Poly(lactic acid-co-glycolic acid)–poly(ethylene glycol)–poly(lactic acid-co-glycolic acid) thermogel as a novel submucosal cushion for endoscopic submucosal dissection

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A B S T R A C T

Endoscopic submucosal dissection (ESD) is a clinical therapy for early stage neoplastic lesions in the gastrointestinal tract. It is, however, faced with a crucial problem: the high occurrence of perforation. The formation of a submucosal fluid cushion (SFC) via a fluid injection is the best way to avoid perforation, and thus an appropriate biomaterial is vital for this minimally invasive endoscopic technique. In this study, we introduced an injectable thermogel as a novel submucosal injection substance in ESD. The hydrogel synthesized by us was composed of poly(lactic acid-co-glycolic acid)–poly(ethylene glycol)–poly(lactic acid-co-glycolic acid) (PLGA–PEG–PLGA) triblock copolymers. The polymer/water system was a low-viscosity fluid at room temperature and thus easily injected, and turned into a non-flowing gel at body temperature after injection. The submucosal injection of the thermogel to create SFCs was performed in both resected porcine stomachs and living minipigs. High mucosal elevation with a clear margin was maintained for a long duration. Accurate en bloc resection was achieved with the assistance of the thermogel. The mean procedure time was strikingly reduced. Meanwhile, no obvious bleeding, perforation and tissue damage were observed. The application of the thermogel not only facilitated the ESD procedure, but also increased the efficacy and safety of ESD. Therefore, the PLGA–PEG–PLGA thermogel provides an excellent submucosal injection system, and has great potential to improve the ESD technique significantly.

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1. Introduction

The incidence of gastric cancer is high in many countries, especially in Eastern Asia. In Japan and Korea, early gastric cancer accounts for up to 50% of all of gastric cancer cases [1]. For early gastrointestinal neoplasms, endoscopic submucosal dissection (ESD) is now acknowledged as a preferred treatment modality [1–3]. A definite en bloc resection is believed to offer an accurate histological assessment and thus lower the risk of neoplastic recurrence. However, the use of electrocautery in ESD leads to a high risk of perforation [1–3]. To make mucosal excision easier and safer, the injection of a fluid into the submucosa to create a sufficient submucosal fluid cushion (SFC) between a lesion and the proper muscle layer is required.

A routine method to elevate mucosa involves a submucosal injection of normal saline solution (NS). It is, however, not easy to produce a high mucosal elevation and to maintain the desired height due to the rapid absorption of NS by the surrounding tissue. The low mucosal elevation makes operation difficult, and the electrocautery damage of the muscularis still results in perforation. Therefore, various substances, including glycerol, hyaluronic acid (HA) and hydroxypropyl methylcellulose, have been explored to achieve sustained mucosal elevation and avoid perforation [4–7]. These materials maintain the same condensed state before and after injection. In our opinion, a biomaterial system with in situ gelling after injection might be a better SFC. It would be ideal if the gelling was not triggered by any chemical reaction and the building blocks of the material were to have been approved by the US Food and Drug Administration. The present study intro-
duces a temperature-sensitive polymer hydrogel as the submucosal injection agent in ESD. The polymer is composed of poly(ethylene glycol) (PEG) and poly(D,L lactic acid-co-glycolic acid) (PLGA), both of which have been applied clinically. The aqueous solution of the block copolymer with appropriate composition and concentration is a free-flowing sol at ambient temperature, and able to be transformed into a hydrogel at body temperature free of any chemical reaction.

Hydrogels are three-dimensional polymeric networks, which can absorb a significant amount of water but do not dissolve in water, resembling natural living tissues and presenting excellent biocompatibility [8–14]. In the past decade, in situ gel-forming polymers have attracted much attention as injectable biomaterials [15–21]. Some biodegradable block copolymers undergo a reversible sol–gel transition in water with increasing temperature, including PEG and PLGA [22,23], PEG and poly(ε-caprolactone) [24,25], PEG and poly(ε-caprolactone-co-lactide) [26,27], PEG/polypeptide [28,29] and poly(phosphazenes) [30]. These systems have been applied in sustained drug delivery, tissue engineering and the prevention of post-operative adhesion [25,30–34], yet have never been tried as a submucosal injection agent in ESD, to the best of our knowledge.

