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Engineering Surface Adhered Poly(vinyl alcohol) Physical Hydrogels as Enzymatic Microreactors

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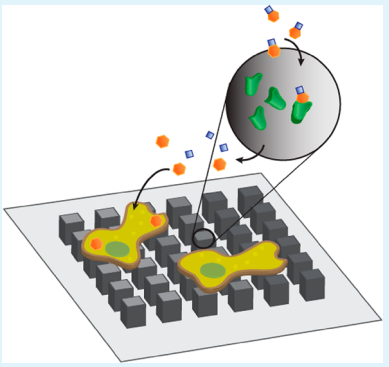
ABSTRACT: In this work, we characterize physical hydrogels based on poly(vinyl alcohol), PVA, as intelligent biointerfaces for surface-mediated drug delivery. Specifically, we assemble microstructured (μS) surface adhered hydrogels via noncryogenic gelation of PVA, namely polymer coagulation using sodium sulfate (Na_2SO_4). We present systematic investigation of concentrations of Na_2SO_4 as a tool of control over assembly of μS PVA hydrogels and quantify polymer losses and retention within the hydrogels. For polymer quantification, we use custom-made PVA with single terminal thiol group in a form of mixed disulfide with Ellman's reagent which provides for a facile UV−vis assay of polymer content in coagulation baths, subsequent washes in physiological buffer, and within the hydrogel phase. Polymer coagulation using varied concentrations of sodium sulfate afforded biointerfaces with controlled elasticity for potential uses in investigating mechano-sensitive effects of mammalian cell culture. For surface mediated drug delivery, we propose a novel concept termed Substrate Mediated Enzyme Prodrug Therapy (SMEPT) and characterize μS PVA hydrogels as reservoirs for enzymatic cargo. Assembled functional interfaces are used as matrices for cell culture and delivery of anticancer drug achieved through administration of a benign prodrug, its conversion into an active therapeutic within the hydrogel phase, and subsequent internalization by adhered hepatic cells. Taken together, the presented data contribute significantly to the development of novel matrices for surface-mediated drug delivery and other biomedical applications.



KEYWORDS: *hydrogel, enzyme, enzyme prodrug therapy, poly(vinyl alcohol), micropatterning, biointerfaces*

INTRODUCTION

Rational design and engineering of intelligent interfaces between a biomaterial and adhering cells allows controlling behavior and fate of the latter across the scales, from cell ensembles (i.e., patterning and directed proliferation) to individual cells, and elicit subcellular effects.[1−3] The available toolbox for creating biointerfaces consists of cues of chemistry, geometry, and physics, and includes controlled fouling, specific ligands,[1] surface topography,[4] and mechanics of the substrate for cell adhesion.[5] Of the diverse materials employed in these undertakings, hydrogels appear to be stand-alone materials that offer arms of control over each of the aforementioned cues. In particular, matrix elasticity is effectively engineered via appropriate cross-linking density and swelling of a hydrogel,[5,6] a feature that is not available for solid materials. Fine control over water content makes hydrogels truly unique biomaterials with utility in diverse biotechnological and biomedical applications, from biomass conversion[7,8] and enzymatic catalysis using immobilized proteins[9−11] to drug delivery[12−14] as well as production of tissue mimics[15−17] and matrices for tissue regeneration.[18,19] Specifically for assembly of intelligent biointerfaces, recently we presented surface adhered hydrogels based on poly(vinyl alcohol), PVA, and demonstrated control over surface topography,[20] matrix elasticity,[21] and cell adhesion.[21] We focused on physical hydrogels, i.e., materials with their integrity maintained via noncovalent linkages. This choice was made based on a favorable toxicity profile of physical hydrogels over their chemical counterparts as well as a friendly nature toward fragile biological cargo.[22−24]

In our previous work, we developed noncryogenic methods of stabilization of microstructured (μS) surface adhered PVA physical hydrogels using coagulating kosmotropic salt (aqueous sodium sulfate), aqueous isopropanol, and oligo(ethylene glycol) and demonstrated that the choice of stabilization milieu is decisive in controlling the Young's modulus of the resulting hydrogel.[21] The latter was varied from 50 kPa to 2 MPa, with polymer molecular weight providing further control over matrix elasticity. This range of Young's moduli is similar to the elasticity of soft human tissues[25] and was previously shown to be relevant in controlling (sub)cellular effects mediated by the matrix for cell adhesion.[5,26,27] The aim of this work is to further investigate the possibility to engineer PVA physical hydrogels with programmed matrix elasticity, specifically using concentrations of coagulating salt as tool in the design of hydrogels.

Another aspect of this investigation relates to a persistent shortcoming of hydrogels as matrices for drug delivery and

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tissue engineering, namely their high permeability to solutes.[28] Although this feature makes hydrogels excellent tools for immobilization of cells and bioconversion applications,[8,29] it effectively spells a failure for retention and controlled release of incorporated cargo. One successful approach to circumvent this is delivered by composite hydrogels,[14] i.e., swollen matrices that contain drug reservoirs in the form of, e.g., liposomes, microparticles, etc. This approach is successful academically and has documented practical applications and commercial success.[14,30] For surface adhered materials, we have implemented this technique with the use of drug-loaded polymersomes and revealed that for surface mediated drug delivery, this approach delivers a practical benefit and a higher therapeutic response as compared to solution based administration of thiocoraline, a cytotoxic peptidic drug.[31] However, a phenomenological limitation of composite hydrogels is a finite, limited drug loading. In this work, we demonstrate an innovative approach to engineering drug delivery into hydrogel matrices. We propose to equip hydrogel materials with enzymes and achieve an in situ conversion of administered prodrugs within the structure of the hydrogels. In doing so, we capitalize on high permeability of hydrogels to solutes, a characteristic that is envisioned to provide for a high rate of diffusion and exchange of (pro)drugs between the hydrogel and the solution bulk. Enzyme immobilization into hydrogels and PVA hydrogels in particular has been previously developed toward bioconversion applications.[9,10,32] However, to the best of our knowledge, it has never been adopted to equip hydrogel matrices with tools for controlled drug delivery. We term this drug delivery approach Substrate Mediated Enzyme Prodrug Therapy (SMEPT) and demonstrate proof-of-concept therapeutic effect achieved via this concept using surface adhered μS PVA hydrogels. Specifically, we use enzyme containing substrates for adhesion of mammalian cells, perform enzymatic conversion of prodrugs of SN-38, a potent anticancer therapeutic, and quantify cytotoxic effects in adhering cells. Taken together, we believe that this work contributes significantly to the development of intelligent biointerfaces with engineered opportunities in controlled drug release. We anticipate that these results will prove useful in the design of therapeutic implants and matrices for tissue engineering.

EXPERIMENTAL SECTION

All chemicals were obtained from Sigma-Aldrich and used without purification, unless stated otherwise. Fluorescein diglucuronide was purchased from Invitrogen. SN-38 glucuronide was purchased from Toronto Research Chemicals (Canada). Differential interference contrast (DIC) images were obtained using a Zeiss Axio Observer Z1 microscope. Solution fluorescence and UV−vis absorbance was quantified using an EnSpire Multilabel Plate Reader. High-purity water with a resistivity of 18.2 MΩ/cm was obtained from an in-line Thermo scientific, Barnstead easypure II. Gel permeation chromatography (GPC) was performed using a system comprising a LC-20AD Shimadzu HPLC pump, a Shimadzu RID-10A refractive index detector, and a DAWN HELEOS 8 light scattering detector along with a SPD-M20A PDA detector, equipped with a HEMA-Bio Linear column with 10 μm particles, a length of 300 mm and an internal diameter of 8 mm from MZ-Analysentechnik providing an effective molecular weight range of 1000−1 000 000.

