Engineering Antioxidant and Oxygen-Releasing Lignin Composites to Accelerate Wound Healing

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ABSTRACT

The application of engineered biomaterials for wound healing has been pursued since the beginning of tissue engineering. Here, we attempt to apply functionalized lignosulfonates to confer antioxidation to tissue microenvironments and to deliver oxygen to accelerate vascularization and healing responses without causing inflammatory responses. The results from fibrosis array shows that thiolated lignosulfonate in methacrylated gelatin can effectively attenuate fibrotic responses of human dermal fibroblasts. Elemental analysis of oxygen releasing nanoparticles shows the positive incorporation of calcium peroxide. Composites including nanoparticles of lignosulfonate and calcium peroxide release around 0.05% oxygen per day at least for 7 days. Stiffness can be precisely modulated to avoid adverse inflammatory responses. Injection of lignin composites with oxygen generation nanoparticles enhanced the formation of blood vessels and promoted infiltration of α-smooth muscle actin^{+} fibroblasts over 7 days. At 30 days after surgery, the lignin composite with oxygen generating nanoparticles remodels the collagen architecture resembling to the reticular pattern of normal collagen and leave minimal scars. Thus, our study shows the potential of functionalized lignosulfonate for wound healing applications requiring balanced antioxidation and controlled release of oxygen.

INTRODUCTION

In spontaneous wound healing of the late fetus and in adult mammals, wound contraction impedes such endogenous regeneration mechanisms. With very few exceptions, e.g. children's digits[1], adult mammals fail to spontaneously regenerate skin or any of their injured organs. In sharp contrast to the late mammalian fetus or adult mammals, several amphibians can regenerate entire limbs spontaneously throughout their lifetime[2].

In recent years, antioxidants have drawn much attention as potential therapeutic interventions due to their ability to fight oxidative stress[3-9]. The main function of antioxidants is to scavenge or neutralize free radical formation and to inhibit the deleterious downstream effects of reactive oxygen species (ROS). However, most antioxidants, taken orally, have limited absorption profile, which leads to low bioavailability and insufficient concentrations at the target site[10,11]. To overcome this issue, research has been focused on developing tissue engineering strategies to provide locoregional delivery of antioxidants. Strategies including nanoparticles made up of inorganic materials such as mesoporous silica, cerium oxide, and fullerene, which exhibit antioxidant activities have been evaluated *in vitro* and in animal models to determine their ability to scavenge free radicals and decrease ROS concentrations to protect cells against oxidative stress[12-15]. ROS-responsive biomaterials have been identified as a type of promising therapeutic substance to alleviate oxidative stress in tissue microenvironments. Hydrogels that release ROS scavengers have been developed to promote cell function and phenotype as a method to mitigate the foreign body response[16]. Currently utilized biomaterials, especially hydrogels, employ relatively complex chemistry, cannot be easily manufactured, are expensive and lack mechanical tunability[17].

Solid inorganic peroxides have been used to support cell growth, survival, tissue regeneration, and bioremediation[18]. Calcium peroxide (CPO) possesses many distinctive properties in comparison with other peroxides when it is used as disinfectant and oxygen generator, including better thermal stability, environmental harmless end products, extended release period of hydrogen peroxide and reasonable cost[19,20]. The amount of oxygen released by inorganic solid peroxides depends on their solubility coefficients and the equilibrium coefficients of calcium and magnesium peroxide are 9.8×10^{-11} and 1.8×10^{-11}, respectively[21]. Calcium and magnesium peroxide have low solubility coefficients (1.65 mg/mL at 20°C and 0.86 mg/mL at 18°C, respectively)[21]. Based on these values, calcium peroxide has a higher oxygen-generation potential than magnesium peroxide[21,22].

For *in situ* application of lignin composites, here we developed injectable lignin compo-

sites with the following components: 1) TLS scavenges ROS from wounds[23], 2) GelMA modulates the mechanical properties of lignin composites to favor M2-like macrophage (Mφ) phenotypes, where integrin $\alpha_2\beta_1$ plays a pivotal role as its inhibition blocks the induction of M2 macrophages[24] and 3) SLS in nanoparticles (NPs)[25] scavenges radicals or excessive H_2O_2 from CPO[25,26] while encapsulating CPO in SLS NPs. First, we assessed the integration of CPO to NPs and the release of O_2 from lignin composites. Then, mechanical properties and swelling/degradation profiles of lignin composites were assessed. Lastly, we applied lignin composites in the wounds of wildtype mice and assessed granulation tissue, neovascularization and wound healing process, inflammatory responses and scaring.

MATERIALS AND METHODS

Synthesis of CPO NPs

The coupling of lignin to PLGA (poly(lactic-*co*-glycolic) acid) was performed by acylation reaction for SLS[25] in a mass ratio of 2 to 1. The final precipitate was collected and dried for 2 days under high vacuum. Then, SLS-*graft*-PLGA was dissolved in ethyl acetate (organic phase) and calcium peroxide (CPO, cat# 466271, Sigma-Aldrich, St. Louis, MO, USA) and dissolved under stirring at room temperature. The organic phase was added to the aqueous phase (low resistivity water) under stirring. The suspension was homogenized with a microfluidizer (Microfluidics Corp., Westwood, MA, USA) at 30 kpsi at 4°C. Next, the organic solvent was evaporated with a rotary evaporator R-300 (Buchi Corp, New Castle, DE, USA). Finally, trehalose was added (1:1 mass ratio) as a cryoprotectant for the lyophilization of SLS:PLGA NPs and stored at -20°C for further characterization and experiments.

Dynamic light scattering (DLS) of NPs

Size, polydispersity and ζ-potential of CPO and TLS NPs (0.2-0.4 mg/mL) was measured by dynamic light scattering (DLS) using Malvern Zetasizer ZS (Malvern Panalytical, Westborough, MA, USA). NPs were filtered through 0.45 µm filter. To prevent the formation of disulfide bonds, TLS sample was prepared in 250 mM Tris(2-carboxyethyl)phosphine hydrochloride (TCEP HCl, Cat# H51864, Alfa Aesar, Haverhill, MA, USA) in PBS.

Elemental analysis of TLS and CPO

We utilized the nuclear microscopy setup at the Louisiana Accelerator Center to probe the calcium concentrations in our samples using particle-induced x-ray emission (PIXE) spectrometry[27]. The samples were placed in a low-pressure environment ($\leq 1\times10^{-6}$ mbar) on electrically conductive non-porous carbon tape attached to the sample holder. A focused (10 µm × 10 µm) 2 MeV proton beam, with a beam current in the range of 10−20 pA, raster scanned the sample region (1 mm × 1 mm) for about 1 h. A silicon drift detector was placed at 135° in front of the sample to detect the characteristic x-rays excited by energetic protons. Analysis of the spectra was performed with the GeoPIXE™ software (v7.3)[28]. The elemental maps and the derived concentrations were generated by the dynamic analysis method using average matrix composition from the whole scanned area.

Formation of lignin composites

TLS and GelMA were synthesized with the previously published protocol[23]. We confirmed the methacrylation of gelatin with ^{1}H nuclear magnetic resonance (NMR, **Figure S1**). Lignin composites were formed by weighing out GelMA, lithium phenyl-2,4,6-trimethylbenzoylphosphinate (LAP, Allevi, Philadelphia, PA, USA), TLS, CPO and CPOc (SLS:PLGA NP without CPO). For the fibrosis array, the concentrations of GelMA and LAP were fixed at 80 and 5 mg/mL, respectively. Any concentrations of TLS higher than 7 mg/mL interfered photo-crosslink-

ing of lignin composites, leading to the reduction of elasticity of lignin composites. All other experiments, the concentrations of GelMA, LAP and TLS were fixed at 50, 5 and 3 mg/mL and the concentrations of CPO or CPOc were varied at 4 or 40 mg/mL. The precursor of lignin composites was prepared by mixing GelMA in PBS at 37°C and the rest of LAP, TLS and CPO or CPOc in PBS. Each precursor was pipetted into a custom PDMS mold (8 mm diameter and 1 mm height, 50 μL) to form lignin composites. Samples were crosslinked using an UV floodlamp (Intelli-Ray 400, Uvitron international, West Springfield, MA, USA) for 120 s at 10 mW/cm^2.

Quantification of O_2 release from lignin composites

The released O_2 was optically measured by a planar O_2 sensor spot (SP-PSt3-SA23-D5-OIW-US, PreSens, Regensburg, Germany) placed at the bottom of a 96-well plate. Lignin composites (5 mm diameter and 1 mm height, 20 μL) were placed on top of the planar O_2 sensor spot and submerged in serum-free medium (37°C/5% CO_2). To prevent evaporation, each well was covered with parafilm and the entire plate was wrapped with parafilm. A polymer optical fiber (POF-L2.5-2SMA, PreSens, Regensburg, Germany) optically senses the concentration of O_2 through the bottom of a well and sends signals to an O_2 sensor (OXY-1 SMA, PreSens, Regensburg, Germany)[29] for measurement.

