Acta Biomaterialia 34 (2016) 125–132

Contents lists available at ScienceDirect

Acta Biomaterialia

journal homepage: www.elsevier.com/locate/actabiomat

Full length article

Microfluidic fabrication of bioactive microgels for rapid formation and enhanced differentiation of stem cell spheroids ☆

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ARTICLE INFO

Article history:
Received 13 October 2015
Received in revised form 11 December 2015
Accepted 12 January 2016
Available online 13 January 2016

Keywords:
Droplet microfluidics
Heparin hydrogel
Microgel
Embryoid body
Endoderm
Nodal

ABSTRACT

A major challenge in tissue engineering is to develop robust protocols for differentiating ES and iPS cells to functional adult tissues at a clinically relevant scale. The goal of this study is to develop a high throughput platform for generating bioactive, stem cell-laden microgels to direct differentiation in a well-defined microenvironment. We describe a droplet microfluidics system for fabricating microgels composed of polyethylene glycol and heparin, with tunable geometric, mechanical, and chemical properties, at kHz rates. Heparin-containing hydrogel particles sequestered growth factors Nodal and FGF-2, which are implicated in specifying pluripotent cells to definitive endoderm. Mouse ESCs were encapsulated into heparin microgels with a single dose of Nodal and FGF-2, and expressed high levels of endoderm markers Sox17 and FoxA2 after 5 days. These results highlight the use of microencapsulation for tailoring the stem cell microenvironment to promote directed differentiation, and may provide a straightforward path to large scale bioprocessing in the future.

Statement of Significance

Multicellular spheroids and microtissues are valuable for tissue engineering, but fabrication approaches typically sacrifice either precision or throughput. Microfluidic encapsulation in polymeric biomaterials is a promising technique for rapidly generating cell aggregates with excellent control of microenvironmental parameters. Here we describe the microfluidic fabrication of bioactive, heparin-based microgels, and demonstrate the adsorption of heparin-binding growth factors for enhancing directed differentiation of embryonic stem cells toward endoderm. This approach also facilitated a ~90-fold decrease in consumption of exogenous growth factors compared to conventional differentiation protocols.

1. Introduction

The study of developmental biology has led to the design of numerous embryonic stem cell (ESC) directed differentiation protocols. For example, the identification of growth factor gradients that drive regional specification during gastrulation has shed light on the biochemical microenvironment needed for *in vitro* production of ESC-derived definitive endoderm (DE), the germ layer of origin for adult tissues including the liver, pancreas, gut, and lung [1]. Directed differentiation protocols for engineering endoderm-derived tissues aim to initially mimic gastrulation-related signaling pathways, involving Nodal, FGF, Wnt, and BMP, to generate DE cells capable of responding to subsequent differentiation signals [1,2]. For instance, mouse ESCs can be differentiated *in vitro* to hepatocyte-like cells by sequentially mimicking certain developmental stages: formation of embryoid bodies (EBs), specification to

☆ Part of the High Throughput Approaches to Screening Biomaterials Special Issue, edited by Kristopher Kilian and Prabhas Moghe.

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http://dx.doi.org/10.1016/j.actbio.2016.01.012
1742-7061/

DE *via* supplementation with Activin A and FGF-2, and finally induction to hepatic differentiation *via* exposure to HGF and other biomolecules produced by non-parenchymal cells of the liver [3]. However, controlling these tightly regulated microenvironmental factors represents a significant challenge when biomedical applications require large quantities of stem cell-derived tissue constructs, such as for cell replacement therapy or high throughput screening. The purpose of this study is to identify a scalable strategy for controlling the stem cell microenvironment using biomaterials. More specifically, we demonstrate the microencapsulation of ES cells in growth factor-binding heparin hydrogels for rapid preparation of EBs and subsequent growth factor mediated directed differentiation.

Microencapsulation is a large-scale cell bioprocessing technique that facilitates rapid nutrient/waste transport, while limiting hydrodynamic stresses associated with stirred suspension culture [4]. ES and iPS cells have been microencapsulated in alginate and other biomaterials to generate 3D aggregates for propagation and differentiation (e.g. EB formation) [5–7]. Of particular interest for directed differentiation applications, droplet microfluidic technologies enable rapid generation of emulsions with excellent monodispersity, a highly desirable feature for EB-based protocols where aggregate size influences cell fate [8–14]. In addition to bio-inert alginate [8,15–18], there are a few examples of biofunctional matrices used for cell microencapsulation with droplet microfluidics. For example, RGD-functionalized polyethylene glycol (PEG) microgels have been shown to support hMSC spheroids in isolation from allogeneic IgG molecules [19]. However, we are unaware of reports describing growth factor-binding microgels for ES cell encapsulation and differentiation.

Herein, we describe a bioactive copolymer microgel system composed of heparin and PEG. Heparins are highly sulfated glycosaminoglycans that regulate cell signaling through reversible sequestration of numerous growth factors (GFs) expressing heparin-binding domains [20,21]. Accordingly, heparin-functionalized synthetic hydrogels have been developed for controlled release of GFs, and as matrices for cell cultivation and differentiation [21–25]. In this work we demonstrate microfluidic fabrication of hydrogel microcapsules containing heparin and explore the possibility of incorporating GFs into the microgels to direct endodermal differentiation of encapsulated mESCs.

