Crosslinkable poloxamer *in-situ* gel as a submucosal cushion for endoscopic submucosal dissection

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Research

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Abstract

Background: Hypertonic saline solution is used for endoscopic submucosal dissection (ESD) as a submucosal fluid cushion (SFC). However, the application of SFC is limited by challenges including a short effect duration as well as increased risk of perforation and bleeding.

Methods: An in-situ gel crosslinked acrylate/thiol Poloxamer 407 (PA-PSH) can be used as a novel SFC for ESD to avoid perforation, and the feasibility and safety of the PA-PSH for ESD was evaluated in vitro and in vivo .

Results: PA-PSH thermal injection led to a longer-lasting elevation with clearer margins as compared with glycerol, with a high mucosal elevation induced by thiol-ene reaction in physiological conditions between acrylate Poloxamer 407 (PA) and thiol-capped Poloxamer 407 (PSH). Precise ESD along the margins of the elevated mucosa was achieved without obvious adverse effects such as bleeding, perforation and tissue damage.

Conclusion: The PA-PSH in-situ gel provides an excellent submucosal injection system, which has great potential to improve the ESD technique.

Background

Endoscopic submucosal dissection (ESD) is a standard treatment modality for gastrointestinal neoplastic lesions. The formation of an adequate submucosal cushion between the lesion and the proper muscle layer can facilitate an en bloc resection and reduce the risk of perforation for ESD. En bloc resections could reduce the risk of local recurrence after the endoscopic resection and provide more accurate histopathological assessments. An ideal submucosal injection solutions could provide a longlasting and sufficiently high submucosal cushion without no obvious adverse effects. They also should be inexpensive, readily available and easy to inject [1–3].

Saline solution is usually used for ESD as a submucosal fluid cushion (SFC). However, saline solution would be rapidly absorbed by the surrounding tissue thus requires repeated injections, making operation difficult. In addition, it is still a challenge to resect lesions larger than 2 cm in diameter completely [4]. Therefore, diverse agents like glycerol, hyaluronic acid, gelatin and so on are applied for ESD and avoid perforation, however, most of these agents are viscous and not easy for injection. [5–13] The *in-situ* gel after injection such as photosensitive gels [14] or thermosensitive gels [15–17] were utilized to avoid the drawbacks. However, there still some limitations. For example, the sol-gel (solution to gel) transformation based on photoinitiated free radical polymerization rely on ultraviolet light, which may be difficult in deep areas *in vivo*. Meanwhile, although thermogels like PLGA-PEG-PLGA [15] and poloxamers [17] demonstrated good efficacy and safety, they are easy to clog inside long delivery tools under human physical body temperature. Therefore, it is necessary for developing more new submucosal agents for ESD.

Here, we exploited an PA-PSH *in-situ* gel as a novel submucosal injection substance for ESD. The application of PA-PSH *in-situ* gel as an SFC in ESD procedure was as shown in Fig. 1. This *in-situ* gel was low sticky solution *ex vivo* but could transform into high condensed gel *in vivo*, which was triggered by thiol-ene reaction and was an ideal material for ESD. Furthermore, it is designed based on poloxamer, a material approved by the US Food and Drug Administration (FDA). The current study assessed the feasibility, durability and tissue biocompatibility of PA-PSH *in-situ* gel in both resected porcine stomachs and living minipigs.

Methods

^{1} H-NMR and FT-IR

The structure of PA, PSH and PA-PSH was confirmed by a Nicolet Magner 550 infrared (IR) spectrophotometer (Thermo Electron Co., Waltham, MA) among 500–4000 cm^{-1} and ^{1}H-NMR at 600 MHz (Varian, CA).

Gel permeation chromatography (GPC)

The average molecular weight (Mw) of samples were measured by GPC (LC-20AD, Shimadzu, Japan) with a refractive index detector, elution solvent: tetrahydrofuran (THF), 1.0 mL/min.

Creating Submucosal Cushion In Resected Porcine Stomachs

One mL the polymeric aqueous solution (17 wt.%) or control solution (PBS or glycerol) (n = 3) were injected into the submucosal layers of the fresh porcine stomachs within 1 min, using a 23-gauge needle while the ambient temperature was kept at 37 °C. Shapes of mucosal lifting induced with different injection agents were assessed at 0, 15, 30 and 60 min after injection.

