

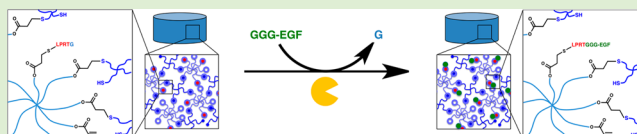
Covalent Modification of Synthetic Hydrogels with Bioactive Proteins via Sortase-Mediated Ligation

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Supporting Information

ABSTRACT: Synthetic extracellular matrices are widely used in regenerative medicine and as tools in building in vitro physiological culture models. Synthetic hydrogels display advantageous physical properties, but are challenging to modify with large peptides or proteins. Here, a facile, mild enzymatic postgrafting approach is presented. Sortase-mediated ligation was used to conjugate human epidermal growth factor fused to a GGG ligation motif (GGG-EGF) to poly(ethylene glycol) (PEG) hydrogels containing the sortase LPRTG substrate. The reversibility of the sortase reaction was then exploited to cleave tethered EGF from the hydrogels for analysis. Analyses of the reaction supernatant and the postligation hydrogels showed that the amount of tethered EGF increases with increasing LPRTG in the hydrogel or GGG-EGF in the supernatant. Sortase-tethered EGF was biologically active, as demonstrated by stimulation of DNA synthesis in primary human hepatocytes and endometrial epithelial cells. The simplicity, specificity, and reversibility of sortase-mediated ligation and cleavage reactions make it an attractive approach for modification of hydrogels.



1. INTRODUCTION

The need for in vitro physiological models and for clinical therapies has driven the development of synthetic biomaterials that recapitulate key features of the extracellular matrix (ECM).¹ Polymer hydrogels are an attractive foundation for synthetic ECM, as their biophysical and biochemical properties can be tuned in modular fashion to control cellular phenotype. Hydrogels built from macromers containing highly flexible, hydrophilic poly(ethylene glycol) (PEG) have found especially widespread use due to their biocompatibility, relative inertness to nonspecific cell interactions, relative ease of functionalization with biomolecular motifs, and commercial availability of reactive PEG macromers of various molecular weights and branching configurations.^{2–5} However, unmodified PEG hydrogels possess limited or no intrinsic biological function and thus may fail to support desired cell behaviors.

Many strategies have been explored to modify PEG hydrogels with ECM-derived biological cues such as adhesion peptides and growth factors,^{6–9} but facile, site-specific attachment of large peptides and proteins in a way that retains bioactivity remains a challenge, particularly if the approaches are intended to be used in the presence of cells. Photopolymerization and conjugation of peptides or proteins through amine groups^{10–14} are straightforward, but lack specificity and may alter protein function. Conjugation through click chemistry allows increased intermediate stability and improved efficiency of hydrogel conjugation; however, this approach is limited by the difficulties associated with producing proteins that incorporate precursors for click chemistry in an appropriate site-specific manner.⁹

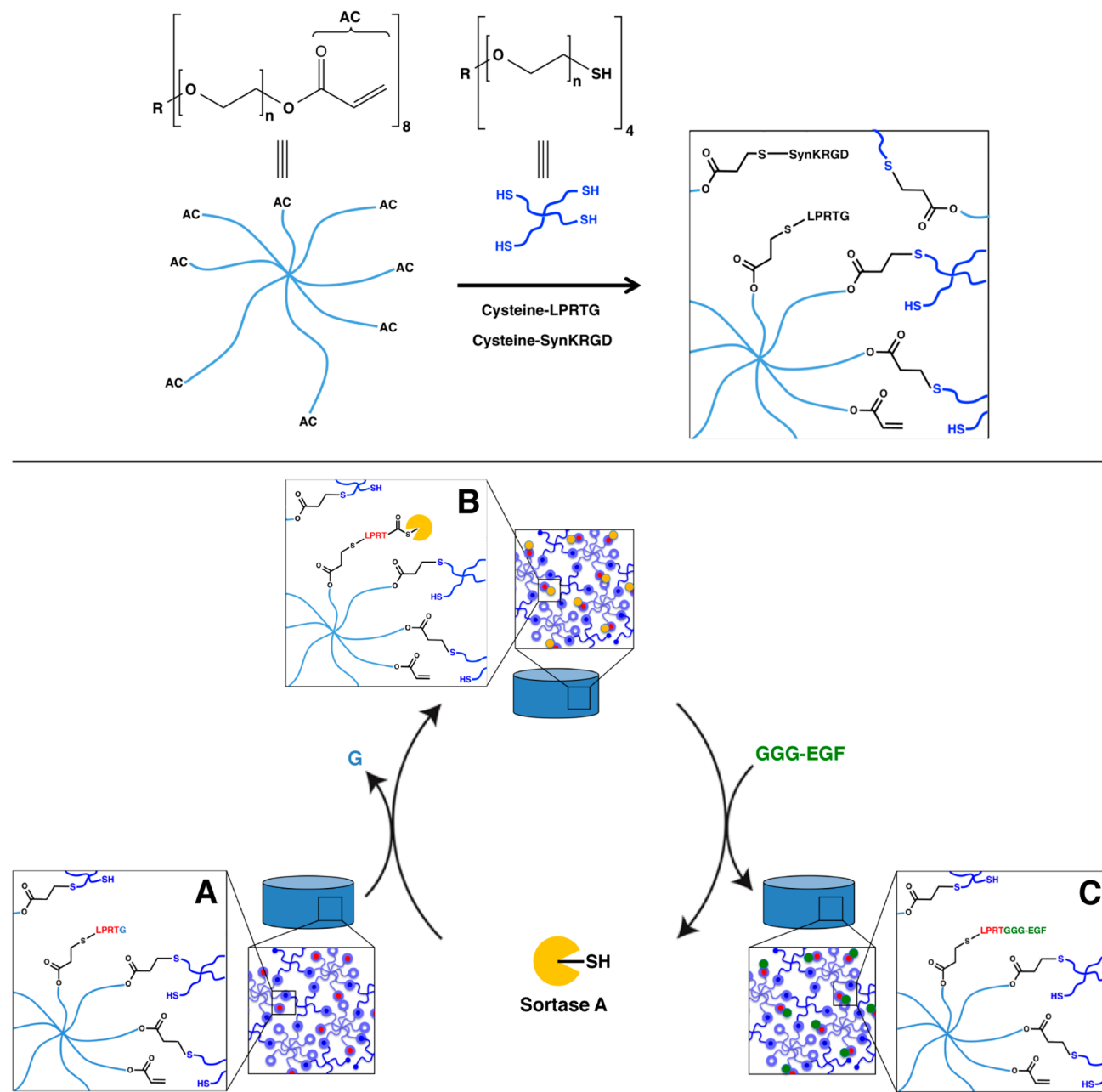
Here, we describe the use of sortase-mediated ligation of epidermal growth factor (EGF) to PEG-based synthetic ECM hydrogels. Sortases are transpeptidases found in Gram-positive bacteria, which anchor surface proteins to the bacterial cell wall in vivo.¹⁵ The enzyme sortase A from *Staphylococcus aureus* recognizes a specific LPXTG motif (X = any amino acid except proline), cleaves the amide bond between the threonine and the glycine, and covalently attaches an oligoglycine nucleophile.¹⁶ Moreover, the formed product maintains the LPXTG motif, allowing controlled release or cleavage of the tethered protein through a second sortase-mediated transpeptidase reaction in the presence of a soluble N-terminal oligoglycine nucleophile (e.g., triglycine, GGG).

