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

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Research

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Abstract

Hypertonic saline solution is used for endoscopic submucosal dissection (ESD) as a submucosal fluid cushion (SFC). However, short duration time and side effects like perforation and bleeding have limited the application of SFC. Here, an *in-situ* gel crosslinked acrylate/thiol Poloxamer 407 (PA-PSH) was used as an SFC for ESD to avoid perforation, and the feasibility and safety of the PA-PSH for ESD was evaluated *in vitro* and *in vivo*. 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 crosslinked acrylate Poloxamer 407 (PA) and thiol-capped Poloxamer 407 (PSH) in physiological conditions. ESD was achieved precisely without obvious adverse effects and tissue damage. The PA-PSH *in-situ* gel provides an excellent submucosal injection system, which has great potential to improve the ESD technique.

Introduction

Endoscopic submucosal dissection (ESD) is usually used for dissection of gastrointestinal tumor lesions. The formation of an submucosal fluid cushion (SFC) between the lesion and the muscle layer can provide guidance for an en bloc resection during the ESD procedure, reducing the risk of perforation and bleeding. An ideal submucosal injection solutions should provide a durable and high SFC, meanwhile, it also should be economic, available and easy to inject without severe side effects [1-3].

Saline solution is usually used for ESD as an SFC. However, saline solution would be rapidly diffused and absorbed by the surrounding tissues thus requires repeated injections, making the ESD procedure complex. In addition, it is hard to resect 2 cm-diameter larger lesions completely [4]. Therefore, diverse agents like glycerol, hyaluronic acid, gelatin and so on are applied during the ESD procedure to avoid side effects, however, the high viscosity limits theirs application. [5-13] The *in-situ* gel such as photosensitive gels [14] or thermosensitive gels [15-17] were utilized to avoid the viscosity problem. However, there still some drawbacks. For example, the sol-gel (solution to gel) transformation based on photosensitive gels rely on prolonged exposure (5 min) under ultraviolet light, which may be difficult to operate *in vivo* especially in deep sites. Meanwhile, although thermogels like PLGA-PEG-PLGA [15] and poloxamers [17] present a comsiderable efficacy and biocompatibility, they are easy to clog inside long delivery channels under 37℃. Therefore, it is necessary for developing more submucosal agents to figure out these limitations.

Here, we exploited an PA-PSH *in-situ* gel as a submucosal injection substance for ESD. The schematic process of PA-PSH *in-situ* gel guided ESD procedure was shown in Fig. 1. PA-PSH *in-situ* gel was a low sticky solution *ex vivo* but could transform into a semi-gel *in vivo* under normal human physiological conditions, which was triggered by thiol-ene reaction between PA and PSH. 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.

Materials And Methods

2.1 ^{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, USA) among 500–4000 cm^{-1} and ^{1}H-NMR at 600 MHz (Varian, USA).

2.2 Gel permeation chromatography (GPC)

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

2.3 *In vitro* evaluation of PA-PSH *in-situ* gel

One mL PA-PSH (17 wt.%) or control solution (PBS or glycerol) (n = 3) were submucosally injected into porcine stomachs within 1 min, using a 23-gauge needle at an ambient temperature of 37°C. The elevation of submucosal cushions were assessed at 0, 15, 30 and 60 min after injection.

2.4 *In vivo* ESD procedures

Minipigs were fasted for 2 days with polyethylene glycol electrolytes powder treatment before operation. Anesthetized the minipigs and performed ESD with an endoscope (H180, Olymplus, Japan).

To create SFCs, each group (n = 6) of minipigs was anesthetized with 10 mL 3% sodium pentobarbital solution using a 23-gauge needle. Then, 10 mL PA-PSH or glycerol solution (control) was subcutaneously injected into the fem intern within 1 min respectively, observing the SFCs at different time intervals. All of the injection agents contained 1% methylene blue (v/v). Finally, ESD procedures were implemented after the formation of SFCs in living minipigs.

2.5 *In vivo* degradation and safety of PA-PSH *in-situ* gel

The degradation of PA-PSH was evaluated on 0th and 9th day with an endoscope. Then, all the minipigs were sacrificed and the histological sections of the injection lesions were stained with hematoxylin and eosin (H&E), and observed on a light microscope (Axiovert 200, Zeiss, Germany).

