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Light-Regulated Pro-Angiogenic Engineered Living Materials

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Regenerative medicine aims to restore damaged cells, tissues, and organs, for which growth factors are vital to stimulate regenerative cellular transformations. Major advances have been made in growth factor engineering and delivery like the development of robust peptidomimetics and controlled release matrices. However, their clinical applicability remains limited due to their poor stability in the body and need for careful regulation of their local concentration to avoid unwanted side-effects. In this study, a strategy to overcome these limitations is explored using engineered living materials (ELMs), which contain live microorganisms that can be programmed with stimuli-responsive functionalities. Specifically, the development of an ELM that releases a pro-angiogenic protein in a light-regulated manner is described. This is achieved by optogenetically engineering bacteria to synthesize and secrete a vascular endothelial growth factor peptidomimetic (QK) linked to a collagen-binding domain. The bacteria are securely encapsulated in bilayer hydrogel constructs that support bacterial functionality but prevent their escape from the ELM. In situ control over the release profiles of the pro-angiogenic protein using light is demonstrated. Finally, it is shown that the released protein is able to bind collagen and promote angiogenic network formation among vascular endothelial cells, indicating the regenerative potential of these ELMs.

1. Introduction

Regenerative medicine is a rapidly developing research field focused on accelerating the repair of damaged cells, tissues, and organs to restore normal function and circumvent the need for transplantation.[1] In healing processes, growth factors play a major role in stimulating cells and orchestrate transformations in them necessary for regeneration. They are proteins that are secreted by cells, can bind to the surrounding extracellular matrix (ECM) and interact with the receptors on the surfaces of other cells. These receptor-specific interactions trigger signal transduction pathways that promote events such as cell growth, proliferation, differentiation, cell migration, adhesion and survival.[2–4] For instance, vascular endothelial growth factor (VEGF), and platelet derived growth factor (PDGF) strongly stimulate blood vessel formation by activating integrin, NOTCH and WNT signaling for endothelial cell differentiation and increasing the permeability of endothelial cell layers.[5] Thus, growth factors are powerful tools in regenerative medicine. However, due to their potency in driving cellular transformations, their concentration and localization need to be carefully regulated. Failing to do so can cause overstimulation or off-site differentiation of cells, leading to necrosis or tumorigenesis.[6,7] This is exemplified by the long list of severe side-effects plaguing the few clinically approved growth factor-based medical products like PDGF-based Regnarex (side effects include increased risk of systemic cancer, skin rash and cellulitis).[8] Apart from unwanted side effects, growth factors are also complex bulky proteins often with poor stability, making them expensive to produce, purify, store, and deliver at effective doses[7,9] These technical challenges have thus far limited the clinical applicability of growth factor-based therapies despite over 3 decades of research demonstrating their potential.[10]

These issues are being addressed through 2 major strategies – i) use of drug release systems ensuring localized and controlled supply of the growth factor over time and ii) development of short and robust variants mimicking a desired function of the growth factors. This is particularly exemplified by advances in VEGF-based therapies. This growth factor plays a prominent role in stimulating the sprouting of new blood vessels from existing ones to supply oxygen to tissues suffering from hypoxia.[11,12] VEGF based therapies are being explored for peripheral vascular disease (PVD) that causes severe blockage of arteries of the lower extremities, leads to high limb amputation and mortality rates and generally results in poor prognosis.[13] In animal models of PVD, increasing VEGF levels to enhance collateral flow around blocked blood vessel has been achieved by intramuscular injection and vascular infusion of an adenoviral vector encoding VEGF.[14] Overexpression or overstimulation by VEGF in laboratory animals has been shown to lead to a variety of side-effects like formation of leaky vessels, metabolic dysfunction, transient edema, increase in atherosclerotic plaques and

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uncontrolled neoangiongenesis (increased risk of cancer).[15,16] Controlled release of VEGF through implantable devices is challenged by the protein's complexity and low serum stability, leading to high costs, low packing densities and poor control over its release.[17] Thus, despite its promise, clinical application of VEGF for PVD has been severely limited by these issues.[18,19]

A cheaper, shorter and more robust alternative to VEGF under investigation is the peptidomimetic, QK (KLTWQELYQLKYKGI), that has been shown to promote formation and organization of capillaries both in vitro[20] and in vivo.[21] The peptide has higher potency to drive angiogenic morphogenesis in endothelial cells when immobilized on hydrogel matrices compared to its soluble form.[20,22] This mimics the activity of VEGF when immobilized to heparan sulphate in the ECM. Furthermore, such immobilization ensures that the pro-angiogenic activity is spatially confined. Recently, sustained release of QK from bone graft materials for up to 6 days was shown to induce angiogenic differentiation in vascular endothelial cells.[23] Thus, QK has shown great promise for promoting blood vessel formation similar to VEGF. For effective treatment of PVD with wide variabilities in disease profiles and progression among patients, it is desirable to develop a versatile strategy to deliver QK where and when it is required in a cost-effective manner.

Recently, a unique strategy to achieve low-cost and in situ controllable drug release has been explored in the form of engineered living materials (ELMs). In ELMs, living cells are combined with non-living materials to create composites with programmable and life-like capabilities.[24] ELMs for drug delivery have been made encapsulating within hydrogels, bacteria genetically engineered to produce and release drugs on demand, remotely controlled by external stimuli like chemical inducers or light. The bacteria in these ELMs are expected to thrive on nutrients available at the disease site and can be triggered to produce and secrete drugs at desired doses when needed. Using hydrogels made of agarose, Pluronic F127 or collagen and bacteria like *E. coli*, *L. lactis*, and *B. subtilis*, therapeutic ELMs have been made in the form of discs, films, patches and 3D printed structures to suit different therapeutic needs.[25–29] While most studies report the release of anti-microbial drugs from ELMs, one set of studies from the Salmeron-Sanchez lab demonstrates the release of BMP, controlled by a peptide-inducer, nisin.[27,30]

In this study, an ELM capable of in situ tunable release of a collagen-binding QK fusion protein is described. For the first time, we demonstrate light controlled release of an active growth factor peptidomimetic from ELMs fabricated in a secure bilayer encapsulation format that prevents bacterial escape/outgrowth.[31] We show that QK secretion can be sustained in a physiologically relevant range of concentrations (5 – 25 nM) for at least 9 days in a light-controlled manner and that it can induce angiogenic differentiation in endothelial cells.

2. Results and Discussion

2.1. Light-Responsive Production, Secretion, and Bioactivity of QK

For this study, *ClearColi*, an endotoxin-free variant of *E. coli*, was engineered to light-responsively synthesize and secrete a QK-bearing fusion protein (YCQ) as shown in **Figure 1**A.