Creating Submucosal Cushion And Performing ESD In Living Minipigs

The *in-situ* gel formation test was carried out using minipigs. Minipigs were fasted for 2 days with polyethylene glycol electrolytes powder treatment before operation. ESD was performed in minipigs under general anesthesia, with a endoscope (Olymplus H180, Olymplus Optical Co. Ltd, Japan). All of the injection agents contained a 1% methylene blue (v/v).

To create SFCs, each group (n = 6) of minipigs was submucosally injected with 10 mL 3% sodium pentobarbital solution, through an endoscope accessory channel by a 23-gauge needle. After anesthetizing the animals, 10 mL (1% methylene blue, v/v) PA-PSH or glycerol solution (control) was injected into the femoribus internus subcutaneously within 1 min respectively, observing the SFCs at 0, 15 and 30 min after injection. Besides, ESD procedures were implemented after the formation of SFCs in living minipigs.

In vivo degradation of PA-PSH *in-situ* gel and histologic examination

In vivo degradation of PA-PSH was evaluated endoscopically, comparing with the mucosal elevation on 9th day. All the minipigs were sacrificed after evaluation of the in vivo degradation of PA-PSH and the tissue damage were examined. All the histological sections were stained with hematoxylin and eosin (HE), and observed on a light microscope (Axiovert 200, Zeiss).

Results

IR and ^{1}H-NMR

PA-PSH was synthesized as reported (Fig. S1) [19,20]. The crosslink of PA and PSH was confirmed by FT-IR, which the C = O stretching vibration peak of the acrylate group was appeared at 1732 cm^{-1} (Fig. S2). Furthermore, the chemical structures of PA and PA-PSH conjugates were confirmed by ^{1}H-NMR. ^{1}H-NMR spectrum of PA in Fig. 2a,b clearly showed increased signals at 1.2 ppm and 6.0-6.5 ppm comparing with the spectrum of Poloxamer 407 (P407), which represented the protons from acryl and propylene oxide units.

The synthesized PSH was further confirmed by FT-IR (Fig. S2), the C = O stretching vibration of terminal thio-propionyl groups in PSH was appeared at 1736 cm^{-1}. For disulfide crosslinked P407 (PSS), three peaks appeared on the GPC curve at 21.19, 22.23 and 23.84 min. (Fig. S3). For PSH, the shoulder peak at 21.29 min disappeared, and for PA-PSH mixture, the peak at 24.01 min broadened largely and a remarkable shoulder peak at 25.34 min appeared, which resulted from products with larger molecular weights had formed based on the thiol-ene reaction [20]. All these results suggested the successful synthesis of PA-PSH.

Sol–gel transition

Figure 2c,d presented the sol–gel transition of PSH in a simulated physiological environment. At 25 °C, pH 3.5, PA-PSH solution was a transparent solution (Fig. 2c), while at 37 °C, pH 7.4, the copolymer solution turned into a transparent gel (Fig. 2d).

Submucosal injection of *in-situ* gel in resected porcine stomachs

The feasibility of *in-situ* gel was assessed in fresh resected porcine stomachs in different concentrations, with PBS and glycerol as controls. The results were shown in Fig. 3a. Although adequate mucosal elevations occurred in each group, the *in-situ* gel created SFCs were more durable than that of PBS or glycerol. Moreover, no significant change in size, shape or consistency of SFCs under the submucosa elevated by the *in-situ* gel was observed over 1 h. In contrast, the elevation created with PBS and glycerol collapsed quickly in 15 min. In short, the ability of mucosal elevation and maintenance exhibited a sequence *in-situ* gel > glycerol > PBS.

Submucosal injection of *in-situ* gel in living minipigs

The *in vivo* efficacy in living minipigs were further evaluated, as shown in Fig. 3b. The mucosa was lifted by different agents immediately after injection. However, the SFCs created by glycerol collapsed obviously after 15 min injection and disappeared at 30 min. In contrast, the SFCs of the *in-situ* gel remained unchanged in 30 min. Even after 9 days, the elevation of the *in-situ* gel still presented an apparently visible edge at the injection site without obvious ischemia or ulceration, while the injection sites of the controls was hard to find (data not shown).

PA-PSH-assisted ESD in living minipigs

As shown in Fig. 4a,b, the procedure of ESD was performed with the assistance of the methylene blue labled *in-situ* gel, it could be accomplished in early stage with the guidance of the mucosal elevation created by the *in-situ* gel, with no significant side effects like major bleeding and perforation. All of the procedures were successful and all the minipigs were sacrificed on 9th day after the ESD procedure, and its stomach was dissected with an ulcer displayed at early healing stage.

