Journal of Visualized Experiments

An Injectable and Drug-loaded Supramolecular Hydrogel for Local Catheter Injection into the Pig Heart

--Manuscript Draft--

Manuscript Number:	JoVE52450R3
Full Title:	An Injectable and Drug-loaded Supramolecular Hydrogel for Local Catheter Injection into the Pig Heart
Article Type:	Invited Methods Article - JoVE Produced Video
Keywords:	supramolecular polymers, hydrogels, catheter injection, drug delivery, pH switchability, pig model
Manuscript Classifications:	3.14: Cardiovascular Diseases; 92.23.5: materials (general)
Corresponding Author:	Patricia Y.W. Dankers Eindhoven University of Technology Eindhoven, - NETHERLANDS
Corresponding Author Secondary Information:	
Corresponding Author E-Mail:	P.Y.W.Dankers@tue.nl;M.H.Bakker@tue.nl;A.C.H.Pape@tue.nl
Corresponding Author's Institution:	Eindhoven University of Technology
Corresponding Author's Secondary Institution:	
First Author:	ACH Pape
First Author Secondary Information:	
Other Authors:	ACH Pape
	Maarten Bakker
	Maartje Bastings
	Cheyenne Tseng
	Stefan Koudstaal
	Joost Sluijter
	Imo Hoefer
	Steven Chamuleau
Order of Authors Secondary Information:	
Abstract:	Regeneration of lost myocardium is an important goal for future therapies because of the increasing occurrence of chronic ischemic heart failure and the limited access to donor hearts. An example of a treatment to recover the function of the heart consists of the local delivery of drugs and bioactives from a hydrogel. In this paper a method is introduced to formulate and inject a drug-loaded hydrogel non-invasively and side-specific into the pig heart using a long, flexible catheter. The use of 3-D electromechanical mapping and injection via a catheter allows side-specific treatment of the myocardium. To provide a hydrogel compatible with this catheter, a supramolecular hydrogel is used because of the convenient switching from a gel to a solution state using environmental triggers. At basic pH this ureido-pyrimidinone modified poly(ethylene glycol) acts as a Newtonian fluid which can be easily injected, but at physiological pH the solution rapidly switches into a gel. These mild switching conditions allow for the incorporation of bioactive drugs and bioactive species, such as growth factors and exosomes as we present here in both in vitro and in vivo

	experiments. The in vitro experiments give an on forehand indication of the gel stability and drug release, which allows for tuning of the gel and release properties before the subsequent application in vivo. This combination allows for the optimal tuning of the gel to the used bioactive compounds and species, and the injection system.
Author Comments:	
Additional Information:	
Question	**Response**
If this article needs to be "in-press" by a certain date to satisfy grant requirements, please indicate the date below and explain in your cover letter.	
If this article needs to be filmed by a certain date to due to author/equipment/lab availability, please indicate the date below and explain in your cover letter.	

TITLE:
An Injectable and Drug-loaded Supramolecular Hydrogel for Local Catheter Injection into the Pig Heart

AUTHORS:
Pape, A.C.H.*
Institute for Complex Molecular Systems
Department of Biomedical Engineering
Laboratory of Chemical Biology
Eindhoven University of Technology
Eindhoven, The Netherlands
a.c.h.pape@tue.nl

Bakker, Maarten H.*
Institute for Complex Molecular Systems
Department of Biomedical Engineering
Laboratory of Chemical Biology
Eindhoven University of Technology
Eindhoven, The Netherlands
m.h.bakker@tue.nl

Bastings, Maartje M. C.
Institute for Complex Molecular Systems
Department of Biomedical Engineering
Laboratory of Chemical Biology
Eindhoven University of Technology
Eindhoven, The Netherlands
maartje.bastings@gmail.com

Tseng, Cheyenne C. S.
Department of Cardiology, Division Heart and Lungs
Interuniversity Cardiology Institute of the Netherlands (ICIN)
University Medical Center Utrecht
Utrecht, The Netherlands
c.c.s.tseng-2@umcutrecht.nl

Koudstaal, Stefan
Department of Cardiology, Division Heart and Lungs
Interuniversity Cardiology Institute of the Netherlands (ICIN)
University Medical Center Utrecht
Utrecht, The Netherlands
s.koudstaal@umcutrecht.nl

Sluijter, Joost P. G.

Department of Cardiology, Division Heart and Lungs
Interuniversity Cardiology Institute of the Netherlands (ICIN)
University Medical Center Utrecht
Utrecht, The Netherlands
j.p.g.sluijter@umcutrecht.nl

Hoefer, Imo E.
Department of Cardiology, Division Heart and Lungs
Interuniversity Cardiology Institute of the Netherlands (ICIN)
University Medical Center Utrecht
Utrecht, The Netherlands
I.Hoefer@umcutrecht.nl

Chamuleau, Steven A. J.
Department of Cardiology, Division Heart and Lungs
Interuniversity Cardiology Institute of the Netherlands (ICIN)
University Medical Center Utrecht
Utrecht, The Netherlands
s.a.j.chamuleau@umcutrecht.nl

Dankers, Patricia Y. W.
Institute for Complex Molecular Systems
Department of Biomedical Engineering
Laboratory of Chemical Biology
Eindhoven University of Technology
Eindhoven, The Netherlands
p.y.w.dankers@tue.nl

*These authors contributed equally to this work.

CORRESPONDING AUTHOR: Dankers, Patricia Y. W.

KEYWORDS:
supramolecular polymers, hydrogels, catheter injection, drug delivery, pH switchability, pig model

SHORT ABSTRACT:
Supramolecular hydrogelators based on ureido-pyrimidinones allow full control over the macroscopic gel properties and the sol–gel switching behavior using pH. Here, we present a protocol for formulating and injecting such a supramolecular hydrogelator via a cathether delivery system for local delivery directly in relevant areas in the pig heart.

LONG ABSTRACT:
Regeneration of lost myocardium is an important goal for future therapies because of the

increasing occurrence of chronic ischemic heart failure and the limited access to donor hearts. An example of a treatment to recover the function of the heart consists of the local delivery of drugs and bioactives from a hydrogel. In this paper a method is introduced to formulate and inject a drug-loaded hydrogel non-invasively and side-specific into the pig heart using a long, flexible catheter. The use of 3-D electromechanical mapping and injection via a catheter allows side-specific treatment of the myocardium. To provide a hydrogel compatible with this catheter, a supramolecular hydrogel is used because of the convenient switching from a gel to a solution state using environmental triggers. At basic pH this ureido-pyrimidinone modified poly(ethylene glycol) acts as a Newtonian fluid which can be easily injected, but at physiological pH the solution rapidly switches into a gel. These mild switching conditions allow for the incorporation of bioactive drugs and bioactive species, such as growth factors and exosomes as we present here in both *in vitro* and *in vivo* experiments. The *in vitro* experiments give an on forehand indication of the gel stability and drug release, which allows for tuning of the gel and release properties before the subsequent application *in vivo.* This combination allows for the optimal tuning of the gel to the used bioactive compounds and species, and the injection system.

INTRODUCTION:

Although the treatment of acute myocardial infarction has significantly improved survival rates, the chronic ischemic heart failure population is a major public health problem that progresses with an aging population. There are approximately 6 million heart failure patients in the US with an estimated 25% increase in prevalence in 2030[1,2]. Initial loss of myocardial tissue leads to cardiac remodeling and eventually causes chronic heart failure. Except for heart transplantation, there is no real treatment for this group of patients. The increasing shortcoming of donor hearts emphasizes the need to develop new available therapies to reverse this process of remodeling. Therefore, a goal for future therapies is the regeneration of lost myocardium.

Hydrogels are interesting materials in the field of regenerative medicine because of their biocompatibility, and their sensitivity to external triggers[3]. Injectable hydrogels offer advantages over non-injectable hydrogels in their use in minimal invasive surgery[4]. These injectable hydrogels can be applied through a syringe because of their switchability within physiological conditions[5], and in principle allow for catheter-based injection approaches[6]. Different strategies have been used for injectable materials, ranging from chemical crosslinking after injection to physical crosslinking by either temperature, pH and shear-thinning behaviour[4,7,8]. Although several systems have shown easy injectability via a syringe[9,10], full catheter-compatibility has not been shown often[6].

Hydrogels prepared from supramolecular polymers are formed by non-covalent interactions which can be switched conveniently from a gel to a solution state, and vice versa using environmental triggers[11]. Furthermore, the low molecular weight precursors allow for easy processability[12,13]. The mild conditions required for switching allow the addition of various biological active components such as often difficult to handle growth factors.

Supramolecular transient networks in water based on poly(ethylene glycol) (PEG), end-modified with ureido-pyrimidinone (UPy) moieties[14] have shown the benefits of non-covalent interactions in combination with biomedical applications and have been used as drug delivery system in the heart[6] and under the renal capsule[15]. These networks are formed by dimerization of the UPy-groups shielded from the aqueous environment by alkyl spacers forming a hydrophobic pocket. Urea hydrogen bonding facilitates subsequent stacking of these dimers into nanofibers. Due to the reversible interaction of the UPy-UPy dimer, triggers such as pH and temperature can be used to switch from solutions to gels. The use of a synthetic motif allows for design of the molecule and gel properties by for example tuning length of the PEG-chains and alkyl spacers[14,16].

