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ORIGINAL PAPER

Electrochemical release of hepatocyte-on-hydrogel microstructures from ITO substrates

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Abstract This paper describes a novel platform that utilizes micropatterning and electrochemistry to release cells-on-hydrogel microstructures from conductive indium tin oxide (ITO) substrates. In this approach, UV photopolymerization was employed to micropattern heparin-based hydrogels onto glass substrates containing ITO electrodes. ITO/glass substrates were first functionalized with acrylated silane to promote attachment of hydrogel structures. The surfaces containing hydrogel micropatterns were further functionalized with poly(ethylene glycol) thiol, rendering the regions around the hydrogel structures non-fouling to proteins and cells. After incubating surfaces with collagen (I), primary rat hepatocytes were shown to selectively attach on top of the hydrogel and not on surrounding glass/ITO regions. Electrical activation of specific ITO electrodes (−1.8 V vs. Ag/AgCl reference) was then used to release cells-on-hydrogel microstructures from the substrate. Immunostaining and reverse transcription polymerase chain reaction analysis of albumin, an important indicator of hepatic function, showed that the hepatocyte-on-hydrogel microstructures released from the surface maintained their function at levels similar to hepatocytes remaining on the culture substrate. In the future, switchable conductive substrates described here may be to collect cell samples at different time points and may also be used for harvesting cell-carrying vehicles for transplantation studies.

Keywords Hepatocytes · Micropatterned surfaces · Cell sorting · Electrochemical release · Heparin-based hydrogel

The first two authors contributed equally.

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Introduction

Designing biological interfaces for cultivation of primary cells is essential for cell therapy and tissue engineering applications. In order to improve the understanding of signals contributing to cellular phenotype, it is essential to mimic the complexity of the in vivo microenvironment when creating an in vitro cell culture system [1–3]. One technology proving to be instrumental in this effort is hydrogels. Hydrogels are biomaterials that have been used extensively for designing cellular interactions in vitro [4, 5]. These gels can be biofunctionalized by incorporating short peptide motifs to promote cell attachment/encapsulation or to release of signaling molecules such as growth factors [4–9].

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Recently, the Tae laboratory described a biofunctional hydrogel formed by Michael-type addition reaction of thiolated heparin and acrylated poly(ethylene glycol) (PEG) chains [10, 11]. Heparin is a glycosaminoglycan that is known to sequester various growth factors (GFs) and extracellular matrix proteins via secondary bond formation [10, 12, 13]. Unlike gels functionalized with short peptide motifs, this gel contained intact heparin molecules (MW 12 kDa) and proved to be an excellent matrix for GF release [10, 14, 15] as well as cell encapsulation and cultivation [15, 16]. In a more recent study, heparin hydrogels were polymerized using UV-initiated thiol-ene polymerization reaction. Illuminating the prepolymer with UV light through a photomask resulted in formation of hydrogel microstructures adherent to glass in the exposed regions [17]. These micropatterned hydrogels were able to sequester and release growth factors, and proved suitable for maintenance of difficult-to-culture primary hepatocytes [17].

It is frequently desirable to release cells from the surface for recultivation, transplantation, or downstream analysis. Electrical stimulation is particularly appealing as a method for controlling composition of biointerface [18] and for guiding cell–surface interactions [19–26]. In this approach, minimal handling of the culture substrate is required; cell culture media may serve as an electrolyte, and the site as well as time of stimulation may be controlled precisely through the use of electrode arrays [24, 25, 27]. The majority of electrochemical switching strategies have focused on altering properties of gold substrates modified with self-assembled monolayers [18, 20, 21]. In addition to changing the conformation of the molecules on the conductive substrate, electrical stimulation can be used to remove the coupling layers and any biomolecules associated with the layer. We have employed reductive desorption to remove cells and proteins from conductive gold substrates [25, 28]. Additionally, Inaba et. al showed release of adherent cells, cell sheets, and cellular spheroids by electrical stimulation of alkanethiol molecules from gold substrates [26].

While gold is an excellent electrode material, indium tin oxide (ITO) provides optical transparency in addition to high conductivity, thus making the platform particularly amenable for cell cultivation and observation. Using ITO instead of gold not only eliminates the need for expensive reflective microscope, researchers have shown its use in building transparent cell chambers for visualizing cells over an extended period of time [29, 30]. Several groups, including ours, have demonstrated electrochemical modulation of ITO surfaces to control protein and cell attachment [24, 27, 31–33].

The majority of cell release approaches mentioned above refer to detachment of "naked' cells from the surface. A smaller number of reports described detachment cells atop polymer objects. Allbritton and co-workers developed strategies for laser-mediated [34] as well as physical retrieval [35] of polymer micropallets carrying cells from the culture surface. The present work is different from these excellent published reports in that our goal has been to characterize interactions of hepatocytes with heparin-based hydrogel microstructures and to demonstrate that hepatocytes remained functional atop hydrogels after electrochemical release. The use of micropatterned, addressable ITO electrodes offers a number of advantages over these previous efforts including relative simplicity (compared with laser retrieval) and excellent spatial addressability (compared with physical retrieval).