Swelling ratio and *in vitro* degradation of lignin composites

Swelling ratios of lignin composites were determined by the following equation: $[W_s-W_d]/W_d$, where W_s and W_d represent the weight after swelling in PBS for 24 h and the weight after lyophilization, respectively. *In vitro* degradation of lignin composites was determined by submerging lignin composites in the solution of collagenase type II (0.5 U/mL, cat# CLS-2, Worthington Biochemical) with 1 mM $CaCl_2$ in serum-free culture medium, along with a control group without including the collagenase. Lignin composites were collected at 0, 2, 6 and 24 h and lyophilized to determine the fraction of remaining composites.

Oscillating rheometry of lignin composites

Using a TA Discovery HR-2 rheometer and the previously published methods[23], viscosity of the precursors of lignin composites were measured in a flow ramp setting (shear rate from 1 to 2000 (1/s)) and with a 25 mm parallel plate. Using an 8 mm parallel plate, storage (G′) and loss (G″) moduli of lignin composites were determined by frequency sweeping from 0.62 to 19.9 (rad/s) at 2% strain. In addition, instead of evaluating stiffness at an arbitrary storage modulus (often storage moduli are altered by axial stress applied during measurement), we evaluated the slope of axial stress vs compression, similar to evaluating Young's modulus from the slope of a stress-strain curve[30]. Axial stresses at 0, 10 and 20% of compression were determined, while composite samples were subject to 2% strain and 6.28 rad/s frequency.

Cultures of high and low scar normal human dermal fibroblasts

Patient sample collection: Skin tissue was collected from abdominoplasty patients who provided written informed consent as part of a protocol approved by the Institutional Review Board of Texas at Baylor College of Medicine in accordance with the Declaration of Helsinki. A biobank of scar and matched normal uninjured skin tissue was obtained from abdominoplasty patients controlled for sex, age, ethnicity, surgery type, indication, wound site, and comorbidities. These skin tissues were grouped into 'low scarring' and 'high scarring' phenotypes based on the evaluation of their existing c-section scars using Vancouver Scar Scale (VSS). Skin obtained from patients with 1-3 score on VSS were categorized as 'low scarring' phenotype (N) and patients with 6-9 score were grouped into 'high scarring' phenotype (S). For each group, n=3 skin tissues were pooled. Fibroblasts (FBs) were isolated from scar and matched normal skin cohorts and were called 'low scarring' (LS) and 'high scarring' (HS) normal fibroblasts based on their VSS phenotype and patient demographics. Only LS-N and HS-N human dermal FBs (hdFBs) were used for cell studies, and they are denoted HS and LS for the rest of the

studies. Both LS and HS hdFBs were maintained in DMEM (cat# 10567-014-500mL, Thermo Fisher Scientific, Waltham, MA, USA) supplemented with 10% fetal bovine serum (FBS, cat# 35-015-CV, Corning, Corning, NY, USA), Penicillin/Streptomycin (P/S, cat# 15140-122, Thermo Fisher Scientific, Waltham, MA, USA), and antibiotic/antimycotic (anti/anti, cat# 15240-062, Thermo Fisher Scientific, Waltham, MA, USA). Only passages 8-10 were used for studies and cells were harvested at 80-90% confluency by applying TrypLE Express (Cat# 12604-021, Thermo Fisher Scientific, Waltham, MA, USA) and pelleted at 350 g for 5 min. Supernatant was removed and hdFBs were gently resuspended in cell culture media and counted. Media was changed every other day.

PCR fibrosis array

To determine the effect of lignin composite on their expression of fibrotic phenotype of hdFBs, LS and HS hdFBs were seeded onto composites at 50,000 cells/cm^2 and maintained for 24 h in a CO_2 incubator (5% CO_2/37°C). At specified time points cell-seeded composites were removed and incubated with TrypLE Express to extract RNA by following the procedure from the PureLink RNA kit (Cat# 12183018A, Thermo Fisher Scientific, Waltham, MA, USA). Extracted RNA was evaluated for quantity and purity using a Take3 Micro-Volume plate and Cytation3 spectrophotometer (Biotek, Winooski, VT, USA). cDNA was reverse-transcribed using High-Capacity RNA-to-cDNA kit (Cat# 4387406, Thermo Fisher Scientific, Waltham, MA, USA) following the protocol from the manufacturer. We also used the RT2 Profiler PCR Array human fibrosis kit (Cat# PAHS-120-ZE-4, Qiagen, Hilden, Germany) to detect the expression of 84 fibrosis-related genes. cDNA (400 ng) made with RT2 first strand kit (Cat# 330404, Qiagen, Hilden, Germany) were used with RT2 SYBR Green qPCR Mastermix (Cat# 330501, Qiagen, Hilden, Germany) to run qRT-PCR on Bio-Rad CFX 384 real-time system. Data were analyzed by Qiagen RT2 Profiler PCR array data analysis software. GAPDH was used as a housekeeping gene, and ΔΔCT values for the genes were analyzed. Principal component analysis (PCA) and hierarchical clustering (HC) were performed and visualized with an open source machine learning software (Orange)[31].

Animal model of wound healing

Wounds healing studies were carried out in wildtype (WT) C57BL/6N mice (8- to 10-week-old, female and male). Mice were maintained under pathogen-free conditions with access to food and water ad libitum in the Texas Children's Hospital Feigin Center animal facility. Protocols for animal use were approved by the Institutional Animal Care and Use Committee at Baylor College of Medicine. At the time of wounding, mice were anesthetized using isoflurane, the backs were shaved, and prepped for surgery with three times alternating betadine and 70% isopropyl alcohol scrubbing. Two 6 mm diameter full thickness wounds were made using a 6 mm dermal punch biopsy, excising tissue through the panniculus carnosus (PC) muscle. Immediate skin contraction was controlled through application of a silicone stent with inner diameter of 8mm and outer diameter of 16mm, secured concentric to wound using skin adhesive and 6 simple interrupted 6-0 Proline© sutures (Ethicon, Raritan, NJ). Wounds were maintained in a moist wound environment using a semi-occlusive sterile adhesive dressing (Tegaderm, 3M, St. Paul, MN, USA) and contraction was controlled through application of a silicone stent with inner diameter of 8 mm and outer diameter of 16 mm, placed concentric to wound using skin adhesive and 6 interrupted sutures. Prior to dressing with Tegaderm, each wound received one of four treatments: a standard saline wash (untreated, UNTX), GelMA with TLS nanoparticles (TLS), GelMA-TLS with microparticles of SLS without CPO as a CPO control (CPOc), or GelMA-TLS with oxygen generating CPO/SLS microparticles (CPO). Crosslinking of the treatments was performed immediately after application to the wound bed using a UV floodlamp (B-100AP High Intensity, Blak-Ray) for an exposure time of 30 s.

Wounds were imaged at 1, 3, and 7 days post operatively (**Figure S2**), and harvested at

days 7 and 30. For histology and immunostaining, wounds were bisected in the rostral-caudal plane, fixed overnight in 10% neutral buffered formalin, dehydrated through a series of graded ethanol and xylene, and embedded in paraffin wax. Five-micrometer-thick sections from the paraffin-embedded wounds were collected using a RM 2155 microtome (Leica, Heidelberg, Germany) and used in staining.

Morphometric quantification

At day 7, epithelial gap and granulation tissue areas were measured from Hematoxylin and Eosin (Cat#3801571 and Cat#3801615, respectively; Leica, Heidelberg, Germany) stained sections using morphometric image analysis (LASX, Leica, Heidelberg, Germany). Staining was carried out on 5 µm formalin fixed paraffin embedded (FFPE) sections following deparaffinization and rehydration in graded xylene and ethanol as following the manufacturer's recommendations. Epithelial gap was determined using a full, 4× tile scan of the wound bed, and measuring the distance (in mm) between the leading epithelial margins on either side of the wound. Granulation tissue area was determined using a standardized approach of calculating the entire wound area (in mm^2) above the panniculus carnosus and bounded within the wound edges laterally. If parts of the hydrogel remained devoid of infiltrated cells, that area was excluded from the granulation tissue assessment.

