Enhancing epicardial EMT to repair the heart

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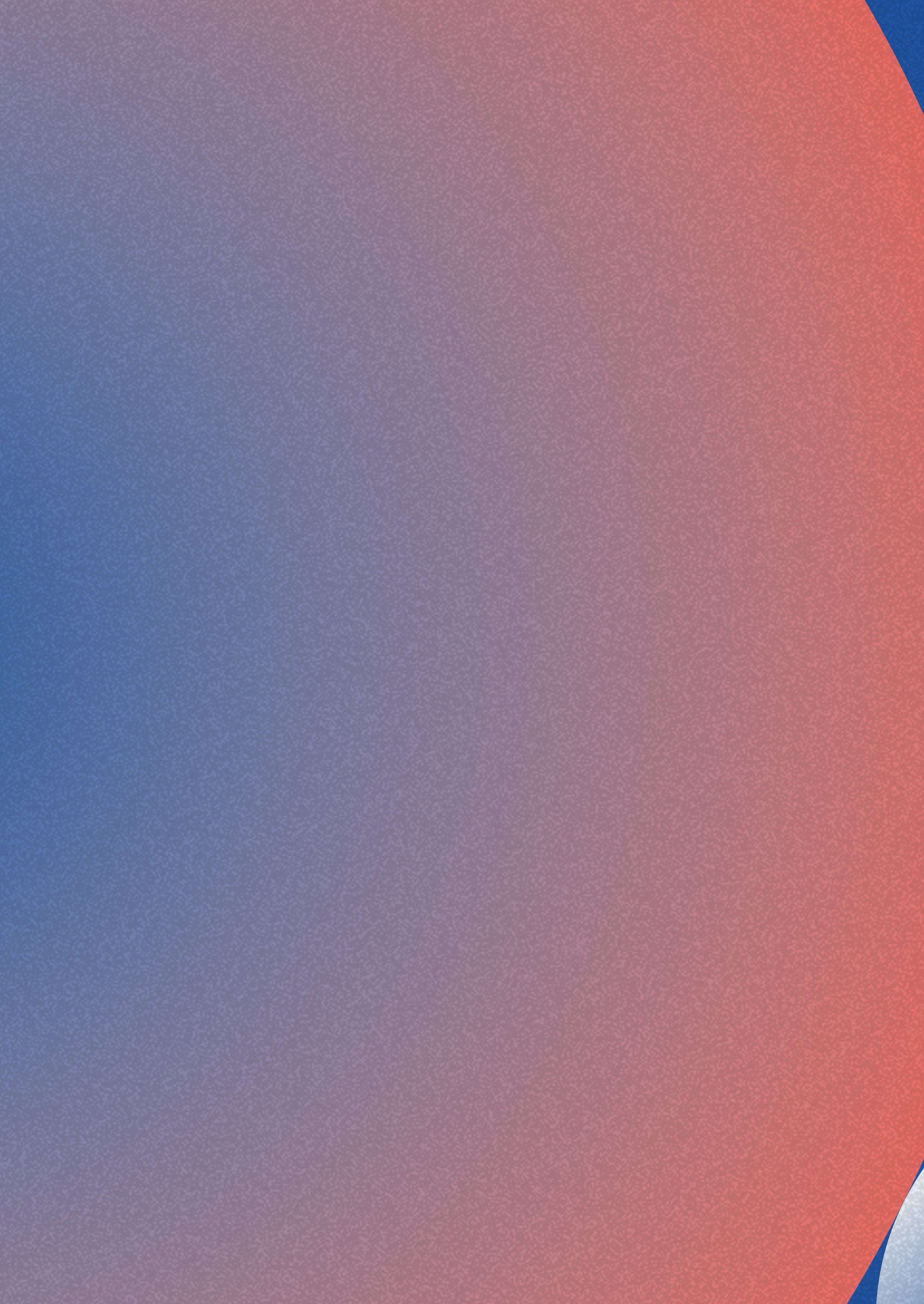

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Optimization of two self-adhering drug delivery patches to target the epicardium of the injured heart

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ABSTRACT

The heart has limited capacity to repair itself. The epicardium, the outer layer of the heart, is an interesting therapeutic target for restoring cardiac tissue. Compounds that activate epicardial cells have been identified. However, a biomaterial is required to secure temporal exposure of the epicardium to these compounds. Therefore, the aim of this study was to optimize a drug delivery patch to target the epicardium of the infarcted heart. We tested six potential epicardial patches. Of these six biomaterials, two hydrogel patches were selected based on the simplicity of the preparation procedure, the ability to provide sustained release for at least one week and the capacity of the patch to adhere to cardiac tissue. When testing these two epicardial patches in a mouse model for myocardial infarction, both patches demonstrated high adherence to cardiac tissue. In conclusion, we have optimized two epicardial patches that are feasible to use in in vivo mice experiments to target the epicardium.