Polymer Synthesis. A sample of PVA with M_n 28 kDa, PDI 1.16 was synthesized as described in detail elsewhere.[33] Briefly, precursor polymer, poly(vinyl acetate), was synthesized via reversible addition−fragmentation chain transfer polymerization (RAFT) using *O*-ethyl *S*-phthalimidomethyl xanthate RAFT agent. Deprotection of the phthalimide end-group using hydrazine afforded polymer chains with a single amine terminal group. Saponification using methanolic NaOH afforded PVA with terminal amino group. Subsequently, in a one-step reaction using Traut's reagent (2-iminothiolane) and Ellman's reagent carried in 0.1 M carbonate buffer pH 8.3, amine terminated PVA was converted into PVA with a single terminal thiol group in a form of a mixed disulfide with Ellman's reagent, PVA_{ER}. Target polymer was isolated via precipitation into methanol.

Microtransfer Molding (μTM) of PVA. Assembly of μS PVA hydrogels via μTM was performed as described in detail in our previous publications.[20,21] Briefly, poly(dimethylsiloxane) (PDMS) elastomeric stamps were prepared using Sylgaard 184 (Dow Corning) and custom-made silicon wafer with 2 μm cuboid pillars. Onto the resulting PDMS stamp with cuboid cavities a drop of polymer solution (12 wt % in Milli-Q water) was deposited together with a microscopy coverslip and clamped at finger tight pressure for 24 h. Detachment of the setup afforded μS PVA thin films adhered to the surface of the coverslip. Coverslips with μS PVA films were then immersed into a solution of sodium sulfate and subsequently incubated in PBS, which afforded the final product, surface adhered μS PVA hydrogels. In all experiments, to ensure reproducibility, μS thin films were assembled on 9 mm coverslips using PDMS stamps with exceeding dimensions. Here and below, unless stated otherwise, for each data point, at least 3 independent runs with at least 3 replicates for each sample were performed and presented as mean ± standard deviation.

Analysis of Polymer Retention. μS films of PVA_{ER} 12 wt %, 28 kDa were assembled as described above using coagulation baths with concentration of Na_2SO_4 ranging from 100 to 1000 mM and duration of coagulation 1 or 24 h and subsequent incubation in PBS for 1 and 24 h. Coagulation and incubation in PBS were conducted at 37 °C. Coagulation media, PBS, and μS films were used to quantify the polymer content using dithiothreitol, DTT. Specifically, solutions were charged with excess DTT (50 g L^{-1} in PBS), which resulted in cleavage of Ellman's reagent terminal functionality. The latter was quantified using UV−vis spectroscopy reading absorbance at 412 nm.

Atomic Force Microscopy (AFM) imaging and Force Curves. For AFM imaging and force curve analysis, μS PVA surface adherent films were prepared via μTM as described above. Specifically, the structures were molded for 24 h, followed by stabilization for 1 or 24 h with Na_2SO_4 of 250, 500, and 1000 mM, and thereafter 24 h incubation in PBS. Both stabilization and PBS incubation was carried out at 37 °C. AFM images were recorded on a Nanowizard II BioAFM (JPK, Germany) using soft contact mode cantilevers (CSC38, no Al, typical value for spring constant 0.08 N m^{-1}, MikroMasch) in PBS. Imaging of the structures was required in order to measure the force curves at the center of the cube. Cantilever calibration was carried out using the JPK software where the sensitivity and spring constant was determined by the thermal noise method. The Hertz model was applied to the force curves in order to derive the Young's modulus using the JPK software assuming a cone shape tip with a 20° half-cone angle and a Poisson's ratio of 0.5. The results are based on at least 10 force curves of at least three independent samples.

Enzyme Immobilization. μS PVA films were prepared as described above using 12 wt % solutions of PVA containing β-glucuronidase (*E. coli*) at a concentration of 1 g L^{-1}. μS PVA films were incubated for 1 and 24 h in stabilization media at 250, 500, and 1000 mM at 37 °C, followed by rehydration for 1 and 24 h in PBS. Stabilization media, PBS, and supernatants above μS films were supplemented with fluorescein diglucuronide (FdG) to a concentration of 0.25 μg mL^{-1} and incubated for 30 min. Fluorescence of the resulting solutions was quantified using excitation and emission wavelengths 495 and 520 nm, respectively. In separate experiment, equivalent amount of β-glucuronidase (β-Glu) as used for assembly of μS hydrogels (ca. 1.5 μg per sample) was used in an assay described above to estimate enzymatic activity of pristine enzyme in solution based prodrug conversion. Enzymatic activity mediated by studied samples (stabilization media, PBS, and hydrated hydrogels) is expressed relative to the above obtained solution based level of β-Glu activity.

Cell Culture. For cell culture experiments, μS PVA films were prepared as described above using 12 wt % solutions of PVA

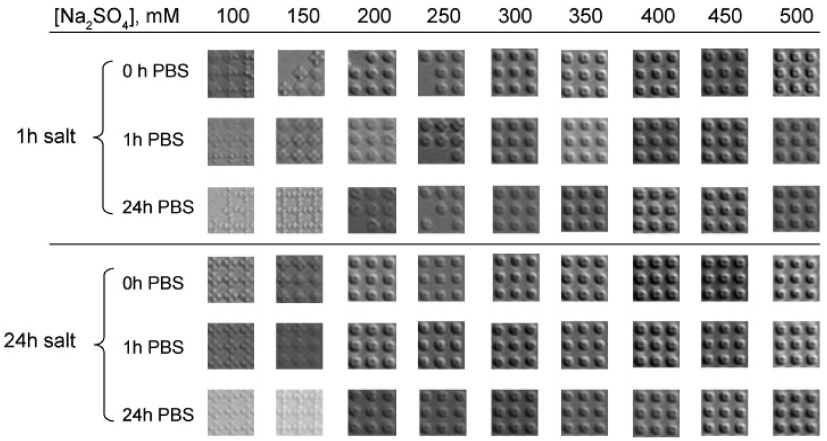

Figure 1. PVA hydrogels were assembled via μTM using PDMS stamps with cuboid cavities (2 μm side) and using sodium sulfate coagulation baths with varied concentration of Na_2SO_4. Hydrogels were stabilized by "salting out" method for 1 or 24 h with subsequent incubation in PBS for 1 or 24 h and visualized in hydrated state in PBS (40× magnification).

supplemented with poly-L-lysine (PLL, 40−60 kDa) to a concentration of 1 g L^{-1}. All samples were submitted to UV sterilization prior to cell culture experiments. Hepatocellular carcinoma cells (HepG2) were seeded on μS PVA films (9 mm coverslip placed in 48 well-plates) at a starting density of 75 000 cells/well and allowed to adhere overnight. For cell viability, the samples were analyzed after a 48 h incubation using Presto Blue cell viability reagent (Invitrogen).

SMEPT. μS PVA films were prepared as described above using 12 wt % solutions of PVA supplemented with poly-L-lysine (PLL, 40−60 kDa) and β-glucuronidase (*E. coli*) to final concentrations of 0.9 g L^{-1} and 1 g L^{-1}, respectively. Cell culture was performed as described above for 48 h in the presence of SN-38 glucuronide after which cell viability was quantified using Presto Blue.

RESULTS AND DISCUSSION

Assembly of hydrogels as surface adhered materials facilitates visualization and analysis of the samples using a suit of microscopy based techniques. Toward this end, we used microtransfer molding technique (μTM) and assembled PVA hydrogels on the surface of microscope coverslips with subsequent stabilization using aqueous kosmotropic salt, sodium sulfate. We have previously shown that sodium sulfate in concentrations of 500 mM and 1 M was equally effective in producing μS PVA hydrogels.[21] Our early observations suggested that high salt concentration is detrimental to the performance of the enzyme chosen for characterization of SMEPT, namely β-glucuronidase. On the other hand, decreasing salt concentration exerts a lower coagulation effect and may compromise performance of PVA matrices as cell adhesion substrates. Herein, we provide a detailed investigation on the concentration of sodium sulfate as a tool for design of PVA physical hydrogels, specifically in the form of surface adhered materials. In the first experiment, μTM was performed using PDMS elastomeric stamps with 2 μm cubic cavities. This was followed by a stabilization of the samples in sodium sulfate baths with salt concentrations varied from 100 to 500 mM and duration of the treatment of 1 or 24 h. Following coagulation treatment, the samples were incubated in PBS for 1 or 24 h and visualized in a hydrated state in PBS using differential interference contrast (DIC) microscopy, Figure 1.