Immunostaining

Immunohistochemistry was carried out on serial 5 µm sections from day 7 FFPE tissues, which were deparaffinized and rehydrated to water using graded xylene and ethanol. Primary antibodies used were αSMA (rabbit anti-mouse, Cat# ab5694, Abcam, Cambridge, United Kingdom, 1:500 dilution) to measure myofibroblast infiltration, CD31 (rabbit anti-mouse, Cat# ab28364, Abcam, Cambridge, United Kingdom, 1:100 dilution) to measure endothelial cells and vascular lumen density, CD45 (rabbit anti-mouse, Cat# ab10558, Abcam, Cambridge, United Kingdom, 1:5000 dilution) to determine pan-leukocyte infiltration, CD206 (rabbit anti-mouse, Cat# ab64693, Abcam, Cambridge, United Kingdom, 1:500 dilution) to measure M2 macrophage levels, F4/80 (rat anti-mouse, Cat# MF48000, Thermo Fisher Scientific, Waltham, MA, USA, 1:5000 dilution) to measure pan macrophage infiltration, and Ly6G (rat anti-mouse, Cat# 551459, BD Biosciences, Franklin Lakes, NJ, USA, 1:5000 dilution) to determine infiltration of monocytes, granulocytes, and neutrophils.

Following deparrafinization and rehydration to water, the sections were then immersed in target antigen retrieval solution (Cat #K800521-2, Agilent, Santa Clara, CA, USA) and treated following the protocol within the DAKO PT Link Rinse Station (Cat #PT10930, Leica, Heidelberg, Germany). Following antigen retrieval, wound sections were stained using the DAKO autostainer (AS480, Leica, Heidelberg, Germany). First, the sections were buffered in DAKO wash buffer (Cat #K800721-2, Agilent, Santa Clara, CA, USA) before incubating in primary antibody diluted in DAKO antibody diluent (Cat# S080983-2, Agilent, Santa Clara, CA, USA) for 1 h. The primary antibody was then rinsed, and the sections were washed in wash buffer before incubation in the appropriate the secondary antibody system, either HRP-Rabbit (Cat# K400311-2, Agilent, Santa Clara, CA, USA) or HRP-Rat (Cat#D35-110, GBI Labs, Bothell, WA) for 20 min. The secondary antibody was then rinsed off using wash buffer, and the appropriate visualization system was applied – DAKO DAB (Cat# K346811-2, Leica, Heidelberg, Germany) for all but CD206 which utilized AEC (Cat# K400511-2, Leica, Heidelberg, Germany). A hematoxylin (Cat #K800821-2, Agilent, Santa Clara, CA, USA) counterstain was then applied, and sections were dehydrated in graded xylene and ethanol before cover slipping using a xylene based mounting media (Cat#23-245691, Thermo Fisher Scientific, Waltham, MA, USA) – except for those sections using AEC as the visualization system which required an aqueous mounting media (Cat#108562, Merck KGaA, Darmstadt, Germany).

Staining was quantified using images taken on the Leica DMI8 camera. For all data, the

percentage of positive cells were determined by counting the number of positive cells per High Powered Field (HPF, 40× magnification) and dividing by the total number of cells in that HPF as determined by the hematoxylin counterstain. Final values were determined by the average of 4-6 HPFs per wound. Total number of vessel lumens were counted per HPF. These percent values or vessel counts were then averaged from 6 images taken across the wound bed to determine the final value.

Scar assessment

Serial 5 μm sections from D30 FFPE tissue were deparaffinized and rehydrated to water using graded xylene and ethanol. The sections were then stained using Gomori's Trichrome (blue) as following the manufacturer's recommendations (Cat# 38016SS2, Leica, Heidelberg, Germany). Once stained and cover slipped, collagen content per HPF in the dermis of the scars was measured using established methods with color-thresholding in ImageJ in which color segmentation is used to isolate only blue pixels, representing collagen fibers, thus allowing quantification of the amount of collagen within the selected area[32]. Gross images of the wound at day 30 were also obtained, and a subjective assessment of scarring was performed.

Statistical analysis

For multiple comparisons, one-way ANOVA with Tukey's *post hoc* tests or with Kruskal-Wallis test followed by Dunn's test was performed, where p values <0.05 were considered significant. At least three independent experiments were performed.

RESULTS

3D and TLS composites attenuated the differential expression of fibrotic genes of HS and LS hdFBs.

Our previous work[23] showed that the antioxidation capacity of TLS attenuated the expression of *COL1A1*, *ACTA2*, *TGFB1*, and *HIF1A* and resulted in the similar extent of expression of the fibrotic markers comparable to proliferative LS hdFB phenotypes. To further confirm that the attenuation of fibrotic phenotypes of hdFBs by TLS composites, we analyzed 84 genes associated with fibrosis using a fibrosis PCR array. We hypothesized that exposure of LS and HS hdFBs to engineered TLS composites attenuates fibrotic responses of HS to the levels comparable to those of LS. As shown in **Figure 1a**, the expression of fibrotic genes in HS hdFBs on composites (regardless of incorporating TLS) was significantly altered to mimic those in LS. In **Figure 1b**, heatmaps showing unsupervised hierarchical clustering (HC) of the expression of fibrotic genes of LS and HS hdFBs on composites (GelMA and GelMA-TLS) show subsets of genes that are significantly altered in HS to mimic LS. These results and the previous works[23] suggest that optimizing the hdFB microenvironment using lignosulfonate may reduce the effect of inherent differences in hdFBs on their overall fibrotic functions during wound repair.

Elemental analysis confirmed the incorporation of CPO in SLS NPs.

Our effort to apply antioxidant capability of SLS to the engineered microenvironments would be synergistic with other pro-regenerative stimuli. We hypothesized that controlled release of oxygen while scavenging ROS in the engineered microenvironments will accelerate wound healing. Thus, we attempt to incorporate CPO in SLS NPs[25] and found that the size of SLS NP with CPO is not significantly altered, even when compared to SLS, as shown in **Table 1**. In comparison to SLS, the average diameter of CPO is increased while the PdI of CPO is significantly smaller than CPO or CPOc. In contrast, the average diameter of TLS is significantly smaller than SLS or CPO or CPOc NPs. After adding TCEP to remove disulfide formation, the average diameter is further reduced. SLS is natural biomaterial with potential lot-to-lot variability.

However, the synthesis of NP with CPO slightly increased the average diameter and significantly reduced PdI upon the incorporation of CPO. In contrast, CPOc NPs were formed only with ethyl acetate, forming irregular shapes and possible with cavity. As evidenced in **Table 1**, TLS formed NPs with a broader distribution. The formation of CPO or CPOc NPs requires stirring at room temperature, while the thiolation of SLS is completed via acid-catalyzed esterification at 80°C. In PBS (pH 7.4) forms particles via disulfide formation, but adding TCEP to PBS dissociates TLS NPs into smaller NPs. This could be an advantage to form lignin composites with homogeneous distribution of TLS in the matrix of GelMA. After the degradation of GelMA, TLS can be cleared by glomerular filtration (5-10 nm).

Due to the size (diameter) of NPs, we assessed the incorporation of CPO using particle-induced x-ray emission (PIXE) spectrometry. CPO and its control CPOc (without CPO) NPs showed similar composition of elements while the normalized concentration of Ca in CPO is about 17 times higher than that of CPOc in the quantitative molecular spectroscopy (**Figure 2**).

Table 1. DLS of NPs with and without CPO incorporated.

Particles	Average dia. ± st. dev. (nm)	PdI_{DLS} (st. dev./mean dia.)^2
SLS	126.6 ± 7.2	0.585 ± 0.017
CPO	140.8 ± 0.5	0.0597 ± 0.0199
CPOc	171.6 ± 1.9	0.204 ± 0.025
TLS	58.01 ± 13.54	0.258 ± 0.056
TLS with TCEP	7.728 ± 0.090	0.442 ± 0.005

dia: diameter
st.dev.: standard deviation

Oxygen released from CPO composites was maintained for 7 days

To measure the release of O_2 from lignin composites, we used a planar O_2 sensor with optical measurement. These planar sensors can measure the concentration of O_2 in liquid or gas. The difference (Δ) of the area under curve (AUC) between composites with CPO and CPOc is calculated over 1440 min for 7 days. As shown in **Figure 3**, the difference is around 500 ppm (equivalent to 0.05% O_2) per day. As the statistical difference is not detected, the oxygen release is maintained up to day 7. We observed that the swelling of lignin composites over first 24 h contributed to the slightly higher ΔAUC in day 1 than that in day 2 since lignin composites were added to serum-free medium without achieving equilibrium swelling.