Moreover, several bioactive components can be incorporated by simply mixing the supramolecular hydrogelator solution before injection, with drugs or bioactive species, such as growth factors or exosomes, respectively. Exosomes are small membrane vesicles that contain cytosolic derivatives. They are secreted by many cells and are involved in intercellular communication. Exosomes derived from cardiomyocyte progenitor cells are suggested to play a role in cardiac protection[17].

Here, we describe the protocol of formulation, and *in vivo* myocardial injection of such a bioactive supramolecular hydrogel. *In vitro* experiments are described which give on forehand an indication of gel stability and drug release, which allows for tuning of the gel and release properties before application *in vivo.*

PROTOCOL:

All *in vivo* experiments were conducted in accordance with the *Guide for the Care and Use of Laboratory Animals* by the Institute of Laboratory Animal Resources. Experiments were approved by the Animal Experimentation Committee of the Medicine Faculty of the Utrecht University, the Netherlands.

1. Formulation of the hydrogel

1.1) To prepare 1 ml of the 10 wt% gel, dissolve 100 mg of the hydrogelator in a vial in 900 µl PBS pH 11.7 by stirring at 70 °C for 1 hour using a magnetic stirrer. Afterwards cool the viscous solution down to room temperature. The solution should now have a pH of approximately 9.0. This solution can be stored for several days.

1.2) Pipette the appropriate amount of drug or biomolecule that is dissolved in neutral PBS into the viscous solution and stir for 10 minutes to reach a uniform distribution. If the solution becomes too viscous, shortly warm it with hot water.

1.3) Place the solution for 1 hour under an UV-lamp to sterilize.

2. Analysis of hydrogel

2.1) Rheological assessment of the solution

2.1.1) Before loading the gel, mount the 25 mm plate-plate geometry in the rheometer, set the temperature to 20 °C and load the plate with water to prevent evaporation of the gel during measurement.

2.1.2) Pipette 300 µl of the solution onto a 25 mm plate-plate geometry on a rheometer maintained at 20 °C and lower the plates to obtain a 0.5 mm gap distance.

2.1.3) Record shear viscosity as function of shear stress from 0.1 to 500 Pa with 10 points per decade.

2.2) Rheological assessment of the gel

2.2.1) Pipette 300 µl of the solution onto the plate and pipette a total of 4.2 µl of 1 M HCl at different places on the solution to induce the gel formation.

2.2.2) Lower the plates to a gap distance of 0.5 mm and let the gel cure for approximately 30 minutes. During this curing process, measure the storage and loss moduli at low frequency and strain, for example respectively 1 rad/s and 0.5%.

2.2.3) After the gel has cured (after approximately 30 minutes), record storage and loss moduli as function of the frequency (0.1 – 100 rad/s) and subsequently as function of the strain (0.1 – 1000%).

3. Erosion and release experiments

3.1) Transfer 100 µl of the viscous solution containing the drug or biomolecule into a poly(ethylene terephthalate) hanging cell culture insert for 24-wells plate with pore size 8.0 µm. To prevent leakage of the polymer solution while in the liquid phase, cover the bottom of the inserts with parafilm (**Figure 2A**).

3.2) Immediately afterwards pipette 1.4 µl of 1 M HCl on top of the viscous solution to reduce the pH to approximately 7.0 - 7.2 and let the gel cure inside the insert for about 30 minutes.

3.3) Remove the parafilm from the inserts, place the insert in a 24-wells plate and fill the well with 800 µl PBS pH 7.4. Incubate the plate at 37 °C with slow rocking or shaking movement. To prevent evaporation of the solvent, fill remaining empty wells with PBS and seal the 24-wells plate with parafilm (**Figure 2B**).

3.4) Periodically refresh the PBS and analyze the removed PBS for released UPy erosion product or drug/biomolecule.

3.4.1) Quantify UPy erosion products or pirfenidone by measuring UV absorbance at 265 nm or 320 respectively. For fluorescent protein mRuby2 measure fluorescence emission at 587 nm after excitation at 559 nm.

3.4.2) Translate measured absorption/emission values to concentrations via predetermined calibration curves.

3.4.2.1) Prepare calibration curves dissolving a series of known concentrations of the analyte in buffer and measure the UV absorbance or fluorescent emission of these samples. Interpolate the data using a linear function to determine the concentration of the unknown samples. For non-fluorescent proteins use ELISA detection[6].

4. Local injection via a catheter

4.1) Induction of myocardial infarction

4.1.1) After 12 hours of fasting, excluding water, sedate the pig in its stable by injecting midazolam 0.4 mg/kg, ketamine 10 mg/kg and atropine 0.014 mg/kg intramuscularly.

4.1.2) Administrate sodium thiopental 5 mg/kg intravenously to induce anesthesia and intubate the pig with an endotracheal tube. Perform balloon-ventilation at a rate of 12/min if needed while transporting the animal to the operating theater.

4.1.3) On arrival at the operation theater immediately start mechanical positive pressure ventilation with FiO_2 0.50, 10 ml/kg tidal volume, and a frequency of 12/min under continuous capnography. Use vet ointment on the eyes to prevent dryness.

4.1.4) Start balanced anesthesia by continuous intravenous infusion of midazolam 0.5 mg/kg/hr, sufentanil 2.5 µg/kg/hr and pancuronium bromide 0.1 mg/kg/hr. To ensure proper anesthesia continuously monitor ECG, arterial blood pressure, temperature and capnography.

4.1.5) Intravenously infuse 4.3 mg/kg amiodarone and place the intracardiac defibrillation catheter in the right ventricle using the venous sheeth[18].

4.1.6) Occlude the left anterior descending artery (LAD) distal to the second diagonal branch by intracoronary balloon occlusion, for 90 minutes, in accordance to previously described protocol[18].

4.2) Electromechanical mapping

4.2.1) At four weeks after myocardial infarction, plan the mapping procedure. Prepare the system (**Figure 4**) in the cathlab for 3D electromechanical mapping (EMM) of the left ventricle. With this system viable, hibernating and infarcted myocardium can be identified without fluoroscopic guidance. To construct such an EM-map acquire a series of points at multiple

locations on the LV endocardial surface by using an ultralow magnetic-field energy source and a sensor-tipped catheter[19,20].

4.2.2) Anesthetize the pig, following protocol steps 4.1.1 – 4.1.4.

4.2.3) Place the external reference patch on the pig's back.

4.2.4) Secure vascular access (a. carotis, v. jugularis) according to protocol[18].

4.2.5) After obtaining a biplane left ventricular angiogram in the 25° right anterior oblique (RAO) and 40° left anterior oblique (LA) view to estimate left ventricular size, give 75 U/kg of heparin.

4.2.6) Advance an 8 French-mapping (D or F curve) catheter under fluoroscopic guidance to the descending aorta, aortic arch and across the aortic valve into the left ventricle (LV).

4.2.7) Orientate the tip of the catheter to the apex of the LV to acquire the first data, followed by outflow tract, lateral and posterior points to form a 3D silhouette, defining the borders of the ventricle.

4.2.8) Obtain subsequent points until all endocardial segments have been sampled by dragging the mapping catheter over the endocardium and sequentially acquiring the location of the tip while in contact with the endocardium[21,22].

4.2.9) Define the target area, that is where electrical activity is (near) normal and mechanical movement impaired, so-called hibernating myocardium (**Figure 6**).

4.3) Intramyocardial injection

4.3.1) Replace the mapping catheter by the intramyocardial injection catheter which is composed of a 27-gauge needle and a core lumen inside an 8 French catheter (**Figure 5A+B**). To deliver specific amounts, load a volume-graded syringe with approximately 2 ml of the hydrogel solution.

4.3.2) Adjust the needle extension at 0° and 90° flex and place 0.1 ml of the hydrogel solution to fill the needle dead space. Then, place the injection catheter tip across the aortic valve and into the target area.

4.3.3) Meet the following criteria for an injection position inside the target area determined in 4.2.9: (1) perpendicular position of the catheter to the LV wall; (2) excellent loop stability (<4 mm) as calculated by the EMM-system; and (3) underlying voltage > 6.9 mV[21].

4.3.3.1) Advance the needle into the myocardium, (4) confirmed by a premature ventricular contraction of the LV, and inject 0.1-0.3 ml of the hydrogel in a bolus at a constant rate of

approximately 0.4-0.5 ml/min. Repeat this at 6-10 different positions as diffuse as possible. The natural pH of the tissue will neutralize the solution after injection, upon which the hydrogel is formed.