Sortase-mediated ligation has found numerous applications in purification, modification and immobilization of proteins.^{17,18} It has been used to conjugate proteins to different types of surfaces,^{19–27} liposomes²⁸ and peptides.^{29,30} Here we extend sortase-mediated ligation to the modification of synthetic ECM hydrogels. Compared to other enzymatic approaches,³¹ sortase-mediated ligation offers the advantage of enhanced catalytic rate due to the engineering of mutant sortases,³² enhanced diffusion rate due to the relatively small size of the sortase (23.5 kDa), high yield of recombinant expression and relatively modest propensity to modify known mammalian proteins.³³

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Scheme 1. Process Used to Tether GGG-EGF to the Hydrogel^a

^aTop: Hydrogels are formed by Michael-type additions of 8-arm PEG-acrylate stars cross-linked with 4-arm PEG-thiol stars. Adhesion peptides (SynKRGD) and sortase motif peptides (LPRTG) can also be cross-linked into the hydrogel if they contain a cysteine residue to react with the acrylate of the 8-arm PEG star. Bottom: (A) Sortase-mediated ligation of GGG-EGF to pre-formed PEG hydrogels containing LPRTG peptide. (B) Sortase diffuses into the hydrogel, cleaves the peptide bond between the threonine and the glycine of the LPRTG peptide, releasing G, then the N-terminus of GGG-EGF, which has diffused into the hydrogel, is ligated to the C-terminus of the LPRTG-motif (C). The same sequence of steps can be used to cleave EGF once it is ligated to the hydrogel, by adding sortase and the simple peptide GGG; GGG will displace tethered EGF, releasing it into solution.

In this study, we ligated EGF to relatively soft, permeable PEG hydrogels prefunctionalized with cell adhesion motifs. The biophysical and cell adhesion properties of hydrogels were designed to support physiological culture of primary human epithelial cells. We formed hydrogels by copolymerizing acrylate-terminated multiarm PEG with a thiol-terminated LPRTG peptide and thiol-terminated multiarm PEG through Michael-type addition (Scheme 1) as reported earlier.³ This cross-linking was carried out in the presence of a cell adhesion peptide bearing a thiol reactive group to render the resulting

hydrogels adhesive to primary human epithelial cells. An evolved triple mutant of the sortase A enzyme (P94S/D160N/K196T) developed by Liu and co-workers³² was used to tether the previously reported N-terminal Gly₃-tagged human epidermal growth factor (GGG-EGF)³⁰ to the hydrogel (Scheme 1). Characterization of the system included sortase-mediated cleavage of the tethered EGF for quantification in solution. To test the bioactivity of the tethered EGF, we investigated DNA synthesis with EGF-responsive human cryopreserved hepatocytes and primary human endometrial

epithelial cells. Our results illustrate several features of the sortase-mediated ligation reaction as implemented for modification of synthetic ECM hydrogels, including the ability to efficiently release tethered proteins via a sortase-mediated reaction with a small soluble oligoglycine substrate, GGG.

2. EXPERIMENTAL SECTION

2.1. Materials. Commercially available chemicals, including triglycine (GGG), were purchased from Sigma-Aldrich and used without further purification unless otherwise noted. PEG macromers (10 kDa 8-arm PEG-acrylate and 5 kDa 4-arm PEG-thiol) were purchased from JenKem. All custom peptides were synthesized by Boston Open Laboratories, USA, as follows: Ac-CRGLPRTGG-CONH₂ (*LPRTG*), Ac-CRGLPRTGGK(ϵ -fluorescein)-CONH₂ (*LPRTG*-fam), PHSRNGGGK(ϵ GGG ERCG-Ac)-GGRGDSPY (*synKRGD*) and PHSRNGGGK(ϵ GGGERCG-Ac)-GGRGDSPY (scrambled *synKRGD*). Hydrogels were formed in 96-well angiogenesis plates (0.125 cm²; Ibidi). The recombinant sortagable GGG-EGF (human EGF fused to a triglycine sequence at the N-terminus) and the evolved triple mutant (P94S/D160N/D165A) of the sortase A enzyme³² were expressed and purified as previously reported.³⁰ Reagents from the DuoSet EGF ELISA kit (R&D Systems, DY236-05) were used for EGF detection. Cryopreserved hepatocytes (Human Plateable Hepatocytes, Induction Qualified, lot# Hu1663) and hepatocyte media were purchased from Life Technologies. Human wild type EGF (hEGF) was obtained from Invitrogen (PHG0313).

2.2. Hydrogel Fabrication and Sortase-Mediated Ligation of GGG-EGF. Using Michael-type addition as described earlier³ nominal (preswelling) 5% w/v polymer hydrogels with 1:1 thiol:acrylate ratio were synthesized by preincubating PEG-acrylate macromers with *LPRTG* and adhesion peptides in phosphate buffered saline (PBS) at pH 6.9 for 20 min. Adhesion peptides (*synKRGD* or scrambled *synKRGD*) were added at a nominal concentration of 500 μ M and *LPRTG* nominal concentrations varied from 0 to 250 μ M. PEG-thiol cross-linking macromers were added and 10 μ L hydrogels were formed within the inner wells of a 96-well angiogenesis plate. After gelation [around 1 h at room temperature (RT)], hydrogels were covered with PBS and allowed to swell for 90 min at 4 °C with PBS changes every 30 min. They were further allowed to swell in intermediate buffer (50 mM HEPES, 150 mM NaCl, pH 7.9) for 90 min at 4 °C with buffer changes every 30 min. Hydrogels were blocked with 70 μ L/well calcium buffer (50 mM HEPES, 150 mM NaCl, 10 mM CaCl₂, pH 7.9) containing 0.5% purified bovine casein for 1 h at RT. Tethering solution containing 2 or 20 μ M GGG-EGF and 15 μ M sortase enzyme in calcium buffer was added to the hydrogels (50 μ L per well) and the reaction was allowed to proceed for 1 h at RT with constant agitation at 30 rpm. Tethering solution without sortase was used to test for nonspecific binding of GGG-EGF to hydrogels and calcium buffer was used for control and in soluble EGF conditions. The sortase reaction was stopped by addition of 5 μ L/well of ethylenediaminetetraacetic acid (EDTA; 300 mM), then supernatants were collected and frozen at -80 °C for EGF quantification, and hydrogels were extensively washed (3 \times immediately and every 30 min for 3 h) with 70 μ L/well intermediate buffer and soaked overnight at 4 °C.

2.3. Hydrogel Fluorescence Measurements for Quantification of Incorporated or Sortase-Cleaved *LPRTG*. Hydrogels containing a mix of 96% *LPRTG* and 4% *LPRTG*-fam peptides with varying total nominal peptide concentrations of 0, 20, 50, 100, or 250 μ M were cross-linked in 96-well angiogenesis plates as described above. After 6 \times 30 min washes at 4 °C, intermediate buffer (50 μ L) was added on top of each hydrogel and fluorescence ($\lambda_{\text{ex}} = 485$ nm; $\lambda_{\text{em}} = 530$ nm) was measured using a microplate reader (SpectraMax M2^c, Molecular Devices). A linear standard curve was established by measuring the fluorescence of hydrogels containing 0, 20, 50, 100, or 250 μ M of total *LPRTG* peptide (Supporting Information Figure 1). After incubation for 1 h with either 2 μ M or 20 μ M GGG-EGF in the presence of 15 μ M sortase the reaction was stopped with 5 μ L/well EDTA (300 mM) and hydrogels were extensively washed (3 \times immediately, every 30 min for 3 h and soaked overnight at 4 °C)

before fluorescence reading. In order to correct for photobleaching, decreased fluorescence of control hydrogels was measured in the absence of sortase (Supporting Information Figure 2).