Swelling ratios and rate of degradation were not significantly different with respect to the presence of CPO or the concentrations of CPO

Injected lignin composites are subject to swelling and enzymatic degradation for remodeling. Thus, we assessed the extent of swelling of lignin composites by varying the concentration of CPO NPs and their control NP (without CPO). As shown in **Figure 4a**, the swelling ratios between CPO and CPOc lignin composites were not significantly different at both 4 and 40 mg/mL. At higher concentration of NP, the swelling ratios were slightly reduced by around 7% (CPO) and 8% (CPOc) without any statistical difference by Student's *t*-test. Apparently, the 10 times higher mass fraction of NPs (regardless CPO or CPOc) in lignin composites contributed to the swelling behavior with marginal difference. To determine the fraction of remaining lignin composites, TLS, CPO (4 and 40 mg/mL) and CPOc (4 and 40 mg/mL) composites were submerged either in collagenase/$CaCl_2$/serum-free medium or $CaCl_2$/serum-free medium. Less than 50% of TLS (28%) and CPOc (38% at 40 mg/mL and 23% at 4 mg/mL) lignin composites was remained after 24 h while the CPO composites retained around 60% at both 4 and 40 mg/mL (**Figure 4b-d**). All control groups showed minimal (up to 7%) to no changes in the remaining fraction of lignin composites. The concentrations of matrix metalloproteinase (MMP) in inflamed

tissue vary from patients to patients. For example, the concentration of MMP-1/18/13 in chronically inflamed dental pulp tissue is 3 ng/mL[33] and MMP-3 in rheumatoid arthritis patients in 350 ng/mL while the health controls have 75 ng/mL[34]. The concentration of collagenase type II was 0.5 U/mL or 430 µg/mL, which is several orders of magnitude higher than the physiological conditions. Native or wounded skin has a plethora of MMPs, their inhibitors and serum proteins, leading to a tighter control of degradation and thus, we expect much slower degradation of lignin composites *in vivo*. Nevertheless, we expect a certain extent of degradation of lignin composites (primarily, GelMA) *in vivo* to transiently protect CPO NPs from rapid, enzymatic and mechanical degradation while promoting antioxidant activity from lignosulfonate (both TLS and SLS in CPO or CPOc).

NP did not alter the viscosity of the lignin composite precursors while quantity and type of NP modulated the viscoelasticity of lignin composites

Since lignin composites are delivered to the wounded areas, we assessed the mechanical properties of lignin composites before and after thiol-ene crosslinking. Viscosity of all five different types of composites was tested and we found no significant difference (**Figure 5a**). Apparently, crosslinked lignin composites exhibited similar viscoelasticity. However, the compressive modulus of elasticity was significantly different upon incorporating CPO and CPOc NPs. As shown in **Figure 5b** and **Table 2**, TLS composites exhibited the highest modulus of elasticity. Upon incorporating CPO NPs at 4 or 40 mg/mL, moduli of elasticity were decreased to around 18-19 kPa. While TLS is thiolated SLS to apply thio-ene crosslinking[23], SLS in CPO or CPOc NPs was not functionalized for crosslinking. Instead, CPO NPs harness calcium peroxide proximal to PLGA chains[35] while NPs of CPOc were formed without any CPO, which may form cavities during lyophilization. This possibly resulted in lowering the elasticity from the compression test during oscillating rheometry. Consequently, higher quantity of CPOc (40 mg/mL) showed much lowered slope down to 7.27 kPa in comparison to that of CPO or TLS composites. In **Figure 5c**, storage moduli of TLS and CPO were similar to each other while those of CPOc were significantly different from those of TLS or CPO. However, loss tangent (G″/G′) of all lignin composites ranged from 0.01 to 0.07 (equivalent to δ (phase lag) ranging from 0.57° to 4°), indicative of well-crosslinked viscoelastic composites (**Figure 5d**). Collectively, the reduction of stiffness by non-crosslinkable CPO is potentially significant around 40 mg/mL, thus we continued our investigation of oxygen generating capability from lignin composites with 4 mg/mL of CPO or CPOc and with mouse models of wound healing.

Table 2. Elastic modulus estimated by the slope of axial stress vs compression.

	Slope (Elastic modulus, kPa)	Intercept (kPa)	R^2
TLS	24.9	0.430	0.8960
CPO 4	18.7	0.286	0.9265
CPO 40	18.4	0.329	0.8661
CPOc 4	11.8	0.304	0.9557
CPOc 40	7.27	0.409	0.9290

The area of granulation tissue is modestly increased with lignin composites with CPO

To determine the effect of lignin composites on the progression of wound healing, wounds were harvested at day 7 post-wounding to examine re-epithelialization and granulation tissue formation. After 7 days of wounding, epithelial gap and the area of tissue granulation were assessed. Untreated wounds (UNTX in **Figure 6a**) showed no sign of re-epithelialization as expected. Lignin composites with TLS or CPOc in **Figure 6a** still showed the separation of

GelMA matrix and tissue (insets in **Figure 6a**), while the lignin composite with CPO facilitates enhanced integration of the lignin composite into the wounded tissue over 7 days. There was no difference of the rate of the wound closure, as determined by epithelial gap (the distance between the arrows), between UNTX and TLS (5.0±1.4 vs. 5.8±1.2 mm, $p>0.05$), CPOc (5.0±1.4 vs. 4.7±0.8 mm, $p>0.05$), or CPO (5.0±1.4 vs. 4.4±0.7 mm, $p>0.05$) treated mice (**Figure 6b**). We observed a trend of increasing granulation tissue, especially with CPO treatment (1.4±0.2 vs. 2.5±1.0 mm^2, $p<0.05$) (**Figure 6c**), which is indicative of healthy healing progress.

Increased vascularity is promoted by the lignin composite with CPO over 7 days

In addition to the increase of granulation tissue, neovascularization is promoted by the lignin composites with CPO. Quantification of neovascularization (**Figure 7a**) showed that UNTX has a significantly higher number of $CD31^+$ endothelial cells while the formation of blood vessel was delayed at day 7 (**Figure 7b**). Staining with the endothelial cell marker (CD31) revealed a greater number of individual $CD31^+$ cells that are not associated with vessels in UNTX wounds (**Figure 7b**) per HPF than TLS- (36.4±19.8 vs. 11.6±6.3%, $p<0.05$), CPOc- (36.4±19.8 vs. 4.2±4.3%, $p<0.01$) or CPO- (36.4±19.8 vs. 15.1±7.7%, $p<0.05$) treated wounds. However, there were significantly more vessel lumens (**Figure 7c**) per HPF in the CPO- (17.3.2±7.6 vs. 17.3±7.5 vessels/HPF, $p<0.05$) treated wounds as compared to UNTX wounds.

As shown in **Figure 7d**, αSMA^+ cells (brown staining) were limited to the edge of wounds in UNTX and TLS. Lignin composites with either CPO or CPOc showed more αSMA^+ cells in the wounds. However, the lignin composite with CPO showed a higher numbers of blood vessel formation both at edge and middle of wounds (**Figure 7b**). As several literature showed that the expression of αSMA^+ implicates the development of vasculature[36,37] and expresses in pericytes on capillaries[38]. While the extent of difference at either edge or middle sections was not significantly different (**Figure S3**), the overall trend was an increase with CPO NPs in lignin composites (**Figure 7e**). We observed similar trends in the number of $CD31^+$ cells (**Figure 7b**) and αSMA^+ cells (**Figure 7e**). The expression of αSMA^+ by myofibroblasts (myoFB) is also shown to underlie tissue regeneration in the skin, and the number of αSMA^+ cells decrease as the regeneration process is completed[39]. Thus, neovascularization is promoted, and the wound healing is still in progress by day 7 with the injection of the lignin composite with CPO.

Lignin composites did not cause significantly inflammatory responses over 7 days

Following the quantification of granulation tissue and neovascularization, inflammatory responses were assessed by immunohistochemical staining (**Figure 8**). CD45 is expressed by common leukocytes except platelet and red blood cells. Ly6G is expressed by monocytes, granulocytes and neutrophils. F4/80 is used to identify murine tissue macrophages (Mφ). CD206 is normally expressed on the M2 (alternatively activated) Mφ. Over 7 days, no significant changes in the expression of the four markers were noticed. There was no significant difference in percentage of $CD45^+$ (pan-leukocyte) cells/HPF across all tested lignin composites when compared to UNTX wounds (UNTX 40.3±5.7 vs. TLS 33.3±1.5 vs. CPOc 38.7±11.7 vs. CPO 34.0±8.2%, $p>0.05$) (**Figure 8b**). A similar trend was observed when looking at monocyte, granulocyte, and neutrophil infiltration as determined through Ly6G staining (**Figure 8c**). There were no significant differences in the percentage $Ly6G^+$ cells/HPF among treatment groups (UNTX 24.0±11.0 vs. TLS 29.1±8.1 vs. CPOc 29.3±6.3 vs. CPO 25.1±10.5, $p>0.05$). Mφ, particularly alternatively-activated macrophages, are important mediators of successful wound healing[40]. F4/80 and CD206 staining showed that UNTX wounds had no statistically significant differences in the levels of $F4/80^+$ cells per HPF (UNTX 22.3±10.0 vs. TLS 21.9±9.6 vs. CPOc 22.8±7.3 vs. CPO 35.6±12.0%, $p>0.05$) or $CD206^+$ cells per HPF (UNTX 49.9±10.5 vs. TLS 42.8±8.9 vs. CPOc 44.0±5.5 vs. CPO 47.0±7.2%, $p>0.05$). As shown in **Figure 8a**, the interface between wounds and lignin composites does not show (indicated by white wedges) significant infiltration of

$CD45^+$ cells. Recently, the stiffness of GelMA is known to directs Mφ phenotypes. Soft GelMA matrix (stiffness less than 2 kPa) is more favorable for priming Mφ toward anti-inflammatory phenotypes with a decreased capacity for spreading in comparison to stiff (over stiffness 10 kPa) GelMA matrix[41]. The lignin composites are in the range of 1-2 kPa of storage modulus (**Figure 5c**), thus likely priming Mφ toward anti-inflammatory phenotypes.