Biosafety of PA-PSH *in-situ* gel

The *in vivo* biodegradation of PA-PSH *in-situ* gel was examined in minipigs (Fig. 4c). An obvious elliptic protrusion appeared at the injection site after injection and the integrity of the mucosal elevation remained at least 9 days. The height and size of the protrusion decreased due to the degradation of the matrix *in vivo*. The degradation of the *in-situ* gel was attributed the hydrolysis of ester bonds in polyoxypropylene segments, and the final degradation products were biocompatible [21]. The gel protrusion was clearly observed at 0th and 9th day, separately (Fig. 4c). Additionally, after injection of *in-situ* gel solution, neither apparent epithelial damage nor inflammation cells were observed in the injection site (Fig. 4d). After 9 days of injection, granulation tissues were appeared on the postoperative ulcer bed and the edge of the ulcer was re-epithelialized (Fig. 4e). All these results confirmed the considerable biocompatibility of the PA-PSH *in-situ* gel as a submucosal injection agent.

Discussion

ESD is an effective procedure in the treatment of gastrointestinal tumors, with low trauma. However, because of the using of electrocautery in ESD procedure, the incidence rates of bleeding and perforation could reach to 16% and 10%, respectively [22]. To reduce the incidence of side effects especially perforation, submucosal agent is used to guide the ESD procedure by creating an enclosed cushion. Good submucosal agent should possess features, including longlasting, biocompatible, economically viable, safe and easy to inject and form SFCs, *etc*. Saline solution is a commonly used submucosal agent, however, it is easy to diffuse at the injection site. Hypertonic saline solution could present better lesion-lifting ability and longer duration than saline solution, for the reason of its high osmotic pressure, it still cause visible tissue damage and inflammation [23]. Besides, photo-crosslinked chitosan hydrogel could form SFCs *in-situ*, however, it should be irradiated with UV light for 5 min to form an elevated mucosa, which was not feasible *in vivo* experiments [24]. Additionally, the high viscosity of normal gel like Cook

Medical's submucosal lifting gel and ORISE™ Gel Submucosal Lifting Agent also limits its application in ESD [25, 26].

In this study, the PA-PSH *in-situ* gel was a low viscous sol at room temperatuere (RT) and transformed into hydrogel under physiological conditions, which could easily be injected by a 23-gauge disposable catheter injection needle (Fig. 2c,d). During the ESD procedure, it is vital for the mucosal solution to maintenance a longlasting and high enough mucosal elevation. Although the two control groups formed a SFCs after injection, the protrusions disappeared quickly. In contrast, the PA-PSH *in-situ* gel remained a satisfactory mucosal lifting at all examined time points (Fig. 3). Besides, the submucosal elevation formed by the *in-situ* gel maintained shape in 9 days (Fig. 3b, Fig. 4c). There was no obvious tissue damage at the injection site. Moreover, the mucosal elevation created by the in-situ gel made the ESD procedure more simple. As the guidance of the longlasting mucosal elevation, an en bloc resection of the lesion could be operated quickly and successfully without bleeding and peforation, and only one single injection of the PA-PSH solution is needed (Fig. 4a,b). In addition, photographs of HE-stained tissue sections further demonstrated the safety of the in-situ gel without damage in muscularis propria (Fig. 4d,e). It is worth noting that, only healthy animals were used in this study. The efficacy of the PA-PSH in-situ gel in the ESD of the animals with gastric lesions might be examined in a future study.

Conclusions

In conclusion, the developed PA-PSH *in-situ* gel was a low viscous aqueous solution at RT and could quickly transform into a semi-gel under human normal physiological environment (37 °C, pH7.4). After submucosal injection into resected pig stomach or in living minipigs, it could provide a long-lasting and high submucosal cushion, assisting and guiding the operation of ESD procedures with no perforation and bleeding. Moreover, the *in-situ* gel solution had good injectability and good biocompatibility, which can be developed as an ideal submucosal injection solution.

Abbreviations

ESD, endoscopic submucosal dissection; SFC, submucosal fluid cushion; NS, saline solution; FDA, Food and Drug Administration; IR, infrared; GPC, gel permeation chromatography; Mw, molecular weights; THF, tetrahydrofuran; IT, insulation-tripped.