2. Materials and methods

2.1. Heparin-methacrylate synthesis

Methacrylate-modified heparin (heparin-MA) was prepared as previously reported [26]. Briefly, 2 g/L heparin sodium salt (16–18 kDa MW) (Sigma) was dissolved in DI water with methacrylic anhydride (5-fold molar equivalent). The reaction solution was adjusted to pH 8.5 with 5 N NaOH, stirred for 2 days at RT, then dialyzed and lyophilized.

2.2. Microfluidic device fabrication

Microfluidic flow focusing devices were fabricated by standard soft lithographic techniques: PDMS elastomer (Sylgard) was poured over SU-8 master molds and cured for 1 h at 70 °C. The PDMS chips were O_2 plasma bonded to glass, then treated with Aquapel™ solution for ~30 s and perfused with air to render channel surfaces hydrophobic. For droplet characterization experiments, flow focusing channel dimensions (H × W) were 45 × 45 μm or 100 × 100 μm.

2.3. Microgel fabrication

Acrylate/methacrylate- and thiol-modified polymer solutions were prepared separately, then mixed on-chip immediately prior to microfluidic flow focusing to generate crosslinked hydrogel droplets (Fig. 1). These solutions consisted of (i) 2× concentration of PEGDA (6 kDA) (Creative PEGworks) and Heparin-MA (16–18 kDA) dissolved in PBS or cell culture medium with 15 mM sodium carbonate (pH 7.4) and (ii) 2× concentration of 8-arm PEG-thiol (10 kDa) (Creative PEGworks) dissolved in PBS or culture medium. Microgels (4%, 7%, and 10% w/v) were prepared by mixing precursor solutions at PEGDA:Heparin-MA:8-arm PEG-thiol ratio of 3.5:2:1.5 to maintain stoichiometric equivalence of reactive groups (i.e. thiol and acrylate groups). Upon mixing, prepolymer solutions were flow focused with an immiscible carrier phase composed of HFE-7500 oil (3 M) with 2% v/v PEGylated fluorosurfactant (Ran Biotech). For droplet characterization experiments, oil and aqueous phase flow rates were varied from 5 to 50 μL min^{-1} and 0.5 to 10 μL min^{-1}, respectively. Microdroplets were collected and incubated off-chip at 37 °C for 10–20 min for gelation. Finally, emulsions were destabilized by replacing carrier oil with a 20% v/v solution of perfluorooctanol (Sigma Aldrich) in HFE-7500, and crosslinked microgels were partitioned into aqueous media.

2.4. Rheometry

Shear moduli were measured with a Discovery HR2 hybrid rheometer (TA Instruments) with parallel-plate geometry in time sweep and strain sweep modes. For gelation kinetics experiments, heparin-MA, PEGDA, and 8-arm PEGSH were mixed and immediately pipetted between instrument plates. Time sweeps were performed by holding constant strain and frequency at 10^{-3} rad and 10 rad s^{-1}, respectively. For steady state rheometry experiments, shear moduli were measured in 8 mm prefabricated hydrogel disks (n = 3) under strain sweep test mode (0–4% strain).

2.5. Growth factor-heparin binding experiments

2.5.1. Surface plasmon resonance

Surface plasmon resonance (SPR) experiments were performed using a four-channel BIAcore T3000 instrument. For surface immobilization on bare gold SPR chips, heparin oligomers were modified with thiol moieties (~40% conversion of COOH groups) as previously described [27]. Thiolated heparin was dissolved in PBS to 1 μM concentration and injected into the SPR instrument at a flow rate of 5 μL min^{-1} for 5 min. Heparin immobilization was followed with surface passivation by flowing 3 mM mercaptohexanol (MCH) in PBS at 20 μL min^{-1} for 30 s. Recombinant Activin A and Nodal (100 ng/mL in PBS) (R&D) were then sequentially introduced into the instrument at 5 μL min^{-1} for 5 min.

2.5.2. GF sequestration in heparin microgels

Growth factor sequestration in heparin microgels was assayed by determining the relative depletion of GFs added to microgel suspensions. In brief, recombinant FGF-2, Activin A, and Nodal (1 ng/mL) were added to DMEM containing a 1:6 volume fraction of microgel droplets (120 μm, 7% w/v) and incubated at RT for 16 h. Supernatant was then collected and analyzed by ELISA (R&D [Activin A and FGF]); MyBioSource [Nodal]) (n = 3), wherein remaining GF concentration was measured and normalized to control samples prepared in DMEM.