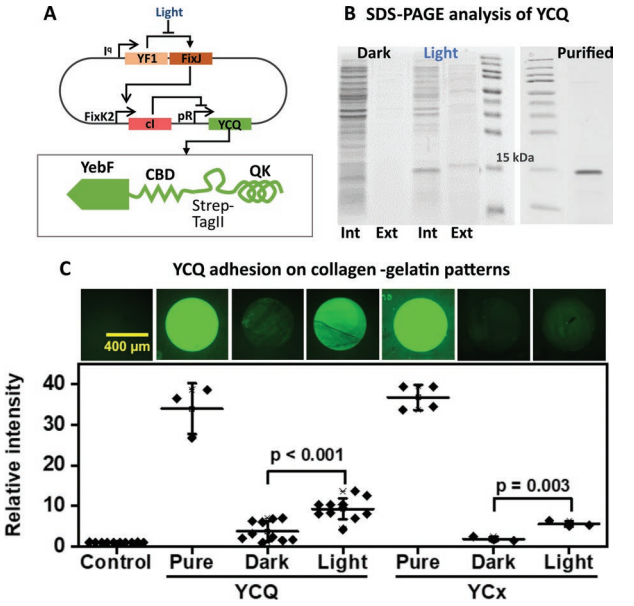

Figure 1. Design and activity of YCQ: A). Graphical representation of the pDawn-based optogenetic circuit for light-responsive production of YCQ along with scheme highlighting the different domains of the fusion protein. B). SDS-PAGE images of the intracellular (Int) and extracellular (Ext) fractions of light-regulated production and secretion of YCQ from engineered bacterial cultures, along with purified YCQ for comparison. C). Fluorescence microscopy images and pattern intensity quantification of the Col-Gel photopatterning assay to verify the ability of YCQ and YCx to adhere to collagen. Staining was done with an Anti-YebF primary antibody and a fluorescently labelled anti rabbit AF-488 secondary antibody. In the Control condition, the patterned Col-Gel surfaces were incubated with supernatants from unmodified *ClearColi* cultures exposed to light. The symbols in the graph represents the intensity of individual patterned spots from 2 independent experiments (N = 2). The intensities have been normalized to the mean of the control sample. Two sample t-tests were performed between YCQ dark/light and YCx dark/light data sets and p values are given above indicating that their differences are statistically significant.

This YCQ fusion protein contained i) YebF, a carrier protein aiding secretion from *E. coli*,[32,33] ii) a collagen-binding domain (CBD, sequence – WREPSFMVLS)[34] to facilitate immobilization of QK to the extracellular matrix, iii) a Strep-TagII peptide (WSHPQFEK) for purification and staining[35] and iv) the QK peptide. Notably, this design allowed for YebF to be at the N-terminal and QK at the C-terminal, which is necessary for their functionality. As a negative control, a similar fusion protein, YCx, was designed bearing a scrambled-QK peptide (GLKEQSPRKHRLG) at the C-terminal, previously shown to be inactive.[36] The gene corresponding to the 18.9 kDa YCQ or YCx protein was inserted into the multiple cloning site of an optogenetic plasmid, pDawn,[37] to achieve light responsive production and secretion. This was confirmed through SDS-PAGE by running the extracellular and cellular fractions from bacterial cultures grown either in white light or in the dark (Figure 1B). In the light exposed cultures, a band ≈15 kDa was observed in both the cellular and extracellular fractions as well as after purification. This lower molecular weight was expected as a consequence of cleavage of the 2.2 kDa N-terminal signal peptide of YebF during its secretion into the periplasm through

the sec pathway. Western Blot analysis confirmed that this band contained the StrepII-tag (Figure S1, Supporting information). MALDI-TOF mass-spec analysis of the protein purified from the cellular fraction revealed a clear peak at 16.7 kDa confirming cleavage of the signal peptide suggesting that most of the intracellular protein resided in the periplasm (Figure S1, Supporting Information). These results confirmed that YCQ can be light-responsively produced and secreted from *ClearColi*. We proceeded to test the capability of the protein to bind collagen and trigger angiogenic differentiation in vascular endothelial cells. For these assays, we used a collagen-gelatin mixture (Col-Gel) in a 3:1 ratio by weight to form a thin film gel that has been reported to mimic the extracellular matrix in wounds where collagen is often degraded to remodel the tissue.[38] Binding of YCQ to collagen was verified by incubating the protein on a photo-patterned substrate of Col-Gel, after which it was immunostained using a primary antibody specific to YebF. Both purified and secreted YCQ and YCx were observed to preferentially adhere to Col-Gel patterned regions (Figure 1C). Analysis of the stained patterns revealed that the intensities of the patterned spots associated with proteins secreted in the presence of light were on average 2.5 fold higher than those cultured in the dark. However, the intensities of spots from light-state protein secretions were on average 3 to 7 fold lower than spots made with 10 nM purified proteins, suggesting that secretion from the overnight cultures was relatively low.

Next, we tested the ability of YCQ-bound Col-Gel matrices to induce angiogenic differentiation in Human Umbilical Vein Endothelial Cell (HUVEC) cultures using a network formation assay.[38] In this assay, Col-Gel matrices mimic disrupted collagen networks found in wounds and are used as model substrates to study angiogenesis-related events. A tell-tale sign of angiogenic differentiation in endothelial cells in 2D is the transformation of their culture from a monolayer into networks with larges gaps in between. Col-Gel matrices have been used to show that such network formation with HUVECs occurs in the presence of collagen-bound QK.[38] This assay can be used to identify genes and pathways that are involved in the promotion or inhibition of angiogenesis in a rapid, reproducible, and quantitative manner. In our study, cultures of bacteria that light-responsively secrete YCQ and YCx were grown in dark until they reached OD_{600nm} of 0.5 and then under light or dark conditions overnight. Cell-free supernatants (SN) from these cultures were then collected, filter sterilized and incubated on Col-Gel surfaces for the proteins to bind with the matrix (Figure S2, Supporting Information). As positive controls, 10 nM of purified YCQ and 10 ng μL^{-1} VEGF[39] were similarly incubated on the Col-Gel surfaces. When HUVECs were seeded on these Col-Gel surfaces, it was observed that those incubated with the negative control conditions of YCx dark, YCx light- and YCQ dark-state supernatants, cells retained monolayer cobblestone morphology over the same period (**Figure 2**). Additional control substrates incubated with unmodified ClearColi supernatants, pure YCx 10 nM, and pure YCQ 1 nM were also found to yield similar results (Figure S3, Supporting Information). On the other hand, those modified with light-activated YCQ supernatants, pure YCQ and VEGF promoted the cells to undergo a modest degree of network formation in 16 h. A much stronger and quicker (3 h) network formation was observed when the Col-Gel matrices modified with a higher YCQ concentration of 100 nM (Figure S4, Supporting Information), similar to what has been observed by others.[39] This indicates that YCQ immobilized on Col-Gel matrices is able to promote network formation in HUVEC cultures but only to a modest degree at the concentrations released by the bacterial cultures. Immunostaining analysis revealed a moderate upregulation of PECAM-1 in cells that formed networks compared to the those that formed a monolayer (Figure 2). These features correlate with the initiation of the angiogenesis process, in line with previous reports.[40,41] All together, these results confirmed that YCQ performed the secretion, collagen-binding and angiogenesis functions it was designed for.

