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Title: The Usefulness of Re-attachability of Anti-adhesive Cross-linked Gelatin Film and The Required Physical and Biological Properties

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Abstract:

BACKGROUND: To overcome the unfavorable issues associated with conventional anti-adhesive HA/CMC film, we developed an anti-adhesive thermally cross-linked gelatin film. **OBJECTIVE:** We tried to clarify the re-attachability of the film and the required properties concerning the film thickness, stiffness and anti-adhesion effect. **METHODS:** To determine the optimal thickness, 5 kinds of the thickness of gelatin film and the conventional film were analyzed by tensile test, shearing test, buckling test and the tissue injury test. Finally, using the optimal film thickness, we tried to clarify the anti-adhesion effect of the reattached film. **RESULTS:** The tensile and shearing test showed gelatin films $\geq$ 30 µm thick had greater tensile strength and a smaller number of film fractures, than the conventional film. The buckling and tissue injury test showed gelatin films $\geq$ 60 µm thick had higher buckling strength and worse injury scores than the conventional film. The anti-adhesive effect of re-attached gelatin film using optimal thickness (30-40 µm) found the anti-adhesion score was significantly better than that of the control. **CONCLUSIONS:** Provided it has an optimal thickness, gelatin film can be reattached with enough physical strength not to tear, safety stiffness not to induce tissue injury, and a sufficient anti-adhesion effect.

Key words: anti-adhesion, re-attachability, gelatin film, thermally cross-linked, tissue injury

1.Introduction

Postoperative abdominal adhesion often occurs after abdominal and gynecological surgery and often causes several complications, such as bowel obstruction, chronic pain, and female infertility [1-7]. In order to prevent adhesion, various kinds of anti-adhesion material has been developed and some has been used clinically [8-11]. Currently, conventional anti-adhesive film made of hyaluronate and carboxy-methyl-cellulose (HA/CMC) has been used widely in clinical situations [12-15]. However, it has serious unfavorable issues, such as controversial clinical effectiveness for preventing adverse events of adhesion and a potential risk for leakage at the site of intestinal anastomosis [16]. To overcome these problems, we have developed a thermally cross-linked gelatin film and previously reported that it has a significantly greater anti-adhesion effect without any cytotoxicity than the conventional film [17]. It could also be used safely for intestinal anastomosis without inducing leakage [18].

The conventional film has another serious clinical problem: easy tearing due to its fragility [19]. Actually, in usual clinical practice, surgeons often place the conventional film on incorrect sites due to technical error or difficulty of delivery. While the film should ideally be able to be detached and re-attached to the correct site again without tearing, it is quite difficult to re-attach the conventional film due to too fragilie [20, 21]. In our previous report, we noted that the gelatin film has better physical strength than the conventional film [17]. Therefore, we are very interesting to determine whether the gelatin film can be reattached with sufficient physical strength in clinical use or not.

Generally, the thicker anti-adhesion film may be stronger physically [22]. However, excessively thick films may be so stiff to induce the tissue-injury around the target tissues. Thus, it is also very important to adjust the thickness and stiffness of the gelatin film in order to avoid causing tissue injury. In the present study, to determine the optimal thickness of the thermally cross-linked gelatin film that would have enough physical strength and safety stiffness for clinical use, we conducted the tensile test, shearing test, buckling test and the tissue injury test on dog liver for gelatin films of varying thickness. Finally, using the gelatin film of the thickness determined to be optimal, we tried to clarify whether or not the reattached film had a sufficient anti-adhesion effect, using rat adhesion models.

2.Materials and methods

2-1.Preparation of gelatin film and conventional film. The thermally cross-linked gelatin film was prepared as described in our previous report [17, 18]. In brief, we used alkali-treated gelatin extracted from porcine skin (type I - collagen, Medigelatin®; Nippi Co. Ltd., Shizuoka, Japan) with an isoelectric point of 5. To make gelatin films of varying thickness (20.0 ± 1.3, 30.0 ± 1.3, 40.0 ± 1.3, 60.0 ± 3.2, and 90.0 ± 6.9 μm thick), each gelatin solution was prepared and cast in plastic plates (Kanto Chemical Co., Tokyo, Japan) at 2.2, 3.5, 4.2, 5.5 and 7.0 mg/cm2, respectively. For the anti-adhesion test, 3.8 mg/cm^2 gelatin solution was cast in plastic plate (38.0 ± 1.2 μm) Those films were then allowed to dry in a clean bench for two days. Finally, the films were thermally cross-linked by a vacuum oven (AVO-250N, As One, Osaka, Japan) for 3.5 h at 140 °C. For further *in vitro* and *in vivo* examinations, the films were sterilized with ethylene-oxide gas (0.43 g/L at 40 °C for 4 h).