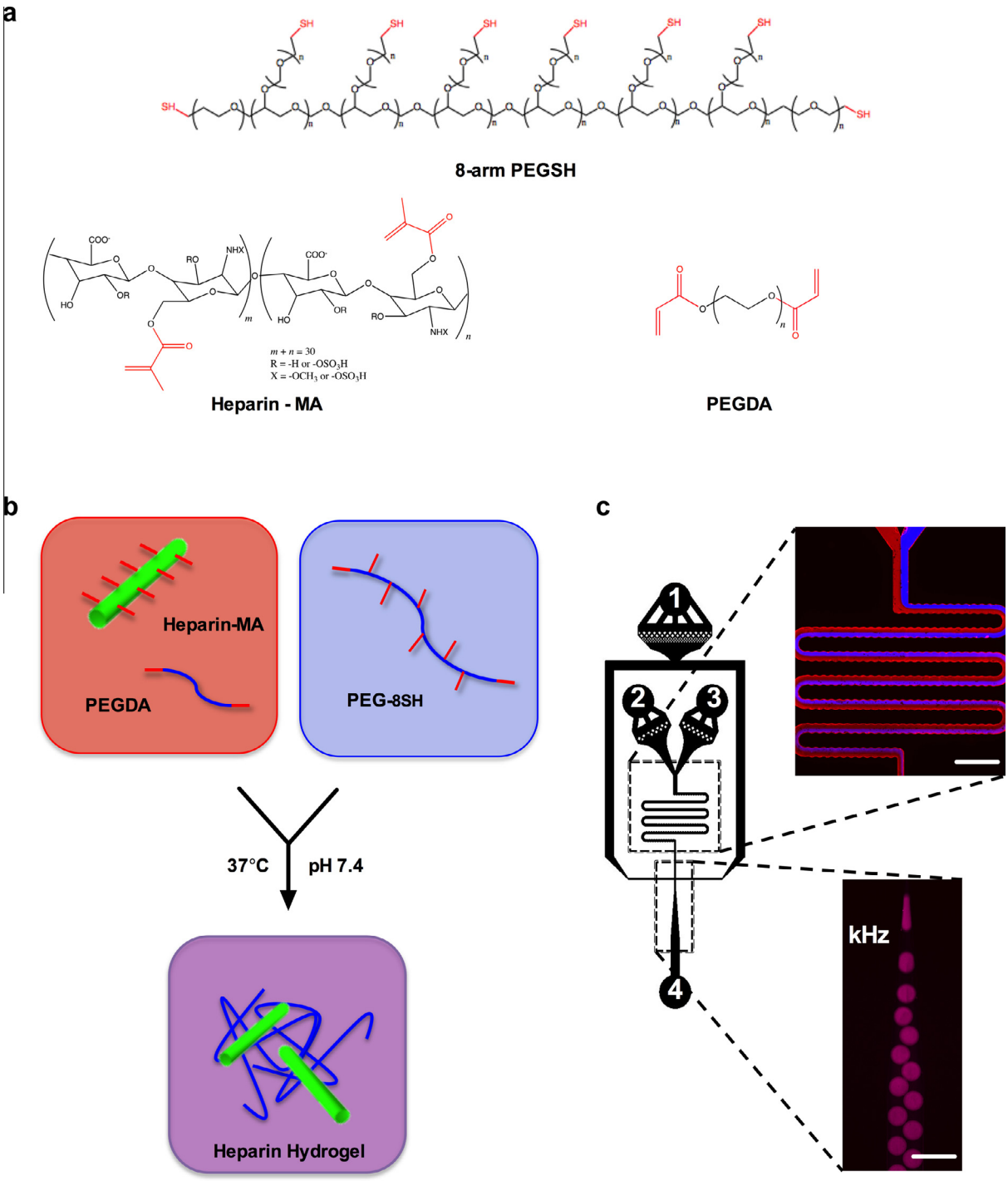

Fig. 1. Heparin microgel fabrication with microfluidic flow focusing. (a and b) Heparin-methacrylate, PEG-diacrylate, and 8-arm PEG-thiol undergo Michael addition crosslinking to form heparin hydrogels. (c) Microfluidic flow focusing device for mixing and emulsifying hydrogel precursors. Numbers represent inlets for (1) carrier oil, (2–3) hydrogel precursors, and (4) collection outlet. Scale bars = 300 μm.

2.6. mESC encapsulation, differentiation and analysis

2.6.1. mESC culture

D3 mouse ESCs (ATCC) were propagated on irradiated mouse embryonic fibroblast (CF-1 MEF, GlobalStem) feeder layers in self-renewal medium consisting of mESC Basal media (ATCC) supplemented with 15% ES-qualified FBS, 1000 U/mL leukemia inhibitory factor (LIF), and 100 nM 2-mercaptoethanol at 37 °C, 5% CO_2 and 90–95% humidity, with medium change every day. mESCs were subcultured every 2–3 days onto fresh feeders using trypsin–EDTA.

2.6.2. Cell encapsulation experiments

mESCs were suspended at a concentration of 25 (10^6) cells mL^{-1} in a prepolymer solution composed of 8% w/v 8-arm PEGSH in media supplemented with 20% v/v Optiprep density gradient medium (Sigma), and mixed on-chip with 7% w/v PEGDA and 3% w/v heparin-MA precursor solution. For all cell encapsulation

experiments, oil and aqueous phase flow rates were held constant at 20 μL min^{-1} and 4 μL min^{-1}, respectively.