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

Results

3.1 IR and ^{1}H-NMR

PA-PSH was synthesized as reported (Fig. S1) [19,20]. PSH as well as the crosslink of PA and PSH was confirmed by FT-IR, the C=O stretching vibrations of terminal thio-propionyl groups in PSH and PA-PSH

were appeared at 1736 cm^{-1} and 1732 cm^{-1}, respectively (Fig. S2). Furthermore, the structures of PA and PA-PSH were confirmed by ^{1}H-NMR. As shown in Fig.2a,b, the increased signals at 1.2 ppm and 6.0-6.5 ppm comparing with the spectrum of Poloxamer 407 (P407) was the protons from acryl and propylene oxide units of PA.

For disulfide crosslinked P407 (PSS), three peaks appeared on the GPC curve at 21.19, 22.23 and 23.84 min; for PSH, the shoulder peak at 21.29 min disappeared; and for PA-PSH, the peak at 24.01 min broadened largely and a remarkable shoulder peak at 25.34 min appeared, which resulted from the larger molecular weight products based on the thiol-ene reaction (Fig. S3), suggesting the successful synthesis of PA-PSH [20].

3.2 Sol–gel transition

As shown in Fig. 2c,d, at 25 °C, pH 3.5, PA-PSH solution was a transparent solution (Fig. 2c), while at 37 °C, pH 7.4 (simulation of physiological conditions), the copolymer solution turned into a transparent gel (Fig. 2d).

3.3 Submucosal injection of PA-PSH *in-situ* gel in resected porcine stomachs

As shown in Fig. 3a, although adequate mucosal elevations occurred in each group, the PA-PSH *in-situ* gel had longer duration time than that of PBS or glycerol. Moreover, there was no significant collapse of SFCs elevated by the PA-PSH *in-situ* gel over 1 h. In contrast, the elevation created with PBS and glycerol dissapeared quickly in 15 min. Therefore, the ability of mucosal elevation and maintenance exhibited a sequence PA-PSH *in-situ* gel > glycerol > PBS.

3.4 Creation of SFCs in living minipigs

As shown in Fig. 3b, each agents elevated the SFCs immediately after injection. However, the SFCs in glycerol group collapsed fastly in 15 min and disappeared in 30 min. Contrastly, the SFCs of the PA-PSH *in-situ* gel maintained a clear shape, height and size in 30 min. Moreover, the elevation of the PA-PSH *in-situ* gel could be witnessed even after 9 days with no obvious ischemia or ulceration, while the SFCs of the controls was hard to find (data not shown).

3.5 *In vivo* ESD procedures

As shown in Fig. 4a,b, the ESD procedure could be accomplished in early stage with the guidance of the mucosal elevation created by the methylene blue labeled PA-PSH *in-situ* gel, without heavy bleeding or perforation. All of the procedures were successful and all the minipigs were sacrificed on 9th day, and the stomachs were dissected with ulcers displayed at early healing stage.

3.6 Biosafety of PA-PSH *in-situ* gel

The *in vivo* biodegradation of PA-PSH *in-situ* gel was examined in minipigs (Fig. 4c). The protrusion of SFCs created by PA-PSH *in-situ* gel maintained integrity even on 9th day, only with the reduction in height and size for the reason of the degradation of the matrix. The degradation was attributed the hydrolysis of ester bonds in polyoxypropylene segments, and all the final products were biocompatible [21]. Additionally, there was neither obviously epithelial damage nor inflammatory infiltration was observed in the injection site (Fig. 4d). After 9 days of injection, the postoperative site was healed and re-epithelialized (Fig. 4e). All these results suggested that the PA-PSH *in-situ* gel was a biocompatible submucosal injection agent.