2.4. GGG-EGF Detection via Direct ELISA on Hydrogels.

Hydrogels were blocked with Odyssey Blocking Buffer (Li-Cor Biosciences) diluted 1:1 with PBS (OBB-PBS) for 1 h at RT and washed 3 \times with 0.1% Tween-20 in PBS. Hydrogels were incubated with biotinylated goat antihuman EGF detection antibody at a concentration of 50 ng/mL in OBB-PBS for 2 h at RT with constant agitation at 30 rpm. The washing steps were repeated and hydrogels were incubated with streptavidin-HRP diluted 1:40 in OBB-PBS for 20 min at RT with constant agitation at 30 rpm and protected from light. After washes, hydrogels were incubated with substrate solution consisting of a 1:1 mixture of Color Reagent A (H₂O₂) and Color Reagent B (tetramethylbenzidine) (R&D Systems, DY994) for 20–30 min at RT protected from light. The reaction was stopped with 1 M H₂SO₄. Aliquots (20 μ L) of supernatant were transferred to a clear bottom Nunc MaxiSorp 384-well plate (Thermo Scientific) and absorbance at 450 nm was measured using a microplate reader (SpectraMax M2^c, Molecular Devices). Absorbance at 540 nm was subtracted to account for optical imperfections of the plate.

2.5. Quantification of Sortase-Mediated Cleavage of Tethered EGF from Hydrogels.

A modified sandwich ELISA was used to measure the amount of EGF cleaved from hydrogels in the presence of sortase A and GGG peptide. GGG-EGF-tethered hydrogels were soaked in calcium buffer for 1 h at 4 °C and incubated for 48 h at 4 °C with constant agitation in 50 μ L/well cleavage solution containing 20 mM GGG and 200 μ M sortase in calcium buffer. Supernatants were collected and frozen. A clear bottom Nunc MaxiSorp 384-well plate (Thermo Scientific) was coated with mouse antihuman EGF capture antibody diluted at the recommended concentration of 4.0 μ g/mL in sterile PBS. The plate was covered with an adhesive strip, spun at 1500 rpm for 3 min and incubated overnight at 4 °C with constant agitation. Three washing steps with 100 μ L/well 0.1% Tween-20 in PBS were performed at RT using an automatic plate washer (405 Touch Microplate Washer, BioTek). The plate was blocked with 100 μ L/well OBB-PBS and incubated for 2 to 6 h at RT with constant agitation at 30 rpm. Samples were first diluted with calcium buffer containing 1% BSA in 1.5 mL Protein LoBind tubes (Eppendorf) and then serially diluted in 0.5 mL Protein LoBind tubes. Standard curves were made by serially diluting GGG-EGF in 1% BSA in calcium buffer. After the washing steps, samples were plated and the plate was covered with an adhesive strip, spun at 1500 rpm for 3 min and incubated for 2 h at RT with constant agitation. This process was repeated with the biotinylated goat antihuman EGF detection antibody diluted at the recommended working concentration of 50 ng/mL in OBB-PBS. The plate was washed, incubated with streptavidin-HRP diluted 1:40 in OBB-PBS and spun at 1500 rpm for 3 min protected from light. After 20 min incubation at RT with constant agitation, the plate was washed and incubated with substrate solution for 20–30 min at RT protected from light. The reaction was stopped with 1 M H₂SO₄ and absorbance at 450 nm was measured.

2.6. Human Cryopreserved Hepatocyte Culture on Hydrogels.

After a PBS wash and UV-sterilization, hydrogels were soaked in human hepatocyte seeding medium (hHSM; Williams E medium supplemented with 5% FBS, 1 μ M hydrocortisone, 1% penicillin/streptomycin (P/S), 4 μ g/mL human recombinant insulin, 2 mM GlutaMAX, 15 mM HEPES (pH 7.4); CM3000) with or without 20 ng/mL hEGF for 1 h. Cryopreserved hepatocytes were quickly thawed in a 37 °C water bath, transferred into a 50 mL falcon tube with 25 mL prewarmed Cryopreserved Hepatocyte Recovery Medium (CHRM; CM7000) and centrifuged at RT at 100g for 8 min. Warm hHSM (1 mL) was added to the cell pellet and cells were gently rocked, counted (Countess Cell Counter, Invitrogen) and placed on ice. Hepatocytes were seeded on hydrogels and on collagen I-coated (BioCoat, BD Biosciences) wells in 96-well angiogenesis plates at a density of 60 000 cells/cm² (7500 cells/well) in 50 μ L/well hHSM. Hepatocytes were incubated at 37 °C, 95% air, 5% CO₂. Twenty-four hours after cell seeding, medium was switched to serum-free human hepatocyte maintenance medium (hHMM; Williams E medium supplemented

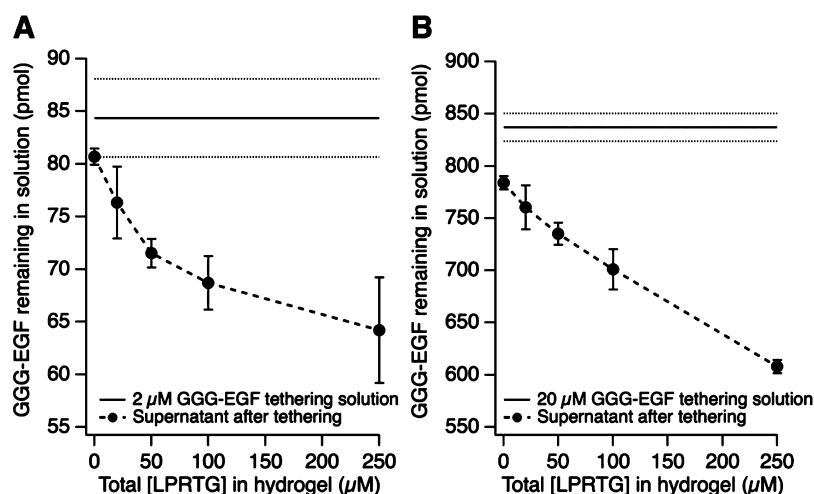


Figure 1. Amount of GGG-EGF peptide nucleophile remaining in solution as a function of total LPRTG concentration in hydrogel. GGG-EGF is quantified by sandwich ELISA before and after sortase-mediated ligation of GGG-EGF to preformed hydrogels containing LPRTG, using nucleophile concentrations of 2 μM (A) or 20 μM (B). The amount of GGG-EGF remaining in solution after sortase-mediated ligation decreases with increasing LPRTG concentration in hydrogel. Error bars represent standard error of the mean ($n = 2$).

with 0.1 μM hydrocortisone, 0.5% P/S, ITS+ (human recombinant insulin (6.25 $\mu\text{g}/\text{mL}$), human transferrin (6.25 $\mu\text{g}/\text{mL}$), selenous acid (6.25 ng/mL), bovine serum albumin (1.25 mg/mL), linoleic acid (5.35 $\mu\text{g}/\text{mL}$), 2 mM GlutaMAX, 15 mM HEPES (pH 7.4); CM4000) with or without 20 ng/mL hEGF and cells were incubated for 24 h.

2.7. Endometrial Biopsy Collection and Isolation. Eutopic endometrial biopsies were obtained from two premenopausal women in the proliferative phase of the menstrual cycle, who were undergoing surgery for benign gynecological diseases. Selective criteria included that the patients had regular menstrual cycles (26 to 35 days) and were not using hormonal treatment for at least 3 months prior to surgery. A standardized questionnaire was used to document all clinical data. Tissues were collected with the approval of the Partners Human Research Committee and the Massachusetts Institute of Technology Committee on the Use of Humans as Experimental Subjects and with the informed consent of each patient. Endometrial Pipelle biopsies were dissociated and cells purified as described by Osteen and co-workers³⁴ with some modifications (Supporting Information).