While collagen content is not significantly modulated, the lignin composite with CPO exhibits mature collagen architecture over 30 days

Tissue sections are prepared to assess collagen architecture and content over 30 days of healing process from trichrome staining. In rodents, normal tissue has a reticular collagen pattern, whereas the collagen in scar tissue forms large parallel bundles at approximately right angles to the basement membrane[42]. The lignin composite with CPO exhibits more pronounced, bundled mesh network as opposed to relatively straight fibers found in TLS or CPOc (**Figure 9a**). Despite qualitative difference of collagen architecture, the quantity of collagen content over 30 days becomes similar to each other (**Figure 9b**). No differences were observed in the overall collagen density between UNTX and TLS- (187.0±23.8 vs. 170.0±26.4 pixels/HPF, $p>0.05$), CPOc- (187.0±23.8 vs. 179.6±21.5 pixels/HPF, $p>0.05$), or CPO- (187.0±23.8 vs. 180.9±29.4 pixels/HPF, $p>0.05$) treated wounds. However, reduction of collagen content in a scar has less impact on scarring outcome compared to the structure of matrix deposited in the dermis[43]. Further, wounds treated with CPO left minimal scar as evidenced in photographs taken at 30 days after surgery (**Figure S4**).

DISCUSSION

Application of lignin to wound healing and tissue engineering applications is increasing

Application of lignin is ever increasing in a number of industrial and biomedical applications, including UV absorption[44], Cu^{2+} chelating in water[45], skin protectants[46], wound dressing with chitosan[47], self-healing smart biomaterials[48], durable and repeatable adhesive[49], antibacterial agent[50,51] with metallic NPs[52], anti-proliferative effect to cancer cells without cytotoxicity[53]. In tissue engineering, lignin composites can be applied to enhance mechanical properties with good protein adsorption capacity and wound compatibility[54], to provide 3D microenvironments of mesenchymal stromal cell (MSC) culture[47], to confer anti-inflammatory properties by reducing gene expression of iNOS and IL-1β of inflamed mouse Mφ[55], to apply for wound-dressing with biocompatibility and nontoxicity[56], to improve mechanical properties and wound healing capabilities on incision-induced Sprague-Dawley rats[57], to provide nanofibers for the cultures of human dermal fibroblasts (hDFs)[58], to promote localized and prolonged antioxidant capabilities for wound healing applications[59] and to achieve enhanced mechanical properties and viability of hDFs for direct ink writing 3D bioprinting[60].

Nevertheless, toxicological studies of lignin have only carried out through simple cytotoxicity testing, which cannot accurately simulate the human body environment. Thus, the biological effects of lignin with *in vivo* animal models should be tested to assess the long-term stability and potential negative effects of lignin and lignin-derived biomaterials. In addition to toxicological studies, another difficulty and key point for the development of lignin-based biomaterials is the heterogeneity of lignin. Although lignin displays good potential for biomedical applications[61], its broad molecular weight distributions and extremely complex structures represent big hurdles for deep studies, standardization, and scale-up. Thus, lignin chemistry and de/polymerization techniques still need to be continuously developed[62-65].

CPO is widely applied for oxygen generating compounds for tissue regeneration

The injectable lignin composite can be a unique platform to confer antioxidation by TLS

and oxygen generation from CPO NPs without incorporating additional enzymes. Application of CPO into NP of SLS hydrophilic outer layer and PLGA hydrophobic core[26] allows CPO to be complexed with PLGA. CPO is a white to yellowish powder which has been extensively applied in water treatment, seed disinfection and food processing[66]. When reacted with water at pH lower than 12 (**Figure S5A**), CPO can be decomposed into hydrogen peroxide, hydroxide ions and carbonate, and the generated hydrogen peroxide will further decompose into highly reactive superoxide and hydroxyl radical[67].

The catalase enzyme can decompose the intermediate hydrogen peroxide into water and oxygen. While catalase is an enzyme found in the blood and liver of mammals, this enzyme has to be available *in situ*, not from peroxisome to prevent damages from ROS. This intermediate step eliminates any potential cytotoxic ROS[68]. Without catalase, the cytotoxic byproduct H_2O_2 may lead to cell damage.[69] Although the actual role of catalase in the oxygen-release process is unclear, decomposition is suggested to take place through the Modified Fenton chemistry – dissociation of H_2O_2 to OH radicals with $Fe^{2+}/^{3+}$[70]. These oxidants react with everything within diffusion limit layer while their half-lives are short. In wound healing, the availability of catalase is limited. Even though the level of expression of catalase mRNA is not changed during wound healing[71], the protein level of catalase is decreased[72]. For example, catalase concentration is reported to be in the range of 50 – 100 U/mL in the oxygen generating gelatin section[73] or 1 mg/mL in the matrix of polycaprolactone (PCL)[74]. In the mixture of CPO, catalase and SLS or TLS, the dissociation of dissociation of H_2O_2 is predominantly facilitated by catalase and partly by TLS and to less extent by SLS (**Figure S5B**). The native antioxidant properties of SLS or TLS (**Figure S6A**) is maintained at levels of 80% or above of the native antioxidant up to 50 µg/mL of CPO (**Figure S6B**).

Several cases of CPO incorporation to biomaterials are reported to date. Injectable cryogels are formed with methacrylated hyaluronic acid and CPO[75]. Cumulative release of H_2O_2 is up to 468 µmol over 3 h. Microparticles (MP) are formed with the encapsulation of CPO in PCL, then formed composites with GelMA[76]. Over 2 weeks, 2.5 µM of H_2O_2 is released without the formation of MPs while 1.0 µM with the formation of MPs. With 5 to 20 mg/mL of CPO, the cumulative release of O_2 is 25% without MP and up to 30% with MP. Some cases include catalase (100 U/mL) and CPO (up to 30 mg/mL)[77,78]. GelMA (50 mg/mL)-CPO (30 mg/mL) with catalase (100 U/mL) releases O_2 up to 5 days[77]. Another type of hydrogel utilizes Ca^{2+} from CPO (up to 10 mg/mL) to crosslink gellan gum (anionic polysaccharide) with catalase[79]. In the matrix of silk/keratin/gelatin, CPO up to 200 mg/mL release O_2 over 2 weeks with additional antibacterial properties for urethral tissue engineering applications[80]. Thiolated gelatin (27 to 63 mg/mL) with CPO (25 to 100 mg/mL) and catalase (2000-5000 U/mg) forms hyperbaric oxygen generating biomaterials.[81] The question is how much CPO is required to efficiently deliver O_2 to tissue microenvironments without causing cytotoxicity. CPO concentrations higher than 10 mg/mL exhibit cytotoxicity to 3T3 FBs[78]. In addition, due to low solubiliuty in water, it is difficult to disperse CPO in aqueous buffers. Further, Ca^{2+} released from these composites influence cell cytotoxicity and bacterial adhesion[82].

To assess the kinetics of O_2, *in situ* oxygen measurement with patch sensors are used[74,81,83]. In the case of H_2O_2, Cu(II)-neocuproine spectrophotometric method[84] or free radical analyzer[85] are used. To delineate the difference, the release kinetics of O_2 and H_2O_2 from CPO in water with varying pH and temperature is probed and modeled.[86] The release of H_2O_2 follows pseudo-zero order (PZO) reaction. Once CPO is dissolved in water, Ca^{2+}, O_2^{2-} and H^+ are formed. Of these, $2H^+$ and O_2^{2-} form H_2O_2. With increase of H^+ (decrease of pH), the solubility of CaO_2 is increased. Temperature has minimal to no effect in the kinetics. In contrast, the release of O_2 follows pseudo-first order (PFO) reaction, meaning that the concentration of CPO directly affects the dissociation of CPO to O_2. With the decrease of pH, the release of O_2 is decreased. With the increase of temperature, the release of O_2 increases. From this study of kinetics, H_2O_2 is not necessarily a precursor of O_2.

Locoregional delivery of antioxidation and oxygen generation could be beneficial to accelerate wound healing

WT mice are programmed to heal physiologically with minimal alterations in perfusion and ROS, thus we did not see significantly different results in our measures of wound healing, vessel formation, and inflammation between treatment groups. The translatable benefit of this lignin composite may be more readily apparent in disease states such as diabetic wound healing.