4.4) Sacrifice

4.4.1) Post-procedure, humanely sacrifice the animal by exsanguination. Cut the inferior caval vein and remove blood with a suction device. Induce ventricular fibrillation by placing a 9 V battery on the apex.

REPRESENTATIVE RESULTS:

Typical results obtained from the oscillatory rheological measurements on both the solution and the gel are shown in **Figure 1**. For injection through a long catheter, a Newtonian fluid with low viscosity is desirable. Viscosity was measured as function of shear rate, showing that at pH 8.5 the solution is shear thinning but at pH 9.0 and 9.5 the solutions behave as Newtonian fluids as evidenced by the constant viscosity of 0.54 and 0.36 Pa·s, respectively (**Figure 1A**). After neutralizing the samples, the samples show a solid-like response observed by a storage modulus G' which is larger than the loss modulus G'' and therefore a tan $\delta = G''/G' < 1$ (**Figure 1B**). The gel obtains its final strength within 30 minutes. Oscillatory rheological measurements show a typical solid-like response with G' almost independent of the angular frequency and G'>G" for all frequencies measured (**Figure 1C**).

[Place Figure 1 here]

Essential for the use as drug delivery system is the erosion of the hydrogel over time. The supramolecular interactions are inherently dynamic and allow for a slow erosion of the gel *in vitro*. Erosion and release experiments are performed at 37 °C using porous well inserts (**Figure 2A+B**). By tuning the length of the hydrophobic and hydrophilic block[14], a gel that erodes over a period of several weeks can be obtained (**Figure 3A**). The gel erodes 25% in 2 weeks with an initial erosion of 10% in the first day, presumably due to initial swelling of the hydrogel. As example, both the release of a small molecule drug (pirfenidone), and the release of a model fluorescent protein (mRuby2) was studied. A fluorescent model protein allows for an easy readout; however, *in vitro* release experiments can also be performed on other proteins using ELISA for quantification[6]. The small molecule drug is released within a day, while bigger molecules such as proteins are gradually released over 1 week (**Figure 3B**). Fitting the release profile of mRuby2 up to 60% release with the semi-empirical Korsmeyer-Peppas model indicates release due to diffusion ($n = 0.44$)[23]. The absence of an offset in the (adapted) Korsmeyer-Peppas model shows that there is no burst release present for mRuby2[24]. Because of the limited amount of data points with a release lower than 60% for pirfenidone, no fitting was performed on this release profile.

[Place Figure 2 here]

[Place Figure 3 here]

The catheter navigation system consists of a communication unit console, a workstation **(Figure 4)**, a triangular location pad (generating a low magnetic field) with an external reference patch, and two catheters, the sensor-tipped mapping and the injection catheter **(Figure 5)**.

[Place Figure 4 here]

[Place Figure 5 here]

After post-processing analysis has filtered unstable points the 3D endocardial reconstruction of the LV is updated in real time with the acquisition of each new data point and is continuously displayed as unipolar and bipolar voltage potentials on a graded color scale (**Figure 6A)**. The local linear shortening (LLS) function quantifies regional wall motion by obtaining the average change in distance between sample site and adjacent points at end-systole and end-diastole. The mean voltage and LLS values are calculated for each segment and displayed in the polar map. (**Figure 6B)**. The presence of an abnormal or low unipolar potential (≤ 6mV) and impaired mechanical activity (LLS ≤4%) characterizes infarcted areas[22].

[Place Figure 6 here]

Figure 1: Rheological assessment of the solutions and gels. A) Viscosity as a function of shear rate for the solutions at different pH. For the sample at pH 8.5 shear thinning is observed but for the samples at pH 9.0 and 9.5 constant viscosities are obtained, showing the Newtonian behavior of these solutions. B) Gel curing followed by plotting tan δ as a function of time. C) Frequency sweep for a neutralized sample after 2 hours curing. Error bars show standard deviations of 3 independent measurements, indicating a typical experimental error.

Figure 2: Setup for degradation and release experiments. A) Poly(ethylene terephthalate) well insert covered with parafilm to prevent leakage during preparation. B) 24-wells plate with inserts, wrapped with parafilm to prevent evaporation of the solvent.

Figure 3: Erosion and Release. A) Erosion of the hydrogel over time. Gradual erosion of the gel for at least 2 weeks is observed. B) Release of a small molecule drug and a model protein. While the small molecule is released within a day, the model protein is gradually released over a week without a significant burst release. The line shows the fit of the Korsmeyer-Peppas model to the initial stage of the release.

Figure 4: The catheter navigation system. Communication unit console with NOGA XP Cardiac Navigation System.

Figure 5: A) The intramyocardial injection catheter with syringe attached. B) Detail of injection needle.

Figure 6: Unipolar voltage and LLS map. A) Unipolar map, LAO view (top) and bulls eye (below).

Red color indicates low unipolar voltage values at myocardial base (normal) with loss of electrical activity posterolateral. Blue indicates normal myocardium, whilst green and yellow colors indicate decreased viability. B) LLS map, LAO view (top) and bulls eye (below). Red color indicates akinesia in the posterolateral wall, green and yellow indicate decreased wall motion. The mapping points are shown by white dots. The drawn white line shows the area of interest, characterized by decreased unipolar voltages and impaired wall motions. Brown points represent the injection sites.

DISCUSSION:

A key challenge is to obtain a solution which is injectable through a long catheter while keeping the solution compatible with the bioactive compounds. Although the pH should be increased to increase injectability, bioactive compounds such as growth factors are fragile molecules that should be handled carefully. We monitor the pH of the solution closely using a pH meter after adding the hydrogelator to confirm it is pH 9.0 before adding any bioactive components. Initially, several rounds of adjusting the starting pH of the PBS were necessary to end with the right pH. For *in vitro* experiments, the solution was gelated by neutralizing the solution with HCl, while *in vivo* this is done by the natural pH of the tissue. Therefore, it is important to add the right amount of HCl to prevent an overshoot in pH. The diffusion of this acid is probably the limiting factor in the gelation of the hydrogel in *in vitro* experiments; however, *in vivo* the liquid would have a high contact surface area with neutralizing tissue, which will most likely result in a faster and more evenly gelation compared to dropwise addition of concentrated acid. Moreover, the gel switching is much faster with this mild procedure as compared to previously used methods (0.5 h vs 2 h)[25]. Using the body's natural pH for switching of the material properties is very appealing since the transition is swift, reversible, cannot occur inside the catheter and is *in vivo* fully automatic. These properties give advantages over *e.g.* thermal switchable gels[26], where risk of gelation in a catheter due to temperature changes is present, gels that require photo-induced polymerization, which is challenging due to limited light penetration and radical formation[27], or gels that require co-injection of a polymerization initiator or accellerator[28].

Successful release of a drug from the hydrogel largely depends on the size of the drug. As shown, the small molecule drug is released immediately while the gradual release of the model protein over 1 week shows the promise of these hydrogels as delivery systems for growth factors. In general, hydrogels are more promising as delivery tool for larger objects such as proteins, exosomes and cells[29,30].

The 3-D electromechanical mapping and injection procedure provides a clinically validated catheter-based delivery approach for various myocardial regenerative therapies, such as hydrogels. The added value of this technology compared to other non-surgical delivery techniques is the treatment planning, making it possible to differentiate normal, infarcted and hibernating myocardium and to guide therapies in the area of interest. Drawbacks of this approach concern the required technical skills and the time consuming and expensive procedure[20]. In the presented porcine model of myocardial infarction electromechanical mapping was followed by guided intramyocardial injections with the bioactive supramolecular

UPy-hydrogel. Other combinations with regenerative therapies have to be tested *in vitro* and *in vivo* to gain more success in this emerging field.

ACKNOWLEDGMENTS:
This work was funded by the Ministry of Education, Culture and Science (Gravity program 024.001.035), the Netherlands Organisation for Scientific Research (NWO), the European Research Council (FP7/2007-2013) ERC Grant Agreement 308045 and conducted within the LSH TKI framework. This research forms part of the Project P1.03 PENT of the research program of the BioMedical Materials institute, co-funded by the Dutch Ministry of Economic Affairs. This project was supported by ICIN - Netherlands Heart Institute (www.icin.nl) and the "Wijnand M. Pom Stichting". The authors would like to thank Henk Janssen and Joris Peters for the synthesis of the UPy-hydrogelator and Remco Arts for providing the mRuby2. We thank Bert Meijer, Tonny Bosman, and Roxanne Kieltyka for the many useful discussions and Marlijn Jansen, Joyce Visser, Grace Croft and Martijn van Nieuwburg for technical assistance.

DISCLOSURES:
The authors have nothing to disclose.

