

Development of a *Janus* tissue adhesive hemostatic patch based on hydrophobically-modified Alaska pollock gelatin

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Abstract

Uncontrollable hemorrhage from trauma and open surgery leads to a high percentage of death. Even though some patch-type hemostatic materials have been used in the clinic, sufficient tissue adhesion property and the management of tissue adhesion and anti-adhesion have been the challenges. In this report, we designed *Janus* tissue adhesive hemostatic patch, consisting of Alaska pollock gelatin (Org-ApGln) as a support layer and decanoyl group-modified ApGln (C10-ApGln) with pentaerythritol poly(ethylene glycol) ether tetrasuccinimidyl glutarate (4S-PEG) as an adhesive layer, named as the C10-ApGln patch. The C10-ApGln patch adhered onto blood vessel surface by the activation 4S-PEG and hydrophobic groups in C10-ApGln through the covalent bond formation and physical interaction. The burst strength of the C10-ApGln patch was optimized in terms of the degree of substitution, the molecular weight of 4S-PEG, the concentration of C10-ApGln, and the NHS/NH₂ ratio. The optimized C10-ApGln patch showed significantly higher burst strength with commercially available TachoSil®. The C10-ApGln patch showed enzymatic degradability in a buffer solution with collagenase. In a rat liver hemorrhage model, the C10-ApGln patch acted as a sealant on the hemorrhage site and exhibited competitive hemostatic property to TachoSil®.

Keywords: hemorrhage; hemostatic patch; *Janus* structure; Alaska pollock gelatin; hydrophobic interaction

1. Introduction

The management of uncontrollable hemorrhage caused during trauma and open surgery is of paramount importance because hemorrhage accounts for 80% of all deaths in the hospital [1]. Among the causes of death in hospitals, one-third are due to surgical procedures and two-thirds are derived from trauma [2, 3]. Because the normal blood coagulation system in the body does not work well to stop hemorrhage, effective and rapid hemostasis using hemostatic materials is required to save lives.

To date, various kinds of hemostatic materials have been commercialized to stop hemorrhage, such as powder type [4], adhesive type cured by two liquids [5], and, patch type [6]. Among them, the patch-type hemostatic materials are easy to use, offering package-to-patient readiness and direct application. In addition, hemostatic patches may provide tissue-sealing properties [7]. As examples of commercially available hemostatic patches, Hemopatch[®] (N-hydroxysuccinimide-terminated polyethylene glycol (NHS-PEG)/collagen) [8-10], Veriset[™] (NHS-PEG/oxidated cellulose/trilysine) [11-13], TachoSil[®] (fibrinogen/thrombin) [6, 14, 15], and HemCon patch[®] (Chitosan) [16, 17] have been used in clinic. TachoSil[®] works by a blood coagulation mechanism with fibronectin and thrombin to form fibrin on the hemorrhage site. However, these fibrin-based adhesives showed a brittle property, low matrix toughness, and low adhesion

energies [18, 19], therefore, TachoSil[®] would be vulnerable to debonding by deformation of soft tissue where the hemorrhage occurs. On the other hand, tissue adhesive patches such as Hemopatch[®] and Veriset[™] employ activated ester (NHS-PEG) to form covalent bonds with proteins in the tissue surface. However, most of these patches have adhesion on both sides, which inevitably led to undesired postsurgical tissue adhesion and scar tissue formation due to their indiscriminate adhesion property to surrounding tissues/organs in the abdominal cavity [19]. To satisfy these requirements for hemostatic materials, design of a hemostatic patch with opposite properties like adhesion and anti-adhesion is required.

One of the solutions to address these requirements was to fabricate a *Janus* patch where the adhesive side and anti-adhesive side are present in the same patch. Wan et al. reported a tissue adhesive *Janus* hemostatic patch using activated ester and synthetic vinyl polymer on the adhesive side and zwitterionic polymer on the anti-adhesive side [19]. Even though the *Janus* tissue patch showed sufficient tissue adhesive/burst strength, the toxicity of the residual vinyl monomer and the degradation by-products of synthetic vinyl polymer will be limited the use in clinic. Therefore, the design tissue adhesive patch with a *Janus* structure using biopolymer is required for adequate hemorrhage and wound healing.

In this report, we presented a tissue adhesive hemostatic patch with *Janus* structure,

consisting of a support layer with Alaska pollock gelatin (Org-ApGln) and an adhesive layer with hydrophobically-modified ApGln (hm-ApGln) and pentaerythritol poly(ethylene glycol) ether tetrasuccinimidyl glutarate (4S-PEG), named as the hm-ApGln patch (**Fig. 1**). In the adhesive layer, the 4S-PEG was impregnated in the hm-ApGln matrix to absorb the blood or fluid on the tissue surface to adhere tissue surface when the patch is applied on the tissue. Then, phosphate buffer salt, which was strategically added to the support layer, dissolved into the adhesive layer to increase the pH and promote the reaction of NHS groups of 4S-PEG with amine groups of hm-ApGln and tissue, leading to tissue adhesion. It is known that the hydrophobic groups of hm-ApGln in the adhesive layer enhanced the tissue adhesion with hydrophobic molecules in the tissue via hydrophobic interaction [20, 21]. Therefore, the hm-ApGln in the patch will effectively enhance the tissue adhesive and sealing properties. On the other hand, the support layer was composed of non-modified Org-ApGln, which has no adhesion property with tissue, hence, leading to less risk of adhesion to untargeted tissues.

Here, we demonstrated the tissue adhesion, sealing ability, enzymatic degradability, and hemostatic properties of the hm-ApGln patch through the burst strength measurement, in vitro degradation test, and in vivo hemostasis test using rat's liver hemorrhage model.

