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Bioresorbable Surface-Adhered Enzymatic Microreactors Based on Physical Hydrogels of Poly(vinyl alcohol)

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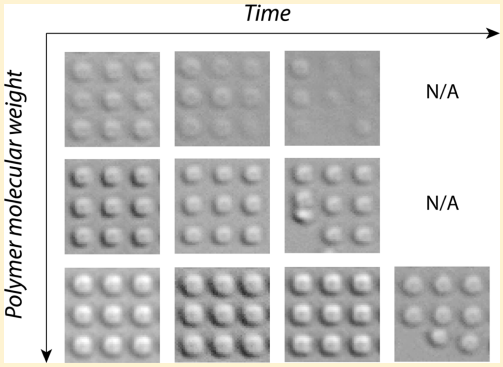
ABSTRACT: Hydrogel biomaterials based on poly(vinyl alcohol), PVA, have an extensive history of biomedical applications, yet in their current form suffer from significant shortcomings, such as a lack of mechanism of biodegradation and poor opportunities in controlled drug release. We investigate physical hydrogels of PVA as surface-adhered materials and present biodegradable matrices equipped with innovative tools in substrate-mediated drug release. Toward the final goal, PVA chains with narrow polydispersities (1.1−1.2) and molecular weights of 5, 10, and 28 kDa are synthesized via controlled radical polymerization (RAFT). These molecular weights are shown to be suitably high to afford robust hydrogel matrices and at the same time suitably low to allow gradual erosion of the hydrogels with kinetics of degradation controlled via polymer macromolecular characteristics. For opportunities in controlled drug release, hydrogels are equipped with enzymatic cargo to achieve an in situ conversion of externally added prodrug into a final product, thus giving rise to surface-adhered enzymatic microreactors. Hydrogel-mediated enzymatic activity was investigated as a function of polymer molecular weight and concentration of solution taken for assembly of hydrogels. Taken together, we present, to the best of our knowledge, the first example of bioresorbable physical hydrogel based on PVA with engineered opportunities in substrate-mediated enzymatic activity and envisioned utility in surface-mediated drug delivery and tissue engineering.



INTRODUCTION

Hydrogels are water swollen three-dimensional polymer networks with utilities in diverse areas of biotechnology and biomedicine, specifically tissue engineering.[1−4] These materials have significant advantages over solid matrices comprised of water-insoluble organic polymers[5] in that a well-hydrated environment favors stability and function of immobilized biological cargo, including enzymes and cells.[6−8] Furthermore, water content and mechanical properties of the hydrogels can be tuned via cross-linking density[9] to match the elasticity of human tissues,[10,11] a feature warranted in the context of the creation of cell mimics and matrices for tissue regeneration. A wide choice of constituting polymers, both natural and synthetic,[12,13] delivers diverse properties which can be engineered into the hydrogel matrices, such as cell adhesiveness or low-fouling behavior.

Among diverse candidate polymers for production of hydrogels, poly(vinyl alcohol), PVA, stands out as a polymer with extensive characterization in diverse biomedical applications, specifically tissue engineering.[3,14−16] An attractive feature of PVA is that both chemical and physical hydrogels can be assembled, thus offering diversity in available routes of manufacturing and properties of resulting materials. While chemical hydrogels dominate the field, physical hydrogels are more advantageous from the standpoint of toxicity of materials to cells[17] and protein cargo.[18] Regretfully, decades of extensive characterization of PVA matrices did not translate into superior control over hydrogel properties as tools of biomedical engineering. Utility of PVA matrices in biomedicine is predominantly limited by two fundamental limitations, namely a lack of a mechanism of degradation and poor opportunities in controlled retention of cargo and sustained drug release. As a result, PVA hydrogels currently do not appear in the focus of biomedical research, specifically in the domain of nanobiomedicine. Nevertheless, given the history of uses and FDA approval for diverse applications,[19] re-engineering of PVA to endow the materials derived thereof with a mechanism for degradation and tools for controlled drug release appears highly rewarding.

As a first step toward this goal, we recently introduced microtransfer molding (μTM) as a tool to assemble surface-adhered, microstructured (μS), micrometer-thick PVA thin films and hydrogels.[20−22] This provided a facile way to monitor hydrogel assembly and stability, as well as means to quantify the cargo retention using readily available microscopy techniques, such as differential interference contrast (DIC) and fluorescence microscopy, atomic force microscopy (AFM), and force−distance measurements, etc. In contrast to a rather limited toolbox available for characterization of bulk hydrogels, the above techniques allowed us to investigate PVA hydrogels

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on the micro- and nanoscale level. We characterized several noncryogenic techniques to afford hydrogelation of PVA, specifically via polymer coagulation using solutions of kosmotropic salt (sodium sulfate), aqueous isopropanol, or liquid oligo(ethylene glycol).[21] Each of these techniques afforded excellent control over topography design with (sub-)micrometer precision, thus presenting novel opportunities in engineering intelligent biointerfaces. To engineer advanced tools of controlled drug release into PVA matrices, we assembled "composite hydrogels" and used polymersomes as reservoirs for the peptidic anticancer drug, thiocoraline.[20] Resulting surface-adhered matrices afforded a greater therapeutic response than the drug with solution-based administration, revealing the potential of μS PVA hydrogels for surface-mediated drug delivery. In this work, we specifically address the factors limiting utility of PVA hydrogels as matrices for tissue engineering, namely a lack of mechanism for biodegradation and poor opportunities in controlled drug release.

Opportunities in creating biodegradable hydrogels are highly warranted in the context of tissue regeneration in that resorbable matrices disintegrate upon playing their successful role and are eliminated from the body. Current techniques in biodegradation include the use of biodegradable polymers, natural[13] or synthetic,[5] or synthetic polymers associated through degradable linkages,[9,23] peptidic or hydrolytically degradable. However, another mechanism of hydrogel degradation remains largely unexplored, namely slow gradual release of polymer chains from a physical hydrogel maintained via labile, noncovalent linkages. Possible points of advantage of these materials include facile assembly under conditions nondamaging to biological cargo. Good case in point, physical hydrogels based on PVA accommodate fragile cargo including mammalian cells and allow maintenance of their viability.[24,25] However, conventional PVA matrices are nondegradable, a feature which is well-documented in the state of art. We hypothesized that if assembled using polymer chains with suitably low molecular weight, affinity of individual macromolecules would be sufficiently low to allow gradual erosion of the material. In this work, we provide the first illustration of this approach to design bioresorbable materials.

To accomplish this, we developed the synthesis of PVA via reversible addition−fragmentation chain transfer (RAFT) polymerization, a powerful tool in the arsenal of controlled radical polymerization techniques.[26−29] Polymerization of vinyl acetate into poly(vinyl acetate), PVAc, the latter being a polymer precursor to PVA, is among the most widely employed reactions in the studies of mechanisms and kinetics of radical polymerization.[26] Despite this, examples of saponification of PVAc and production of PVA with superior quality of polymer samples with regard to polydispersity are scant,[30−34] and there is no report to date, to the best of our knowledge, specifically aimed at the synthesis of PVA with systematically varied macromolecular characteristics. Data presented in this manuscript therefore have fundamental value for polymer and materials science associated with PVA.

Poor opportunities in controlled drug release is a persistent shortcoming of PVA-based materials and hydrogel biomaterials in general.[35,36] Indeed, due to high swelling, hydrogels present a weak barrier to diffusion of solutes and are therefore ill-suited for retention of cargo and its release with fine-tuned kinetics. This shortcoming is addressed with the use of electrostatic association of charged cargo molecules with hydrogels bearing counter-charged ionizable groups,[37,38] affinity-based immobilization of (biological) solutes,[39] and chemical conjugation of drugs to the hydrogel matrix.[40] Powerful in their own right, these methods are applicable to particular systems and are not readily generalized to suit diverse therapeutic cargo. Superior control over drug release is engineered into hydrogels via the use of composite hydrogels (i.e., matrices with supramolecular drug depots, such as liposomes, microparticles, etc.).[41−43] Despite this progress, there is still a call for more general and flexible platforms to engineer drug delivery into hydrogels as matrices for cell culture and specifically tissue engineering.[36]

In pursuit of novel opportunities in controlled drug release associated with hydrogel materials, we proposed a strategy to achieve an in situ synthesis of the drug within the hydrogel phase, an approach we termed substrate-mediated enzyme prodrug therapy (SMEPT).[44,45] The key to this concept is an enzyme immobilized within a cell-culture substrate to perform conversion of benign prodrugs into active therapeutic molecules for delivery to adhering or adjacent cells. SMEPT is poised to capitalize on high diffusivity of hydrogels to small solutes, the latter feature ensuring fast exchange of (pro)drugs between the hydrogel and solution bulk. Further, SMEPT is envisioned to combine the benefits of solution-based administration of a drug (i.e., facile dosage and design of regimen) and surface-mediated drug delivery, namely localized drug release at the site of action. For the proof-of-concept of SMEPT, we used the β-glucuronidase enzyme (β-Glu) and glucuronide prodrugs and demonstrated control over the rate of enzymatic reaction, and an overall amount of generated cargo achieved over several orders of magnitude.[44] With the use of a glucuronide of SN-38, a potent anticancer drug, we demonstrated that the efficacy of SMEPT is comparable to that achieved via a solution-based administration of the drug[44] and achieved facile dose response via administration of the judiciously chosen concentration of the prodrug.[44,45] Taken together, our prior work presented initial characterization of SMEPT, specifically with regard to the feasibility of the concept.