REFERENCES:
1. Levy, D., *et al.* Long-Term Trends in the Incidence of and Survival with Heart Failure. The *New England Journal of Medicine.* **347** (18), 1397-1402 (2002).
2. Roger, V. L., *et al*. Heart disease and stroke statistics—2012 update: a report from the American Heart Association. *Circulation*. **120** (1), 2-220 (2012).
3. Peppas, N. A., Huang, Y., Torres-Lugo, M., Ward, J. H. & Zhang, J. Physicochemical foundations and structural design of hydrogels in medicine and biology. *Annual Review of Biomedical Engineering.* **2** (1), 9–29 (2000).
4. Olsen, B. D., Kornfield, J. A. & Tirrell, D. A. Yielding Behavior in Injectable Hydrogels from Telechelic Proteins. *Macromolecules.* **43** (21), 9094–9099 (2010).
5. Guvendiren, M., Lu, H. D. & Burdick, J. A. Shear-thinning hydrogels for biomedical applications. *Soft Matter.* **8** (2), 260 (2012).
6. Bastings, M., *et al*. A Fast pH-Switchable and Self-Healing Supramolecular Hydrogel Carrier for Guided, Local Catheter Injection in the Infarcted Myocardium. *Advanced Healthcare Materials.* **3** (1), 70–78 (2014).
7. Pawar, G. M., *et al.* Injectable Hydrogels from Segmented PEG-Bisurea Copolymers. *Biomacromolecules.* **13** (12), 3966–3976 (2012).
8. Yoon, H.-J. & Jang, W.-D. Polymeric supramolecular systems for drug delivery. *Journal of Materials Chemistry.* **20** (2), 211–222 (2009).
9. Christman, K. L. & Lee, R. J. Biomaterials for the treatment of myocardial infarction. *Journal of the American College of Cardiology.* **48** (5), 907–913 (2006).
10. Yu, L. & Ding, J. Injectable hydrogels as unique biomedical materials. *Chemical Society Reviews.* **37** (8), 1473–1481 (2008).
11. Krieg, E. & Rybtchinski, B. Noncovalent Water-Based Materials: Robust yet Adaptive. *Chemistry – A European Journal.* **17** (33), 9016–9026 (2011).
12. Davis, M. E., *et al.* Injectable self-assembling peptide nanofibers create intramyocardial

microenvironments for endothelial cells. *Circulation.* **111** (4), 442–450 (2005).
13. Li, J., Ni, X. & Leong, K. W. Injectable drug-delivery systems based on supramolecular hydrogels formed by poly(ethylene oxide)s and alpha-cyclodextrin. *Journal of Biomedical Materials Research. Part A.* **65** (2), 196–202 (2003).
14. Dankers, P. Y. W., *et al.* Hierarchical formation of supramolecular transient networks in water: a modular injectable delivery system. *Advanced materials.* **24** (20), 2703–2709 (2012).
15. Dankers, P. Y. W., *et al.* Development and in-vivo characterization of supramolecular hydrogels for intrarenal drug delivery. *Biomaterials.* **33** (20), 5144–5155 (2012).
16. Kieltyka, R. E., *et al.* Mesoscale modulation of supramolecular ureidopyrimidinone-based poly(ethylene glycol) transient networks in water. *Journal of the American Chemical Society.* **135** (30), 11159–11164 (2013).
17. Vrijsen, K. R., *et al.* Cardiomyocyte progenitor cell-derived exosomes stimulate migration of endothelial cells. *Journal of Cellular and Molecular Medicine*. **14** (5), 1064-1070 (2010).
18. Koudstaal, S., *et al*. Myocardial infarction and functional outcome assessment in pigs. *Journal of Visualized Experiments*. (86), (2014).
19. Koudstaal, S., *et al*. Sustained delivery of insulin-like growth factor-1/hepatocyte growth factor stimulates endogenous cardiac repair in the chronic infarcted pig heart. *Journal of Cardiovascular Translational Research.* **7** (2), 232-241 (2014).
20. van der Spoel, T. I., *et al*. Non-surgical stem cell delivery strategies and in vivo cell tracking to injured myocardium. *International Journal of Cardiovascular Imaging.* **27** (3), 367-383 (2011).
21. Gepstein, L., Hayam, G., Shpun, S. & Ben-Haim, S. A. Hemodynamic evaluation of the heart with a nonfluoroscopic electromechanical mapping technique. *Circulation.* **96** (10), 3672-3680 (1997).
22. Gyöngyösi, M. & Dib, N. Diagnostic and prognostic value of 3D NOGA mapping in ischemic heart disease. *Nature Reviews Cardiology.* **8** (7), 393-404 (2011).
23. Siepmann, J. & Siepmann, F. Modeling of diffusion controlled drug delivery. *Journal of Controlled Release : Official Journal of the Controlled Release Society.* **161** (2), 351–362 (2012).
24. Kim, H. & Fassihi, R. Application of binary polymer system in drug release rate modulation. 2. Influence of formulation variables and hydrodynamic conditions on release kinetics. *Journal of Pharmaceutical Sciences.* **86** (3), 323–328 (1997).
25. Pape, A. C. H., *et al.* Mesoscale characterization of supramolecular transient networks using SAXS and rheology. *International Journal Of Molecular Sciences.* **15** (1), 1096–1111 (2014).
26. Lee, B. H. & Vernon, B. In Situ-Gelling, Erodible *N*-Isopropylacrylamide Copolymers. *Macromolecular Bioscience*. **5** (7), 629-635 (2005).
27. Annabi, N., *et al.* 25th Anniversary Article: Rational Design and Applications of Hydrogels in Regenerative Medicine. *Advanced Materials*. **26** (1), 85-124 (2014).
28. Asai, D., *et al.* Protein polymer hydrogels by *in situ,* rapid and reversible self-gelation. *Biomaterials*. **33** (21), 5451-5458 (2012).
29. Peppas, N. A., Hilt, J. Z., Khademhosseini, A. & Langer, R. Hydrogels in Biology and

Medicine: From Molecular Principles to Bionanotechnology. *Advanced Materials.* **18** (11), 1345–1360 (2006).

30. Lutolf, M. P. & Hubbell, J. A. Synthetic biomaterials as instructive extracellular microenvironments for morphogenesis in tissue engineering. *Nature Biotechnology.* **23** (1), 47–55 (2005).