2.8. Endometrial Epithelial Cell Culture on Hydrogels. Before cell seeding, hydrogels were washed with PBS, UV-sterilized for 15 min and soaked in DMEM/F12/FBS (mixture of Dulbecco's Modified Eagle's Medium and Ham's F-12 (Gibco) supplemented with 1% penicillin/streptomycin and 10% v/v dextran/charcoal treated fetal bovine serum (Atlanta Biologicals) with or without 20 ng/mL hEGF for 1 h. Cultured endometrial epithelial cells (EECs) were trypsinized and seeded on hydrogels and on standard tissue-culture polystyrene (TCPS) in 96-well angiogenesis plates at a density of 20 000 cells/cm² (2500 cells/well) in 50 μL /well DMEM/F12/FBS. EECs were incubated at 37 $^{\circ}\text{C}$, 95% air, 5% CO₂. Twenty-four hours after cell seeding, medium was switched to serum-free medium DMEM/F12 with or without 20 ng/mL hEGF and cells were incubated for 16 h.

2.9. DNA Synthesis Assay. Click-iT EdU Alexa Fluor 488 kit (Life Technologies) was used to quantify cells actively synthesizing DNA. Forty-eight hours after cell seeding, hepatocytes were incubated in hHMM with 10 μM of 5-ethynyl-2'-deoxyuridine (EdU) in with or without 20 ng/mL (3.3 nM) hEGF for 24 h at 37 $^{\circ}\text{C}$, 95% air, 5% CO₂. Similarly, EECs were incubated 40 h post seeding with 10 μM EdU in DMEM/F12 with or without 20 ng/mL (3.3 nM) hEGF for 24 h. Both cell-types were fixed with 3.7% formaldehyde in PBS for 15 min at RT. Cells were washed twice with 3% BSA in PBS and permeabilized with 0.5% Triton X-100 in PBS for 20 min at RT. Click-iT reaction cocktail was prepared as described by the manufacturer and 20 μL per well was added. Cells were incubated for 30 min at RT protected from light. After cells were washed once with 3% BSA in

PBS and once with PBS, they were incubated with Hoechst 33342 diluted 1:2000 in PBS for 30 min at RT protected from light. Cells were finally washed twice with PBS and imaged using a Leica DMI 6000 microscope and Oasis Surveyor software. Images were processed, and cell nuclei were counted using ImageJ64 software. The percentage of cells synthesizing DNA was computed as the ratio of cells positively stained with Alexa Fluor 488 divided by the total number of cells given by Hoechst counter-staining.

3. RESULTS AND DISCUSSION

We first evaluated the ability of the evolved triple mutant of the sortase A to tether the solution-phase GGG-EGF nucleophile to LPRTG substrate motifs that were covalently incorporated in PEG hydrogels (LPRTG-gel). Compared to the wild-type sortase, this mutant displays a 3-fold improvement in turnover ($k_{\text{cat}} = 4.8 \pm 0.6 \text{ s}^{-1}$) and a 14-fold improvement in the affinity for the LPXTG substrate ($K_{\text{m LPXTG}} = 0.56 \pm 0.007 \text{ mM}$), resulting in a 43-fold increase of catalytic efficiency ($k_{\text{cat}}/K_{\text{m LPXTG}} = 8600 \pm 1500 \text{ M}^{-1} \text{ s}^{-1}$), although the affinity for the N-terminal GGG substrate is less favorable.³² These enhanced properties were predicted to allow efficient catalysis at the relatively low LPXTG substrate concentrations in the hydrogels.

Sortase-mediated ligation was characterized by three complementary methods: measurement of the consumption of the GGG-EGF nucleophile from the reaction solution, measurement of the consumption of the LPRTG-gel substrate, and measurement of GGG-EGF released from the hydrogel after sortase-mediated cleavage of tethered EGF from the hydrogel. We also used a direct on-gel ELISA approach to assess EGF accessibility. The results of these experiments were used to define suitable tethering conditions to create synthetic ECM for modulation of epithelial cell DNA synthesis.

3.1. The Amount of GGG-EGF Consumed from Solution in the Presence of Sortase Depends on LPRTG-Gel Concentration. We first measured the disappearance of the GGG-EGF nucleophile from the supernatants of PEG hydrogels fabricated with systematically varied LPRTG-gel concentrations (0, 20, 50, 100, or 250 μM , corresponding to 0, 200, 500, 1000, and 2500 total pmol LPRTG per hydrogel) and then exposed to coupling solutions containing 15 μM (750

pmol) sortase and GGG-EGF at concentrations of either 2 μM (100 pmol total peptide) or 20 μM (1000 pmol total peptide) to tether EGF via Scheme 1. The amount of GGG-EGF present in the reaction supernatant was measured by sandwich ELISA at the start and end of the reaction.

For control hydrogels (i.e., LPRTG-gel = 0 μM), GGG-EGF diffuses into the hydrogel but does not react even in the presence of sortase. The observed reduction in GGG-EGF concentration in the supernatant of control hydrogels due to this partitioning was 6% for both the 2 and 20 μM GGG-EGF conditions (Figure 1A,B), a value about half that expected based on simple dilution due to the volume of liquid in the hydrogel and consistent with the protein-repulsion properties of PEG. For both the 2 μM and 20 μM GGG-EGF coupling concentrations, GGG-EGF depletion from the coupling solution increased with increasing concentrations of LPRTG-gel (Figure 1A,B) in a manner consistent with enzyme catalysis of the reaction. For the 2 μM GGG-EGF coupling condition (Figure 1A), total LPRTG-gel is always in stoichiometric excess to total GGG-EGF (200–2500 pmol LPRTG: 100 pmol GGG-EGF); hence, the incomplete depletion of GGG-EGF substrate (~20% at each condition) indicates a kinetic or thermodynamic limit to coupling under these conditions. Interestingly, for the 20 μM GGG-EGF coupling condition (Figure 1B), the proportion of GGG-EGF depleted for each LPRTG-gel concentration is comparable to that in the 2 μM GGG-EGF case; i.e., within the error of the ELISA, 10 times as much GGG-EGF is consumed in the 20 μM versus 2 μM GGG-EGF coupling condition (Figure 1A,B).

This apparent first-order dependence of the reaction on GGG-EGF concentration is consistent with the reported ping-pong bi-bi mechanism for sortase A,³⁵ where a thioacyl intermediate is resolved by the N-terminus of an oligoglycine nucleophile—provided that there is sufficient oligoglycine to render the competing reaction, hydrolysis,¹⁶ relatively unimportant. However, the interpretation of the results from this pilot experiment is inherently limited by the convolution of two rate processes—enzyme kinetics and enzyme/substrate diffusion—occurring simultaneously with a complex reaction mechanism, which can include not only ligation but also hydrolysis. Based on size, the diffusion coefficients of GGG-EGF and sortase in water are about $2.5 \times 10^{-6} \text{ cm}^2/\text{s}$ and $1.5 \times 10^{-6} \text{ cm}^2/\text{s}$, respectively,³⁶ and diffusion of molecules of this size in the hydrogels would be expected to be hindered by 50–90%.^{36–38} We can thus estimate the characteristic diffusion times, τ_D , for GGG-EGF and sortase in the hydrogel in the absence of reaction as 0.4–1.8 h and 0.6–3 h, respectively, using the well-established relationship $\tau_D \sim L^2/4D_{A\text{-hydrogel}}$ where L is the thickness of the hydrogel (0.8 mm) and $D_{A\text{-hydrogel}}$ is the effective diffusion coefficient for either GGG-EGF or sortase in the hydrogel. These characteristic diffusion times represent a lower boundary, as interactions with the LPRTG-gel substrate would be expected to retard diffusion and create gradients in reaction rate during the 1 h incubation. In summary, this pilot experiment provides compelling evidence that sortase is capable of tethering relatively large peptides (~6 kDa) to PEG hydrogels, motivating further characterization of the reaction process.