In this article, we describe a novel approach involving micropatterning and electrochemistry to release cell-coated bioactive hydrogels from conductive ITO substrates. Unlike the previous reports describing release of single cells or cell sheets from either self-assembled monolayers [25, 26] or polyelectrolyte layers [32, 33], the present paper focuses on the release of three-dimensional bioactive micro-scaffolds that can be used as cell carriers for future in vivo implantation studies. This study builds on several preceding reports from our group that describe desorption of silane molecules from ITO electrodes upon application of reductive potential [24, 31] and electrochemical detachment of millimeter-sized hydrogels anchored to ITO via silane coupling layer [36]. The present study demonstrates fabrication of heparin hydrogel microstructures on glass substrates containing ITO electrodes, cultivation of hepatocytes atop hydrogels, and release of hepatocyte-on-hydrogel microstructures upon electrical stimulation of conductive substrate. Significantly, primary hepatocytes remained functional atop heparin hydrogel microstructures 6 days after detachment from the surface suggesting that (1) electrochemical release did not damage the cells and (2) heparin hydrogels provided an excellent environment for maintenance of fragile primary cells. In the future, the electrochemical release strategy described here may be used to harvest cells along with microstructured gels for transplantation or for cell analysis.

Materials and methods

Chemicals and materials

ITO-coated glass slides (75×25 mm) were obtained from Delta Technologies (Stillwater, MN, USA). The ITO-coated glass slides had a sheet resistance of 4–8Ω with nominal transmittance of >82% and an ITO thickness of 1,500–2,000 Å. 3-(Acryloxypropyl) trichlorosilane was purchased from Gelest, Inc. (Morrisville, PA, USA). Poly(ethylene glycol) diacrylate (PEG-DA, MW 3.4 and 6 kDa, 98% degree of substitution) and methoxyl poly(ethylene) glycol thiol (PEG-SH, MW 5 kDa) were purchased from SunBio

Inc. (Anyang, Korea). 4-(2-Hydroxyethoxy) phenyl-(2-hydroxy-2-propyl) ketone (Irgacure 2959) was purchased from Ciba Specialty Chemicals Inc. (Basel, Switzerland). Sulfuric acid, hydrogen peroxide, hydrochloric acid, nitric acid, ethanol, acetone, anhydrous toluene, collagen from rat tail (type I), epidermal growth factor (EGF), collagenase type IV, bovine serum albumin (BSA), and toluidine blue O were purchased from Sigma-Aldrich (St. Louis, MO, USA). Glucagon and recombinant human insulin were obtained from Eli Lilly and Company (Indianapolis, IN, USA), and hydrocortisone sodium succinate was obtained from Pfizer Inc. (Ann Arbor, MI, USA). Concentrated phosphate-buffered saline (10× PBS) was purchased from Lonza (Walkersville, MD, USA). Dulbecco's modified Eagles' medium (DMEM), minimal essential medium (MEM), sodium pyruvate, non-essential amino acids, fetal bovine serum (FBS), and penicillin/streptomycin were purchased from Invitrogen Life Technologies (Carlsbad, CA, USA). Cell proliferation reagent WST-1 was purchased from Roche Ltd (Basel, Switzerland). Sheep anti-rat albumin was obtained from Bethyl Laboratories (Montgomery, TX, USA). Formalin was purchased from Fisher (Pittsburgh, PA, USA). Goat anti-sheep antibody conjugated with fluorescein isothiocyanate was purchased from Santa Cruz Biotechnologies, Inc. (Santa Cruz, CA, USA). Mounting medium with 4',6-diamidino-2-phenylindole (DAPI) was purchased from Vector Laboratories, Inc. (Burlingame, CA, USA).

Microfabrication of ITO electrodes

The microfabrication of ITO electrodes involved photoresist lithography followed by wet etching. ITO-coated glass substrates were dehydrated at 200 °C for 24 h prior to photoresist patterning. Positive photoresist (AZ 5214-E) was spin-coated on ITO substrate at 800 rpm for 10 s followed by 4,000 rpm for 30 s. The photoresist-coated slide was soft-baked on a hot plate at 100 °C for 105 s. After baking, the photoresist layer was exposed to UV light (10 mW/cm^2) for 45 s using a Canon PLA-501 F Mask Aligner. Exposed photoresist was then developed for 5 min in AZ 300 MIF developer solution, briefly washed with deionized (DI) water to remove residual developing solution, and dried under nitrogen. The substrates were then hard baked for 30 min at 120 °C and placed in ITO etchant for 20 min. The composition of ITO etchant was 20% *v/v* hydrochloric acid, 5% *v/v* nitric acid, and 75% DI water. After etching, the remaining photoresist was removed from the substrate via sonication in acetone for 20 min. The substrate was then washed with DI water and dried with nitrogen. This resulted in the fabrication of individually addressable ITO regions on glass.

Surface modification of ITO electrodes

Prior to silane modification, the glass slides, silicon pieces, and ITO/glass substrates were cleaned in an oxygen plasma chamber (YES-R3, San Jose, CA, USA) at 300 W for 5 min. Surfaces were then placed for 10 min in a 0.05% solution of 3-acryloxypropyl trichlorosilane diluted in anhydrous toluene. The silanization reaction was carried out in a glove bag under nitrogen purge to eliminate atmospheric moisture. After modification, glass slides or ITO electrodes were rinsed with fresh toluene, dried under nitrogen, and cured at 100 °C for 2 h. Silane-modified substrates were placed in a desiccator until further use. Contact angle measurements (Rame-Hart goniometer) were routinely performed to assess the silane quality. In addition, silane assembly was characterized using ellipsometry (LSE Stokes ellipsometer, Gaertner Scientific). In these experiments, 4-in. silicon wafers (Wafer World) were diced into smaller pieces (0.5×0.5 in.) and identically modified using silane. Presence of the acrylated silane layer was determined using optical constants of clean silicon substrate and taking refractive index to be 1.45. Ellipsometry measurements from at least three regions of the same substrate were collected to obtain an average thickness for each sample. The number of samples tested for contact angle and ellipsometry experiments was $n=3$.