The advanced glycation end products (AGEs) present in diabetic tissue generate ROS leading to fibroblast apoptosis via the NLRP3 signaling pathway, thus impairing wound repair[87]. Some systemic oral anti-hyperglycemics currently used to treat diabetes mellitus (DM) also have antioxidant and beneficial wound healing effects. Metformin, for example, is a commonly used medication used for glucose control in DM patients which has antioxidant effects and demonstrated benefits on angiogenesis and wound closure in diabetic mice[88]. However, as previously mentioned, pure systemic antioxidants have a poor enteric absorption profile. Locally applied antioxidant hydrogels have been demonstrated to accelerate diabetic wound healing while promoting M2 Mφ differentiation and reducing IL-1β production[89]. Further, topical oxygen delivery to ischemic wounds is shown to accelerate healing and promote granulation tissue formation[90]. The strategy of combining these two wound-beneficial attributes into one local therapy for pathologic wounds has the potential to greatly benefit the wound healing in at-risk patients.

CONCLUSIONS

Lignin has been applied to many different tissue engineering applications. Wound healing applications of lignin has been somewhat limited to doping NPs into a matrix of biomaterials. First, we functionalized lignosulfonate to form lignin composites with TLS. TLS maintained antioxidation capacity in GelMA to attenuate fibrotic responses of hdFBs. Then, we designed CPO NPs (SLS:PLGA NPs with CPO) to deliver O_2 without cytotoxicity to accelerate wound healing in addition to the antioxidation properties conferred by TLS. The release of O_2 from lignin composites with CPO NPs and TLS was around 0.05%/day at least for 7 days. Mechanical properties of lignin composites were not significantly different, but the stiffness set for the *in vivo* applications was amenable to direct M2 Mφ phenotypes. Swelling and enzymatic degradation of lignin composites *in vitro* were verified before the animal experiments. The area of granulation tissue was modestly increased by the lignin composite with CPO while the rate of wound closure was not significantly different by 7 days after surgery. However, the lignin composite with CPO increased neovascularization as evidenced by significantly increased blood vessel formation and by the infiltration of αSMA^+ cells to the wound treated with the lignin composite with CPO over 7 days. We observed minimal inflammatory responses by 7 days after surgery. For scar assessment, we sacrificed mice at 30 days after surgery. Although we did observe no difference in the overall collagen density, the collagen architecture treated by the lignin composite with CPO exhibited more pronounced, bundled mesh network and left minimal scar. Thus, soft matrix with antioxidation (conferred by TLS) and synergistic oxygen release (CPO NPs) could be applied to wound healing applications with enhanced tissue granulation, vascularization and maturation of collagen architecture without significant inflammatory responses. Further optimization on the balance between antioxidation and O_2 is in progress, aiming to tackle diabetic wound healing with infection.

AUTHOR CONTRIBUTIONS

JPJ and SB conceived the experiments. JAB, CAE and DS synthesized lignin composites. NTD performed PXIE. SEJ performed swelling, degradation and rheometry of lignin composites. ACG and JCG quantified O_2 and H_2O_2 release from lignin composites. JAB performed fibrosis array. SB, WDS, BWP, TJP and FF did animal surgery and animal data collection.

WDS, BWP, TJP, FF, AK, and OSJ analyzed the results of animal experiments. JPJ, SB, MK, WDS and BP wrote the manuscript and all authors approved the final manuscript.

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DISCLOSURE

None

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FIGURES

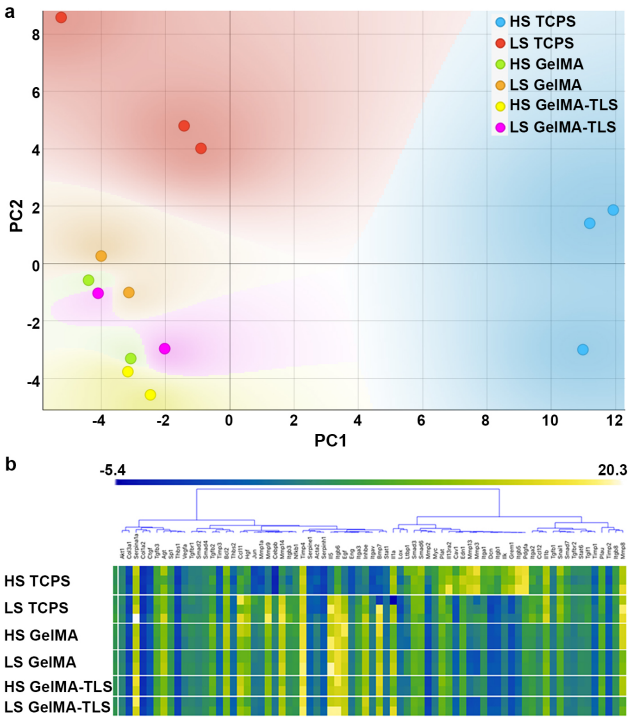

Figure 1. Fibrosis array of human dermal fibroblasts (hdFBs). (a) PCA of 84 fibrosis-related genes from high scar (HS) and low scar (LS) hdFBs cultured on three different substrates (TCPS, GelMA and GelMA-TLS) (b) HC of 84 fibrosis-related gene. LS exhibits a proliferative phenotype and GelMA or GelMA-TLS composites modulates the profiles of the fibrosis-related genes.

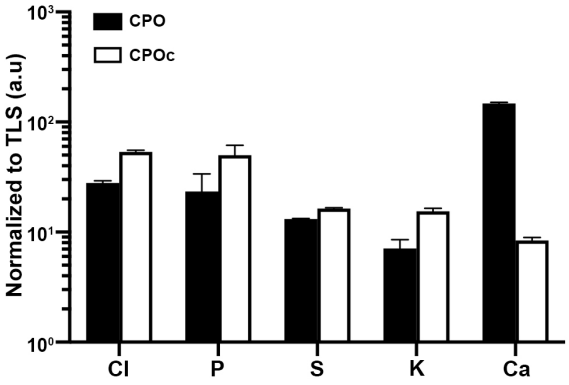

Figure 2. Elemental analysis of CPO and CPOc NPs by microPXIE. The concentration (arbitrary units, a.u.) of Cl, P, S, K and Ca in CPO and CPOc (NPs without CPO) NPs normalized to TLS NPs. The bars represent counting uncertainties (1σ) in a single measurement analyzed by GeoPIXE software; mean±SEM.

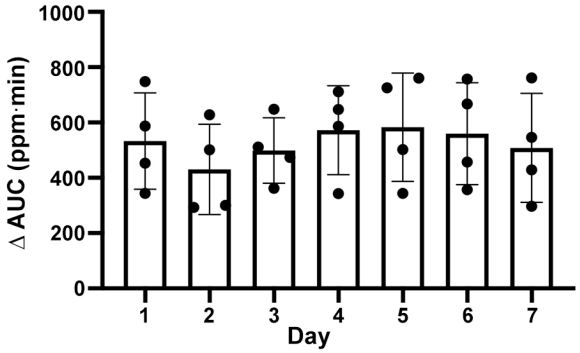

Figure 3. Quantification of O_2 from CPO composites. Oxygen released from CPO composites was measured using a planar O_2 sensor attached to the bottom of a well in a 96-well plate. Area under curve (AUC) was calculated and the differential between CPO and CPOc (4 mg/mL) over 1440 min is reported. One-way ANOVA with Tukey's *post hoc* test, no significant difference to each other; mean±SD, n=4.

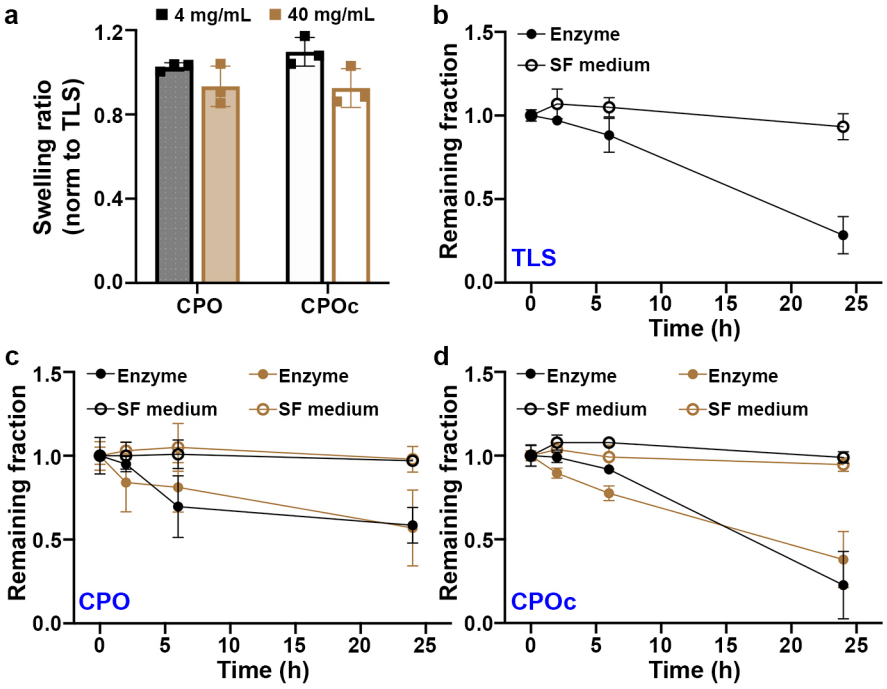

Figure 4. Swelling ratios and degradation of CPO and CPOc composites. (a) Swelling ratios normalized to TLS. No statistical difference between CPO and CPOc at the same concentration and between two different concentrations at each composite type. Student's *t*-test showed no statistically significant difference; mean±SD, n=3. Degradation of lignin composites, TLS (b), CPO (c) and CPOc (d), in the solution of 0.5 U/mL collagenase (enzyme) or serum-free medium (SF medium). In (c) and (d), black and brown symbols represent CPO or CPOc concentrations of 4 and 40 mg/mL, respectively; mean±SD, n=3.