Discussion

ESD is an effective procedure in the treatment of gastrointestinal tumors, however, the risk of bleeding and perforation could reach to 16% and 10%, respectively [22]. To reduce the side effects, submucosal agents are applied to guide the ESD procedure by creating an SFC, which are usually biocompatible, economically viable, safe and easy to inject and form SFCs such as saline solution. However, saline solution is easy to spread at the injection site. Hypertonic saline solution is a better alternative for its could longer duration and better SFC-lifting ability, it still inevitably causes considerable tissue damage and inflammation yet [23]. Besides, photo-crosslinked chitosan hydrogel could form SFCs *in-situ*, however, it is not feasible enough as it should be irradiated with ultraviolet light for at least 5 min to form an SFC [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 temperature (RT) and could transform into hydrogel under physiological conditions (Fig. 2c,d). It is important to maintenance a longlasting and high enough SFC during the ESD procedure. Although the two control groups formed a SFC after injection, the protrusions disappeared quickly. In contrast, the PA-PSH *in-situ* gel remained a high mucosal lifting at all intervals, and it could even maintain a good shape in 9 days *in vivo* (Fig. 3, Fig. 4c). Moreover, the mucosal elevation created by the *in-situ* gel made the ESD procedure more simple. As the guidance of the durable mucosal elevation created by PA-PSH *in-situ* gel, an en bloc resection of the simulated lesion was operated fast and successfullywithout obvious side effects, and only one single injection was needed (Fig. 4a,b). In addition, H&E results further demonstrated the safety of PA-PSH *in-situ* gel *in vivo (*Fig. 4d,e). It is worth noting that, only healthy animals were studied in thisn research. The efficacy of the PA-PSH *in-situ* gel in the ESD procedure of the animals with gastric lesions might be examined in a further 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.

Declarations

Data Availability

The data used to support the findings of this study are included within the article.

Conflicts of Interests

The authors declare that there is no conflict of interests 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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Supplementary materials

Materials, method of synthesis of PA-PSH and Figure S1-S3 were provided in Supplementary information.

Fig. S1. Illustration of the synthesis route of PA (A), PSH (B) and PA-PSH conjugate (C).

Fig. S2. FT-IR of P407, PA and PSH.

Fig. S3. GPC results of P407, PSS, PSH and PA-PSH.