Micropatterning of hydrogels on silane-modified surfaces

Heparin-based hydrogels were prepared by UV-initiated polymerization of thiolated heparin (Hep-SH) and diacrylated poly (ethylene glycol) (PEG-DA). Forty percent thiolated Hep-SH, prepared as reported previously [11], and 6 kDa PEG-DA (1:1 molar ratio of thiol group and acrylate group) were dissolved in PBS containing 1% *w/v* photo-initiator, 4-(2-hydroxyethoxy) phenyl-(2-hydroxy-2propyl) ketone (Irgacure 2959) in 70% *v/v* ethanol. This precursor solution was pipetted onto the silane-treated glass and then covered with a cover slip (25×25×0.13 mm) to create a uniform prepolymer layer sandwiched between the two substrates. Next, a photomask was placed on top of the liquid prepolymer layer, and the substrate was exposed to 365 nm, 18 W/cm^2 UV light for 10 s using an OmniCure series 1,000 light source (EXFO, Vanier, Quebec, Canada). The regions exposed to UV light underwent thiol-ene polymerization and became cross-linked, while unexposed prepolymer was dissolved after immersion in DI water. Manual dispensing of prepolymer allowed us to conserve custom-synthesized thiolated heparin (only 10 μL prepolymer solution was needed to micro-pattern each 1×1 in. glass piece). However, exposure of the prepolymer layer through a 130-μm thick glass cover slip limited hydrogel feature resolution to ~30 μm in diameter and ~25 μm in thickness. The use of spin-coating and elimination of cover slip from the process should allow

fabrication of much smaller features (~5 μm) as described in our previous work [37]. Toluidine blue O staining was performed as described by Gosey et al. [38]. Because toluidine blue O stains negatively charged biomolecules such as heparin, this staining was used to visualize hydrogel micropatterns. Zeiss Axiovert 40 (Carl Zeiss, NJ, USA) was used for optical microscopy.

Attachment of PEG-SH on micropatterned glass surfaces

To prevent cell adhesion on regions around the heparin hydrogel micropatterns, substrates were treated with a solution of 10 mg/mL methoxyl poly(ethylene) glycol thiol (PEG-SH, MW 5 kDa) dissolved in 1× PBS solution. This treatment was expected to result in Michael-type addition reaction of PEG-thiol with acrylated silane molecules on glass surface. The micropatterned surfaces were incubated in PEG-SH solution for 24 h at 37 °C in the dark, and then were washed three times in 1× PBS solution and stored in PBS solution until further use. Contact angle measurements and ellipsometric measurements were carried out to check attachment of PEG-SH on acrylated silane. Deposition of PEG-SH layer on top of acrylated silane was also examined by the secondary ion mass spectrometry method (SIMS).

The SIMS setup consisted of a custom-built C_{60}^{+} ion source coupled with time-of-flight (ToF) mass spectrometer. C_{60}^{+} ions were accelerated to +10 keV towards a negatively biased target (−5 keV), which resulted in a total impact energy of 15 keV. C_{60}^{+} projectiles impacting the target stimulate emission of the secondary ions [39]. The secondary ions were mass selected and detected by ToF mass spectrometer. The observations were made in the event-by-event bombardment-detection mode at the limit of single projectile impacts (super static regime of bombardment) [40]. Each spectrum is a summation of at least 2×10^{6} impact events over an impact area of ~10^{-2} mm^2. ToF–SIMS analysis was carried out to check for deposition of collagen on acrylate, PEG-SH, and heparin-based hydrogel regions of micropatterned surfaces.

Adhesion of cells on hydrogel micropatterns

Hepatoma cells (HepG2 cells) were maintained in MEM supplemented with 10% FBS, 200 U/mL penicillin, 200 μg/mL streptomycin, 1 mM sodium pyruvate, and 1 mM non-essential amino acids at 37 °C in a humidified 5% CO2 atmosphere. Our studies also employed primary rat hepatocytes. Cells were isolated from adult female Lewis rats (Charles River Laboratories, Boston, MA) weighing 125–200 g, using a two-step collagenase perfusion procedure as described previously [41]. Typically, 100–200 million hepatocytes were obtained with a viability of >90% as determined by trypan blue exclusion. Primary hepatocytes were maintained in DMEM supplemented with EGF, glucagon, hydrocortisone sodium succinate, recombinant human insulin, 200 units/mL penicillin, 200 μg/mL streptomycin, and 10% FBS. Prior to cell seeding, hydrogel micropatterns were formed on silane-coated ITO electrodes using the protocol described earlier. Regions around the micropatterns were passivated with a non-fouling PEG-SH layer. The substrates were then incubated with collagen I solution (0.1 mg/mL) for 45 min, washed in 1× PBS solution, and rinsed in DI water.

The surfaces were diced into 1×1-in. pieces and placed into a six-well plate and exposed to 3 mL of rat primary hepatocytes suspended in culture medium at a concentration of 1×10^{6} cells/mL. After 1 h of incubation at 37 °C, hepatocytes became localized on collagen regions on top of the hydrogel but did not attach around the hydrogel, as these regions were protected by PEG-SH. The samples were then washed twice in PBS to remove unbound hepatocytes, and fresh media was added. HepG2 cells were incubated on the surface using the same protocol as described earlier.

