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Thermally responsive hydrogel for atrial fibrillation related stroke prevention

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A B S T R A C T

Atrial fibrillation induced stroke accounts for up to 15% of all strokes. These strokes are caused approximately 90% of the time by clot formation in the left atrial appendage (LAA). To prevent these clots, the most common approach is to administer blood thinners. However, contraindications prevent some people from being able to have blood thinners. Devices have been developed to seal the LAA to prevent clot formation in these patients. Current devices, such as the LARIAT® tie off the LAA theoretically preventing blood from entering the LAA. These have had limited clinical success mainly due to failure to completely close the LAA leaving holes and orifices for thrombi to form. To overcome this lack of complete closure, many surgeons use off-label approaches, classically filling the LAA filamentous coils, to cover these holes. Although this usually helps largely cover the holes, placement is challenging, the coils can migrate, the holes are not fully closed as there is space within and around the coils that don't fully mold to the LAA geometry. Furthermore, the coils can develop device related thrombi defeating their purpose. Therefore, these are not fully sufficient to complement the closure techniques in closing the LAA. To address limitation of the closure devices and coil sealing of remaining holes, we developed a thermally responsive hydrogel (Thermogel) that solidifies once injected into the LAA to uniformly and fully close off the LAA thus preventing clot formation and device related thrombi. This Thermogel consists of three portions: 1) a structural component composed of thiolated Pluronic F127 for gel to solid transition following injection, 2) Heparin for anticoagulation, and 3) Dopamine for adhesion to the surrounding endothelium in the turbulent flow encountered in cardiovascular applications. Here we have demonstrated that Thermogel, in conjunction with the LARIAT®, is capable of filling the defects in small and large animals through catheter injection. Thermogel was biocompatible and led to atrophy of the LAA at 5 weeks in a large animal model. Given the advantages of this Thermogel for sealing this defect and ability to be delivered through an endovascular approach, Thermogel presents a viable adjuvant to current occlusion-based treatments for sealing cardiovascular defects.

1. Introduction

Atrial fibrillation (AF) is the most common heart rhythm disorder in adults with a 1 in 4 lifetime risk [1]. AF significantly increases the risk of stroke and mortality, and roughly 90% of strokes in patients with AF derive from the formation of thrombi in the left atrial appendage (LAA) [2,3]. Traditional preventive strategies have relied on oral anti-coagulation (OAC), either with warfarin or with newer anticoagulant agents [4]. OAC increases the risk of bleeding, requires frequent monitoring, has significant drug and food interactions, and has a high discontinuation rate [5], particularly in high-risk patients who need it the most. Mechanical approaches to percutaneously exclude or occlude the LAA have been investigated as alternatives to long-lasting OCA. Several devices have been developed with the WATCHMAN® being the only currently FDA approved device for stroke prevention as an alternative to OAC [6]. The WATCHMAN® device consists of a self-expanding nitinol frame and a fabric cap, and occludes the LAA from an endovascular approach, which prevent flow in and out of the LAA. Other device

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designs being tested (Amulet®, Wavecrest®, etc.), all entail deploying a foreign body to close the LAA ostium. Extravascular LAA ligation with the LARIAT® device is designed to ligate the LAA through the delivery of a surgical suture via a combined *trans*-septal and subxiphoid approach with the final aim to completely suture off the LAA [7]. However, this device has yet to receive FDA approval for LAA closure. Incomplete ligation, which is not always able to be detected during the initial catheterization, occurs in up to one-third of patients and leads to flow leak into the LAA. Although initial clinical investigations proved the feasibility of both occlusion and ligation approaches, several limitations have become apparent. Occlusion devices can embolize [8], have incomplete occlusions [9,10] and form thrombi when incompletely endothelialized [11]. Altogether, incomplete closures and leaks can occur in up to 24% of surgeries performed with occlusion devices [12]. To overcome the incomplete closure experienced with these devices, there have been many proposed off label solutions to seal the incomplete closures. One popular approach among surgeons is to insert filamentous fibers to seal off the open orifice, which can seal up to 83.3% of these leaks [13]. However, these filaments can migrate, leave extra space, and are pro thrombotic. Therefore, an approach with a similar design of filling in the partially sealed off atrial appendage but is antithrombotic and non-migratory would provide an ideal adjuvant to make these LAA closure devices more efficacious.

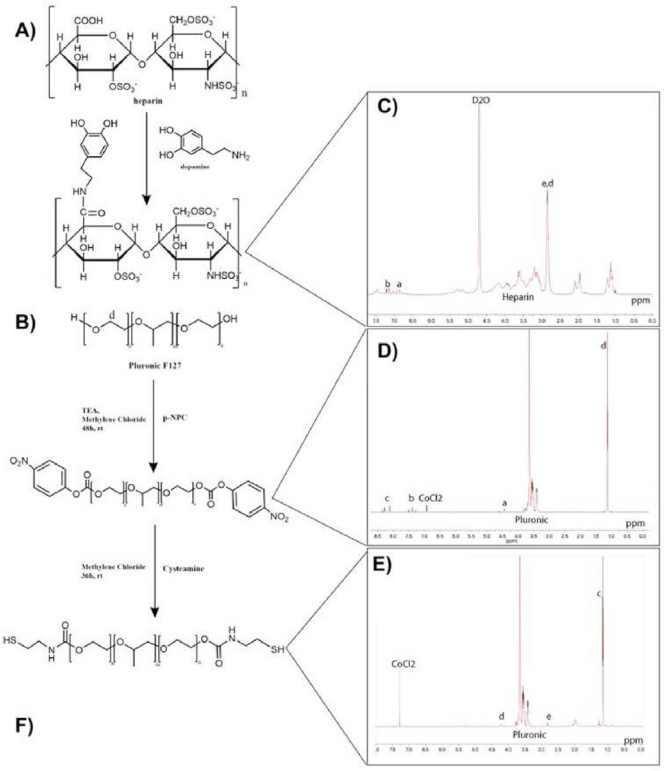
Due to the limitations of current occlusive devices, we developed a strategy to create an injectable, radiopaque, anticoagulant, adhesive, and thermally responsive biocompatible polymer inspired by the mussel's ability to adhere to wet surfaces in the presence of turbulent flow. Marine mussels are known to adhere onto boats and other substrate in wet conditions despite rapid, turbulent flow. This was discovered to be due to the amino acid 3,4-dihydroxy-L-phenylalanine (DOPA) that is overexpressed in specialized mussel adhesive proteins secreted at the interface between the mussel's adhesive pad and the opposing surface [14]. The DOPA catechol functional group forms a strong covalent and noncovalent bond with a variety of surfaces, particularly in wet conditions [15,16]. This unique functionality lends towards the use of dopamine in biomedical designs for adherence to tissue under turbulent flow commonly experienced in cardiovascular applications.

