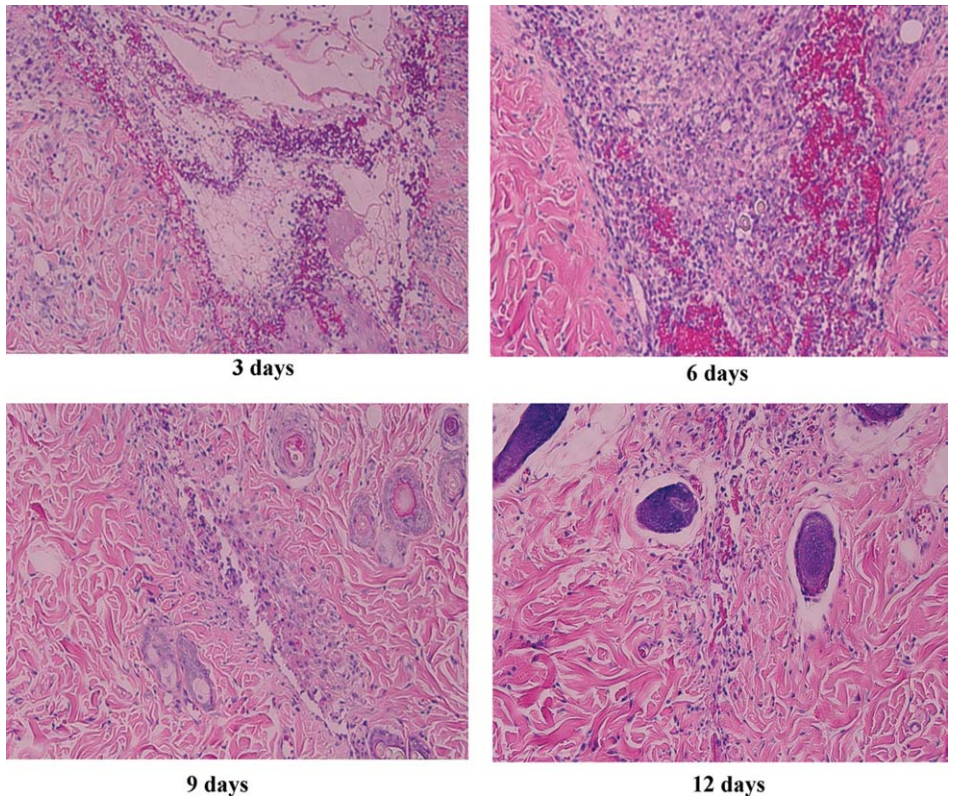

FIGURE 3. HE histological examination for chit-dext at different healing time. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