Desorption of heparin-based hydrogel from ITO electrodes

Experiments aimed at desorbing acrylated silane and releasing heparin hydrogel micropatterns from ITO electrodes were carried out in a custom-made Plexiglass electrochemical cell. After making heparin micropatterns on silane modified ITO electrodes, a steel wire was attached to contact pads of the electrodes using temperature curable conductive (silver) epoxy (EPO-TEK®, Billerica MA). The electroactive substrate was then secured in an electrochemical cell and immersed in 500 μL of 1× PBS acting as an electrolyte solution. Ag/AgCl reference and Pt counter electrodes were positioned in the same electrochemical cell with ITO region serving as a working electrode. In silane desorption studies, a potentiostat (CH Instruments) was used to apply −1.8 V for 60 s to an ITO electrode of interest. After silane stripping, the ITO substrate was washed with 1× PBS to release the hydrogels from the surface. The same protocol was used to release cell-coated hydrogels from ITO.

Analysis of hepatic function

Proliferation of HepG2 cells on top of heparin hydrogel microstructures was assessed using standard WST-1 assay. At each measurement point, WST-1 was added for 4 h, and the colorimetric absorbance of the produced formazan at 450 nm was measured using a microplate reader (Thermo max microplate reader, Molecular Devices, Sunnyvale, CA, USA). For detecting intracellular albumin, samples with primary hepatocytes were first fixed with 4% formalin

solution for 30 min followed by three washes with 1× PBS solution. The samples were then blocked with a mixture of 1% BSA solution and 0.3% TritonX 100. The samples were then incubated with sheep anti-rat serum albumin antibody (1:100 dilution in 1× PBS) for 1 h at room temperature. After incubation, samples were washed twice in 1× PBS solution. The samples were then stained with anti-sheep IgG conjugated with fluorescein isothiocyanate (1:200 dilution in 1× PBS) for 1 h at room temperature followed by a two-time rinse in 1× PBS solution. Finally, samples were mounted using a mounting medium containing DAPI to determine the locations of nuclei. Stained cells were visualized and imaged using a confocal microscope.

For real-time reverse transcription polymerase chain reaction (RT-PCR) experiments, hepatocytes were cultured on hydrogel micropatterns for 2 days and were then released from the surface. After detachment, cells-on-gels were cultivated in a six-well plate for up to 6 days. For analysis, cells-on-gels were stabilized in 200 μL lysis buffer and stored at −20 °C. Total RNA was extracted from the cell lysates using absolute total mRNA isolation microprep kit (Stratagene) according to the manufacturer's instructions. cDNA was synthesized using 12 μL of DNAse pretreated total mRNA according to the instructions from the QuantiTect Reverse Transcription kit (Qiagen). Quantitative real-time PCR was performed using SYBR Green PCR Master Mix (Applied Biosystems). Primers for rat albumin (forward: 5′-CATCCTGAACCGTCTGTGTG and reverse: 5′-TTTCCACCAAGGACCCACTA) and GAPDH (forward: 5′-AGACAGCCGCATCTTCTTGT and reverse: 5′-CTTGCCGTGGGTAGAGTCAT) genes were selected from a database (http://medgen.ugent.be/rtprimerdb). Primer (Sigma Genosys) concentrations were optimized before use. SYBR Green Master Mix (1×) was used with 1 μL of forward and reverse primers in a total volume of 12 μL that also included 1 μL cDNA. All PCR reactions were done in duplicates. PCR amplification was performed as follows—95 °C for 10 min, 40 cycles of 95 °C for 15 s, 60 °C for 10 s, and 68 °C for 1 min on Masterecycler Realplex (Eppendorf). The comparative *Ct* value method, using housekeeping gene (GAPDH) as an internal standard, was employed to determine relative levels of albumin gene expression. In a parallel experiment, cells cultured on top of hydrogel microstructures adherent to glass slides were trypsinized form the surface and lysed to perform RT-PCR experiments as described above.

Results and discussion

This paper describes electroactive substrates with hydrogel microstructures for cultivation and release hepatocytes (see Fig. 1). The electrochemical sorting approach may be used in the future to harvest cell/hydrogel constructs for the purposes of transplantation or cell analysis.

Electrochemical desorption of heparin-based hydrogels from ITO electrodes

Initially, we wanted to demonstrate release of hydrogel microstructures without cells. Heparin hydrogels were fabricated on ITO/glass substrates by photolithography-like process [17] involving exposure of the photosensitive prepolymer to UV through a photomask. Figure 2a, d show 300 μm diameter rings and 500 μm diameter hydrogel discs fabricated on ITO/glass substrates. These microstructures were stained with toluidine blue O dye (red/purple color) that stains negatively charged biomolecules such as heparin. The staining of microstructures in Fig. 2a, d is due to presence of negatively charged heparin molecules in the hydrogel. Gels formed without heparin were not stained.

Based on our previous work, detachment of the hydrogels was expected to occur through electrochemical cleavage of silane anchors holding the gel structures to the substrate [24, 36]. A reductive potential of −1.8 V was applied to the ITO electrode to desorb the underlying silane layer, thereby unmooring hydrogel microstructures from the surface of the electrode. Electrochemical experiments were performed using a three-electrode set-up with Ag/AgCl reference, Pt counter, and ITO working electrodes. In all desorption experiments, the potential was held constant for 60 s. Figure 2b, e show typical ITO substrates after removal of hydrogel rings and disks upon electrical activation. Note that brown color occurred in regions where ITO substrate was free to interact with electrolyte during reductive desorption. This color change has been noted by others after voltage has been apply to ITO and has been attributed to Sn4+ in the ITO being reduced to the low-hydroxide state of Sn which attached to the surface, giving it the brown granular look [42]. The white regions (rings and disks in Fig. 2b and e, respectively) correspond to ITO regions initially covered by hydrogel microstructures during desorption experiments. Figure 2c, f show hydrogel constructs released from the surface upon electrochemical stimulation. Electrochemical desorption of the microstructures occurred at a high success rate of ~90%. As the hydrogel structures are covalently bound to the ITO surface through acrylated silane, microstructure detachment did not occur without electrical stimulation (over the course of 6 days).