Among those thermo-reversible hydrogels, the thermogelling PLGA–PEG–PLGA triblock copolymer system is a very promising biomedical material due to its convenient synthesis and good safety profile [23,35,36]. The gelation and degradation properties of PLGA–PEG–PLGA thermogels can be adjusted via block length, polymer concentration, sequence of PLGA segment and even end group [23,37–40]. To date, the biomedical applications of PLGA–PEG–PLGA thermogels have been focused on drug delivery [36,41–45]. For instance, PLGA–PEG–PLGA formulation containing paclitaxel (an anticancer agent) can sustain the release of drug ex vivo for up to 50 days [36]. In clinical trials, this formulation (OncoGel) exhibited excellent efficacy against human esophageal cancer [46].

Herein, we tried the thermogelling PLGA–PEG–PLGA triblock copolymers as a submucosal injection substance to create SFCs, as schematically presented in Fig. 1. The current study assessed the feasibility, safety, durability and tissue biocompatibility of SFCs created with the PLGA–PEG–PLGA thermogel in both resected porcine stomachs and living minipigs.

2. Materials and methods

2.1. Materials

PEG with molecular weight (MW) 1500 and stannous octoate of purity 95% were products of Sigma–Aldrich. D,L-Lactide (LA) and glycolide (GA) were purchased from Purac and used as received. An injection of glycerol (10 wt.%), fructose (5 wt.%) and sodium chloride (0.9 wt.%) abbreviated as glycerol was acquired from Cisen Pharmaceutical Co. Ltd (PR China). HA (1 wt.%, MW 600–1500 KDa) was obtained from FREDA Group (PR China), and diluted via glycerol to 0.125 wt.% before use. Other reagents were used without further purification.

2.2. Animals

Five minipigs (~30 kg) were provided by the Experimental Animal Center of the Second Military Medical University. The animal experiments were approved by the Animal Use and Care Committee of the Second Military Medical University.

2.3. Polymer synthesis

PLGA–PEG–PLGA triblock copolymers were synthesized via a bulk ring-opening copolymerization method in the presence of LA and GA using PEG as an initiator. The detailed procedure has been provided in our previous publications [37,38]. Briefly, PEG (15 g) was added into a three-necked flask and dried under vacuum with stirring at 120 °C for 4 h. Then, LA (28 g), GA (4.4 g) and the initiator, stannous octoate (0.035 g) were added. The reaction system was further heated at 150 °C for 12 h under an argon atmosphere. Next, the temperature of the oil bath was reduced to 120 °C, and unreacted monomers were removed under vacuum.
for 60 min. Crude polymer products were washed with 80 °C deionized water five times, and the residual water in the polymer was removed by lyophilization. The final products were stored at −20 °C until use.

2.4. Physico-chemical characterization

The proton nuclear magnetic resonance (1H-NMR) spectrum of the triblock copolymer was recorded using a Bruker spectrometer (DMX 500) with CDCl3 as solvent in the presence of tetramethylsilane as internal standard. Gel permeation chromatography (GPC, Agilent 1100) was used to determine the polymeric MW and its distribution. Measurements were performed at 35 °C with tetrahydrofuran as eluent at a flow rate of 1.0 ml min−1. The MWs were calibrated with polystyrene standards.

2.5. Determination of sol–gel transition

The sol–gel transition was determined via the vial inverting approach [37,38]. The PLGA–PEG–PLGA triblock copolymers were dissolved in NS. The 2 ml vials containing 0.5 ml polymer solutions were stored at 4 °C overnight. Next, the vials were immersed in a water bath. The sol–gel transition temperatures were determined from 15 to 60 °C with a temperature increment of 1 °C per step. At each temperature, the samples were kept for 15 min and then inverted 180° for 30 s. If no obvious flow was observed, the sample was regarded to form a gel.