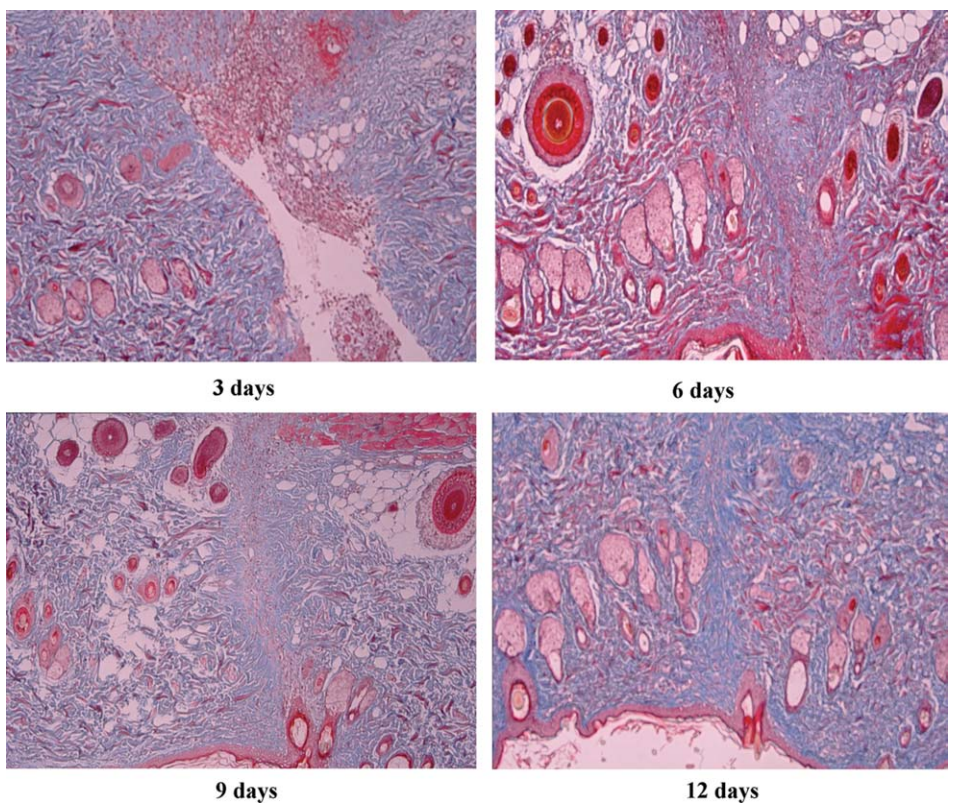

FIGURE 4. MTC histological examination for fibrin glue at different healing time. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

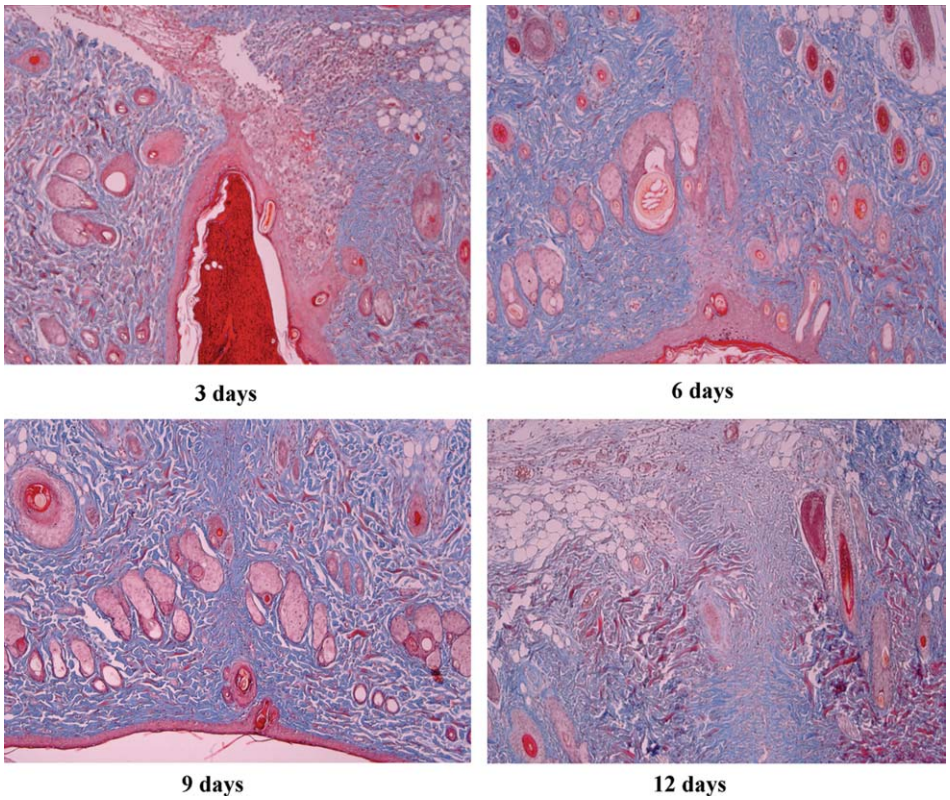

FIGURE 5. MTC histological examination for gel-dext at different healing time.