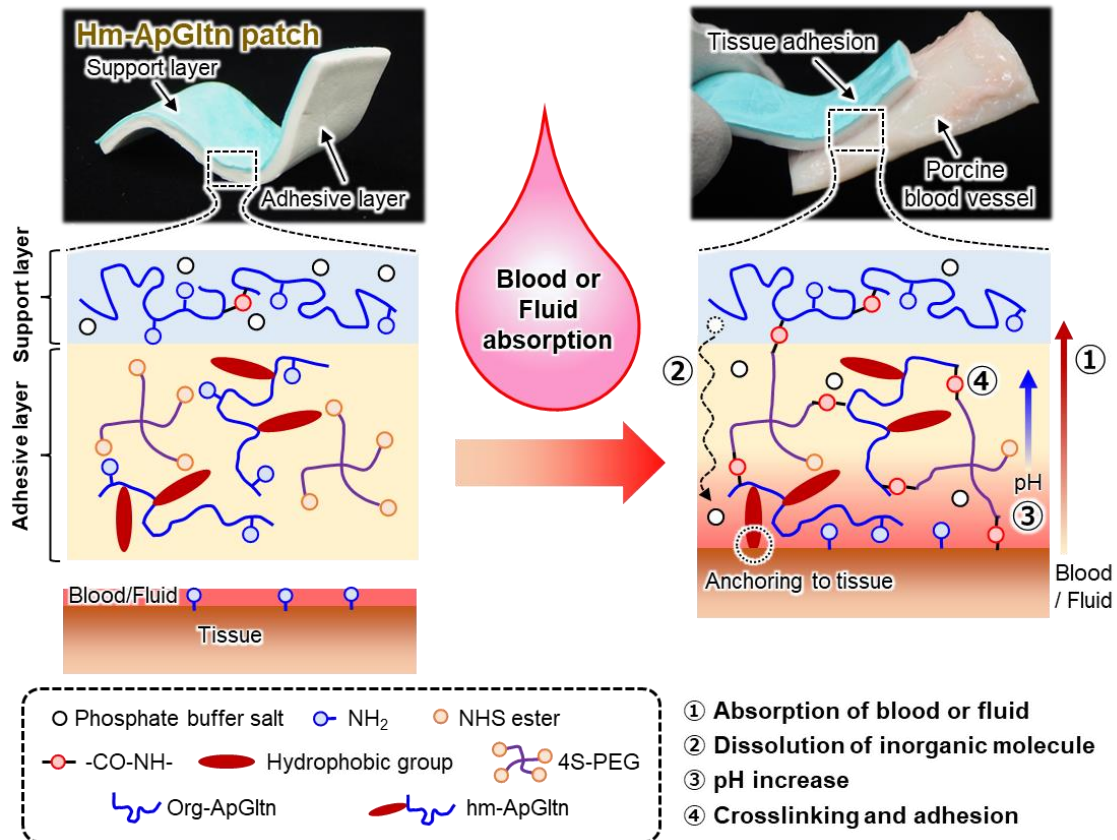


Fig. 1. Mechanism of tissue adhesion and hemostatic property of the *Janus* hm-ApGln patch. Hm-ApGln patch consisted of two layers: a support layer and an adhesive layer. The support layer is composed of thermally-crosslinked Alaska pollock gelatin (Org-ApGln) and inorganic salts (phosphate buffer salt (pH8)) to increase pH in the adhesive layer with a buffering effect. The adhesive layer included hydrophobically-modified ApGln (hm-ApGln) and pentaerythritol poly(ethylene glycol) ether tetrasuccinimidyl glutarate (4S-PEG). When the adhesive layer contacts with tissue, the adhesive and support layer absorbs blood or body fluid on the soft tissue to stop hemorrhage. Then, phosphate ions are released into the adhesive layer to raise pH, promoting crosslinking formation between 4S-PEG and hm-ApGln as well as 4S-PEG and wet tissue. Hydrophobic groups on the hm-ApGln also contribute to the adhesion to tissues via hydrophobic interaction.

2. Materials and Methods

2-1. Materials

Alaska pollock gelatins (ApGltns) with a molecular weight of 40,000 or 85,000 Da with amino group content of 316 $\mu\text{mol/g}$ or 379 $\mu\text{mol/g}$ were purchased from Nitta Gelatin Inc. (Osaka, Japan). Pentaerythritol poly(ethylene glycol) ether tetrasuccinimidyl glutarate (4S-PEG) (SUNBRIGHT[®] PTE-200GS, MW = 5,000 and 20,000) was purchased from NOF Corporation (Tokyo, Japan). Octanoic anhydride (C8), decanoic anhydride (C10), dodecanoic anhydride (C12), 10% formalin neutral buffer solution, 2,4,6-trinitrobenzene sulfonic acid (TNBS), 4% paraformaldehyde phosphate buffer solution, dimethyl sulfoxide (DMSO), ethanol, and triethylamine (TEA) were purchased from Wako Pure Chemical Industries, Ltd. (Osaka, Japan). Ethyl acetate was purchased from Kishida Chemical Co., Ltd. (Osaka, Japan). Saline (0.9% w/v sodium chloride) was purchased from Otsuka Pharmaceutical Co., Ltd. (Tokyo, Japan). Porcine blood vessel was purchased from Tokyo Shibaura Zouki (Tokyo, Japan).

2.2. Synthesis of hydrophobically-modified ApGltns (*hm-ApGltns*)

Hm-ApGltns were synthesized through a reaction with primary amine of ApGltin and alkyl anhydride (C8, C10, and C12). Briefly, ApGltin (10 g) was dissolved in 35 mL of

0.01 M borate buffer solution (pH 9) at 55°C. After the addition of 10 mL of ethanol, 0.474 mmol of alkyl anhydride (15 mol% of amine groups in ApGltN) dissolved in 5 mL of ethanol was added into the reaction solution. The resulting solution had a 20 w/v% ApGltN concentration (water: ethanol = 35:15 (mL)). After stirring for 1 h at 55°C, the obtained solution was dropped into 500 mL of cold ethanol to separate hm-ApGltNs from by-products such as alkyl carboxylic acids. The precipitate was then washed three times with 250 mL of ethanol to remove residual alkyl carboxylic acids. The precipitate was washed three times with 1,250 mL of ethanol and then vacuum dried overnight (< 3 mbar) to obtain hm-ApGltN powder.

2.3. Characterization of hm-ApGltNs

The degree of substitution (DS) of the alkyl groups into ApGltNs were determined using the TNBS method as described previously [20]. The introduction of the alkyl groups in hm-ApGltNs was also confirmed by fourier transform infrared spectroscopy (FT-IR) (FTIR-8400S, Shimadzu Ltd, Japan) and proton nuclear magnetic resonance (¹H-NMR) (JNM-AL400, JEOL Ltd, Japan).

