Modulation of hepatocyte phenotype in vitro via chemomechanical tuning of polyelectrolyte multilayers

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ABSTRACT

It is increasingly appreciated that since cell and tissue functions are regulated by chemomechanical stimuli, precise control over such stimuli will improve the functionality of tissue models. However, due to the inherent difficulty in decoupling these cues as presented by extracellular materials, few studies have explored the independent modulation of biochemical and mechanical stimuli towards the manipulation of sustained cellular processes. Here, we demonstrate that both mechanical compliance and ligand presentation of synthetic, weak polyelectrolyte multilayers (PEMs) can be tuned independently to influence the adhesion and liver-specific functions of primary rat hepatocytes over extended in vitro culture (two weeks). These synthetic PEMs exhibited elastic moduli E ranging over $200\,\text{kPa} < E < 142\,\text{MPa}$, as much as one thousand-fold more compliant than tissue-culture polystyrene ($E \sim 2.5\,\text{GPa}$). The most compliant of these PEM substrata promoted hepatocyte adhesion and spheroidal morphology. Subsequent modification of PEMs with type I collagen and the proteoglycan decorin did not alter substrata compliance, but enhanced the retention of spheroids on surfaces and stabilized hepatic functions (albumin and urea secretion, CYP450 detoxification activity). Decorin exhibited unique compliance-mediated effects on hepatic functions, down-regulating the hepatocyte phenotype when presented on highly compliant substrata while up-regulating hepatocyte functions when presented on increasingly stiffer substrata. These results show that phenotypic functions of liver models can be modulated by leveraging synthetic polymers to study and optimize the interplay of biochemical and mechanical cues at the cell–material interface. More broadly, these results suggest an enabling approach for the systematic design of functional tissue models applied to drug screening, cell-based therapies and fundamental studies in development, physiology and disease.

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1. Introduction

Tissue functions depend on the reciprocal and dynamic interactions of cells with their surrounding microenvironment or niche, which includes biochemical and mechanical stimuli defined by neighboring cells and extracellular matrices. Accordingly, it is becoming increasingly clear that the development of functional in vitro models of tissue patho/physiology depends on the ability to understand, predict, and harness the chemical and mechanical properties of extracellular substrata [1,2]. Several descriptive studies have highlighted the cooperative effects of ligand presentation and substrata stiffness on cellular functions ranging from adhesion and motility to morphogenesis and remodeling [3,4]; furthermore, these findings have led to the development of new synthetic substrata offering improved control over independent biochemical and mechanical cues [5–12]. In particular, poly (acrylamide) (PA) hydrogels of approximate elastic modulus (E) of 10^1–10^5 Pa have been surface-functionalized with adhesion proteins or ligands, and used extensively to study chemomechanical effects on a variety of cell fate processes including fibroblast migration and contractility [5], endothelial cell adhesion [6,7], myotube formation [8], stem cell differentiation [9], and hepatocyte spreading [10]. Polyethylene glycol (PEG)-based substrata exhibiting similar E comparable to those of PA hydrogels have also been used to evaluate the effects of mechanical

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compliance on cellular morphology and phenotype [11]. However, because changes in composition or extent of crosslinking in natural and aforementioned synthetic systems may also affect surface ligand density, configurations, and distensibility [12], the interplay between biochemical and mechanical cues on cellular fates has not yet been fully decoupled. A system amenable to independent modulation of chemical composition, stiffness, and ligand presentation has the potential to help elucidate the mechanisms of cooperative chemomechanical feedback, as well as aid in the development of highly functional in vitro models of tissues.

Weak polyelectrolyte multilayers (PEMs) comprising poly (acrylic acid)/poly(allylamine hydrochloride) are ionically cross-linked hydrogels that serve as a robust model system with unique advantages for decoupling the effects of chemical and mechanical stimuli on cellular processes. Distinct from PA or PEG synthetic systems, the elastic modulus of these weak PEM substrata is varied over several orders of magnitude ranging 10^5–10^8 Pa through control of layer-by-layer (LbL) assembly pH [7]. LbL assembly is performed by alternately dipping substrates (e.g., glass or tissue-culture polystyrene, TCPS) in solutions of polyanion and polycation chains with defined pH; for these weak PEMs, the degree of ionic crosslinking between the polyanion and polycation chains, and thus the mechanical stiffness defined by E, increases as pH increases from pH 2.0 to pH 6.5. Extensive characterization of these PEM films, hydrated at near-neutral pH in water or buffered cell media, has confirmed that assembly pH reliably modulates mechanical stiffness over this range, without concurrent or statistically significant differences in surface roughness, surface charge, or hydrophobicity/philicity as determined by total interaction energy [13]. We have previously employed these weak PEMs to show that increasing the stiffness of a given PEM composition via increased crosslinking can enhance the adherence of vascular endothelial cells [7] and fibroblasts [14], and that ligand functionalization of such PEMs can be achieved to avoid inadvertent alteration of the substrate [14]. Therefore, these PEMs are uniquely suited for the independent modulation of substrata mechanical compliance and ligand presentation for cell types less amenable to sustained in vitro manipulation and function.