2.2. Engineered Living Material Design and Fabrication

To fabricate our ELMs, we encapsulated the bacteria in acrylate modified Pluronic F127 hydrogels, commonly used in medically relevant ELM reports.[31,42] ensure bacterial survival but prevent their escape to the surroundings, 30% wv^{-1} Pluronic F127-diacrylate (PluDA) hydrogels were fabricated in a bilayer format, with bacteria encapsulated in a core layer and surrounded by a protective shell layer. To make the ELMs compatible for microscopy imaging and biochemical assays, they were made in the form of discs bonded to acryloxypropyl silane coated glass cover slips. These constructs were fabricated in a stepwise manner, wherein the bacterial gel was first formed on the glass then coated with the shell hydrogel (**Figure 3**A,B). Chemical cross-linking of the acrylate groups in the gels was done using a photo-initiator (Irgacure 2959) that could be activated using 365 nm light, which is orthogonal to the blue light required to activate YCQ production in the bacteria. The duration (1 min) and power (6 mW cm^{-2}) of the 365 nm irradiation was selected based on a previous report [31] that identified conditions, which ensured complete cross-linking, while minimally affecting the bacteria. To ensure that the ELMs had well-defined and reproducible dimensions, ring-shaped PDMS moulds (Figure S5, Supporting Information) were used to form the core (dia 6 mm, h 0.8 mm) and shell (dia 13 mm, h 1.2 mm). In this format, the bacterial gel has a volume of almost 23 µL with an initial bacterial density of 0.1 OD_{600nm}, resulting in an initial population of $\approx 10^6$ bacterial cells (1 $OD_{600nm} = 8 \times 10^8$ cfu mL^{-1} for *E. coli*). To test the performance of the ELMs in conditions suitable for mammalian cells, we decided to incubate them in HUVEC-compliant M199 medium. However, the bacteria were found to not grow in this medium, even in liquid culture. To support bacterial growth, the increasing glucose and sodium chloride concentrations were tested and the final concentrations in the medium were increased from 0.1 to 0.5% w/v for glucose and from 0.68 to 1.56% w/v for sodium chloride (Figure S5, Supporting Information). Incubation of the ELMs in this medium at 37 °C resulted in the entrapped bacteria growing from single cells to spherical colonies in 24 h (Figure 3C), in line with our previous reports studying bacterial growth in PluDA hydrogel constucts.[31,40] No leakage or outgrowth of the bacteria into the medium was observed for at least 15 days, verified by brightfield microscopy (Figure S6, Supporting Information). Bacterial colonies producing YCQ were stained with fluorescently labelled

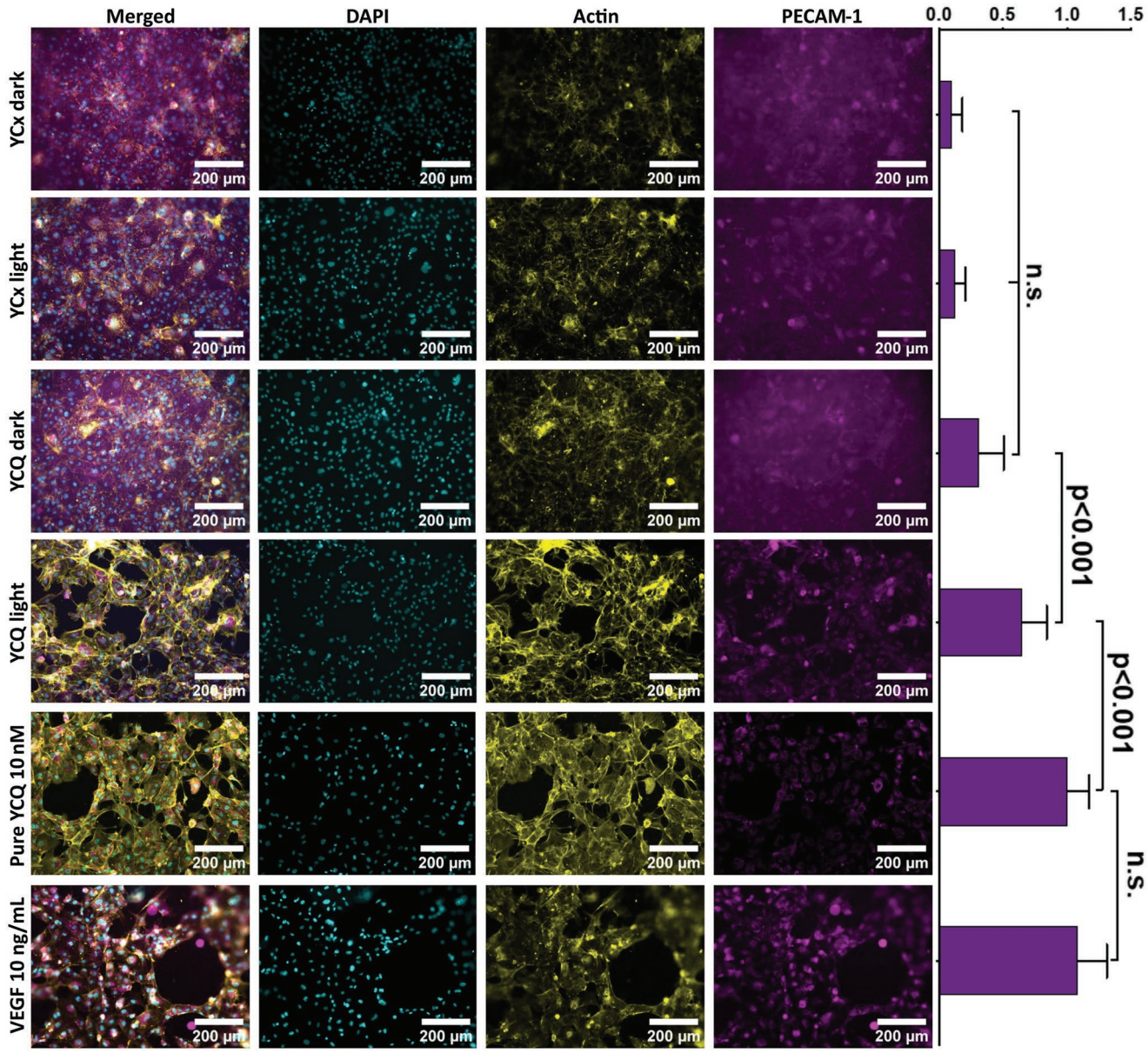

Figure 2. Fluorescence microscopy images of HUVECs grown for 16 h on Col-Gel surfaces incubated with supernatants of different bacterial cultures grown with or without light and with purified YCQ and VEGF as positive controls. The cells were stained for PECAM-1 (magenta), actin (yellow), and DNA (cyan). Brightness and contrast of each channel has been adjusted for optimal visualization. The graph represents intracellular PECAM-1 signal intensity after 16 h of seeding HUVECs from experimental duplicates. Bar lengths represent mean values and whiskers represent standard deviation from at least 2 experimental replicates and 2 images in each replicate. The data was normalized to the mean value of Pure YCQ 10 nM. ANOVA was performed to test for statistical significance of differences between different samples (n.s. = no significance).

streptavidin and could be observed as diffuse patches by fluorescence microscopy. These patches represent staining of high concentrations of secreted YCQ around the bacterial colonies (Figure 3D). In comparison, no such patches were observed in gels containing unmodified bacteria (Control).

2.3. Light-Regulated Release of Active YCQ from ELMs

Next, we tested the capability of switching and tuning YCQ release from the ELMs using light. After preparation, the ELMs were incubated in dark at 28°C for 16 h, allowing the bacteria to grow into colonies before inducing them with light. Induction of protein expression was maintained by pulsed irradiation with blue light (2 s on, 1 min off, 450 nm wavelength, 200 $\mu W.cm^{-2}$ power) (**Figure 4**A). The release of YCQ from these bilayer ELM constructs was quantified using a sandwich ELISA assay with a Streptactin-coated plate and a primary antibody specific to YebF (Figure S7, Supporting Information). To test the possibility of switching YCQ release ON and OFF, one set of constructs were exposed to blue light for 3 days and another set was left in the dark for the same time at