Declarations

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The authors appreciate to everyone who participated and assisted in this study.

Author's contributions

SG and XW conceived and designed the project. ZT and YL participated in collecting the data, YG, CH and JC collected, analyzed and interpreted the data as well as drafted the manuscript. All authors read and approved the final manuscript.

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Availability of data and materials

The data and materials used in the current study are available from the corresponding author upon reasonable request.

Ethics approval and consent to participate

All experiments were performed according to guidelines established by the Second Military Medical University.

Consent for publication

Not applicable.

Competing interests

The authors declare that there is no conflict of interests in this study.

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Figures

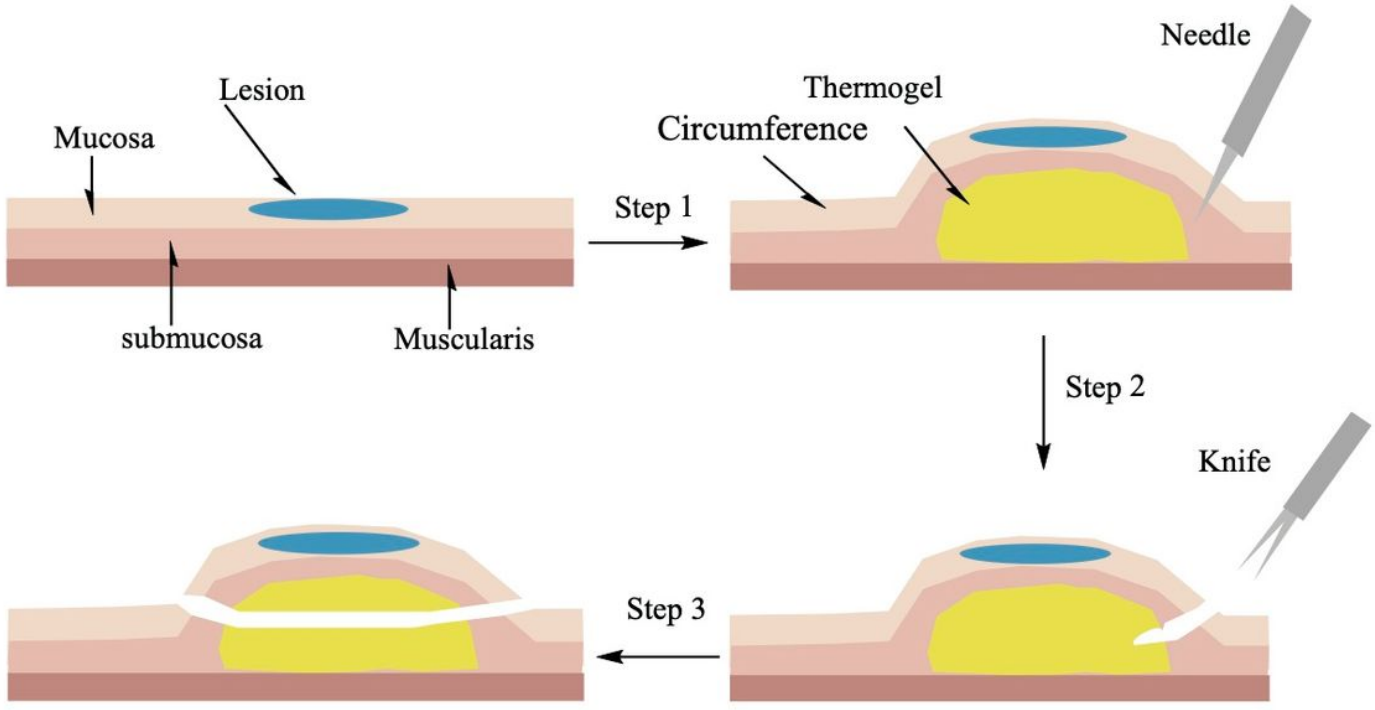

Figure 1

Schematic diagram illustrating the application of PA-PSH in-situ gel as an SFC in ESD procedure.