Figure 1
Click here to download high resolution image

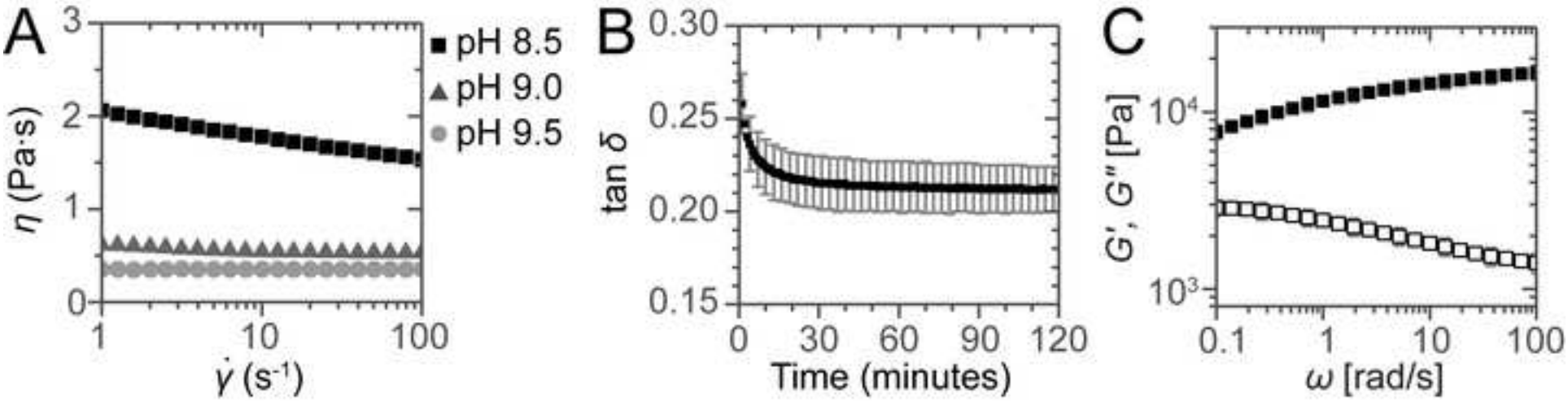


Figure 2
Click here to download high resolution image

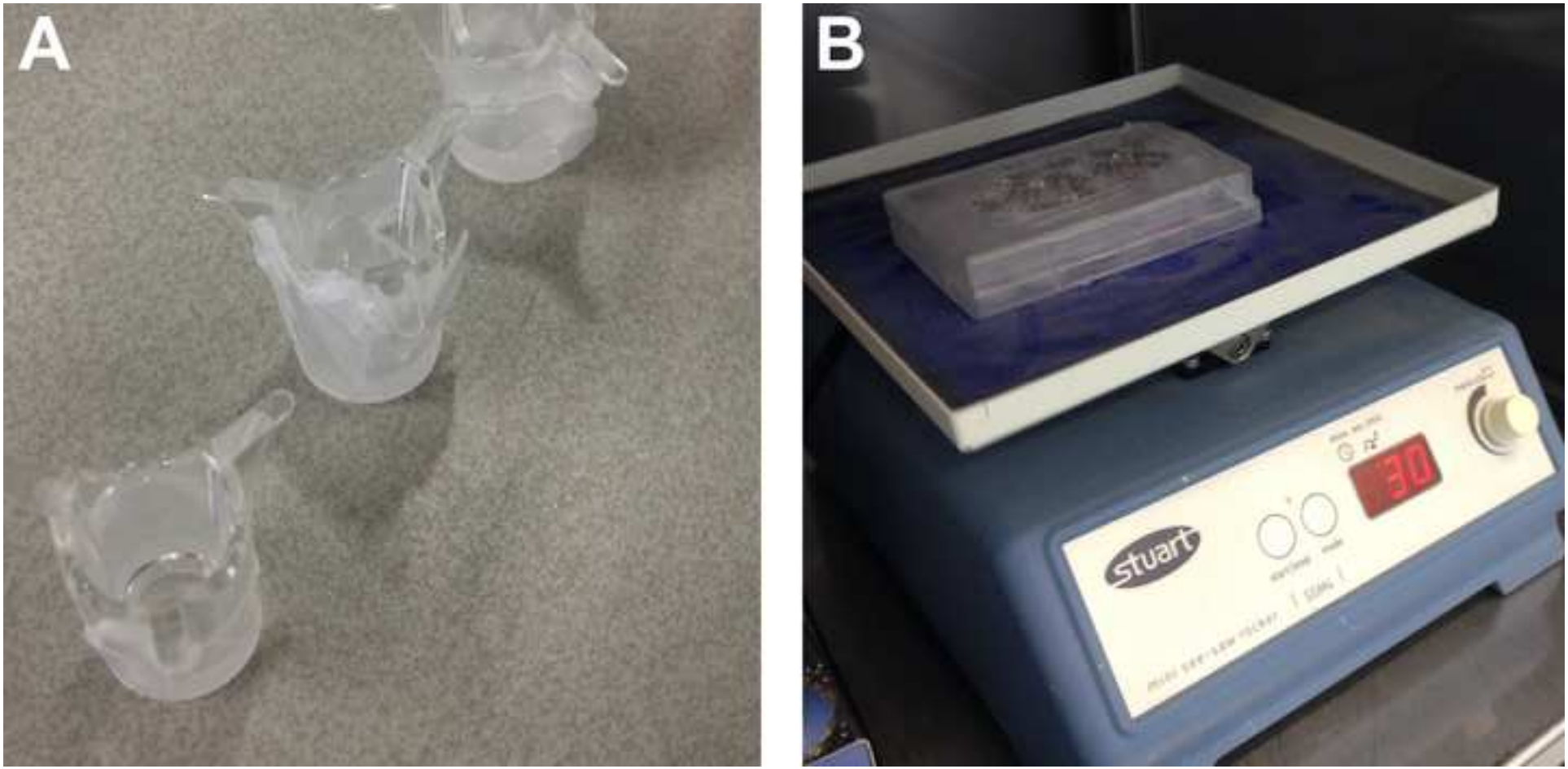


Figure 3
Click here to download high resolution image

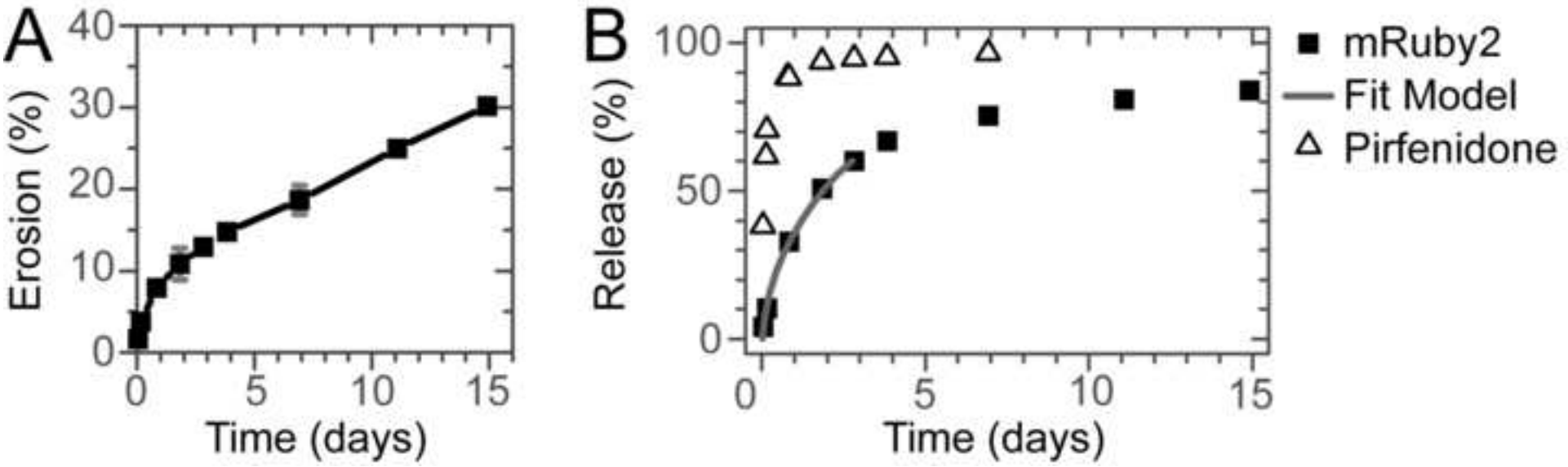


Figure 4
Click here to download high resolution image

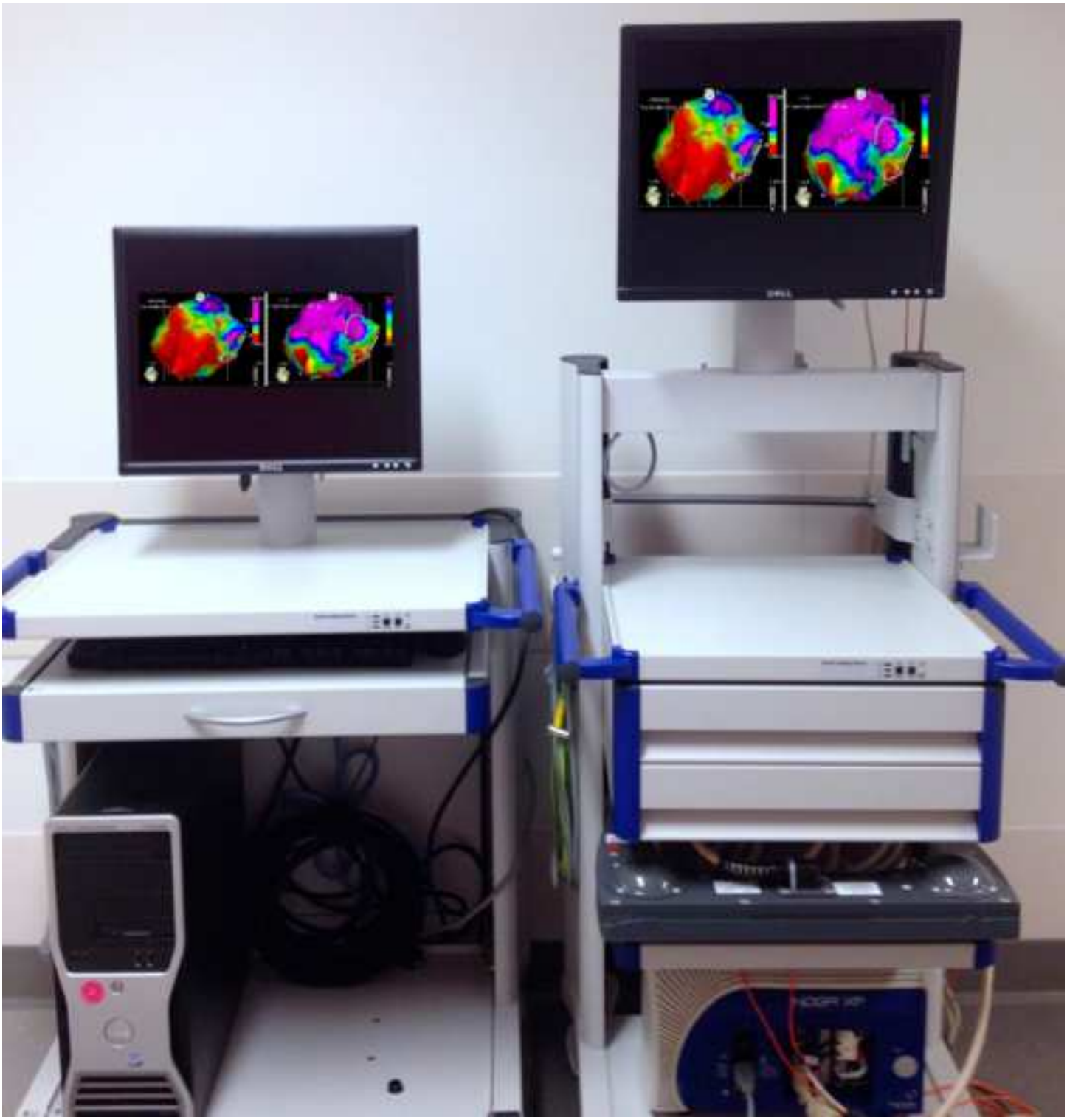


Figure 5
Click here to download high resolution image

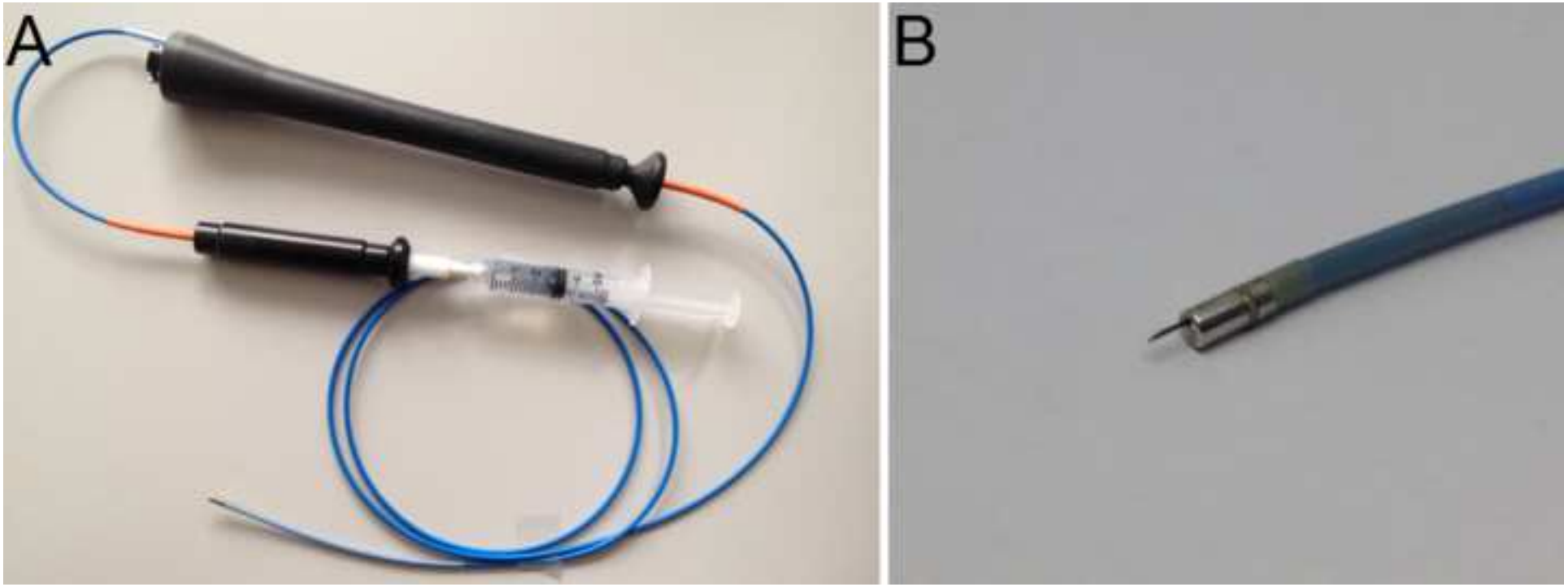
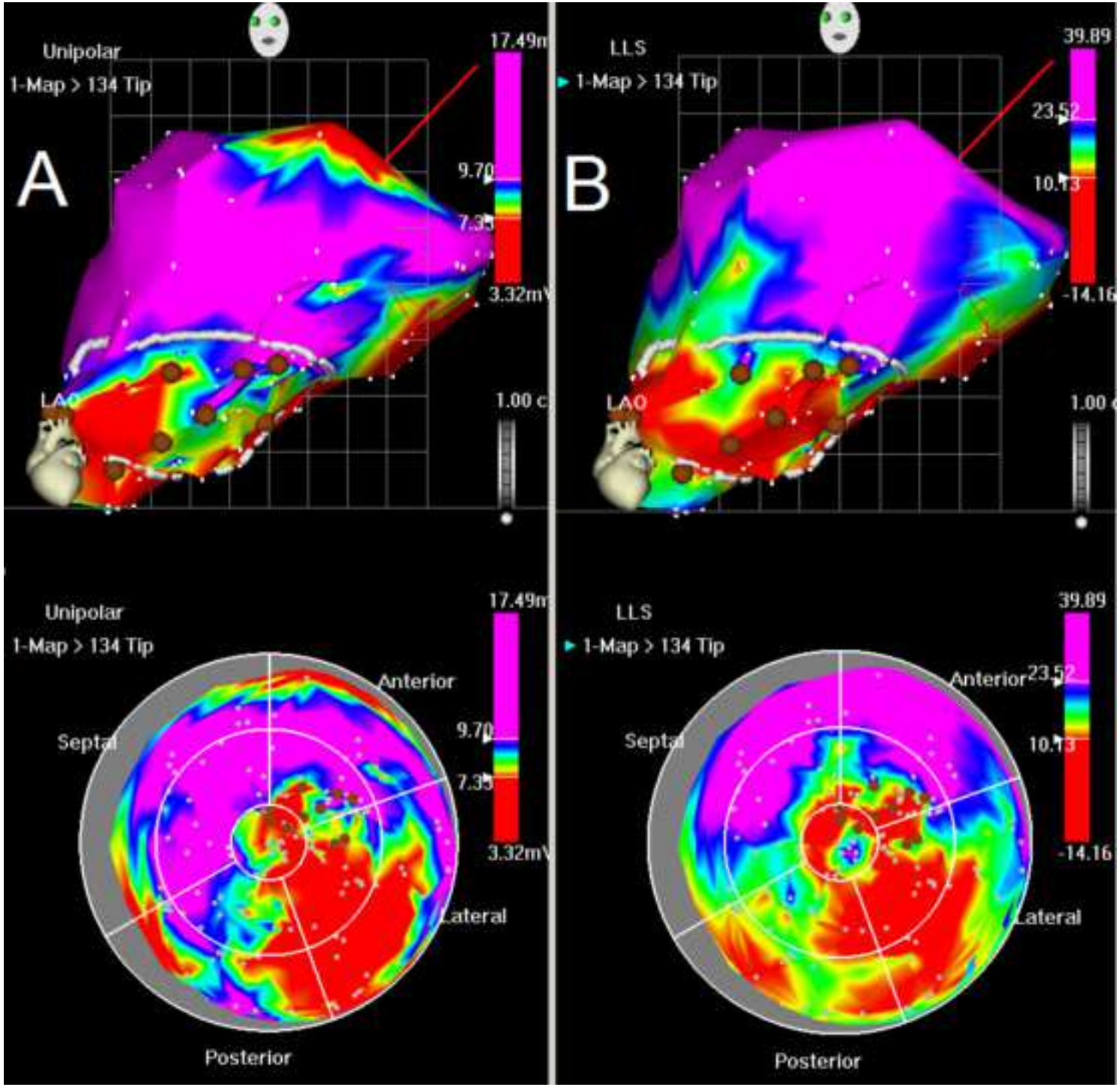


Figure 6

Click here to download high resolution image



Excel Spreadsheet- Table of Materials/Equipment

Click here to download Excel Spreadsheet- Table of Materials/Equipment: Pape-Bakker-JoVE_TableOfMaterials.xls

Name of Material/ Equipment	Company	Catalog Number
1M HCl		
1M NaOH		
Polystyrene 24-wells plate	Falcon	353047
Amiodarone	Cordaron I.V. (Sanofini)	
Anton Paar Physica MCR501	Anton Paar GmbH	
Atropine	PCH	
Balloon ventilator		
Cary 50 Scan UV-Visible Spectrophotometer	Varian	
Cary Eclipse Fluorescence Spectrophotometer	Varian	
Defibrillation patches		
DMSO	Biosolve	44705
Endotracheal tube	Covidien	
Heparin		
Ketamine	Narketan 10 Vétoquinol	
Mapping catheter 115cm	Biosense Webster	