Here, we leverage PEMs to systematically study the effects of mechanical and biochemical cues on primary rat hepatocytes towards the development of a functional in vitro model of liver tissue. Freshly isolated primary hepatocytes are widely considered to be ideal for construction of liver tissue models useful in fundamental biological studies [15,16], bio-artificial liver devices [17], and drug screening [18]; yet these cells rapidly (hours to a few days) lose viability and phenotypic functions upon isolation from the native in vivo microenvironment of the liver [15,19]. Several studies have enhanced the phenotypic functions of hepatocytes in vitro by modifying the chemomechanical microenvironment via gels formed from natural proteins (e.g., Matrigel® [20–22] and collagen gel sandwiches [23]). However, natural gel- and sandwich-culture systems are not ideal for systematic chemomechanical manipulation and testing due to variability among protein batches and challenges in decoupling or systematically varying the mechanical and biochemical properties. Synthetic polymeric systems do not generally suffer from such disadvantages but, to date, have not sustained hepatocytes for longer than one week without additional stromal cells or ligands requiring chemical conjugation to the polymer backbone [24–26]. Furthermore, compliance of PEMs used in previous reports was not quantified independently of added cellular or extracellular matrix cues; some surface chemistry modifications can inadvertently increase the mechanical stiffness of PEMs [14], such that the actual compliance of substrate after chemical modification should be confirmed. Thus, the systematic and reproducible investigation of biochemical and mechanical stimuli on pure hepatic phenotype has not been fully

explored towards facile engineering of defined PEM microenvironments which influence broad classes of liver-specific functions over extended in vitro culture.

In this study, weak PEM substrata with elastic moduli ranging from 10^5 to 10^8 Pa were used to evaluate the independent and synergistic effects of multiple biochemical cues (type I collagen, proteoglycan decorin) and mechanical compliance on the adhesion, morphology and phenotypic functions of primary rat hepatocytes. Hepatocyte functions were evaluated on substrata that were chemomechanically optimized to promote the attachment and retention of hepatic spheroids, which have previously been shown to stabilize several liver-specific functions [27–29]. More specifically, we demonstrated retention of broad classes of hepatic functions (albumin secretion, urea synthesis and CYP450 1A activity) for two weeks on optimized PEMs, as compared to the well-known loss of phenotype of primary hepatocytes on collagen-coated TCPS and to shorter retention durations achieved via other synthetic hydrogels. We also observed that substrata stiffness modulated the functional effects of substrata-bound decorin ligand on the duration and levels of hepatic functions. Decorin, previously shown to induce functions in primary rat hepatocytes when presented on collagen-coated TCPS [15], retained such behavior on stiff PEMs; however, this proteoglycan down-regulated hepatic functions when presented on highly compliant PEMs, a previously unreported finding. We thus conclude that liver-specific functions are modulated strongly by the coupling between ligand presentation and mechanical compliance of the synthetic substrata, over a wide range of elastic moduli achievable in a scalable synthetic substrata platform.

2. Materials and methods

2.1. Preparation of PEM substrata

Poly(acrylic acid) (PAA, Polysciences; $M_w \sim 70,000$ g/mol) and poly(allylamine hydrochloride) (PAH, Sigma–Aldrich; $M_w \sim 90,000$ g/mol) were prepared as dilute solutions of polyelectrolytes (0.01 M by repeating unit molecular weight) in deionized water, and pH adjusted to 2.0, 4.0, or 6.5 using HCl or NaOH. Layer-by-layer (LbL) assembly was employed, using a programmable slide stainer (Zeiss) to coat multi-well tissue culture-treated polystyrene plates (TCPS, Becton Dickinson) and glass coverslips (VWR International) with alternating layers of PAA and PAH adjusted to the same pH, resulting in ionically crosslinked PEMs [7]. Substrata are denoted by assembly pH, e.g., "PEM 2.0" indicates that the substrata were assembled for PAA and PAH solutions both adjusted to pH = 2.0, with PAA as the last dipping solution. The number of layers was varied to obtain a uniform hydrated thickness of ~100 nm: PEM 2.0, 4.0, and 6.5 samples contained 11, 15, and 42 bilayers, respectively [7]. Prior to cell seeding, all surfaces were sterilized in 70% EtOH for 1 h, followed by 3× rinses with sterile ddH₂0. Substrates were coated with 100 μg/mL collagen I or 100 μg/mL collagen I pre-mixed with 25 μg/mL decorin (Sigma) for 2–3 h at 37 °C.

2.2. Rat hepatocyte isolation and culture

Hepatocytes were isolated from 2–3 month old adult female Lewis rats (Charles River Laboratories) using a modified procedure of Seglen [30] and seeded at 0.3×10^6 cells per well (12-well plates modified with PEMs) in serum-free culture medium comprising high glucose DMEM, 0.5 U/mL insulin, 7 ng/mL glucagons, 7.5 μg/mL hydrocortisone, 10 U/mL penicillin, and 10 μg/mL streptomycin. Cells were cultured in serum-free medium at 37 °C, 5% CO₂ for 6–8 h to allow for attachment, followed by removal of unattached cells and replacement with serum-supplemented (10% FBS) medium. Culture medium was sampled and replaced daily.

2.3. Quantification of hepatocyte adhesion and functions

Quantification of cell adhesion was performed by counting cells in phase contrast micrographs (Nikon Eclipse TE200 and CoolSnap-HQ Digital CCD camera) taken 6–8 h after cell seeding. Six 10× magnification fields of cells per condition were averaged for each condition and normalized to the average number adhered to positive control substratum (collagen-modified TCPS). Albumin content in conditioned media was measured using an enzyme linked immunosorbent assay (ELISA) with horseradish peroxidase detection and peroxidase substrate 3,3′,5,5′-tetramethylbenzidine as described previously [15]. Urea concentration was quantified using a colorimetric endpoint assay based on acid- and heat-catalyzed condensation of urea with diacetylmonoxime (Stanbio Labs). Cytochrome P450 (CYP1A1) enzymatic activity was measured by quantifying the amount of resorufin produced from the

CYP-mediated cleavage of ethoxyresorufin *O*-deethylase (EROD). EROD (5 μM) was incubated with cell cultures for 30 min, medium was collected, and resorufin fluorescence was quantified at 571/585 nm (excitation/emission wavelengths).