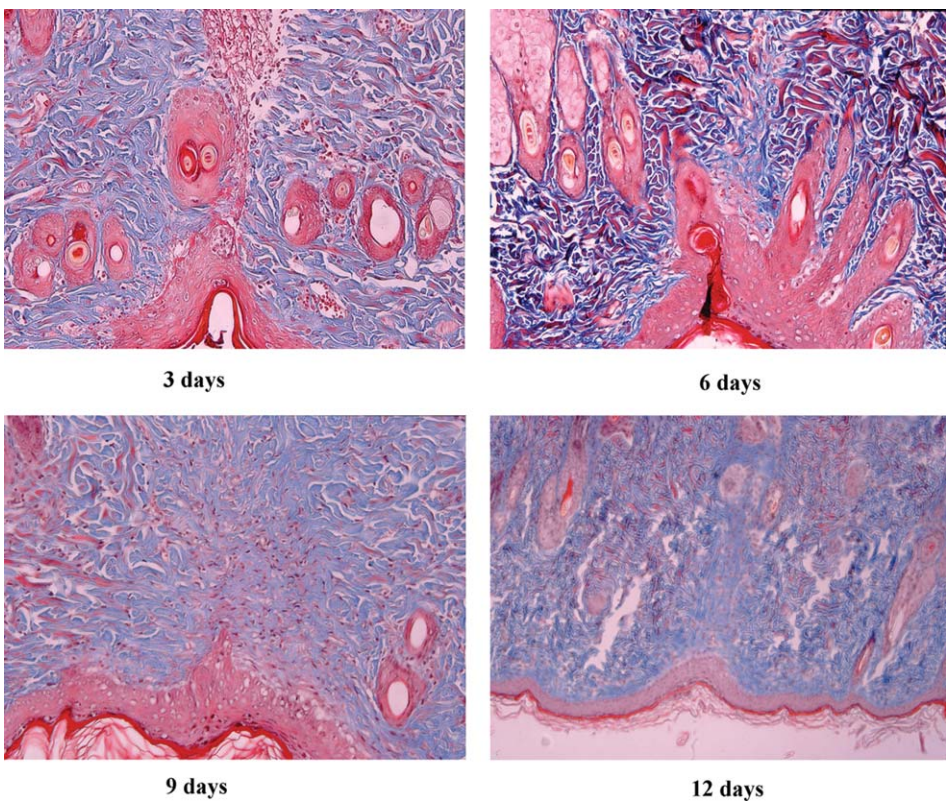

FIGURE 6. MTC histological examination for gelatin-HES at different healing time.

TABLE II. Hemostatic Ratio Varied with the Gelation Time

Sample	Gelation Time (s)	Hemostatic Ratio (%)
Without glue		0
Gel-HES-1	8	39
Gel-HES-2	2	50

MTC histology of healed skins. Masson's trichrome method (MTC) is specially used to see the collagen in skin, which could be stained into green or blue color.

Figure 4 showed the MTC histology of the cross section of the incision sealed by fibrin glue. The blue network represents the skin collagen. At 3 days after implantation there is still a big gap in the incision, no new skin collagen growths inside, and the fibrin glue is still exist in the gap with normal inflammation; at 6 and 9 days the collagen network have formed and crossed the incision, the fibrin glue may have been degradated; at 12 days the new formed collage have adjusted themselves and looks similar to the normal tissue collagen.

Figure 5 showed the MTC histology of skin cross section sealed by gel-dext glue; Figure 6 showed the histology of skin cross section sealed by gel-HES glue. Similar results can be seen as that of fibrin glue. At 12 days the glue has been degradated and new collagen has grown to connect each other from both side of incision.

Figure 6 showed the MTC histology of skin cross section sealed by chit-dext glue. Until 6 days still a big gap exist in the incision. Even at 12 days the incision was still not completely crossed by new formed collagen. It seams the chit-glue is still exist in the incision without completely degradated. Comparing with other three kinds of adhesive glues chit-dext glue shown lower healing speed to the skin incision. One reason is the serious inflammation of chit-dext glue caused by chitosan acetic acid solution (Fig. 3), another reason may because of the lower biodegradability of chitosan compared with gelatin and fibrin glue (Fig. 6).[16] That is also the reasons why the adhesive strength of chitosan-dextran glue is lower.

In vivo *biodegradation of the adhesive glues.* The *in vivo* biodegradation of different adhesive glues can be observed clearly from the histology examination from Figures 2–6. Fibrin glue shows the most fast degradation speed, it disappeared at day 6 after implantation as in Figure 4; chit-dext glue shows the slowest degradation speed, which still slightly be seen at day 12 after implantation as in Figure 3. Gel-dext and gel-HES show middle degradation speed, which disappeared at day 9 after implantation as in Figures 2 and 6.

Hemostatic ratio

New developed two-composite adhesive glue from modified gelatin and polysaccharides can also be used as hemostatic agent. When the two-composite solution were injected out from a syringe to syringe connector to a cut liver surface, the glue will quickly cover the all surface and at same time crosslink to form a gel, which can tightly adhere to the bleeding surface to stop bleeding. As a hemostatic agent gelation time is a very important effect, as if the gelation time is too slow, when the two components were put on the bleeding surface they will be flowed with bleeding. Shorter gelation time will be suitable as a hemostatic agent. Table II shown the hemostatic ratio varied with the gelation time of gel-HES glue.

CONCLUSION

The two-composite adhesive glue of modified gelatin and modified polysaccharides can be used as both safe, and effective adhesive and hemostatic agent. The bonding strength to porcine skin and adhesive strength to rat skin for gel-HES and gel-dext has been certificated higher than that of chit-dext and fibrin glue.

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