Midazolam	Actavis	
MilliQ	MD Milipore MilliQ Integral Water Purification System	
mRuby2		
NaCl 0.9% 500cc	Braun	
NOGA guided Myostar injection catheter	Biosense Webster	
NOGA-RefStar EFO-patch	Biosense Webster	
Pancuronium bromide		
Parafilm	VWR	IKAA3801100
PBS	Sigma Aldrich	P4417
PET millicel	Millipore	PIEP12R48
Pirfenidone	Sigma Aldrich	P2116
Sodiumthiopental	Inresa	
Sufentanil	Sufentanil-Hameln	
Tegaderm		
UPy-PEG10k		
UV-Lamp		
Vet ointment		

Visipaque contrastfluid 100cc

Comments/Description

Equipped with a parallel-plate geometry (25 mm)

Used from 100mM stock in DMSO

jove JOURNAL OF VISUALIZED EXPERIMENTS
1 Alewife Center #200
Cambridge, MA 02140
tel. 617.945.9051
www.jove.com

ARTICLE AND VIDEO LICENSE AGREEMENT

Title of Article: An injectable and drug-loaded supramolecular hydrogel for local catheter injection into the pig heart

Author(s): Pape, Bakker, Bastings, Tseng, Noordstaal, Sluijter, Hoefer, Chamuleau, Dankers

Item 1 (check one box): The Author elects to have the Materials be made available (as described at http://www.jove.com/publish) via: ☐ Standard Access ☒ Open Access

Item 2 (check one box):

☒ The Author is NOT a United States government employee.