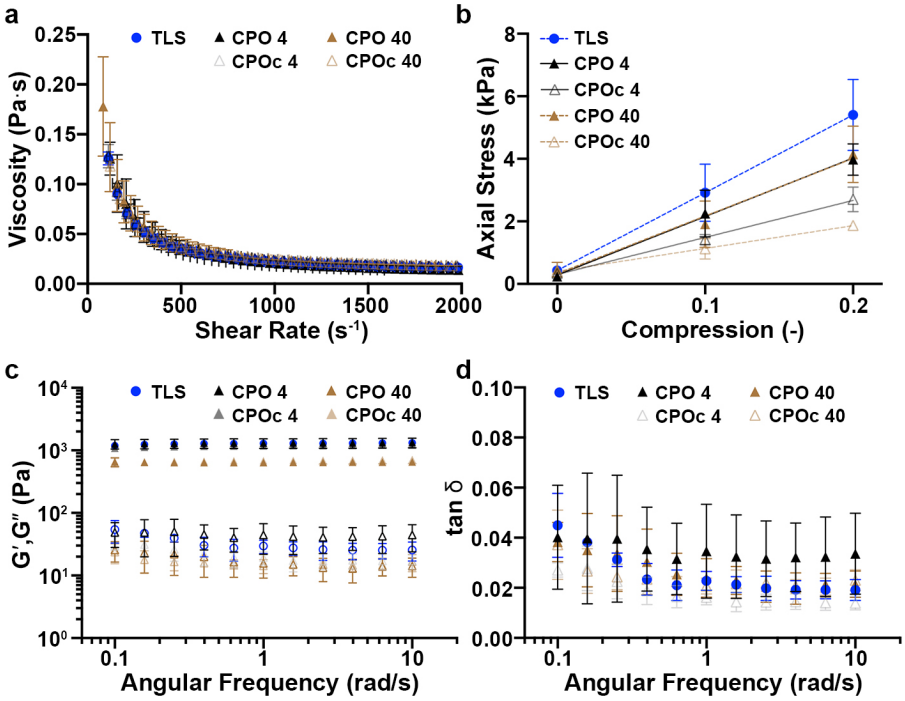

Figure 5. Oscillating rheometry of lignin composites. (a) Viscosity of each precursor before photo-crosslinking. (b) Axial stresses are plotted against compression varying from 0 to 20%. (c) Frequency sweeping of lignin composites. Solid and open symbols represent G′ (storage modulus) and G″ (loss modulus), respectively. (d) Loss tangent (δ) of lignin composites from 0.1 to 10 rad/s. mean±SD, n=3 for all samples.

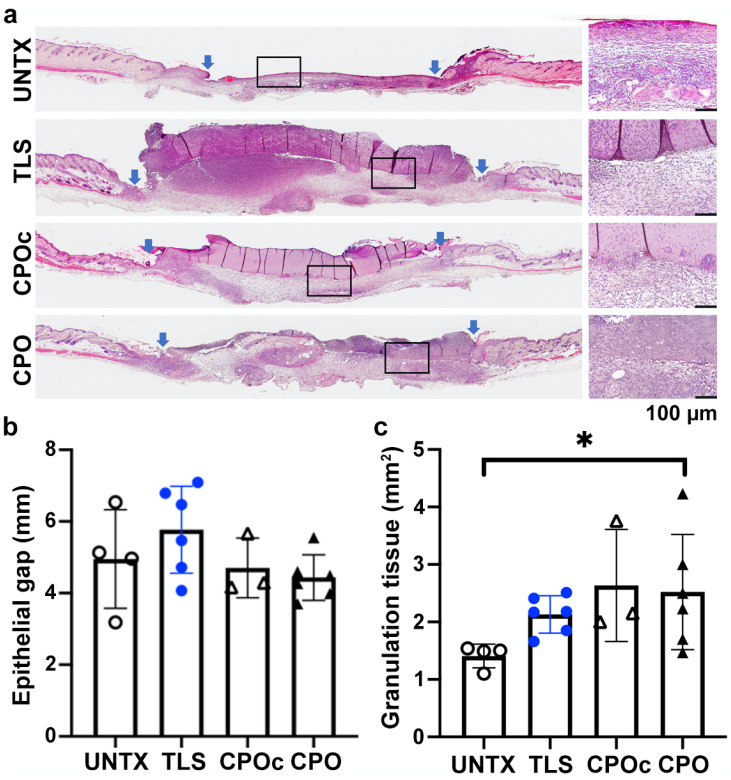

Figure 6. Morphometric analysis of wounds treated with lignin composites at 7 days after surgery. Wounds in WT C57BL/6N mice were treated with lignin composites (a) to measure epithelial gap (b) and granulation tissue area (c). In (a), boxed areas are provided in inset images at higher magnification of hematoxylin (blue, nuclei) and eosin (red, ECM and cytoplasm) staining. UNTX, untreated; TLS, GelMA-TLS; CPOc, GelMA-TLS-CPOc (CPO control); CPO, GelMA-TLS-CPO. Scale bar, 100 µm. One-way ANOVA with Kruskal-Wallis test followed by Dunn's multiple comparison test. *$p<0.05$. $3 \leq n \leq 6$, mean±SD.