The overall aim of this work was set on assembly and characterization of biodegradable surface-adhered enzymatic microreactors, i.e., eroding SMEPT matrices with set lifetimes and investigating polymer molecular weight as a tool of control over properties of the hydrogels. We characterize assembly losses and retention of polymer chains within the hydrogel matrix and monitor enzymatic activity mediated by the hydrogels as prepared using polymer samples with varied molecular weights and concentrations. In particular, we quantify polymer chains within the hydrogel phase and quantitate enzymatic conversion as performed by PVA hydrogels for short- and medium-term performance of SMEPT (i.e., from 1 h to 7 days in physiological media). Taken together, these data contribute significantly to the development of drug-delivery techniques associated with hydrogel biomaterials with envisioned utility in biomedicine, specifically tissue engineering and regenerative medicine.

EXPERIMENTAL SECTION

All chemicals were purchased from Sigma-Aldrich and used without purification, unless stated otherwise. Fluorescein diglucuronide (FdG) was obtained from Invitrogen. A Zeiss Axio Observer Z1 microscope was employed for DIC imaging; solution fluorescence and absorbance was quantified using an EnSpire Perkin-Elmer multilabel plate reader.

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Polymer Synthesis. Vinyl acetate (23.4 g, 272 mmol), *S*-phtalimidomethyl-*O*-ethyl xanthate (0.39 g, 1.40 mmol), and AIBN (23.0 mg, 0.14 mmol) were combined in a Schlenk tube, degassed by four freeze-pump-thaw cycles and incubated at 60 °C for 13.5 h. A crude NMR at this point showed 80% conversion. The solution was diluted with acetone to reduce the viscosity and precipitated into 1.5 L heptane. Collected solids were dried in vacuo to yield PVAc as a brittle, white solid (15.8 g). GPC analysis (solvent: tetrahydrofuran) revealed a M_n of 20.6 kDa and a PDI of 1.3.

PVAc (10.14 g) was dissolved in 100 mL methanol; hydrazine hydrate (1.21 g, 24.1 mmol) was added, and the solution was stirred at 60 °C for 0.5 h. At this point, 3 mL of 40 wt % NaOH (aq) was added, and the solution was stirred at room temperature overnight. The resulting white precipitate was collected by filtration and washed with methanol to yield amine-terminated PVA (4.9 g). GPC analysis (solvent: water) revealed a M_n of 10.4 kDa and a PDI of 1.1. Samples of PVA with molecular weights of 5 and 28 kDa were synthesized according to the protocol described above and monomer to RAFT ratios were used, as indicated in Table 1.

Table 1. Experimental Details of Synthesis of PVAc and Resulting PVA[a]

	M/ RAFT	time (hours)	conversion (%)	Mn (kDa) (PVAc)	PDI (PVAc)	Mn (kDa) (PVA)	PDI (PVA)
1	100/1	16	73	9.1	1.2	5.3	1.1
2	194/1	13.5	80	20.6	1.3	10.4	1.1
3	498/1	21	90	66.0	1.5	28.1	1.2

[a]For brevity, throughout the text molecular weights of the polymers are rounded to an even thousand.

PVA_{ER}. Two hundred milligrams of 10.4 kDa PVA was dissolved in 2.2 mL of 0.1 M $NaHCO_3$ buffer (pH 8.3), by heating and shaking. A solution of Ellman's reagent (211 mg, 0.53 mmol in 9 mL buffer) was added, followed by the addition of a freshly prepared solution of iminothiolane (61 mg, 0.44 mmol in 1 mL buffer). The mixture became deep orange and was left to stir for 16 h. The target polymer was recovered via precipitation into excess methanol and filtration, followed by trituration with copious amounts of methanol (yield: 135 mg). Samples of PVA_{ER} with molecular weights of 5 and 28 kDa were synthesized via the same protocols as described above.

Assembly of μS PVA Hydrogels. For assembly of surface-adhered μS PVA hydrogels, solutions of PVA were heated to 50 °C for 5 min to homogenize the solution and brought to 37 °C for 5 min. 1.5 μL of PVA solution was placed between a glass coverslip and a poly(dimethylsiloxane) (PDMS) mold with 2 μm cubic cavities and pressed at finger-tight pressure for 24 h. Upon detachment of the PDMS mold, surface adhered PVA films were stabilized for 1 h with 0.5 M Na_2SO_4 (hereafter referred to as "coagulation bath") and immersed into PBS for 24 h, unless stated otherwise.

Polymer Distribution. For quantitative analysis of the distribution of the polymer during the fabrication process, μS hydrogels were assembled using PVA_{ER} of 5, 10, and 28 kDa. Samples were stabilized with sodium sulfate and further incubated in PBS washing solution, as described above. Collected volumes of coagulating sodium sulfate solution and PBS wash solutions were charged with dithiothreitol (DTT) to a final concentration of 5 g/L. Hydrogel samples were replenished with fresh PBS containing DTT at 5 g/L. This procedure leads to a cleavage of mixed disulfide formed by a polymer chain and Ellman's reagent. As a result, 2-nitro-5-thiobenzoate (NTB) is released into solution bulk in a quantity directly proportional to the amount of polymer chains in solution or within the hydrogel. Incubation for 10 min was allowed before quantifying absorbance of solutions at 412 nm using a plate reader. Resulting values were compared to the total polymer content used for assembly of individual hydrogel samples, the latter obtained via dissolution of μS PVA hydrogels immediately after μTM, i.e., without coagulation treatment, and analysis using DTT as described above. Herein and below, 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. Statistical significance was analyzed using a Students *t* test with $*p < 0.05$, $**p < 0.01$, and $***p < 0.001$.

Assembly of Enzymatic Microreactors. For preparation of enzyme-loaded samples, the stock solution of β-Glu (1 μL) was mixed with solutions of PVA (9 μL) to a final enzyme concentration of 1 g/L via pipet-assisted mixing and μS hydrogels were assembled as described above. For brevity throughout the text, the concentration of PVA is quoted as the original stock solutions, disregarding dilution due to the addition of the enzyme. Enzyme activity in coagulation bath, PBS, and resulting hydrogel samples were quantified using FdG added to respective collected volumes or solution above hydrogels to a 0.25 μg/mL concentration and allowing 30 min for enzymatic conversion, unless stated otherwise.

For analysis of enzyme release from μS hydrogels following coagulation treatment, samples were immersed into PBS wherefrom aliquots were drawn at time points of 1, 2, 4, and 24 h, after which time the buffer solution above hydrogels was exchanged with fresh PBS. Collected volumes and hydrogel samples were analyzed for enzymatic activity using FdG, as described above (0.25 μg/mL, 30 min conversion time).

Analysis of the retention of enzyme activity over time was performed using μS PVA hydrogels fabricated using polymer samples with molecular weights of 5, 10, and 28 kDa and 12 wt % polymer solutions with 1 g/L content of β-Glu. Samples were stabilized via coagulation and immersed into PBS for time periods of 1 h, 24 h, 3 and 7 days. At specified time points, supernatants above the μS PVA hydrogels were exchanged to fresh PBS containing FdG, and the fluorescence of solutions was quantified using a multilabel plate reader.

Hydrogel Degradation. For quantitative analysis of hydrogel degradation, μS PVA_{ER} hydrogels were fabricated using 12 wt % solutions of PVA with molecular weights of 5, 10, and 28 kDa. Samples were stabilized via coagulation and immersed into PBS for 1 h, 24 h, 3 days, or 7 days. At specified time points, supernatants above hydrogels were exchanged with fresh PBS containing 5 g/L DTT. Following 10 min of incubation, absorbance of collected volumes was quantified using a plate reader. Remaining hydrogels were visualized in a hydrated state using DIC microscopy (40× magnification).