As expected, at the lowest concentrations of the kosmotropic salt used (100 mM), coagulation did not afford robust hydrogels and μS films dissolved upon contact with PBS. With increased concentration, at 250 mM sulfate, μS thin films exhibited a poorly controlled adhesion to the glass slide yet the structures were clearly visible and well-defined. We conclude that with 1 h stabilization, at least 300 mM salt concentration is required to produce robust hydrogels which remain visually stable upon an incubation in PBS over 24 h. With extended stabilization time, as low as 200 mM salt reliably produced μS PVA hydrogels which remained stable in PBS for at least 24 h. Increased duration of a coagulation treatment therefore affords more robust hydrogels. In turn, extended incubation in PBS leads to a noticeable dissolution of structures produced via coagulation, (c.f. the samples prepared using 300 mM sulfate and 1 h coagulation after PBS incubation for 1 and 24 h treatment). Taken together, images in Figure 1 qualitatively reveal a broad set of conditions that afford μS PVA films.

To gain quantitative information on the incorporation of PVA into the μS hydrogels, we capitalized on our recent success in producing PVA polymer samples via reversible addition−fragmentation chain transfer polymerization (RAFT).[33] This technique affords polymers with well-defined molecular weights, narrow polydispersities, as well as opportunities to define polymer terminal groups.[34] "Classical" engineering of terminal groups for RAFT derived polymers, i.e., a cleavage of thioester into a terminal thiol,[34] is not applicable to PVA due to side reactions and elimination of the thiol functionality.[35] As a partial solution to this synthetic limitation of PVA, we developed an alternative RAFT-based design, specifically using phthalimide (Phth) containing chain transfer reagent. The Phth group was part of the "R" group and thus was not eliminated during saponification, making this approach to PVA end group design effective. Removal of Phth using hydrazine afforded amine-functionalized PVA chains[33] which are significantly advantaged over pristine PVA with regard to, for example, bioconjugation.

In this work, we further developed the outlined strategy to PVA functionalization. Amine-containing PVA was treated with 2-iminothiolane (Traut's reagent) to produce thiol-functionalized polymer chains which were then in situ protected using Ellman's reagent (Figure 2A). The final polymer, PVA_{ER}, therefore contains single terminal thiol group that is protected from oxidation and is activated toward thiol-disulfide exchange.[36] Further to this, treatment of this polymer-bound mixed disulfide with e.g. dithiotreitol (DTT) affords release of a chromophore with a high extinction coefficient. The latter reaction is fast and quantitative, as is well documented in the

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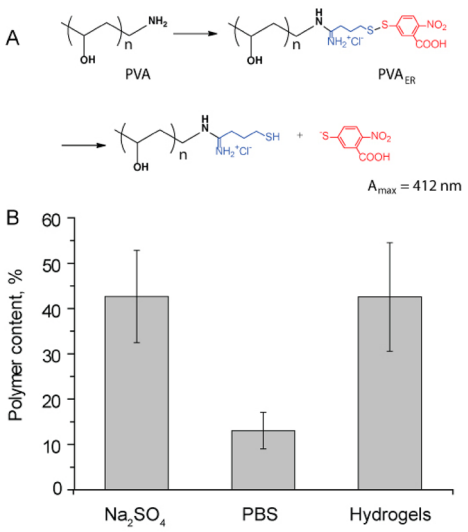

Figure 2. (A) Schematic illustration for the synthesis of PVA with a terminal thiol in a form of a mixed disulfide with Ellman's reagent, PVA$_{ER}$. Upon a treatment with DTT, resulting polymer releases a chromophore, 2-nitro-5-thiobenzoate, with a high extinction coefficient, which provides a basis for quantification of polymer chains in solution and within the hydrogel phase. (B) Representative results of quantification of PVA in the coagulation bath, PBS, and within the surface adhered μS PVA hydrogels. μS PVA hydrogels were prepared using 12 wt % solution of PVA$_{ER}$ using 1000 mM Na_2SO_4 coagulation bath and duration of coagulation 1 h and subsequent incubation in PBS for 1 h.

field of bioconjugation, and can be used to quantify PVA polymer chains. We hypothesized that this approach is applicable to PVA both in solution and within a hydrogel phase, in the latter case because of a high hydration of the material and excellent diffusivity of solutes through the PVA matrices.[28,29,37] To verify this hypothesis, samples of μS PVA hydrogels were assembled as described above and subjected to a thiol quantification test through addition of excess DTT to each of the following, coagulation bath, PBS wash medium, and final hydrogel preparation. For each sample, polymer content was quantified via a UV-vis spectrum and normalized to the total amount of polymer used initially to prepare the sample. The latter value was obtained via dissolution of nonstabilized samples in DTT-containing PBS. Representative results are presented in Figure 2B for a sample obtained using a 1000 mM sodium sulfate stabilization (1 h treatment) and subsequent incubation in PBS for 1 h. Presented data indicate that a sum of polymer content in the three samples within experimental error matches the amount of polymer used in the sample preparation. Averaged for >100 samples analyzed, the additive of three readings was established at 89 ± 18% thus verifying utility of the method and also demonstrating the associated error of the measurements. We believe these data justify the proposed method to polymer quantification, specifically within the hydrogel phase.

Next, polymer quantification assay was performed as described above for the samples obtained via coagulation with varied sodium sulfate concentrations for 1 or 24 h and subsequent incubation in PBS for 1 or 24 h, i.e. conditions matching those used in Figure 1. For comparison, we also performed this analysis using 1 M of sodium sulfate, i.e., coagulation conditions used in our prior reports.[21] For ease of data presentation, for each sample, total detected polymer content (coagulation bath, PBS, hydrogels) was set at 100%. The first observation made in Figure 3 is that for all stabilization conditions, more than half of the polymer initially taken for the preparation of the hydrogels escaped gelation and was lost into the solution phase. This notion is rather expected and corresponds well to our previous observations[21] as well as prior reports on PVA cryogels,[38] i.e., hydrogels obtained via

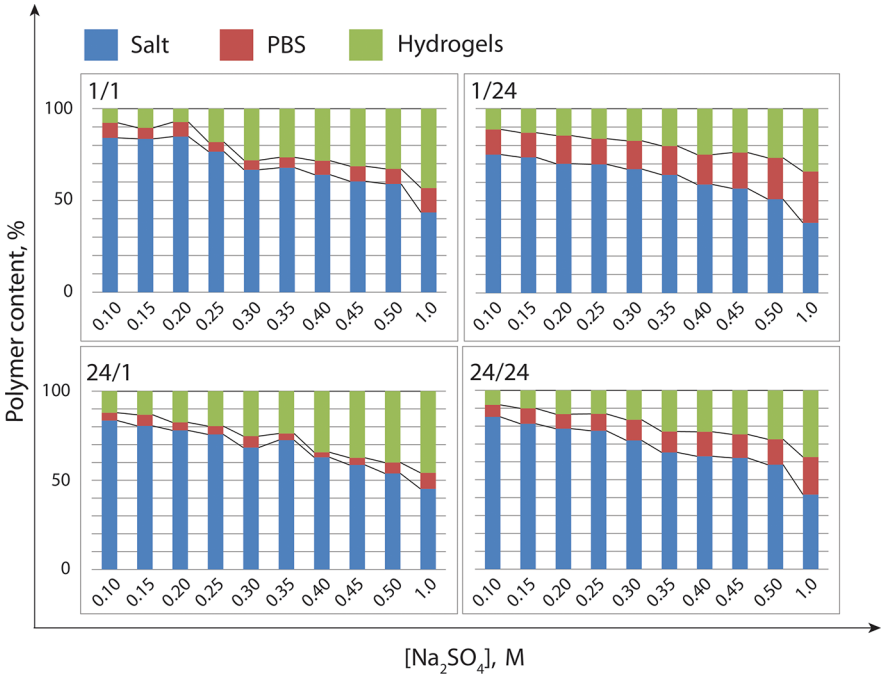

Figure 3. Distribution of polymer between coagulation bath, PBS, and resulting μS hydrogels surface adhered samples prepared using coagulation baths with varied concentration of sodium sulfate, duration of coagulation 1 or 24 h and subsequent incubation in PBS 1 or 24 h. For brevity, assembly conditions are abbreviated as X/Y, where X and Y denote duration of coagulation treatment and incubation in PBS, respectively.