2.6.3. Differentiation experiments

To generate embryoid bodies (EBs) in microgel droplets, encapsulated cells were cultured for 6 days in "incomplete" media (self-renewal media without LIF). Control EBs were generated by the hanging drop method with identical media.

Definitive endoderm differentiation methods were adapted from a published protocol for hepatic differentiation of mESC-derived embryoid bodies [3]. Undifferentiated mESCs were encapsulated in microgels containing FGF-2 (100 ng/mL) and Nodal (1 μg/mL), and then suspended in "Embryoid Body Medium" (DMEM supplemented with 15% ES-qualified FBS, 1× non-essential amino acids, 1× penicillin/streptomycin, and 100 nM 2-mercaptoethanol) at a volume fraction of ~1:30 (droplet volume : media volume). Control samples included mESCs cultured as monolayers on fibronectin-coated tissue culture plastic, or cultured in suspension in ultra-low binding dishes for 48 h (to form EBs), then transferred to matrigel-coated plates as previously described [3]. During the initial 48 h of culture, both control samples were supplemented with low concentrations of FGF-2 (3.3 ng/mL) and Nodal (33.3 ng/mL) to account for total amount of GFs added to microgel precursor solutions. After 48 h all samples were switched to "Differentiation Medium" (DMEM-F12 supplemented with 1% FBS, 1× non-essential amino acids, 1× penicillin/streptomycin). Control samples were supplemented during this time with 100 ng/mL FGF2 and 1 ug/mL Nodal, with media changes every other day.

2.6.4. qRT-PCR

Total RNA was extracted from encapsulated cells using an RNeasy Plant Mini Kit (Qiagen) per manufacturer instructions. cDNA was synthesized with a Quantitest Reverse Transcription Kit (Roche), and quantitative real-time RT-PCR was performed with universal SYBR Green Master (Roche) in a StepOne qPCR instrument (Thermo Fisher). Relative gene expression was calculated using the comparative threshold cycle (C_t) method with glyceraldehyde 3-phophate dehydrogenase (GAPDH) as a housekeeping gene. qPCR reactions were performed in biological triplicate ($n = 3$).

2.7. Statistical analysis

Mean and standard deviation were calculated from cell encapsulation, rheometry, growth factor sequestration, and gene expression data. The Student's *t*-test was used to compare unpaired data for statistical significance.

3. Results

3.1. Heparin microgel synthesis and characterization

Fig. 1a schematically illustrates microfluidic mixing of heparin hydrogel precursors and dripping *via* flow focusing, resulting in monodisperse microgel emulsions. Copolymers composed of PEGSH and heparin-MA crosslink *via* Michael addition under physiologic conditions. However, we found this copolymer formulation to result in mechanically unstable microgels unless heparin was heavily modified (>60% conversion of COOH groups), which drastically decreases innate bioactivity [26]. By including PEGDA as a crosslinker, microgel stiffness is not limited by the degree of heparin modification, thereby decoupling mechanical properties from gel bioactivity. Upon crosslinking, these microgels could be collected and transferred to aqueous media. Dynamic shear rheometry revealed a viscous-to-elastic transition, indicated by an abrupt increase in storage modulus and decrease in stress/strain phase lag, at approximately 6 or 12 min after mixing polymers at 37 °C or 23 °C, respectively, at pH 7.4 (Fig. 2a and b). The final steady state hydrogel storage modulus was modulated between ~2 and 10 kPa by varying total polymer concentration from 4% to 10% w/v (Fig. 2c). With these data, we estimate a microgel storage modulus of ~6.5 ± 1 kPa for all cell encapsulation experiments (in which a 7% w/v polymer concentration was used). The number of encapsulated cells per droplet can be controlled by initial cell loading density and droplet volume, and is generally considered to follow a Poisson distribution. For differentiation experiments, we used a density of 10–15 (10^6) cells per mL of hydrogel precursor solution and generated 120 μm droplets (~1 nL), which corresponded to a mean of 12 ± 3 cells per droplet (Fig. 2d and e). Furthermore, droplet diameter could be adjusted by tuning oil/aqueous flow rates or channel geometry. Fig. 2f and g depicts droplets spanning a range of ~60–160 μm in diameter. Broader size ranges could be achieved by further modulating cross-sectional area of flow focusing channels (data not shown). 120 μm microgels were used for all subsequent cell encapsulation experiments.

3.2. mESC encapsulation and embryoid body development

Calcein staining indicated >95% viability of encapsulated mESCs at droplet production rates up to ~1 kHz (Fig. 3a). When cultured in "incomplete" media, these cells rapidly proliferated over the course of 6 days to form spheroids greater than 200 μm in diameter. We observed that although cells did not migrate through nor degrade hydrogels, the growing cellular spheroids caused microgels to expand and distort (Fig. S1), eventually breaking open around day 5–6, at which point spheroids could be collected by centrifugation (Fig. 3b). RT-PCR analysis indicated these constructs expressed tri-lineage germ layer markers similar to embryoid bodies generated by the hanging drop method (Fig. 3c).