AFM Force–Distance Curves. AFM force curve analysis was carried out on μS PVA films assembled by μTM. Polymer solutions of 12 wt % (28 kDa) were clamped for 24 h, stabilized for 1 h, and subsequently incubated in PBS for 1 h, 24 h, and 3 days. Both stabilization and PBS incubation were 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, Estonia). The samples were imaged in PBS using a contact mode, in order to obtain force curves at the center of the structures. Calibration of the cantilever was performed by JPK SPM, and the sensitivity and spring constant were determined by the thermal noise method. The Hertz model in JPK SPM was utilized, in order to derive the Young's modulus from the force curves assuming a cone-shaped tip having 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.

RESULTS AND DISCUSSION

Polymer Synthesis. For investigation of PVA physical hydrogels and the impact of the polymer molecular weight on the properties of the gels, polymer samples with narrow polydispersities were synthesized via RAFT polymerization. Specifically, vinyl acetate (VAc) was polymerized using *O*-ethyl and *S*-phthalimidomethyl xanthate RAFT agents.[46] The latter provides good to excellent control over VAc polymerization and affords polymer chains with phthalimidomethyl terminal groups, which are easily removed with hydrazine hydrate to yield amine-terminated polymers. Hydrazinolysis of the phthalimide and deacetylation to yield PVA was performed as

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a one-pot reaction. GPC traces of the synthesized polymers (Mn 5, 10, and 28 kDa) are presented in Figure 1. For all

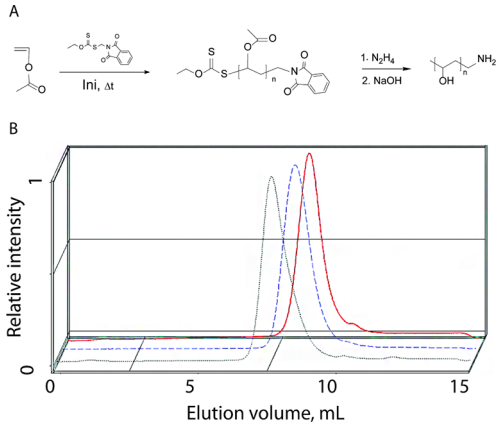

Figure 1. (A) Schematic representing synthesis of amine-terminated samples of PVA through RAFT polymerization of vinyl acetate, subsequent treatment with hydrazine to remove the phthalimide group, and saponification to produce the final polymer. (B) GPC traces for the synthesized polymers with molecular weights of 5, 10, and 28 kDa.

samples, PDI values were 1.1−1.2 (Table 1) and, to the best of our knowledge, this is the first example of judicious control over the molecular weight of PVA achieved via controlled radical polymerization. We note that qualitatively, PVA gelation (specifically, physical gelation) is favored by high polymer molecular weight and typically samples with a MW of at least ∼100 kDa are used in the production of robust PVA hydrogel samples. The latter are typically perceived as nondegradable, as is well-documented in the state of art. Nevertheless, our prior experiments,[21,46] as well as other reports on noncryogenic gelation of PVA,[47] revealed that chains with relatively low molecular weight, such as those used in this work, are effective in producing 3D polymer matrices (i.e., gels). We hypothesized that when using RAFT and well-defined polymer samples, it would be possible to obtain polymer samples with molecular weights sufficiently high to afford robust hydrogels, yet sufficiently low to allow a gradual erosion of the hydrogel. For this reason, in this work we have focused on relatively low molecular weight samples and hydrogels derived thereof.

Assembly of μS Hydrogels. For assembly of surface-adhered PVA hydrogels, we used microtransfer molding (μTM), an approach to topography design through replication of cavities within elastomeric molds (Figure 2A). A drop of polymer solution was placed between a PDMS stamp with 2 μm cubic cavities and a coverslip (step ii) and clamped at finger-tight pressure for 24 h (step iii). During this time, the samples underwent partial dehydration, and disassembly of clamps afforded surface-adhered, μS PVA thin films (step iv). Without further treatment, these samples dissolve upon contact with water or aqueous buffered solutions, and for the production of hydrogels require post-treatment using kosmotropic electrolytes (sodium sulfate), aqueous isopropanol, or liquid oligo(ethylene glycol).[21] Throughout this work, all samples were produced via polymer coagulation using 0.5 M sodium sulfate and 1 h for the duration of treatment. Resulting samples were subsequently hydrated in PBS to afford the final preparation, surface-adhered PVA physical hydrogels, which were visualized in a hydrated state in PBS using DIC

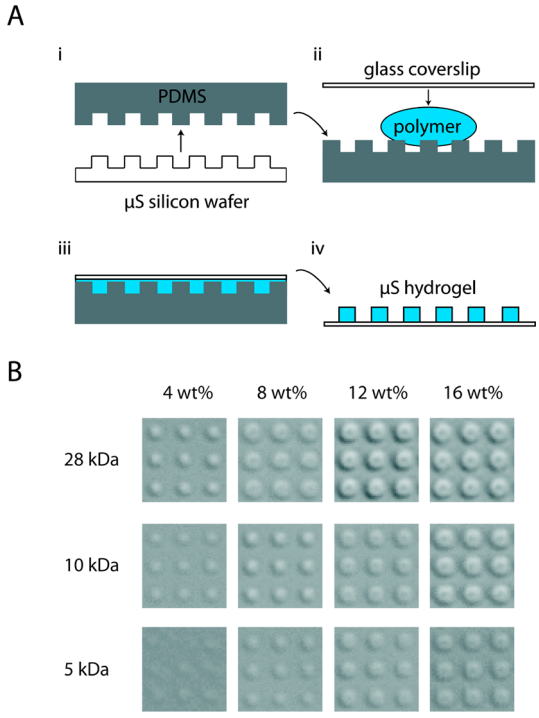

Figure 2. (A) Schematic illustration of the assembly of μS PVA hydrogels via μTM. (i) PDMS stamps are produced on the matrix of a silicon wafer with 2 μm sized cuboid features, (ii) the polymer solution is then placed between a PDMS stamp and a coverslip, (iii) fills the cavities of the stamp, and (iv) undergoes initial dehydration affording coverslip-adhered μS PVA thin films upon detachment of the coverslip. Subsequent polymer hydrogelation is achieved via a noncryogenic method, namely polymer coagulation using aqueous sodium sulfate. (B) DIC micrographs of surface-adhered μS PVA hydrogels prepared using polymers with varied molecular weights (5, 10, and 28 kDa) and solutions with a polymer content from 4 to 16 wt %.

microscopy (Figure 2B). The first observation from this data set is that hydrogel samples assembled using the same PDMS stamp reveal a significantly different size and shape. When assembled using low polymer concentrations, μS hydrogels do not exhibit a cubic shape of the parent elastomeric stamp, and individual hydrogel pillars are significantly smaller than the cavities within the stamp. Presented images further illustrate that PVA samples with molecular weights as low as 5 kDa are effective in producing surface-adhered hydrogels with structural stability retained over at least 24 h of incubation in physiological buffers. For this polymer sample, 16 wt % solutions afford well-defined μS hydrogels, whereas with decreasing polymer content, topography features become ill-pronounced. Similar observations can be made for polymer samples with molecular weights of 10 and 28 kDa, and overall it holds true, qualitatively, that increased polymer concentration affords visually better-defined hydrogels. At a constant polymer concentration (e.g., 12 wt %), increasing polymer molecular weight also favors assembly of well-defined μS hydrogels. We note that these observations are similar to those typically made upon hydrogelation of PVA. However, we strongly believe that this is the first report on PVA physical hydrogels as surface-adhered materials prepared using polymer samples with molecular weights as low as 5 kDa.

Quantitative Analysis of μS Hydrogels. For quantitative analyses, we synthesized the PVA chains with a single terminal

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thiol group in the form of a mixed disulfide with Ellman's reagent, PVA_{ER} (Figure 3A). In contrast to a pristine polymer,

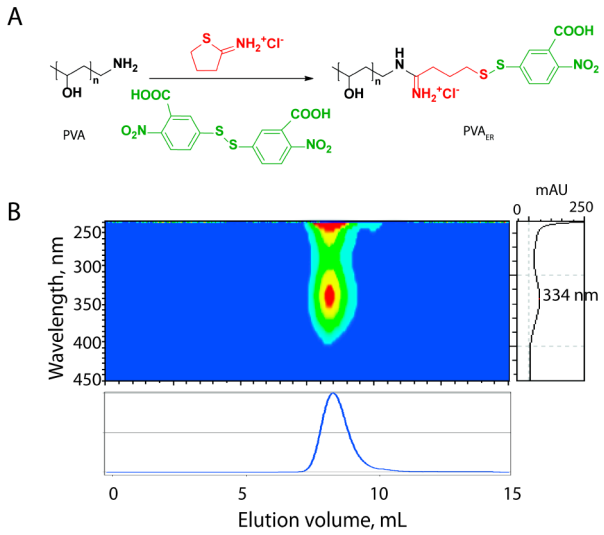

Figure 3. (A) Schematic illustration of the synthesis of PVA_{ER}, polymer sample with a single terminal thiol group in a form of a mixed disulfide with Ellman's reagent. Synthesis was accomplished as a one-pot reaction using amine-terminated PVA, iminothiolane, and Ellman's reagent. (B) Pseudo-3D gel permeation chromatography elution plot for a sample of PVA_{ER} (10 kDa) and analysis using a combination of MALS and full spectrum UV−vis detectors. The graph shows elution of a polymer with an expected retention time and associated chromophore with a UV−vis signature of Ellman's reagent, thus demonstrating success of a terminal-group conversion.