INTRODUCTION

As the heart has a limited potential to repair itself, the loss of myocardial tissue due to a myocardial infarction causes irreversible damage. Currently, a major goal in cardiac research is to identify therapeutic targets to restore cardiac tissue. One of these targets is the epicardium, the outer layer of the heart, which is a source for progenitor cells during cardiac development and repair. In order to participate in the cardiac tissue formation, epicardial epithelial to mesenchymal transition (epiMT) is an essential step. It is a process which allows cells to detach from the epicardial layer, invade underlying tissue, and contribute to multiple cardiac cell lineages (1). Therefore, epiMT has been studied extensively to identify targets to further enhance the contribution of the cell population to cardiac repair. Over time, multiple factors have been identified in vitro that activate epiMT, such as Activin (2) and TBBz (unpublished, chapter 4 of this thesis). Investigating the effect of these compounds on epiMT in in vivo models for myocardial infarction requires a method to administer the factor to the epicardium. Previous studies have shown that simply injecting a fluid into the myocardium leads to wash out of the fluid within a few contractions of the heart muscle (3). Given that epithelial to mesenchymal transition is also related to pathological conditions such as cancer and fibrosis, it is vital that an epiMT stimulating factor is locally applied and does not spread throughout the body. Furthermore, in mice, the epicardium is most active between 3-7 days after MI (4) and therefore the epiMT inducing factor should be available for at least one week to be fully effective. Because of the convenient location of the epicardium at the outside of the heart, it is possible to target these cells directly by applying a drug-loaded patch on top of the epicardial cells.

The aim of this study was to generate and optimize a drug releasing patch that can be applied on the outside of the injured heart to deliver factors to the epicardium. Ideally, the patch should meet the following demands. Firstly, the patch should adhere to the cardiac tissue, but not to the surrounding (lung) tissue, preferably through self-adhesive properties instead of previously described sutures. Secondly, the patch should release the factor over at least a week and both the patch and its derivatives should not be toxic to the surrounding tissue. Thirdly, to obtain feasible and reproducible results, it is essential that the patch can easily be prepared and that it is compatible with multiple factors (e.g. proteins, small molecules, viruses) and solvents (e.g. PBS and DMSO).

In this study, we describe the optimization of two epicardial patches that are easy to prepare, release a drug over time, adhere to cardiac tissue and are feasible to use in vivo studies in the infarcted mouse heart.

MATERIALS AND METHODS

Cell culture

Human primary epicardial cells were isolated and cultured as described (5). Briefly, cells were isolated from human heart auricles and cultured in a medium consisting of a mix of Dulbecco's modified Eagle's medium (DMEM low-glucose, Gibco) and Medium 199 (M199, Gibco) mixed in a 1:1 ratio, supplemented with 10% fetal bovine serum (heat inactivated for 25 minutes at 56 °C, Biowest), 100 U/mL penicillin (Roth) and 100 mg/mL streptomycin (Roth). Cells were cultured in the presence of 10 μM SB431542 (SB, Tocris) at 37 °C in 5% CO2. Experiments were performed in cell culture medium without SB.

HT1080 cells, stably transfected with a CAGA-luciferase reporter construct (6), were used as TGFβ reporter cell line. Cells were maintained in Dulbecco's modified Eagle's medium (DMEM high-glucose, Gibco) supplemented with 10% fetal bovine serum (Biowest), 100 U/mL penicillin (Roth) and 100 mg/mL streptomycin (Roth) at 37 °C in 5% CO_2.

Preparation of UPy-catechol patch

UPy-catechol was synthesized as described (7). The polymer was dissolved in 100 μl PBS and pH was adjusted with 1.25 μl 1N NaOH up to a pH of 8, while stirring at 50 °C for 15 minutes. When completely dissolved, 5 μl of TBBz (stock concentration of 10 mM in DMSO), or 5 μl of DMSO was added and mixed for 5 minutes at 50 °C. Subsequently, 15 μl of the hydrogel mixture was applied onto an electrospun supramolecular mesh with a 5 mm diameter (as described (8)). Gelation was initiated by 2 drops of 0.75 μl sodium periodate, one directly applied onto the mesh and one on top of the hydrogel. The mesh and hydrogel were immediately applied onto the heart.