3.2. LPRTG Cleavage Product Is Released from the Hydrogel in the Presence of GGG-EGF and Sortase. In order to elucidate how many LPRTG molecules were effectively processed by sortase, 4% of the LPRTG peptides incorporated into the hydrogels were labeled with fluorescein (LPRTG-fam).

When the sortase enzyme cleaves the peptide bond between the threonine and the glycine of the LPRTG-fam motif, fluorescein is released, thus resulting in a decrease of fluorescence associated with the hydrogel. Fluorescence intensities after washing for hydrogels before and after incubation for 1 h with either 2 μM or 20 μM GGG-EGF in the presence or absence of 15 μM sortase are shown in Supporting Information Figure 2. After correction for photobleaching, fluorescence intensities were converted to pmol LPRTG in hydrogel (see Experimental Section), and the amount of reacted LPRTG was calculated as the difference between the values before and after the sortase reaction. As shown in Figure 2, LPRTG consumption increased with

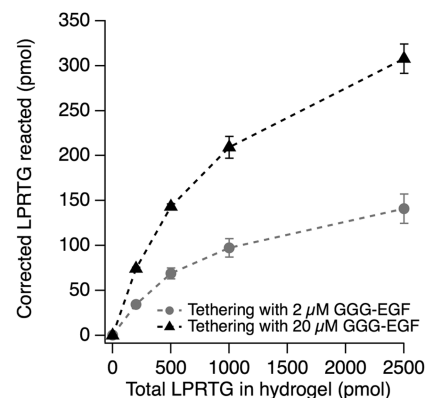


Figure 2. Amount of reacted LPRTG as a function of total initial LPRTG amount in hydrogel. After correction for photobleaching, fluorescence intensities were converted to pmol LPRTG in the hydrogel and the amount of reacted LPRTG was calculated as the difference between the values before and after sortase-mediated ligation. Error bars represent standard error of the mean ($n = 2$).

increasing initial amount of LPRTG in the hydrogels and was greater for the 20 μM GGG-EGF than the 2 μM GGG-EGF condition. This pattern is qualitatively consistent with the trends seen for consumption of GGG-EGF (Figure 1). This confirms that the triple mutant sortase is capable of recognizing the LPRTG motif when it is incorporated in PEG hydrogels; i.e., sortase can cleave the peptide bond between the threonine and the glycine in order to release the fluorescein from the hydrogel.

Interestingly, whereas the amount of GGG-EGF consumed from the supernatant in the 20 μM condition was 10-fold higher than the amount consumed in the 2 μM condition, the amount of reacted LPRTG at the 20 μM GGG-EGF condition was only about 2-fold higher than the reacted LPRTG at the 2 μM condition (Figure 2). We speculate that this discrepancy arises from a difference in the relative rates of hydrolysis and transpeptidation for the different GGG-EGF concentrations. Considering the greatest LPRTG concentration as a representative case, the number of moles of GGG-EGF consumed (20 pmol and 230 pmol for the 2 μM and 20 μM concentrations, respectively) is substantially lower than the amount of reacted LPRTG (140 pmol and 310 pmol for the 2 μM and 20 μM GGG-EGF concentrations, respectively), and the ratio of reacted LPRTG:GGG-EGF consumed is much higher for the lower GGG-EGF concentration. Because the sortase and LPRTG concentrations compared here are identical and only the GGG-EGF nucleophile concentration changes, this observation is consistent with nucleophile starvation at the

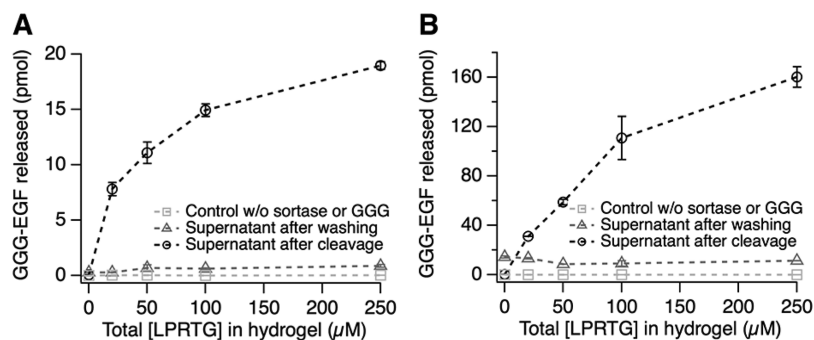


Figure 3. Amount of GGG-EGF released in solution as a function of total LPRTG concentration in hydrogel. GGG-EGF is quantified by sandwich ELISA after hydrogel washes and sortase-mediated hydrogel cleavage following tethering of GGG-EGF at either 2 μM (A) or 20 μM (B). The amount of GGG-EGF released in solution after sortase-mediated cleavage increases with increasing LPRTG concentration in hydrogel. Error bars represent standard error of the mean ($n = 2$).

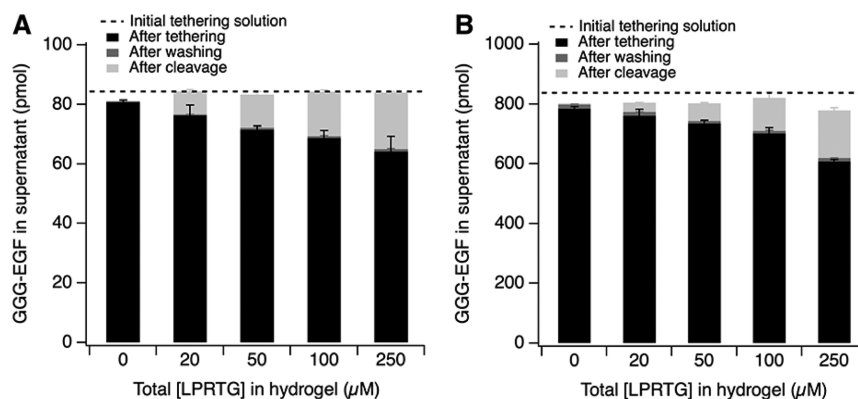


Figure 4. Nucleophile mass balance for sortase-mediated ligation of GGG-EGF to LPRTG-containing hydrogels as a function of LPRTG concentration in the hydrogel and for GGG-EGF concentrations of either 2 μM (A) or 20 μM (B). The amount of GGG-EGF present in initial tethering solution and in hydrogel supernatant after washes and after cleavage was quantified by sandwich ELISA. Error bars represent standard error of the mean ($n = 2$).

lower GGG-EGF concentration leading to proportionately greater hydrolysis compared to ligation. This interpretation is plausible as the $K_{m,\text{GGG}}$ value is in the millimolar range, and thus the reaction would be expected to be in a regime that depends on the GGG-EGF concentration.

3.3. Specific Tethering of GGG-EGF Increases with LPRTG Concentration in Hydrogels and GGG-EGF Concentration in Solution. A noteworthy aspect of sortase-mediated ligation is that the product formed contains an LPRTGGG sequence that becomes itself a potential substrate if a GGG-containing nucleophile is available. During ligation of GGG-EGF to the hydrogel through the LPRTG motif covalently incorporated into the hydrogel, this secondary reaction is minimized by the presence of a substantial stoichiometric excess of LPRTG substrate compared to GGG-EGF. However, this feature can also be exploited to cleave EGF from the hydrogel following ligation, by adding a high concentration (20 mM) of the simple nucleophile GGG, thus driving the reaction toward replacement of EGF by GGG and freeing EGF to the solution phase. Subsequent measurement of EGF in solution via ELISA allows the amount of ligated (vs nonspecifically associated) EGF to be ascertained.

