Published in final edited form as:

Small. 2020 February 01; 16(8): e1904806. doi:10.1002/sml.201904806.

Injectable cardiac cell microdroplets for tissue regeneration

Idan Gal^{#1}, Reuven Edri^{#1}, Nadav Noor^{1,2}, Matan Rotenberg², Michael Namestnikov¹, Itai Cabilly, Assaf Shapira¹, Tal Dvir^{1,2,3,4,*}

¹The School for Molecular Cell Biology and Biotechnology, Faculty of Life Sciences, Tel Aviv University, Tel Aviv 6997801, Israel

²Department of Materials Science and Engineering, Faculty of Engineering, Tel Aviv University, Tel Aviv 6997801, Israel

³The Center for Nanoscience and Nanotechnology, Tel Aviv University, Tel Aviv 6997801, Israel

⁴Sagol Center for Regenerative Biotechnology, Tel Aviv University, Tel Aviv 6997801, Israel

These authors contributed equally to this work.

Abstract

One of the strategies for heart regeneration includes cell delivery to the defected heart. However, most of the injected cells do not form quick cell-cell or cell-matrix interactions, therefore, their ability to engraft at the desired site and improve heart function is poor. Here we report on the use of a microfluidic system for generating personalized hydrogel-based cellular microdroplets for cardiac cell delivery. To evaluate the system's limitations, a mathematical model of oxygen diffusion and consumption within the droplet was developed. Following, the microfluidic system's parameters were optimized and cardiac cells from neonatal rats or iPSCs were encapsulated. The morphology and cardiac specific markers were assessed, and cell function within the droplets was analyzed. Finally, the cellular droplets were injected to mouse gastrocnemius muscle to validate cell retention, survival and maturation within the host tissue. These results demonstrate the potential of this approach to generate personalized cellular microtissues, which can be injected to distinct regions in the body for treating damaged tissues.

Keywords

hydrogel; microfluidics; droplets; micro-scale; cell delivery

1 Introduction

Heart transplantation is the only treatment available today for end-stage myocardial infarction. As heart donors are scarce, there is a need to develop new regenerative technologies to treat the diseased heart. One of the strategies for heart regeneration includes cell delivery to the defected heart. However, most of the injected cells do not form quick

*Correspondence to Tal Dvir (tdvir@tauex.tau.ac.il).

cell-cell or cell-matrix interactions, therefore, their ability to engraft at the desired site and improve heart function is poor [1].

A promising strategy to protect the injected cells is their encapsulation within 3D hydrogels prior to injection. If the hydrogel contains cell binding motifs, it facilitates cell matrix interaction through cell-surface receptors and prevents anoikis [2, 3]. Biological motifs in natural hydrogels also affect cell behavior, including growth, migration, maturation and differentiation. Recently, our group has developed a personalized hydrogel, based on the omentum extracellular-matrix (ECM) [4]. The omentum is a highly vascularized fatty tissue with a well-documented regenerative capabilities, that extends from the stomach overlying the abdomen [5–7]. Since the omentum can be removed from patients by simple microsurgery techniques without affecting any physiological function, its ECM can be utilized to serve as a personalized material that fits the immunological profile of the same individual. As a strong immune response to implants may jeopardize the regenerative process, personalized hydrogels may be useful for tissue engineering applications.

Another important issue that affects implant survival is the degree of pre-vascularization. Without proper blood vessel networks within the implants, oxygen transfer is limited and cells in a thick patch cannot survive [8].

One strategy to improve cell survival is cell microencapsulation in small-scale spherical beads. In these spheres the cells are protected from the external environment and maintain their cellular functions [9, 10]. The spherical nature of the beads maximizes the surface area and their small volume facilitates efficient biomolecular transport [11]. In this way, the microenvironment of every single hydrogel bead is controlled in a precise manner, resulting in equal distribution of oxygen, nutrients, etc. In particular, the microbeads may provide a substrate for cell adhesion, and control over the localization site in vivo after injection. To date, there are several methods to encapsulate cells, including extrusion (electrostatic spray [12], air flow nozzle, and vibrating nozzle), emulsion/thermal gelation and a microfluidics-based approach [13]. Such encapsulations of cells were reported using different natural hydrogels, including hyaluronic acid [14], chitosan-collagen [15], agarose [16] and collagen-gelatin [17].

Here we report on the use of a microfluidic system for generating personalized hydrogel-based microdroplets for cardiac cell delivery. Previously we have shown the ability to produce personalized tissue implants from the omentum. In this approach a biopsy of the omentum is taken from a patient and the cellular and a-cellular materials are separated. After processing the ECM to generate a personalized hydrogel the stromal cells of the omentum are reprogrammed to pluripotent stem cells and cultured within. We have shown that any tissue implant can be generated in this approach [4]. Here, as a proof of concept, pigs' omental tissues were extracted and the ECM was decellularized and processed to generate a 1% (w/v) thermoresponsive hydrogel. In parallel, human omentum-derived pluripotent stem cells were differentiated to cardiomyocytes. The liquid ECM hydrogel was then mixed with the cells, and flown inside the microfluidic device to generate cellular microdroplets that fully match the immunological and biochemical properties of the patient (Fig. 1). After heating the droplets to 37°C, they solidify to become a viscous gel. To evaluate the system's

limitations, a mathematical model of oxygen diffusion and consumption within the droplet was developed. Following, the microfluidic system's parameters were optimized and cardiac cells from neonatal rats or iPSCs were encapsulated. The morphology and cardiac specific markers were assessed, and cell function within the droplets was analyzed. Finally, the cellular droplets were injected to mouse gastrocnemius muscle to validate cell retention, survival and maturation within the host tissue.