Leveraging this DOPA functionality, we developed a thermally responsive hydrogel (Thermogel) to fill in defects in LAA occlusion treatment thus preventing leaks and subsequent thrombi & AF-related stroke. We aimed to create a gel that can be injected through the currently used catheter, solidify upon injection, and stay in place as the LAA atrophied. To accomplish this, we designed Thermogel to use: 1) a structural component composed of thiolated Pluronic F127 for thermoreversibility following injection, 2) and a functional component Heparin for anticoagulant conjugated with 3) dopamine for adhesion to the surrounding endothelium in the turbulent flow encountered in many cardiovascular applications. This composite polymer creates the necessary features for an AF-stroke therapeutic capable of being delivered via an endovascular approach, adhere, and create sufficient endothelization to seal off desired areas.

2. Results

2.1. Engineering thermally reversible thermogel

We designed multiple thermally reversible hydrogels by thiolating Pluronic F127 (Sigma Aldrich) and combining with Heparin-DOPA (Fig. 1A–E) as described in Methods. The different compositions were in line with previous literature on dopamine, heparin and pluronic based **hydrogels** [17,18]. We increased the Heparin: DOPA ratio to increase adhesion in the initial EDC: NHS crosslinking process to yield more adherent Heparin: DOPA ratios with 50%, 40%, and 30% DOPA in the Heparin: DOPA step for Hydrogel I, Thermogel, and Hydrogel II respectively (Fig. 1F and Supplementary Figure 1). Hydrogel I-II and Thermogel were compared for in vitro experiments to blank gels containing similar



Gel	Dopamine: Heparin	% Pluronic	% Dn-Hep	% PBS
Thermogel	0.67	23.1	4.8	72.1
Control	-	30	0	70

Fig. 1. Synthesis of Thermogel. A) Heparin Dopamine conjugation is performed using EDC NHS crosslinking B) Pluronic F127 is thiolated using a *p*-NPC, Cysteamine amidation reaction C-E) 1H NMR at 600 MHZ showing corollary heparin, pluronic, and thiolated pluronic respectively F) Heparin and Dopamine ratios in Thermogel and Control Pluronic: PBS only.

Pluronic: PBS ratios (Fig. 1F and Supplementary Figure 1). The Heparin: DOPA conjugate was then combined with thiolated Pluronic F127 at a ratio of 10%, 5%, and 5% respectively. We performed NMR to confirm the presence of DOPA on heparin carboxyl group and thiol on the terminal ends and of the thiolated Pluronic F127 (Fig. 1C–E).

2.2. Thermogel is thermally responsive and maintains integrity in vitro

We first assessed Phase Transition as the hydrogel needs to be liquid on ice and solidify upon injection to the body (above room temperature). Only Hydrogel I and Thermogel demonstrated this behavior (Fig. 2B and Supplementary Figure 1). We elected to use Thermogel over Hydrogel I because Hydrogel I did not exhibit shear thinning properties with sufficient decrease in viscosity with increasing shear rate like Thermogel (Fig. 2D and Supplementary Figure 1) indicating it would not perform well upon being injected through a catheter. Thermogel demonstrated maintenance of size with and did not erode away similar swelling ratio as the control over 2 h in PBS. Furthermore, similar to the control, the measured weight of the gel increased over 21 days in vitro suggesting the Thermogel will likely slightly increase in size and fill up the LAA after injection.

Upon injection, the Thermogel needs to be able to stay in place and withstand the stress and energy placed upon it during the contraction and movement of the heart. This was studied by looking at the storage vs loss modulus where the storage modulus (elastic properties) is the ability of the gel to respond to stress and energy whereas the loss modulus (viscous

properties) is its ability to relax to dissipate energy. An ideal gel would have high storage modulus and a low loss modulus upon solidification. When we tested the gels, Thermogel (22378 ± 4316 Pa and 3588 ± 782 Pa respectively) had a high storage modulus and loss modulus (Fig. 2E).

We then determined to make the gel radiopaque so that the gel could be visualized during fluoroscopy, the standard x-ray visualization performed during occlusion device placement. We used different percentages of Iohexol (in PBS) and found the radiopacity 2.5% and above significantly increased in comparison to baseline (Fig. 3A). However, we chose to continue with 25% based on surgeon preference for visibility (Supplementary Figure S1) in comparison to surrounding structures by the surgeons during catheterization (Supplementary Movie S3). All experiments after were used with a 25% Iohexol in PBS solution in lieu of PBS only. When assessing a hydrogel for injection through a catheter, it is important to have a shear thinning gel as characterized by a decrease in viscosity with increasing shear rate. Further rheometry studies were performed to assess for viscosity and flowability under different shear rates. This is performed to assess how the gel handles upon injection and with the flow potentially experienced in the LAA to ensure no parts fall off for the opportunity for thrombi to form. Thermogel demonstrated good shear-thinning behaviors with a decreasing complex viscosity vs shear rate (Fig. 3C) and was capable of being injected through a full-

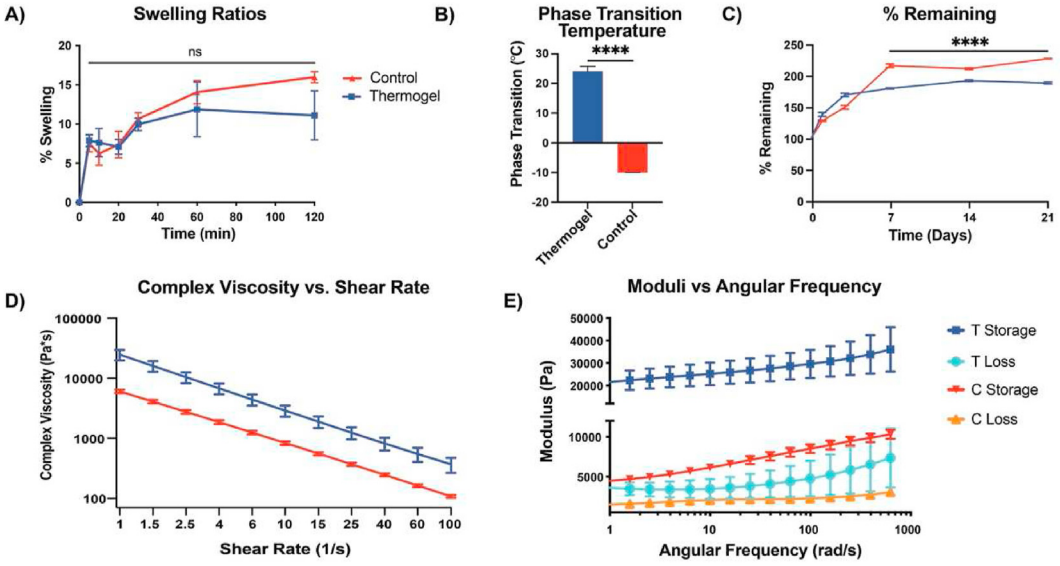


Fig. 2. *Hydrogel Characterization.* A) Swelling Ratios of Thermogel in PBS in comparison to control pluronic hydrogel B) Phase Transition Temperature of Thermogel C) % Remaining of Thermogel when exposed to PBS and shaking at 37 °C D) Rheometry assessment of complex viscosity by shear rate E) Storage and Loss Moduli by angular frequency with T = Thermogel and C= Control.