The sol–gel transition of PLGA–PEG–PLGA triblock copolymers in NS (25 wt.%) was also investigated on a strain-controlled rheometer (ARES Rheometric Scientific) using a Couette cell (Couette diameter, 32.0 and 34.0 mm; height 33.3 mm). Data were collected under an oscillatory frequency of 10 rad s−1 and a heating rate of 0.5 °C min−1.

2.6. In vivo degradation of PLGA–PEG–PLGA thermogel

In vivo gel formation and degradation tests were carried out using a minipig. The polymer aqueous solution (25 wt.%) was sterilized through a 0.22 μm filter and stored at 4 °C in a refrigerator before use. A polymer solution (2.5 ml) was then subcutaneously injected into the femoral internus using a 2.5 ml syringe after anesthetizing the animal with 3% sodium pentobarbital solution. Both thighs were injected once, and the photographs of the injection site were captured by a digital camera every 10 days.

2.7. Creating submucosal cushion in resected porcine stomachs

Fresh porcine stomachs were used. The polymeric aqueous solution (25 wt.%) and two controls (HA and glycerol) (n = 4 for each group) were submucosally injected through an endoscope accessory channel using a 23-gauge injection needle. The dose was 3 ml for each sample. The mucosal elevation at the target sites was observed endoscopically 0, 15, 30 min and 1 week after injection. ESD procedures were implemented after remarkable mucosal elevations were created via an injection of 10 ml solution in living minipigs (n = 8).

Three animals were euthanized after completion of experiments to examine immediate tissue damage. Two other minipigs recovered from general anesthesia and were sacrificed after 1 week to investigate delayed tissue damage. Injection or ESD procedure sites captured from retrieved stomachs were fixed with 10% neutral buffered formalin and embedded in paraffin. Histological sections were stained with Hematoxylin and Eosin, and observed on a light microscope (Axiovert 200, Zeiss).

3. Results

3.1. Synthesis and thermogelling properties of PLGA–PEG–PLGA triblock copolymers

PLGA–PEG–PLGA triblock copolymers were synthesized via a ring-opening copolymerization of LA and GA in the presence of hydroxyl-terminated PEG. The product obtained was characterized by 1H-NMR. On the basis of the 1H-NMR peaks at 1.55, 3.60 and 5.20 ppm [41,47], the average MW of PLGA–PEG–PLGA was determined to be 1740–1500–1740, and the LA/GA molar ratio in PLGA block was calculated to be 5.1:1. A GPC study was performed to measure the distribution of MW, showing a unimodal distribution with a polydispersity index of 1.24.

The phase diagram of PLGA–PEG–PLGA triblock copolymers in NS is presented in Fig. 2. At low or room temperatures, the polymer samples were easily soluble in water. Upon raising temperature, a reversible sol–gel transition occurred, and a no-flowing semi-solid gel was achieved when the concentration of sample was higher than the critical gelation concentration. Further heating led to another transition from a gel to a “sol (suspension)”, which originally presented a sol but eventually precipitated after a sufficiently long time. The upper transition temperature exhibited a stronger concentration dependency than the lower one. The gel window covered body temperature, suggesting that this thermogel system has potential for biomedical applications.

The sol–gel transition temperature Tgel of PLGA–PEG–PLGA triblock copolymers in NS (25 wt.%) was 33 °C, determined via the vial inverting approach. The rheological properties of the thermogel system were also measured. Fig. 3 displays the modulus of the copolymers in NS (25 wt.%) as a function of temperature. The sol–gel transition was accompanied by an abrupt increase in storage modulus (G′) and loss modulus (G″). G′ is an elastic component of the complex modulus, and G″ belongs to its viscous component. Generally speaking, the crossover point between G′ and G″
indicates the sol–gel transition temperature. $T_{gel}$ was 35°C, determined via the criterion of $G' = G''$. In fact, this transition point is usually dependent on oscillatory frequency [24,48]. It is thus not surprising that the sol–gel transition temperature determined via the vial inverting method (33°C) was 2°C different from that determined via the $G' = G''$ standard (35°C) at 10 rad s$^{-1}$. The rheological data of the thermogel presented good reproducibility, which has been demonstrated in our previous publication [37].