2 Results and Discussion

2.1 Oxygen diffusion modelling

One of the main challenges of working with cells that are encapsulated in a bulk hydrogel is the lack of homogenous microenvironment inside the hydrogel. This may lead to a concentration gradient of oxygen and nutrients between the hydrogel's outskirts and core. It may result in heterogeneous morphology of the engineered tissue and cell death in the hydrogel core. As we hypothesized that cell encapsulation within micro-scale droplets could overcome this challenge, we initially modelled oxygen concentration by using COMSOL multi-physics program. We have taken into consideration oxygen diffusion according to Fick's second law, and consumption by the cells according to Michaelis-Menten kinetics [18]. The external concentration of oxygen was considered as the concentration within arterial blood, and oxygen diffusion coefficient was considered as the one in a muscle. Two assumptions were made during our mathematical modelling: 1. Twenty minutes is considered the maximal period of time, in which cardiac cells can survive without oxygen [19]. 2. Cell concentration within the hydrogel was 1×10^8 cells/mL, which is considered a physiological concentration within the heart [20]. At first, oxygen concentration within adjacent droplets was compared to a bulk hydrogel with the same volume. Analyzing oxygen concentration within a $(0.4 \text{ mm})^3$ bulk hydrogel revealed a hypoxic core after 20 min (Fig. 2a). At the same volume, 64 ECM hydrogel droplets with a diameter of $100 \mu\text{m}$ could be tightly arranged (the center spheres have 6 points of contact with the other spheres; Fig. 2b). Here, after 5 s the system had reached a steady state. At this stage oxygen concentration within the core droplets had reached 0.13 mol/m^3 , well above what is considered hypoxic conditions (Fig. 2c). Despite oxygen consumption by the cells, droplets' surface to volume ratio improved oxygen diffusion to the encapsulated cells, and maintained oxygen concentration.

We next sought to evaluate the effect of single droplet diameter on the level of oxygen within. Figure 2d reveals the distribution of oxygen throughout droplets with different diameters, ranging from 60 to $500 \mu\text{m}$. As shown, up to droplets with a diameter of $160 \mu\text{m}$ only a slight decrease in oxygen concentration was observed. Above this size a drop of 10% in oxygen concentration was observed for every $30 \mu\text{m}$ (supplementary Fig. 1). Such drop in oxygen level is explained by the limitation of mass transfer within a tissue. These results are consistent with the empirical data presented in the literature, indicating that oxygen can penetrate only 100 to $200 \mu\text{m}$ into a tissue.

2.2 Microfluidics droplet system design and characterization

In order to generate monodispersed cellular droplets, we designed and fabricated a flow-focusing droplet microfluidic system (Fig. 3a). The device includes two inlets, one for oil phase (continuous phase) and one for the cell-containing hydrogel phase (dispersed phase). The system also has a single outlet for the mixture. Initially, the system's parameters were optimized using fibroblasts as model cells. After being retrieved from the outlet (Fig. 3b, and Supplementary movies 1 and 2), the cellular droplets were heated up to 37°C to allow gelation of the omentum-based hydrogel by physical crosslinking (supplementary fig. 2). As shown, the droplets sizes were monodispersed with homogenous cell distribution (Fig. 3c). We next sought to evaluate the parameters affecting droplet diameter. Therefore, the system was operated under various oil and cell-containing hydrogel phase flow rates. Initially, the flow rate of the oil phase was set to 40, 80 and 120 $\mu\text{L h}^{-1}$, while the hydrogel phase was fixed to 40 $\mu\text{L h}^{-1}$ (Fig. 3d). As shown, droplet diameter mean size, using 40, 80 and 120 $\mu\text{L h}^{-1}$ oil phase velocities were 96, 87.7 and 78.3 μm , respectively. As oil phase flow rate increased, droplets were strained quickly and their mean diameter decreased. To evaluate the effect of hydrogel solution flow rate on droplet size, the flow rate of the oil phase was fixed to 80 $\mu\text{L h}^{-1}$ and the cell-containing hydrogel was flown at a flow rate of 20, 40 and 60 $\mu\text{L h}^{-1}$. As shown, droplets diameter mean size, was 75.7, 78.7 and 98.7 μm , respectively (Fig. 3e). Using velocities of 20 $\mu\text{L h}^{-1}$ (supplementary Fig. 3a) and 40 $\mu\text{L h}^{-1}$ (Fig. 3f), a narrow Gaussian distribution was achieved. The higher velocity (60 $\mu\text{L h}^{-1}$, supplementary Fig. 3b) resulted in a wider Gaussian distribution, reaching to a diameter of 150 μm , probably due to the high speed of the hydrogel and the coalescence of adjacent droplets. Consequently, we fixed the system flow rates to 80 and 40 $\mu\text{L h}^{-1}$, for oil and hydrogel phases, respectively.