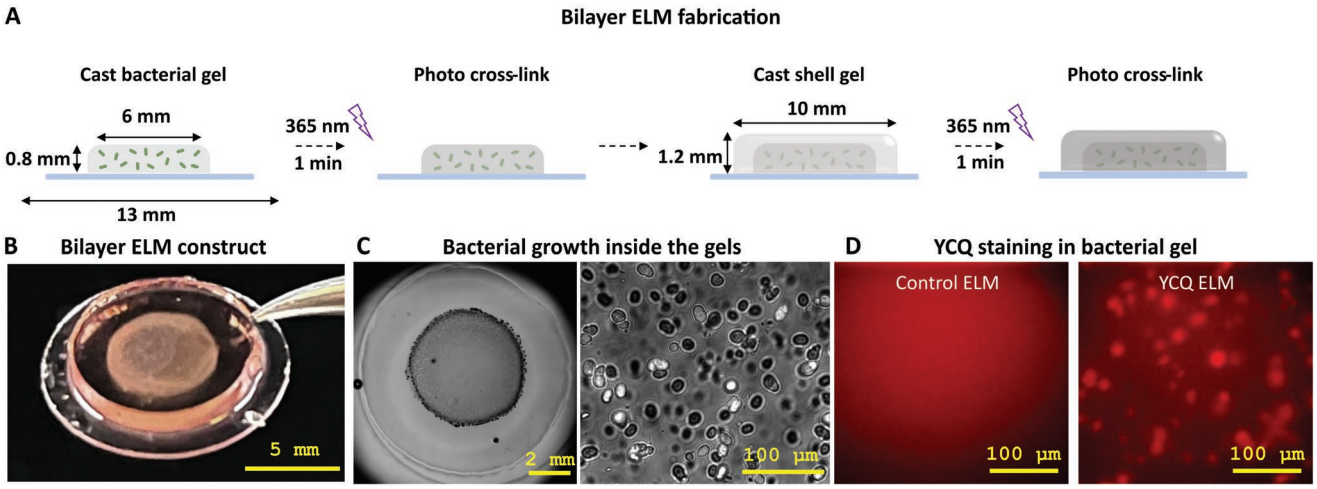

Figure 3. Engineered living material design and fabrication. A). Graphical representation of manual fabrication of ELM. The scheme depicts the steps involved in the making of the bilayer ELMs, along with relevant dimensions of the core and shell layers. B). Macroscopic image of the ELM held by a tweezer. C). Brightfield microscopy stitched image of the ELM with bacteria grown in the core layer (left) along with a magnified image of 6 day grown bacterial colonies. D). Qdot streptavidin staining of YCQ-producing ELM. *ClearColi* YCQ colonies are stained red indicating YCQ production within ELM; control ELM has unmodified *ClearColi*.

37 °C (Figure 4B). On day 3, YCQ quantification revealed that the amount released in light (≈1.4 nM) was only slightly higher than what was released in the dark (≈0.6 nM). The low level of YCQ release in light might be due to bacterial growth continuing to occur in the gel during this period or partial trapping of the protein in the gel, as previously reported by us.[31,43] After this, the ELMs that had been exposed to light pulses were placed in the dark and vice versa for another 3 days. At this point, a major difference in production levels were seen between ELMs exposed to light pulses (≈4 nM) compared to those left in the dark (~0.7 nM). Once again, the irradiation conditions were switched for these ELM samples for another 3 days and it was clearly seen that those exposed to light pulses released considerably more YCQ in light (≈3.8 nM) compared to those in dark (≈1.1 nM). Furthermore, these results clearly demonstrated that the ELMs could be switched from OFF to ON or ON to OFF state using light. To test the sensitivity of YCQ release to different light intensities, ELMs were induced with pulsed blue light (2 s on, 1 min off) with intensities of 0, 80, 105, or 125 µW cm^{-2} for 6 days (Figure 4C). The media surrounding ELMs was collected on day 6 from Control (unmodified bacteria) and YCQ-releasing ELMs and was analyzed with ELISA (Materials and Methods Section: E, Figure S4, Supporting Information). ELMs kept in dark (0 µW cm^{-2}) released <1 nM YCQ, those exposed to 80 µW cm^{-2} released 8–15 nM, 105 µW cm^{-2} released 14–19 nM, and 125 µW cm^{-2} released 11–20 nM of YCQ (figure 4C). Statistical analysis (ANOVA) revealed significant differences in YCQ release between the 0, 80, and 105 µW cm^{-2} conditions. This suggests that the ELMs are highly responsive to light within this range and precise

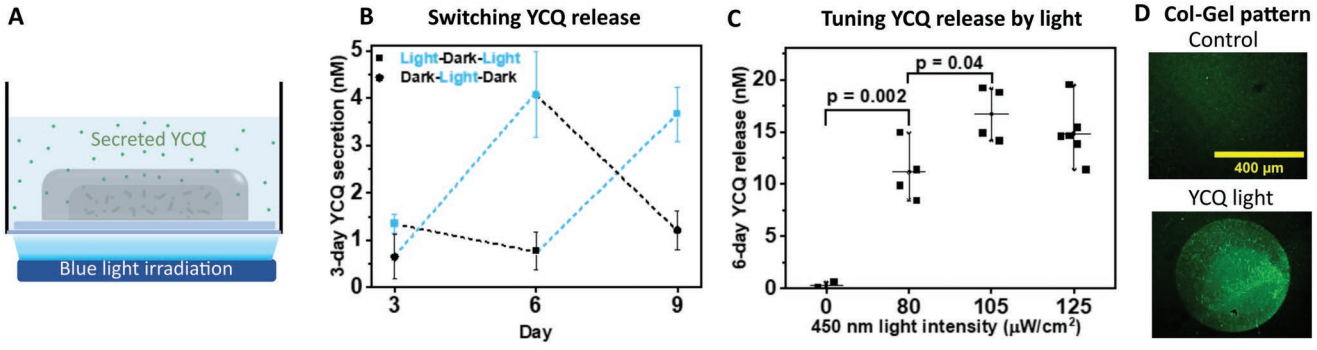

Figure 4. Light regulation of YCQ release from ELMs: A). Graphical representation of the ELM setup including blue light irradiation from below. The ELM constructs were placed in 24 well-plate wells containing 300 uL of optimized M199 medium on top of an optowell device for pulsed irradiation at 450 nm (2 s On, 1 min OFF) B). Light switchable control over YCQ release from ELMs. The lines in blue represent durations when samples were placed in light and the lines in black represent durations when samples were placed in the dark. The first blue and black symbols at 3 days were placed in light and dark, respectively till the measurement was made. Symbols represent means values from individual ELM samples and whiskers represent standard deviation (N = 2, n = 2) C). Tuneable release of YCQ by varying intensities of pulsed blue light irradiation from ELMs. Intensities of 0, 80, 105, and 125 µW cm^{-2} were used. Symbols represent individual ELM samples; horizontal bars represent means and whiskers represent standard deviation. D). Fluorescence microscopy images of the Col-Gel photopatterning assay to verify the ability of ELM-secreted YCQ to adhere to collagen. Control refers to Col-Gel surfaces incubated with supernatants from unmodified *ClearColi* ELMs exposed to light.

control over release concentrations by light intensities could be achieved when the variance in the output from different constructs is minimized. We expect that experimental variabilities can be minimized by switching to an automated ELM fabrication method (e.g., 3D printing[28]) and increasing the rate of YCQ secretion, which has been discussed in the conclusions section. Furthermore, the increased variance and lower mean in the 125 μW cm^{-2} condition compared to 105 μW cm^{-2} suggests a possible detrimental effect of blue light on bacterial performance beyond this intensity. This is further supported by the low release concentrations observed in Figure 4B where a light intensity of 200 μW cm^{-2} was used. While further optimization is possible, these results are evidence that YCQ release profiles can be remotely controlled in situ using light. Finally, using the Col-Gel patterning assay, we confirm that YCQ released from the light-irradiated ELM constructs are able to bind to these matrices (Figure 4D).