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Figures

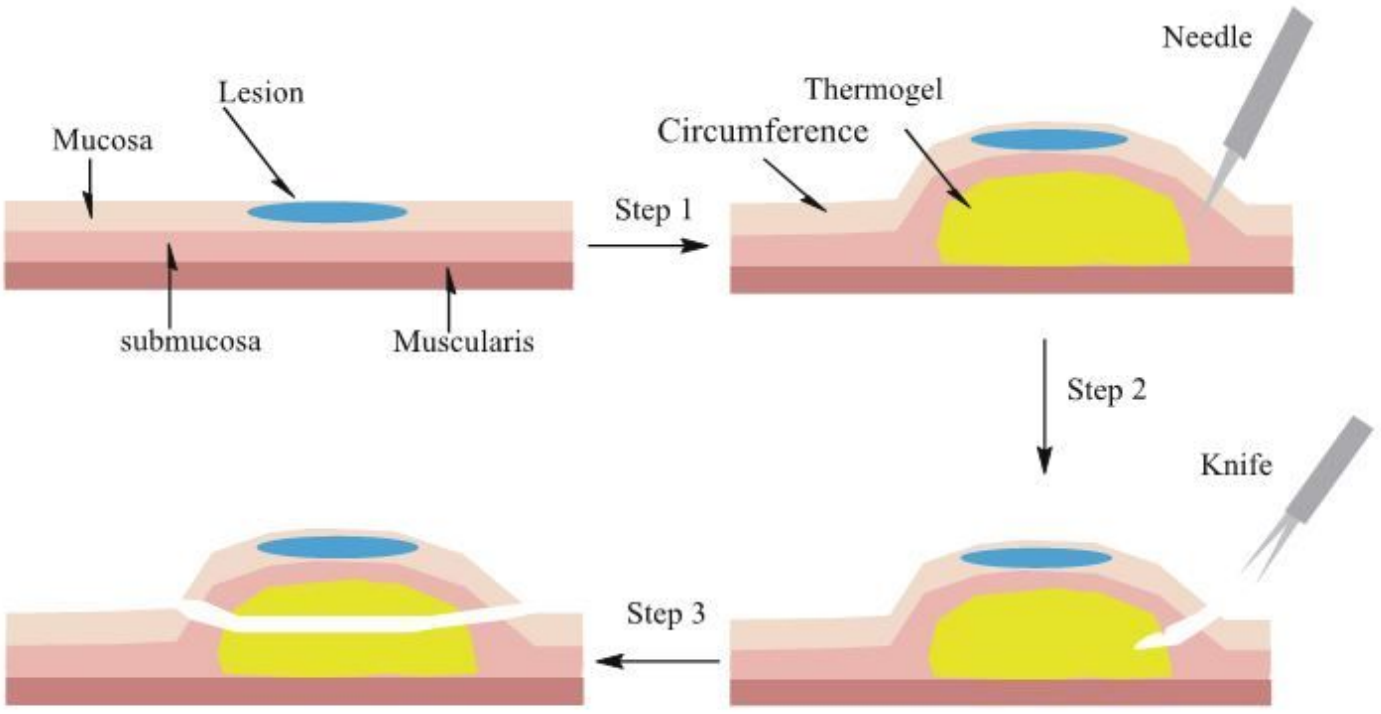
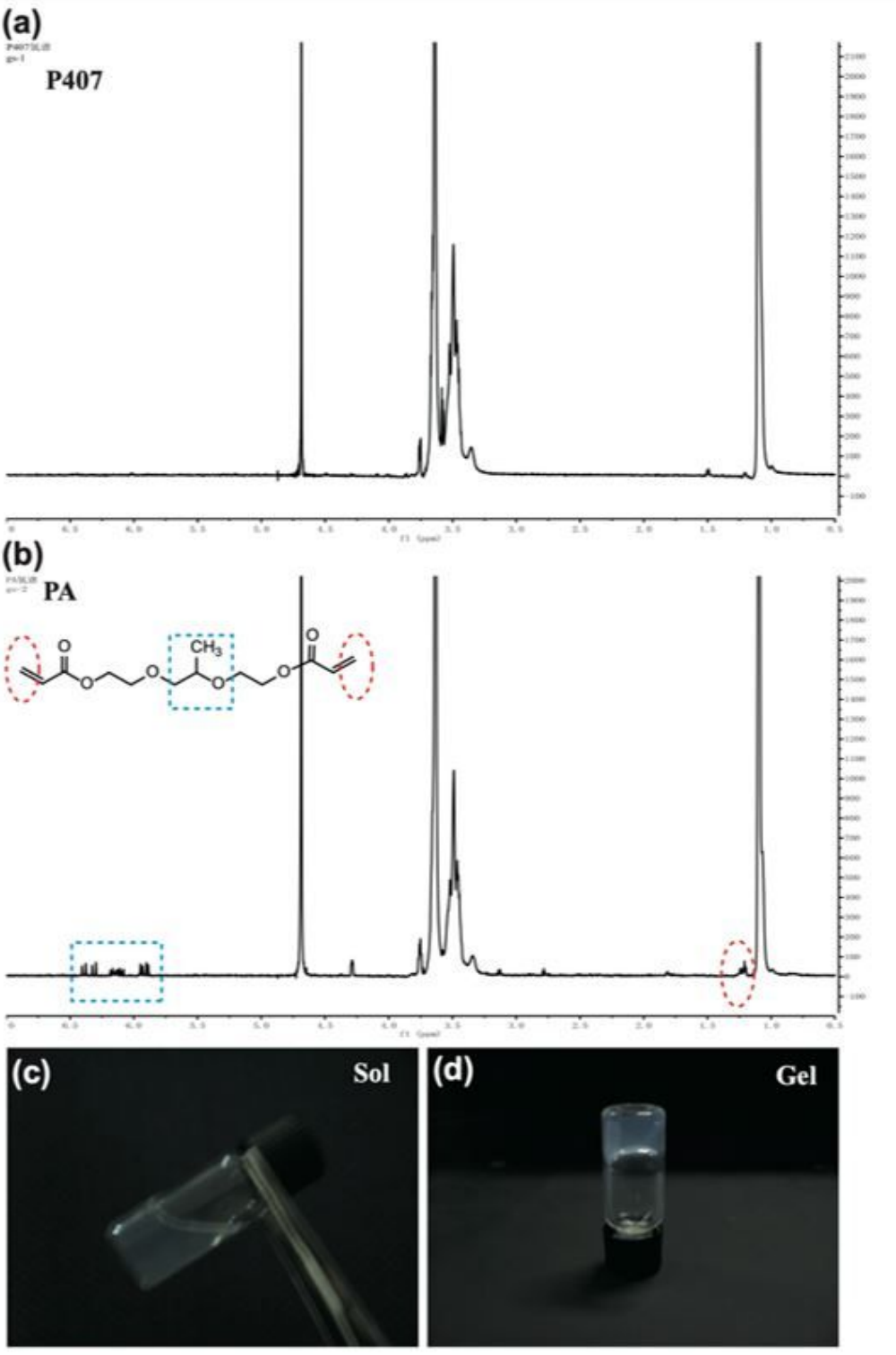


Figure 1

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 (c) 25°C, pH3.5 (sol); (d) 37°C, pH7.4 (gel).