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repetitive freeze−thawing of polymer solutions. For the latter, as much as half of the polymer chains can be lost in the first freeze−thaw cycle and even after 6 cycles as much as 25% of the polymer may escape gelation.[38] For noncryogenic approach developed herein, a surprising observation is that polymer loss largely occurred during incubation in the coagulation bath with a fraction of polymer extracted during subsequent incubation in PBS being relatively minor. This conclusion holds true regardless of the concentration of sodium sulfate, including the highest concentration tested (1 M). We believe that this observation will prove useful for optimization of PVA hydrogels with regard to the amount of polymer incorporated into the gel matrix.

Further analysis of the data presented in Figure 3 allows making the following conclusions. Supporting visual observations, 100−200 mM were ineffective in retaining the polymer with virtually an entire polymer payload dissolving in the coagulation bath. With increased sodium sulfate concentration, a progressively lower amount of the polymer was registered in the coagulation bath and correspondingly increased content of the polymer was established with the gel phase. From a different perspective, upon an increased incubation in PBS from 1 to 24 h, an increasing fraction of the polymer escaped hydrogel phase and was registered in the PBS solution. This observation is true for the samples which underwent coagulation treatment for 1 as well as 24 h and suggests that PVA physical hydrogels possibly undergo gradual degradation upon incubation in physiological media. Counterintuitively, an increase in polymer content in PBS in time is more pronounced for the samples obtained through coagulation using higher sulfate concentrations. This is plausibly explained by that these samples also have higher initial polymer content, i.e., less polymer lost during coagulation.

For a detailed analysis of polymer retention within the hydrogels, Figure 4 presents quantitative data on this parameter

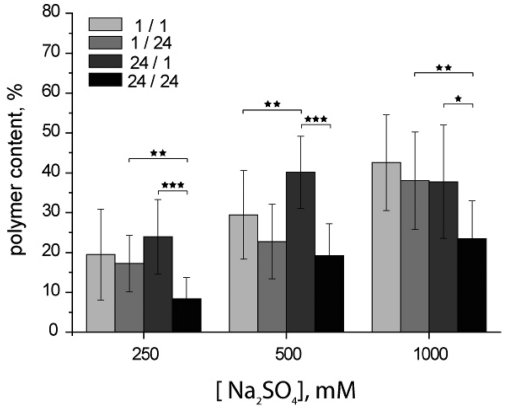

Figure 4. Polymer content within μS PVA hydrogels prepared using coagulation baths with varied concentration of sodium sulfate, duration of coagulation 1 or 24 h, and subsequent incubation in PBS for 1 or 24 h.

obtained using three sodium sulfate concentrations (250, 500, and 1000 mM) and 4 coagulation/PBS incubation regimes (1 and 24 h coagulation; 1 and 24 h incubation in PBS). In agreement with the analysis put forward in Figure 3, for each coagulation concentration, the overall amount of polymer retained in μS PVA hydrogel was well below 50%. Contrary to expectations, increase in the duration of coagulation treatment did not result in a significant increase in the amount of retained polymer. Furthermore, with 1 h coagulation, polymer loss into PBS with increased incubation from 1 to 24 h is relatively minor. In contrast, for 24 h stabilization treatment, PBS washing bath extracted a significant amount of PVA. Nevertheless, for each of the conditions considered in Figure 4, Figure 1 clearly shows that the structures were robust and well-defined. As will be shown below, despite a loss of significant amount of the polymer, μS PVA hydrogels remain intact, serve as efficient hosts to enzymatic cargo, and sustain adhesion and proliferation of mammalian cells.

To provide further characterization for the μS PVA films obtained via coagulation treatment and using varied amounts of sodium sulfate and duration of coagulation treatment, the samples were analyzed using atomic force microscopy (AFM) for visualization (Figure 5) and toward evaluation of their mechanical properties, in the latter case through force−distance measurements (Figure 6). In hydrated state, μS PVA hydrogels appeared to be uniform, with smooth surface of the micro features. While all the samples were produced using PDMS stamps with 2 μm side cuboid cavities, none of the samples exhibited a 2 μm height (Figure 5, C). A plausible explanation to this is an incomplete filling of the cavities with the polymer solution; we are currently optimizing this aspect of μTM technique. Interestingly, while all samples exhibited a decreased height, most samples were also characterized with a diameter well exceeding the expected 2 μm value (Figure 5, B). These data indicate that the hydrogel features underwent a pronounced lateral swelling. We note that hydrogel dimensions are indicative of cross-linking density.[39,40] However, because of observed irregular lateral and height swelling of the samples, as well as the change in μS dimensions as a result of increased coagulation time, at present we cannot draw sound conclusions regarding this material characteristic. Recently, we revealed that PVA hydrogelation exerted via treatment with aqueous isopropanol is an interplay between polymer dissolution, precipitation, and true hydrogelation.[21] It appears plausible that similar considerations hold true for μS PVA hydrogels obtained via sodium sulfate coagulation. Full understanding of these phenomena requires further investigation, which is subject of ongoing research.

Although hydrogel dimensions have proven to be rather similar for all coagulation treatment regimes, analysis of hydrogel elasticity revealed that judicious choice of coagulation treatment, both in terms of the sodium sulfate concentration and duration of treatment, present opportunities to fine-tune the Young's modulus of PVA matrix (Figure 6). In agreement with the data in Figure 1, 1 h coagulation using 250 mM sodium sulfate did not provide samples with reliable adhesion and therefore did not afford reproducible AFM force−distance measurements. With 24 h duration of coagulation, sodium sulfate concentration has proven to be a powerful tool to define elasticity of the noncryogenic, physical PVA hydrogels. From 250 to 500 mM and further to 1 M treatment, Young's modulus exhibited a progressively increasing value covering the range from 75 to 500 kPa. Duration of coagulation treatment is a second tool of control with increased length of event affording matrices with higher Young's moduli.

Interestingly, comparing the data in Figures 4 and 6 reveals that changes in the values for Young's moduli may not reflect the changes in polymer content within the hydrogels. For instance, increase in coagulation time from 1 to 24 h with constant 24 h incubation in PBS did lead to an increase in

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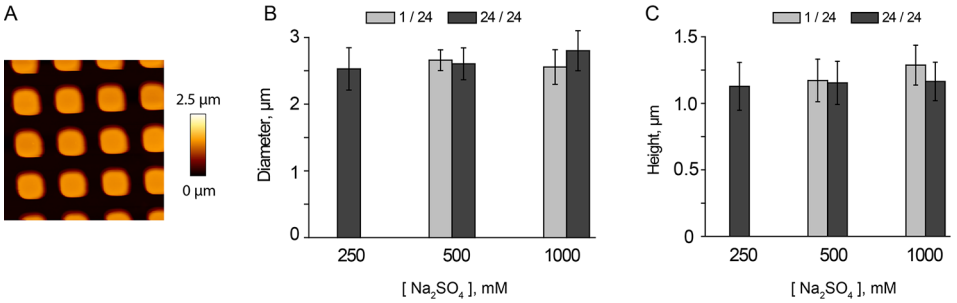


Figure 5. (A) Representative atomic force microscopy image of μS PVA hydrogels, (B) average diameter and (C) height of μS PVA hydrogels prepared using a PDMS stamp with 2 μm cuboid cavities and PVA as a function of concentration of sodium sulfate in coagulation bath and duration of coagulation treatment (1 or 24 h; image A, 500 mM, 1 h) and subsequent incubation in PBS for 24 h. Hydrogels were imaged and analyzed in hydrated state in PBS.