Preparation of TISSEEL patch

For the TISSEEL patch, TBBz (10 mM in DMSO) was mixed with PBS in a 1:1 ratio. Subsequently, the TBBz/PBS solution was mixed with Thrombin solution (TISSEEL, Baxter) in a 1:1 ratio. To form the patch, 15 μl adhesion solution was pipetted on a piece of parafilm and 15 μl of the thrombin/compound mixture was pipetted into the droplet

of adhesion solution. The TISSEEL patch was cured for 5 minutes before application onto the heart. To secure proper adherence to the tissue, 4 µl of adhesion solution was applied onto the heart and 4 µl of thrombin solution was applied on the patch before administration.

Release studies in vitro

To study drug release from patches, patches were prepared and applied onto the upper side of a transwell insert (6.5 mm, 8.0 µm Pore Polycarbonate Membrane Insert, Transwell, Corning). The insert was placed in a 24 wells plate well containing 500 µl releasing medium. For experiments with epicardial cells, epicardial cell medium was used. For experiments with HT1080-CAGA cells, DMEM without FBS was used, which was supplemented with 1%BSA. BSA was added to prevent binding of TGFβ to the plate surface. After the indicated duration of 1 hour or 1 day, the transwell was moved to the next well containing 500 µl fresh medium. This process was repeated every day up to the end of the experiment. Because of practical reasons, occasionally the medium was not refreshed daily which is indicated in the figure (e.g. 5-7 days). Releasing medium was collected and stored at -20 °C.

TGFβ reporter assay

Ht1080-CAGA cells were seeded in 24 wells plate with a density of 50.000 cells/well. After two days, cells were washed with PBS and exposed to releasing medium samples which were supplemented with TGFβ (1 ng/mL). After 6 hours incubation, cells were lysed in 50 µl passive lysis buffer (Promega) and luciferase activity was measured using the Luciferase Assay System (Promega). Luciferase activity was normalized by total protein concentration of the samples, which was determined using the Pierce BCA Protein Assay Kit (Thermo Scientific).

EpiMT assay

Epicardial cells were seeded. When confluency reached 50%, cells were stimulated with releasing medium samples for 5 days.

Mice pilot study and immunostaining

All animal experiments were performed according to protocols approved by the animal welfare committee of the Leiden University Medical Center and conform the guidelines from Directive 2010/63/EU of the European Parliament on the protection of animals used for scientific purposes. Wt1CreERT2/+;R26RmTmG/+ mice, both male and female of 12-15 weeks old, were injected with 4x2 mg tamoxifen, two days before and two days after MI. MI was induced by permanent ligation of the left anterior

descending artery (LAD ligation) under isoflurane anesthesia, as described previously (9). Immediately after MI, the patch was applied onto the heart. All surgical procedures were performed by a blinded investigator. After 7 days, mice were sacrificed by cervical dislocation. Hearts were flushed by injecting 3 mL of PBS in the right ventricle and subsequently isolated and fixed in 4% PFA for 24 hours. Tissue was embedded in paraffin and sectioned in 6 um slices. Immunostaining was performed as described (10) using the following antibodies: α-GFP (Abcam, ab13970), α-Tropomyosin (Sigma-Aldrich, T9283) and α-Wt1 (Abcam, ab89901)

RESULTS

We defined a list of potential patch materials based on literature and on collaborative efforts (Table 1). We identified six materials that we considered to fulfill our criteria. We prepared the patches and established for each patch 1) the feasibility of the protocol, 2) the coherence of the patch, 3) their compatibility with multiple factors and solvents, such as DMSO and PBS, and 4) their adhesive abilities. We started by preparing the collagen patch described by Wei et al. (11) but due to technical issues we were not able to fully repeat their protocol and produce a proper biomaterial to be used as a patch. Next, we tested Pluronic F-127 polymer, which can form a gel-like structure and is very easy to work with. Unfortunately, it did not result in a coherent patch that can be handled with forceps. Then, we tested an electrospun mesh developed by Putti et al. (8) which consists of a sheet of electrospun fibers generating a layer of randomly aligned thin threads appearing like soft woven cloth. The core of this thread contains the factor of interest, which is encapsulated in a hydrophilic shell of ureido-pyrimidinones poly(ethylene glycol) (UPy-PEG) that regulates its release. The mesh is very easy to handle, and because of its flexible characteristics it can cover the heart without interfering with contraction. One disadvantage of the spinning procedure is that only small molecules can be incorporated in this patch. In addition, the patch is not adhesive and will need a suture or glue to apply it to the outside of the heart. The TachoSil patch is a bilayered sponge that is used in surgeries to stop local bleeding. This material has been exploited as an epicardial patch before (12). The TachoSil patch is ordered as a dry material, consisting of a collagen layer at the outside that serves as a mechanical carrier for the inner lining of thrombin and fibrinogen that requires activation with fluid (e.g. blood or PBS). In theory, the application of a solution containing the factor of interest will cause the thrombin and fibrinogen to form an adhesive layer thereby incorporating the compound. This approach is highly robust and very easy to execute. Moreover, the collagen layer,