Next, to encapsulate heart cells in the hydrogel, cardiac cells were isolated from the ventricles of neonatal rats and flown in the microfluidics system. Following the cellular droplets were characterized. As shown, cell viability was not affected by the encapsulation process (Fig. 3g,h). The number of cells within the droplets varied in correlation with the cell concentration within the hydrogel phase (Fig. 3i-l). The ability to adjust the cell number is essential, as different tissues in the body contain different cell types with varying densities.

2.3 Growth, morphology and function of encapsulated cardiac cells

To evaluate cell morphology and function, cardiac cell-containing droplets were cultured for 7 days. As shown, during this period, an increase in cell number was observed, probably due to the proliferation of cardiac fibroblasts (Fig. 4a) while no significant change in droplet diameter was observed during this period (supplementary fig. 4). Scanning electron microscope analysis of the cellular droplets at day 0 revealed rounded cells, well-wrapped by the ECM fibers. This tight encapsulation within the hydrogel fibers allowed for efficient cell-cell and cell-matrix interactions (Fig. 4b). Such interactions were previously shown to promote essential physiological processes, such as proliferation, differentiation and maturation [21–23]. On day 7, the cells have occupied the entire volume of the droplet and tightly packed, spread cells could be observed on the surface of the droplet (Fig. 4c). Sarcomeric α -actinin immunostaining on day 7 revealed encapsulated cardiomyocytes spread and elongated all across the droplet volume exhibiting significant striation. In

addition, connexin 43 protein was highly expressed within the cellular droplets, indicating on the ability of adjacent cells to form electrical coupling (Fig. 4d). Immunostaining of the cells with anti-troponin T protein and collagen revealed an intimate interaction between the cardiac cells and the ECM scaffolding material within the droplet (Fig. 4e). Due to the close cell-cell interactions within the droplet, the entire cellular droplet contracted (supplementary movies. 3 and 4). Analysis of the electrical signal within the droplets was performed by calcium imaging, indicating on signal transfer throughout the droplet (Fig. 4f). Calcium signal propagation map and quantification (supplementary fig. 5) at 6 distinct locations within the droplet revealed synchronous cell contraction, indicating on the cells ability to form electrical coupling post encapsulation and to communicate with each other (supplementary movie 5).

In order to explore in vitro cell migration outside the droplet, cardiac cell-containing droplets were cultured for 15 days, and migration distances were microscopically quantified (Figure 5 and Supplementary fig. 6). Cells migrated to an average distances of 168, 355, 573 and 667 μm on days 3, 6, 11 and 15, respectively (Fig. 5a), indicating that the encapsulating ECM posed no barrier to interaction with the outside environment. In vitro functionality and migration into cellular environment was further investigated by placing fluorescently labeled encapsulated cardiac cells on a pre-seeded cardiac cells sheet. As shown in Fig. 5b, encapsulated cardiac cells migrated outside the droplets, assimilating into the surrounding cell population. Analysis of the electrical signal, performed by calcium imaging, revealed signal transfer throughout the droplet, migrating cells and cells sheet (Fig. 5c and Supplementary movie 6). Quantification of the electrical signal in 5 different points revealed a shift in latency, before and after passage through the droplet (Fig. 5d-f), indicating on electrical coupling between the droplet and its surroundings. Both cell migration and electrical coupling is demonstrated in figure 5g-i and supplementary movie 7, showing two adjacent droplets electrically coupled by cells that migrated and settled between them. The signal propagation indicates on the ability of encapsulated cardiac cells to migrate and communicate with each other to form functioning network.

We next sought to engineer cardiac cell droplets from human cells. In theory, both the hydrogel and the cells can be derived from the same piece of omentum extracted from the same patient. Here, for the proof of concept human induced pluripotent stem cells (iPSCs) derived from omental cells were encapsulated within pig's omentum hydrogel. Stromal cells from human omentum were isolated and reprogrammed to become iPSCs. In our approach, after differentiation within a bulk hydrogel, the cells were isolated by collagenase digestion and re-encapsulated in the hydrogel droplets (Fig. 6a). As shown, prior to differentiation, the cells exhibited the pluripotency marker OCT4 and the proliferation marker Ki67 (Fig. 6b). The cells were then efficiently differentiated to cardiomyocytes, exhibiting actinin, connexin-43, troponin T and Nkx2.5 markers (Fig. 6c,d) [4, 24]. Following re-encapsulation, the cells were spread throughout the droplet, expressing troponin T (Fig. 6e). Furthermore, contractions with regularly-spaced spikes, and beating frequency of $\sim 1\text{-}2\text{ Hz}$ could be clearly seen within the droplets (Fig. 6f and supplementary movie 8).