2.4. Fabrication of hm-ApGltN patches

Hm-ApGln patches consist of two layers such as a support layer and an adhesive layer. For the preparation of the support layer, Org-ApGln (85,000 Da) was first dissolved in various concentrations (0.1, 0.15, and 0.2 M) of phosphate buffer at pH 8. Then, 10 mL of obtained Org-ApGln solution was poured into a Unipac (size G) and sandwiched between two glass plates with a silicone rubber spacer with 0.5 mm. After freeze-drying, the obtained support layer was thermally crosslinked at 150°C for 5 h. For preparation of the adhesive layer, hm-ApGln (40,000 Da) was dissolved in ultrapure water, and adjusted to pH 5 using 1N-HCl. A silicone sheet frame (inner length: 50 mm × 50 mm, thickness: 0.5 mm) was placed on the support layer, which was cut into 70 mm x 70 mm. The hm-ApGln solution was poured onto the support layer and immediately fixed with a silicone sheet plate. After pre-freezing, the solution was freeze-dried. Finally, 4S-PEG was dissolved in ethyl acetate and the obtained 4S-PEG solution was permeated into the hm-ApGln layer and air-dried in a draft for overnight.

2.5. SEM observation

The surface morphology of the obtained hm-ApGln patch was assessed using scanning electron microscopy (SEM; JSM-5600, JEOL Ltd., Japan) after sputtering with gold for 5 min.

2.6. Burst strength measurement

The burst strength of the obtained hm-ApGln patch was measured according to the ASTM F2392-04 with minor modifications. The fresh porcine blood vessel was used as an adherend to assess the burst strength of the hm-ApGln patch. The porcine blood vessel was cut into a 30 mm-diameter circular shape with a 1-mm pinhole in the center, and the hm-ApGln patch with 15 mm in diameter was then placed to close the pinhole, followed by waiting for 3 min under 0.9 N-intermittent force. Then, water pressure was applied to the pin-hole and the maximum burst strength was measured when the hm-ApGln patch broke or peeled off. The commercially available Tachosil[®] was also used as a control adhesive patch. After the burst strength measurements, the blood vessel tissue with hm-ApGln patch was fixed in 10% formalin neutral buffer solution, followed by paraffin embedding, and stained with hematoxylin and eosin (HE). The resulting section was observed to assess the interface between the vessel and the hm-ApGln patch after the burst strength measurements.

2.7. Enzymatic degradation study

The 10C10-ApGln tissue adhesive patch (diameter, 8 mm; thickness, 1.0 mm) was

immersed in the 5 µg/mL collagenase/Tris-HCl buffer solution (pH 7.5, 2.5 mM CaCl₂). Then, the 10C10-ApGln patch in the collagenase solution was incubated for up to 24 h at 37°C. The remaining weight (%) was calculated by measuring the weight after immersion compared with the weight before immersion using the following equation:

$$\text{Remaining weight (\%)} = \frac{W_t}{W_0} \times 100$$

where W_t and W_0 are the weights of the 10C10-ApGln patch before and after immersion, respectively.

2.8. In vivo hemostatic property of hm-ApGln patch using a rat liver hemorrhage model

All animal experiments were conducted with the approval of the Institutional Animal Care and Use Committee of National Institute for Material Science (approval number: 76-2023-3). The hemostatic effect of the 10C10-ApGln patch was assessed using a rat liver hemorrhage model, following a previous report [22]. Male SD rats (7 weeks old, $n = 5$ to 8) were anesthetized through the inhalation of isoflurane. Following ethanol disinfection, the abdominal wall was opened to expose the liver. A liver injury was prepared by creating a defect with 2 mm in depth and 4 mm in diameter using a circular punch. After free

bleeding for 5 s, the 10C10-ApGltN patch with 7 mm in diameter and 1 mm in thickness was applied to the bleeding area using a tweezer. Blood was collected for 5 min using a pre-weighed filter paper to measure blood loss. Org-ApGltN patch and TachoSil® were used as control materials. Following this experiment, the damaged livers after treatment with patches were collected and fixed with a 10% formalin buffer solution for the HE staining.

2.9. Statistical analysis

All data are presented as mean $\pm$ standard deviation (SD), calculated from 3, 4, 5, 6, or 8 independent experiments. Statistical analysis was conducted using GraphPad Prism v.8.0 (GraphPad Software). We conducted One-way ANOVA, followed by Tukey's multiple comparison test and multiple t-test as post hoc test. Significance levels are denoted as follows: $*P < 0.05$, $**P < 0.01$, $***P < 0.001$, and $****P < 0.0001$.

3. Results and Discussion

3.1. Synthesis and characterization of hm-ApGltns

Hm-ApGltns with different alkyl chain lengths were successfully synthesized by the reaction of amine groups in Org-ApGltN (Mw: 40,000 Da) with octanoic, decanoic, and dodecanoic anhydride under high pH condition (**Fig. 2(a)**). The DS of obtained hm-ApGltns were 12 mol% for C8 (12C8)-ApGltN, 10 mol% for C10 (10C10)-ApGltN, and 8 mol% for C12 (8C12)-ApGltN, respectively (**Table S1**). We also synthesized C10-ApGltN with high DS (58 mol%) in order to evaluate the effect of DS on the adhesive strength. Considering the molecular weight of ApGltN: 40,000 Da and the number of residual amine groups: 316 mmol/g, the number of amine groups in one ApGltN molecule was calculated as 12.64. Therefore, the number of alkyl groups in the hm-ApGltN was estimated as 1.51, 1.26, and 1.01 for 12C8-, 10C10-, and 8C12-ApGltN, respectively. The decreased DS for longer alkyl chain length was due to the steric hindrance of longer alkyl anhydride and the same tendency was seen in the previous report [23]. The introduction of the alkyl chain was further confirmed by FT-IR and ¹H-NMR. FT-IR spectra in **Fig. S1(a)** showed the increased -NH- stretching peak at 3,284 cm⁻¹ and -CH₃ stretching peak at 2,935 cm⁻¹ in hm-ApGltNs compared with Org-ApGltN. ¹H-NMR spectra in **Fig. S1(b)** showed the increased alkyl CH₂ peak at 1.294 ppm. From these results, the introduction

of alkyl groups into the ApGln molecule was confirmed.