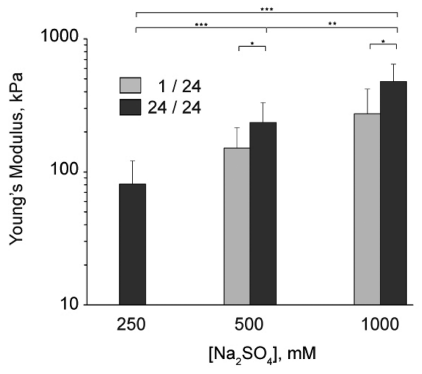


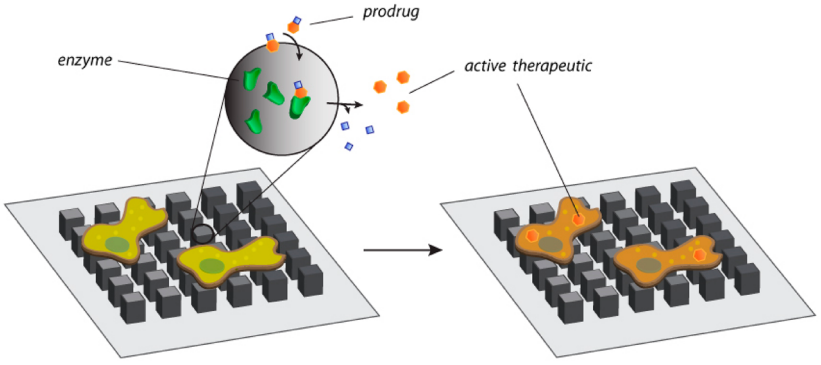
Figure 6. Young's moduli of μS PVA hydrogels prepared using coagulation baths with varied concentration of sodium sulfate, duration of coagulation 1 or 24 h, and subsequent incubation in PBS 1 or 24 h.

hydrogel elasticity but did not lead to an increased polymer content. We have previously suggested that 2 μm cuboid PDMS masks afford μS PVA hydrogels with a core–shell structure, i.e., nonuniform distribution of the polymer within the hydrogel and accumulation of the polymer at the interface. Total polymer content considers both, macromolecules in the shell area and within the hydrogel core. In turn, Young's modulus is likely defined by the density (polymer content) of the shell layer. This implies that mechanics of the hydrogel may not be a function of the total polymer content, which is indeed observed in our experiments.

For biomedical applications, the data in Figure 6 present PVA hydrogels as convenient tools for elucidation of mechanosensitivity of mammalian cells and effects exerted by the cell substrate matrices onto adhered cells. While elasticity of soft human tissues is lower than the range of Young's moduli covered in this work,[25] it has recently been shown that it is this range which allows controlling cell adhesion and proliferation.[26,27] Furthermore, substrate elasticity within the range covered in Figure 6 was used to control intracellular processes, specifically DNA replication and transcription.[41] Although it is now understood that these effects are cell- and material-specific, in particular with regard to the exact numerical values of matrix elasticity,[27] we believe that PVA matrices lend themselves for these applications. In typical approaches, elasticity of hydrogel–substrate for cell adhesion is controlled through the cross-linking density, e.g., mediated by carbodiimide coupling agents.[27] The latter treatment, as well as most covalent modification approaches, can have detrimental effects on incorporated fragile biological cargo. In contrast, controlling elasticity of PVA hydrogels through the concentration of sodium sulfate appears to be a convenient, benign approach. Our first results on elucidating utility of PVA matrices for cell culture will be presented below.

A persistent limitation of hydrogels as matrices for controlled drug delivery relates to poor capability of the hydrogels to retain cargo, specifically low molecular weight. This phenom-

Scheme 1. Schematic Illustration of PVA Hydrogels as Surface Adhered Enzymatic Microreactors and Substrate Mediated Enzyme Prodrug Therapy (SMEPT)[a]



[a]A cell culture substrate contains an enzyme that is able to convert an inactive prodrug into the active product, which is then internalized by adhering cells.

enon is beneficial and contributes to the overall success of e.g. matrices with immobilized cells or enzymes for biomass conversion,[29,42] but effectively spells a failure of these materials in applications requiring gradual release of immobilized cargo with fine-tuned kinetics of release. Partial solution to this is found through the creation of composite hydrogels, i.e. matrices which contain drug depots (liposomes, microparticles, etc), wherein the former provide a structural carcass and the latter afford a controlled drug release profile.[14] However, to the best of our knowledge, this strategy has documented only a limited success in the context of surface mediated drug delivery. We hypothesized that a high rate of diffusion of low molecular weight cargo can be used as a beneficial feature and define the success of controlled drug release mediated by hydrogel biomaterials. To achieve this, we propose a strategy termed Substrate Mediated Enzyme Prodrug Therapy, SMEPT, whereby hydrogel matrix is functionalized with embedded enzyme and the latter accomplishes a conversion of benign prodrugs into active therapeutic substances, Scheme 1. In an implementation like this, high diffusivity of solutes is a desired feature, and hydrogels are therefore materials of choice for this undertaking. We note that PVA hydrogels have been extensively used for enzyme immobilization;[9,10] also, surface coatings were functionalized with enzymes for prodrug conversion;[43,44] however, to the best of our knowledge, there are no prior reports on enzyme-containing matrices toward controlled drug release for uptake by adhering cells, as proposed herein.

The first step toward accomplishing the set goal is engineering sound matrices equipped with enzymes to achieve conversion of the prodrug. Literature survey[45] revealed a promising candidate enzyme, β-glucuronidase (β-Glu), which is well characterized as an antibody-linked component of ADEPT (antibody directed enzyme prodrug therapy).[46,47] The latter strategy delivers the enzyme to the tumor site. Subsequently administered prodrug is benign and has no therapeutic action, but once converted by β-Glu, it has a cytotoxic, anticancer activity. Conversion of the prodrug occurs only at the location of the enzyme, i.e. at the tumor, which ensures a low systemic and a high local concentration of the drug, both features being highly favorable for an overall success of the treatment.

To be successful, PVA matrices for SMEPT need to be structurally sound and maintain the level of enzymatic activity mediated from within the hydrogel, i.e. ensure effective protein retention. In initial experiments, we monitored enzymatic activity mediated by μS PVA hydrogels prepared via 1 h of polymer coagulation treatment, 24 h incubation in PBS wash bath and using solutions of PVA supplemented with β-Glu to 1 g L^{-1}. Specifically, using a fluorogenic substrate, fluorescein diglucuronide (FdG), we ascertained kinetics of enzymatic conversion in the coagulating salt and PBS wash baths as well as mediated by the enzyme within the gel phase. In a separate experiment, enzymatic activity of β-glucuronidase taken in an amount as used for assembly of μS hydrogels (ca. 1.5 μg per sample) was analyzed as described above and this provided the level of enzymatic activity of this enzyme in solution based prodrug conversion. Normalizing β-Glu activity of studied samples by that exhibited by the enzyme in solution afforded relative enzymatic activity, Figure 7. For low concentration of sodium sulfate, the highest level of enzymatic activity was registered within the coagulation bath indicating a pronounced loss of the protein during stabilization treatment. Increasing concentration of salt to 500 mM and 1 M eliminated this effect

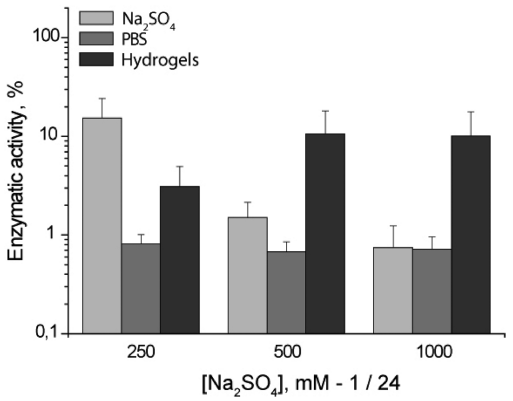


Figure 7. Quantification of enzymatic activity within the coagulating bath, PBS wash solution, and resulting μS PVA hydrogels. μS PVA hydrogels were prepared using 12 wt % solution of PVA supplemented with β-Glu to 1 g L^{-1}, 1 h coagulation treatment using coagulation baths with varied concentration of sodium sulfate and subsequent incubation in PBS for. [FdG] = 0.25 μg mL^{-1}; 30 min reaction time.

and coagulation baths revealed negligible conversion of the fluorogenic substrate. Upon subsequent incubation in PBS for 24 h, wash baths also revealed only minor enzymatic activity. We note that two phenomena may contribute to this effect, namely a near-full retention of the protein within the hydrogel phase as well as inactivation of the protein upon release into the solution bulk. Full understanding of this was beyond the scope of this investigation. Important final observation is that μS hydrogels exhibited a pronounced level of enzymatic activity mediated from within the hydrogel making these matrices promising for applications in SMEPT.