2.4 Injection of the cellular droplets

We next tested the durability of the cellular droplets after injection through a 25 G needle. While the internal diameter of the needle (260 μm) is larger than the droplet size; in high density, several droplets can pass together through the needle (supplementary movie 9) and may be harmed due to the applied shear stress. To test that, the droplets were supplemented with fluorescent nanoscale liposomes. If the droplets are damaged by the shear stress during injection, pieces of the hydrogel could be disrupted and fluorescence will be seen outside the droplets. As shown, droplets shape was not changed after injection and spherical structures, containing liposomes could be observed before and after injection (Fig. 7a). Furthermore, cell viability within the droplets was not affected by the injection (Fig. 7b).

Finally, we sought to evaluate the capacity of the system to deliver cells into a pre-defined sites in a living animal, while ensuring their retention and survival. To this end, cardiac cells were pre-stained with cytopainter, encapsulated within the hydrogel droplets and injected into the gastrocnemius muscle of mice. On day 2, the pre-stained cells could be observed at the injection site (Fig. 7c). The cells were spread at the injection site, exhibiting striation of sarcomeric actinin and connexin 43, indicating on their physiological state and ability to form interactions with their close surroundings (Fig. 7d,e). These can be attributed to the protective and supportive nature of the hydrogel, allowing the cells to interact with each other and with the ECM fibers until interactions with the host are made [25].

3 Conclusions and future directions

Encapsulation of the cells in small-scale droplets increases mass transfer into the core of the engineered tissue generated from multiple droplets and thus may provide an appropriate atmosphere for cell growth. The use of a microfluidic system to generate the cellular droplets allows to control droplet size and cell concentration in a reproducible manner. We have shown that the encapsulation process is safe for the cells and that injection of the droplets do not damage the cells or rupture the hydrogel. Moreover, in vivo injection of these droplets could easily be performed and the support layer around the cells protected them and promoted cell spread and survival. Obviously, further in vivo experiments should be conducted in order to evaluate the capacity of the system to regenerate specific defected organs. Finally, the use of a personalized hydrogel with the patient's own cells was previously shown to provoke significantly lower immune response, compared to hydrogels originated from animals' ECM [4]. The use of fully personalized cellular droplets where both the cells and the biomaterials are used for encapsulation may have an immunological advantage over the use of other materials.

Looking forward, while some diseases, such as myocardial infarction or liver cirrhosis may require engraftment of large tissue patches, treatments for other diseases, such as Parkinson's disease or amyotrophic lateral sclerosis (ALS), may mostly benefit from delivery of cells to distinct regions of the CNS. However, when cells are injected directly to target sites, they do not form rapid cell-matrix interactions, and most of the injected cells die [1, 26]. In these cases, cell encapsulation in a supporting matrix is needed to maximize the efficacy of the treatment and to provide a supporting microenvironment to the cells [27].

Another use for the droplet system may be for developing efficient differentiation microenvironments for stem cells [28, 29]. The encapsulation of iPSCs within a picoliter volume may allow to study the differentiation process of the cells in a more controlled environment. In the small scale environment, the cells are not exposed to gradients of the small molecules and growth factors that are used for dictating their fate.

4 Experimental section

Oxygen concentration mathematical modeling

Oxygen concentration mathematical modeling was performed using COMSOL multi-physics program. General assumptions and mass balance equations are detailed in the supplementary section.

Omentum hydrogel preparation

Omenta were decellularized as previously described [4]. Omenta from healthy pigs (Kibutz Lahav, Israel) were washed with phosphate buffered saline (PBS) (At least 5 pig omenta were used). Then, transferred to hypotonic buffer (10 mM Tris, 5 mM ethylenediaminetetraacetic acid (EDTA) and 1 μ M phenylmethanesulfonyl-fluoride (PMSF), pH 8.0) for 1 hour. Next, tissues were frozen and thawed 3 times in the hypotonic buffer. The tissues were washed gradually with 70% (v/v) ethanol and 100% ethanol for 30 min each. Lipids were extracted by three, 30 min washes of 100% acetone, followed by 24h incubation in a 60/40 (v/v) hexane: acetone solution (solution was exchanged 3 times in 24h). The defatted tissue was washed in 100% ethanol for 30 min and incubated O.N. at 4°C in 70% ethanol. Then, the tissue was washed four times with PBS (pH 7.4) and incubated in 0.25% Trypsin–EDTA solution (Biological Industries) O.N. The tissue was washed thoroughly with PBS and incubated in 1.5 M NaCl (solution was exchanged 3 times in 24h), followed by washing in 50 mM Tris (pH 8.0), 1% triton-X100 (Sigma-Aldrich) solution for 1 h. The decellularized tissue was washed in PBS followed by double distilled water and then frozen (20°C) and lyophilized. The dry, decellularized omentum was ground into powder (Wiley Mini–Mill, Thomas Scientific, Swedesboro, NJ). The milled omentum was then enzymatically digested for 96 h at RT with stirring, in a 1 mg/ml solution of pepsin (Sigma-Aldrich, 4000 U mg⁻¹) in 0.1 M HCl. Subsequently, pH was adjusted to 7.4 using 5M NaOH and either DMEM/F12 X10 or PBS X10 (Biological industries). The final concentration of decellularized omentum in the titrated solution was 1% (w/v).