2.4. Statistical analysis

Statistical significance was determined using two-tailed *t*-test or one-way ANOVA (analysis of variance) with Tukey's post-hoc test. Unless otherwise noted, error bars represent SEM (standard error of mean), with sample size (n) and *p*-value for each data set indicated in the corresponding figure captions.

3. Results

3.1. Effects of substrata compliance on hepatocyte adhesion and morphology

In order to evaluate the effect of mechanical compliance on the adhesion and morphology of primary rat hepatocytes, we assembled weak polyelectrolyte multilayer films (PEMs) of ~100 nm hydrated thickness, comprising ionically crosslinked polycationic poly(acrylic acid) (PAA) and polyanionic poly(allylamine hydrochloride) (PAH), onto tissue-culture polystyrene (TCPS); see Fig. 1A for general schematic. PEM substrata are typically described by the cation/anion pair and assembly pH for each polyelectrolyte, e.g., PAA/PAH 2.0/2.0 indicates a PEM assembled at pH 2.0 for both polyelectrolytes [7], but are denoted herein as PEM 2.0, etc. The assembly pH of the PEMs (e.g., PEM 2.0, 4.0, or 6.5) determines the extent of ionic crosslinking between the polycation and polyanion chains, and thus the extent of swelling and the mechanical stiffness (or, inversely, mechanical compliance) of the PEMs in solvents of pH ~ 7 (e.g., water and cell culture medium). Physical properties [13] and mechanical stiffness of these PEMs have been extensively characterized by our group, and indentation elastic modulus E was

confirmed for the substrata assembled in this study via atomic force microscopy-enabled nanoindentation (see Supplementary data) to range from 10^5 Pa (PEM 2.0) to 10^8 Pa (PEM 6.5). TCPS of $E \sim 10^9$ Pa [7] served as the rigid substratum control for our studies.

Primary rat hepatocytes were first seeded onto unmodified substrata, i.e., substrata that were not coated with proteins such as collagen, in serum-free culture medium (to avoid cell attachment via serum proteins that adsorb onto the PEMs). Cell adhesion was quantified by counting cells in phase contrast micrographs acquired 6–8 h after initial seeding. Attachment of hepatocytes on collagen-coated TCPS was used to normalize all subsequent adhesion values. Our results in Fig. 1B indicate that, relative to collagen/TCPS controls, hepatocyte attachment was maximal (~100%) on unmodified PEM substrata of low compliance (PEM 6.5: $E \sim 142$ MPa), followed by substrata of intermediate compliance (PEM 4.0: $E \sim 1.7$ MPa and ~91% attachment relative to collagen/TCPS). Negligible hepatocyte attachment (~5% of collagen/TCPS) was observed on the most compliant PEM substrata (PEM 2.0: $E \sim 200$ kPa) used in this study. Hepatocyte attachment on rigid, unmodified TCPS ($E \sim 2.5$ GPa) was ~67% of that seen on collagen-coated TCPS. Furthermore, hepatocytes formed spheroidal structures on unmodified PEMs 6.5 and 4.0, while cells spread upon attachment to collagen-adsorbed TCPS (Fig. 1C).

3.2. Chemomechanical modulation of hepatic adhesion, morphology and phenotypic functions

In order to evaluate the effect of chemomechanical stimuli on hepatocyte behavior, we modified the two PEM substrata of maximally disparate mechanical compliance (PEM 6.5 of $E \sim 142$ MPa and PEM 2.0 of $E \sim 200$ kPa) with type I, rat-tail collagen or with collagen pre-mixed with the small proteoglycan
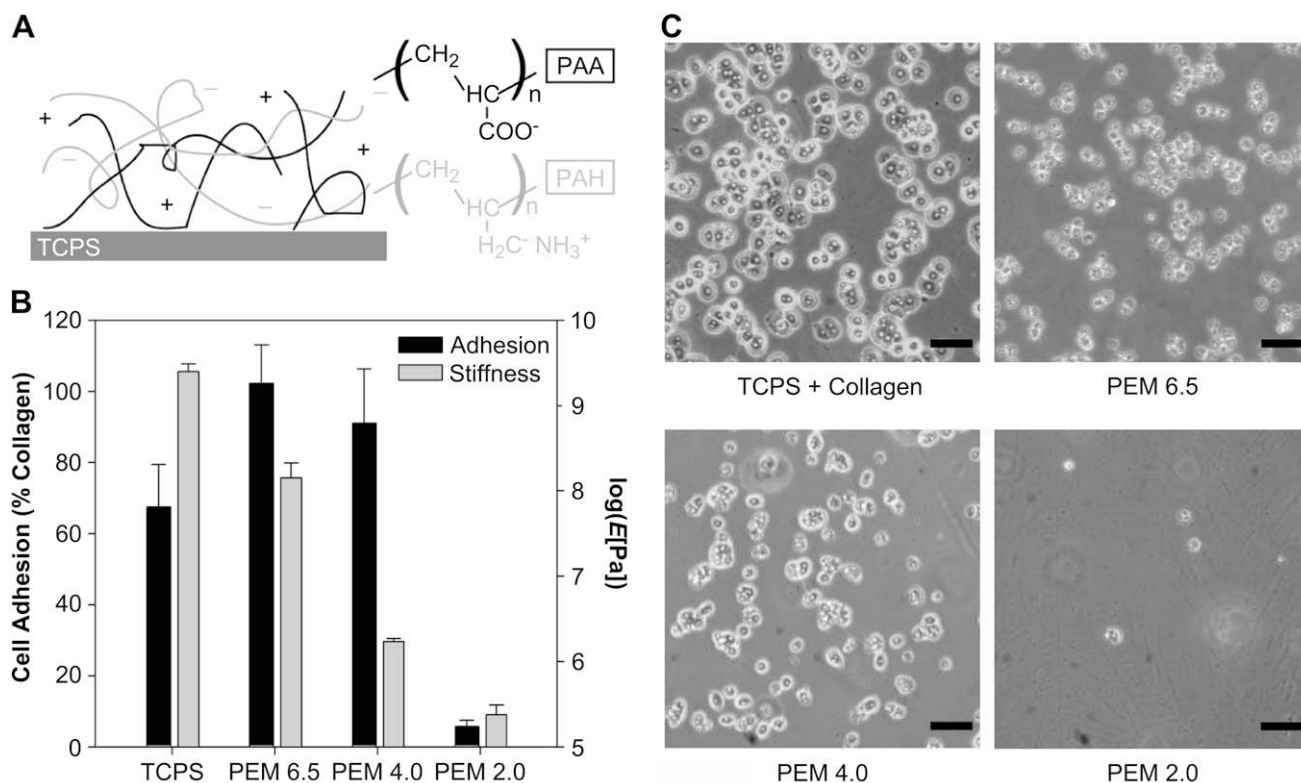