It should be noted that hydrogel detachment reported in this paper was performed from two types of conductive substrates: (1) uniform ITO layer serving as an electrode and (2) patterned ITO electrodes. The hydrogel detachment

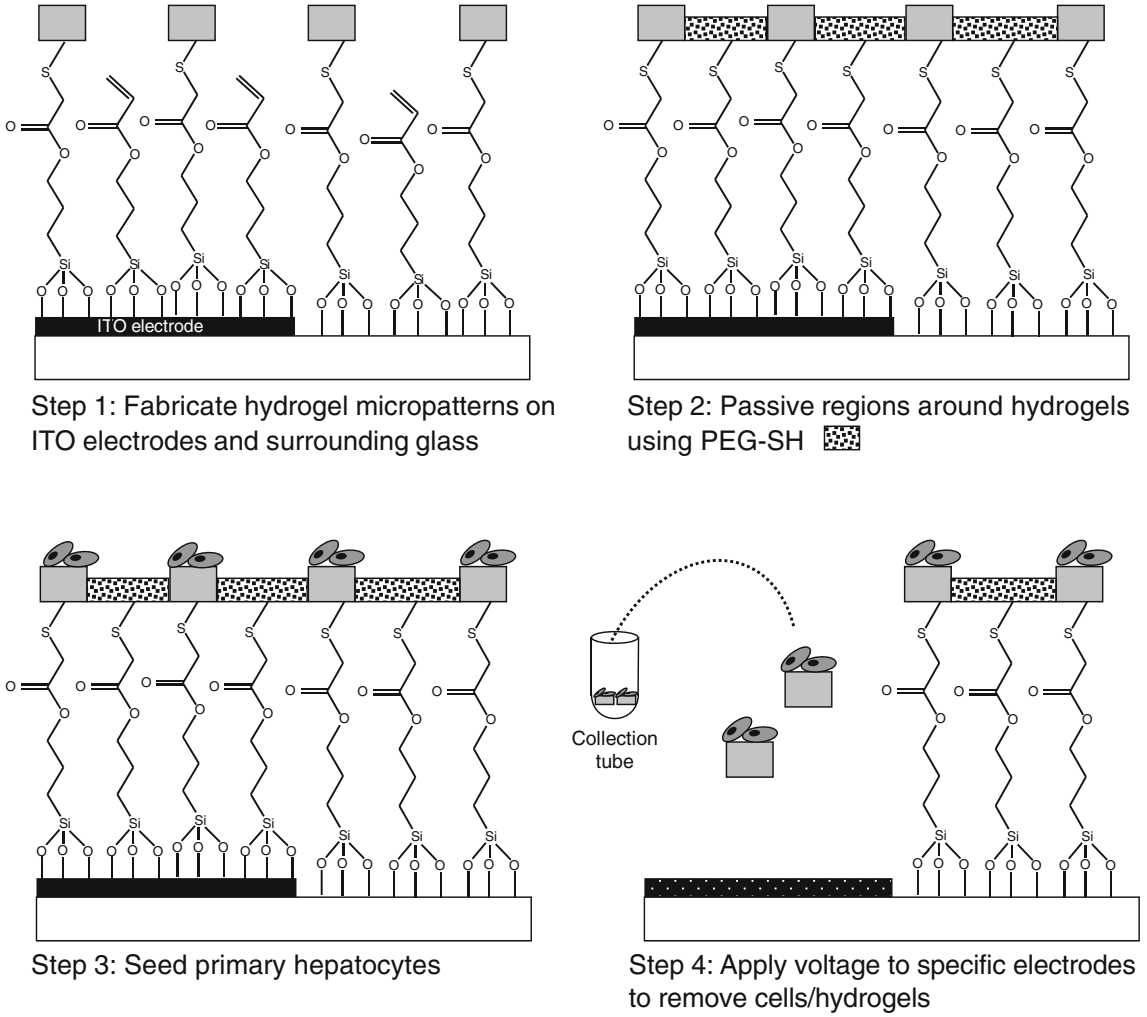

Fig. 1 Diagrammatic representation of electrochemical release of cell-coated bioactive heparin hydrogels. *Step 1*: Heparin-based hydrogels are micropatterned using photopolymerization on top of silane modified glass and ITO electrodes. Heparin hydrogel binds to the underlying silane layer via Michael-type addition. *Step 2*: The regions not covered with the hydrogel are passivated with a non-fouling layer of PEG-SH. PEG-SH attaches to the underlying silane layer via Michael addition. *Step 3*: The substrates are then incubated with cells resulting in selective attachment on top of heparin hydrogel microstructures. *Step 4*: Applying reductive potential (−1.8 V vs Ag/AgCl reference) resulted in the release of cell-coated hydrogels from selective ITO electrode

results reported in Fig. 2 were obtained using uniform ITO electrodes whereas cell-on-gel detachment described in the sections that follow was performed using patterned electrodes.