3.2. Preparation and characterization of hm-ApGln patches

Hm-ApGln patches were prepared by drying the Org-ApGln and hm-ApGln solutions to fabricate the support and adhesive layer with the 4S-PEG (**Fig. 2(b)**). To prevent undesired adhesion to other tissues in the abdominal cavity, the support layer was composed of Org-ApGln, which did not show tissue adhesion property. Furthermore, Org-ApGln with high molecular weight (Mw: 80,000 Da) was chosen for support layer and thermally crosslinked to increase the mechanical stability of the support layer. On the other hand, the adhesive layer consisted of hm-ApGln (Mw: 40,000 Da) and 4S-PEG (Mw: 5,000 or 20,000 Da) for tissue adhesive layer formation, where the pH of hm-ApGln solution maintained low to prevent crosslinking reaction during the preparation process of the patches. In addition, we strategically impregnated phosphate buffer salt (pH8) in the support layer to increase pH so as to promote a crosslinking reaction once the support layer absorbs blood or body fluid. We expected that the phosphate buffer salt will dissolve into the adhesive layer after the absorption of blood or body fluid and will increase the pH of the adhesive layer, boosting the *in situ* crosslinking between hm-ApGln, 4S-PEG, and tissue to form an adhesive layer. The presence of hydrophobic

groups on hm-ApGln can also contribute to robust tissue adhesion by the hydrophobic interactions with tissue. The surface morphology of the adhesive layer on the hm-ApGln patch was characterized by SEM (**Fig. 2(c)**). The adhesive layer with hm-ApGln and 4S-PEG showed a porous structure. This porous structure contributes to the permeation of blood or body fluid from soft tissues.

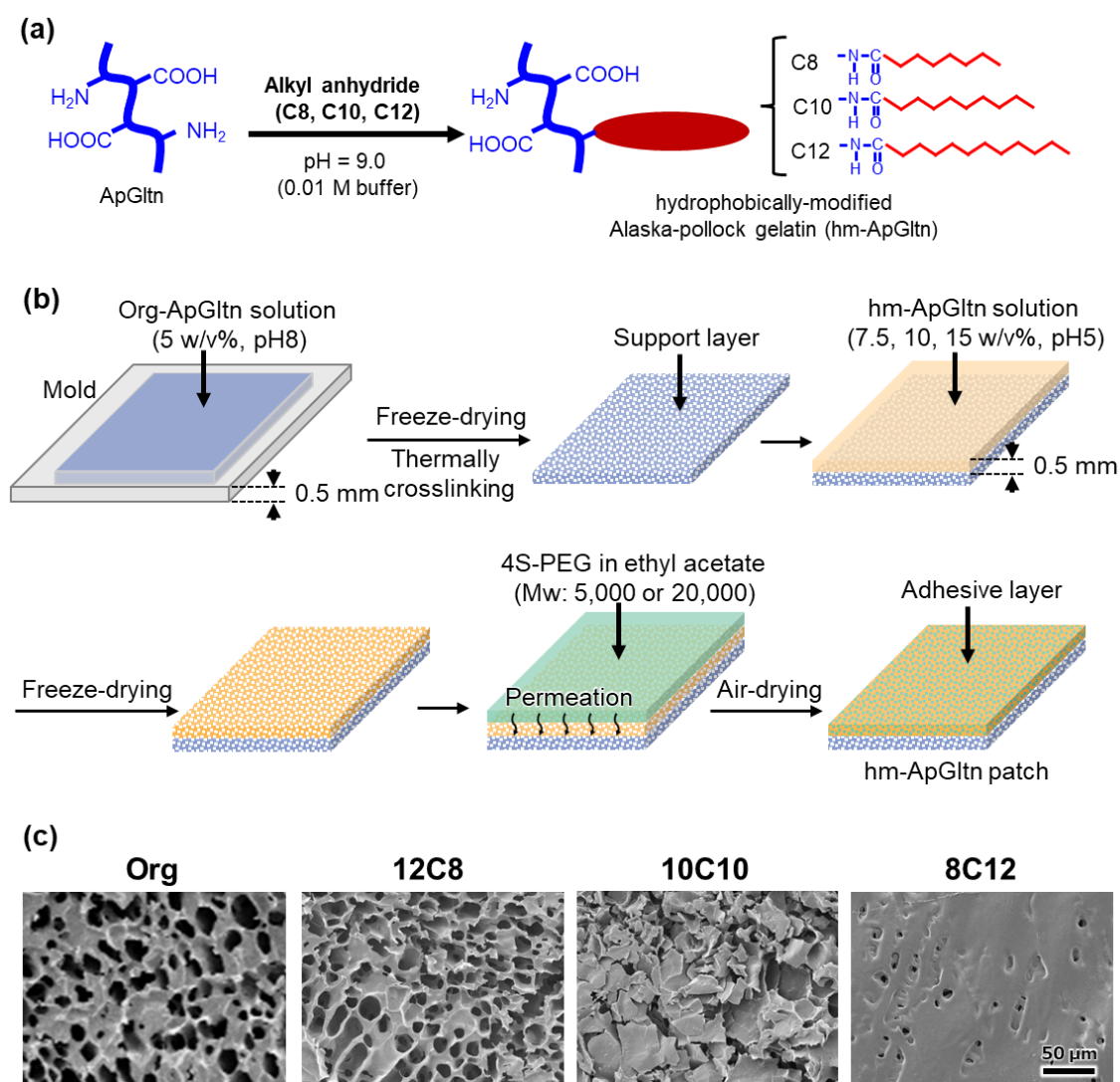


Fig. 2. Preparation and characterization of hm-ApGln patch. (a) Synthesis of hm-ApGln. Hm-ApGln was synthesized by the reaction of amine groups of ApGln with alkyl anhydride in a high pH condition. (b) Preparation method of the hm-ApGln patch. Org-

ApGln solution (pH: 8 in phosphate buffer salt) was freeze-dried to prepare the support layer. One side of the obtained support layer was covered by hm-ApGln solution (pH 5) and then freeze-dried. 4S-PEG dissolved in ethyl acetate was applied onto the hm-ApGln layer and air-dried to prepare the adhesive layer. (c) SEM images of the adhesive layer of Org-, 12C8-, 10C10-, and 8C12-ApGln patch. The scale bar represents 50 μm .