Fig. 1. Adhesion and morphology of primary rat hepatocytes on polyelectrolyte multilayers (PEMs). (A) Schematic depicting coating of tissue-culture polystyrene (TCPS) with PEMs comprising interpenetrating poly(acrylic acid), PAA, and poly(allylamine hydrochloride) PAH. (B) Quantification of hepatocyte adhesion on rigid TCPS and PAA/PAH PEMs of varying compliance (assembly pH 6.5, 4.0 and 2.0). All data normalized to hepatocyte adhesion on collagen-coated TCPS. Indentation elastic modulus E for each substratum (measure of stiffness) also shown. Error bars represent SEM ($n = 6$–8). (C) Phase contrast micrographs showing hepatocyte morphology ~24 h after seeding onto substrates of varying compliance. Scale bars = 100 μm. Error bars represent SEM.

decorin, previously demonstrated by our group to induce hepatic functions on collagen-modified TCPS [15]. We have shown that decorin alone is insufficient to promote hepatocyte attachment; hence, mixing with collagen was required. Prior to hepatocyte culture, we verified that this adsorption of proteins (collagen ± decorin) did not significantly alter the thickness, roughness, or compliance of the PEMs (Supplementary Fig. 1). Furthermore, collagen and decorin surface density on each PEM were shown to be statistically indistinguishable via antibody staining of protein-modified PEMs (Supplementary Fig. 2). As with unmodified surfaces, primary rat hepatocytes were seeded onto the protein-modified surfaces in serum-free culture medium and attachment was quantified via phase contrast micrographs taken 6–8 h after initial seeding. Our results indicated that protein modification of the most compliant substratum (PEM 2.0) led to enhanced hepatocyte attachment which was statistically similar to the stiffer, unmodified and protein-modified substrata (Fig. 2A). As Supplementary Fig. 1 shows, protein modification of PEMs did not alter mechanical compliance of each substrata type, suggesting that differences in hepatocyte attachment can be attributed to collagen I modification of the compliant PEM 2.0 substratum. Similar cell attachment across the collagen I-modified surfaces of varying

compliance thus enabled culture and compliant-dependent phenotypic evaluation of hepatocytes for two weeks in vitro.