PVA_{ER} is equipped with a chromophore group with a high extinction coefficient. For analysis of polymer synthesis, we employed gel permeation chromatography (GPC) and a combination of multiangle light scattering and full-spectrum UV−vis detectors. While GPC analysis provides quantitative information on the polymer molecular weight, UV−vis spectra provide information on the presence of chromophore groups in the polymer solution. When used individually, these methods of analysis cannot distinguish between conjugated samples or polymer samples with impurities of the Ellman reagent. In contrast, combination setup reveals coelution of a chromophore with a UV−vis signature matching Ellman's reagent and polymer chains with expected molecular weights (Figure 3B) (i.e., the presence of end-group modified chains of PVA).

Synthesized polymer samples were used to monitor incorporation of PVA from assembly solutions into physical hydrogels. While quantification of polymer chains within micro- and nano-sized hydrogels is challenging, samples of PVA_{ER} present a unique opportunity to cleave the reversible disulfide linkage at the polymer terminal group and release 2-nitro-5-thiobenzoate (NTB) into the solution bulk (Figure 4A). Polymer content within the hydrogel can therefore be quantified via monitoring of UV−vis spectrum of the supernatant, and a high extinction coefficient of NTB (14300 $M^{-1}cm^{-1}$) ensures that even micrometer-thick surface-adhered polymer thin films afford good signal-to-noise ratios and reproducible results.[45] μTM was performed as described above using samples of PVA_{ER}. To ensure a uniform surface area of the hydrogels and minimize sample-to-sample variation, here and below, hydrogels were prepared using 9 mm glass coverslips and PDMS stamps with exceeding dimensions.

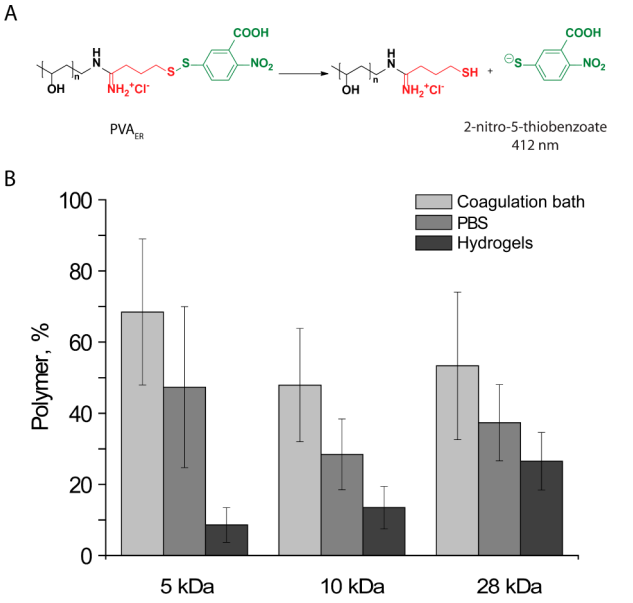

Figure 4. (A) Schematic illustration of reaction between PVA_{ER} and DTT. This quantitative conversion releases 2-nitro-5-thiobenzoate, an anion with a high extinction coefficient and serves as basis for the solution phase, UV−vis-based quantification of polymer chains in solution and/or in the hydrogel matrix. (B) Experimental data illustrating polymer content registered in the coagulation bath, PBS washing solution, and final μS hydrogels for samples prepared using 12 wt % solutions of PVA with varied molecular weights (5, 10, and 28 kDa).

Collected volumes of coagulation bath (sodium sulfate) and PBS-washing solutions, as well as the volume of PBS above the resulting hydrogels, were charged with DTT to a final concentration of 5 g/L upon which test samples attained a characteristic yellow color, indicating the release of NTB. For each sample, the total absorbance for the coagulation bath, washing solution, and the supernatant aspirated from the hydrogel was set to 100% and used to ascertain the polymer fraction, contained in respective volumes. Representative results are presented in Figure 4B for hydrogel samples prepared using polymers with varied molecular weights and a constant solution concentration of 12 wt %. The first observation from this data set is that for each sample, the polymer fraction contained within the final hydrogel preparation does not exceed ca. 25%. In other words, an overall majority of polymer chains escapes gelation and is registered in the solution bulk. Of these, a greater amount of polymer chains is localized in the coagulation bath while the PBS washing solution effects further extraction of the polymer. The pronounced escape of polymer chains from gelation is not unique to noncryogenic gelation employed herein. Conventional freeze−thaw treatment of 10 and 20 wt % solutions of PVA with a molecular weight of 94 kDa documented incorporation of ca. 50% chains after single cycle and escape of ~25% of chains upon 6 freeze−thaw cycles.[48] Nevertheless, despite a relatively low level of incorporation of polymer chains into the hydrogel phase, Figure 2B presents images of robust hydrogels as surface-adhered materials.

To ascertain the role of polymer macromolecular characteristics and solution concentration on incorporation of chains into the final μS hydrogels, similar experiments were performed using PVA with molecular weights of 5, 10, and 28 kDa and solutions in the range of polymer content from 4 to 16 wt %. As expected, the absolute polymer content in the hydrogel phase

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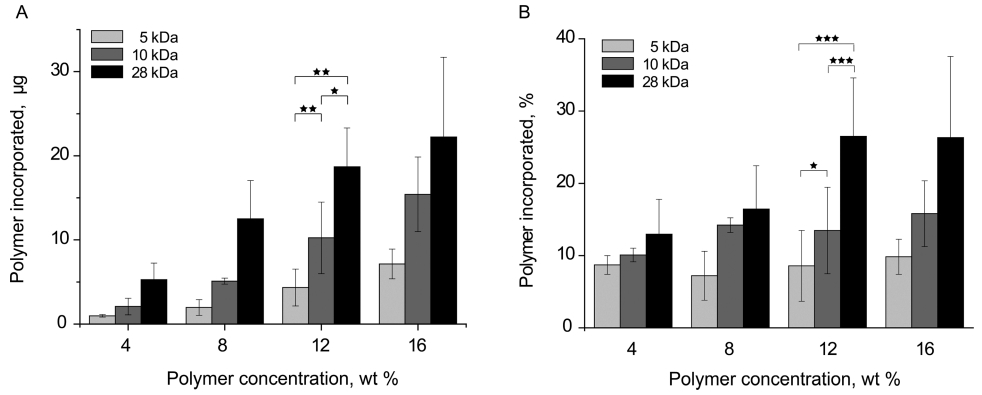

Figure 5. Absolute (A) and (B) relative content of polymer chains within μS PVA hydrogels prepared using the polymer samples with molecular weights of 5, 10, and 28 kDa and solution concentrations from 4 to 16 wt %. Absolute polymer content is expressed as polymer mass per hydrogel sample (μg); relative content is calculated therefrom via a normalization by the total polymer mass used for the assembly of the sample and using the polymer with the respective molecular weight and solution concentration. Presented data reflect polymer content in samples after a 24 h incubation in PBS.

increased linearly with the polymer concentration used in the assembly of hydrogel samples (Figure 5A). For each molecular weight, a 2×, 3×, and 4× increase in polymer concentration from 4 wt % resulted in a corresponding ca. 2-, 3- and 4-fold increase in the polymer content experimentally registered in the hydrogel phase. These data demonstrate that the concentration of the polymer solution used for assembly of μS hydrogels is a facile tool of control over the content of solids in the final preparation. The influence of polymer molecular weight on the assembly of μS hydrogels was also well-pronounced and at each solution concentration, the increase in Mn affords a statistically significant increase in polymer content in the hydrogel phase (Figure 5A). In other words, aliquots with identical mass and volume afforded hydrogels with a greater content of solids for polymers with increased chain lengths. Interestingly, at a given concentration (e.g., 12 wt %), polymers with higher molecular weight also exhibited significantly increased fraction of chains incorporated into the gel (Figure 5B). Thus, not only is a lower polymer mass registered in the hydrogel but also fewer relative amounts of chains are retained in the matrix for 5 kDa of PVA as compared to 28 kDa counterpart. These data quantitatively illustrate that polymer molecular weight favors hydrogelation of PVA. In turn, polymer concentration affords facile control over polymer content in the hydrogel, yet a higher concentration does not favor gelation, and at a given molecular weight, the fraction of polymer chains incorporated into the gel remains nearly constant (Figure 5B). For the 10 and 5 kDa samples, this fraction was unchanged in the concentration range from 4 to 16 wt %; for the 28 kDa polymer sample, results do indicate an increased polymer fraction registered in the gel phase with progressively higher solution concentration, yet this effect was not statistically significant.