3.3. Growth factor sequestration in heparin microgels

Charged sulfate groups on crosslinked heparin oligomers were visualized by their interaction with a metachromatic Toluidine Blue stain, which remained stably incorporated in microgels for several weeks. Inert PEG microgels (lacking heparin) served as negative controls (Fig. 4a).

SPR analysis indicated that Nodal, but not Activin A, could bind to heparin immobilized on gold surfaces (*via* Au-thiol chemistry) (Fig. 4b). These results are not unexpected in light of the fact that heparin-binding domains have been mapped to the propeptide region of Activin A (which is removed from the commercially available protein) [28]. However, it is important to note that heparin used for microgel fabrication was modified with methacrylates, and could potentially exhibit lesser GF affinity or mobility when incorporated into a hydrogel mesh. To measure sequestration in *crosslinked* heparin-MA, we spiked heparin microgel suspensions with GFs and measured their relative depletion from the supernatant by ELISA. In agreement with SPR results, these ELISA experiments revealed greater retention and slower release of Nodal and FGF-2 (but not Activin A) in heparin microgels compared to inert PEG microgels (Fig. 4c).

3.4. Definitive endoderm directed differentiation in heparin microgels

Given the affinity of heparin microgels for FGF-2 and Nodal, we tested the effects of encapsulation on inducing mESCs to definitive endoderm (Fig. 4d). Undifferentiated mESCs were encapsulated in heparin microgels and subjected to a DE differentiation protocol based on FGF-2 and Nodal/Activin supplementation [3,23]. After