3.3. Burst strength measurement

The burst strength of the resulting hm-ApGln patches was measured using porcine blood vessels according to ASTM F2392-04 with minor modifications (**Fig. S2**). First, we investigated the effect of the alkyl chain length (C8, C10, and C12) of hm-ApGln on the burst strength (**Fig. 3(a)**). The burst strength raised as the alkyl chain length increased and maximum burst strength was obtained in the C10-ApGln patch. This result was because the hm-ApGln patch with longer alkyl chain length enhanced hydrophobic interaction with the blood vessel. We previously reported that the hydrophobic groups such as alkyl groups could enhance the interaction with hydrophobic molecules like fibronectin and phospholipid cell membranes [21]. Similar to our previous reports using adhesive particles [23-29] or in situ hydrogels [20, 30, 31], we observed that the introduction of the alkyl group in the patch effectively enhanced the adhesive strength to the soft tissues. On the other hand, the burst strength of the C12-ApGln patch was comparable to that of the C10-ApGln patch. This was due to the fact that the higher hydrophobicity of C12-ApGln delayed the hydration of the patch inducing the formation of a heterogeneous adhesive layer. From these results, we employed C10-ApGln as the optimal alkyl chain length for further experiments. We also evaluated the effect of phosphate buffer salt concentration in the support layer on the burst strength (**Fig. S3**).

As a result, the burst strength of the 10C10-ApGln patch with 0.15 M of buffer concentration tended to increase burst strength compared to that with 0.1 M, indicating the high concentration of buffer contributed to increasing the pH in the adhesive layer to promote crosslinking formation among 10C10-ApGln, 4S-PEG, and tissue. From these results, we fixed the phosphate buffer salt concentration in the support layer as 0.15 M for further experiments. Next, we evaluated the effect of 4S-PEG molecular weight and DS of C10-ApGln on the burst strength (**Fig. 3(b)**). 4S-PEGs with 5,000 and 20,000 Da were used to compare the effect of 4S-PEG molecular weight on the burst strength. Also, the effect of DS of C10-ApGlns on the burst strength was evaluated. From these results, the C10-ApGln patch with 10 mol% in DS and 5,000 Da in 4S-PEG molecular weight showed maximum burst strength compared to other preparation conditions. This was because the patch with lower molecular weight 4S-PEG (5,000 Da) and 10C10-ApGln showed lower hydrophobicity than the other patches prepared using 58C10-ApGln. Therefore, the 10C10-ApGln patch could hydrate and form an adhesive layer more quickly than the 58C10-ApGln patch. We also investigated the effect of 10C10-ApGln concentration (7.5, 10, and 15 w/v%) and NHS/NH₂ ratio (50, 100, and 200 mol%) in the adhesive layer on the burst strength (**Fig. 3(c)**). The result showed the patch with 10 w/v% of 10C10-ApGln concentration and 100 mol% of NHS/NH₂ ratio exhibited the highest

burst strength. The reason for this result was due to the fact that the lower concentration of 10C10-ApGln caused a lack of molecule density, leading to lower mechanical strength and adhesion strength after hydration. On the other hand, the higher concentration of 10C10-ApGln prevented quick hydration of the patch. In terms of the NHS/NH₂ ratio, 50 mol% in NHS/NH₂ made lowered crosslinking density in the adhesive layer, while, the presence of excessive 4S-PEG molecules with 200 mol% condition might prevent the adequate covalent crosslinking. From these results throughout the burst strength measurement, 10C10-ApGln with 5,000 Da in the 4S-PEG molecular weight, 10 w/v% in the 10C10-ApGln concentration, and 100 mol% in the NHS/NH₂ ratio was determined as a optimal condition. We further compared the burst strength among Org-, 10C10-ApGln patch, and commercially available TachoSil[®] (**Fig. (d) and (e), supporting videos 1 to 3**). When saline was applied to the bottom of the patches, the 10C10-ApGln patch near the defect expanded compared to the Org-ApGln patch and TachoSil[®], indicating the higher burst strength of the 10C10-ApGln patch. As well as the tendency in the images, the 10C10-ApGln patch showed the highest burst strength (89.9 ± 29.9 mmHg). The increase of burst strength in the 10C10-ApGln patch compared to the Org-ApGln patch (57.5 ± 34.0 mmHg) was due to the presence of hydrophobic groups at the interface between the patch and blood vessel, forming physical interactions with

hydrophobic molecules in blood vessel. Because the average systolic blood pressure and portal blood pressure in the liver were reported as 127 mmHg [32] and 5 to 10 mmHg [33], the 10C10-ApGln patch is expected to withstand the blood pressure when applied on the liver hemorrhage site. Meanwhile, TachoSil[®] is known as a hemostatic sealant, which uses a fibrin gel formation mechanism by human fibrinogen and human thrombin. However, the fibrin gel does not have enough tissue adhesion property and is expected to easily peel off from the surface of blood vessels in the presence of water and blood [18]. Therefore, the 10C10-ApGln with an enhanced sealing property has an advantage over TachoSil[®], especially for patients whose blood coagulation does not work. The cross-sectional images of the patches were compared to observe the interface between the blood vessel tissue and the patch (**Fig. 3(f)**). The 10C10-ApGln patch showed that the adhesive layer closely adhered to the blood vessel, whereas the Org-ApGln patch and TachoSil[®] detached from the surface of the blood vessel, indicating that the 10C10-ApGln patch had higher tissue adhesion property with the contribution of hydrophobic interaction derived from introduced decanoyl groups. Generally, ideal hemostatic patches should have opposite tissue adhesion properties where one side of the patch adheres to tissue and another side does not, preventing postoperative adhesion [19, 34]. Even though the adhesive layer of the 10C10-ApGln adhered to the blood vessel tissue, the support layer

did not show burst strength because the patch detached during hydration (**Fig. 3(g)**). This result indicated that the adhesive layer of the 10C10-ApGln will perform the anti-adhesion property avoiding the adhesion to other tissues after hydration. By the optimization of hydrophobicity, 4S-PEG molecular weight, 10C10-ApGln concentration, and NHS/NH₂ ratio of the patch, we successfully fabricated the *Jauns* 10C10-ApGln patch, which exhibited higher burst strength than the commercially available TachoSil[®].