As a comparative anti-adhesion material, we used the conventional HA/CMC film (Seprafilm®; Genzyme Co., Cambridge, MA, USA). The details of this film are described in the product information (available online at; http//www.genzyme.com). In our preliminary study, the thickness of the conventional film was approximately 52 ± 3 μm using a thickness gage (Dial

Thickness Gauge G-7C; OZAKI MFG., CO., LTD., Tokyo, Japan).

2-2.Animals. Six female beagle dogs (about 2 years of age, weighing 10.5 ± 1.0 kg) and 24 female Wistar/ST rats (7 weeks of age, weighing 203.5 ± 17.8 g) were purchased from Shimizu Animal Laboratory (Kyoto, Japan) a week before the examination. The dogs and rats were kept in a specific-pathogen-free condition room with a 12h light-dark cycle, mean temperature of 23 °C and mean humidity of 50%. Standard laboratory rodent and dog chow and water were freely available. All animal experiments, animal care, housing and surgical procedures complied with the instructional guidelines of the Committee for Animal Research of Doshisha University (the ethical approval no. A15021).

2-3.Tensile test. Gelatin films of each thickness and the conventional film were cut into an oblong-shaped piece 10×50 mm in size. Next, each film piece was placed into two folders of the testing apparatus (CPU gauge: MODEL-RX10, TESTSTAND: MODEL-1323R; Aikoh Engineering, Osaka, Japan) by grasping the film ends at a distance of 3 cm. Each piece was then drawn automatically in opposite directions at a fixed speed of 5 mm/min until the film tore (Figure 1-a). The maximum tensile load was recorded for six pieces of each film. The statistical comparisons relative to conventional film were made using a non-paired Student's *t*-test. A p value of less than 0.05 was considered to be significant.

2-4.Buckling test. Gelatin films of each thickness and the conventional film were cut into an oblong-shaped piece 10×30 mm in size. Next, each film piece was placed into two folders of the testing apparatus (CPU gauge: MODEL-RX10, TESTSTAND: MODEL-1323R; Aikoh Engineering, Osaka, Japan) by grasping the film ends at a distance of 1 cm. Each piece was then compressed automatically at a fixed speed of 5 mm/min until the film buckled (Figure 1-b). The maximum buckling load was recorded for six pieces of each film. The statistical comparisons relative to conventional film were made using a non-paired Student's *t*-test. A p value of less than 0.05 was considered to be significant.

2-5.Shearing test. Thirty large intestines samples (each 10 cm in length) were resected from 6 beagle dogs. The intestines were placed sideways on the testing machine of horizontal type (CPU gauge: MODEL-1016 C, TESTSTAND: MODEL-2152VCE, Aikoh Engineering). Gelatin film of each thickness and the conventional film were cut into an oblong-shaped piece 10×40mm in size. Each film piece was then held using the testing rig by grasping one film end at a distance of 1 cm. The other 3 cm of the film was pasted onto the intestinal wall. After 30 or 120 seconds, each piece was pulled at an angle of 45° to the pasted intestine at a fixed speed of 5 mm/sec until the film had completely sheared or torn away (Figure 1-c). The number of torn films was recorded for six pieces of each film. The statistical comparisons relative to conventional film were made using a Pearson's chi-squared test. A p value of less than 0.05 was considered to be significant.

2-6.Tissue injury test. Gelatin films of each thickness and the conventional film were cut into an oblong-shaped piece 10×20 mm in size. For 6 beagle dogs, the liver was exposed under intravenous pentobarbital anesthesia (40 mg/kg of body weight). The serosal surfaces of the livers were rubbed with the corner of each film. The degree of the injured liver was then scored using a tissue injury score (Table 1) [23]. Six pieces of each film were examined. The statistical comparisons relative to conventional film were made using a non-paired Mann-Whitney U test.

A *p* value of less than 0.05 was considered to be significant.