on the outside of the patch protects the thoracic organs. We combined the excellent release capacities of the supramolecular UPy-PEG hydrogel with that of the TachoSil. Unfortunately, the application of the UPy-PEG hydrogel massively impaired the adhesive capabilities of the patch and using this combined patch in an in vivo experiment therefore would require stitches or glue. TISSEEL is a tissue glue which is routinely used in the clinic. It has previously been described for atrial epicardial application for encapsulating viruses (13) and cells (14). TISSEEL is a two-compound glue consisting of an adhesion solution and a thrombin solution that require mixing, after which solidification occurs resulting in a patch that can easily be picked up using forceps. To add releasing properties to this glue, the factor of interest can be mixed in either the adhesion solution or the thrombin solution which subsequently forms a hydrogel patch upon combining the solutions. The patch can be prepared ex vivo, according to a straight-forward protocol and produces a coherent patch. We were able to mix the TISSEEL with adenoviruses, conditioned medium, PBS and DMSO, although the latter did have some effect on the structure of the patch. Because the ex vivo prepared TISSEEL patch once gelified is not adherent, it will not stick to the surrounding tissue, but also not to the heart. To facilitate this, a fresh drop of liquid TISSEEL was applied directly on to the heart and on the basis of the drug-loaded patch which allowed firm adhesion onto the outside of the heart. Lastly, we tested a UPy-catechol patch, which is based on a synthetic UPy-PEG hydrogel which has been functionalized with a catechol group that provides strong binding in wet environments (7). To be able to apply the hydrogel to the heart and to protect surrounding tissue, the hydrogel was prepared on an electrospun mesh made of UPy fibers. The mesh allows for application of the patch onto the heart, and provides a shield between the patch and surrounding tissue and allows for unidirectional release, e.g. towards the heart. The preparation of the patch is more elaborate than the TISSEEL patch, but still straightforward. The UPy-catechol preparation includes a final step where the hydrogel mixture requires to be maintained at 50 °C to prevent premature gelification. This means that the factor of interest that is mixed into the biomaterial should keep its functionality at this temperature. Furthermore, the catechol groups can bind to the included factor (e.g. to TGFβ) thereby preventing its release and limiting wide use of this patch. However, the adhesiveness of this patch is outstanding and the best that we have observed in this study. In summary, all patches we have tested in this study have strong and weak points summarized in Table 1. We continued our experiments with the two most promising patches, the TISSEEL patch and the UPy-Catechol patch.

Table 1 Overview of tested patches

	Feasibility protocol	Coherent patch	Compatibility with factors/solvents	Adherence to heart tissue	Timed release	Biocompatible	Unidirectional release
Collagen (Wei et al.)	-	-	NA	NA	NA	NA	NA
Pluronic gel	-	-	NA	NA	NA	NA	NA
Electrospun patch	+	+++	+/-	-	NA	+	+/-
Tachosil (+UPy-PEG hydrogel)	++	+++	+	+/-	NA	+	+/-
TISSEEL patch	++	++	++	++	++	++	-
UPy-catechol patch	++	+++	+	+++	++	+/-	+

Next we determined if these two patches were effective drug release materials by performing in vitro experiments to assess the release profile. We prepared patches containing DMSO (control) or the ALK4/5/7 kinase inhibitor SB431542 (SB), placed them in transwells and determined the release of compound over time into the cell culture medium (Fig. 1A). The medium was collected after 1 hour, refreshed and subsequently collected daily. To establish the release of SB, TGFβ reporter cells (HT1080-CAGA luciferase cells) were stimulated with TGFβ combined with the collected medium. Release of SB from the material into the medium would inhibit the TGFβ induced CAGA reporter activity. Quantification of the luciferase activity demonstrated the release of SB for at least 8 days (Fig. 1B) for both the TISSEEL and the UPy-catechol patch since patches containing SB demonstrate a lower luciferase response along the total timeline compared to the DMSO patches. Interestingly we also prepared a TISSEEL patch containing TGFβ, and using the same assay demonstrated release of this growth factor at a similar level of luciferase activity as the positive control (ctrl medium + TGFβ). The UPy-catechol patch is not compatible with TGFβ and was therefore not tested. In summary, both patches serve as a drug releasing system for at least a week in vitro.