Incorporation of cells onto hydrogels

In order to localize cells onto the hydrogel microstructures, the surrounding surface must be rendered non-fouling while the gels themselves need to be rendered amenable to cell attachment. To do so, we take advantage of the varied chemistry present on our surfaces. We first utilized the available acryloxy groups present on the regions surrounding the hydrogel microstructures. We assembled a layer comprised of short PEG molecules (MW 5 kDa) with thiol groups at one end (methoxyl poly(ethylene) glycol thiol, denoted PEG-SH) to the surface. The presence of the thiol group of the PEG-SH allows assembly onto acrylated silane via Michael addition between the thiol and the acrylate groups present on the surface self-assembled monolayer (SAM). The surface was exposed to a 10 mg/mL PEG-SH solution overnight at 37 °C. Ellipsometer and contact angle measurements were taken to confirm deposition of PEG-SH onto the silane layer. After PEG-SH modification, the water contact angle of the ITO surface decreased from 59.3±2.7° to 40.3±3.1°, indicating the presence of hydrophilic PEG-SH on ITO. Companion samples were prepared on silicon substrates to monitor assembly of PEG-SH using ellipsometry. Ellipsometry data indicate a film thickness growth of 6.0±0.1 nm after

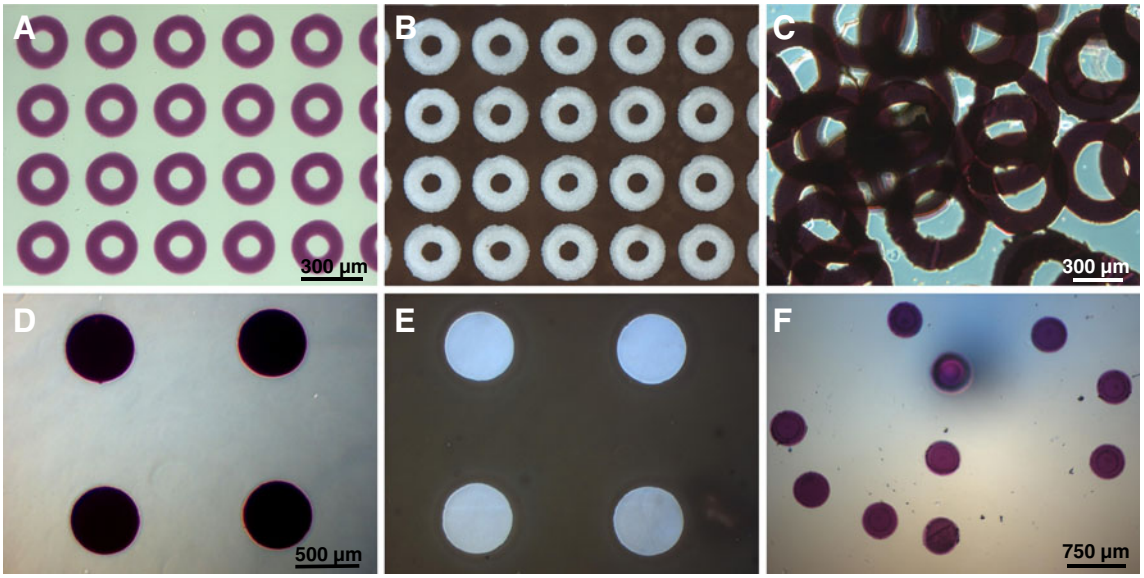

Fig. 2 Release of heparin hydrogel microstructures. **A** Photopolymerization was used to form heparin hydrogel ring microstructures (stained purple using toluidine dye). **B** Glass substrates after removal of ring structures. Application of reductive potential resulted in the release of ring structures. **C** Released hydrogel rings. Similarly, disk structures (**D**) of heparin-based hydrogels were released after application of voltage (**D**) and gathered in a collection tube (**F**)

PEG-SH incubation, consistent with the thickness expected from the molecular size of PEG-SH. After protection of the surrounding areas, the hydrogel microstructures needed to be rendered adhesive to hepatocytes. This was accomplished by incubating PEG-modified micropatterned surfaces in collagen. Importantly, protein adsorption occurred mainly on hydrogel regions and not on PEG containing glass regions. Deposition of proteins on these micropatterned surfaces was characterized using ToF SIMS discussed below.

It is worth noting that our previous studies described selective attachment of cells on glass around heparin hydrogels [17], whereas the present paper talks about cell attachment on top of the hydrogel. These reports are not contradictory and may be explained as follows. In our previous study, we have described how surfaces containing regions of acryloxy SAMs rapidly adsorb collagen while the adsorption on heparin-containing hydrogels is limited during short incubation times [17]. We found that, when surfaces were incubated with collagen solution for a short period of time (~20 min), protein deposited on silanized glass whereas longer incubation period led to protein adsorbing to both gel and glass region of the substrate. Taking advantage of this variability, we use a concentration of 0.1 mg/mL solution for 45 min to adsorb collagen to the hydrogel microstructures.

After characterizing the assembly process, we sought to examine the non-fouling properties of the surface. To this end, additional analysis of the PEG-SH deposition and its ability to prevent adhesion of proteins, namely collagen, was characterized using ToF–SIMS. These results, discussed in detail in supplemental section (Fig. 1S), demonstrate that significant amounts of protein adsorb to both the acryloxy SAM and to the heparin-containing hydrogel while the PEG-SH functionalized surface shows only negligible protein adsorption. These results confirm the non-fouling nature of the PEG-SH layer and the addition of adhesion-promoting collagen to the hydrogel microstructures. To further test the ability of PEG-SH to resist cell adhesion, human hepatoma cells (HepG2) were cultured on ITO substrates containing hydrogel micropatterns. The results (described in Fig. 2S supplemental section) showed HepG2 cells selectively adhering on top of the hydrogel micropatterns but not on the surrounding regions glass/ITO regions.

Upon seeding, primary rat hepatocytes were observed to adhere on top of the hydrogel microstructures (Fig. 3a). As seen in Fig. 3a, the hepatocytes were observed to selectively attach on top of hydrogels with minimal attachment of cells around the hydrogel structures in the regions functionalized with PEG-SH. Immunostaining for intracellular albumin was carried out to characterize functionality of hepatocytes. Figure 3b shows a strong signal associated with the presence of albumin in the cytoplasm of hepatocytes (green fluorescence) residing on top of heparin hydrogel microstructures after 5 days culture. Figure 3c shows a combination of the DAPI staining of nuclei, albumin staining, and brightfield image of the hepatocytes on top of the gel structures.