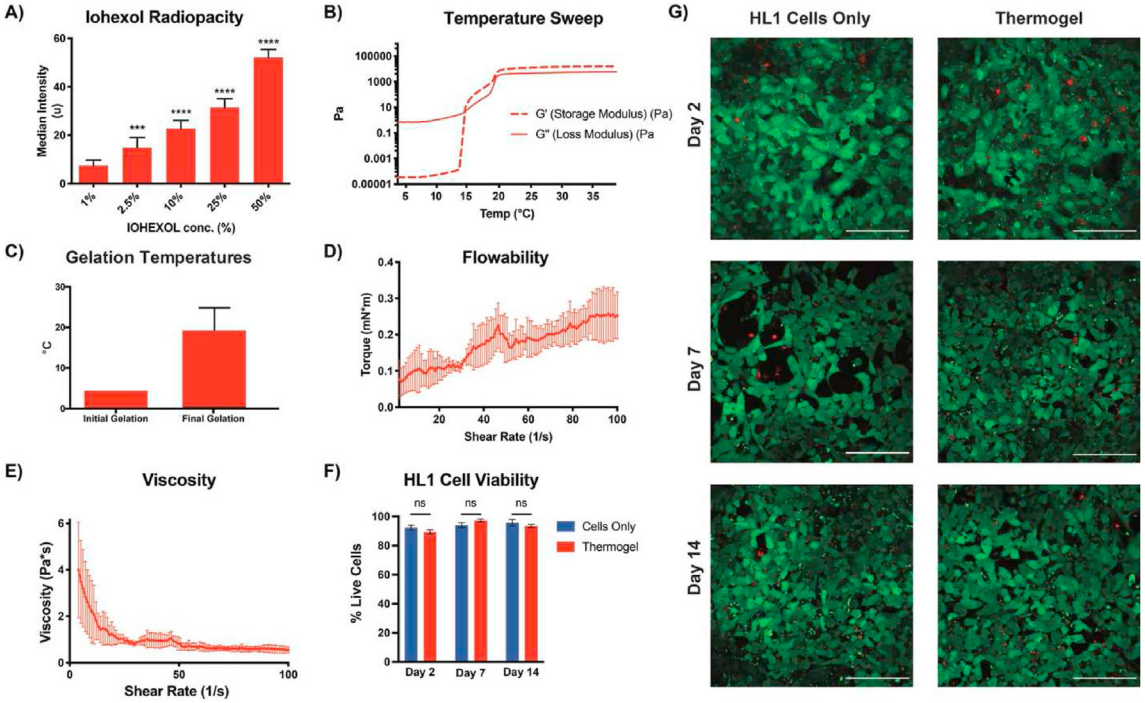


Fig. 3. *Thermogel Characterization.* A) Iohexol Radiopacity by increasing Iohexol concentration in Thermogel B) Representative Thermogel II rheometry temperature sweep C) Rheometry Gelation Temperatures and Specific Heat Capacity of Thermogel D)Rheometric flowability of Thermogel E) Thermogel Viscosity by shear rate F) HL1 Cell Viability at Days 2–14 via Live Dead Assay with and without Thermogel G) Representative images of Cell only and Thermogel on Days 2,7 and 14.

length catheter under 37 °C (Supplementary Figure S2). Additionally, a temperature sweep of this Thermogel with 25% Iohexol was performed to ensure the added Iohexol instead of pure PBS did not alter the Thermogel's liquid to solid transition. The initial gelation was at 4.1 ± 0.1 °C (Fig. 3B) and final gelation at 18.9 ± 5.9 °C (Fig. 3E). This is similar to the Thermogel with PBS only indicating similar properties (24 ± 2 °C) (Fig. 3C). HL-1 mouse atrial cell line was cultured and Live/Dead assays and MTT were performed to assess cell viability in comparison with the cells only. Cells maintained viability over 14 days with no difference seen between cell only and cells cultured in the presence of Thermogel via live dead assay (Fig. 3). Furthermore, cellular proliferation in line with HL-1 cells only was seen in Thermogel treated cells via AlamarBlue Assay (Supplementary Figure 3).

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2.3. Ex vivo thermogel II stability and tissue adhesion

Instron testing was performed to assess the adhesive abilities of Thermogel to isolated LAA tissue of pig hearts. Thermogel was placed between two pieces of LAA tissue and warmed to 37 °C to adhere the tissues and solidify (Fig. 4A). The pieces were then clamped (Fig. 4B) and pulled apart (Fig. 4C) until the tissues were no longer able to adhere to each other (Fig. 4D). The stress strain curves for samples (n = 3) were plotted (Fig. 4E) and the point where the tissues couldn't adhere to each other anymore, the adhesion yield stress, was calculated to be 13.49 ± 2.91 kPa.

To demonstrate the ability of the Thermogel to stay within an appendage while under flow conditions, pig hearts were obtained for LAA appendage Thermogel injections in a Langendorff perfusion. A suture was placed, as outlined in Methods, around the left atrial appendage. The pig heart was cannulated (Fig. 4G), Thermogel was then injected into the LAA of ex vivo pig hearts and continuously perfused (Fig. 4H) as described in Methods. No thrombi or emboli were observed in the perfusion system. Thermogel was visualized to be adherent in the dissected LAA even upon compression (Fig. 4H and Supplementary Movie S1) and SEM was performed to assess Thermogel intercalation into tissue as the Thermogel is not biologically integrated into the heart tissue upon injection upon injection. This demonstrated the Thermogel intercalated into the tissue which increases the adhesive interface between Thermogel and heart tissue (Fig. 4J).