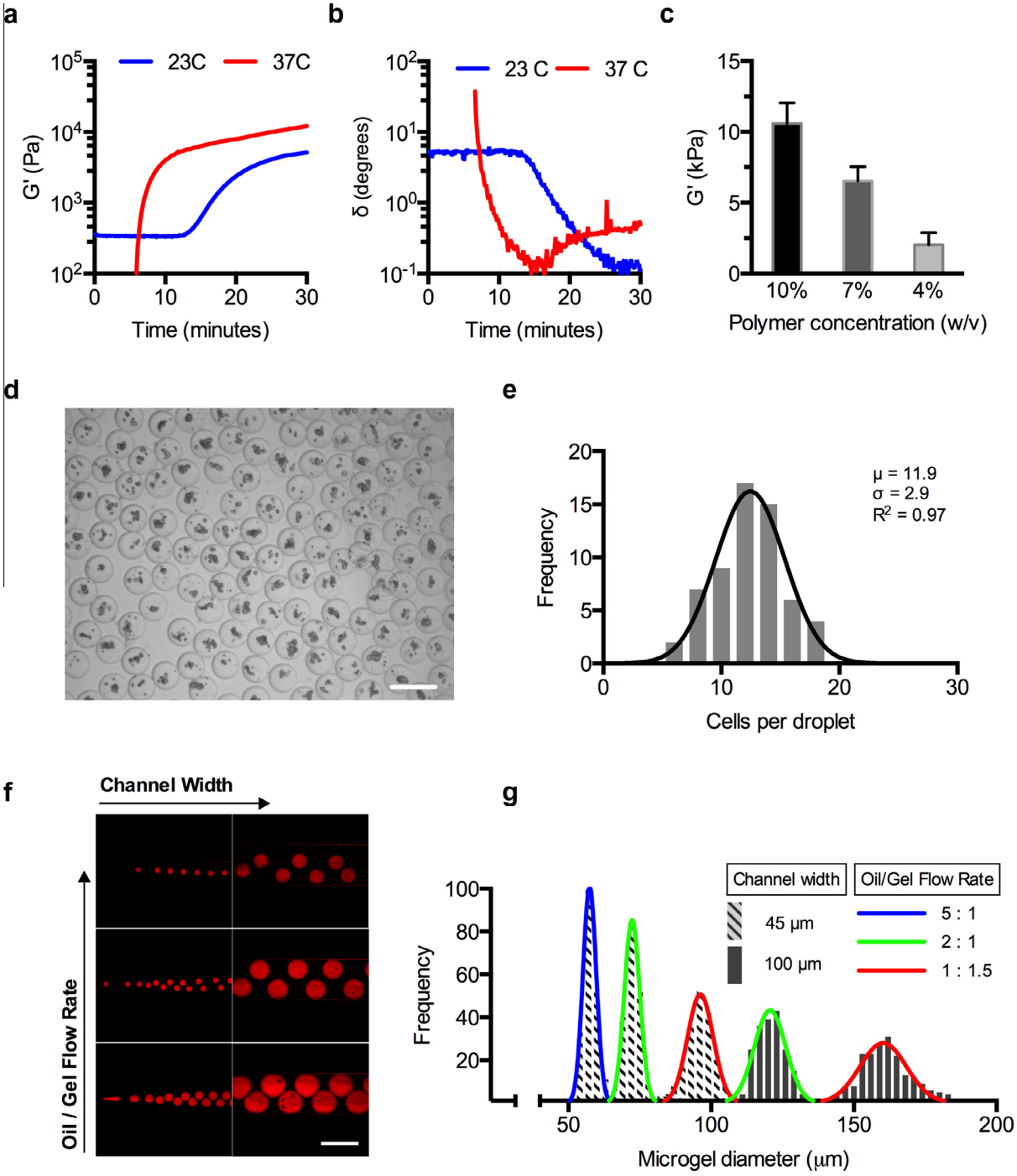

Fig. 2. Mechanical, geometric and encapsulation characterization of heparin microgels. (a) Dynamic shear modulus and (b) stress–strain phase lag of 7% w/v heparin hydrogel as a function of time and temperature. (c) Steady state shear modulus as a function of total polymer concentration. (d and e) Representative micrograph and frequency distribution of mESC encapsulation number. (f and g) Microgel diameter as a function of microfluidic channel dimensions and relative flow rates. Scale bars = 200 μm.

5 days, qRT-PCR showed cells encapsulated with a one-time dose of Nodal and FGF-2 cells expressed a 33-fold increase in FoxA2 and 65-fold increase in Sox17 compared to undifferentiated mESCs. Control cells cultured in monolayers on fibronectin with continuous Nodal/FGF-2 supplementation expressed 2-fold and 5-fold increases in FoxA2 and Sox17 expression, respectively, while 3D spheroids cultured on matrigel expressed a 10-fold and 11-fold increase. ESCs encapsulated without FGF-2 or Nodal expressed only 3-fold and 7-fold increase of FoxA2 and Sox17, respectively (Fig. 4d).

4. Discussion

By enabling parallel assays to be performed in discrete, miniaturized compartments, droplet microfluidic technologies have become a powerful tool for high throughput biology [29]. However, their use in high throughput fabrication and/or screening of biomaterials has been limited. We describe in this report a biomaterials-based droplet microfluidics approach to rapidly encapsulate mESCs in a well-defined, DE-inducing microenvironment. In our design, heparin-MA and PEGDA macromers are mixed