Assembly of Enzymatic Microreactors. Performance of μS hydrogels as surface-adhered enzymatic microreactors requires that the polymer matrix exhibits efficient retention of protein cargo. Conventional, cryogenic PVA matrices by design are macroporous materials and allow diffusion and exchange of solutes between the hydrogel and solution bulk. This characteristic ensures success of PVA hydrogels in applications such as biomass conversion.[8,25] However, for performance of hydrogel-supported enzymatic microreactors, unhindered protein diffusion presents an arresting limitation and for these applications, enzymes are typically immobilized via covalent association with anchoring polymers[49] and/or cross-linking into larger-sized multimers.[50] In our work, for the development of enzymatic microreactors we chose β-glucuronidase (β-Glu), a relatively large-sized protein with a molecular weight of ∼300 kDa and achieved immobilization of the enzyme into PVA hydrogel matrix in the pristine form of the protein (i.e., using no cross-linking agents). Solutions of PVA (12 wt %) were mixed with a stock solution of the enzyme via pipet-assisted mixing to afford a final protein concentration of 1 g/L and used in μTM, as described above. Hydrogelation was afforded via polymer coagulation using 0.5 M Na_2SO_4 and a 1 h coagulation time, after which the samples were immersed in 1 mL PBS wherefrom aliquots were drawn at specified time points over a total of 24 h of incubation. Finally, the supernatant above μS samples was exchanged to fresh PBS, and samples were analyzed for the presence of an active enzyme within the hydrogel phase, alongside collected volumes of buffered solutions. Enzymatic activity was quantified using a fluorogenic substrate, fluorescein diglucuronide (FdG), conversion of which affords a highly fluorescent product, fluorescein. Collected volumes of coagulation baths and PBS equilibration buffers, as well as supernatant above hydrogels, were supplemented with FdG to a final concentration of 0.25 μg/mL, and levels of solution fluorescence were quantified following a 30 min incubation time using a multilabel plate reader. In separate experiments, conversion of FdG was achieved in PBS using 1.5 μg of β-Glu (i.e., an amount of the protein used for production of each individual hydrogel sample), which provided a reference activity of a pristine enzyme in the solution phase. Fluorescence of supernatants aspirated from μS hydrogels and collected volumes of coagulation and washing solutions was normalized by that achieved in the reference experiment to afford the values of relative enzymatic activity (Figure 6).

For each sample, coagulation baths revealed non-negligible levels of enzymatic activity indicating a loss of the enzyme from μS thin films during polymer coagulation (Figure 6). This observation appears to be rather expected given a significant dissolution of polymer samples during the sodium sulfate treatment (i.e., release of polymer chains from the thin films into the coagulation solution). Enzyme loss is the most pronounced for the sample with the lowest molecular weight, diminishing for the 10 kDa sample and being only minor for

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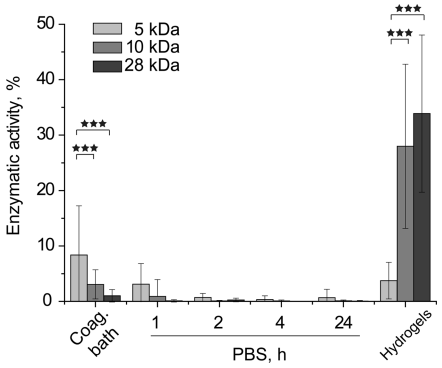

Figure 6. Enzymatic activity experimentally determined in coagulation baths, PBS equilibration buffers over initial 24 h incubation time, and hydrogels for samples prepared using 12 wt % solutions of PVA with molecular weights of 5, 10, and 28 kDa. β-Glu: 1 g/L in the initial polymer solution; [FdG] = 0.25 μg/mL.

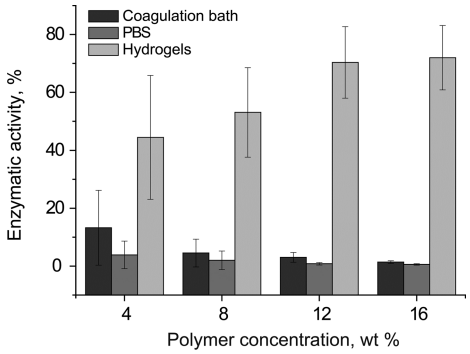

Figure 7. Enzymatic activity in the coagulation baths, PBS washing solutions, and the final hydrogel preparations for μS PVA hydrogels prepared using a 28 kDa sample of PVA and solutions with varied concentrations, from 4 to 16 wt %.

the 28 kDa sample of PVA. Subsequent incubation in PBS over 24 h did not register significant enzymatic activity in PBS washing solutions, as indicated by minimal levels of solution fluorescence resulting from enzymatic conversion. We note that these data do not rule out enzyme release, and low levels of enzymatic activity may be due to enzyme deactivation. Indeed, when speaking of this scenario, a 5 kDa sample exhibits a non-negligible β-Glu activity in PBS after an initial 1 h incubation, which gradually disappears upon subsequent incubation. Nevertheless, the most significant observation is that for 5, 10, and 28 kDa samples, at least 5×, 300×, and 600× higher enzymatic conversion, respectively, is mediated by the enzyme within the hydrogel compared to that in PBS aspirated immediately prior to the analysis of respective samples. In other words, while the protein release and enzymatic conversion in the solution phase above the μS hydrogels cannot be fully ruled out, data in Figure 6 demonstrate that for all the samples, substrate conversion is predominantly achieved within the hydrogel phase.

On the basis of the results presented in Figure 5A, increased concentration of the polymer solution taken for the assembly of μS hydrogels translates into higher polymer content within the final samples and this, in turn, may contribute to a greater retention of the enzyme within the structure of surface-adhered reactors. To verify this, μS PVA hydrogels were assembled using a 28 kDa polymer sample and solutions with varied polymer content, from 4 to 16 wt %. Samples underwent coagulation treatment and subsequent rehydration in PBS, after which time enzymatic activity in the coagulation bath, PBS washing solution aspirated immediately prior to analysis, and the final hydrogel samples were quantified using FdG, as described above. Activity of β-Glu in the coagulation baths exhibited a gradual decrease with an increasing polymer concentration (Figure 7). In contrast, μS hydrogels revealed progressively higher enzymatic activity with increased polymer content, although this effect was relatively minor. Importantly, at each polymer concentration, β-Glu activity within the μS hydrogel was significantly higher than that revealed by the PBS solutions aspirated from above the hydrogels prior to enzyme quantification. The magnitude of this effect was ca. 10× for the 4 wt % polymer concentration and reached >100× for the 16 wt % polymer concentration. This observation is imperative for the interpretation of data and applications of this platform in enzymatic catalysis and SMEPT, as it reveals that an overall majority of substrate conversion is accomplished by the enzyme within the hydrogel structure.

To further elucidate the effect exerted by the polymer macromolecular characteristics on the retention of β-Glu, substrate conversion mediated by the enzyme within μS hydrogels was quantified using a fluorogenic substrate and using hydrogels prepared from polymer solutions with concentrations from 4 to 16 wt % and molecular weights of 5, 10, and 28 kDa. In agreement with the above presented data, for each polymer molecular weight, an increased polymer concentration results in an increased enzymatic activity (with a varied degree of statistical confidence) (Figure 8). At each

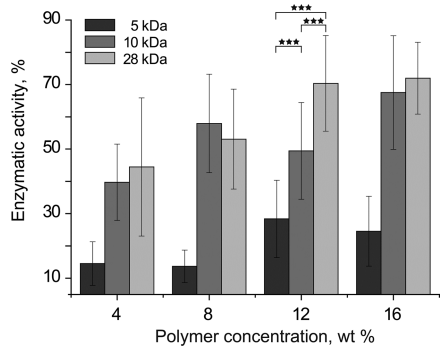

Figure 8. Enzymatic activity revealed by μS PVA hydrogels prepared using PVA with molecular weights of 5, 10, and 28 kDa and polymer solutions from 4 to 16 wt %.

polymer concentration, PVA with a 5 kDa molecular weight exhibited the lowest β-Glu activity, whereas 10 kDa and 28 kDa revealed similar levels of substrate conversion. As reasoned above, we believe that higher polymer content affords hydrogels with increased cross-linking density and this, in turn, affords an increased protein retention. However, another phenomenon may also contribute to the experimental observations presented in Figures 7 and 8. Previously, we observed that sodium sulfate elicits a non-negligible deactivating effect on β-Glu; in contrast, the presence of PVA exerted a stabilization effect on the enzyme. Specifically, in solution, high concentrations of sodium sulfate (1 M) led to a complete loss of activity for β-Glu within 1 h. In contrast, when mixed with a polymer solution, the enzyme withstood hydrogel stabilization under these conditions and retained at least 10−20% activity mediated from within the hydrogel phase.[45] It is plausible that higher polymer content

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provides a greater stabilization effect on the enzymatic cargo, as indeed is observed in our experiments (Figure 8). We note, however, that the revealed trend may not be monotonous with further increase in polymer content, as the latter is expected to exert a limitation on the performance of hydrogel-based enzymatic microreactors via a build-up of steric hindrance to the exchange of solutes between the hydrogel and solution bulk.