2-7.Anti-adhesion effect test. A total of 32 female Wistar/ST rats were divided randomly into 4 groups of 8 rats: the gelatin film (gf) group, reattached gelatin film (r-gf) group, conventional film group, and control group. Under general anesthesia, a 4-cm-long incision was made at the midline of the abdomen. A 15-mm-diameter area of abrasion was then created on the cecum using a dental sanding tip (Sharp-Mini; Ohki Chemical Co., Hiroshima, Japan) until small blood drops appeared. In the r-gf group, each film was attached to the serosal aspect of the anterior wall and after 2 min, the film was detached from the anterior wall and re-attached to the abraded cecum. Another 15-mm-diameter region of abrasion was then made on the right lateral internal abdominal wall 2 cm from the midline incision on the abdominal wall, directly opposite the abraded cecum. In the gf and conventional film groups, after the anterior wall and the cecum had been abraded as described above, the abraded cecum was wrapped manually with each film covering the entire abrasion area. In the control group, the rats received no wrapping film treatment. In all groups, the two abraded surfaces were approximated with 6/0 Prolene® sutures (Ethicon Inc., Tokyo, Japan) before closing the abdomen in order to induce tight adhesion between the two sites.

Twenty-one days after the procedure, all animals were sacrificed, and the status of the abdominal cavity and abraded sites was observed macroscopically, including each piece of remaining material. The extent and severity of adhesion were graded and scored numerically, according to the adhesion grading scale (Adhesion Scores, (Table 2)) described previously [24]. The evaluation was performed by a researcher blinded to the animal assignments. The statistical comparisons relative to control were made using a non-paired Mann-Whitney U test. A *p* value of less than 0.05 was considered to be significant.

3.Results

3-1.Tensile test. The results of the tensile test are shown in Figure 2-a. The maximum tensile loads of the gelatin film were 18.75 ± 4.05, 36.09 ± 4.20, 43.30 ± 5.70, 61.65 ± 14.23 and 78.70 ± 6.85 N at 20, 30, 40, 60 and 90 μm thickness, respectively. That of the conventional film was 19.66 ± 2.86 N. The gelatin films ≥ 30 μm thick showed higher tensile strength than the conventional film.

3-2.Buckling test. The results of the buckling test are shown in Figure 2-b. The maximum buckling loads of the gelatin film were 0.06 ± 0.03, 0.16 ± 0.05, 0.24 ± 0.04, 0.50 ± 0.15 and 0.96 ± 0.08 N at 20, 30, 40, 60 and 90 μm thickness, respectively. That of the conventional film was 0.22 ± 0.05 N. The gelatin films of 20 μm thick showed lower buckling strength while those of ≥60 μm thick showed higher buckling strength than the conventional film.

3-3.Shearing test. The number of fractured films is shown in Figure 3-a. The number of fractured gelatin films at 30 seconds was 5/6, 0/6, 0/6, 0/6 and 0/6 pieces at 20, 30, 40, 60 and 90 μm thickness, respectively. The number of fractured gelatin films at 120 seconds was 5/6, 1/6, 0/6, 0/6 and 0/6 pieces at 20, 30, 40, 60 and 90 μm thickness, respectively. The number of the fractured conventional films was 6/6 and 6/6 at 30 and 120 seconds, respectively. The gelatin films ≥30 μm thick showed a significantly smaller number of fractures than the conventional film at both 30 and 120 seconds. ($p<0.01$)

3-4.Tissue injury test. The tissue injury scores of each gelatin film are shown in Figure 3-b. The tissue injury scores of the gelatin films that were 20, 30 and 40 μm thick were almost 0, while the scores of the gelatin films that were 60 and 90 μm thick were 1.5 ± 0.55 and 2.83 ± 0.41, respectively. That of the conventional film was 0 ± 0. The gelatin films $\geq$ 60 μm thick showed higher injury scores than conventional film. ($p<0.01$)

3-5.Anti-adhesion effect test. After postoperative three weeks, there was no morbidities or mortalities associated with the operation or the application of either film. No remaining film was found macroscopically in the abdominal cavity of the animals in any experimental groups. The anti-adhesion scores of each group are shown in Figure 4. The scores of both the gf and r-gf were significantly lower than those of the control in the categories of both extent and severity ($p<0.05$). However, the conventional film showed no significant difference compared with the control.

4.Discussion

As described in the introduction, one of the most important properties required for an anti-adhesive film's re-attachability is its thickness, which will determine whether or not it has enough physical strength not to tear. To examine the relationship between the physical strength and the thickness of the film, a tensile test and a shearing test using a canine model were performed. As expected, the results showed that the maximum tensile loads increased with the film thickness [22]. The gelatin films ≥30 μm thick showed a significantly higher maximum tensile load than the conventional film. The shearing test also showed that nearly all gelatin films ≥30 μm thick remained unbroken at examination times of both 30 seconds and 120 seconds. In contrast, all of the conventional films were broken at both time points. From these results, it is considered that gelatin films ≥30 μm thick were needed in order to ensure enough physical strength for re-attachment.