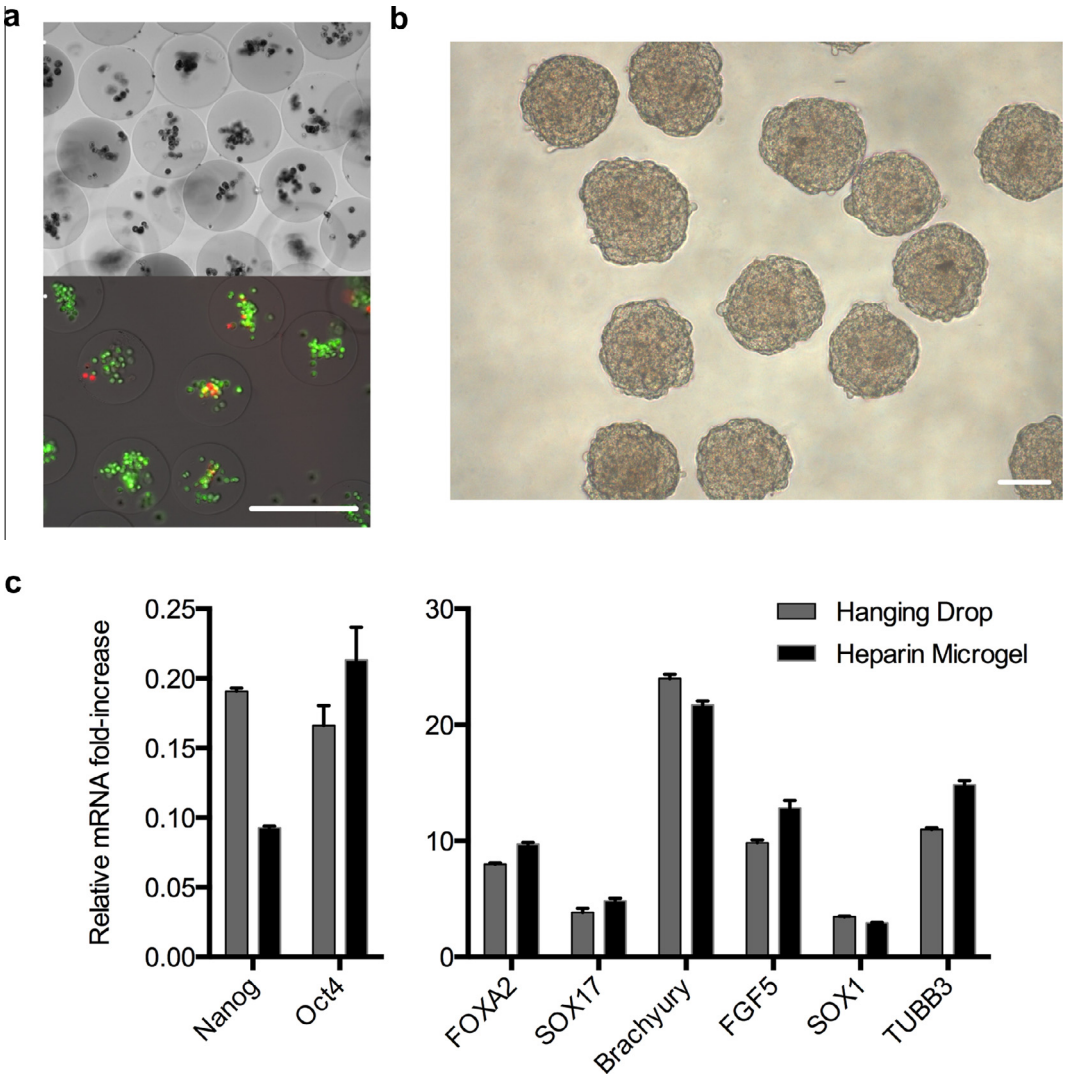

Fig. 3. Encapsulated mESC viability and EB formation. (a) Representative Live/Dead staining of mESCs 2 h after encapsulation. (b) Embryoid body formation and (c) gene expression of germ layer markers after 6 days of differentiation. Scale bar = 200 μm.

with 8-arm PEG-thiol and are subsequently emulsified in a flow-focusing microfluidic device at kHz frequency. Once emulsified into droplet templates, these polymers undergo gelation *via* a Michael-type addition reaction. Crosslinking schemes of this type are popular for cell encapsulation because they are specific, cyto-compatible, and do not rely on free radicals [19,30]. However, the kinetics of crosslinking becomes an important design criterion for internally gelled droplets, as changes in polymer viscosity affect the balance of forces (i.e. *Ca* number) that govern the hydrodynamic parameters of dripping [31]. In our case, the time scale of acrylate-thiol coupling enabled an efficient cell encapsulation workflow, by ensuring that crosslinked microgels could be quickly collected and transferred to aqueous media, yet did not interrupt flow regimes by crosslinking prematurely in the microfluidic device. Importantly, we do not anticipate our fabrication technique to be limited to the specific polymers described herein. We speculate that many thiol- and acrylate- modified polymers with similar mechanical properties and crosslinking kinetics to those described here could be easily adapted to our system. Future studies with degradable polymers (e.g. photolabile or protease cleavable PEG) would be of interest for applications that require triggered release for further cell analysis (e.g. immunohistochemistry or FACS).

In order to demonstrate the utility of heparin microgels for stem cell differentiation, we sought to characterize their affinity to GFs associated with endoderm – germ layer cells serving as precursors to such organs as liver, lung and pancreas. FGF-2 has been shown to synergize with Nodal signaling in generating mESC-derived Sox17 + endoderm, [32] and is known to bind to several variants of heparin-based hydrogels [21]. However, the interaction of Nodal with heparin hydrogels has not been previously examined. The Nodal/Activin signaling pathway is an inimitable regulator of DE differentiation. It is activated by Nodal ligand binding with epidermal growth factor receptor-Cripto-FRL1-Cryptic co-receptor (Cripto) and Activin type I and II serine/threonine kinase receptors (ALK4 and ActRIIB, respectively), which results in phosphorylation of Smad2 and subsequent downstream gene regulation [33]. Activin A is a related TFG-β superfamily member, and can bind to nearly the same set of receptors as Nodal with the exception of Cripto. Although both Nodal and Activin A generate DE with similar global gene expression *in vitro*, Chen et al. recently showed that Nodal-derived DE contributed to *functionally* superior adult tissues (liver and pancreas) *in vivo* compared to Activin-derived endoderm [34]. Despite these differences, Activin A is still widely used to mimic Nodal/Smad2 signaling in DE differentiation protocols due to its higher potency and relatively lower cost. Ours and several