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2.4. Thermogel occlusion of major vessels in vivo

To better understand the Thermogel's capabilities beyond the LAA appendage, we sought to look at the ability of Thermogel to resist flow in a different cardiovascular setting. Although the primary purpose of the proposed Thermogel is for LAA appendage, additional applications can be used medically to occlude flow in vessels such as for varicose veins. Furthermore, this allows for an in vivo assessment of Thermogel's ability to withstand nonpulsatile and pulsatile flow. *In vivo* rabbit injections into the iliac artery (IA) and inferior vena cava (IVC) were performed (n = 3) to demonstrate the ability of Thermogel to occlude both venous and the highly pulsatile arterial flow. For venous injections (Fig. 5A–G), a catheter was placed in the right femoral vein and advanced to the IVC. Thermogel (500 μL) was injected, and the catheter drawn back. Contrast was injected into femoral vein distal to the Thermogel delivery site and flow was assessed (Fig. 5 B, D). No contrast was observed going into the IVC, where the Thermogel was placed, and injection forced retrograde flow into the left (contralateral) femoral vein. The experiment was repeated in the left iliac artery to demonstrate stability under pulsatile flow (Fig. 5E–H). Both femoral arteries were cannulated. Thermogel was injected via the left femoral artery into the left iliac artery and the cannula was retracted to record blood pressure from the femoral artery distal to the injection site. Thermogel injection resulted in no flow into the left iliac artery following in all rabbits as shown by contrast injection in the descending aorta via the right femoral artery and by absent blood pressure and pulse in the left femoral artery (Fig. 5I–J).

2.5. Injection into LAA in large animal model

We performed initial ex vivo experiments on rabbit hearts and demonstrated the Thermogel was injectable and able to stay in place in the LAA (n = 3) (Supplementary Movie S2). However, an initial experiment was performed in a dog model (n = 1, data not shown) to assess the Thermogel only in a large animal model. The gel did solidify to close off

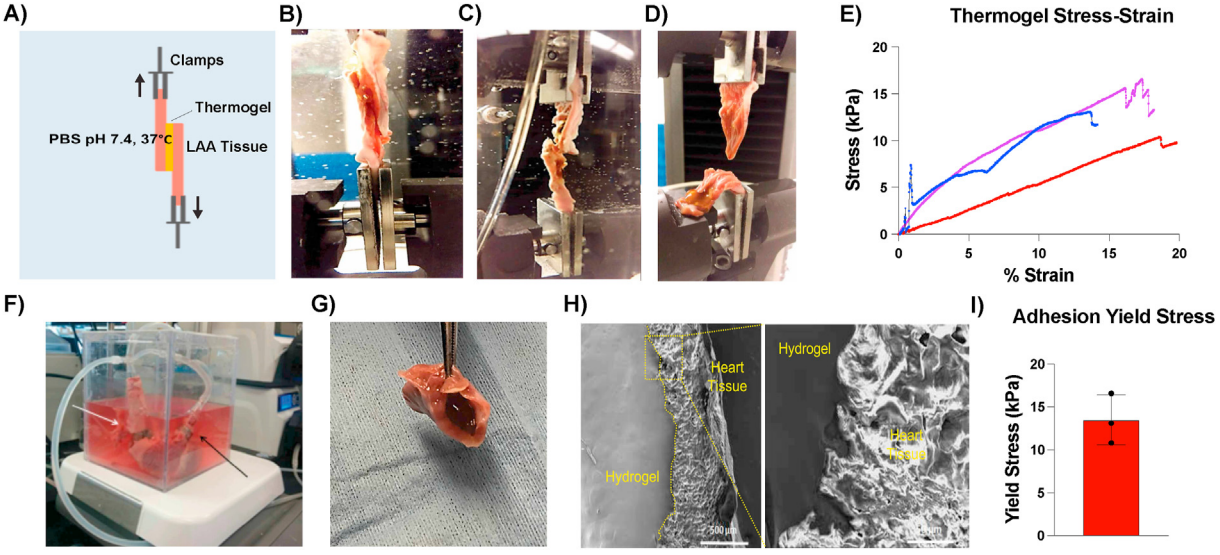

Fig. 4. Pig Langendorff Injection. A) Schematic of Instron adhesion testing design with two symmetric LAA Pig samples bonded by Thermogel prior to clamping and placing in PBS solution at 37 °C B) Samples were tightened to an initial stretch point and then C) stretched until the D)adhesion failed which is termed the adhesion yield stress E) Instron Thermogel Stress-Strain curves for Instron testing F) Pig ex vivo heart Langendorff setup G) Excised LAA following 4h perfusion H) SEM of excised LAA following Langendorff demonstrating Thermogel-heart tissue interaction and adherence with gel intercalating into heart I)Adhesion Yield Stress of LAA tissue in Instron testing with an average adhesion yield stress (n = 3) 13.49 ± 2.91 kPa tissue.