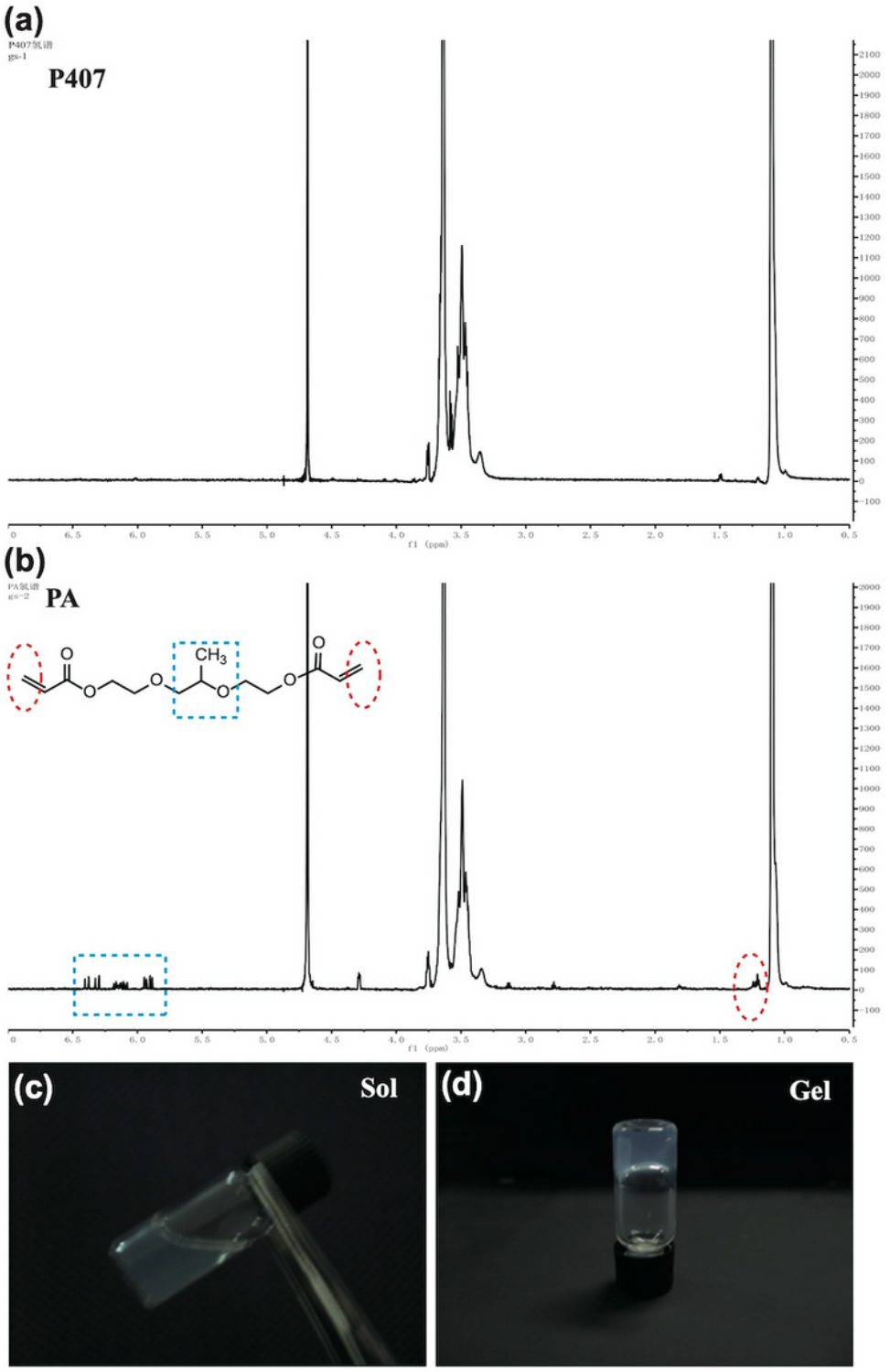

Figure 2

(a,b) 1H-NMR of P407 and PA; (c-d) Images of PA-PSH in-situ gel aqueous solution with concentration of 14wt% at different condition. (c) 25°C, pH3.5 (sol); (d) 37°C, pH7.4 (gel).