2.4. Angiogenesis Promoting Capabilities of Light-Responsively Secreted YCQ

Next, we tested the bioactivity of YCQ released from the ELMs after 6 days of pulsed blue light irradiation at 105 μw cm^{-2} and concentration >10 nM. As controls, ELMs containing unmodified bacteria and YCx-producing bacteria were also made. The supernatants from the ELMs were incubated with Col-Gel surfaces after which the network formation assay using HUVECs was performed. On 3 out of 4 Col-Gel surfaces incubated with light-exposed YCQ-releasing ELMs, HUVECs were seen to form networks in 16 h, while this did not occur in all other conditions, where HUVECs were seen to grow as monolayers with cobblestone morphology (**Figure 5**; Figure S8, Supporting Information). Notably, this cobblestone morphology was also seen on Col-Gel surfaces incubated with dark-state YCQ-releasing ELMs (<1 nM YCQ), indicating that the light-induced fold change of

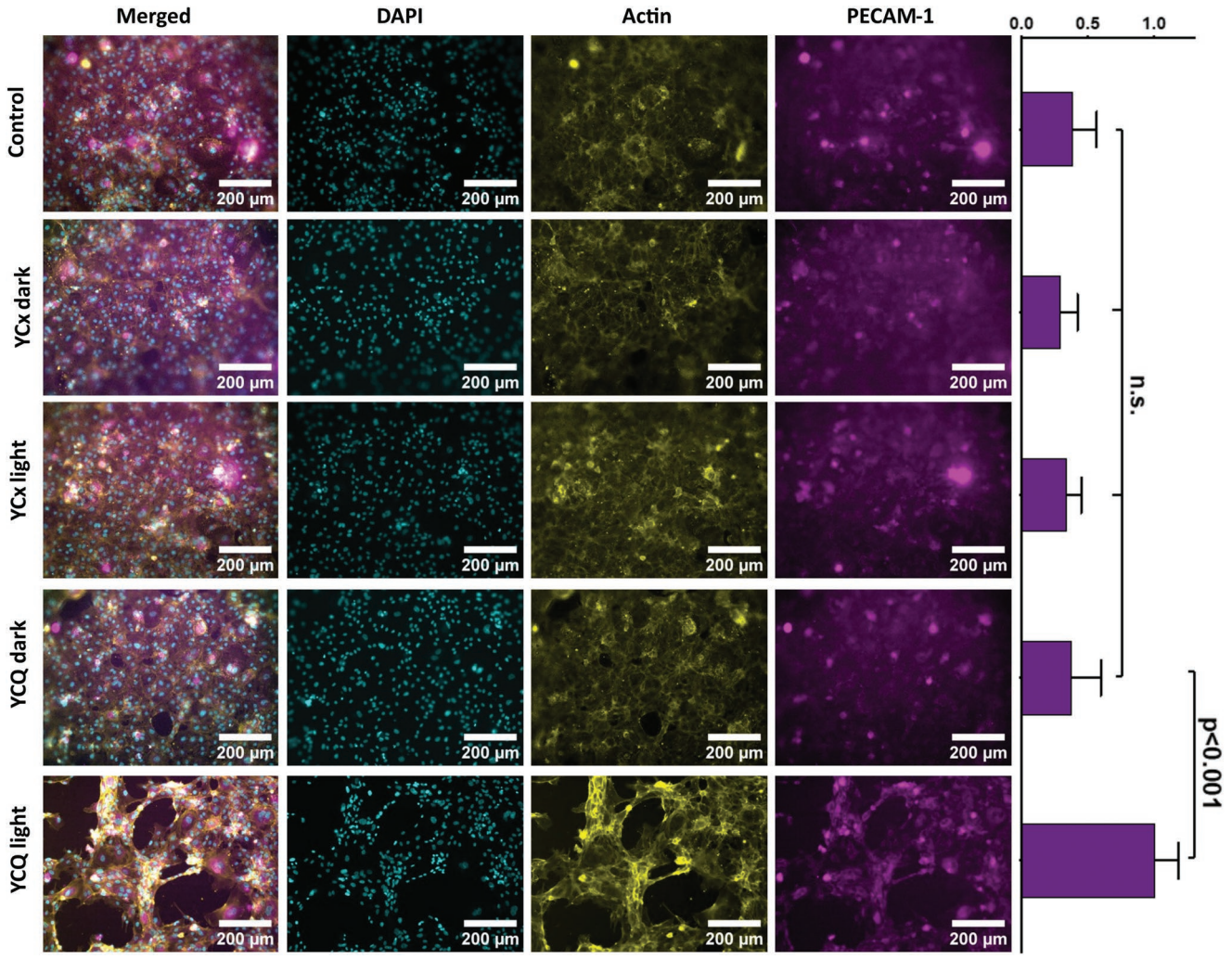

Figure 5. Epifluorescence images of HUVECs cultured for 16 h on Col-Gel surfaces that were incubated in supernatants from ELMs containing unmodified *ClearColi* (Control), YCx-expressing and YCQ-expressing bacteria kept in dark or exposed to blue light pulses (2 s ON, 1 min OFF; 105 μW cm^{-2}) for 6 days. The cells were stained for PECAM-1 (magenta), actin (yellow), and DNA (cyan). Brightness and contrast of each channel has been adjusted for optimal visualization. The graph represents intracellular PECAM-1 signal intensity after 16 h of seeding HUVECs from experimental triplicates (supernatant derived from 3 individual ELM constructs). Bar lengths represent mean values and whiskers represent standard deviation. The data was normalized to the mean value of YCQ light.

YCQ release was sufficient to elicit different responses from the HUVECs. Based on imaging of immunostained cells, the HUVECs that underwent network formation showed an increase in the expression PECAM-1 when compared to the control samples (Figure 5). While there are a few small regions in the control conditions like YCQ dark where the PECAM-1 fluorescence intensity is comparable to that in the YCQ light condition, the quantification reveals that the differences between these conditions are significant. This is a clear indication that the cells were undergoing angiogenic differentiation, in agreement with the results from previous reports.[40,44]

3. Conclusion

This study demonstrates the possibility to develop remote-controlled ELMs capable of promoting angiogenic differentiation of vascular endothelial cell cultures in a light regulated manner. This was realized by optogenetically engineering bacteria to produce and secrete a collagen-binding VEGF peptidomimetic fusion protein, YCQ, in response to blue light and securely encapsulating this strain in bilayer hydrogel constructs. With this ELM, we were able to demonstrate the possibility to repeatedly switch ON and OFF the release of YCQ up to 9 days and vary the amount released with different light intensities within the range of 1 – 20 nM in 6 days. The released YCQ was able to adhere to Col-Gel matrices that simulate the ECM of healing wounds and promote network formation in HUVEC cultures, indicating the cells underwent angiogenic differentiation. Notably, the negligible leaky expression of YCQ in dark was insufficient to promote such angiogenic differentiation, thereby validating the capability of controlling the ELM within relevant ranges of YCQ's functionality.

However, it is desirable to improve the precision and rate of protein released from these ELMs. Variabilities in the system are attributed to experimental errors that can arise during manual fabrication of the ELM constructs despite our strategies to minimize them. This can be further minimized using automated fabrication methods like 3D printing,[28] which will be explored in future studies. The low levels of protein release are expected to be caused by 2 factors – i) *E. coli* is a poor secretor of proteins[45] and ii) the PluDA hydrogels have a relatively high polymer content of 30%, possibly resulting in slow protein diffusion.[46] In future studies, both these aspects will be addressed by i) using gram-positive probiotic bacteria like *B. subtilis* or lactic acid bacteria, which are prolific at protein secretion[47] although their genetic toolboxes are poorly equipped compared to *E. coli* and ii) constructing bilayer hydrogels with lower polymer concentrations of PluDA or introducing soluble fillers that increase matrix porosity.[48] Despite these limitations, the results in this study clearly highlight the unique advantages that ELMs can offer for regenerative therapies in terms of long-term release of therapeutic proteins and in situ control over release profiles.