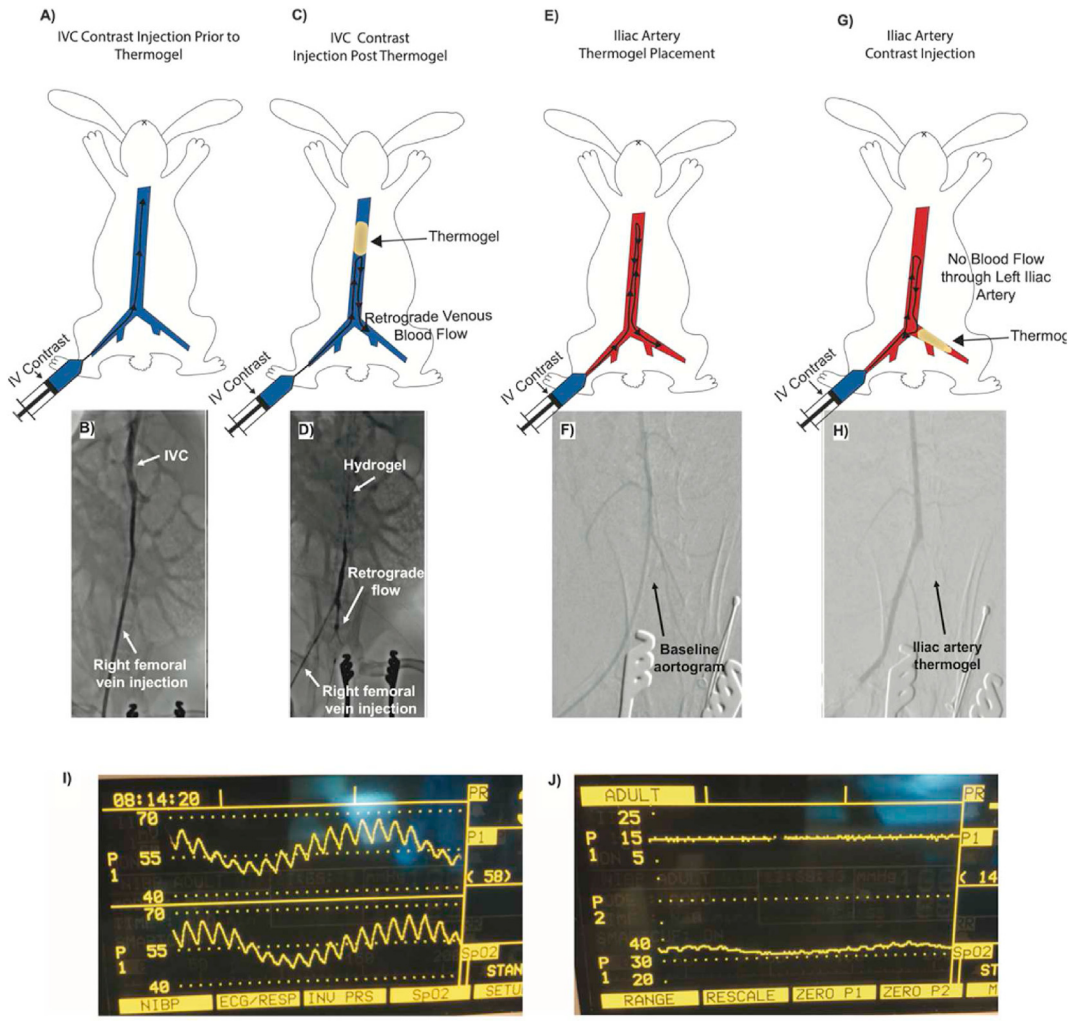

Fig. 5. Thermogel occlusion of major vessels. A,C,E,G) Schematic of injections in rabbit and corresponding fluoroscopy. Injections were performed at the Internal Iliac, and Right femoral vein (n = 3). Pre and post Thermogel injection contrast fluoroscopies were performed in the right femoral vein with B,D) retrograde flow into the left femoral vein F,H) Pre and post Thermogel injection contrast fluoroscopy into the left iliac artery I) Pulsatile wave flow in the iliac artery prior to injection and no flow after Thermogel injection into iliac artery.

the appendage. However, likely due to the movement of the heart during contraction, the whole solidified Thermogel moved out of the appendage resulting in embolic stroke. Therefore, we chose to use the Thermogel in conjunction with the appendage occlusive device the LARIAT® going forward.

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We performed the LARIAT® surgery on dogs (n = 4) followed be injection of Thermogel into the LAA to seal off the LAA and any orifices (Supplementary Movie S3). Briefly, the LARIAT®, FindrWIRE is placed in the pericardial sac while the endocath FindR wire is placed from the femoral vein, across the interatrial septum into the LAA. Once the magnets are in close proximity, they snap together, forming a rail that allows the LARIAT® snare to be advanced over the LAA base (Fig. 6A). In these experiments, we snared the LAA while a sheath (8.5F, 2.8 cm diameter) was still in the LAA, for the purpose of having an incomplete closure (Fig. 6B). The LARIAT® snare was tightened, and the suture was deployed. We then injected Thermogel through the 8.5F sheath already in the LAA (Fig. 6C–D) by attaching a precooled syringe filled with Thermogel to the catheter and injecting through the catheter.

LAA were examined 1 h and 5 weeks after the surgery demonstrating endothelialization, LAA necrosis and closing of the LAA over the Thermogel (Fig. 6I-P). No thrombi were visualized during the procedure via fluoroscopy, with no symptomatic or histological evidence of embolization following surgery in the dogs. Fluoroscopy was performed without leakage of dye into the LAA demonstrating complete closure of the LAA (Figure 6L). All 5 dogs survived surgery with no major changes in heart rate or blood pressure. One animals passed away from surgical complications on day 21. However, all 5 animals had the Thermogel intact with no evidence of leakage or embolization at the end of experiments (Fig. 6 and Supplementary Figure 3, 4, 5) and there was evidence of inflammatory reaction and endothelization as assessed blinded by an independent pathologist.

3. Discussion

Atrial fibrillation is the most common sustained heart rhythm disorder, and is associated with increased risk of stroke, dementia, death [19]. Strokes most commonly develop because of cardioembolism arising from thrombus in the LAA [20]. Implantable devices to occlude the LAA can prevent these events but are limited by incomplete seals and risk of thrombosis as the device surface-blood interaction leads to an activation of the coagulation system. LAA ligation via epicardial clipping or snaring can often be incomplete and lead to potential thrombus formation and embolization [21]. Incomplete ligation by either endocardial occluder devices or by extrinsic ligation can be treated with coils or additional