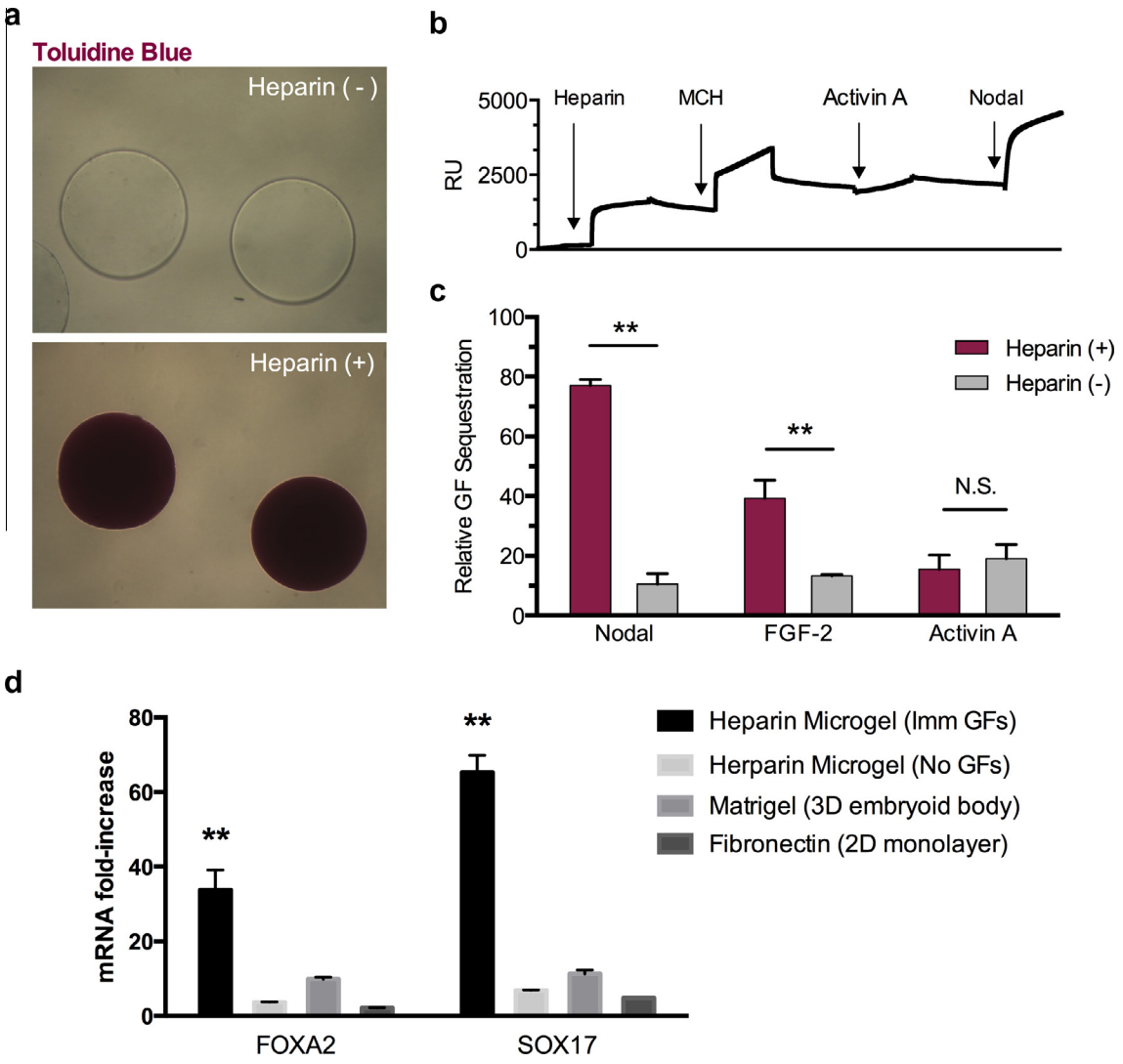

Fig. 4. Growth factor adsorption and mESC-to-DE differentiation in microgels. (a) Toluidine blue staining shows charged sulfate groups in heparin containing microgels. (b) SPR plot for thiol-modified heparin challenged with recombinant Activin A and Nodal. (c) ELISA analysis of growth factor sequestration in heparin vs PEG microgels. (d) Definitive endoderm marker expression after 5 days differentiation with or without Nodal and FGF-2. Error bars represent standard deviation for $n = 3$ samples. Significance indicated at the $p < 0.05$ level.

other groups previously demonstrated that growth factor signaling can be enhanced *in vitro* by ligand immobilization, thus mitigating the consumption of expensive recombinant reagents [35,36]. Although heparin-binding moieties are present on both Nodal and Activin A, these regions are removed from the commercially available recombinant form of Activin A, which likely explains the disparity in observed microgel affinity (Fig. 4b and c) [28,34,37]. Nevertheless, we demonstrate that a one-time dose of Nodal and FGF-2 (added to polymer solution) is sufficient to induce significantly higher Sox17/FoxA2 expression in encapsulated mESCs, when compared to standard 2D or 3D differentiation protocols with continuous Nodal and FGF-2 supplementation. Notably, this strategy conserved total Nodal/FGF-2 consumption by ~90-fold, on a per cell basis, over the 5-day period of differentiation.

5. Conclusions

Microgels composed of PEG and heparin were fabricated with a microfluidic flow-focusing device. Size, elastic modulus, and bioactivity of these hydrogel particles could be easily tuned, and mild crosslinking conditions facilitated the encapsulation of sensitive mouse ESCs with high viability. Nodal and FGF-2 were sequestered by heparin-containing microgels, and drove enhanced expression of definitive endoderm markers in encapsulated mESCs, compared to a gold standard directed differentiation protocol. While this study focused specifically on endoderm directed differentiation, the versatility and simplicity of fabrication suggests this system could be implemented as a scalable platform for general EB-based differentiation applications, or for the development of tailored cell delivery vehicles.

Acknowledgments

This work was supported by NIH-R01-DK079977 awarded to AR and NIH T32-GM008799 awarded to CS.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.actbio.2016.01.012.

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