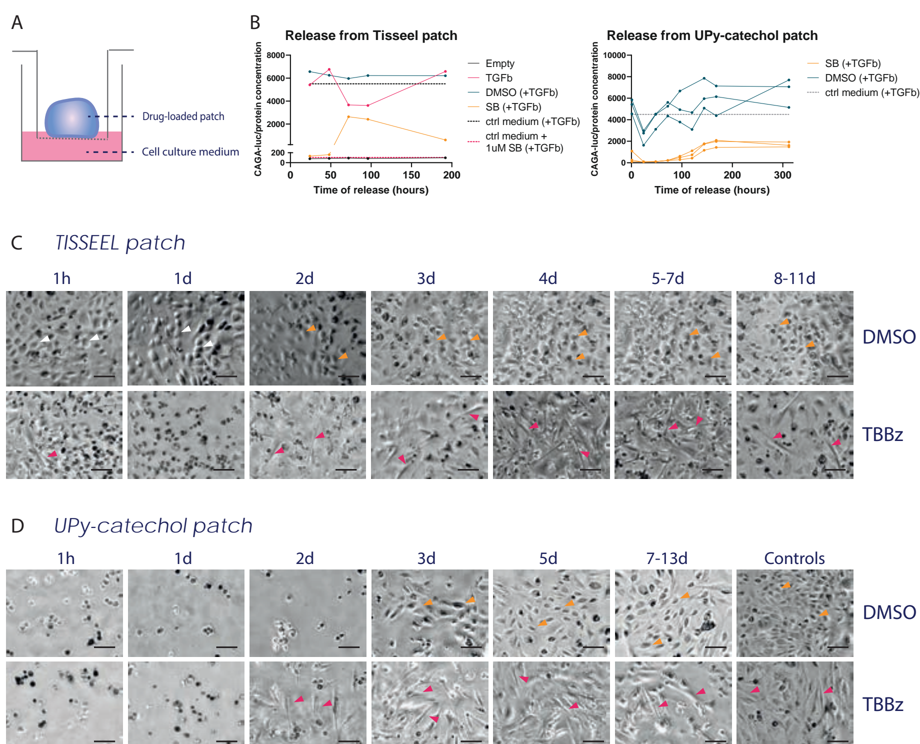

Fig. 1 | TISSEEL and UPy-catechol patch release SB and TBBz for at least 7 days in vitro. (A) Schematic overview of set-up to obtain releasing medium. (B) Temporal release of TGFβ and SB from patches measured by CAGA-activity. Release was evaluated by the ability of the released TGFβ to initiate CAGA-luciferase response (only for the TISSEEL patch(n=1)) compared to the response of an empty patch and the ability of the released SB to inhibit the TGFβ induced CAGA-luciferase response (TISSEEL (n=1), UPy-catechol (n=3)) compared to a DMSO loaded patch. As controls for the luciferase assay, medium supplemented with TGFβ and medium supplemented with TGFβ+SB were included. (C) and (D) Temporal release of TBBz from the patches measured by an epiMT assay. Release was evaluated by the morphology of epicardial cells, indicating epiMT. Orange arrows indicate examples of epithelial cells while pink arrows indicate examples of mesenchymal cells. Scale bar: 100 µm.

The next step was to determine if there was release of an epiMT inducing compound, TBBz (unpublished, chapter 4 of this thesis), and if it retained its activity. Therefore, we generated TBBz-containing patches and used the same transwell set-up to collect media of DMSO (control) and TBBz patches. To study epiMT, human primary epicardial cells were exposed to the collected media. For both the TISSEEL and the UPy-catechol TBBz patches, the collected medium of the first hour and day were toxic to the cells (Fig. 1C and 1D) indicating a burst of release of TBBz resulting in toxic levels. The

medium collected from the control UPy-catechol DMSO patch was also toxic during the first two days. However, after this initial toxic burst, the TBBz medium derived from both patches clearly induced a morphological change in the epicardial cells that indicated epiMT at all measured time points (Fig. 1C and 1D). Interestingly, for both patches, this effect lasted for more than 7 days.