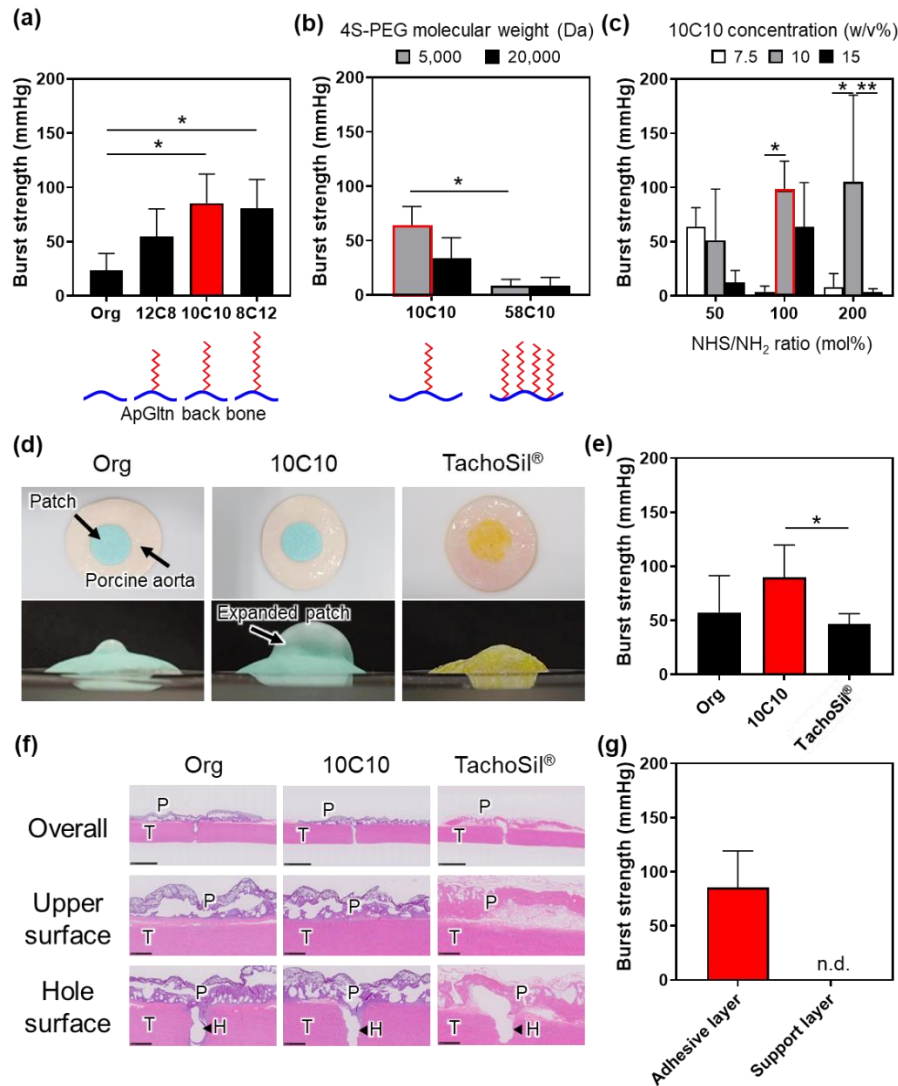


Fig. 3. Burst strength measurement using hm-ApGltN patches with different conditions. (a) Burst strength of hm-ApGltN patches with different alkyl chain lengths: Org, C8, C10, and C12. $*p < 0.05$; statistical significance was analyzed using Tukey's multiple comparison test. (b) Burst strength of hm-ApGltN patches with different 4S-PEG molecular weights: 5,000 and 20,000 Da, and different DSs: 10 and 58 mol%. $*p < 0.05$; statistical significance was analyzed using the multiple t-test. (c) Burst strength of hm-ApGltN patch with different concentrations of 10C10-ApGltN: 7.5, 10, and 15 w/v%, and NHS/NH₂ ratios: 50, 100, and 200 mol%. $*p < 0.05$, $**p < 0.01$; statistical significance was analyzed using Tukey's multiple comparison test. (d) The upper row images show the Org- and 10C10-ApGltN patch and TachoSil[®] that adhered to porcine blood vessel before the burst strength test. The lower images show the patches just before detaching from the blood vessel by saline pressure. (e) Comparison of burst strength among Org- and 10C10-ApGltN patch and TachoSil[®]. $*p < 0.05$; statistical significance was analyzed using Tukey's multiple comparison test. (f) Cross-sectional HE staining images of the Org-, 10C10-ApGltN patch, TachoSil[®], and porcine blood vessel. The upper, middle, and lower rows represent overall images, upper surface images, and pin-hole surface images, respectively. Abbreviations, T: porcine blood vessel tissue, P: Org- or 10C10-ApGltN patch, and TachoSil[®]. The scale bars represent 2.5 mm for overall images and 250 μ m for upper surface and pin-hole surface images. (g) Burst strength of 10C10-ApGltN patch with adhesive or support layer adhered to blood vessel.

3.4. *In vitro* degradation of 10C10-ApGltN patch

To assess the enzymatic degradation behavior of the patch, we immersed the 10C10-ApGltN patch in 5 μ g/mL collagenase/ Tris-HCl buffer solution (**Fig. 4(a) and (b)**). We employed a collagenase as a typical matrix metalloproteinase (MMP) which plays an important role in tissue regeneration [35]. The 10C10-ApGltN patch swelled for the first 2 h and then started to degrade. Eventually, complete degradation was observed at 24 h of immersion time, meaning that the 10C10-ApGltN patch shows enzymatic degradation

during a wound healing process. It is known that MMPs (gelatinase A and B), which degrade gelatin, are expressed in soft tissues such as the heart, liver, and intestines [36]. Therefore, the 10C10-ApGltN patch will degrade with those enzymes after closing the defect on the soft tissues.