Another important property required for the re-attachability is the film stiffness, as tissue injury should be avoided. To assume tissue injury by film edge, we performed a buckling test and a tissue injury test using dog liver for gelatin films of varying thickness. Since the degree of the buckling of films is closely related to its stiffness, it can be used as an index to estimate the possibility of injuring adjacent tissues along the film edges. The results indicated that the maximum buckling loads increased with the film thickness. It also showed that gelatin films ≥60 μm thick showed a significantly higher buckling load than the conventional film. The tissue injury test using dog liver also shows that almost all of the gelatin films ≥60 μm thick damaged the dog's liver, although the gelatin films ≤40 μm thick and the conventional film did not. These results indicate that the gelatin films ≥60 μm thick have carried potential risks of tissue injury while those ≤40 μm thick had little risk of tissue injury.

Taken together with above findings, it is considered that gelatin film 30 to 40 μm thick may be optimal with enough physical strength and not for inducing tissue injury. However, the most important point is whether or not the re-attached gelatin film (r-gf) with such the estimated optimal thickness has sufficient anti-adhesion effect. Therefore, we examined an anti-adhesion test using re-attached gelatin film with a thickness between approximately 30 and 40 μm. The anti-adhesion scores of not only the gf but also r-gf were significantly lower than those of the control and the r-gf could be also found great peritoneum regeneration. Thus, this gelatin film with the optimal thickness can be considered to be re-attachable without tearing or inducing injury to the surrounding tissues and to retain sufficient anti-adhesion effect even after being re-attached.

To our knowledge, there have been no reports describing the re-attachability among previous anti-adhesive materials. The re-attachability of this gelatin film has a benefit for reducing the loss of the films. In addition, we think that it is more advantageous for use in the operations with more severe condition such as laparoscopic surgery. Indeed, the conventional film has been reportedly used in laparoscopic surgery [19-21]. However, inserting or handling such a fragile film in a tight abdominal cavity is quite difficult, so the film often makes contacts with incorrect sites or tear easily. Otherwise, the use of mesh type material such as oxidized regenerated cellulose (Interceed®) contributes to usability for surgeon due to its flexibility and reduces the rate of postoperative adhesion, especially in gynecological surgery. However, such mesh type materials shows a lower anti-adhesive effect than the film type materials because of its less physical barrier function to adjacent tissues. In addition, the adhesive force to objective tissues and also the anti-adhesive effect are decreased remarkably under hemorrhagic conditions [25-27]. Recently, the gel-spray type material, N-hydroxysuccinimide-modified carboxymethyl dextrin (Adspray®) has been reported as an alternative anti-adhesive material for laparoscopic surgery due to its simple usability [28, 29]. However, such gel-spray type material has some issues for less adhesive to objective tissues with lower anti-adhesive effect because of its property to flow away from the target areas. [11]. Actually, there may be the possibility that the optimal thickness clinically depends on the dimension of the target organ or its mechanical property. Although such further examinations are needed, this gelatin film may be more useful and effective for anti-adhesion including re-attachbility than the conventional and other comparative materials.

5.Conclusion

Provided it has an optimal thickness, gelatin film can be reattached with enough physical strength not to tear, safety stiffness not to induce tissue injury, and a sufficient anti-adhesion effect. This re-attachability of the gelatin film probably improves its usability with greater clinical benefits.

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Tables

TABLE 1. Tissue Injury Scores

Description	Score
Minor (No Injury)	0
Moderate (A little hemorrhage with small tissue injury)	1
Serious (Middle hemorrhage with tissue injury)	2
Severe (Hemorrhage with sharp tissue injury)	3

TABLE 2. The Adhesion Scores

Category and Description	Score
(Extent)	
No Involvment	0
≤25% of the site involved	1
≤50% of the site involved	2
≤75% of the site involved	3
≤100% of the site involved	4
(Severity)	
No adhesion present	0
Adhesions fall apart	1
Adhesions can be lysed with traction	2
Adhesions requiring <50% sharp dissection	3
Adhesions requiring <50% sharp dissection	4

Figure captions

FIGURE.1

The schematic illustrations of Tensile test (a), Buckling test (b) and Shearing test (c).