To test the feasibility of preparing and handling these patches for in vivo application in experimentally induced myocardial infarction (MI), we performed a pilot experiment using Wt1CreERT2/+;Rosa26mTmG mice that express GFP in epicardial (Wt1+) cells once tamoxifen is administered (Fig. 2A). This lineage tracing approach allows for studying epicardial behavior. To induce MI, mice were subjected to a left anterior descending coronary artery (LAD) ligation, causing major cell death in downstream tissue. Directly after the LAD ligation, the patches containing DMSO or TBBz were applied to the outside of the heart (Fig. 2B and 2D).

While performing these in vivo mouse experiments, we observed that the time needed to prepare the TISSEEL patch is very short and the protocol is robust, as temperature management and timing are flexible. However, the TISSEEL patch leaves behind some of the fluid after preparation indicating that not all the compound may be incorporated. The procedure to prepare the UPy-catechol patch is a little more sensitive to error but once applied, this patch is highly adhesive. Furthermore, the hydrogel sometimes got scraped from the electrospun patch when moving the patch along the ribs to apply it to the outside of the heart.

Seven days after application of the patch, the mice were sacrificed, hearts were collected and embedded in paraffin for further analysis. Immunofluorescent analysis demonstrated that both patches could be easily identified and were located on top of the infarcted area (Fig. 2C and 2E). We observed that the GFP-labeled epicardial cells were still present underneath the patches, and found mainly around the edges of the patch (Fig. 2C''' and 2E'''). For the TISSEEL patch, we did not observe any sign of toxicity, e.g. disintegrated nuclei or a large influx of immune cells. Underneath the UPy-catechol patch, some unidentified structures were observed in 2 out of 4 hearts (Fig. S1A). To conclude, the TISSEEL and UPy-catechol patch can readily be prepared and subsequently applied in vivo. Moreover, both patches demonstrated sufficient adherence and were easily located afterwards.