Assessment of hepatic albumin secretion (a marker of liver-specific protein synthesis [31]) indicated increased hepatic function on protein-modified surfaces as compared to unmodified controls (Fig. 2B). Furthermore, albumin secretion decreased with decreasing substrata mechanical compliance: secretion was highest on collagen I-modified PEM 2.0 substrata, lower on collagen I-modified PEM 6.5, and lowest on collagen I-modified TCPS. Consistent with our previous studies [15], we verified here that decorin pre-mixed with collagen induced hepatocyte functions on rigid TCPS ($\sim$150% of collagen/TCPS controls). We found that decorin induced hepatic functions on the stiffer PEM 6.5 to a similar extent as on rigid TCPS; however, functions were down-regulated on the more compliant PEM 2.0 ($\sim$80% of collagen/TCPS controls). Urea synthesis (data not shown), a surrogate marker of liver-specific nitrogen metabolism, showed trends similar to those seen for albumin secretion in Fig. 2B. Quantification of hepatocyte DNA, to assess how well cells and multi-cellular spheroids were retained over time on PEM surfaces, confirmed that protein-modified PEM 2.0 surfaces maintained hepatocyte attachment over at least two weeks, while hepatocytes were only weakly adhered to and
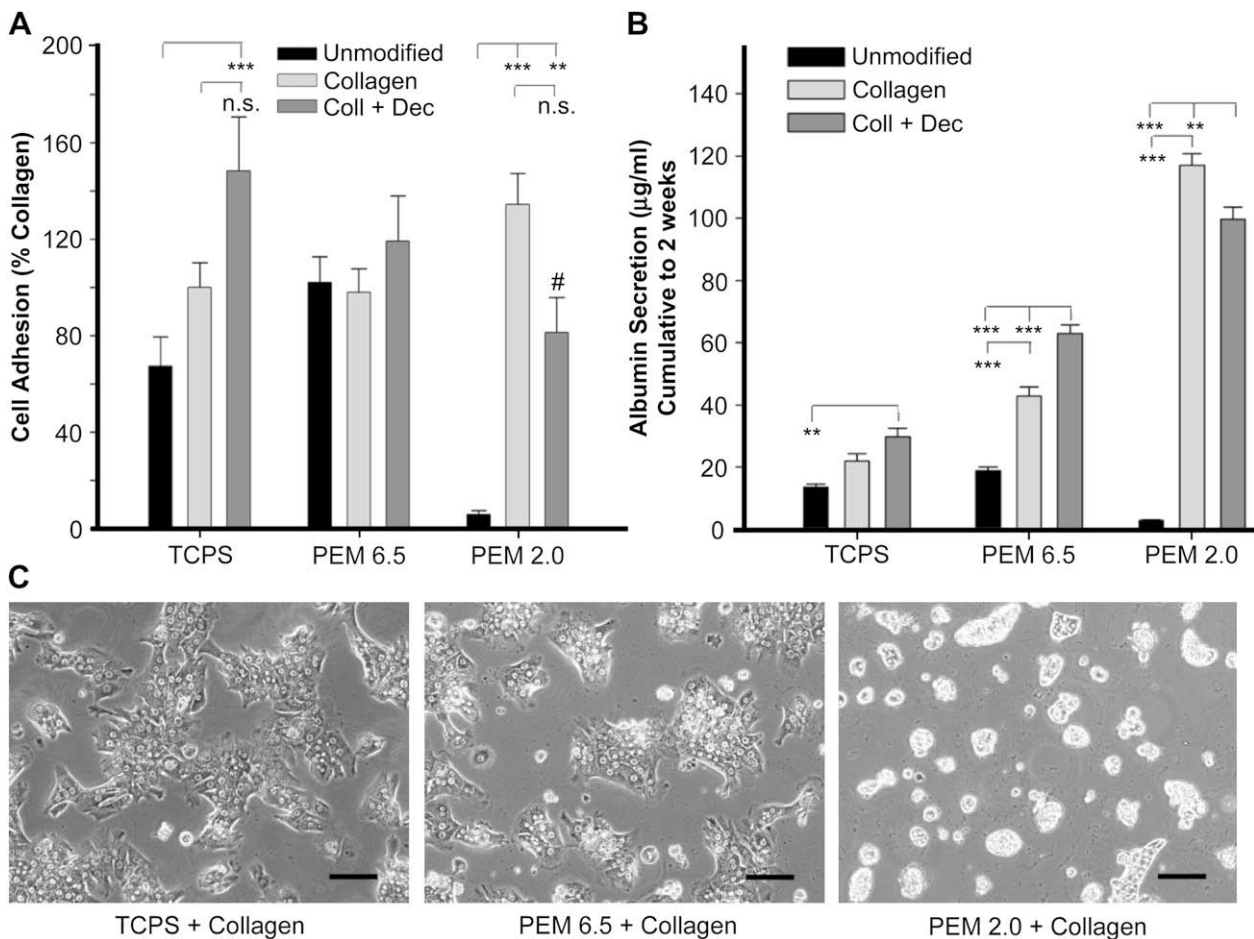

Fig. 2. Adhesion, morphology and phenotypic functions of primary rat hepatocytes on polyelectrolyte multilayers (PEMs) modified with extracellular matrix proteins. (A) Quantification of hepatocyte adhesion on substrates modified with either type I collagen (100 μg/mL) or collagen mixed with the proteoglycan decorin (25 μg/mL). All data are normalized to hepatocyte adhesion on collagen-coated TCPS. Error bars are SEM ($n = 6$–8). Pairwise differences among collagen-modified substrates of varying compliance were not statistically significant (n.s.). #$p < 0.01$ vs. 'TCPS + Coll + Dec', **$p < 0.01$, ***$p < 0.001$, for One-way ANOVA with Tukey's post-hoc test. (B) Quantification of hepatocyte functions on protein-modified substrates: cumulative albumin secretion over two weeks. Error bars are SEM ($n = 3$). Pairwise differences among unmodified surfaces were not statistically significant (n.s.), among collagen-modified surfaces $p < 0.001$, and among collagen + decorin-modified surfaces $p < 0.001$. **$p < 0.01$, ***$p < 0.001$ for One-way ANOVA with Tukey's post-hoc test. (C) Hepatocyte morphology on collagen-coated substrata of varying compliance, 2 days post-seeding. Hepatocyte morphology on substrata modified with collagen + decorin was similar. Scale bars = 100 μm. Error bars represent SEM.

released within one week from PEM 6.5 surfaces (Supplementary Fig. 3). Lastly, hepatocytes formed stable, spheroidal aggregates of approximately 50–100 μm diameter on protein-modified PEM 2.0 surfaces, while the extent of cell spreading increased with reduction in substrata compliance (Fig. 2C).

3.3. Retention of hepatic spheroids and functions on collagen-coated PEMs

We measured functional kinetics of hepatocytes on collagen-modified PEM 2.0 surfaces over two weeks in order to evaluate the longevity and phenotypic stability of hepatocytes interfaced with PEMs. Hepatocytes cultured on collagen-modified TCPS were considered as declining controls, as is conventional for in vitro hepatic studies [15,19]. Hepatic albumin secretion (Fig. 3A), urea synthesis (Fig. 3B), and cytochrome P450 1A activity, a marker of liver-specific detoxification (Fig. 3C), were well retained on collagen-modified PEM 2.0 substrata for 2 weeks, whereas a monotonic decline was confirmed on collagen-modified TCPS. Furthermore, over this extended culture, hepatocytes organized into stable spheroids maintained at approximately 50–100 μm

diameter on the compliant PEM 2.0 substrata, whereas cells spread on rigid TCPS to adopt a fibroblastic morphology, characteristic of unstable hepatocytes (Fig. 3D).