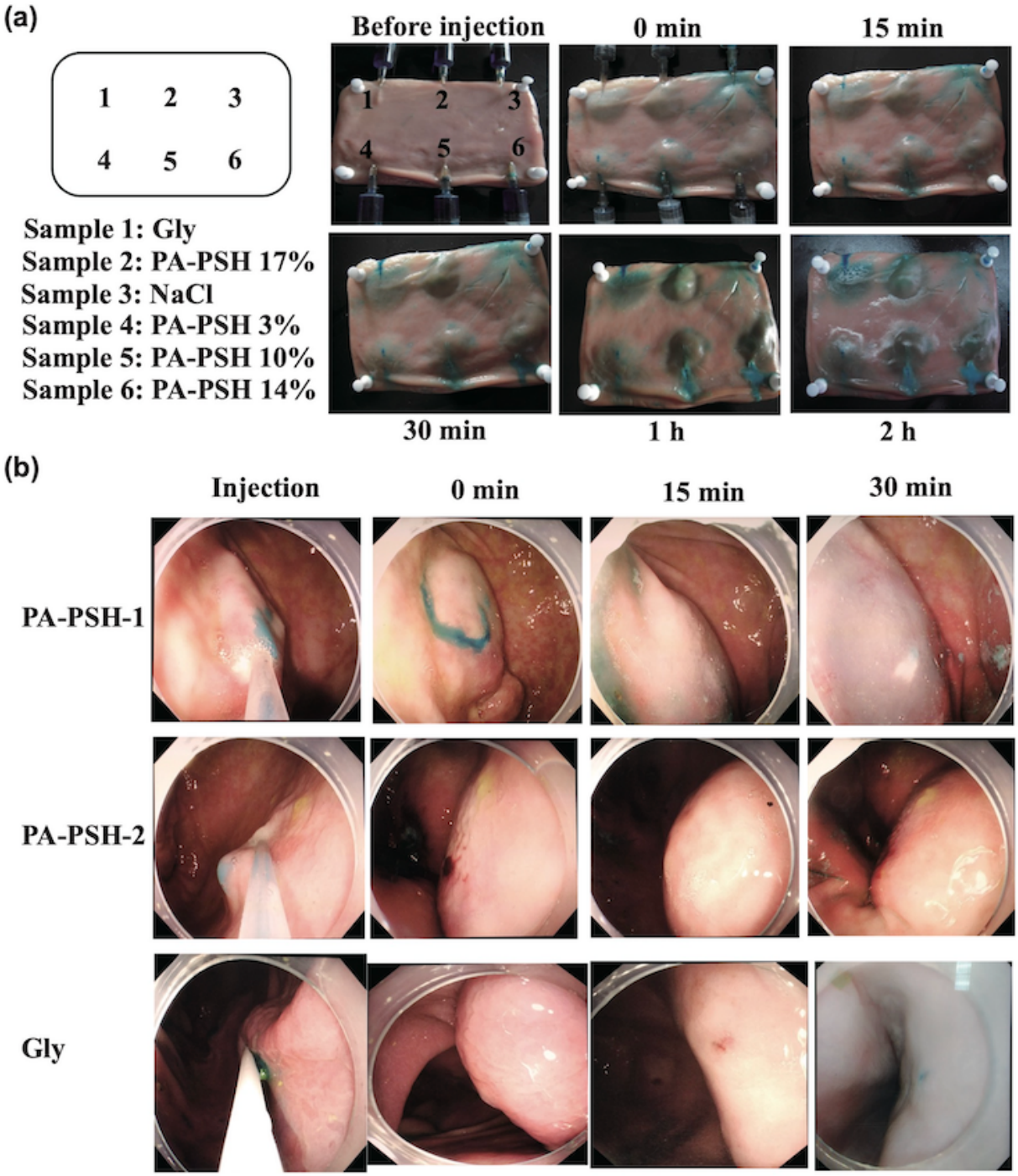

Figure 3

(a) Change in mucosal elevation as a function of time after injection of indicated 6 samples into a resected porcine stomach. The methylene blue was mixed as the color agent; (b) endoscopic changes of mucosal elevation shapes as a function of time after injection of PA-PSH in-situ gel (PA-PSH-1, 17 wt% ; PA-PSH-2, 14 wt%) and control. The methylene blue was mixed as the color agent.