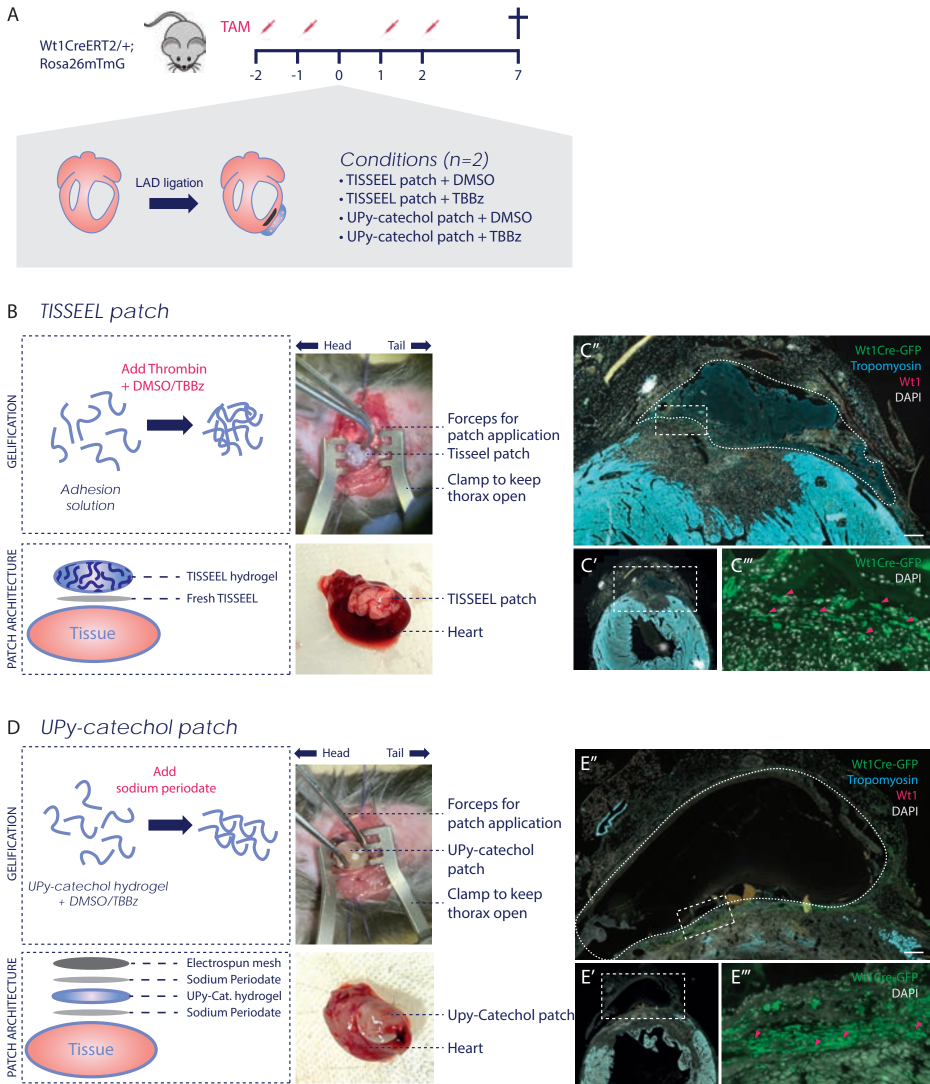

Fig. 2 | TISSEEL and UPy-catechol patch are feasible to use in in vivo study. (A) Schematic overview of study design of mouse study. (B) Schematic overview of preparation of the TISSEEL patch. Thrombin solution was mixed with the compound and combined with adhesion solution to form a patch. The patch was applied onto the heart with a drop of fresh TISSEEL. (C) Immunostaining with antibodies against Wt1Cre-GFP, Tropomyosin and Wt1 in the infarcted mouse heart treated with a TISSEEL patch. C″ shows a magnification of the TISSEEL patch on top of the infarcted area, highlighted by a dotted line. Scale bar: 200 μm. C‴ demonstrates the epicardial Wt1Cre-GFP cells underneath the patch. (D) Schematic overview of preparation of the UPy-catechol patch. UPy-catechol hydrogel was mixed with the compound. A droplet of sodium

periodate was applied onto a 5 mm electrospun mesh. Subsequently, the hydrogel was applied and another droplet of sodium periodate to initiate gelification. The total of mesh and hydrogel was directly applied onto the heart. (E) Immunostaining with antibodies against Wt1Cre-GFP, Tropomyosin and Wt1 in the infarcted mouse heart treated with a UPy-catechol patch. E'' shows a magnification of the UPy-catechol patch on top of the infarcted area, highlighted by a dotted line. Scale bar: 200 μm. E''' demonstrates the epicardial Wt1Cre-GFP cells underneath the patch.

DISCUSSION

In this study, we identified and optimized two self-adhering drug-eluting hydrogel patches and demonstrated 1) easy and robust protocols that gave rise to patches with 2) a sustained release of compounds in vitro for at least 7 days, and 3) adherence to the beating mouse heart in vivo.

Using biomaterials to repair the heart is a fast-emerging research field aiming to support the heart in its reparative response, and to deliver drugs or cells to the myocardium (15). In this study, we aimed to place a patch to the outside of the heart to target the epicardium. For this, it is essential that the epicardium itself is not harmed during the procedure, which is more likely when using stitches or aggressive glue. Therefore we explored different self-adhering patch materials. Although several epicardial patches have been described (15), we were aiming for a simple procedure that would not require complicated methods or specific equipment which is undesirable during a mouse experiment. After compiling a list of patch materials to deliver drugs to the epicardium, we identified two highly promising patches in which we observed gradual release over time of the incorporated factor in vitro and a strong adherence to mouse hearts in vivo.

The main difference between the two patches is their nature, the TISSEEL being a natural hydrogel and UPy-catechol being a synthetic one. TISSEEL is based on the coagulation system and consists of a mixture of sealer protein solution (fibrinogen and aprotinin) and thrombin solution (human thrombin and calcium chloride dihydrate). Upon mixing these two solutions, a sealant is formed that is used in patients to stop internal bleeding. Besides its clinical use, this hydrogel has been used before to deliver AAVs to the atria in rats (13), and as engineered tissue construct to support the infarcted mouse heart with cardiac adipose tissue-derived progenitor cells (14). This shows the all-round applicability of this hydrogel and it also demonstrates that the protocol is robust in multiple laboratories. Similarly, we observed that the TISSEEL protocol is highly straight forward. The main disadvantage of the TISSEEL patch is its

poor tolerance for DMSO, demonstrated by fluid that was left after picking up the patch, showing that not all the liquid was incorporated in the patch. In general, we found that all hydrogels displayed significant structural changes upon mixing with DMSO. We therefore aimed to keep the DMSO concentrations as low as possible and attempt to use other solvents when applicable.