4. Experimental Section

Construction of Plasmids and Bacterial Strains: The YebF-CBD-strep-QK fragment (DNA sequence in Supporting information) was ordered as gBlock from Eurofins Genomics, and inserted into the plasmid, pDawn (pDawn was a gift from Andreas Moeglich – Addgene plasmid# 43 796; http://n2t.net/addgene:43796; RRID:Addgene_43 796) using NEBuilder® HiFi Assembly cloning kit (NEB, E5520S) using following primers-

pDawn Fwd 5′-ataaaagcttAACAAAGCCCGAAAGGAAG-3′,

pDawn Rev 5′-ctcttttttttCATGGTATATCTCCTTCTTAAAGTTAAAC-3′,

YebF-CBD-QK Fwd-5′-atataccatgAAAAAAAGAGGGGCGTTTTTAG-3′

YebF-CBD-QK Rev-5′-gggctttgttAAGCTTTTATTTCAGGGTC-3′.

This yielded the pDawn-YCQ plasmid which was then transformed into *ClearColi* BL21(DE3) electrocompetent cells as specified by the provider (BioCat 60810-1-LU). The recombinant pDawn-YCQ was used as a template to construct the pDawn YebF-CBD-Scrambled QK (YCx) mutant (DNA sequence in supporting information) using the NEBuilder® HiFi DNA Assembly Cloning Kit with the following primers:

pDawn Fwd 5′-CTAGCATAACCCCTTGGG-3′,

pDawn Rev 5′-CTAGTAGAGAGCGTTCAC C-3′,

YebF-CBD-Scrambled QK Fwd-5′-cggtgaacgctctctactagAGTCACACT GGCTCACCTTC-3′

YebF-CBD-QK Scrambled Rev-5′-gccccaaggggttatgctagTTATTGCTCA GCGGTGGC-3′.

pDawn-YCx was then transformed in *ClearColi* BL21(DE3) electrocompetent cells as specified by the provider. For storage at −80 °C, glycerol stocks with 30% of glycerol for both clones were made from bacterial cultures grown overnight at 37 °C, 250 rpm in the dark from single colonies.

Bacterial culture for protein purification and secretion: *ClearColi* BL21(DE3) pDawn-YCQ or pDawn-YCx cultures of 250 mL were grown in dark for 37 °C, 250 rpm in LB Miller medium supplemented with 50 µg mL^{-1} of Kanamycin to an OD_{600nm} between 0.4 and 0.8. The culture was then induced for 12 h by exposing it to white light for production of YCQ and YCx at 37 °C, 250 rpm

Bacterial cultures for ELMs: *ClearColi* BL21(DE3) cultures were grown for 16 h at 37 °C, 250 rpm in LB Miller medium supplemented with 50 µg mL^{-1} of Kanamycin to an OD_{600nm} ≈0.8. All procedures were performed either in the dark or under orange light.

Purification of YCQ and YCx: *ClearColi* BL21(DE3) with pDawn-YCQ or pDawn YCQx were cultured in LB Miller medium supplemented with Kanamycin. YCQ/YCx production was induced with white light for 16 h. For harvesting the cells, cultures were transferred to 50 mL flacon tubes and centrifuged with an Avanti J-26S XP centrifuge (Beckman Coulter, Indianapolis, USA) using the JLA-10.500 rotor for 20 min at 4000 rpm and 4 °C. The supernatants were discarded and pellets weighed and stored at −80 °C until further use. For protein extraction, the bacterial pellets were thawed on ice and resuspended in a volume of lysis buffer (100 mM TriCl pH8, 150 nM NaCl and 1 mM PMSF) having mL magnitude equivalent to 5x the cell pellet weight in g. To lyse the cells, a sonicator (Branson ultrasonics, Gehäuse SFX150) was used with sonication cycles having pulse "ON" for 3 s, "OFF" for 5 s at 20% power over 6–8 mins. The sonicated solutions were centrifuged at 14 000 rpm for 15 mins at 4 °C and the supernatants were collected for further purification by affinity-based column chromatography. Supernatants and cell debris were stored for analysis by Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). Since the proteins engineered with a StrepII-tag, columns containing Strep tactin beads (Quiagen, 30 004) were used for purification of the protein by affinity chromatography modifying the protocol given by the manufacturer (Quiagen) to optimize pure protein yields. 4 mL of the strep-tactin bead solution was pipetted into a 15 mL falcon tube and was centrifuged at 100 rpm for 2 mins to obtain a strep-tactin bed volume of 2 mL. The beads were washed three times with 5 mL of lysis buffer; the lysis buffer was removed each time by centrifuging the beads at 100 rpm for 2 mins. Cell lysate of 5 mL was added onto the strep tactin beads and this assembly was incubated at 4 °C on a rotary shaker for 30 mins to facilitate optimal contact between the beads and cell lysate. The beads were centrifuged at 100 rpm for 2 mins to remove the unbound protein and washed with 5 column volumes (CV) of wash buffer (100 Mm TrisCl, 150 Mm NaCl, and 0.5% tween 20) 3 times by adding 5 CV of wash buffer to the 15 mL falcon

and rotating it upside down manually each time; the wash buffer was removed each time by centrifugation of the beads at 100 rpm for 2 mins. The protein was eluted using an elution buffer (100 mM TrisCl, 150 mM NaCl, and 2.5 mM Desthiobiotin). The eluted protein was rebuffered in 5 volumes PBS pH 7 and concentrated by using 3 kDa-cutoff centrifugal filter units and centrifugation parameters – 4000 rpm for 45 mins at 4 °C. The protein yields for YCQ and YCx were in the range of 6 to 20 mg L^{-1}

Patterning of Col-Gel Matrix on a Glass Surface to Assess Binding of YCQ: Coating coverslips with PLL-PEG: Glass coverslips were cleaned by treating them with oxygen plasma in a plasma oven (Harrick Plasma, Ithaca, NY, USA) for 5 mins. 50 µL of 0.1 mg mL^{-1} PLL-PEG (PLL (20)-g [3.5]-PEG (5), SuSoS AG, Dübendorf, Switzerland) solution in PBS was placed on a sheet of parafilm. The plasma-treated coverslips were inverted on the drop of PLL-PEG and incubated for 1 h at room temperature (RT).

Cleaning of the photomask: The patterned surface of the photomask was cleaned with acetone, water, ethanol, and water by dipping in each solution. It was air dried gently and placed in UVO cleaner (Jelight Company Inc, model no.42) for 5 min, in such a way that the patterned side faced up.

Making patterns using the photomask: After the incubation, the coverslip was released from the parafilm gently by infusing deionized water under the coverslip in such a way that it starts floating. It was rinsed by dipping the patterned surface 5 times in PBS pH 7 solution and dried by draining the excess solution onto a paper towel. 5 µL deionized water was placed on the patterned side of the photomask in order to reduce friction between the coverslip and patterned surface. The coverslip was carefully picked up with forceps and placed on the drop of water on the photomask. This assembly was placed in UVO cleaner for 5 mins in a way that the pattered surface in contact with the coverslip faces down. UV exposure cleaves PEG chains at the site of exposure on the glass coverslip. The coverslip was released gently by infusing 100 µL PBS pH 7 beneath it after which it was washed with PBS by dipping it in PBS 5 times. The coverslip was then placed on a 25 µL Col-Gel solution which was prepared by modifying a reported protocol.[38] All stock solutions were made in PBS pH 7. The gels were prepared by mixing 75% v/v of 600 µg mL^{-1} collagen (gibco, A1048301), and 25% v/v of 600 µg mL^{-1} gelatin (Sigma–Aldrich, 9000-70-8) solution. The two solutions were mixed by manually stirring with the pipette tip in the Eppendorf tube without pipetting it. The gels were allowed to polymerize at 37 °C, 5% CO_2 for 1 h followed by 4 °C incubation overnight. The coverslips were released from the parafilm surface by infusing 200 µL deionized water and washed by dipping them10 times in 1X PBS with the help of a tweezer.