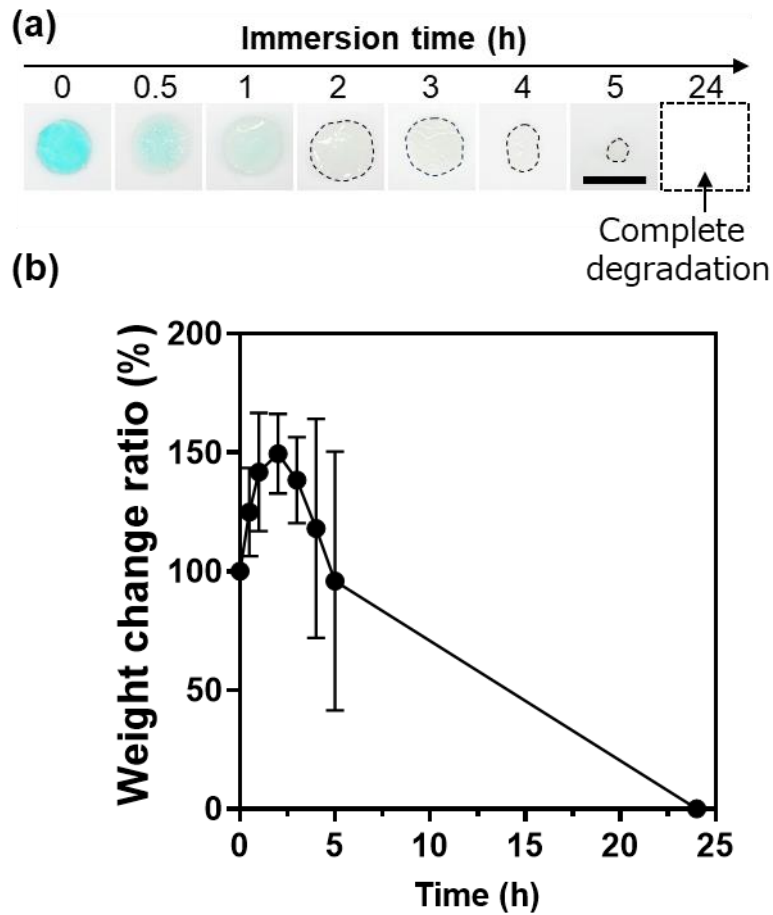


Fig. 4. Degradation behavior of the 10C10-ApGltN patch in collagenase / Tris-HCl buffer solution. (a) Images of the 10C10-ApGltN patch after immersion in 5 $\mu\text{g/mL}$ collagenase/ Tris-HCl buffer solution for up to 24 h. The scale bar represents 10 mm. (b) Weight change ratio of the 10C10-ApGltN patch as the function of immersion time.

3.5. Investigation of hemostatic property of the 10C10-ApGln patch using rat's liver hemorrhage model

We also evaluated the hemostatic property of the 10C10-ApGln patch using a rat liver hemorrhage model according to the previous report [37]. The hemorrhage model was prepared by creating a defect on the rat liver with 4 mm in width and 2 mm in depth. Then, the Org-, 10C10-ApGln patch, and TachoSil® were applied to the defect to stop the hemorrhage (**Fig. 5(a)**). From the images of applied patches, Org-, 10C10-ApGln patch, and TachoSil® adhered to the defect on the liver with the blood permeating to the patch (**Fig. 5(b)**). The result of blood loss from the liver revealed that the Org- and 10C10-ApGln patch groups significantly decreased blood loss compared to the control group (non-treated) (**Fig. (c)**). Even though the TachoSil® group did not show a significant difference with the 10C10-ApGln patch, the 10C10-ApGln tended to inhibit hemorrhage more than TachoSil®. From the cross-sectional image of the liver and patch after the experiment, the 10C10-ApGln patch closely adhered to the liver surface with less blood leakage (**Fig. 5(d)**). On the other hand, a blood coagulation layer was observed between TachoSil® and the liver surface, indicating less adhesiveness and less sealing effect. As TachoSil® has a less tissue adhesion property, it is considered that TachoSil® peeled off from the liver surface due to the blood flow. On the other hand, the 10C10-ApGln patch

can form the adhesive layer on the tissue by the formation of covalent bonds of 4S-PEG with amine groups of tissue and also hydrophobic interaction driven by decanoyl (C10) groups in 10C10-ApGln acting as a sealant to close the defect properly and led to less blood loss. (**Fig. 6**). It is known that the hydrophobic groups incorporated into chitosan interacted with the phospholipid membrane of blood cells by hydrophobic interaction, crosslinking blood cells to facilitate blood coagulation. [38]. **Therefore, we expected the interaction of decyl groups in 10C10-ApGln with blood cells as well as the sealing effect of the 10C10-ApGln patch contributed to the hemostatic effect.** Not only the acute hemostatic property but also the biodegradability and biocompatibility of hemostatic materials are important. Our previous study reported that hydrogels made of hydrophobically modified ApGln and 4S-PEG showed no cytotoxicity and were degraded without severe inflammation in rat back [20, 30, 39]. Therefore, it is expected that this material is also biodegradable and biocompatible.

In this report, we successfully fabricated the *Janus* tissue adhesive hemostatic 10C10-ApGln patch based on 4S-PEG and 10C10-ApGln with opposite properties like tissue adhesion and anti-adhesion properties. The animal experiment using the rat liver hemorrhage model showed that the 10C10-ApGln patch had a hemostatic property. However, because the acute hemostatic property of the 10C10-ApGln patch was just only

clarified by the rat liver hemorrhage model, further experiments will be required using chronic hemorrhage model or large animal models in the future.