3.2. In vivo degradation of PLGA–PEG–PLGA thermogel

The in situ-forming thermogel and its in vivo degradation were examined in a large mammal for the first time, with the results presented in Fig. 4. After a subcutaneous injection of the copolymer solution into the femoribus internus of a minipig, an obvious elliptic protrusion appeared at the injection site due to a rapidly thermo-induced gelation (∼30 s) at the physiological condition. Its integrity remained over 1 month, and the height and size of the protrusion decreased slowly due to the degradation of the gel matrix in vivo. The degradation of the thermogel was attributed to the hydrolysis of ester bonds in PLGA segments, and the final degradation products were lactic acid, glycolic acid and PEG [35,36]. The acidic products were absorbed by metabolism and nondegradable PEG with low MW was cleared by kidney [35,49]. It is worth pointing out that the gel protrusion was not only distinctly touched by hand but also clearly observed by eyes at different time points. Considering the low contrast ratio of the gel margins in Fig. 4, dashed lines were added to emphasize the gel regions. Furthermore, no significant adverse reaction, such as edema and necrosis, was observed. Thus the thermogel system has great potential as an injectable and biodegradable biomaterial.

3.3. Submucosal injection of thermogel in resected porcine stomachs

The feasibility of the submucosal injection of the thermogel was first assessed in fresh resected porcine stomachs, with HA and glycerol as two controls. The results are shown in Fig. 5. Although adequate mucosal elevations occurred after submucosal injection of all three samples, the SFC created with the thermogel was more durable than those obtained with HA or glycerol. Compared with the initial injection of the thermogel, no significant change in size, shape or consistency of mucosal lifting was observed over 1 h, which was attributed to the formation of a semi-solid gel under the submucosa. In contrast, the elevation created with HA and glycerol gradually collapsed 15 min later. In short, the ability of mucosal elevation and maintenance exhibited a sequence thermogel > HA > glycerol.

3.4. Submucosal injection of thermogel in living minipigs

We further evaluated the in vivo efficacy in living minipigs. Fig. 6 shows the endoscopic views at 0, 15 and 30 min. Injection of the PLGA–PEG–PLGA aqueous solution or commonly used substances into the submucosal layer led to mucosal elevation. The mucosa was lifted by different agents immediately after injection, and no significant difference was initially observed among the three groups. However, the height of elevation had obviously collapsed after injection of HA or glycerol for 15 min. At 30 min, both the elevations created with the two controls were not visible under the endoscope. In contrast, the shape of mucosal lifting with a clear
border produced via injection of the thermogel remained unchanged, even at 30 min. The feasibility of the thermogel as a submucosal injection agent was thus confirmed.

After 1 week, another endoscopic examination was performed. A striking mucosal elevation with a clearly defined edge was still present at the target site without ischemia or ulceration, as shown in Fig. 7A. In contrast, it was difficult to find the injection sites of the two controls (data not shown). Then, the remained minipig was sacrificed and the necropsy was performed. The mucosal elevation was visible at the injection site, with the result shown in Fig. 7B.

We also examined the histological response to the submucosal injection of the thermogel. From the histological micrographs obtained immediately after the submucosal injection, neither apparent epithelial damage nor inflammation cells were observed (Fig. 8A and B). After 1 week following the injection, a fibrin network separating the thermogel from the submucosa was formed, associating with some inflammation cells such as eosinophils and lymphocytes at the edge of the hydrogel (Fig. 8C and D). Meanwhile, the connective tissue had infiltrated into the hydrogel. These observations confirmed the acceptable biocompatibility of the thermogel as a submucosal injection agent.