Neonatal cardiac cell isolation

Neonatal cardiac cells were isolated according to Tel Aviv University ethical use protocols from intact ventricles of 1 to 3-day-old neonatal Sprague-Dawley rats as previously reported [30]. Cells were isolated using 6 cycles (37°C, 30 min, each) of enzyme digestion with collagenase type II (95U/mL) and pancreatin (0.6 mg/mL) in DMEM (Biological industries, Beit-Haemek, Israel). After each round of digestion, cells were centrifuged (600 g, 5 min) and re-suspended in M-199 culture medium supplemented with 0.6 mM CuSO₄•5H₂O, 0.5 mM ZnSO₄•7H₂O, 1.5 mM vitamin B12, 500 U/mL penicillin and 100 mg/mL streptomycin, and 0.5% fetal bovine serum (FBS). To enrich the cardiomyocyte population, cells were suspended in culture medium with 5% FBS and were pre-plated twice for 45 min.

Cell number and viability were determined by a hemocytometer and trypan blue exclusion assay.

iPSCs Culture

iPSCs were generated from omental stromal cells and were a kind gift from Dr. Rivka Ofir, Ben Gurion University. The undifferentiated cells were cultivated on culture plates, pre coated with Matrigel™ (BD, Franklin Lakes, New Jersey), diluted to 250 µg/mL in DMEM/F12 (Biological Industries, Beit HaEmek, Israel). Cells were maintained in NutriStem™ (Biological Industries) medium containing 1% Penicillin/Streptomycin (Biological Industries) and cultured under a humidified atmosphere at 37°C with 5% CO₂. Medium was refreshed daily and cells were passaged weekly by treatment with 1 U/mL dispase (Stemcell Technologies, Vancouver, Canada) followed by mechanical trituration.

Cardiomyocyte differentiation from iPSCs

Cells were differentiated as previously described [4, 24]. Briefly; Growth media (NutriStem™) was refreshed daily until iPSCs reached 100% confluence. The undifferentiated cells were mixed homogenously with 1.5% (w/v) omentum hydrogel solution that was heated to 37°C to crosslink the 3D hydrogel. Culture medium was refreshed daily (NutriStem™) until iPSCs reached 100% confluence (1-3 days). At this point (day 0) medium was changed to RPMI (Biological Industries) supplemented with 0.5% glutamine (Biological Industries), B27 minus Insulin (X50, Invitrogen, Carlsbad, California) and 10 µM CHIR-99021 (Tocris, Bristol, UK). Medium was refreshed every other day. At day 2, CHIR-99021 was removed from media. At day 4, 5µM IWP-2 (Tocris) was added to the media and was removed on day 6. At day 8, contracting implants were observed and medium was changed to a medium supplemented with 0.5% glutamine, B27 minus retinoic acid (X50, Invitrogen), and 1µM retinoic acid (Sigma-Aldrich). After day 10, medium was changed to M-199 (Biological Industries), supplemented with 500 U/mL penicillin, 100 mg/mL streptomycin, 5% fetal bovine serum (FBS, Biological Industries), 0.6 mM CuSO₄•5H₂O, 0.5 mM ZnSO₄•7H₂O, 1.5 mM vitamin B12 (Sigma-Aldrich), this media was refreshed every other day.

Isolation of iPSCs-derived cardiac cells from the hydrogel

Cells from the omentum hydrogel were isolated using 6 cycles (30 min each) of enzyme digestion with collagenase type II (95U/mL, Worthington, Lakewood, new Jersey) and pancreatin (0.6 mg/mL, Sigma-Aldrich) in DMEM (37°C, 30 min). After each round of digestion, cells were centrifuged (100 g, 5 min) and re-suspended in M-199 culture medium supplemented with 0.6 mM CuSO₄•5H₂O, 0.5 mM ZnSO₄•7H₂O, 1.5 mM vitamin B12, 500 U/mL penicillin, 100 mg/mL streptomycin and 0.5% (v/v) FBS.

Microfluidics

Microfluidic device design: Microfluidic devices were fabricated by soft lithography. Negative photo resist SU-8 (3050, MicroChem, Corp.) was spin coated onto a clean silicon wafer (300 µm thick, University Wafer, Boston, MA) at 50 µm thickness and patterned by UV exposure through a transparency photomask. A mixture of Sylgard 184 poly

(dimethylsiloxane) (PDMS) (Dow Corning Corp, Midland, Michigan) and cross-linker (10:1) was poured, degassed and cured at 65°C for 1 hour. PDMS molds were peeled and the device inlets and outlets were punctured using a 0.75 mm diameter biopsy punch (World Precision Instruments, Sarasota, FL). PDMS replicas were bonded to a glass slide after oxygen-plasma activation of both surfaces using oxygen plasma (Diener Electronic GmbH & Co, Ebhausen, Germany). Finally, the device channels (50 µm in height and width) were washed using Aquapel (PPG Industries, Pittsburgh) and air dried immediately.