☐ The Author is a United States government employee and the Materials were prepared in the course of his or her duties as a United States government employee.

☐ The Author is a United States government employee but the Materials were NOT prepared in the course of his or her duties as a United States government employee.

ARTICLE AND VIDEO LICENSE AGREEMENT

1. Defined Terms. As used in this Article and Video License Agreement, the following terms shall have the following meanings: **"Agreement"** means this Article and Video License Agreement; **"Article"** means the article specified on the last page of this Agreement, including any associated materials such as texts, figures, tables, artwork, abstracts, or summaries contained therein; **"Author"** means the author who is a signatory to this Agreement; **"Collective Work"** means a work, such as a periodical issue, anthology or encyclopedia, in which the Materials in their entirety in unmodified form, along with a number of other contributions, constituting separate and independent works in themselves, are assembled into a collective whole; **"CRC License"** means the Creative Commons Attribution-Non Commercial-No Derivs 3.0 Unported Agreement, the terms and conditions of which can be found at: http://creativecommons.org/licenses/by-nc-nd/3.0/legalcode; **"Derivative Work"** means a work based upon the Materials or upon the Materials and other pre-existing works, such as a translation, musical arrangement, dramatization, fictionalization, motion picture version, sound recording, art reproduction, abridgment, condensation, or any other form in which the Materials may be recast, transformed, or adapted; **"Institution"** means the institution, listed on the last page of this Agreement, by which the Author was employed at the time of the creation of the Materials; **"JoVE"** means MyJove Corporation, a Massachusetts corporation and the publisher of *The Journal of Visualized Experiments;* **"Materials"** means the Article and / or the Video; **"Parties"** means the Author and JoVE; **"Video"** means any video(s) made by the Author, alone or in conjunction with any other parties, or by JoVE or its affiliates or agents, individually or in collaboration with the Author or any other parties, incorporating all or any portion of the Article, and in which the Author may or may not appear.

2. Background. The Author, who is the author of the Article, in order to ensure the dissemination and protection of the Article, desires to have the JoVE publish the Article and create and transmit videos based on the Article. In furtherance of such goals, the Parties desire to memorialize in this Agreement the respective rights of each Party in and to the Article and the Video.

3. Grant of Rights in Article. In consideration of JoVE agreeing to publish the Article, the Author hereby grants to JoVE, subject to **Sections 4** and **7** below, the exclusive, royalty-free, perpetual (for the full term of copyright in the Article, including any extensions thereto) license (a) to publish, reproduce, distribute, display and store the Article in all forms, formats and media whether now known or hereafter developed (including without limitation in print, digital and electronic form) throughout the world, (b) to translate the Article into other languages, create adaptations, summaries or extracts of the Article or other Derivative Works (including, without limitation, the Video) or Collective Works based on all or any portion of the Article and exercise all of the rights set forth in (a) above in such translations, adaptations, summaries, extracts, Derivative Works or Collective Works and (c) to license others to do any or all of the above. The foregoing rights may be exercised in all media and formats, whether now known or hereafter devised, and include the right to make such modifications as are technically necessary to exercise the rights in other media and formats. If the "Open Access" box has been checked in **Item 1** above, JoVE and the Author hereby grant to the public all such rights in the Article as provided in, but subject to all limitations and requirements set forth in, the CRC License.

jove JOURNAL OF VISUALIZED EXPERIMENTS
1 Alewife Center #200
Cambridge, MA 02140
tel. 617.945.9051
www.jove.com

4. Retention of Rights in Article. Notwithstanding the exclusive license granted to JoVE in **Section 3** above, the Author shall, with respect to the Article, retain the non-exclusive right to use all or part of the Article for the non-commercial purpose of giving lectures, presentations or teaching classes, and to post a copy of the Article on the Institution's website or the Author's personal website, in each case provided that a link to the Article on the JoVE website is provided and notice of JoVE's copyright in the Article is included. All non-copyright intellectual property rights in and to the Article, such as patent rights, shall remain with the Author.

5. Grant of Rights in Video – Standard Access. This **Section 5** applies if the "Standard Access" box has been checked in **Item 1** above or if no box has been checked in **Item 1** above. In consideration of JoVE agreeing to produce, display or otherwise assist with the Video, the Author hereby acknowledges and agrees that, Subject to **Section 7** below, JoVE is and shall be the sole and exclusive owner of all rights of any nature, including, without limitation, all copyrights, in and to the Video. To the extent that, by law, the Author is deemed, now or at any time in the future, to have any rights of any nature in or to the Video, the Author hereby disclaims all such rights and transfers all such rights to JoVE.

6. Grant of Rights in Video – Open Access. This **Section 6** applies only if the "Open Access" box has been checked in **Item 1** above. In consideration of JoVE agreeing to produce, display or otherwise assist with the Video, the Author hereby grants to JoVE, subject to **Section 7** below, the exclusive, royalty-free, perpetual (for the full term of copyright in the Article, including any extensions thereto) license (a) to publish, reproduce, distribute, display and store the Video in all forms, formats and media whether now known or hereafter developed (including without limitation in print, digital and electronic form) throughout the world, (b) to translate the Video into other languages, create adaptations, summaries or extracts of the Video or other Derivative Works or Collective Works based on all or any portion of the Video and exercise all of the rights set forth in (a) above in such translations, adaptations, summaries, extracts, Derivative Works or Collective Works and (c) to license others to do any or all of the above. The foregoing rights may be exercised in all media and formats, whether now known or hereafter devised, and include the right to make such modifications as are technically necessary to exercise the rights in other media and formats. For any Video to which this Section 6 is applicable, JoVE and the Author hereby grant to the public all such rights in the Video as provided in, but subject to all limitations and requirements set forth in, the CRC License.

7. Government Employees. If the Author is a United States government employee and the Article was prepared in the course of his or her duties as a United States government employee, as indicated in **Item 2** above, and any of the licenses or grants granted by the Author hereunder exceed the scope of the 17 U.S.C. 403, then the rights granted hereunder shall be limited to the maximum rights permitted under such statute. In such case, all provisions contained herein that are not in conflict with such statute shall remain in full force and effect, and all provisions contained herein that do so conflict shall be deemed to be amended so as to provide to JoVE the maximum rights permissible within such statute.

8. Likeness, Privacy, Personality. The Author hereby grants JoVE the right to use the Author's name, voice, likeness, picture, photograph, image, biography and performance in any way, commercial or otherwise, in connection with the Materials and the sale, promotion and distribution thereof. The Author hereby waives any and all rights he or she may have, relating to his or her appearance in the Video or otherwise relating to the Materials, under all applicable privacy, likeness, personality or similar laws.

9. Author Warranties. The Author represents and warrants that the Article is original, that it has not been published, that the copyright interest is owned by the Author (or, if more than one author is listed at the beginning of this Agreement, by such authors collectively) and has not been assigned, licensed, or otherwise transferred to any other party. The Author represents and warrants that the author(s) listed at the top of this Agreement are the only authors of the Materials. If more than one author is listed at the top of this Agreement and if any such author has not entered into a separate Article and Video License Agreement with JoVE relating to the Materials, the Author represents and warrants that the Author has been authorized by each of the other such authors to execute this Agreement on his or her behalf and to bind him or her with respect to the terms of this Agreement as if each of them had been a party hereto as an Author. The Author warrants that the use, reproduction, distribution, public or private performance or display, and/or modification of all or any portion of the Materials does not and will not violate, infringe and/or misappropriate the patent, trademark, intellectual property or other rights of any third party. The Author represents and warrants that it has and will continue to comply with all government, institutional and other regulations, including, without limitation all institutional, laboratory, hospital, ethical, human and animal treatment, privacy, and all other rules, regulations, laws, procedures or guidelines, applicable to the Materials, and that all research involving human and animal subjects has been approved by the Author's relevant institutional review board.