Ureido-pyrimidinone (UPy) is a synthetic supramolecular hydrogel: a group of materials that consists of polymers that are cross linked by non-covalent interactions. Supramolecular materials are tunable and considered to mimic the biological environment. The UPy hydrogel used in this study was functionalized with catechol groups to introduce adhesiveness, inspired by mussels that demonstrate exceptional adhesiveness under wet conditions (7). In our current set-up we used an electrospun mesh as a carrier of the gel, allowing for easy handling. This electrospun mesh can also be modified. This creates a window of opportunity, e.g. when applying an impermeable mesh that allows for unidirectional release of the hydrogel towards the epicardium. Similar to the TISSEEL patch, a small difference was observed between the addition of DMSO or PBS to the hydrogel. We observed that the structure of the hydrogel is different on a microscopic level in PBS patches compared to DMSO patches (Fig. S1B-C), validating our observation that DMSO intervenes with the patch structure during the formulation. However, except for the fact that the gelification appeared to be a little weaker with DMSO compared to PBS patches, we did not find any functional differences, both patches adhered well onto the heart in vivo.

In this study, the data regarding the biocompatibility of this patch was inconclusive. In vitro, release during the first 48 hours was highly toxic to the cells, indicating either a burst release of DMSO (we expect this amount of cell death in a concentration higher than 2%) or a toxic effect of the degraded hydrogel. Comparing an empty UPy-catechol patch with a DMSO loaded patch will provide insight in the origin of the toxicity. In vivo, we observed some unidentified structures beneath the patch. Further examination of the structures should reveal if this is a sign of any harmful events (e.g. excessive immune influx or toxicity). Potential toxicity may be reduced by using a replacement for sodium periodate to initiate gelification of the hydrogel.

In conclusion, the TISSEEL patch is most widely applicable for epicardial application due to its tolerance for all types of compounds, and its robust and easy preparation. The UPy-catechol patch requires a more elaborate protocol but can be fully adjusted to one's needs and shows the best adhesiveness. Which patch should be used depends on the requirements of the specific application. Here, we have used both

patches to study the effect of small molecules on epiMT. The numbers in this study are not sufficient to compare the effect of the two patches. However, we demonstrated the feasibility of the experiment. In addition to this, one could think of other applications such as viral knock down or overexpression to study a specific pathway or mix cell specific conditioned medium into the patch.

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SUPPLEMENTAL FIGURES

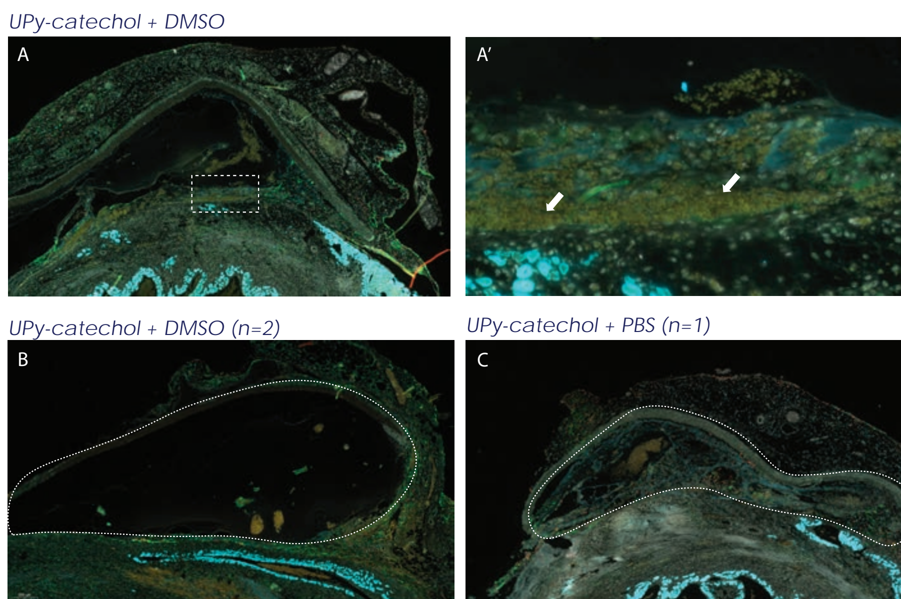

Fig. S1 | Observations of UPy-catechol patch in infarcted mouse heart. (A) Infarcted mouse heart treated with a UPy-catechol patch with DMSO. In the magnification in A', arrows point to unindentified structures below the patch, that may indicate toxicity. (B) Infarcted mouse heart treated with a UPy-catechol patch with DMSO. The patch is highlighted by a dotted line. (C) Infarcted mouse heart treated with a UPy-catechol patch with PBS. The patch is highlighted by a dotted line.