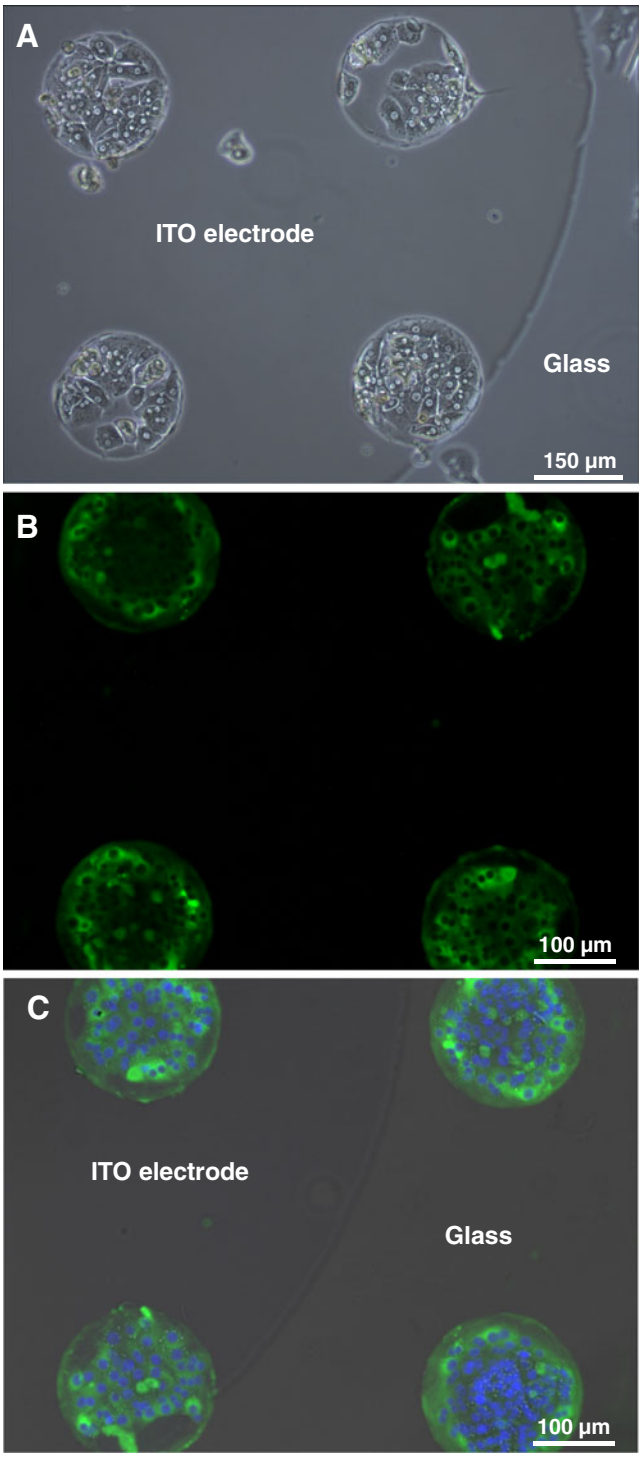

Fig. 3 **A** Selective attachment of rat primary hepatocytes on top of heparin hydrogel structures micropatterned on top of ITO electrodes. **B** Intracellular albumin immunostaining of primary hepatocytes residing on top of heparin-based hydrogel microstructures at day 5 in culture. **C** A combination of images showing albumin production by cells on top of gel structures. *Green* color—intracellular albumin and *blue* color—DAPI staining of nuclei

Selective release of cell-coated hydrogels

Electrical activation was used to release cell-coated hydrogels from conductive ITO electrodes at specified times and spatial locations. By applying a short reductive potential (−1.8 V vs Ag/AgCl reference, for 60 s), we were able to disrupt the silane layer responsible for the hydrogel adhesion [24, 36] and selectively detach the heparin-containing hydrogels along with the hepatocytes residing on top.

Figure 4a shows this ability to address and release a specific subset of cell-coated hydrogels by applying voltage to the desired ITO electrode. After the application of a reductive potential, the substrate was washed with 1× PBS solution to release the hydrogels from the surface and to accumulate them in a collection tube (Fig. 4b). As seen in these images, the cell-coated heparin hydrogel microstructures were released from the activated ITO electrode but remained attached on the surrounding glass substrate and inactivated electrodes.

In addition to on-demand release from the culture surface, it was important to determine effects of electrochemical desorption on hepatocyte function. To assess this, cells atop released hydrogel microstructures were kept in culture and presence of albumin, hallmark liver product[5, 43], was monitored for up to 6 days post-release (8 days of culture per and post-release). Albumin immunostaining is shown in Fig. 4c where fluorescence (green) signal is due to presence of intracellular albumin present in the cytoplasm of hepatocytes residing on top of released hydrogels. Note that one of the released discs in Fig. 4c is facing down while the other is facing up so that cells on one of the gel discs (left) are out of focus. The albumin in released cells was comparable to the signal observed from cell-coated microstructures before electrical stimulation (Fig. 3b).