3.5. Performing the ESD procedure in living porcine stomachs

ESD was successfully performed using the thermogel as a submucosal injection substance, as shown in Fig. 8. The presence of the thermogel neither complicated the procedure nor changed the electrocautery settings. What is more, the ESD procedure was accomplished in easy stages due to the long-lasting maintenance of the mucosal elevation created with the thermogel. After the protrusion was cut open via an insulation-tripped (IT) knife, the circumferential resection was made conveniently (Fig. 9A). Next, the suction of the in situ-forming gel under the mucosa was readily performed via the working channel of the endoscope, and a clear cavity under the mucosa was generated, as seen in Fig. 9B. Finally, the lesion was resected en bloc (Fig. 9C), and any repeated injection was not required during dissection.

Minor bleeding occurred and stopped spontaneously without treatment. There were no significant complications including major bleeding and perforation during the procedure. A minipig was euthanized 7 days after the ESD procedure, and its stomach was dissected. An artificial ulcer displayed an early healing stage (Fig. 9D). In the present study, eight ESD operations were accomplished in four animals, and all of the procedures were successful.

Histological examinations were also performed after dissection. A clean separation between the mucosal layer and the muscularis layer in a living porcine stomach was achieved due to the thermogel-assisted resection, as shown in Fig. 10A. No sign of mucosal or muscularis propria damage was observed. Meanwhile, an intact mucosal specimen was obtained via the ESD procedure (Fig. 10C), which provided more accurate histologic assessment and reduced the risk of neoplastic recurrence. Histological examination 1 week after the procedure affirmed that granulation tissues were generated on the artificial ulcer bed, and re-epithelialization occurred at the ulcer edge (Fig. 10E). Although inflammatory cells such as lymphocytes and neutrophils were observed in the regenerated granulation and epithelialization tissues (Fig. 10F), the inflammatory response did not happen in the deeper proper muscle layer.

4. Discussion

ESD is an effective therapeutic modality in the treatment of gastrointestinal tumors with minimal surgical wounds and preservation of the organ. However, due to the use of electrocautery during the ESD procedure, the incidence rates of bleeding and perforation were 0.1–15.6% and 1.2–9.7%, respectively [1]. The most effective approach to reduce these common complications, especially perforation, is to elevate mucosa for a sufficient time via injecting submucosal agents, namely, to create an enclosed cushion. An ideal submucosal agent in ESD should provide a reliable, long-lasting mucosal lifting without damaging the surrounding tissue. Moreover, some factors pertaining to expense and difficulty in preparing, storing and submucosally delivering the agent should be taken into account as well.

NS is often used to induce mucosal lifting, yet it diffuses quickly at the target site. Multiple injections are thus required for larger lesions, resulting in a prolonged time of the ESD procedure. Although hypertonic solutions present better lesion-lifting ability and longer duration than NS, significant tissue damage was observed after the submucosal injection because of their high osmotic pressure [5]. HA solution is one of the best available solutions in creating an SFC, and has been approved as an injection fluid for endoscopic mucosal resection in Japan in 2007 [7]. One of its disadvantages is a high cost ($49.50–128.00 per ml) [4]. What is more, mammal-resource materials including HA are, due to risk of infection, strictly controlled as medical biomaterials in many countries, and a large amount of HA is required to create an SFC. In addition,

![Fig. 6. Endoscopic photographs showing the change in mucosal elevation as a function of time after injection of indicated samples into a living porcine stomach. The thermogel contained 25% copolymer in NS. The methylene blue was mixed as the color agent.](image)

![Fig. 7. (A) An in vivo endoscopic observation 1 week after creation of a submucosal cushion with the thermogel; (B) an in vitro photograph of the submucosal cushion made with the thermogel after euthanasia of the minipig.](image)
possible tumor stimulation by HA should also be taken into account [50]. A photo-crosslinked chitosan hydrogel has recently been investigated as a submucosal injection agent in ESD [51]. Mucosal elevation was created by the injection of a chitosan solution, and then polymerization was triggered, resulting in the in situ-formed hydrogel. However, the homogeneity of the hydrogel cannot be guaranteed, and the chemical reaction in the animal is inconvenient in the process “the elevated mucosa was irradiated with UV light for a total of 5 min (30 s each at 10 different places by using an UV light-fiber through the endoscopic accessory channel and UV lamp system)” [51]. Immediately before submitting our paper, we found that another gel made by Cook Medical has been suggested as a submucosal injection substance [52]. The composition of this gel was not released by the company, yet a special injection device with a 19-gauge needle was required to deliver this high viscous gel [52].