4. Discussion

Cell fate processes are influenced not only by cell-autonomous programs, but also by the local microenvironment or "niche", which is comprised of variable biochemical stimuli and mechanical cues presented by both neighboring cells and extracellular materials. Thus, development of functionally robust models of tissues for in vitro and therapeutic applications will require precise control over such cues at multiple length and time scales. In this study, we have utilized synthetic, weak polyelectrolyte multilayer substrata (PEMs) to evaluate the independent and synergistic effects of biochemical and mechanical stimuli on the adhesion, morphology, and phenotypic functions of primary hepatocytes, which are considered highly important for liver tissue engineering and in vitro drug screening yet are difficult to maintain in culture [15].

The compatibility of primary rat hepatocytes with purely synthetic hydrogels, strong PEMs comprised of poly(diallyldimethylammonium
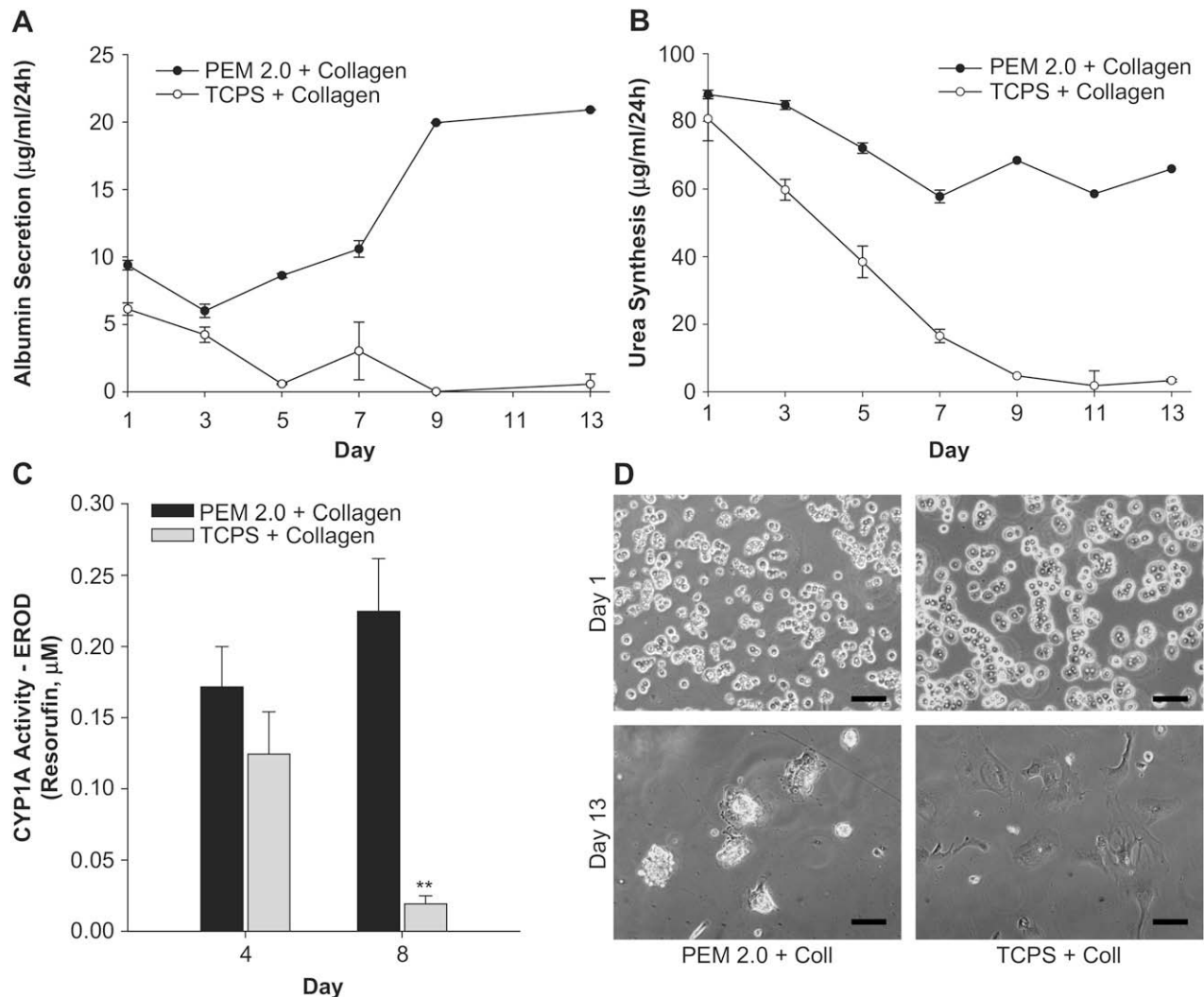

Fig. 3. Comparison of hepatocyte morphology and phenotypic functions on tissue-culture polystyrene (TCPS) and compliant polyelectrolyte multilayers (PEM 2.0) modified with type I collagen. (A) Rate of albumin secretion (marker of liver-specific protein synthesis) in hepatocytes on collagen-modified substrates over two weeks. (B) Rate of urea synthesis in hepatocytes on collagen-modified substrates over two weeks. (C) Activity of cytochrome P450 1A (CYP1A, marker of detoxification function) as measured via ethoxyresorufin O-dealkylation (EROD) in hepatocytes, 4 and 8 days after seeding onto collagen-modified substrates. Error bars represent SEM ($n = 3$). $**p < 0.05$ vs. 'PEM 2.0 + Collagen (Day 8)' for One-way ANOVA with Tukey's post-hoc test. (D) Morphology of hepatocytes 1 and 13 days after seeding onto collagen-modified substrates. Scale bars = 100 μm. Error bars represent SEM.