Next, enzymatic activity mediated by the μS PVA hydrogels was ascertained using samples prepared using varied coagulation concentrations of sodium sulfate, duration of coagulation treatment and duration of subsequent incubation in PBS, Figure 8. With 24 h stabilization time, as low as 250 mM salt

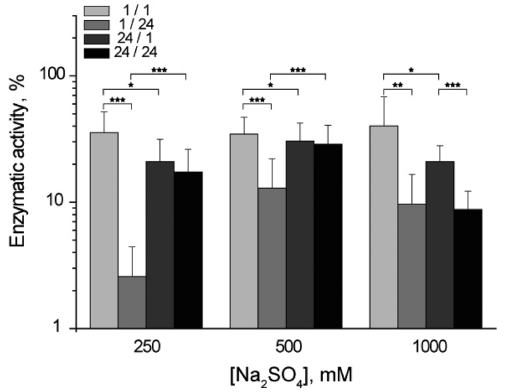


Figure 8. Activity of the β-Glu immobilized within μS PVA hydrogels prepared using coagulation baths with varied concentration of sodium sulfate, duration of coagulation 1 or 24 h, and subsequent incubation in PBS 1 or 24 h.

produced hydrogels with pronounced enzymatic activity. Upon incubation in PBS (1–24 h), with exception of 250 mM coagulation bath and 1 h duration of treatment, μS hydrogels revealed no significant drop in the enzymatic activity. The latter observation is surprising in that retention of enzymatic activity occurred despite the loss of as much as half of the polymer

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constituting the hydrogel matrix. For 24 h coagulation and incubation in PBS, 500 mM bath appears to provide optimized levels of enzymatic activity, whereas further increase in the concentration of sodium sulfate in the coagulation bath was detrimental. The latter observation correlates with our early observation on enzyme of deactivation in the presence of high concentrations of sodium sulfate. We note that in solution phase, β-Glu undergoes a complete loss of activity in the presence of 1 M sodium sulfate within 1 h. Within the μS hydrogels, the enzyme revealed significant levels of activity, which implies that PVA hydrogels exerted a stabilization effect on the enzyme, a notion highly beneficial for the performance of SMEPT as well as broader biomedical applications of μS physical hydrogels based on PVA.

Having established utility of PVA matrices as reservoirs for embedded enzyme, i.e., surface adhered microreactors, we next aimed to verify their suitability as substrates for cell adhesion. Pristine PVA hydrogels are well-characterized as non-cell adhesive materials,[22] a feature which finds use in biotechnology (e.g., patterning[48]) as well as biomedicine (e.g., prevention of postoperative tissue adhesion[49]). To promote adhesion of mammalian cells, documented strategies include covalent surface modification[50] as well as blending of PVA with pro-cell adhesive biomacromolecules,[22,51,52] e.g., chitosan. In our prior report, we have shown that μS PVA hydrogels support cell adhesion when prepared using cogelation of PVA with poly-L-lysine (PLL) or collagen.[21] Herein, we build on these data and use PLL-containing μS hydrogels as matrices for SMEPT. In separate experiments we verified that the presence of PLL (1 g L^{-1} in 120 g L^{-1} PVA in solution used for the preparation of μS hydrogels) provides a minor interference with the enzymatic conversion and resulting hydrogels sustained efficient conversion of glucuronide prodrugs into their respective products (data not shown).

For a study of cell adhesion to μS PVA hydrogels and SMEPT, we chose to use a hepatic cell line, HepG2. Hepatic cultures and cocultures[53,54] are widely employed in metabolic studies and drug screening,[55] as well as in tissue engineering toward regeneration of liver.[56] In these experiments, coculture of hepatocytes with other cell types is pivotal to maintain hepatic function whereas monocultures lose specific hepatic activity (e.g., secretion of albumin).[54−56] Advanced efforts in culture and specifically coculture of hepatocytes are particularly successful on microstructured substrates and using cell patterning techniques.[53,55] We envision that μS PVA hydrogels may present a novel, adaptable platform for these applications and, together with an innovative drug delivery strategy, i.e., SMEPT, deliver broader opportunities for these biomedical applications. These notions justify the use of hepatic cell line and μS PVA hydrogels in this study.

In contrast to pristine μS PVA hydrogels, PLL-containing counterparts were effective in sustaining adhesion of mammalian cells. This conclusion holds true for the substrates prepared using varied concentration of coagulating salt (250, 500, and 1000 mM) and coagulation treatment (1 or 24 h), Figure 9. In each case, viability of HepG2 was identical to that of these cells cultured in conventional 48-well plates at a matched density of cell seeding. This result is encouraging yet unexpected in that matrices in the range of Young's moduli as documented in Figure 6 were shown to exert an influence on cell adhesion and rate of proliferation. However, these effects are cell- and material-specific[27] and to be registered on μS PVA hydrogel may require even softer materials; we are now investigating this

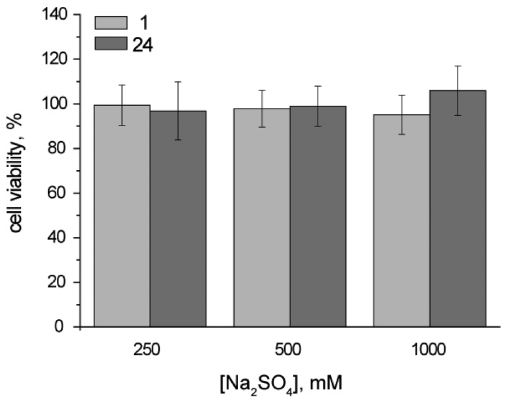

Figure 9. Viability of hepatocellular carcinoma cells, HepG2, cultured on PLL containing μS PVA hydrogels. Samples were prepared using 12 wt % solutions of PVA supplemented with PLL to a concentration of 1 g L^{-1}, coagulation baths with varied concentration of sodium sulfate, duration of coagulation 1 or 24 h and subsequent incubation in PBS 1 or 24 h. Cell viability assay performed using PrestoBlue after 48 h cell culture and expressed relative to viability of nontreated cells cultured in 48-well plates at matched initial cell seeding density.

aspect of materials design. Nevertheless, the above results reveal a successful assembly of μS PVA hydrogels which sustain adhesion and proliferation of mammalian cells and contain enzyme toward performance of SMEPT as a novel tool in surface mediated drug delivery.

To provide an initial verification of therapeutic potential of SMEPT, we assembled enzyme- and PLL-equipped μS PVA hydrogels as described above and employed a glucuronide prodrug of a potent anticancer agent, SN-38, a clinically tested metabolite of irinothecan (Figure 10). In experiments using HepG2 and cell cultures in standard 48-well plates we established an IC_{50} value of SN-38 at 0.1 μM, whereas addition of SN-38 glucuronide in the absence of enzyme revealed minimal cytotoxic effect. For SMEPT, the cells were allowed 24

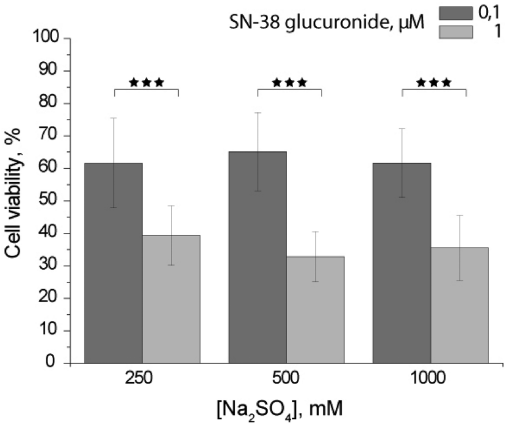

Figure 10. Viability of hepatocellular carcinoma cells, HepG2, cultured on μS PVA hydrogels under SMEPT conditions. Samples were prepared using 12 wt % solutions of PVA supplemented with PLL and β-Glu to a concentration of 1 g L^{-1} each, coagulation baths with varied concentration of sodium sulfate, duration of coagulation 24 h and subsequent incubation in PBS for 24 h. Cell culture media was supplemented with glucuronide prodrug of SN-38 to a concentration of 0.1 or 1 μM. Cell viability was quantified using Presto Blue reagent after 48 h cell culture and expressed relative to viability of nontreated cells cultured in 48 well-plate at matched initial cell seeding density.