Incubation of protein solutions and staining of the patterned surface: Supernatants from overnight induced (light) and uninduced (dark) YCQ and YCx were collected by centrifuging the bacterial culture at 4000 rpm for 20 mins. The supernatants were filter sterilized using 0.4 µm syringe filters (Carl roth, SE2M230104). The coverslips with Col-Gel patterns were then incubated with either filtered supernatants or 10 nM purified solutions of YCQ and YCx by inverting the coverslips on 25 µL drops of the protein solutions. This assembly was incubated for one hour at RT. After the incubation, glass coverslips were released gently from the photomask by infusing 100 µL of PBS between glass surface and the parafilm. The coverslips were picked up with forceps and dipped in PBS 5 times to remove the unbound protein. The patterned glass surfaces with immobilized proteins were then incubated with anti-YebF primary antibody (Athena ES, AES-0313) by placing the patterned coverslip on 25 µL 1:500 diluted antibody (in 1% BSA) on parafilm. After incubation at RT for 1 h, the coverslips were released as mentioned before and all the patterned surfaces were then incubated with1:500 diluted AF-488 Goat anti rabbit secondary antibody (ThermoFischer, A-11008) for 1 h at RT. Patterned surfaces were washed with PBS as mentioned before and were placed on a glass slide by placing 10 µL of PBS on the glass slide and inverting the patterned surface on it. YCQ or YCx stained Col-Gel patterns were visualized using a Nikon Ti-Eclipse microscope (Nikon Instruments Europe B.V., Germany).

Fabrication of ELM: Silanizing glass coverslips: Glass coverslips (13 mm) were arranged in a Teflon holder with a removable handle (custom made) for washing steps in a beaker. The Teflon holder with coverslips was suspended in a beaker with 99% ethanol and was sonicated for 10 mins. Then, it was washed with ultrapure water followed by 99% ethanol. The Teflon holder handle was removed and the base holding the cover slips was slid into a 50 mL falcon tube containing 20 mL of 95% ethanol, 4% ultrapure water, and 1% 3-APS (3-(Trimethoxysilyl)propyl acrylate, Sigma–Aldrich, 4369-14-6) such that coverslips were completely immersed in the solution. After incubation overnight at RT, the coverslips were washed 3 times with deionized water to remove excess APS and transferred into a beaker with ultrapure water and stored in it till further use.

Preparation of Polydimethylsiloxane (PDMS) molds: A beaker in which PDMS molds were to be made was sonicated for 3 mins with 99% ethanol in it. 10 g SYLGARD™ 184 silicone elastomer base (Dow chemicals, USA) was added to beaker on a weighing balance and (1 g) SYLGARD™ 184 silicone elastomer curing agent (cross linker) (Dow chemicals, USA, 1 023 993) was also added and mixed thoroughly using a spatula. The Beaker with the PDMS-crosslinker mixture was placed in a vacuum-desiccator (DN 150 Duran) for up to 10 mins to remove air from the mixture. The beaker was then removed from the desiccator after confirmation of absence of bubbles in mixture. The volume of the PDMS mixture was calculated to obtain molds of dimensions depicted in Figure 2 and Figure S4 (Supporting Information) and poured into glass petri dishes. These were then placed in a hot air oven at 95 °C for 2 h for polymerization. They were taken out after 2 h and left undisturbed at room temperature overnight. Solidified PDMS molds were scraped out from glass flasks and holes of desired diameter were punched with wad punch set (BOEHM, 832 100).

Preparation of Pluronic diacrylate solution: Pluronic diacrylate (PLU-DA) was obtained from the group of Prof. del Campo, who synthesized it as previously reported.[31] 30% w/v Pluronic diacrylate (PLU-DA) + 0.02% w/v IRGACURE 2959 (2-Hydroxy-4′-(2-hydroxyethoxy)-2-methylpropiophenone, Sigma–Aldrich, 410896-10G) solution was prepared for construct fabrication. 3 g PLU-DA and 20 mg IRGACURE 2959 photo initiator were dissolved in ultrapure water in an amber colored glass bottle and the final volume was adjusted to 10 mL. The PLU-DA solution was kept on a rotary shaker at 4 °C overnight for complete dissolution of contents. PLU-DA was stored in the same amber glass bottle at 4 °C until further use.

Fabrication of ELMs: Bacterial cultures of 5 mL at their exponential phase (OD_{600} = 0.5–0.8) were centrifuged at 4000 rpm for 10 mins at room temperature. Pellets were resuspended in M199 cell culture media and was made up to an OD_{600} of 1 after measuring it in a Nanodrop One device. Bacterial suspensions were prepared by mixing 90% v/v PLU-DA and 10% v/v bacterial culture (OD_{600} = 1) such that the final density of bacteria in PLU-DA mixture was 0.1 OD_{600}. Solutions containing PLU-DA were always handled on ice to prevent their physical gelation. 3-APS coated coverslips were placed on disinfected paraffin paper in a petri dish. A cylindrical PDMS mold with 6 mm diameter was placed on the coverslips with conformal contact. 22 µL bacterial gel solutions were pipetted into the PDMS molds to make a core layer of radius 3 mm and height of 0.7 mm. It was left undisturbed for 2 mins at room temperature for the PLU-DA to form a physical gel. The PDMS + coverslip setup was transferred to a Gel-Doc (Biozym Scientific GmbH, FluorChemQ) and the PLU-DA-Bacterial mixture was cross-linked by irradiating it with low intensity UV transillumination (365 nm) for a duration of 60 s. The whole setup was transferred back to a sterile hood and left undisturbed for 2 mins after which the PDMS mold was removed from the coverslip. A PDMS mold of 10 mm diameter was then placed on the same coverslip to produce a protective PLU-DA shell to prevent the leakage of bacteria from the ELM. 60 µL PLUDA was pipetted onto the PLU-DA bacterial core layer such that it formed a shell layer covering the entire surface of the core with a diameter of 10 mm and height of 1 mm. The PDMS and coverslip setup was transferred to the Gel-Doc and cross-linked by UV transillumination for a duration of 90 s. The whole setup was transferred back to the sterile hood and the PDMS mold was removed from the

coverslip. This ELM construct was transferred into a well in a 24-well plate and 300 µL M199 cell culture media supplemented with additional 0.4% w/v glucose and 0.88% w/v NaCl was pipetted into the well. The 24-well plates with hydrogel constructs was placed in an incubator (28 °C) for 16 h in dark. For reversible light switching experiments.: one 24-well plate was kept in light (blue light irradiation device; pulse: 2 s ON, 1 min OFF;200 µW cm^{-1}) for 3 days and then moved to dark for 3 days followed by a period of light again for 3 days. The other 24 well plate was kept in dark for 3 days and then switched to light (same irradiation parameters as mentioned before) for 3 days followed by a period of dark for 3 days. Surrounding media was collected from each well after every 3 days and ELMs were replenished with 300 µL fresh media. For light tuning experiments: the 24 well plate containing ELMs was kept on an optoWELL irradiation device (Opto biolabs), which was programmed to irradiate wells with blue light pulses (2 s ON; 1 min OFF, 450 nm) of intensities of 0, 80, 105, and 125 µW cm^{-2} (corresponding to 3%, 6%, and 9% in the device settings) for 6 days. The volume of media surrounding ELM was decreased in 3 days from 300 µL to 250 µL due to evaporation. It was replenished by adding 50 µL fresh media to it. The surrounding media containing secreted protein was collected at day 6 for quantification with ELISA (see point vi)