chloride) (PDAC) and poly(sulfonated styrene) (PSS), was first demonstrated by Kidambi et al. [25]. The authors showed that PSS-terminated substrata promoted hepatocyte adhesion and spreading; yet liver-specific functions (albumin and urea production) on these synthetic substrates declined over one week in culture and were comparable to those seen on unmodified tissue-culture polystyrene [25]. Furthermore, the authors did not evaluate the dependence of hepatocyte morphology and functions on varying substrata mechanical compliances. In a follow-up study, the authors created co-cultures of hepatocytes and fibroblasts on protein-free surfaces by utilizing micropatterned domains of PEMs adhesive to either hepatocytes or fibroblasts [26]. The authors verified the previously reported and well-known "co-culture effect" on their surfaces by showing that 3T3 murine embryonic fibroblasts were able to induce functions in primary rat hepatocytes via heterotypic signaling [16,32,33]. It was unclear from this study, however, whether there were any synergistic effects of substrate mechanical compliance and heterotypic cell–cell interactions on liver-specific functions of hepatocytes. More recently, Janorkar et al. functionalized polyacrylic acid (PAA)/polyethyleneimine (PEI) PEMs with extracellular matrix-like polypeptides to enhance liver-specific functions [24]. Despite these experimental developments using PEMs, substrata compliance in the aforementioned reports could not be tuned via assembly pH and was not quantified independently of adjunct cells or ligands. Using PA hydrogels, Semler et al. polymerized, cut, and adhered to tissue-culture plate surfaces a 9-condition array of substrates with varying elastic moduli and densities of immobilized fibronectin, identifying high compliance regimes ($E = 1.9$ kPa) in which hepatocyte cell–cell interactions dominated over hepatocyte–fibronectin interactions [10]. While highlighting the impact of mechanical and biochemical cooperative signaling on liver-specific functions and gene expression, the conditions of this study were limited to relatively small variations in mechanical stiffness (5.6–19 kPa) and a single protein (fibronectin). Furthermore, this study required multiple tedious steps for system assembly, which limits the facile, reproducible investigation of multiple stimuli towards engineering defined microenvironments for hepatocytes or other cells.

We probed hepatocyte attachment on fully synthetic PEM substrata that varied in compliance over several orders of magnitude (elastic moduli E ranging from 0.2 to 2500 MPa), using serum-free culture medium to avoid cell attachment due to serum proteins pre-adsorbing onto the substrate. We found that hepatocytes displayed maximal attachment ($\sim$100% of adhesion to TCPS/collagen control, $E \sim 2500$ MPa) on substrata of lower mechanical compliance (PEM 6.5, $E \sim 142$ MPa; and PEM 4.0, $E \sim 1.7$ MPa), while negligible attachment was observed on the most compliant substrata (PEM 2.0, $E \sim 0.2$ MPa). We note that it is possible these substrata are sufficiently thin such that traction exerted by hepatocytes at adhesion sites could increase the effective or perceived stiffness of these substrata. Although calculation of such effective stiffness requires several assumptions and estimates of cell-generated forces and adhesion site radii that have not yet been well quantified for hepatocytes, our recent model of reduced adhesion site displacement on coatings of finite thickness [34] indicates that this effective stiffness would increase by the same factor (much less than 10-fold) for each of these three PEM substrata of identical thickness $\sim$100 nm. The inverse correlation between unfunctionalized substrata compliance and percentage of cell adhesion/spreading is consistent with previous observations for endothelial cells [7] and fibroblasts cultured on these PEMs [35], as well as for other adherent tissue cell types on polymer hydrogels [36]. While the mechanism of this protein-free hepatocyte adhesion to unfunctionalized, weak PEM substrata is currently unknown, differential adhesion of hepatocytes on varying compliance cannot be attributed to surface charge, energy, or roughness of these PEMs,

as we have shown that these physical properties are statistically indistinguishable for these substrata [13]. Lack of hepatocyte attachment to PEM 2.0 was therefore most likely due to reduced cell–substratum adhesion via unstable focal contact or adhesion complexes, as observed with other adherent tissue cell types such as fibroblasts [1]. On the stiffer PEM substrata (PEM 6.5 and 4.0), however, the balance of cell–cell and cell–substrata interactions stabilized formation of hepatic aggregates for several days in culture. In comparison, as expected from our previous work [15], hepatocytes on collagen-modified, rigid TCPS rapidly spread to adopt a fibroblastic morphology. Thus, the results of our primary hepatocyte adhesion studies are consistent with others showing that a variety of adhesive tissue cell types, including fibroblasts, cardiomyocytes and endothelial cells, adhere poorly to highly compliant hydrogels. The magnitude of substrata compliance that is sufficient to significantly reduce (or promote) cell adhesion depends on the cell type, substrata composition, and protein/ligand coating density, such that this trend is typically reported for internal comparisons for a given substrata system and cell type [1,7,10].