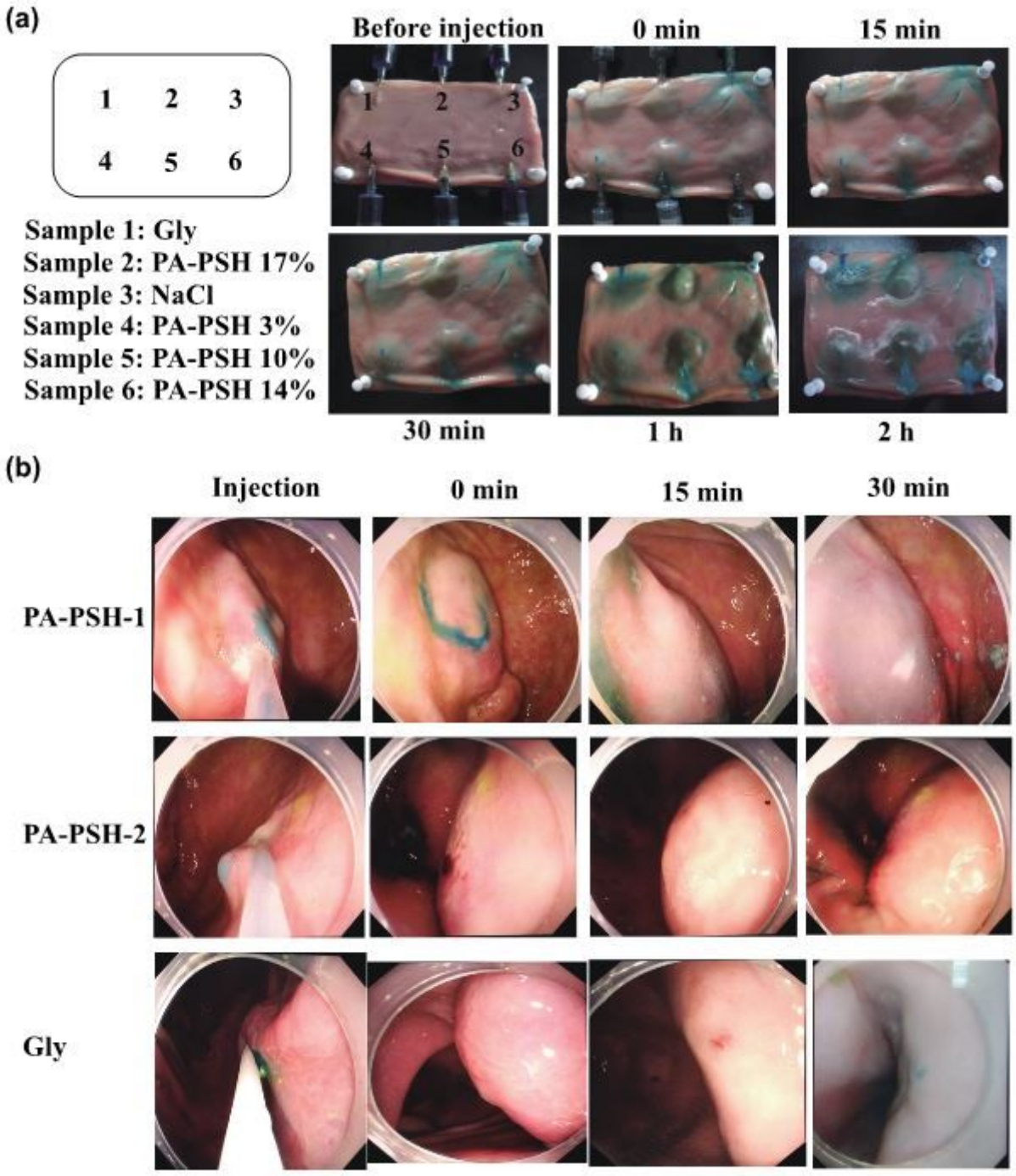


Figure 3

(a) Change in mucosal elevation as a function of time after injection of indicated 6 samples (mixed with methylene blue) into a resected porcine stomach; (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 (mixed with methylene blue).