Droplets generation

Cells were counted, centrifuged and suspended in 4:1 omentum-hydrogel: culture medium mix to a final concentration of 0.8% (w/v) omentum ECM (final cell concentration: $50 \times 10^6 \text{ mL}^{-1}$). The hydrogel mixture was then loaded into a plastic 1 ml syringe (pic solution, Grandate, Italy). A mixture of 2% Pico-Surf 2 surfactant in Novec-7500 oil (Sphere fluidics, Cambridge, UK) was used as outer phase. Two Fine Bore Polythene tubes (Smiths Medical International Ltd, Ashford, UK) with an outer diameter of 1.09 mm and an inner diameter of 0.38 mm were used to connect the device inlets with the syringes. Flow rates were controlled by an NE-1000 syringe pump (New era pump systems, Farmingdale, NY). Droplet generation was monitored using a Dino lite edge digital microscope (Dino-lite digital microscope, New Taipei City, Taiwan). Flow rates of $80 \mu\text{L h}^{-1}$ (oil phase) and $40 \mu\text{L h}^{-1}$ (hydrogel phase) were used to obtain desired droplet size of 80 µm. Generated droplets were transferred immediately to 37°C for 10 min, for gelation. After gelation, droplets were mixed with 20 % (v/v) 1H,1H,2H,2H-perfluoro-1-octanol (PFO, Sigma Aldrich, 370533) in perfluoro-compound FC-40 (Sigma aldrich, 51142-49-5), which is used as an emulsion destabilizer. After addition of M-199 medium (water phase), the supernatant was transferred to 40 µm nylon mesh strainer (located in 6-well plate) and droplets were cultured for 7-15 days at 37°C.

Immunostaining, confocal imaging and calcium imaging

Cells/tissues were fixed in 4% formaldehyde, permeabilized with 0.05% (v/v) triton X-100, blocked with PBS containing 1% bovine serum albumin (BSA) and 10% FBS and stained with primary antibodies followed by secondary antibodies (as indicated in the antibody list-supplementary information). Cells/tissues were imaged using an upright confocal microscope (Nikon ECLIPSE NI-E) and inverted fluorescence microscope (Nikon ECLIPSE TI-E). Images were processed and analyzed using NIS elements software (Nikon Instruments). Representative images from at least 3 different biological experiments were chosen. For calcium imaging, the cardiac droplets were incubated with 10 µM fluo-4 AM (Invitrogen) and 0.1% Pluronic F-127 (Sigma-Aldrich) for 45 min at 37 °C. Cardiac droplets were then imaged using an inverted fluorescent microscope (Nikon Eclipse TI). Videos were acquired with an ORCA-Flash 4.0 digital complementary metal-oxide semiconductor (CMOS) camera (Hamamatsu Photonics) at 100 frames/s.

Migration assay

Neonatal cardiac cells were isolated and encapsulated using the microfluidics system. Some cellular droplets were placed on 24 tissue culture well plates and migration distances of cells outside the droplets were quantified based on BF images taken on days 3, 6, 11 and 15 (4

perpendicular lines were used to quantify the average migration distance outside the droplet, for each image). On day 1, Other Cellular droplets were stained using Vybrant DiI cell-labeling solution (V22885, Thermo Fisher scientific) for 30 min. droplets were washed with fresh M-199 medium and placed on neonatal cardiac cells sheet (500,000 cardiac cells seeded on 24 well on the day of isolation). On day 7 of culture, Calcium imaging was performed.

Viability assay

Cell viability was determined using a Live/Dead fluorescent staining with fluorescein diacetate (Sigma-Aldrich, 7 µg/mL) and propidium Iodide (Sigma-Aldrich, 5 µg/mL) for 10 min at 37°C. The number of live and dead cells was determined by manual counting using NIS Elements software (Nikon) from at least 3 different microscopic field (n = 3 in each experiment), visualized by inverted fluorescence microscope (Nikon Eclipse TI).

Prestoblué viability assay

Cells were incubated with Prestoblué™ cell viability reagent in M-199 cell culture medium (1:19) for 1.5 hour at 37°C. Cell viability was determined by following fluorescently Prestoblué™ reagent at 560 nm excitation and 590 nm emission using Tecan platereader-infiniteM200pro. All values were normalized to day 0.

Scanning electron microscopy (SEM)

Encapsulated CMs samples on days 0 and 7 of culture were fixed with 2.5% glutaraldehyde (24 h at 4°C), followed by graded incubation series in ethanol–water solutions (25–100% (v/v)). All samples (n = 3) were critical point dried, sputter-coated with gold in a Polaron E 5100 coating apparatus (Quorum technologies, Lewis, UK) and observed under JSM-840A SEM (JEOL, Tokyo, Japan).

Statistical Analysis

Statistical analysis data are presented as means ± s.d. Differences between samples were assessed by student's t-test. $p < 0.05$ was considered significant. ns denotes not significant. Analyses were performed using GraphPad prism version 6.00 for windows (GraphPad Software).

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

T.D. received support from European Research Council Starting Grant 637943, the Slezak Foundation, the Israeli Science Foundation (700/13), the Israel Ministry of Science, Technology and Space (3-12587) and Moxie Foundation.