In order to establish reaction conditions that would lead to complete cleavage of the tethered molecules, we used cleavage of LPRTG-fam as a surrogate measure for reaction progress. As noted above, sortase-mediated reactions occurring in the hydrogel require diffusion of the enzyme and substrate into

the hydrogel, a process that likely requires several hours longer than the 1 h reaction time used for ligation. After the initial ligation reaction with 2 or 20 μM GGG-EGF, hydrogels containing 0, 20, 50, 100, or 250 μM of total LPRTG-containing peptide at a ratio of 4% LPRTG-fam and 96% LPRTG were incubated with 20 mM GGG and 200 μM sortase for 48 h at 4 $^{\circ}\text{C}$. Fluorescence was measured as previously described. After this cleavage step (i.e., cleavage of previously tethered GGG-EGF plus LPRTG and LPRTG-fam), the percentage of residual fluorescence dropped to a similar level of 9–12% of initial fluorescence for all LPRTG concentrations and for both GGG-EGF concentrations (Supporting Information Figure 3). Residual fluorescence is likely caused by adsorption of fluorescein to the plastic of the plate or to the hydrogel, or less likely, by inaccessibility of certain LPRTG-fam molecules to cleavage. The relatively similar low level of residual fluorescence observed for all the conditions suggests that previously tethered GGG-EGF was released completely.

Hydrogel supernatants were collected after all reaction steps, including washes following sortase-mediated ligation and after hydrogel cleavage, in order to quantify GGG-EGF with sandwich ELISA. Figure 3 shows the amount of released GGG-EGF as a function of LPRTG concentration for sortase-mediated ligation at 2 and 20 μM GGG-EGF respectively. For both concentrations, the amount of GGG-EGF released during washes after tethering is low, constant, and proportional to the initial concentration of GGG-EGF used (Figure 3). Cleaved

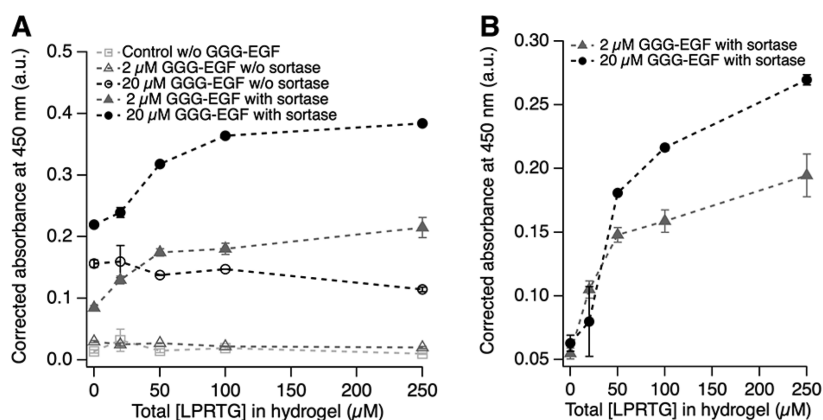


Figure 5. Detection of GGG-EGF on hydrogels as a function of total LPRTG concentration in hydrogel after sortase-mediated ligation of 2 or 20 μM GGG-EGF. (A) GGG-EGF nonspecific binding was tested after incubation of the hydrogels with 2 or 20 μM GGG-EGF in the absence of sortase. (B) Measurements performed without sortase were subtracted from the ones performed in the presence of the enzyme. Error bars represent standard error of the mean ($n = 2$).

EGF increased with LPRTG concentration in the hydrogels and is also proportional to the GGG-EGF in the initial tethering solution, a trend consistent with previous experiments. The sum of the amounts of GGG-EGF left in solution after ligation, extracted in washes, and released by cleavage should be equal to the amount of GGG-EGF present in the original coupling solution. Indeed, this is the case for most of the conditions, as shown in Figure 4. For sortase-mediated ligation at 2 μM GGG-EGF, a maximal amount of 19 pmol of GGG-EGF was found to be cleaved from hydrogels (Figure 3A), equivalent to the 20 pmol obtained from quantification of depletion from the original reaction solution with a sandwich ELISA after the reaction (Figure 1A). Further, this suggests that at this nucleophile concentration, all the consumed GGG-EGF is tethered to the hydrogel with imperceptible nonspecific binding. At 20 μM GGG-EGF initial concentration, a maximal amount of ~ 160 pmol released GGG-EGF was detected, for hydrogels with 250 μM LPRTG-gel (Figure 3B). This means that from the estimated 230 pmol GGG-EGF that disappeared from the initial ligation reaction supernatant (Figure 1B), 160 pmol GGG-EGF was tethered to the hydrogel. Further, ~ 11 pmol was recovered from washes (i.e., presumably partitioned into the aqueous phase of the hydrogel without binding), leaving a balance of 59 pmol that is presumably adsorbed to the hydrogel through noncovalent binding mechanisms (Figure 4B). Noncovalent binding, as estimated by the gap between the sum of the recovered material and that in original ligation solution, seems to be higher and more uniform relative to the LPRTG-gel concentration for the 20 μM GGG-EGF condition (Figure 4B).

3.4. Direct ELISA Reveals Nonlinear Dependence of Surface-Tethered EGF on Substrate Concentration. The results of ELISA analysis on the reaction solutions (Figure 1) and the cleavage products (Figures 3 and 4) provide strong evidence for covalent sortase-mediated ligation of GGG-EGF to PEG hydrogels bearing the LPRTG motif, and suggest that the total amount of EGF tethered to the hydrogel is approximately proportional to the GGG-EGF concentration in the ligation solution. As the objective of tethering EGF to the hydrogels is to stimulate the EGF receptor of cells adhering to the hydrogel on a sustained basis, thus influencing downstream phenotypic responses, we further characterized the EGF tethered to the hydrogel using a direct ELISA approach to quantify the relative

amounts of accessible EGF tethered under the different substrate conditions. Direct ELISA was conducted by incubating hydrogels with an anti-EGF antibody for 2 h, and thus it is expected that antibody binding would be restricted to near the surface of the hydrogels due to the slow diffusion of large proteins in the hydrogels. In this analysis, hydrogels incubated with GGG-EGF in the absence of sortase were included as a control for nonspecific binding.

The relative amounts of EGF detected by direct ELISA, as a function of initial LPRTG-gel concentration, GGG-EGF concentration in the ligation solution, and the presence or absence of sortase, are shown in Figure 5A as the net signal after subtraction of background. In agreement with the analysis presented in Figure 4, nonspecific interaction of GGG-EGF with the hydrogel is undetectable at the 2 μM GGG-EGF concentration, but is significant at the 20 μM GGG-EGF concentration, as evidenced by the detectable signal for hydrogels incubated with GGG-EGF in the absence of sortase, the lack of clear dependence of this signal on LPRTG-gel concentration, and the significant increase in the signal for hydrogels incubated with 20 μM compared to 2 μM GGG-EGF (Figure 5A). Interestingly, ELISA-detectable EGF increases with addition of sortase even in the absence of LPRTG-gel (i.e., comparison of the points along the vertical axis for LPRTG-gel = 0 μM in Figure 5A). The magnitude of this increase is comparable for the 2 μM and 20 μM GGG-EGF conditions. A plausible explanation is that sortase adsorbs nonspecifically to the hydrogels and acts as an affinity capture molecule for GGG-EGF.