While data in Figures 6−8 reflected end-point analyses based on readings taken upon a 30 min enzymatic conversion, the next experiment demonstrates continuous performance of μS hydrogels as enzymatic microreactors. Figure 9 shows kinetic

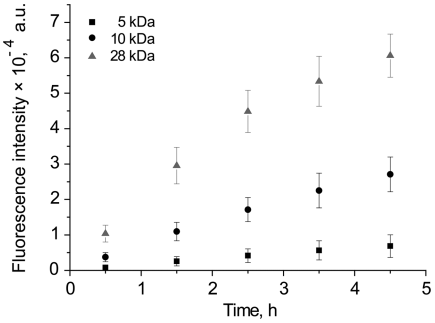

Figure 9. Kinetic data on enzymatic activity revealed by μS PVA hydrogels prepared using 12 wt % polymer solutions and PVA samples with molecular weights of 5, 10, and 28 kDa and incubated in PBS for 24 h prior to the administration of FdG.

curves for conversion of FdG by a hydrogel-immobilized enzyme obtained using samples differing in polymer molecular weights and initiation of conversion after a 24 h incubation in PBS. For each sample, solutions reveal a steady increase in fluorescence, indicating continuous substrate conversion and retained enzymatic activity. In agreement with the above-discussed data, enzymatic activity mediated by the hydrogels based on 5 kDa PVA is lowest, while prodrug conversion mediated by hydrogels assembled using 28 kDa of polymer sample is the most pronounced. For a 28 kDa sample, the fluorescence curve reveals a tendency toward saturation, which suggests depletion of the substrate. For hydrogels with lower activity (10 kDa and 5 kDa polymer samples), evolution of fluorescence is near linear, demonstrating a possibility to achieve a zero-order kinetics of release for model cargo.

Degradation of μS Hydrogels and Enzymatic Microreactors. For further characterization of these novel surface-adhered enzymatic microreactors, we aimed to ascertain durability of enzymatic activity, i.e., monitor prodrug conversion achieved by samples upon their extended incubation in physiological media. To achieve this, μTM and hydrogel stabilization was performed as described above using polymer samples with varied molecular weight and constant concentrations of the polymer and the enzyme. Samples underwent coagulation and were subsequently immersed in PBS for periods of time up to 1 week at 37 °C. At specified time points, the supernatant above the samples was exchanged for a fresh buffer and enzymatic activity mediated by the enzyme-equipped hydrogels was quantified using the fluorogenic prodrug, FdG. These data reveal that for all the samples tested, enzymatic activity mediated by the hydrogel matrix decreases with incubation in physiological media (Figure 10). A further important observation is that the durability of enzymatic

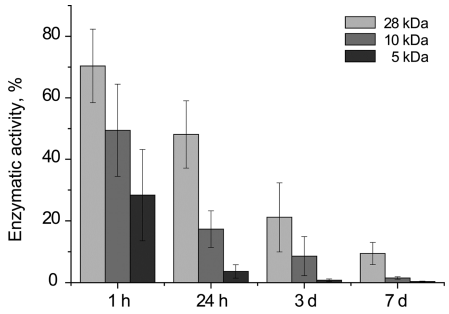

Figure 10. Enzymatic activity for μS PVA hydrogels prepared using polymer samples with molecular weights of 5, 10, and 28 kDa and substrate conversion initiated at specified time points: 1 h, 24 h, 3 and 7 days.

activity is governed by the molecular weight of the polymer taken for assembly of μS hydrogels. For a 5 kDa sample, enzymatic conversion of fluorogenic substrate becomes almost ineffective at a 24 h incubation time point. In contrast, for 10 kDa PVA, hydrogels derived thereof sustain enzymatic activity for at least 3 days. Finally, 28 kDa polymer chains comprise hydrogels with pronounced β-Glu activity observed even after 7 days. The above data demonstrate a possibility to assemble surface-adhered enzymatic microreactors with a predetermined lifetime, at least within 7 days of performance. Presented data strongly suggest that with higher polymer molecular weights, it is possible to engineer hydrogel matrices with significantly extended lifetimes. This hypothesis is supported by our recent data on μS PVA hydrogels assembled using commercial PVA with a MW of ∼90 kDa, for which we observed no drop in the enzymatic activity over the first 24 h of incubation in the physiological environment.[44]

The two plausible approaches to explain the data in Figure 10 relate to (i) gradual deactivation of the enzyme within the hydrogel matrix and (ii) gradual erosion of the μS hydrogels and loss of enzymatic cargo from their structure. Both phenomena are likely to contribute to an overall effect. Of these, degradation of the PVA matrix can be directly quantified using samples of PVA_{ER}, as used above to quantify polymer loss during preparation of hydrogel samples. Twelve wt% solutions of PVA_{ER} were used in μTM with hydrogel stabilization using sodium sulfate, as described above. Subsequently, μS hydrogels were incubated in PBS at 37 °C, taking readings of polymer retention at 1 and 24 h, and 3 and 7 days (i.e., time points matching those used in Figure 10). At each time point, hydrogels were also visualized using DIC microscopy (Figure 11). For a 5 kDa polymer sample, incubation of μS hydrogels in physiological buffer resulted in a decrease in polymer content to <10% within 24 h. With an increase in polymer molecular weight to 10 kDa, hydrogel samples derived thereof appear structurally sound upon 24 h and even 3 days of incubation in PBS, after which time polymer content falls below a detectable level and hydrogels cannot be found on the surface. Finally, a 28 kDa polymer sample affords hydrogels which appear robust after 3 days of incubation in PBS. In this case, even after 7 days of incubation, samples reveal significant retention of the polymer within surface-adhered hydrogels and could readily be visualized using DIC. Taken together, these data illustrate, qualitatively and quantitatively, a possibility to engineer gradually eroding PVA hydrogels as enzyme-loaded surface-adhered materials toward utility as biodegradable enzymatic microreactors. A plausible mechanism by which increased

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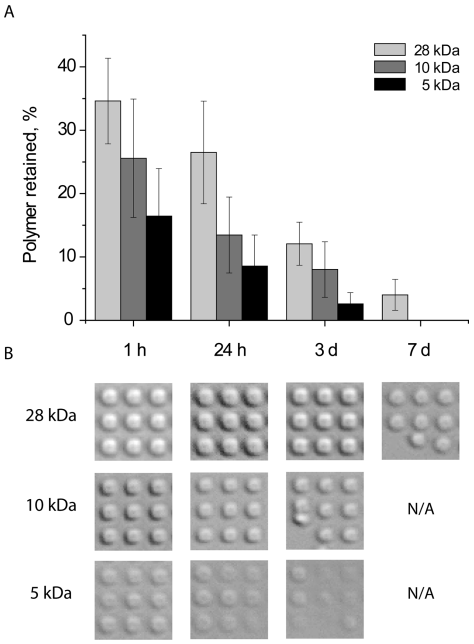

Figure 11. (A) Quantitative evaluation of polymer retention within hydrogel structures after 1 h, 24 h, 3 and 7 days of incubation in PBS. (B) DIC images for μS PVA hydrogel films prepared using PVA samples with molecular weights of 5, 10, and 28 kDa and 12 wt % polymer solutions and subsequent incubation in PBS for 1 h, 24 h, 3 and 7 days.