FIGURE.2

(a): The maximum tensile loads of each thickness of gelatin film and the conventional film. (b): The maximum buckling loads of each thickness of gelatin film and the conventional film. Statistically significant relative to conventional film (*:$p<0.05$, **:$p<0.01$)

FIGURE.3

(a): The fracture number of each thickness of gelatin film and the conventional film in shearing test. (b): The tissue injury scores of of each thickness of gelatin film and the conventional film. Statistically significant relative to conventional film (**:$p<0.01$)

FIGURE.4

The anti-adhesion scores of anti-adhesion effect test with 30 μm thickness of gelatin film, re-attached gelatin film and the conventional film. Statistically significant relative to control (*:$p<0.05$)

Figures

FIGURE.1

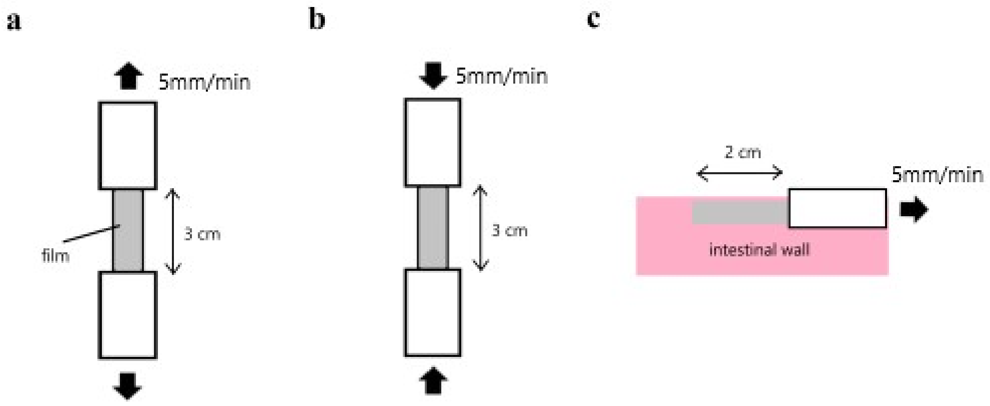

FIGURE.2

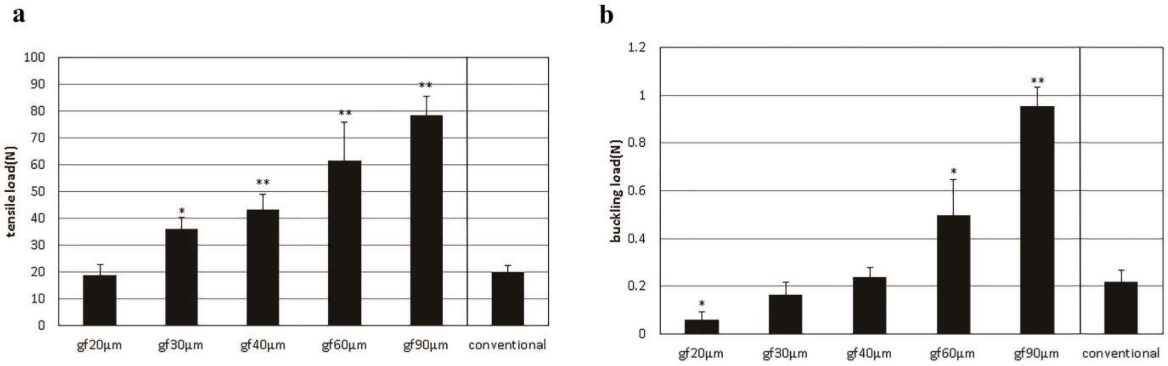

FIGURE.3

a

	gf20µm	gf30µm	gf40µm	gf60µm	gf90µm	conventional
30 sec	5/6	$0/6^{**}$	$0/6^{**}$	$0/6^{**}$	$0/6^{**}$	6/6
120 sec	5/6	$1/6^{**}$	$0/6^{**}$	$0/6^{**}$	$0/6^{**}$	6/6

b

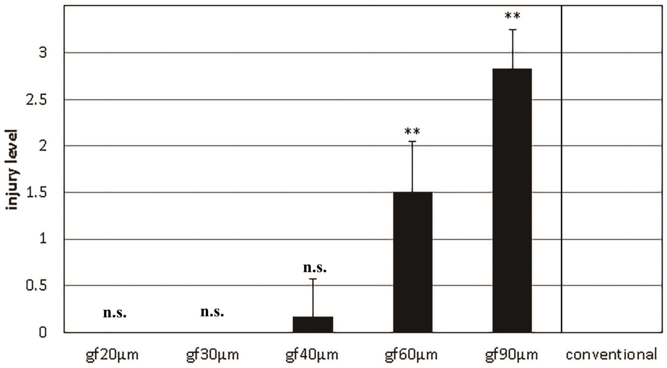

FIGURE.4