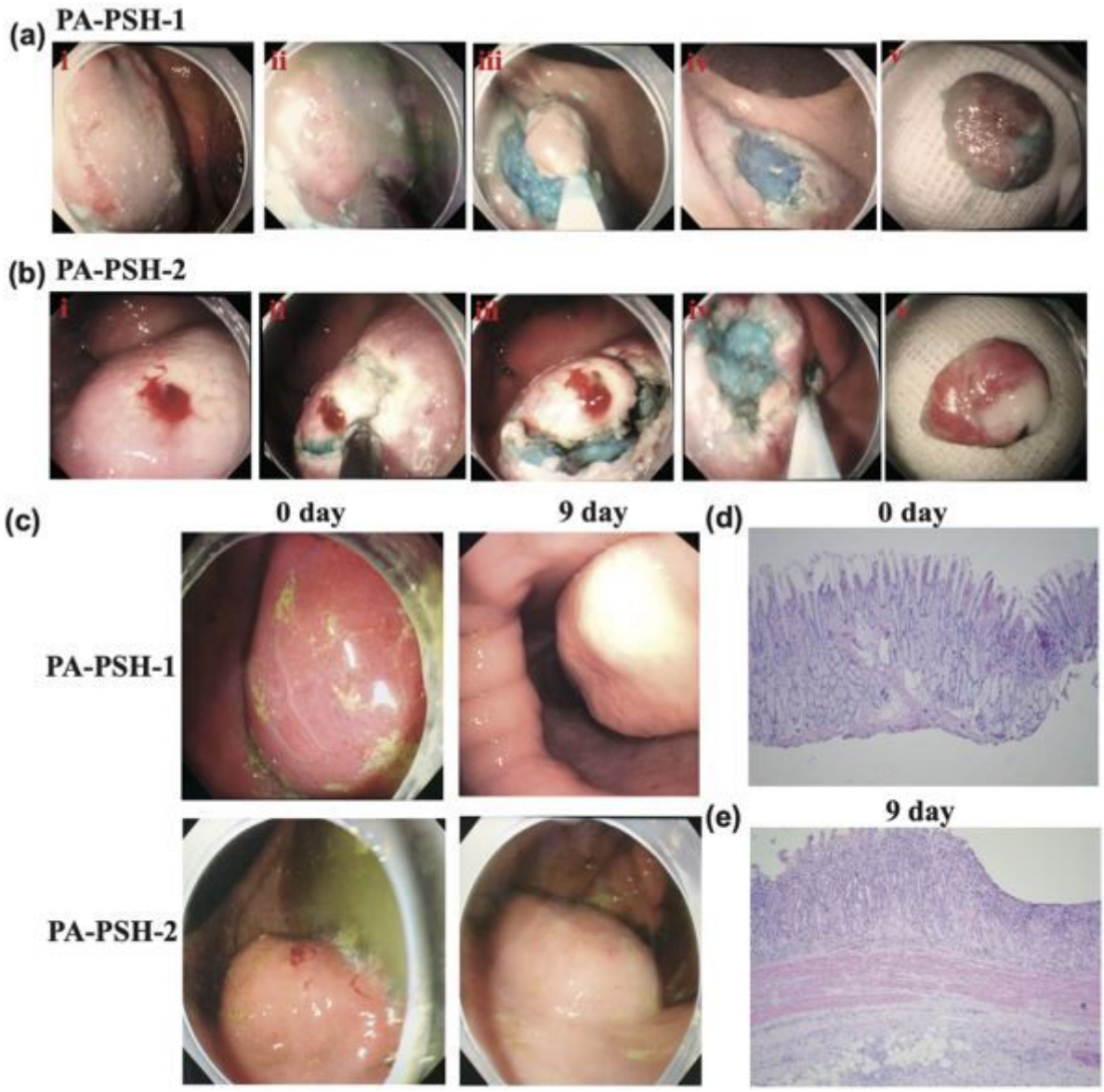


Figure 4

(a,b) Photographs of ESD produce; (i) the mucosal elevation created with the PA-PSH in-situ gel (PA-PSH-1, 17% w/w; PA-PSH-2, 14% w/w)(mixed with methylene blue); (ii,iii) mucosal circumcision with an IT knife; (iv) a submucosal cavity formed via suction of the in-situ gel underneath mucosa; (v) lesion resected by ESD; (c) endoscopic images of submucosal cushion with the PA-PSH in-situ gel (PA-PSH-1, 17% w/w; PA-PSH-2, 14% w/w)(mixed with methylene blue) on 0th and 9th day; (d,e) H&E results of the mucosal lifting produced with the PA-PSH-1 on 0th (d) and 9th (e) day.

Supplementary Files

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- ESDSI.docx