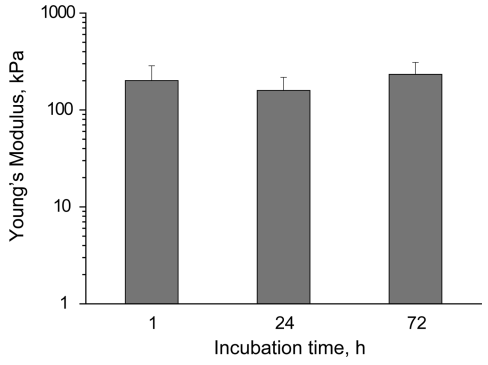

Figure 12. Young's moduli established for μS hydrogels prepared using 12 wt % solution of PVA with a molecular weight of 28 kDa and incubated in PBS over 72 h.

polymer molecular weight contributes to a greater stability of samples to erosion is that PVA physical hydrogels are maintained via labile, noncovalent linkages (hydrogen bonds; van der Waals interactions), and it is reasonable to expect that with increased molecular weight, chains have a higher chance to form interpolymer contacts. It is plausible that polymer concentration would exert a similar effect and at higher concentrations, the chains would appear close to one another, have a higher chance to form interchain bonds, and afford materials with enhanced stability in time. We are now investigating this possibility in detail. We note that surface-mediated drug delivery documented utility of eroding surfaces with kinetics of degradation from several hours to several months.[51−53] Controlling performance and kinetics of degradation of biointerfaces from 24 h to 7 days is therefore highly warranted and is expected to find use in diverse biomedical applications.

Interestingly, analysis of gradual erosion of the hydrogels using force−distance readings conducted using atomic force microscopy did not reveal noticeable change in substrate elasticity with time. For a 28 kDa sample, readings taken at 1, 24, and 72 h of hydrogel incubation in PBS yielded similar values as that of Young's moduli (Figure 12). With subsequent incubation, hydrogels exhibited poor adhesion to the glass slide which prevented conducting force−distance measurements at this time point. A plausible explanation to the above finding is a core−shell structure of μS hydrogels with 2 μm cubic features. We have alluded to this possibility in our previous publications, and data in Figure 12 appear to further support this hypothesis. Dominant release of the polymer from within the hydrogel structure and maintenance of stability of the shell may lead to an overall depletion of polymer content within the sample which is not accompanied by pronounced changes in the properties of the surface, and this is indeed observed in our experiment. Biointerfaces with properties like this may be highly advantageous in that throughout the lifetime of the material, its size and mechanical characteristics are maintained at a near constant level, which ensures uniform performance of the sample in time. We note, however, that the core−shell structure is not inherent with PVA physical hydrogels as such and this characteristic is likely a result of μTM employed as an assembly technique. Application of PVA solutions onto μS PDMS stamps and incubation within a mold results in structurally stable μS polymer thin films, a notion which suggests that the polymer undergoes partial dehydration and gelation within the mold. It is plausible that during this time polymer chains exhibit a tendency to accumulate at the interface and thus produce a core−shell structure. Indeed, crystallization of PVA at interfaces was described previously and resulted in surface-adhered hydrogels.[47,54] It is further likely that 2 μm cubic features exhibit a greater tendency to producing core−shell structures due to increased surface area of the mold (i.e., greater surface of the interface).

CONCLUSIONS

We presented the first example of biodegradable enzymatic microreactors based on surface-adhered physical hydrogels of PVA. Toward the final goal, we accomplished synthesis of PVA chains with molecular weights which are suitably high to support polymer gelation and at the same time suitably low to afford gradual erosion of the matrix. Polymer gelation was characterized quantitatively using custom-made samples of PVA and an innovative strategy to enumerate chains within the hydrogel using solution-based UV−vis readouts. Retention of enzymatic cargo within μS hydrogels and conversion of model prodrugs was characterized in depth using polymer molecular weight and concentration of the polymer solution used for assembly of hydrogels as tools of control over properties of the matrix. Assembled biointerfaces exhibited controlled degradation and durability of enzymatic activity with the lifetime of materials of at least one week and the possibility to tune this using polymer molecular weight.

Further to the factors of control identified in this work, we have previously investigated, in detail, opportunities in design of μS enzymatic microreactors using coagulation conditions as tools to fine-tune enzymatic catalysis and mechanical properties of the hydrogels.[45] Taken together, our reports establish PVA-based surface-adhered hydrogels as biointerfaces with multi-armed control over surface topography,[22] elasticity,[21] cell adhesive properties,[20,21] and innovative opportunities in

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substrate-mediated drug delivery.[20,44,45] We anticipate that these biomaterials will prove useful for diverse applications in biotechnology and biomedicine, specifically surface-mediated drug delivery and tissue engineering.

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Notes

The authors declare no competing financial interest.

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REFERENCES

(1) Seliktar, D. Designing cell-compatible hydrogels for biomedical applications. *Science* **2012**, *336*, 1124−1128.

(2) Lee, K. Y.; Mooney, D. J. Hydrogels for tissue engineering. *Chem. Rev.* **2001**, *101*, 1869−1879.

(3) Hassan, C. M.; Peppas, N. A. Structure and applications of poly(vinyl alcohol) hydrogels produced by conventional crosslinking or by freezing/thawing methods. *Adv. Polym. Sci.* **2000**, *153*, 37−65.

(4) Slaughter, B. V.; Khurshid, S. S.; Fisher, O. Z.; Khademhosseini, A.; Peppas, N. A. Hydrogels in regenerative medicine. *Adv. Mater.* **2009**, *21*, 3307−3329.

(5) Uhrich, K. E.; Cannizzaro, S. M.; Langer, R. S.; Shakesheff, K. M. Polymeric systems for controlled drug release. *Chem. Rev.* **1999**, *99*, 3181−3198.

(6) Parascandola, P.; Branduardi, P.; de Alteriis, E. PVA-gel (Lentikats®) as an effective matrix for yeast strain immobilization aimed at heterologous protein production. *Enzyme Microb. Technol.* **2006**, *38*, 184−189.

(7) Wilson, L.; Illanes, A.; Pessela, B. C. C.; Abian, O.; Fernandez-Lafuente, R.; Guisan, J. M. Encapsulation of crosslinked penicillin G acylase aggregates in lentikats: Evaluation of a novel biocatalyst in organic media. *Biotechnol. Bioeng.* **2004**, *86*, 558−562.

(8) Lozinsky, V. I.; Plieva, F. M. Poly(vinyl alcohol) cryogels employed as matrices for cell immobilization. 3. Overview of recent research and developments. *Enzyme Microb. Technol.* **1998**, *23*, 227−242.

(9) Kloxin, A. M.; Kloxin, C. J.; Bowman, C. N.; Anseth, K. S. Mechanical properties of cellularly responsive hydrogels and their experimental determination. *Adv. Mater.* **2010**, *22*, 3484−3494.

(10) Levental, I.; Georges, P. C.; Janmey, P. A. Soft biological materials and their impact on cell function. *Soft Matter* **2007**, *3*, 299−306.

(11) Engler, A. J.; Sen, S.; Sweeney, H. L.; Discher, D. E. Matrix elasticity directs stem cell lineage specification. *Cell* **2006**, *126*, 677−689.

(12) Drury, J. L.; Mooney, D. J. Hydrogels for tissue engineering: Scaffold design variables and applications. *Biomaterials* **2003**, *24*, 4337−4351.

(13) Van Vlierberghe, S.; Dubruel, P.; Schacht, E. Biopolymer-based hydrogels as scaffolds for tissue engineering applications: A review. *Biomacromolecules* **2011**, *12*, 1387−1408.

(14) Baker, M. I.; Walsh, S. P.; Schwartz, Z.; Boyan, B. D. A review of polyvinyl alcohol and its uses in cartilage and orthopedic applications. *J. Biomed. Mater. Res., Part B* **2012**, *100B*, 1451−1457.

(15) Alves, M. H.; Jensen, B. E. B.; Smith, A. A. A.; Zelikin, A. N. Poly(vinyl alcohol) physical hydrogels: new vista on a long serving biomaterial. *Macromol. Biosci.* **2011**, *11*, 1293−1313.

(16) Chaouat, M.; Le Visage, C.; Baille, W. E.; Escoubet, B.; Chaubet, F.; Mateescu, M. A.; Letourneur, D. A novel cross-linked poly(vinyl alcohol) (PVA) for vascular grafts. *Adv. Funct. Mater.* **2008**, *18*, 2855−2861.

(17) Gough, J. E.; Scotchford, C. A.; Downes, S. Cytotoxicity of glutaraldehyde crosslinked collagen/poly(vinyl alcohol) films is by the mechanism of apoptosis. *J. Biomed. Mater. Res.* **2002**, *61*, 121−130.

(18) Cascone, M. G.; Sim, B.; Downes, S. Blends of synthetic and natural polymers as drug-delivery systems for growth-hormone. *Biomaterials* **1995**, *16*, 569−574.

(19) Nair, B.; panel, C. I. R. E. Final report on the safety assessment of polyvinyl alcohol. *Int. J. Toxicol.* **1998**, *17*, 67−92.

(20) 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. Surface-adhered composite poly(vinyl alcohol) physical hydrogels: Polymersome-aided delivery of therapeutic molecules. *Advanced Healthcare Materials* **2012**, *1*, 791−795.