References

1. Mitrousis N, Fokina A, Shoichet MS. Nature Reviews Materials. 2018; 3(11):441–456. DOI: 10.1038/s41578-018-0057-0

2. Foyt DA, Norman MD, Tracy T, Gentleman E. *Advanced healthcare materials*. 2018; 7(8)
3. Walters NJ, Gentleman E. *Acta biomaterialia*. 2015; 11:3–16. [PubMed: 25266503]
4. Edri R, Gal I, Noor N, Harel T, Fleischer S, Adadi N, Green O, Shabat D, Heller L, Shapira A. *Advanced Materials*. 2018
5. Patel RS, Makitie AA, Goldstein DP, Gullane PJ, Brown D, Irish J, Gilbert RW. *Head & Neck: Journal for the Sciences and Specialties of the Head and Neck*. 2009; 31(5):655–663.
6. Shelton EL, Poole SD, Reese J, Bader DM. *Journal of tissue engineering and regenerative medicine*. 2013; 7(6):421–433. [PubMed: 22318999]
7. Collins D, Hogan AM, O'Shea D, Winter DC. *Journal of Gastrointestinal Surgery*. 2009; 13(6):1138–1146. [PubMed: 19291335]
8. Stevens K, Kreutziger K, Dupras S, Korte F, Regnier M, Muskheli V, Nourse M, Bendixen K, Reinecke H, Murry C. *Proceedings of the National Academy of Sciences*. 2009; 106(39):16568–16573.
9. Wan J. *Polymers*. 2012; 4(2):1084–1108.
10. Chan HF, Zhang Y, Leong KW. *Small*. 2016; 12(20):2720–2730. [PubMed: 27038291]
11. Schmidt JJ, Rowley J, Kong HJ. *Journal of Biomedical Materials Research Part A: An Official Journal of The Society for Biomaterials, The Japanese Society for Biomaterials, and The Australian Society for Biomaterials and the Korean Society for Biomaterials*. 2008; 87(4):1113–1122.
12. Leslie SK, Nicolini AM, Sundaresan G, Zweit J, Boyan BD, Schwartz Z. *Journal of Materials Chemistry B*. 2016; 4(20):3515–3525.
13. Zhang W, He X. *Journal of healthcare engineering*. 2011; 2(4):427–446. [PubMed: 22180835]
14. Searle, S, Porras, AM, Barbe, L, Shi, L, Ossipov, D, Trau, D, Tenje, M. *Hyaluronic acid based hydrogel droplets: A potential injectable cell culture scaffold*. Vol. 2018. EUROoC; Stuttgart, Germany: 2018 May 24 and 35.
15. Wang L, Rao RR, Stegemann JP. *Cells Tissues Organs*. 2013; 197(5):333–343. [PubMed: 23571151]
16. Kumachev A, Greener J, Tumarkin E, Eiser E, Zandstra PW, Kumacheva E. *Biomaterials*. 2011; 32(6):1477–1483. [PubMed: 21095000]
17. Ma S, Natoli M, Liu X, Neubauer MP, Watt FM, Fery A, Huck WT. *Journal of Materials Chemistry B*. 2013; 1(38):5128–5136.
18. Mattei G, Magliaro C, Giusti S, Ramachandran SD, Heinz S, Braspenning J, Ahluwalia A. *PloS one*. 2017; 12(3):e0173206. [PubMed: 28267799]
19. Thygesen K, Alpert JS, White HD. *Journal of the American College of Cardiology*. 2007; 50(22):2173–2195. [PubMed: 18036459]
20. Radisic M, Euloth M, Yang L, Langer R, Freed LE, Vunjak-Novakovic G. *Biotechnology and bioengineering*. 2003; 82(4):403–414. [PubMed: 12632397]
21. Ahearne M. *Interface focus*. 2014; 4(2)
22. Ferreira SA, Motwani MS, Faull PA, Seymour AJ, Tracy T, Enayati M, Taheem DK, Salzlechner C, Haghighi T, Kania EM. *Nature communications*. 2018; 9(1)
23. Jansen, K, Atherton, P, Ballestrem, C. *Mechanotransduction at the cell-matrix interface* *Seminars in cell & developmental biology*. Elsevier; 2017. 75–83.
24. Lian X, Hsiao C, Wilson G, Zhu K, Hazeltine LB, Azarin SM, Raval KK, Zhang J, Kamp TJ, Palecek SP. *Proceedings of the National Academy of Sciences*. 2012; 109(27):E1848–E1857.
25. Lev R, Seliktar D. *Journal of The Royal Society Interface*. 2018; 15(138)
26. Müller-Ehmsen J, Krausgrill B, Burst V, Schenk K, Neisen UC, Fries JW, Fleischmann BK, Hescheler J, Schwinger RH. *Journal of molecular and cellular cardiology*. 2006; 41(5):876–884. [PubMed: 16973174]
27. Marquardt LM, Heilshorn SC. *Current stem cell reports*. 2016; 2(3):207–220. [PubMed: 28868235]
28. Wilson JL, Najia MA, Saeed R, McDevitt TC. *Biotechnology and bioengineering*. 2014; 111(3):618–631. [PubMed: 24166004]

29. Allazetta S, Lutolf MP. Current opinion in biotechnology. 2015; 35:86–93. [PubMed: 26051090]
30. Dvir T, Benishti N, Shachar M, Cohen S. Tissue engineering. 2006; 12(10):2843–2852. [PubMed: 17518653]

Figure 1. Concept schematics.