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h for initial adhesion to μS PVA hydrogels after which time the media in the wells was changed to fresh and charged with SN-38 glucuronide. Change of media was implemented to ensure removal of β-Glu possibly leaked from the hydrogels into the supernatant. At 0.1 μM concentration of the prodrug, viability of cultured cells was decreased to 60%. Increase in the prodrug concentration to 1 μM resulted in a pronounced, statistically significant decrease in cell viability to 30−40%. For either concentration, attained viability levels match closely those observed for HepG2 cells cultured in conventional 48-well plates at the same initial cell seeding density and using pristine SN-38 with solution administration. We note that the above observations were similar for PVA matrices prepared using varied concentrations of coagulating salt and at matching prodrug concentrations difference between the samples was insignificant. This reveals that in the current setup, HepG2 cells did not reveal mechano-sensitivity[57,58] and properties of cell culture substrate were not decisive for the effectiveness of the treatment. We further note that exchange of media and washing procedures cannot eliminate all enzyme from the solution bulk and a fraction of the prodrug is converted by the residual enzyme in the solution phase above the hydrogel. However, the data in Figure 7 strongly suggest that an overall majority of the prodrug conversion is achieved with the μS hydrogels, i.e., via SMEPT.

From a different perspective, the above data demonstrate therapeutic effect achieved via SMEPT and a facile dose control achieved via the choice of prodrug concentration. In the context of surface mediated drug delivery, current high bar in controlled release of small cargo is set by degrading matrices comprising organic, non-water-soluble polymers.[59] Gradual degradation or erosion of the polymer matrix affords well-defined drug dosage, and this strategy has a well-established biomedical relevance and commercial success.[60,61] However, a limitation of this paradigm is that this mode of drug delivery is engineered into the degrading matrix, but provides no control over drug delivery once matrix erosion has commenced. Once initiated, drug release cannot be temporarily slowed or accelerated, altogether arrested or else modified to achieve a personalized treatment. In contrast, design of SMEPT allows overcoming this limitation. The data in Figure 10 present a proof-of-concept dose control gained for drug eluting matrices with a constant loading of enzyme and achieved externally via the choice of the prodrug concentration.

As an outlook, we note that performance of surface adhered enzymatic microreactors is limited by stability of the hydrogels and maintenance of levels of enzymatic activity, i.e. enzyme deactivation. Our preliminary observations reveal that for, for example, samples prepared using 28 kDa PVA, 12 wt % polymer solution, 500 mM sodium sulfate, and 1 h coagulation conditions, μS hydrogels undergo a gradual degradation with kinetics exceeding 7 days of incubation. Over this time, samples also exhibit a gradual decrease of enzymatic activity and following 72 h incubation, as employed in the current presentation, conversion of fluorogenic substrate is mediated at a ca. 30% level of activity relative to the original μS hydrogel. These observations provide indicative timeframes for performance of SMEPT using matrices as described in the presentation. We anticipate that stability of the hydrogels as well as durability of enzymatic activity can be controlled by polymer molecular weight, concentration of polymer solution and coagulation conditions employed for production of hydrogels. From a different perspective, above-mentioned data provide early evidence of an intriguing possibility to obtain μS PVA hydrogels with set lifetime, i.e. biodegradable surface adhered microreactors. We are now investigating these research opportunities in detail and these results will be presented in upcoming publications.

CONCLUSIONS

In this work, we provided a systematic investigation into assembly of surface adhered μS PVA hydrogels as intelligent matrices for biotechnological and biomedical applications. Through innovative macromolecular and materials design, we quantified incorporation and retention of PVA within the structure of physical hydrogels. Variation of conditions for polymer coagulation was used as a tool to assemble μS hydrogels with tunable Young's moduli, specifically in the range previously shown to elicit (sub)cellular responses in cultured mammalian cells. We then introduced a novel biomedical concept, Substrate Mediated Enzyme Prodrug Therapy (SMEPT), according to which generation of the drug is achieved within the substrate for cell culture using immobilized enzymes and biologically benign prodrugs which are administered externally. We accomplished SMEPT using the glucuronide prodrug of SN-38, a potent anticancer therapeutic, and achieved a dose response using administered prodrug. We envision that the latter feature is essential to engineer possibility for personalized treatment into surface mediated drug delivery and tissue engineering, which is the subject of our ongoing research.

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Notes

The authors declare no competing financial interest.

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REFERENCES

(1) Kong, H. J.; Hsiong, S.; Mooney, D. J. *Nano Lett.* **2007**, *7*, 161−166.
(2) Kong, H. J.; Mooney, D. J. *Nat. Rev. Drug Discovery* **2007**, *6*, 455−463.
(3) Teo, B. K. K.; Goh, S. H.; Kustandi, T. S.; Loh, W. W.; Low, H. Y.; Yim, E. K. F. *Biomaterials* **2011**, *32*, 9866−9875.
(4) Alves, N. M.; Pashkuleva, I.; Reis, R. L.; Mano, J. F. *Small* **2010**, *6*, 2208−2220.
(5) Lo, C. M.; Wang, H. B.; Dembo, M.; Wang, Y. L. *Biophys. J.* **2000**, *79*, 144−152.
(6) Engler, A. J.; Sen, S.; Sweeney, H. L.; Discher, D. E. *Cell* **2006**, *126*, 677−689.
(7) Lozinsky, V. I.; Galaev, I. Y.; Plieva, F. M.; Savinal, I. N.; Jungvid, H.; Mattiasson, B. *Trends Biotechnol.* **2003**, *21*, 445−451.
(8) Lozinsky, V. I.; Zubov, A. L.; Titova, E. F. *Enzyme Microb. Technol.* **1997**, *20*, 182−190.
(9) Parascandola, P.; Branduardi, P.; de Alteriis, E. *Enzyme Microb. Techmol.* **2006**, *38*, 184−189.
(10) Wilson, L.; Illanes, A.; Pessela, B. C. C.; Abian, O.; Fernandez-Lafuente, R.; Guisan, J. M. *Biotechnol. Bioeng.* **2004**, *86*, 558−562.