(21) Jensen, B. E. B.; Alves, M.-H.; Fejerskov, B.; Stadler, B.; Zelikin, A. N. Surface adhered poly(vinyl alcohol) physical hydrogels as tools for rational design of intelligent biointerfaces. *Soft Matter* **2012**, *8*, 4625−4634.

(22) 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. Poly(vinyl alcohol) physical hydrogels: Noncryogenic stabilization allows nano- and microscale materials design. *Langmuir* **2011**, *27*, 10216−10223.

(23) Lin, C. C.; Anseth, K. S. PEG hydrogels for the controlled release of biomolecules in regenerative medicine. *Pharm. Res.* **2009**, *26*, 631−643.

(24) Lozinsky, V. I.; Zubov, A. L.; Titova, E. F. Swelling behavior of poly(vinyl alcohol) cryogels employed as matrices for cell immobilization. *Enzyme Microb. Technol.* **1996**, *18*, 561−569.

(25) Lozinsky, V. I.; Zubov, A. L.; Titova, E. F. Poly(vinyl alcohol) cryogels employed as matrices for cell immobilization 0.2. Entrapped cells resemble porous fillers in their effects on the properties of PVA-cryogel carrier. *Enzyme Microb. Technol.* **1997**, *20*, 182−190.

(26) Boyer, C.; Bulmus, V.; Davis, T. P.; Ladmiral, V.; Liu, J. Q.; Perrier, S. Bioapplications of RAFT polymerization. *Chem. Rev.* **2009**, *109*, 5402−5436.

(27) Moad, G.; Rizzardo, E.; Thang, S. H. Living radical polymerization by the RAFT process: A second update. *Aust. J. Chem.* **2009**, *62*, 1402−1472.

(28) Boyer, C.; Stenzel, M. H.; Davis, T. P. Building nanostructures using RAFT polymerization. *J. Polym. Sci., Part A: Polym. Chem.* **2011**, *49*, 551−595.

(29) Bulmus, V. RAFT polymerization mediated bioconjugation strategies. *Polym. Chem.* **2011**, *2*, 1463−1472.

(30) Tong, Y. Y.; Dong, Y. Q.; Du, F. S.; Li, Z. C. Block copolymers of poly(ethylene oxide) and poly(vinyl alcohol) synthesized by the raft methodology. *J. Polym. Sci., Part A: Polym. Chem.* **2009**, *47*, 1901−1910.

(31) Tong, Y. Y.; Wang, R.; Xu, N.; Du, F. S.; Li, Z. C. Synthesis of well-defined azide-terminated poly(vinyl alcohol) and their subsequent modification via click chemistry. *J. Polym. Sci., Part A: Polym. Chem.* **2009**, *47*, 4494−4504.

(32) Stenzel, M. H.; Cummins, L.; Roberts, G. E.; Davis, T. R.; Vana, P.; Barner-Kowollik, C. Xanthate mediated living polymerization of vinyl acetate: A systematic variation in MADIX/RAFT agent structure. *Macromol. Chem. Phys.* **2003**, *204*, 1160−1168.

(33) Kostov, G.; Boschet, F.; Buller, J.; Badache, L.; Brandsadter, S.; Ameduri, B. First amphiphilic poly(vinylidene fluoride-*co*-3,3,3-trifluoropropene)-*b*-oligo(vinyl alcohol) block copolymers as potential nonpersistent fluorosurfactants from radical polymerization controlled by xanthate. *Macromolecules* **2011**, *44*, 1841−1855.

(34) Stenzel, M. H.; Davis, T. P.; Barner-Kowollik, C. Poly(vinyl alcohol) star polymers prepared via MADIX/RAFT polymerisation. *Chem. Commun.* **2004**, 1546−1547.

(35) Hassan, C. M.; Peppas, N. A. Structure and applications of poly(vinyl alcohol) hydrogels produced by conventional crosslinking or by freezing/thawing methods. In *Biopolymers/PVA Hydrogels/*

Anionic Polymerisation Nanocomposites; Springer-Verlag: Berlin, 2000; Vol. *153*, pp 37−65.
(36) Ladewig, K. Drug delivery in soft tissue engineering. *Expert Opin. Drug Delivery* **2011**, *8*, 1175−1188.
(37) Sato, T.; Uchida, R.; Tanigawa, H.; Uno, K.; Murakami, A. Application of polymer gels containing side-chain phosphate groups to drug-delivery contact lenses. *J. Appl. Polym. Sci.* **2005**, *98*, 731−735.
(38) Andrade-Vivero, P.; Fernandez-Gabriel, E.; Alvarez-Lorenzo, C.; Concheiro, A. Improving the loading and release of NSAIDs from pHEMA hydrogels by copolymerization with functionalized monomers. *J. Pharm. Sci.* **2007**, *96*, 802−813.
(39) Wang, N. X.; von Recum, H. A. Affinity-Based Drug Delivery. *Macromol. Biosci.* **2011**, *11*, 321−332.
(40) Bouhadir, K. H.; Kruger, G. M.; Lee, K. Y.; Mooney, D. J. Sustained and controlled release of daunomycin from cross-linked poly(aldehyde guluronate) hydrogels. *J. Pharm. Sci.* **2000**, *89*, 910−919.
(41) Gulsen, D.; Li, C. C.; Chauhan, A. Dispersion of DMPC liposomes in contact lenses for ophthalmic drug delivery. *Curr. Eye Res.* **2005**, *30*, 1071−1080.
(42) Galeska, I.; Kim, T. K.; Patil, S. D.; Bhardwaj, U.; Chatttopadhyay, D.; Papadimitrakopoulos, F.; Burgess, D. J. Controlled release of dexamethasone from PLGA microspheres embedded within polyacid-containing PVA hydrogels. *AAPS J.* **2005**, *7*, E231−E240.
(43) Hoare, T. R.; Kohane, D. S. Hydrogels in drug delivery: Progress and challenges. *Polymer* **2008**, *49*, 1993−2007.
(44) Fejerskov, B.; Zelikin, A. N. Substrate mediated enzyme prodrug therapy. *PLoS One* **2012**, *7*, e49619.
(45) Fejerskov, B.; Jensen, B.; Jensen, N.; Chong, S.-F.; Zelikin, A. N. Engineering surface adhered poly(vinyl alcohol) physical hydrogels as enzymatic microreactors. *ACS Appl. Mater. Interfaces* **2012**, *4*, 4981−4990.
(46) Smith, A. A. A.; Hussmann, T.; Elich, J.; Postma, A.; Alves, M.-H.; Zelikin, A. N. Macromolecular design of poly(vinyl alcohol) by RAFT polymerization. *Polym. Chem.* **2012**, *3*, 85−88.
(47) Kozlov, M.; McCarthy, T. J. Adsorption of poly(vinyl alcohol) from water to a hydrophobic surface: Effects of molecular weight, degree of hydrolysis, salt, and temperature. *Langmuir* **2004**, *20*, 9170−9176.
(48) Valentín, J. L.; López, D.; Hernández, R.; Mijangos, C.; Saalwächter, K. Structure of poly(vinyl alcohol) cryo-hydrogels as studied by proton low-field NMR spectroscopy. *Macromolecules* **2009**, *42*, 263−272.
(49) Czichocki, G.; Dautzenberg, H.; Capan, E.; Vorlop, K. D. New and effective entrapment of polyelectrolyte-enzyme-complexes in LentiKats. *Biotechnol. Lett.* **2001**, *23*, 1303−1307.
(50) Gröger, H.; Capan, E.; Barthuber, A.; Vorlop, K. D. Asymmetric synthesis of an (*R*)-cyanohydrin using enzymes entrapped in lens-shaped gels. *Org. Lett.* **2001**, *3*, 1969−1972.
(51) Zelikin, A. N. Drug releasing polymer thin films: New era of surface-mediated drug delivery. *ACS Nano* **2010**, *4*, 2494−2509.
(52) Takahashi, H.; Letourneur, D.; Grainger, D. W. Delivery of large biopharmaceuticals from cardiovascular stents: A review. *Biomacromolecules* **2007**, *8*, 3281−3293.
(53) Jewell, C. M.; Lynn, D. M. Multilayered polyelectrolyte assemblies as platforms for the delivery of DNA and other nucleic acid-based therapeutics. *Adv. Drug Delivery Rev.* **2008**, *60*, 979−999.
(54) Kozlov, M.; Quarmyne, M.; Chen, W.; McCarthy, T. J. Adsorption of poly(vinyl alcohol) onto hydrophobic substrates. A general approach for hydrophilizing and chemically activating surfaces. *Macromolecules* **2003**, *36*, 6054−6059.