An omentum specimen is extracted from the patient and while the ECM is processed into a personalized thermoresponsive hydrogel, the cells are reprogrammed to become iPSCs. Next, the cells are encapsulated within the hydrogel and efficiently differentiated to cardiomyocytes, generating functional tissue implants. A microfluidic system is used to generate cell-encapsulating microdroplets (using iPSCs-derived CMs or neonatal cardiac cells) that may be injected in the future to the infarcted heart.

Figure 2. Modelling oxygen concentration.

(a-c) Oxygen concentration modelling in bulk and droplets systems. Oxygen concentration in $(0.4 \text{ mm})^3$ of a bulky hydrogel after 20 min (a) and $(0.4 \text{ mm})^3$ of 100 μm droplets after 0.1 s (b) and 5s (c). (d) Single droplet oxygen concentration distribution within droplets with varying diameters.

Figure 3. Characterization and optimization of the microfluidic system.

(a) The microfluidic device used to generate the cellular droplets. (I) Macroscopic view of the device. (II) The device contained oil and hydrogel inlets (white and black arrows, respectively) and droplet outlet (yellow arrow). (III) Generation of cell-containing hydrogel emulsions in the system. (b) Generated droplets at the outlet of the device. (c) Homogenous cellular droplets. (d), (e) Droplet mean diameter generated in different oil (d) and hydrogel (e) flow rates. (f) Droplets size distribution histogram using 80 and 40 $\mu\text{L h}^{-1}$, oil and hydrogel flow rates, respectively. (g) Viability of encapsulated cells as assayed by live

(green)/dead (red) staining. (h) Viability of cells before and after encapsulation. (i-k). Brightfield images of droplets with 5 (i), 25 (j) and 50 (k) million cardiac cells/mL. (l) Average number of cardiac cells per droplet for the three tested concentrations. Scale bars: 100 μm .

Figure 4. Morphology and contractility of the encapsulated cardiac cells.

(a) Cardiac cell viability over 7 days. (b, c) SEM micrographs of cardiac cells in the hydrogel on day 0 (b) and day 7 (c). (d) Immunostaining of α -sarcomeric actinin (red), connexin-43 (green) and nuclei (blue). (e) Immunostaining of collagen (red), troponin T (green) and nuclei (blue). (f) Electrical signal analysis performed by calcium imaging on day 7. Scale bars: 10 μ m for b (left image), 5 μ m for b (right image), 50 μ m for c (left image), 20 μ m for c (right image), 50 μ m for d-f.

Figure 5. In vitro migration and function of encapsulated cardiac cells.

(a) Quantification of cell migration distances in an empty well plate, over a period of 15 days. (b-f) Cultivation of cellular droplets on cardiac cell sheet. b. Fluorescence image of DiI pre-stained encapsulated cardiac cells (red) on cardiac cells sheet. The green color is the calcium staining. (c) Heat map of calcium signal propagation. (d-f) Quantification of calcium signal. (d) Locations of quantification on the fluorescence image. (e) Analysis of calcium transients in the chosen locations. (f) The delay between the different spots. (g-i) Calcium imaging between two adjacent droplets. (g) Fluorescence image. (h) Heat map of the signal transfer between the droplets. (i) The delay in calcium transfer between the droplets. Scale bars: 100 μm .

Figure 6. Encapsulation of iPSCs-derived CMs.

(a) Scheme of the process: at first, iPSCs were differentiated to CMs inside the hydrogel. Cells were then extracted from the hydrogel using collagenase, mixed with the liquid ECM and were encapsulated in microdroplets using the microfluidic device. (b) Hydrogel implant immunostaining for OCT4 (green), ki67 (blue) and collagen (red), 13 days after iPSCs encapsulation. (c-d) Hydrogel implant immunostaining for α -sarcomeric actinin (pink), connexin-43 (green) and nuclei (blue) (c) and of troponin T (green), Nkx2-5 (red) and nuclei (blue) (d) at day 30 of differentiation. (e) Encapsulated cells were stained for troponin T (green) and nuclei (blue). (f) Electrical signal analysis performed after calcium imaging. Scale bars: 50 μ m for b-e.

Figure 7. Injection of cardiac cell droplets.

(a) Droplets mixed with liposomes before (left image) and after (right image) injection through a 25G needle. (b) Viability of cardiac cells before and after injection of the cellular droplets. (c) gastrocnemius mouse muscle stained for nuclei. Cells cytoplasm was labeled using cytopainter (green). (d-e) gastrocnemius mouse muscle stained for α -sarcomeric actinin (red), connexin-43 (green) and nuclei (blue) on day 2 after injection (d). (e) Colocalization of sarcomeric actinin (red) and the prestained cells (green). Nuclei appear in blue. Scale bars: 100 μ m for a and c, 20 μ m for d-e.