Qdot Streptavidin staining of ELMs: ELMs in 24-well plate wells were washed with PBS thrice. Qdot 655 Streptavidin conjugate (Thermo Fisher scientific, Germany, Q10121MP) was used to stain the hydrogels. The staining solution was prepared in the ratio of 1:500 in 2% w/v BSA. 300 µL staining solution was added to each ELM construct and was incubated at 37 °C for 2 h. The well plate was completely wrapped with aluminum foil to avoid photobleaching during incubation. The antibody solution was pipetted out from wells and constructs were washed with 300 µL PBS thrice. Then constructs were analyzed by Nikon epifluorescence microscope and overlay images of both brightfield as well as the 561 nm red channel were captured.

Analysis of ELM supernatant by ELISA: ELISA was performed using Strep-tactin coated 96 well plates (iba-life sciences) with the following protocol:

An initial blocking step was performed with 100 µL of 2% w/v BSA added to the wells and the well-plate left overnight at 4 °C or for 1 h at 37 °C.

The wells were then washed by pipetting out the blocking solution and washing with 100 µL wash buffer (PBS + 0.1% w/v Tween-20) thrice.

Samples of 100 µL were added to the wells and left to incubate for 1 hour at room temperature followed by 3x washing step.

Another blocking step was performed where 100 µL of 2% w/v BSA was added to wells and left for 1 h at room temperature followed by 3x washing step.

Primary antibody treatment: Rabbit Anti-YebF antibody (Athena enzyme systems, USA) was prepared in the ratio of 1:500 in 2% w/v BSA. 100 µL antibody solution was added to wells and left at room temperature for 1 hour followed by 3x washing step.

Secondary antibody treatment: Goat anti-Rabbit IgG conjugated with horseradish peroxidase (Invitrogen) was used as secondary antibody in the ratio of 1:500 in BSA 1% w/v. Secondary antibody solution of 100 µL was added to wells and left at room temperature for 1 hour followed by 4x washing step.

Substrate treatment: Tetramethylbenzidine (TMB) (Sigma-Aldrich, Germany) substrate of 100 µL was added to each well and was left undisturbed until stable blue color developed.

Stopping step: 1 M Hydrochloric acid (HCl) of 100 µL was added to wells to stop the reaction and it resulted in yellow coloration of solution which was measured within 30 mins.

The absorbance of test samples was measured at 450 nm by using *iControl 2000* software of Tecan plate reader and resulting data was analyzed.

Using serial dilutions of the purified proteins in the optimized M199 medium, a standard curve was plotted. The slope of this standard curve enabled determination of the molar concentrations of ELM-released YCQ or YCx.

HUVEC Network Formation Assay: Cell Culture Conditions: HUVECs (PromoCell, C-12205) were maintained on cell culture flasks coated with gelatin (0.2% w/v). Cells were cultured in M199 medium (Sigma, M4530) supplemented with penicillin (1000 U L^{-1}), streptomycin (100 mg L^{-1}, Sigma), ECGS (Sigma, E2759), sodium heparin (Sigma, H-3393), and 20% w/v fetal bovine serum (FBS, Gibco, 10 270) as previously described.[40] HUVECs between passages 2 to 7 were used for the experiments.

Preparation of Col-Gel surfaces: Col-Gel surfaces were prepared as mentioned previously (Materials and Methods section C ii). This solution of 10 µL was added into the 15 well angiogenesis well plates (ibidi, 81 506). The gels were allowed to polymerize at 37°C, 5% CO_2 for 1 h followed by overnight incubation at 4 °C.

Immobilization of secreted YCQ on Col-Gel surfaces: 10 µL of the SN collected from ELMs were incubated on the Col-Gel surface for 1 h at 37°C in a CO_2 incubator. The supernatants were removed with a pipette and the Col-Gel surfaces were washed with 50 µL PBS once to remove unbound proteins.

HUVEC seeding: (50 µL of 2×10^5 cells mL^{-1}) HUVECs (P3-P7) were seeded on the Col-Gel surfaces and the plate was incubated at 37 °C and 5% CO_2 for 16 h. The culture was then fixed with 4% aqueous PFA solution for 15 mins, washed with PBS and blocked with 5% w/v BSA solution for 1 h. Cells were permeabilized with 0.1% w/v Triton X-100 for 15 mins and incubated with monoclonal goat anti-rabbit PECAM-1 primary antibody (1:500 in 1% w/v BSA, Abcam) overnight and washed with PBS. This was followed by incubation with anti-rabbit Alexa flour-488 secondary antibody (1:500 in water, Thermo Fisher Scientific) for 1 h. Subsequently, cells were washed with PBS and nuclei were stained with DAPI (1:500 in water, Life Technology) and actin fibers were labelled with TRITC-phalloidin (1:500 in water, Thermo Fisher Scientific). Samples were washed thrice with PBS and imaged with a Nikon epifluorescence microscope at 10X magnification using excitation wavelengths of 405 nm (900 ms excitation), 488 nm (100 ms excitation), and 565 nm (100 ms excitation) and 20% incident light intensity. Images were captured using NIS-Elements software and processed as follows for inclusion in the figures: Image processing and analysis was done using Fiji edition of ImageJ (Image J Java 1.8.0). Brightness and contrast for the DAPI (Cyan, 405 nm) and Actin (Yellow, 488 nm) were adjusted in their respective LUT histograms to the same range for all the images in an individual experiment. For PECAM-1, since Anti-PECAM-1 agglomeration at some places created bright spots to different degrees, the brightness and contrast in the LUT histograms were manually adjusted for each image based on the maximum intensity within the cells that were not caused by the agglomerates and the minimum intensity in the cell-free background. This way ensured best possible visualization of PECAM-1 expression in all the cells.

PECAM-1 analysis – Fiji edition of ImageJ (ImageJ Java 1.8.0). was used for quantification PECAM-1 fluorescence intensity. Quantification of the fluorescence intensities was done by determining the mean grey value within the cells excluding the bright spots created by agglomerates (by manual selection) and subtracting the mean grey value of the cell-free background.

Statistical Analysis: All image processing for quantification of PECAM-1 intensities was performed using the Fiji edition of ImageJ on Raw microscopy images. All data processing and analyses were done using Origin 2022 software. Details of sample sizes, replicates and statistical tests for quantified data were provided in the figure captions. Significant differences were determined by testing for the null hypothesis using a Two sample t-test when comparing 2 sample sets (Figure 1C) and using one-way ANOVA with the Tukey test for means comparison when comparing >3 samples sets (Figure 2, 4, and 5). The p values were given directly in the figures and p values above 0.05 were indicated as non-significant (n.s.) in Figures 2 and 5.

Supporting Information

Supporting Information is available from the Wiley Online Library or from the author.

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Conflict of Interest

The authors declare no conflict of interest.

Data Availability Statement

The data that support the findings of this study are available from the corresponding author upon reasonable request.

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angiogenesis, bacteria, engineered living materials, growth factor, optogenetics

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