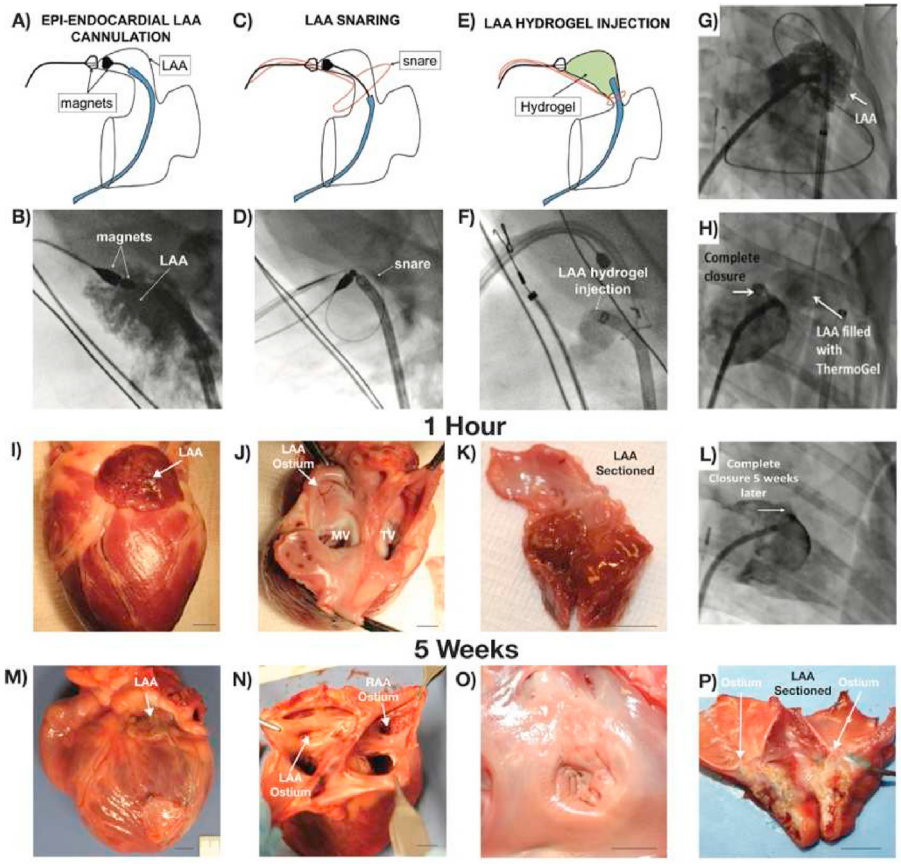

Fig. 6. Procedure for cannulation and fluoroscopic evaluation using LARIAT® procedure and injection of Thermogel in vivo *in* dogs. A) Magnets are exposed and connected epi and endocardially B) The LARIAT®, device is released snaring the LAA C) the LARIAT® snare is tightened and D) Thermogel is injected into the snared LAA occluding the orifice of the LAA E,F) Thermogel solidifies in place following injection and remains in place.

closure devices, but these products can create problems on their own including clotting on the surface [13,22] and typically require systemic anticoagulation treatment. Similarly, device-related thrombus -related to activation of the extrinsic coagulation system on the device surface upon contact with blood-can lead to embolism and potential stroke [23].

Here we developed a thermally reversible hydrogel that can: 1) fill incomplete LAA ligation as it transitions from liquid to solid at body temperature; 2) adheres to the endothelial surface, eliminating potential embolization; and 3) that creates an anticoagulant microenvironment that eliminates risk of local thrombosis.

There have been recent attempts to seal the left atrial appendage, with drawbacks to each. One such approach by *Lang* et al. was a design of a blood resistant glue that was photocurable [24]. The backbone of this hydrogel was a poly (glycerol sebacate acrylate) that could be crosslinked by UV light. This resulted in excellent strength of adhesion and could theoretically be used in conjunction with a patch to seal off the LAA. However, this approach is not feasible in a traditional catheter-based approach as there are multiple components. The gel component, the patch and a source of direct UV light required for the gel to be properly used all require access near the LAA at the same time. Another unique approach was a customized, 3d printed soft occlude by Robinson et al. [25]. This was composed of an inflatable, thick silicone/polyurethane balloon deployed from a catheter from the exterior side of the heart, which requires a thoracotomy and visualization to confirm balloon placement. Additionally, sutures are currently needed to secure the occlude, with portions protruding out of the LAA [25]. Although they are currently working on these problems, there is much work to be done in this field and an alternative solution appear warranted.

We aimed to overcome these limitations by synthesizing an injectable sealant to seal off the holes left behind following incomplete closure. We wanted this sealant to be capable of being injected for ease of use, while solidifying without adding burdensome additional steps into surgery. The sealant needed to be able to stick to the LAA to not embolize, not promote clot formation on the surface as that would put the patient at risk for atrial fibrillation related stroke and be radiopaque for proper visualization and placement confirmation during injection.

We chose to use a four-compound gel that could address these goals. The gel contains: 1) thiolated pluronic for a thermally reversible gel backbone; 2) dopamine for adherence to the LAA under turbulent flow conditions; 3) heparin for preventing thrombi formation on the surface; and 4) Iohexol for radiopacity.

The composition was designed to optimize each parameter providing the optimal injectable hydrogel. We chose three separate composition to test with varying Heparin: DOPA, Pluronic and overall amount of the Heparin: DOPA to test (Fig. 1).

For an ideal surgical injectable, the hydrogel must be liquid during storage and rapidly solidify upon injection but not before being able to be delivered to the injection site. We envisioned the hydrogel being stored on ice until usage for maintenance of low viscosity and then solidified once it gets above room temperature. Thermogel had a final gelation near room temperature (18.9 $\pm$ 5.9 °C) which allows for it to be liquid on storage and solidify rapidly upon injection. We were able to show Thermogel's ability to stay within the LAA using in vivo and ex vivo models with good intercalation into the heart tissue via SEM (Fig. 4). We then demonstrated cells were viable (Fig. 3F) in the presence of gel suggesting the gel would support cellular growth around it and that the gel was capable of being injected (Fig. 3C, F, Supp Fig S2), have a high storage modulus to withstand pressures experienced in the LAA while being able to hold and stay inside the LAA upon injection (Fig. 2E) [26]. Therefore, we elected to use Thermogel for our animal studies.

With the ease of injection through a catheter-based approach we went into a rabbit in vivo model to demonstrate the ability of the Thermogel to withstand turbulent flow. Thermogel was injected into both the IVC and Iliac Artery and was able to stop forward flow through the area in both types of blood vessels (Fig. 5). This demonstrated the Thermogel's ability to properly seal and hold among a variety of vascular conditions.

We chose to look at Thermogel's capabilities to seal the LAA with an occlusion device, in this case the LARIAT®. The LARIAT® procedure has been effective in closing off the LAA but has been associated with ~60% incomplete closure rates (5). Therefore, we aimed to improve upon the LARIAT® procedure. The Thermogel did not work independently on a large animal model (n = 1), which we hypothesize due to the wide orifice of the LAA allowing the fully solidified Thermogel to be pushed out of the LAA during heart contraction. Therefore, we studied it in conjunction with closure of the ostium with either suture or surgical loop like the LARIAT® to implement in an appropriate clinical situation. We demonstrated that Thermogel was capable of solidifying at body temperature and injectable through an endovascular approach (Fig. 6). We tested the Thermogel in combination with a LARIAT®, procedure both ex vivo and in vivo in small and large animals. Thermogel maintained in the LAA, promoted endothelialization and led to necrosis and subsequent atrophy and sealing off the LAA while not leading to clot formation on the injected Thermogel at 5 weeks following surgery. Furthermore, Thermogel was able to occlude and maintain integrity in both venous and arterial blood flow in vivo in a rabbit model (Fig. 5). These results lead us to conclude that the Thermogel is a suitable thermally reversible hydrogel for cardiovascular applications where an injectable, non-thrombotic gel is needed to seal off areas under flow.

4. Limitations

Although the Thermogel was able to occlude vessels safely and stay in and encourage endothelialization over the LAA, there are a few limitations to its application. The primary being that it wasn't able to seal off the LAA on its own without the use of the occlusion device LARIAT®. This was likely due to the contractile nature and flexibility of the LAA resulting in the mass being pushed out over time in the dog model. However, it still fills a large need in the treatment of Atrial fibrillation as there is a significant portion of patients who still experience leakage following the use of occlusion devices. Therefore, we have demonstrated that is a good adjuvant to the occlusion devices as it is able to close off the extra leakage areas that can occur in patients. Another limitation of the Thermogel is that it must be stored on ice prior to injection. This storage allows the Thermogel to have enough warming time where it can be injected into the LAA through the full length of the catheter and then solidify. This need for cooling before can be overcome in surgical techniques and storage on ice during surgery. Overall, the Thermogel provides an excellent adjuvant to occlusion devices in LAA and is also a flexible platform that may be used in other aspects where a vessel needs to be occluded.