10. JoVE Discretion. If the Author requests the assistance of JoVE in producing the Video in the Author's facility, the Author shall ensure that the presence of JoVE employees, agents or independent contractors is in accordance with the relevant regulations of the Author's institution. If more than one author is listed at the beginning of this Agreement, JoVE may, in its sole discretion, elect not take any action with respect to the Article until such time as it has received complete, executed Article and Video License Agreements from each such author. JoVE reserves the right, in its absolute and sole discretion and without giving any reason therefore, to accept or decline any work submitted to JoVE. JoVE and its employees, agents and independent contractors shall have

jove JOURNAL OF VISUALIZED EXPERIMENTS
1 Alewife Center #200
Cambridge, MA 02140
tel. 617.945.9051
www.jove.com

ARTICLE AND VIDEO LICENSE AGREEMENT

full, unfettered access to the facilities of the Author or of the Author's institution as necessary to make the Video, whether actually published or not. JoVE has sole discretion as to the method of making and publishing the Materials, including, without limitation, to all decisions regarding editing, lighting, filming, timing of publication, if any, length, quality, content and the like.

11. Indemnification. The Author agrees to indemnify JoVE and/or its successors and assigns from and against any and all claims, costs, and expenses, including attorney's fees, arising out of any breach of any warranty or other representations contained herein. The Author further agrees to indemnify and hold harmless JoVE from and against any and all claims, costs, and expenses, including attorney's fees, resulting from the breach by the Author of any representation or warranty contained herein or from allegations or instances of violation of intellectual property rights, damage to the Author's or the Author's institution's facilities, fraud, libel, defamation, research, equipment, experiments, property damage, personal injury, violations of institutional, laboratory, hospital, ethical, human and animal treatment, privacy or other rules, regulations, laws, procedures or guidelines, liabilities and other losses or damages related in any way to the submission of work to JoVE, making of videos by JoVE, or publication in *JoVE* or elsewhere by JoVE. The Author shall be responsible for, and shall hold JoVE harmless from, damages caused by lack of sterilization, lack of cleanliness or by contamination due to the making of a video by JoVE its employees, agents or independent contractors. All sterilization, cleanliness or decontamination procedures shall be solely the responsibility of the Author and shall be undertaken at the Author's expense. All indemnifications provided herein shall include JoVE's attorney's fees and costs related to said losses or damages. Such indemnification and holding harmless shall include such losses or damages incurred by, or in connection with, acts or omissions of JoVE, its employees, agents or independent contractors.

12. Fees. To cover the cost incurred for publication, JoVE must receive payment before production and publication the Materials. Payment is due in 21 days of invoice. Should the Materials not be published due to an editorial or production decision, these funds will be returned to the Author. Withdrawal by the Author of any submitted Materials after final peer review approval will result in a US$1,200 fee to cover pre-production expenses incurred by JoVE. If payment is not received by the completion of filming, production and publication of the Materials will be suspended until payment is received.

13. Transfer, Governing Law. This Agreement may be assigned by JoVE and shall inure to the benefits of any of JoVE's successors and assignees. This Agreement shall be governed and construed by the internal laws of the Commonwealth of Massachusetts without giving effect to any conflict of law provision thereunder. This Agreement may be executed in counterparts, each of which shall be deemed an original, but all of which together shall be deemed to me one and the same agreement. A signed copy of this Agreement delivered by facsimile, e-mail or other means of electronic transmission shall be deemed to have the same legal effect as delivery of an original signed copy of this Agreement.

A signed copy of this document must be sent with all new submissions. Only one Agreement required per submission.

CORRESPONDING AUTHOR:

Name: P. Y. W. Dankers

Department: Biomedical Engineering

Institution: Eindhoven Univ. of Technology

Article Title: An injectable and drug-loaded supramolecular hydrogel for local catheter injection into the pig heart

Signature: P Dankers Date: June 30, 14

Please submit a signed and dated copy of this license by one of the following three methods:

1) Upload a scanned copy of the document as a pfd on the JoVE submission site;
2) Fax the document to +1.866.381.2236;
3) Mail the document to JoVE / Attn: JoVE Editorial / 1 Alewife Center #200 / Cambridge, MA 02139

For questions, please email submissions@jove.com or call +1.617.945.9051

List of changes to JoVE52450 'An Injectable and Drug-loaded Supramolecular hydrogel for Local Catheter Injection into the Pig Heart'

Editorial comments:

Changes to be made by the Author(s):

2. Please describe step 4.3.3.1 in more detail. How are the injection locations determined? Is it a pattern? What is done to release the specified injection amount? How is the sample prepared prior to injection? How is the sample injected? In a bolus or while moving the tip of the needle? Better define the criteria and how they are measured.

Please keep in mind the the 2 page limit of highlighted protocol text as filming will be split between two locations.

Reply: Step 4.3.3.1 has been described in more detail. Sample preparation has been described in point 4.3.1. Due to these changes, point 4.3.3 is now obsolete. We thank the editor for these remarks, as we think the procedure is now much clearer. To stay within the 2 page limit, point 3.4.2 has been split and we have partially removed the highlighting.

3. Are there any histology images the authors could show of the hydrogel in the heart tissue?

Reply: Here, we have not chosen to go into detail on the analysis of the heart after sacrifice of the animals but focus on the actual hydrogel formulation and injection methodology. For histology images and more information after sacrificing the animals, please see Bastings and Koudstaal (*Adv. Healthcare Mater.* **2014**, *3*, 70–78).

Reviewers' comments:

Reviewer #1:
Manuscript Summary:
The Authors tried to demonstrate the methodology of formulation and characterizations of a hydrogel, and it's use for drug delivery system in the field of regenerative medicine. The hydrogel was formed by non-covalent interactions in between poly(ethylene glycol) (PEG) and end-modified with ureido-pyrimidinone (UPy) moieties. When tested on the myocardial infraction area of pig heart, this hydrogel helps to release the drug in a controlled manner. This is academically and clinically a very valuable methodology, specifically in the field of regenerative medicine and drug delivery system in the heart.

Reply: We thank the referee for his/her detailed review of our manuscript and shared interest in the methodology; we believe that the points raised by the referee are of great interest and have tried to address these here and in our revised manuscript.

Major Concerns:
1. HCl was used to adjust pH of the solution, and for the in vitro experiments, the solution was

gelated by neutralizing the gel with HCl. However, for in vivo condition, the pH was mainly adjusted from the natural pH of the tissue. It may vary from the cell to cell or tissues and so, it is important to add the right amount of HCl to prevent an overshoot in pH. I am wondering if there is any way of monitoring the pH. In addition, how can you estimate the amount of HCl needed for the in vivo settings?

Reply: We agree with the referee that the right pH is essential to create a good gel. However, for the solutions to become solid, the pH should be lowered below 8. For the in vivo release, we only rely on the buffering capacity of the body. In this case, we do not add any HCl. We have not monitored the pH of the gel in the body, but extract the success of the experiment from the formation of a gel. To make this clearer, we have clarified in point 4.3.2 that a solution is used, and added that it will only form a gel after injection in point 4.3.3.1.

2. The Authors demonstrated the mRuby2 molecule as a model protein as a proof of principle of the methodology. I'm wondering if this procedure is applicable to deliver the growth factors like VEGF or EGF molecules and for how long they can be monitored.

Reply: As the referee points out, it is of much more clinical relevance to study the release of growth factor proteins. However, for demonstration purposes we have chosen for the colored mRuby2 here. In a recent paper from Bastings, Koudstaal, et al. (*Adv. Healthcare Mater.* **2014**, *3*, 70–78) where this methodology is used, the release of hepatocyte growth factor and insulin-like growth factor-1 is measured using ELISA detection.

Minor Concerns:
1. The authors wrote '1 hour' (line no: 165) in one place and '1h' (line no: 173) in the other place. It should be consistent.

2. They assayed the oscillatory rheological and modulus of the formulated hydrogel. In the results section, Standard error has been shown in the graph. It will be good if the authors mention "N", i.e., the number of independent measurements / experiments there.

3. There is typographic error (i.e: Myosatr) in the page of the list of material/equipment.

Reply: We have addressed the minor concerns raised by the referee.

1) We have checked the consistency of the used symbols (track changes)
2) We have added the number of experiments performed in the caption of the figure 1.
3) Typographic errors have been addressed (track changes).

Additional Comments to Authors:
Is it possible to provide some data on growth factors delivery?

Reply: See above.

Reviewer #2:
Manuscript Summary:
It is an interestin new concept with injection into the heart of a biomaterial, which potentially can be mixed with different drugs to improve the stay of the drug in the injected area.

The description of the production of the biomateriale and its handling in some situations are described clearly.
It has been injected into the heart with the NOGA method, which also is used in clinical studies.

Major Concerns:
I have no major concern.

Minor Concerns:
To reach a level where it can be used for clinical therapy, the authors have to look more on the production method, sterility methods etc. However, the present study can be seen as the proof of concept study, which now can be moved into the next research phase for optimization.

Additional Comments to Authors:
N/A

Reply: We would like to thank the referee for the nice words on our manuscript. We do agree that for the use in clinical therapies, several hurdles have to be taken such as the points raised by the referee. Here, we have indeed chosen to present the concept of the methodology. More details on for instance biocompatibility can be found in the paper from Bastings, Koudstaal, et al. (*Adv. Healthcare Mater.* **2014**, *3*, 70–78).