Unmodified PEM substrata of stiffness greater than $E \sim 0.2$ MPa promoted attachment and aggregation of hepatocytes; however, we found that cells detached from these stiffer substrata after only a few days of culture, due ostensibly to the dominance of cell–cell over cell–substrata interactions. We thus modified substrata with type I collagen, an extracellular matrix protein that has been shown to promote hepatocyte attachment for several weeks in culture [15]. We found that protein modification affected neither the thickness nor the mechanical compliance of the stiffest and most compliant PEM substrata. The PEM system therefore enabled independent comparison of hepatocyte functions over several weeks in well-defined chemomechanical microenvironments. Following collagen modification, hepatic spheroid formation was observed on PEMs exhibiting both low (PEM 6.5) and high (PEM 2.0) compliance. Spheroidal aggregates displayed higher levels of broad classes of liver-specific functions (albumin secretion, urea synthesis and CYP450 1A activity) as compared to well-spread hepatocytes on collagen-modified TCPS controls, a finding that is consistent with previously published reports [27–29]. However, previous methods to create hepatic spheroids (e.g., tumor-derived Matrigel®, roller bottles, non-adhesive dishes) are either confounded by coupled changes in ligand density (i.e., Matrigel®), or limited by challenges in handling and processing spheroids as they form and coalesce to become large cellular masses with necrotic cores. In this study, the compliant, collagen-modified PEM 2.0 substrata promoted attachment and long-term (two weeks) retention of hepatic spheroids (approximately 50–100 μm in diameter) over stiffer PEM 6.5 substrata, as evaluated by the quantification of adherent hepatocyte DNA over time. Tethered spheroids eliminated the need for sedimentation steps during culture medium changes for suspended spheroid cultures, and facilitated evaluation of hepatic morphology and functions with varying chemomechanical stimuli on a reproducible and synthetic PEM platform.

Type I collagen typically does not induce liver-specific functions in hepatocytes and is instead used with hepatocytes primarily as an adhesive cue on solid substrates. Although we used type I collagen to promote long-term retention of highly functional hepatic spheroids on PEM substrata, a primary objective of this study was to utilize the tunable PEM system to investigate the incorporation of hepatocyte-stabilizing biochemical cues on mechanically distinct substrata. Several such molecular cues have been previously implicated in induction of hepatic functions when presented alone on solid substrates or in combination with adhesive factors such as collagen [15,16,37]. Using a functional genomic screen on hepatocyte–fibroblast co-cultures, we have previously shown that

decorin, a chondroitin sulfate–dermatan sulfate proteoglycan that binds collagen and is present in the native liver [38,39], can induce phenotypic functions in primary rat hepatocytes adhered to collagen-coated TCPS [15]. In this study, we sought to evaluate the interplay of decorin and substrata mechanical compliance on the hepatic phenotype over extended in vitro culture. We observed that the presence of decorin caused hepatic functions of cultured hepatocytes to be up-regulated similarly on rigid TCPS and PEM 6.5 substrates. However, on highly compliant PEM 2.0 substrata, the presence of decorin led to down-regulation of liver-specific functions in hepatic spheroids, and such down-regulation was not attributable to statistically significant differences in initial attachment of cells or to differential retention of hepatic spheroids on collagen-only controls (evaluated by DNA quantification over time). Furthermore, the inability of decorin to enhance hepatic functions on PEM 2.0 was not due to saturation of albumin secretion, as hepatocytes secrete much higher albumin levels (~ 2 to 3-fold) upon co-cultivation with supporting fibroblasts at the cell seeding densities used in this study [15,39]. Such multifaceted effects of mechanical compliance and ligand type/density are not unexpected [8,20], given the common components of mechanotransductive and other functional signaling pathways. Semler et al. have also reported a monotonic coupling for fibronectin-functionalized polyacrylamide hydrogels [10], noting increased albumin secretion for gels of lower compliance and constant fibronectin concentration; however, the authors considered shorter durations (one week) and a much narrower range of substrata stiffness (shear elastic storage modulus $G' \sim 2$–9 kPa) than those considered in this study.

Although we demonstrated via antibody staining that the extent of collagen and decorin adsorption to PEMs was statistically indistinguishable, it remains possible that ligand orientation and/or altered collagen fibril structure [38,40] may differ among these PEMs (or between the PEMs and the TCPS) in a manner correlative with mechanical compliance. It is further possible that PEM mechanical compliance may not only define the initial microenvironment that modulates cell adhesion, but also the subsequent capacity of cell-generated proteins and ligands to be produced and/or absorbed to these substrata. These correlative factors are very challenging to quantify on hydrogel surfaces and are beyond the scope of the present study, but remain important considerations in the distinction between causal and correlative effects of substrata stiffness on tissue cell function. We also note that the mechanical stiffness of the most compliant hydrogels considered in this study ($E \sim 200$ kPa) is within the range of normal liver tissue measured via various methods ($E \sim 1$–750 kPa) [41–43]; however, it is difficult to draw a direct analogy between the tunable, synthetic PEM platform used in vitro here and microenvironments present in patho/physiological states in vivo. Future studies in our laboratories will further tune and improve hepatic functions in vitro via the use of combinatorial mixtures of polymer-tethered ligands and soluble factors (e.g., growth factors), on two- and three-dimensional materials that display spatial variations in compliance and ligand-tether flexibility.

5. Conclusion

We demonstrate that hepatocyte morphology and broad classes of phenotypic functions can be modulated via independent and synergistic tuning of biochemical and mechanical stimuli as presented on synthetic polymeric substrata. Facile assembly and synergistic effects of high substrata compliance and collagen presentation onto a standard 2D culture surface enabled creation of a robust, pure-hepatocyte tissue model which displayed enhanced liver-specific functions over collagen-modified TCPS controls for two weeks. We also discovered compliance-mediated effects of the proteoglycan decorin on hepatic functions, with hepatocyte functions down-regulated on highly compliant surfaces as compared to collagen-only controls but up-regulated on increasing PEM stiffness. Potential applications of our multi-well platform include medium- to high-throughput evaluation of interactions between exogenous compounds (e.g., drugs, environmental toxicants) and the various microenvironmental cues used to modulate fate processes of primary hepatocytes. Lastly, our approach of modulating chemomechanical cues towards improvement of cellular functions in vitro is amenable to multiple cell types (e.g., stem and precursor cells) for applications such as drug screening, cell-based therapies or the fundamental study of chemomechanical processes underlying tissue function and disease.

Appendix. Supplementary data

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.biomaterials.2008.10.055.

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