5. Materials and methods

5.1. Thermogel synthesis

5.1.1. Heparin: DOPA synthesis

Heparin: DOPA was synthesized based on previously published work [27,28]. Briefly, 2000 mg Heparin (Sigma) was dissolved in 200 mL phosphate buffered saline (PBS) and adjusted to pH using hydrochloric acid. 2246 mg Dopamine and 2876 mg N-(3-Dimethylaminopropyl)-*N*′-ethylcarbodiimide hydrochloride (EDC) are added stirring for 4 h at room temperature. pH was checked every 30 min and adjusted to pH 5.5. The solution is then dialyzed in milliQ water with dialysis changes every 12 h for 3 days. The solution is then lyophilized and placed under UV light overnight for sterilization.

5.1.2. Activation of pluronic F127 with nitrophenyl chloroformate

6 g of Pluronic F127 was dissolved in 42 mL of dichloromethane (DCM). 133 μL of Triethylamine is then added to the Pluronic solution. 576 mg of *p*-nitrophenyl chloroformate are dissolved in 6 mL of DCM in a separate flask. The Pluronic solution is then added to the *p*-nitrophenyl chloroformate solution over 30 min using a cylindrical separatory funnel. The resultant solution is then left under agitation for 48h capped. 50 mL of saturated sodium chloride solution (NaCl) is added to the flask and placed into separatory funnel. Solution is degassed and the organic phase is collected. This was repeated 3 times. 500 mg of Sodium Sulphate is added to final organic phase and filtered using filter paper and allowed to dry for 3 days.

5.1.3. Synthesis of thiolated pluronic

The activated Pluronic is dissolved in 60 mL of DCM while stirring. 1016 mg of Cysteamine (Sigma) is added and allowed to react at room temperature for 24 h. Sample is precipitated using −20 °C 450 mL Diethyl Ether and centrifuged for 10 min at 5000 RPM. Precipitate is then dried in hood for 48 h.

5.1.4. Thermogel synthesis

800 mg of Heparin: DOPA is added to 2 mL (75:25) PBS-Iohexol solution and 800 mg of thiolated Pluronic is added to 2 mL (75:25) PBS-Iohexol solution. Both are rotated overnight at 4 °C. Solutions are combined and rotated overnight at 4 °C. Then rotated at 37 °C. Thermogel II is then stored at 4 °C until use.

5.2. Iohexol optimization

To render the Thermogel radiopaque, Iohexol (Sigma) was added to the gel at increasing percentages. X-ray imaging was performed on gel only and the comparison of gel: background was assessed to find the minimal amount that was statistically different than background (n = 9). This percentage was used for all subsequent experiments.

5.3. Rheometry

Rheological assessment was performed as described per manufacturer's protocols. Briefly, rheological properties of Thermogel II were monitored using a rotating rheometer (Anton Paar Rheometer) equipped with a temperature controller. 500 μL of Thermogel II at 4 °C was placed on rheometer and allowed to equilibrate to start temperature. Frequency was optimized to 1 Hz as determined to be within the linear viscoelastic range using a frequency sweep experiment as per manufacturer's protocol. The contribution of solid-like behavior (elastic modulus (G′)) and liquid-like behavior (viscous modulus (G″)) were recorded with changing temperature using a parallel plate (25 mm). The crossover points of G′ and g'' was considered to be the gelation point.

5.4. SEM

Images of the Thermogel were taken with a FEI Nova NanoSEM, Scanning Electron Microscope. Samples were placed on a specimen mount and imaged at a voltage of 5 kV after sputtering a 7 nm layer of iridium using Cressington 208HR.

5.5. Live dead assay

Live/Dead Viability/Cytotoxicity Kit was performed as per manufacturer's protocols. Briefly, HL-1 atrial cells were plated at 50,000 cells/well as per manufacturer's protocol and incubated at 37 °C with media changes every other day. 500 μL of Thermogel II were placed into a Transwell with 8 μm pore size Transwells (Corning) and incubated for 3h at 37 °C. Transwells were then placed into each well. On corresponding days, cells were washed with PBS. Cells were then treated with 4 μM

EthD-1 and 2 μM Calcein AM in PBS and incubated for 30 min at room temperature. Cells are then washed with PBS three times and imaged via confocal microscopy.

5.6. AlamarBlue assay

AlamarBlue Assay was performed as previously described [29]. HL-1 atrial cells were plated at 50,000 cells/well as per manufacturer's protocols and incubated at 37 °C with media changes every other day. 500 μL of Thermogel II were placed into a Transwell with 8 μm pore size Transwells (Corning) and incubated for 3h at 37 °C. Transwells were then placed into each well. On corresponding days, cells were washed with PBS. Media was combined with AlamarBlue solution as per manufacturer's protocol. Plates were incubated for 4 h at 37 °C and then read at 570 nm with reference at 600 nm. Thermogel treated HL-1 cells were compared to HL-1 cells only.

5.7. Phase transition

Phase transition was performed using the test-tube inversion method as previously described [30]. Briefly, the gels were placed in a 4-mL vial test tube with an inner diameter of 10 mm and heat at temperature intervals of 1 °C until the temperature when solidification occurred. The phase transition was observed by inverting the vial for 1 min and solid was defined as with no significant flow within 1 min. Three measurements were performed and averaged to define the transition point.

5.8. Swelling studies

1 mL of Thermogel samples were accurately weight and placed in a 1.5 mL the test tube. The samples were equilibrated at least for 1 h at 37 °C. The Thermogels were left in a PBS solution (pH 7.4) previously warmed up to 37 °C. The PBS solution was removed at regular intervals (5, 10, 20, 30, 60, 120 min) and the samples was weighed again to determine the uptake of PBS solution. Fresh samples were used for each individual time point. The mixture of 30 w/v % and 67 w/v% of Pluronic F-127 dissolved in PBS (pH 7.4)/IOHEXOL solution was used as controls.