(11) Groger, H.; Capan, E.; Barthuber, A.; Vorlop, K. D. *Org. Lett.* **2001**, *3*, 1969−1972.
(12) Hamidi, M.; Azadi, A.; Rafiei, P. *Adv. Drug Delivery Rev.* **2008**, *60*, 1638−1649.
(13) Lin, C.-C.; Metters, A. T. *Adv. Drug Delivery Rev.* **2006**, *58*, 1379−1408.
(14) Hoare, T. R.; Kohane, D. S. *Polymer* **2008**, *49*, 1993−2007.
(15) Casciaro, S.; Conversano, F.; Musio, S.; Casciaro, E.; Demitri, C.; Sannino, A. *J. Mater. Sci.−Mater. Med.* **2009**, *20*, 983−989.
(16) Demitri, C.; Sannino, A.; Conversano, F.; Casciaro, S.; Distante, A.; Maffezzoli, A. *J. Biomed. Mater. Res. B* **2008**, *87B*, 338−345.
(17) Park, S. K.; Guntur, S. R. A. R.; Il Lee, K.; Paeng, D. G.; Choi, M. J. *IEEE Trans. Biomed. Eng.* **2010**, *57*, 194−202.
(18) Khademhosseini, A.; Langer, R. *Biomaterials* **2007**, *28*, 5087−5092.
(19) Slaughter, B. V.; Khurshid, S. S.; Fisher, O. Z.; Khademhosseini, A.; Peppas, N. A. *Adv. Mater.* **2009**, *21*, 3307−3329.
(20) Jensen, B. E. B.; Smith, A. A. A.; Fejerskov, B.; Postma, A.; Senn, P.; Reimhult, E.; Pla-Roca, M.; Isa, L.; Sutherland, D. S.; Stadler, B.; Zelikin, A. N. *Langmuir* **2011**, *27*, 10216−10223.
(21) Jensen, B. E. B.; Alves, M.-H.; Fejerskov, B.; Stadler, B.; Zelikin, A. N. *Soft Matter* **2012**, *8*, 4625−4634.
(22) Alves, M. H.; Jensen, B. E. B.; Smith, A. A. A.; Zelikin, A. N. *Macromol. Biosci.* **2011**, *11*, 1293−1313.
(23) Gough, J. E.; Scotchford, C. A.; Downes, S. *J. Biomed. Mater. Res.* **2002**, *61*, 121−130.
(24) Cascone, M. G.; Sim, B.; Downes, S. *Biomaterials* **1995**, *16*, 569−574.
(25) Levental, I.; Georges, P. C.; Janmey, P. A. *Soft Matter* **2007**, *3*, 299−306.
(26) Kocgozlu, L.; Lavalle, P.; Koenig, G.; Senger, B.; Haikel, Y.; Schaaf, P.; Voegel, J. C.; Tenenbaum, H.; Vautier, D. *J. Cell. Sci.* **2010**, *123*, 29−39.
(27) Boudou, T.; Crouzier, T.; Nicolas, C.; Ren, K.; Picart, C. *Macromol. Biosci.* **2011**, *11*, 77−89.
(28) Hassan, C. M.; Peppas, N. A. In *Biopolymers/PVA Hydrogels/Anionic Polymerisation Nanocomposites*; Springer-Verlag Berlin: Berlin, 2000; Vol. *153*, pp 37−65.
(29) Lozinsky, V. I.; Plieva, F. M. *Enzyme Microb. Tech.* **1998**, *23*, 227−242.
(30) Gulsen, D.; Li, C. C.; Chauhan, A. *Curr. Eye Res* . **2005**, *30*, 1071−1080.
(31) Hosta-Rigau, L.; Jensen, B. E. B.; Fjesdso, K. S.; Postma, A.; Li, G. X.; Goldie, K. N.; Albericio, F.; Zelikin, A. N.; Stadler, B. *Adv. Healthcare Mater.* **2012**, DOI: adhm.201200092.
(32) Czichocki, G.; Dautzenberg, H.; Capan, E.; Vorlop, K. D. *Biotechnol. Lett.* **2001**, *23*, 1303−1307.
(33) Smith, A. A. A.; Hussmann, T.; Elich, J.; Postma, A.; Alves, M.-H.; Zelikin, A. N. *Polym. Chem.* **2012**, *3*, 85−88.
(34) Moad, G.; Rizzardo, E.; Thang, S. H. *Polym. Int.* **2011**, *60*, 9−25.
(35) Li, Z. C.; Tong, Y. Y.; Dong, Y. Q.; Du, F. S. *J. Polym. Sci., Polym. Chem.* **2009**, *47*, 1901−1910.
(36) Chong, S. F.; Chandrawati, R.; Stadler, B.; Park, J.; Cho, J. H.; Wang, Y. J.; Jia, Z. F.; Bulmus, V.; Davis, T. P.; Zelikin, A. N.; Caruso, F. *Small* **2009**, *5*, 2601−2610.
(37) Hassan, C. M.; Stewart, J. E.; Peppas, N. A. *Eur. J. Pharm. Biopharm.* **2000**, *49*, 161−165.
(38) Valentin, J. L.; Lopez, D.; Hernandez, R.; Mijangos, C.; Saalwachter, K. *Macromolecules* **2009**, *42*, 263−272.
(39) Bell, C. L.; Peppas, N. A. *J. Controlled Release* **1996**, *39*, 201−207.
(40) Kozlovskaya, V.; Kharlampieva, E.; Mansfield, M. L.; Sukhishvili, S. A. *Chem. Mater.* **2005**, *18*, 328−336.
(41) Kocgozlu, L.; Rabineau, M.; Koenig, G.; Haikel, Y.; Schaaf, P.; Freund, J. N.; Voegel, J. C.; Lavalle, P.; Vautier, D. *Biomaterials* **2012**, *33*, 798−809.
(42) Lozinsky, V. I.; Zubov, A. L.; Titova, E. F. *Enzyme Microb. Technol.* **1996**, *18*, 561−569.
(43) Mertz, D.; Vogt, C.; Hemmerle, J.; Mutterer, J.; Ball, V.; Voegel, J. C.; Schaaf, P.; Lavalle, P. *Nat. Mater.* **2009**, *8*, 731−735.
(44) Vogt, C.; Mertz, D.; Benmlih, K.; Hemmerle, J.; Voegel, J. C.; Schaaf, P.; Lavalle, P. *ACS Macro Lett.* **2012**, *1*, 797−801.
(45) Kratz, F.; Müller, I. A.; Ryppa, C.; Warnecke, A. *ChemMedChem* **2008**, *3*, 20−53.
(46) NiculescuDuvaz, I.; Springer, C. J. *Adv. Drug Delivery Rev.* **1997**, *26*, 151−172.
(47) Syrigos, K. N.; Epenetos, A. A. *Anticancer Res.* **1999**, *19*, 605−613.
(48) Nakayama, Y.; Nakamata, K.; Hirano, Y.; Goto, K.; Matsuda, T. *Langmuir* **1998**, *14*, 3909−3915.
(49) Muller, S. A.; Weis, C.; Odermatt, E. K.; Knaebel, H.-P.; Wente, M. N. *Eur. J. Obstet. G. R. B.* **2011**, *158*, 67−71.
(50) Nuttelman, C. R.; Mortisen, D. J.; Henry, S. M.; Anseth, K. S. *J. Biomed. Mater. Res.* **2001**, *57*, 217−223.
(51) Koyano, T.; Minoura, N.; Nagura, M.; Kobayashi, K. *J. Biomed. Mater. Res.* **1998**, *39*, 486−490.
(52) Mathews, D. T.; Birney, Y. A.; Cahill, P. A.; McGuinness, G. B. *J. Biomed. Mater. Res. B* **2008**, *84B*, 531−540.
(53) Kaji, H.; Camci-Unal, G.; Langer, R.; Khademhosseini, A. *BBA-Gen Subjects* **2011**, *1810*, 239−250.
(54) Bhatia, S. N.; Balis, U. J.; Yarmush, M. L.; Toner, M. *FASEB J.* **1999**, *13*, 1883−1900.
(55) Khetani, S. R.; Bhatia, S. N. *Nat. Biotechnol.* **2008**, *26*, 120−126.
(56) Zinchenko, Y. S.; Schrum, L. W.; Clemens, M.; Coger, R. N. *Tissue Eng.* **2006**, *12*, 751−761.
(57) Kong, H. J.; Liu, J. D.; Riddle, K.; Matsumoto, T.; Leach, K.; Mooney, D. J. *Nat. Mater.* **2005**, *4*, 460−464.
(58) Rehfeldt, F.; Engler, A. J.; Eckhardt, A.; Ahmed, F.; Discher, D. E. *Adv. Drug Delivery Rev.* **2007**, *59*, 1329−1339.
(59) Acharya, G.; Park, K. *Adv. Drug Delivery Rev.* **2006**, *58*, 387−401.
(60) Uhrich, K. E.; Cannizzaro, S. M.; Langer, R. S.; Shakesheff, K. M. *Chem. Rev.* **1999**, *99*, 3181−3198.
(61) Liechty, W. B.; Kryscio, D. R.; Slaughter, B. V.; Peppas, N. A. *Annu. Rev. Chem. Biomol.* **2010**, *1*, 149−173.