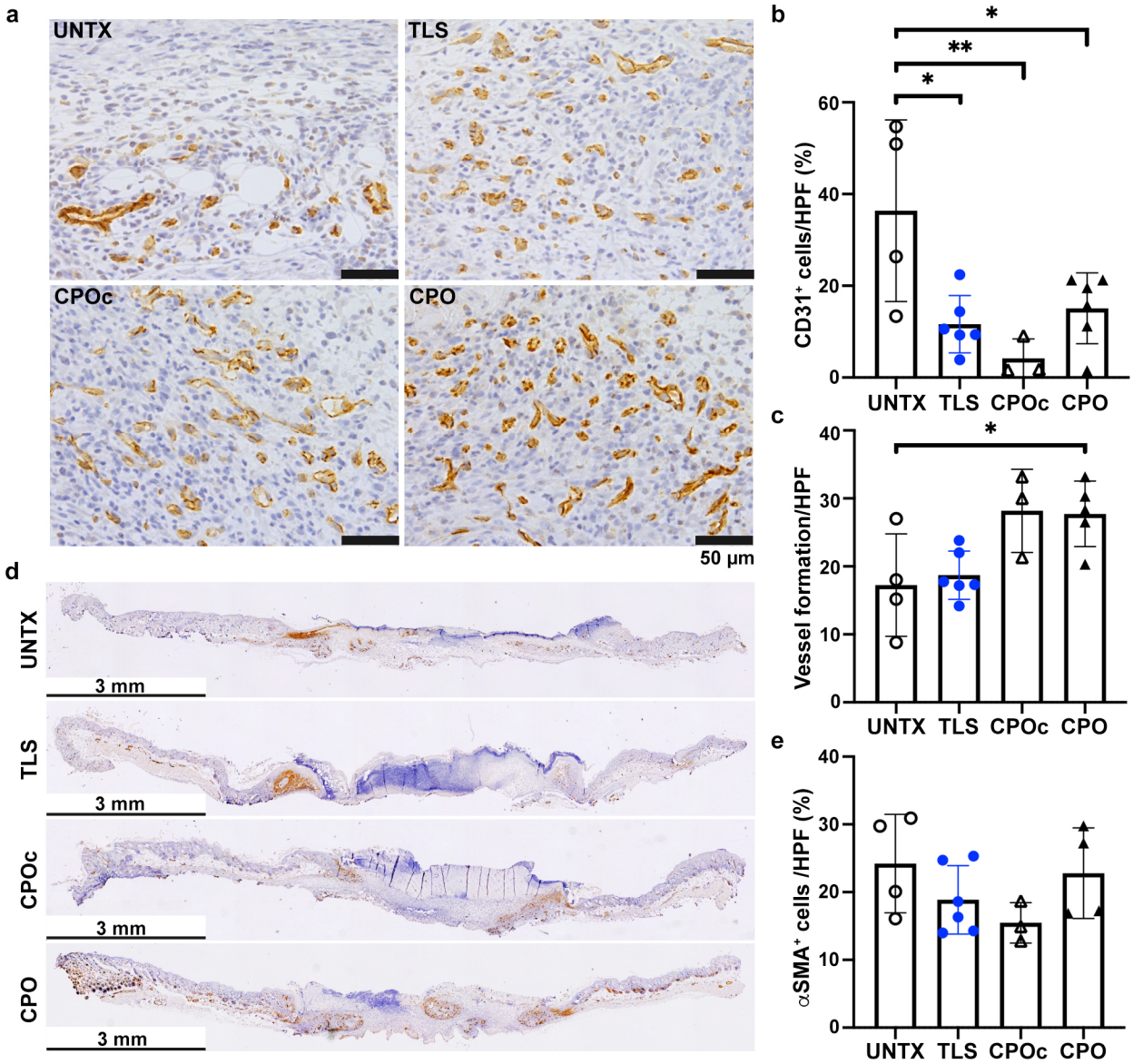

Figure 7. Assessment of vascularization in the wounds treated with lignin composites at 7 days after surgery. Wounds in WT C57BL/6N mice were treated with lignin composites to assess the extent of vascularization with immunostaining (CD31, brown) and hematoxylin counterstaining (blue, nuclei). $CD31^+$ cells (b) and vessel formation (c) per HPF were quantified. The infiltration of αSMA^+ cells to wounds was visualized (d) and quantified per HPF (e). UNTX, untreated; TLS, GelMA-TLS; CPOc, GelMA-TLS-CPOc (CPO control); CPO, GelMA-TLS-CPO. Scale bars, 50 μm in (a) and 3 mm in (d), respectively. One-way ANOVA with Tukey's *post hoc* tests, $^{**}p<0.01$ and $^{*}p<0.05$. $3 \leq n \leq 6$, mean±SD.

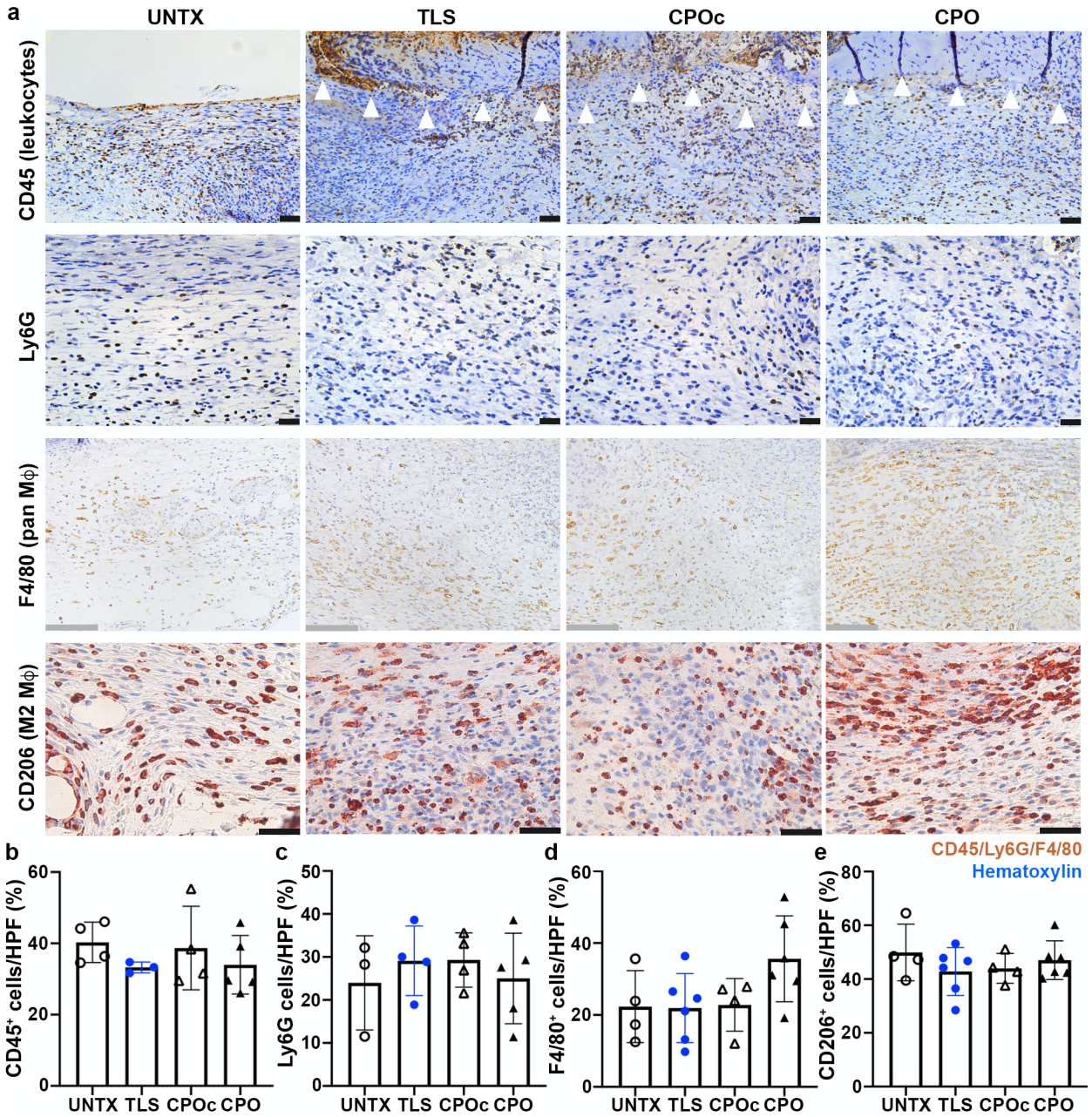

Figure 8. Assessment of inflammatory responses from the wounds treated with lignin composites at 7 days after surgery. Wounds in WT C57BL/6N mice were treated with lignin composites to assess the extent of inflammatory responses (a) with immunohistochemical staining and hematoxylin counterstaining (blue, nuclei). $CD45^+$ leukocytes (b), $Ly6G^+$ monocytes, granulocytes, and neutrophils (c), $F4/80^+$ pan Mφ (d) and CD206 M2 Mφ (e) per HPF were quantified. UNTX, untreated; TLS, GelMA-TLS; CPOc, GelMA-TLS-CPOc (CPO control); CPO, GelMA-TLS-CPO. In (a), black scale bars, 50 μm and grey scale bars, 125 μm, respectively. $3 \leq n \leq 6$, mean±SD.

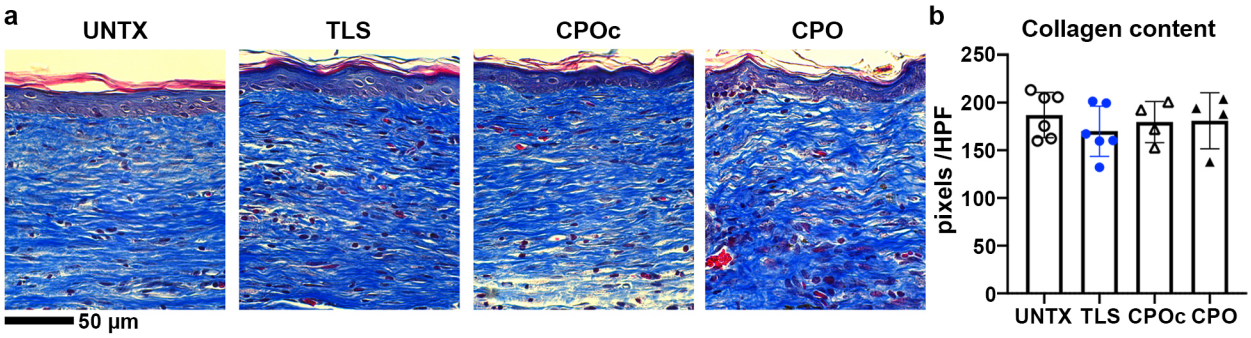

Figure 9. Scar assessment in the wounds treated with lignin composites at 30 days after surgery. Wounds in WT C57BL/6N mice were treated with lignin composites to assess the collagen content with trichrome (blue, collagen) (a). Collagen content was quantified per HPF using color moment thresholding in ImageJ (b). UNTX, untreated; TLS, GelMA-TLS; CPOc, GelMA-TLS-CPOc (CPO control); CPO, GelMA-TLS-CPO. Scale bar, 50 µm. 3≤n≤6, mean±SD.