In order to compare the relative amounts of EGF tethered to hydrogels via sortase-mediated ligation at different LPRTG-gel concentrations for the 2 and 20 μM GGG-EGF tethering concentrations, the signal associated with incubation in the absence of sortase was subtracted from the total signal for each LPRTG-gel concentration for the 2 μM and 20 μM GGG-EGF cases to normalize the data for nonspecific association of GGG-EGF with the hydrogels. The resulting values are plotted in Figure 5B as a function of LPRTG-gel concentration initially present in the hydrogel. This plot shows that the net ELISA signal above background (including nonspecific adsorption) is roughly comparable at low (<50 μM) LPRTG-gel concentrations for both the 2 μM and 20 μM GGG-EGF substrate conditions and at high (>50 μM) LPRTG-gel concentrations,

the net ELISA signal is about 2-fold greater for the 20 μM GGG-EGF than that for the 2 μM GGG-EGF condition. The difference in this result and the data in Figure 4, where measurement of cleaved EGF indicates a 10-fold greater amount of tethered EGF for the higher substrate concentration, can be interpreted in the context of the complex parallel rate processes occurring during the ligation reaction and during the direct ELISA. During reaction, both the substrate GGG-EGF and the sortase enzyme diffuse into the hydrogel, and the ligation reaction competes with a hydrolysis reaction in a manner such that hydrolysis is more prominent at very low GGG-EGF concentrations. Thus, it is likely that the ratio of tethered EGF for the two different substrate concentrations changes with distance from the surface of the hydrogel, such that the ratio of [tethered EGF, 20 μM] to [tethered EGF, 2 μM] increases away from the surface, due to the increase in prominence of hydrolysis in the 2 μM case.

3.5. DNA Synthesis of Primary Human Hepatocytes and Endometrial Epithelial Cells Is Enhanced by Tethered EGF. The central objective of tethering EGF to PEG hydrogels is to present EGF in a mode that engages and activates the EGF receptor on the basal surface of adherent cells in a sustained fashion, and does so in the context of an environment that mimics key adhesion and mechanical features of extracellular matrix. Epithelial cells polarize EGFR and EGFR ligands to the basolateral surface, and disruption of this polarization is associated with disease states.³⁹ Some ligands for EGFR, such as amphiregulin, are strongly matrix binding and may act as pseudotethered ligands, hence presentation of EGF tethered to PEG hydrogels may mimic a mode of natural stimulation that is deficient in synthetic matrix. We thus focused on assessing the DNA synthesis response of primary epithelial cells to tethered EGF as proof-of-principle for the activity of tethered EGF.

Motivated in part by several studies showing the phenotypic effects of soluble versus tethered EGF on primary hepatocytes,^{40–42} we examined the DNA synthesis response of primary human hepatocytes as a metric of tethered EGF activity. We also investigated the DNA synthesis response of primary human endometrial epithelial cells, which express EGFR and respond to EGF stimulation.⁴³ Expansion and differentiation of primary human endometrial epithelial cells in culture is of great interest for study of endometrial biology and disease. Primary human hepatocytes and primary human endometrial epithelial cells are also representative of epithelial cells that are responsive to EGF but relatively refractory toward in vitro proliferation.

For these proof of principle experiments, we aimed to choose an EGF concentration in a regime that would be expected to maximally stimulate DNA synthesis, as human hepatocytes and endometrial epithelial cells exhibit relatively low rates of DNA synthesis and growth in vitro compared to cell lines and other more highly proliferative primary cell types such as mesenchymal stem cells and keratinocytes. The anticipated low rates of DNA synthesis under optimal conditions is further compounded by the potential for the PEG adhesion environment to further diminish the DNA synthesis response capacity of EGF-stimulated cells: even cells that proliferate robustly in response to EGF culture can exhibit diminished proliferation behaviors when the adhesion context is altered by the presence of relatively poorly adsorptive PEG chains. For example, in the challenging application of PDMS surface modification with PEG-tethered EGF for creation of a confluent corneal epithelial

cell monolayer for a biohybrid cornea replacement, tethered EGF improved cell coverage but the PEG chains impeded complete, homogeneous monolayer formation.⁴⁴ We have also observed that DNA synthesis by primary rat hepatocytes cultured on glass substrates with PEG-tethered EGF is diminished when the PEG brush inhibits attachment of adhesion proteins⁴⁰ and that tethered EGF improves, but does not completely rescue, colony formation by primary human bone marrow stromal cells cultured on PEG-tethered minimal adhesion peptides compared to untreated glass.⁴⁵

Given that hydrogels tethered in the presence of 2 μM GGG-EGF exhibited undetectable (<0.5 pmol) noncovalently bound EGF and presented abundant surface-accessible EGF (Figure 4A), we used a tethering concentration of 2 μM GGG-EGF with hydrogels containing 250 μM LPRTG-gel. This concentration ensures that observed effects were due to tethered EGF and not to noncovalently bound EGF leaching off the hydrogel. Further, based on our previous estimates of tethered ligand density required to stimulate maximal rates of DNA synthesis in hepatocytes,⁴⁰ the ligand density in such hydrogels was anticipated to be sufficient to stimulate maximal EGFR signaling on these epithelial cells. Specifically, at bulk concentrations of 250 μM , the LPRTG motif is spaced 19 nm apart on average, yielding an approximate surface density of about 2500 LPRTG/ μm^2 . EGF is tethered to yield a final average bulk concentration of 2 μM (20 pmol for 10 μL hydrogel, data from Figure 1), resulting in an average spacing between EGF of about 95 nm and average of ~ 100 EGF/ μm^2 . However, the surface appears to be somewhat enriched for tethered EGF than the bulk, thus we can estimate that cells spread to a typical size of 1000 μm^2 are exposed to a lower limit of 100 000 and an upper limit of 2 500 000 molecules of EGF (the latter presumed all surface LPRTG are modified by EGF); i.e., comparable to or in excess of the number of cell surface receptors.

During the hydrogel fabrication process, hydrogels were functionalized with a branched peptide containing the PHSRN and RGD sequences derived from the 9th and 10th type III repeats in fibronectin. The canonical RGD motif from the 10th domain interacts primarily with α_v integrins and induces mesenchymal-like behavior in epithelial cells, while inclusion of the 9th domain PHSRN synergy site fosters interactions through integrin $\alpha_5\beta_1$ and a more physiological phenotypic response.⁴⁶ The *synKRGD* sequence⁴⁷ was incorporated into hydrogels via Michael-type addition through the thiol on the GGGERC segment at the time of initial hydrogel synthesis to give a concentration of 0.5 mM, as we observed in pilot studies that this concentration was sufficient to induce robust attachment of the epithelial cell types used in this study.

Human primary hepatocytes and endometrial epithelial cells were cultured on hydrogels containing 0 or 500 μM *synKRGD* adhesion peptide. Because the presence of the adhesion peptide during cross-linking may influence the structure of the hydrogels, we substituted peptides with scrambled adhesion sequences (see Experimental Section) for the active *synKRGD* to create the control “0 μM *synKRGD*” peptide condition. Cells were either unexposed to EGF, presented with EGF tethered at 2 μM on 250 μM LPRTG-gel hydrogels, or presented with soluble hEGF at a concentration of 20 ng/mL (3.3 nM). The K_D for EGF binding to EGFR is 0.2–1 nM,⁴⁰ and the DNA synthesis response is saturated at soluble EGF values >3 nM.⁴⁸ DNA synthesis was assessed over a period of 24h, 48 h after