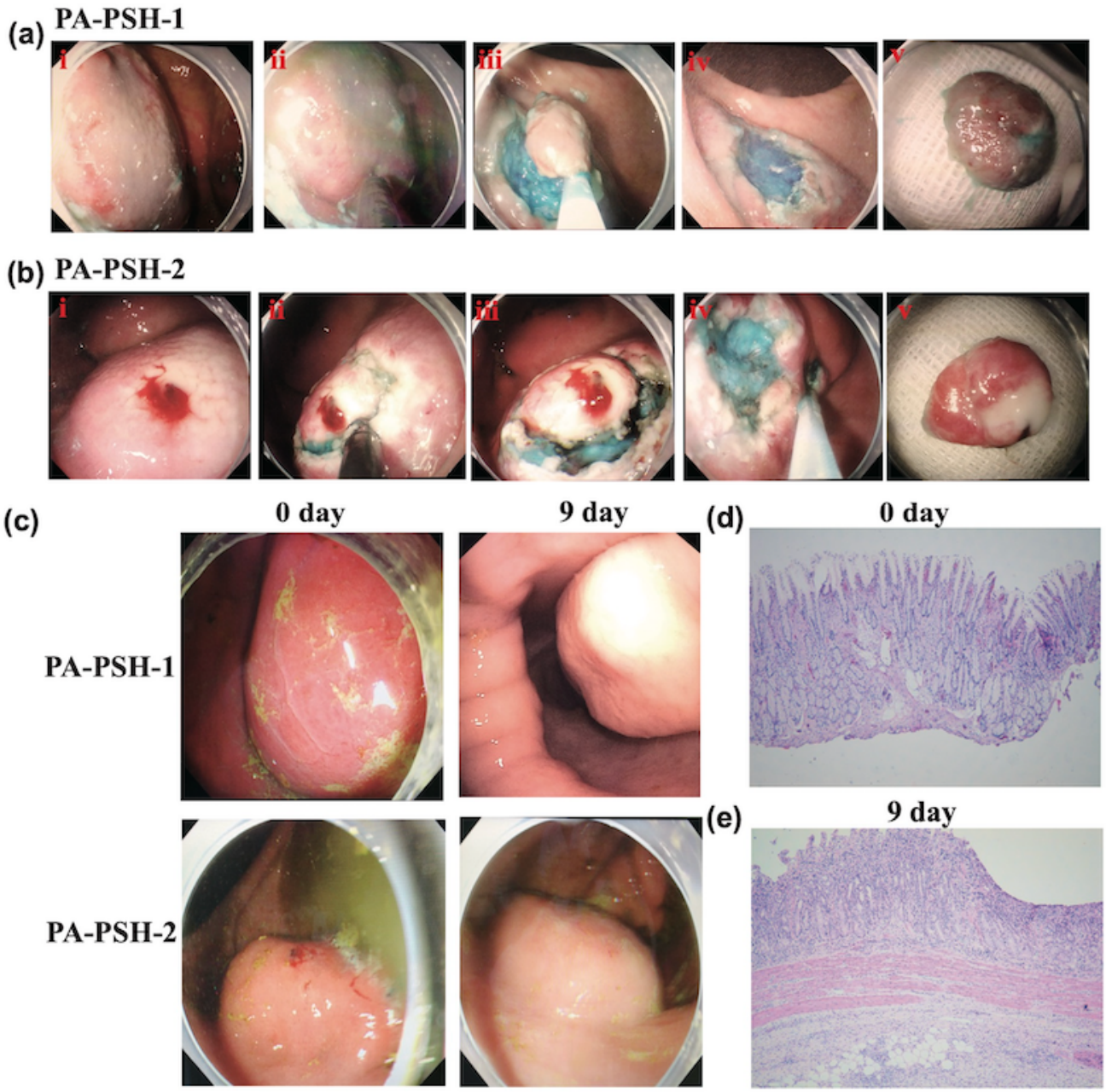

Figure 4

(a,b) Photographs of ESD produce, the methylene blue was added as the color agent; (i) the mucosal elevation created with the PA-PSH in-situ gel (PA-PSH-1, 17% w/w; PA-PSH-2, 14% w/w); (ii,iii) circumferential mucosal cutting using an IT knife; (iv) a submucosal cavity formed via suction of the in-situ gel underneath mucosa after completion of the circumferential mucosal cutting; (v) lesion resected by ESD; (c) an in vivo endoscopic observation 9 days after creation of a submucosal cushion with the PA-PSH in-situ gel (PA-PSH-1, 17% w/w; PA-PSH-2, 14% w/w) and control), the methylene blue was added as

the color agent; (d,e) histological section made immediately after mucosal lifting produced with the in-situ gel (d) and histological section made 9 days after mucosal lifting induced with the PA-PSH-1 (e).

Supplementary Files

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