In this study, a thermogelling PLGA–PEG–PLGA system, which is biodegradable and biocompatible [35,36], was tried as a novel endoscopic injection agent to make mucosal elevation. It is actually not a gel before injection. The polymer/water system was a low viscous sol at low or room temperatures and transformed into a semi-solid hydrogel with increasing temperature (Figs. 2 and 3). The physical gelation happened due to the formation of a percolated micelle network [38,47]. Consequently, the injectability of the PLGA–PEG–PLGA aqueous solution was good, and a disposable 23-gauge catheter injection needle could be used. In ESD procedures, the ability of mucosal lifting and its maintenance are vital to avoid perforation and accomplish en bloc resection of tumors. Although all of the examined solutions led to the mucosal elevation after submucosal injection both in vitro and in vivo (Figs. 5 and 6), the PLGA–PEG–PLGA thermogel remained a satisfactory mucosal lifting at all examined time points. In contrast, the protrusions in the two control groups disappeared quickly, which might be attributed to the rapid diffusion of glycerol and HA out of the injection sites. Different from the controls, the disappearance of the mucosal lifting created with the thermogel was dependent on its in vivo degradation, which was rather slow (Fig. 7). Therefore, the significant difference in maintenance of mucosal lifting arises from the different material mechanisms.

The submucosal cushion created with the thermogel was still visible under an endoscope after 1 week (Fig. 7). Neither ischemia nor ulceration occurred at the injection site. Meanwhile, histological investigations further demonstrated the biocompatibility of the thermogel as a submucosal injection agent (Fig. 8). These features were consistent with the subcutaneous injection at the same mammal model (Fig. 4) and indicated that the PLGA–PEG–
PLGA thermogel might be applied in mucosal healing after the resection process, for instance as a submucosal drug delivery system for mucosal regeneration. One prominent advantage of using the thermogel is to facilitate the ESD procedure. After a single injection of the polymeric aqueous solution, the additional injections during operation were not needed. The in situ-forming polymer gel could be sucked out via the working channel of the endoscope during the ESD procedure, and an en bloc resection of lifted mucosa was successfully achieved (Fig. 9). The use of the thermogel resulted in a significant reduction in mean procedure time. Meanwhile, neither major bleeding nor perforation was encountered during the experiments. In addition, the mucosal specimen obtained via ESD demonstrated a clean submucosal tissue dissection without muscularis propria injury (Fig. 10). It is worth noting that only healthy animals were used in this study. The efficacy of the PLGA–PEG–PLGA thermogel in the ESD of the animals with gastric lesions might be examined in a future study.

5. Conclusions

A thermoreversible PLGA–PEG–PLGA hydrogel was successfully tried as a novel submucosal injection agent in ESD. The aqueous polymer system was a thin liquid at room temperature and spontaneously formed a semi-solid gel due to contacting with warmer surroundings after injection into the submucosal layer of a mammal. The thermogel provided a sustained elevation of the mucosa, which is superior to commonly used substances. Even 1 week after an injection of the thermogel, the mucosal lifting remained without noticeable ischemia or ulceration. En bloc resection of lesions was successfully accomplished just after a single injection, and the mean procedure time of ESD was significantly decreased due to the assistance of the thermogel. No perforation, major bleeding or tissue damage were observed during ESD. Uncovering of the mucosal specimen confirmed a clean submucosal dissection and provided more accurate pathologic assessment. Therefore, the PLGA–PEG–PLGA thermogel is suitable as an ideal submucosal agent to provide higher mucosal elevation and longer duration while minimizing complications. Its unique submucosal dissecting property simplifies operational steps, avoids tedious dissection and thus makes ESD accessible to a large extent.

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Appendix A. Figures with essential colour discrimination

Certain figures in this article, particularly Figs. 1–10 are difficult to interpret in black and white. The full colour images can be found in the on-line version, at http://dx.doi.org/10.1016/j.actbio.2013.12.007.

References