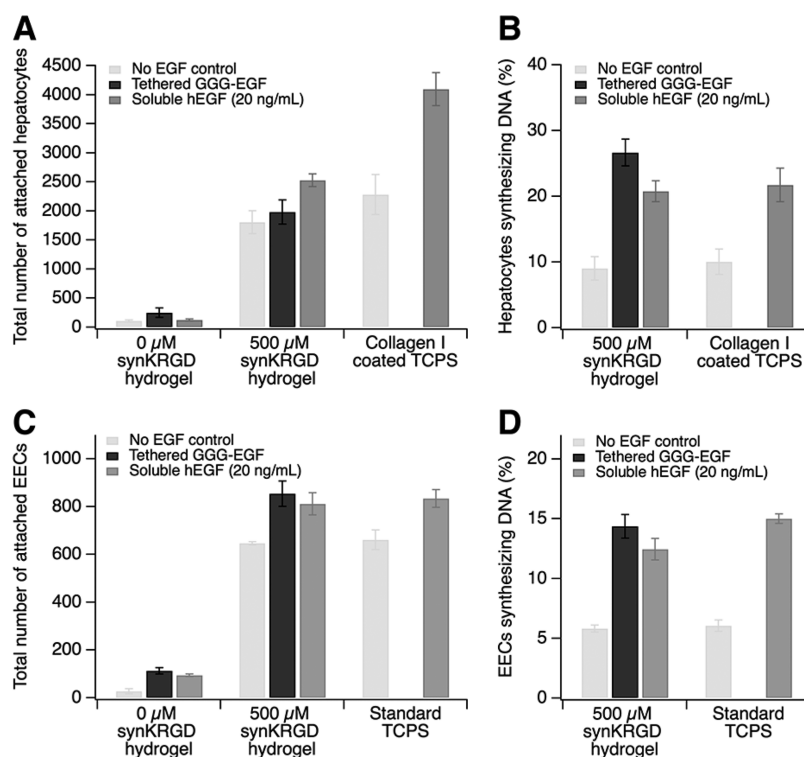


Figure 6. Cell attachment and DNA synthesis of primary human hepatocytes (A, B) and endometrial epithelial cells (C, D). Cells were seeded on hydrogels containing 0 or 500 μ M *synKRGD* and 250 μ M *LPRTG* and on standard culture substrates. After 48 h (A, B) or 40 h in culture (C, D), cells were incubated with 10 μ M EdU for 24 h. Tethered EGF stimulated DNA synthesis compared to unmodified hydrogels or soluble hEGF. Error bars represent standard error of the mean ($n = 3$).

seeding for hepatocytes and 40 h after seeding for endometrial epithelial cells.

In the absence of EGF, while only a small number of cells attached to hydrogels containing 500 μ M scrambled adhesion peptide (“0 μ M *synKRGD*”), cell attachment on 500 μ M active *synKRGD* hydrogels was comparable to positive controls on collagen I or TCPS (Figures 6A and C). Tethered EGF had no effect on hepatocyte attachment, contrary to soluble hEGF, which slightly enhanced attachment on 500 μ M *synKRGD* hydrogels compared to unexposed hydrogels, and which yielded a 1.8 fold increase on collagen I-coated TCP (Figure 6A). In contrast, tethered EGF was as effective as the soluble form in fostering endometrial epithelial cell attachment to 0 and 500 μ M *synKRGD* hydrogels (Figure 6C), and soluble EGF substantially enhanced endometrial epithelial cell attachment on standard TCPS (Figure 6C).

Additionally, soluble EGF considerably increased DNA synthesis of primary hepatocytes compared to control cultures without EGF, from 9 to 21% on 500 μ M *synKRGD* hydrogels and from 10 to 22% on collagen I-coated TCPS (Figure 6B). Stimulation of hepatocyte DNA synthesis by tethered EGF tended to be slightly greater: from 9 to 27% on 500 μ M *synKRGD* hydrogels (Figure 6B). In previous studies of primary rat hepatocytes on EGF tethered to glass⁴⁰ or to self-assembling peptides hydrogels,⁴¹ DNA synthesis rates stimulated by tethered EGF were comparable to or slightly lower than rates stimulated by a saturating amount of soluble EGF on hydrogels or on collagen-coated tissue culture plastic. Human hepatocytes have previously been reported to exhibit a lower rate of DNA synthesis than rat hepatocytes when stimulated by soluble EGF,⁴⁹ hence, the enhanced DNA synthesis response here for EGF tethered to *synKRGD*-modified hydrogels, compared to

stimulation by soluble EGF of hepatocytes on either *synKRGD*-modified hydrogels or collagen I-coated substrates, may reflect a species difference. It may also arise from a more favorable combination of adhesion and EGFR signaling than was achievable in the glass-tethered or peptide-gel-tethered format. Primary endometrial epithelial cells also responded to soluble EGF stimulation by increasing DNA synthesis, from 6 to 12% on 500 μ M *synKRGD* hydrogels and from 6 to 15% on TCPS (Figure 6D). The effect of tethered EGF was comparable to that of soluble EGF in these cells, increasing DNA synthesis from 6 to 14% compared to control hydrogels (Figure 6D).

These results provide encouraging evidence that EGF tethered to functionalized PEG hydrogels via sortase-mediated ligation stimulates biologically relevant responses in primary human epithelial cells. This study thus provides a foundation for future analysis of more detailed phenotypic responses.

4. CONCLUSIONS

In conclusion, we report sortase-mediated ligation of human EGF to preformed PEG hydrogels. This enzymatic approach is not only simple and relatively inexpensive, but it also displays high specificity and modularity, and may be applied to a variety of PEG hydrogels including those cross-linked in situ in the presence of cells. A compendium of analytical approaches were used to show that the amount of grafted growth factor was readily controlled by the amount of *LPRTG* substrate incorporated in hydrogels and the amount of GGG-EGF present in solution. While the sortase enzyme was capable of recognizing and processing *LPRTG* peptides that were incorporated in the hydrogels through Michael-type addition, this method represents a useful tool for specific modification of any type of hydrogel containing the appropriate substrate. The

results of this study underscore the challenges in interpreting reaction data for preformed hydrogels, as the combination of kinetic and diffusion phenomena working in concert during the ligation process may create gradients of enzyme and substrate in the hydrogels; indeed, here, the growth factor appeared to be somewhat enriched at the surface of the hydrogels. This study also underscores the challenges in minimizing noncovalent binding during such tethering processes, as even with the well-established antifouling properties of the PEG polymer as well as enzymatic specificity, significant nonspecific binding appeared to occur for high GGG-EGF coupling concentration. One of the more novel aspects of the study is the exploitation of sortase as a cleavage enzyme. Efficient and near complete release of tethered EGF from the hydrogels through sortase-catalyzed reaction of the LPRTG-containing tether with soluble GGG allows near absolute quantification of the previous tethered protein through well-established solution phase techniques. Finally, biological activity of the tethered EGF was confirmed by the phenotypic response of human primary epithelial cells. The well-studied hepatocyte cell system showed a more robust enhancement to tethered EGF (compared to soluble EGF) than had been observed in previously published studies, while preliminary experiments with endometrial epithelial cells constituted a first example of endometrial epithelial cell culture in the presence of a tethered growth factor. This demonstration of sortase-mediated ligation of bioactive molecules to hydrogels will likely contribute to the development of improved culture systems for in vitro models, by expanding the repertoire of bioactive molecules that can be covalently attached to PEG to include large peptides and proteins.

■ ASSOCIATED CONTENT

● Supporting Information

Protocol for primary human epithelial cells isolation and purification; (S1) standard curve for conversion of hydrogel fluorescence arbitrary units to amount of LPRTG in pmol; (S2) photobleaching controls; (S3) percentage of retained hydrogel fluorescence as a function of LPRTG in hydrogel after sortase-mediated hydrogel cleavage; (S4) micrographs of hepatocyte DNA synthesis on tethered EGF hydrogels; (S5) micrographs of endometrial epithelial cell DNA synthesis on tethered EGF hydrogels. The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acs.biomac.5b00549.

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Author Contributions

[#]These authors contributed equally. E.C. designed and conducted experiments, analyzed data and wrote the manuscript. K.R. conceived and designed experiments, produced recombinant proteins and wrote the manuscript. C.C.A. conceived and designed experiments and contributed to protocol development. C.D.C. conceived experiments with endometrial cells, designed protocols, and interpreted data. C.K. conceived experiments and developed recombinant

protein expression protocols. A.T.K. conceived sortase kinetic experiments, developed protocols, and produced recombinant proteins. B.I. conceived and designed experiments and cosupervised the project. L.G.G. conceived and cosupervised the project and wrote the manuscript. All authors edited the manuscript.

Notes

The authors declare no competing financial interest.

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