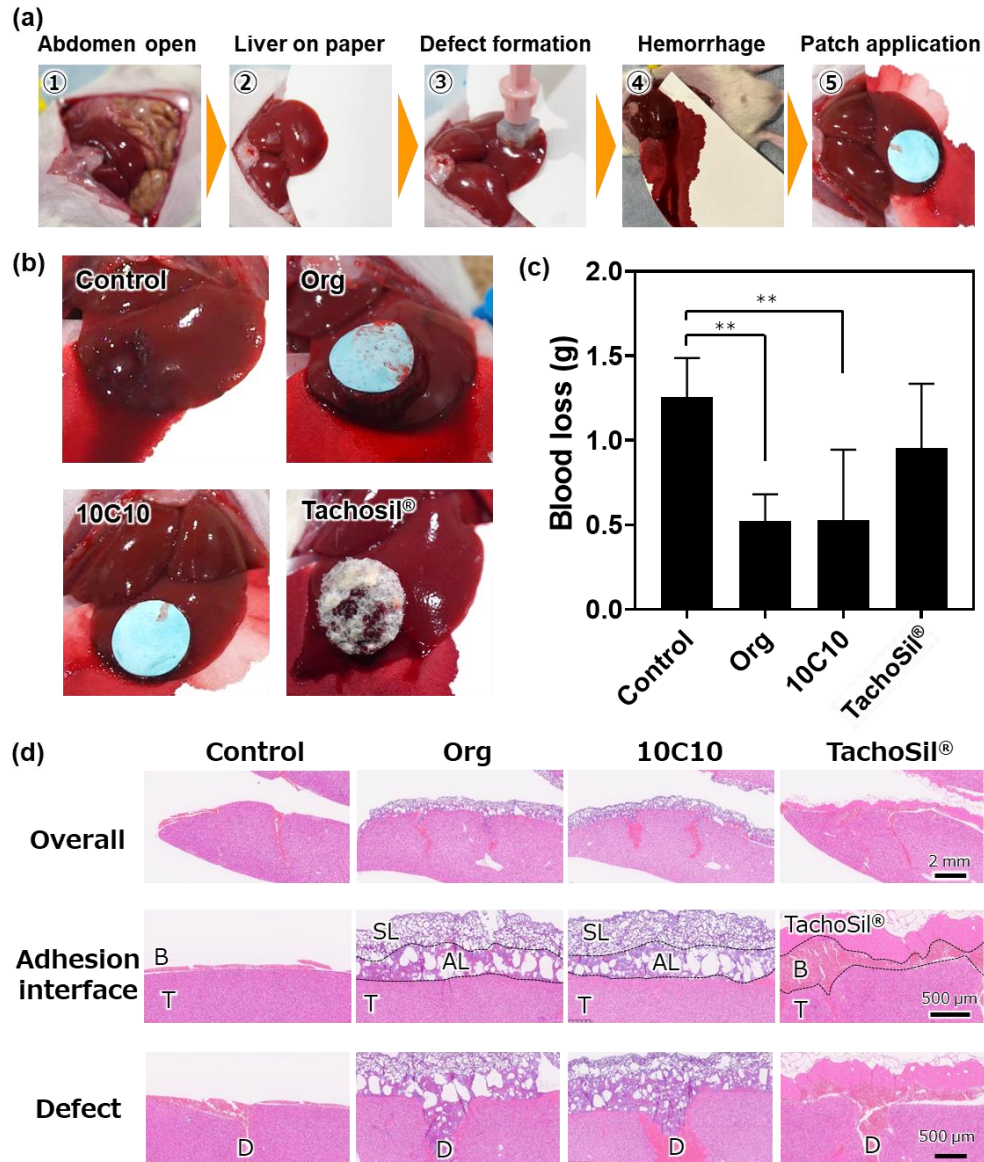


Fig. 5. Hemostatic property of the 10C10-ApGln patch in a rat liver hemorrhage model. (a) Experimental procedure of the hemorrhage model. 1. The abdomen of the SD rat was opened. 2. The filter paper was placed under the liver. 3. A defect with 4 mm in width and 2 mm in depth was prepared using a punch. 4. Blood came out for 5 s. 5. Org-, 10C10-ApGln patch or TachoSil® was applied to the defect to stop the hemorrhage. (b) Images of the liver without any treatment (Control) and with the treatment of the Org-, 10C10-ApGln patch, or TachoSil® for 5 min. (c) The amount of blood loss by 5 min after the

defect was prepared. $n = 8$ for control (untreated), $n = 5$ for Org-ApGln patch, $n = 6$ for 10C10-ApGln patch, $n = 6$ for TachoSil[®]. $**p < 0.01$; statistical significance analyzed using Tukey's multiple comparison test. (d) Cross-sectional HE staining images of the Org-, 10C10-ApGln patch, TachoSil[®], and liver tissue. The upper, middle, and lower rows represent overall images, adhesion interface images, and defect images, respectively. Abbreviations, T: liver tissue, B: blood, SL: support layer of ApGln patch, AL: adhesive layer of 10C10/Org-ApGln patch, and D: defect site filled with blood. The scale bars represent 2 mm for overall images and 500 μm for adhesion interface and defect images.

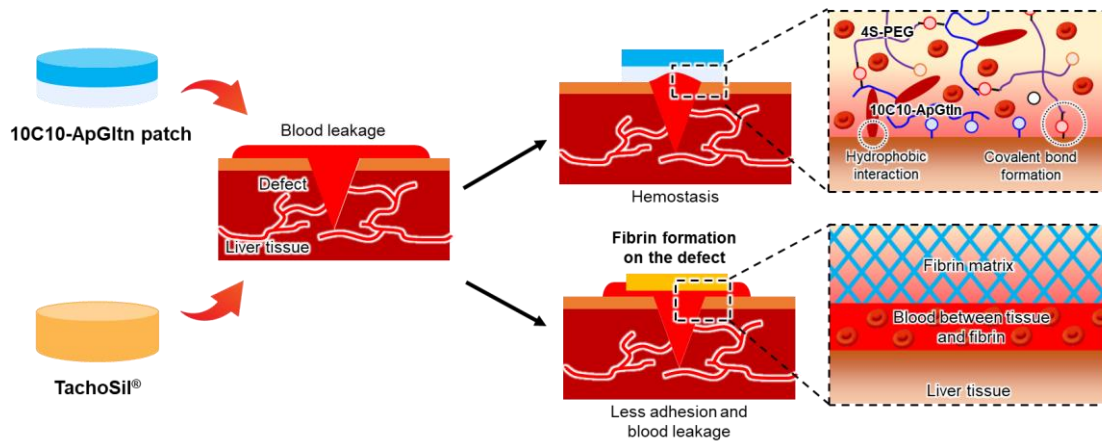


Fig. 6. Hemostatic mechanism of the 10C10-ApGln patch. The 10C10-ApGln patch absorbed surface blood or body fluid and formed covalent bonds with amine groups on the tissue. The hydrophobic interactions between 10C10-ApGln and tissue also contributed to the enhancement of tissue adhesion. This enhanced sealing property of 10C10-ApGln led to the hemostatic effect. Although TachoSil[®] formed fibrin gel on the defect by fibrinogen and thrombin, less adhesion of fibrin gel with tissue caused a peeling from tissue and blood leakage.

4. Conclusion

We fabricated a *Janus*-structured hemostatic patch (10C10-ApGln patch) that has adhesive and anti-adhesive layer to prevent unwanted adhesion relevant to the hemostatic material. 10C10-ApGln consisted of 4S-PEG and 10C10-ApGln in the adhesive layer and Org-ApGln in the anti-adhesive (supportive) layer. The 10C10-ApGln patch

adhered onto porcine blood vessel tissue by just only hydration that activated a crosslinking reaction among 4S-PEG, 10C10-ApGln, and tissue. The hydrophobic groups in the 10C10-ApGln also contributed to the enhancement of tissue adhesion. The maximum burst strength of the patch was obtained by the optimization of the hydrophobicity, 4S-PEG molecular weight, 10C10-ApGln concentration, and NHS/NH₂ ratio. Furthermore, the support layer did not adhere to tissue due to *Janus* structure. Finally, the 10C10-ApGln patch showed comparable hemostatic property to the commercially available hemostatic patch in the rat liver hemorrhage model. From the histological observation, the 10C10-ApGln patch stopped the bleeding by acting as a sealing material on the hemorrhage site.

Data availability

The authors declare that all data supporting the findings of this study are available within the paper and the associated Supporting Information.

Conflicts of interest

The authors have no competing interests to declare.

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Appendix A. Supplementary data

Supplementary data related to this article can be found at

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