To further quantitate albumin production, albumin gene expression was analyzed using RT-PCR (Fig. 4d). Comparing albumin expression in cells that were electrochemically detached from the surface along with hydrogels vs. cells atop hydrogels still adherent on the glass substrates, we note that the application of voltage does not appear to have affected the functionality of the cells. Hydrogels were released off the surface on day 2 after seeding, collected, and cultured for additional 6 days. As seen in Fig. 4d, the expression of albumin transcripts in hepatocytes 3 days after release is comparable to hepatocytes/hydrogels cultured on glass substrates for the same period of time (n=3, p>0.05). Similarly, albumin gene expression in released hepatocytes 6 days post-release was comparable to albumin expression in cells cultured on heparin-containing hydrogels adherent to the substrate. Importantly, albumin gene expression increased

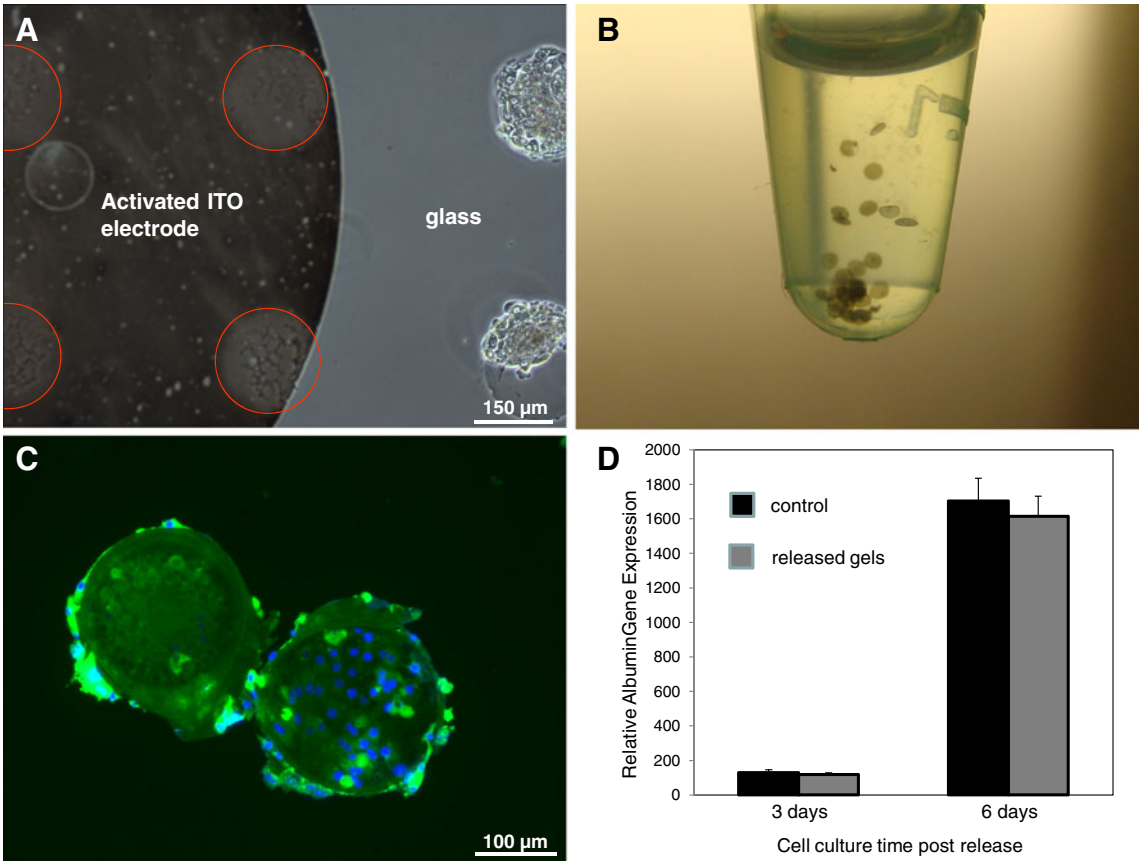

Fig. 4 **A** Selective release of primary hepatocyte-containing heparin hydrogel structures from ITO electrodes (denoted by *red outline*). Note that cell-coated hydrogels from surrounding glass regions are still intact on the surface. **B** Released cell-coated hydrogels acquired in a collection tube. **C** Immunostaining analysis for intracellular albumin shows that cells are functional after application of voltage. **D** Gene expression analysis for albumin also confirms that released cells express similar levels of albumin when compared with cells adherent on the surface 3 and 6 days post-electrical activation

~16-fold from day 3 to day 6 in released cells, highlighting bioactivity of the hydrogel and its ability to maintain phenotype of difficult-to-culture primary hepatocytes. The bioactivity of heparin-based hydrogels in maintaining hepatic phenotype is consistent with our previous reports [16, 17]. The results in Fig. 4c, d highlight the fact that hepatocyte-on-hydrogel microstructures retained the ability to express markers of hepatic differentiation after detachment from the surface.

Conclusion

The goal of this work was to develop a platform for on-cue release of hepatocyte-on-hydrogel microstructures from conductive ITO substrates. Bioactive heparin-based hydrogels were micropatterned on ITO electrodes using a photolithographic process. To guide cell attachment on top of the hydrogel microstructures, the surrounding glass/ITO regions were modified with a non-fouling PEG silane layer, and the hydrogels were functionalized with collagen. Hepatocytes selectively attached on top of heparin–hydrogel microstructures and remained functional for at least 8 days in culture. Importantly, hepatocyte-on-hydrogel microstructures could be released from ITO culture substrates upon electrochemical stimulation. These detached cell/gel constructs expressed albumin at equivalent levels to those expressed by constructs remaining on the surface, demonstrating that the electrical stimulation did not affect function of these fragile primary cells.

This platform provides flexibility for on-cue spatio-temporal desorption of cell-coated biomaterial constructs. Applications of this switchable biointerface will include collection of cell samples at different time points from the same dish for re-cultivation or downstream analysis.

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