5.9. In vitro erosion behavior

The in vitro stability of Hp-DN/Plu-SH Thermogels in aqueous solution was evaluated measuring the mass erosion rates under physiological condition. In briefly, 1 mL of Hp-DN/Plu-SH was prepared in 1.5 mL test tube and incubated at 37 °C for 10 min for thermal stabilization. Pre-warmed PBS solution pH 7.4 was added to the Thermogel and incubated at 37 °C. After predetermined time intervals (1, 3, 7, 14, 21 days), the supernatants were removed and the weights of the remaining solid Thermogels were measured. Fresh samples were used for each individual time points.

5.10. NMR

Thermogel II samples were dried and lyophilized overnight. The synthesis was confirmed by ^{1}H NMR analysis in DMSO-d_6 (Bruker Ascend 600). The degree of dopamine substitution was determined based on the molecular fractional ratio of conjugated carboxylic acid, degree of *p*-NPC.

5.11. Instron testing

Two geometrically equivalent heart segments were isolated from the heart. Compound was added to half of one heart piece, and half of the other heart piece was placed on top of the compound, so that the intimal walls were facing each other. Adjoined pieces were then warmed in an incubator for ~5min. Once warmed the adjoined tissues were attached to the Instron machine calipers at the two compound-free sections of the two heart pieces. The adjoined heart segments were stretched at a rate of 2 mm/min until a preload of 0.05 N was reached on an Instron tensile tester.

5.12. Ex vivo LAA occlusion

Adult New Zealand white rabbits, 3–4 kg, were anesthetized with 50 mg/kg i. p. pentobarbital sodium and anticoagulated with 1000 U/kg i. p. heparin. Hearts were rapidly removed and retrogradely perfused in a Langendorff apparatus (see Figure S1A) with a constant perfusion pressure of 60–65 mmHg at 37 °C with oxygenated Tyrode's solution containing (mM): NaCl 136, KCl 5.4, CaCl2 1.8, MgCl2 1.0, NaH2PO4 0.33, HEPES 10, glucose 10, pH adjusted to 7.2 with NaOH. The perfusion pressure was monitored using an in-line physiological pressure transducer connected to the Bridge Amplifier (AD Instruments Inc., Colorado Springs, CO). ECGs were recorded using the Animal Bio Amplifier (AD Instruments Inc.) from perfusion start through to the experiment end using two wire leads, one connected to the right atrium and the other connected to the right ventricular apex. A suture was placed around the LAA and tightened down around the orifice. Thermogel was then injected into the LAA.

5.13. Rabbit vascular occlusion

All animal experiments performed in this paper adhere to the NIH Guide for the Care and Use of Laboratory Animals in accordance with Houston Methodist Animal Care Guidelines. Rabbits were anesthetized via intramuscular injection of ketamine, midazolam and inhaled fluorane anesthesia. Bilateral inguinal incisions were made to expose the femoral arteries and veins. In 3 rabbits, we tested Thermogel deployment in the venous circulation. The right femoral vein was cannulated with a Micropuncture needle (Cook Medical, Bloomington, IN) and a 4F sheath was inserted in the inferior vena cava (IVC) below the liver under fluoroscopic visualization. Thermogel was injected via the sheath as described. We then retracted the sheath and injected 5 cc radiopaque contrast (visipaque®, GE Healthcare) under fluoroscopy to assess the completeness of IVC occlusion. In 3 rabbits, arterial occlusion was tested. Both femoral arteries were cannulated with 4 F sheaths. Baseline pressure recordings were obtained from both femoral arteries. The left sheath was advanced into the left iliac artery, and the right sheath into the mid-abdominal descending aorta. Through the left sheath, we delivered Thermogel at the level of the left iliac artery and retracted the sheath into the left femoral artery. After Thermogel injection, contrast was injected into the descending aorta to verify left iliac obstruction of antegrade flow.

5.14. Canine acute and chronic experiments

Mongrel dogs (n = 5) were brought to the animal catheterization laboratory, intubated, and sedated under isoflurane anesthesia. Percutaneous pericardial access was obtained via an anterior subxiphoid puncture using a Micropuncture needle, then dilated to insert a Soft-tip 14F sheath (Sentreheart, CA). We then inserted and 8.5F sheath into the femoral vein and advanced it into the left atrium via a *trans*-septal puncture. Baseline angiograms of the LAA were obtained. The magnetic-tip FindrWIRE was advanced in the pericardial space towards the LAA. A second FindrWIRE was advanced over the sheath in the LAA and directed towards the LAA tip endocardially, aiming towards the epicardial wire. Once the magnetic tips snapped together, a LARIAT® snare was advanced towards the LAA base and closed leaving the 8.5F sheath in the LAA cavity. The wires were removed and Thermogel - 5–10 cc, as visualized to fill the LAA cavity-were delivered via the sheath to achieve complete filling of the LAA. The LARIAT® suture was delivered while the sheath was in the LAA, to leave an incomplete ligation of the LAA – an opening of the sheath's diameter or 2.8 mm. The sheath was then retracted. Repeat angiograms were performed to verify complete LAA occlusion despite the incomplete closure of the LAA. Animals were

monitored for thrombi embolization as assessed by vitals, and fluoroscopy during the study with assessment of physical manifestations and through pathology. At 5 weeks, tissue was collected for pathology analysis. From the obtained paraffin blocks, LAA was divided in 4 block as showed in Supplementary figure 3. Three 5 μm thick sections per block were stained with haematoxylin-eosin (HE). The different samples were scored using microscopy analysis following the score method previously reported to look for signs of inflammation, cell infiltration, and neovascularization, among others [31]. In Supplementary figure 4 is reported the table and the evaluation.

6. Statistical analysis

Results are presented with ±standard deviation and number of samples/trials indicated in captions. Quantitative data were compared using student's t-test, one-way and two-way ANOVA, followed by Tukey's test as applicable, where $p < 0.05$ was considered statistically significant (Prism 7 Software, GraphPad, La Jolla, CA).

Author contributions

FT, ET and MV designed the research plan. FT, CL and TH optimize the synthesis. CL performed Thermogel chemical characterization. SH and MM with the help of TH and GB performed the mechanical test. TH performed in vitro studies and rheological characterization. LT, MV, SW performed animal experiments supported by FT, CL and GB. FT, GB and TH with the help of LT performed necropsy and histology. FT, TH and MV wrote the manuscript. All authors discussed and commented on manuscript.

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Declaration of competing interest

The authors declare the following financial interests/personal relationships which may be considered as potential competing interests: Ennio Tasciotti, Francesca Taraballi, Miguel Valderrabano has patent Thermosensitive Hydrogel Sealing of the Left Atrial Appendage to Prevent Thromboembolism in Atrial Fibrillation pending to Ennio Tasciotti, Francesca Taraballi, Miguel Valderrabano.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.mtbio.2022.100240.

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