Phenol red-silk tyrosine cross-linked hydrogels

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1. Introduction:

Two dimensional mammalian cell culture is fundamental for expanding cells towards engineering three dimensional tissues *in vitro*. Commercially available mammalian cell culture media use phenol red (pKa: 7.9) as a visual pH indicator, switching from red to yellow, indicating a change from neutral to acidic pH. However, phenol red bears structural similarity to nonsteroidal estrogens and exhibits weak estrogenic activity on estrogen-sensitive cells (e.g., breast cancer cells) at concentrations found in cell culture media [1, 2]. Removal of lipophilic impurities in phenol red preparations can ameliorate estrogenic activity [3], but phenol red-free medium is often used when working with estrogen-sensitive cell types [4, 5]. Additionally, phenol red is removed from cell culture media during live cell imaging due to its absorbance at 432 nm at pH 7.0, quenching fluorescence from fluorophores emitting in the 400-500 nm range (e.g., Green Fluorescent Protein (GFP), Cyan Fluorescence protein (CFP), Fluorescein isothiocyanate (FITC) etc.). Therefore, there is a need for optical methods of pH sensing when using phenol red free media for cell culture applications.

Optical pH sensing hydrogels are based on pH-dependent changes in absorbance or fluorescence of indicator molecules that are immobilized within the polymeric network. Immobilization of pH indicator molecules is critical in the fabrication process to avoid leaching and can be performed by adsorption, covalent binding or entrapment [6, 7]. Phenol red based pH sensors have been prepared using sol-gel silica glasses via adsorption and entrapment [7-10], however, leakage of the dye is a problem [8]. Addition of surfactants and organosilanes reduced dye leakage from the gel, although extended response times and altered pKa values are reported in these systems [8].

Covalent linkage of phenol red results in leak-proof hydrogels, but covalent binding methods are challenging due to the lack of amino or carboxyl groups on phenol red [6]. Phenol red was covalently linked to diacetylcellulose and polyvinyl alcohol (PVA) membranes using formaldehyde [6]. These hydrogels had rapid response times (2-50 seconds) for pH sensing, marginally altered pKa values (7.9 to 8.37) and had a broad pH response range [6]. However, the cytocompatibility of these sensors is not clear due to the use of formaldehyde in the crosslinking process [6, 11]. Phenol red was copolymerized with

polyacrylamide microspheres to achieve continuous pH monitoring and slightly altered pKa (7.9 to 7.57) values [12]. The phenol red bound polyacrylamide hydrogels were fluorescent when dry, although pH dependent fluorescence and cytocompatibility are not reported and these gels were non-degradable [12]. A microarray based fluorescent pH and oxygen sensor for cell culture applications was generated and employed UV cross-linked poly(ethylene glycol) diacrylate (PEG-DA) hydrogels using a photo-initiator, entrapping either 2',2'-Bis-(2-carboxyethyl)-5-(and-6)-carboxyfluorescein-dextran conjugate (BCECF-dextran) or (dichlorotris (1,10-phenanthroline) ruthenium(II) hydrate (Ru(Phen))) ruthenium complex for detecting changes in pH and oxygen respectively [13]. When cross-linked hydrogels were placed in aqueous solution, non-homogeneous swelling resulted in leaching of ruthenium complex, but not BCECF-dextran due to larger molecular size, raising concerns of ruthenium toxicity [13]. Leaching of ruthenium ceased after the initial swelling and PEG hydrogels with both fluorophores were tested for oxygen and pH sensing in a bioreactor for 2 weeks, however, toxicity of ruthenium remains an issue.

Silk fibroin is a natural protein biopolymer extracted from *Bombyx mori* silkworm with good cytocompatibility and extensive applications as a cell encapsulation matrix for tissue engineering [14]. Previous reports from our laboratory have shown that phenol red dye can be entrapped within silk films for pH sensing [15]. Drying silk films resulted in beta sheet structures and the dye was retained within the matrix. But, leak-proof functionality of this system in aqueous solution was not reported.

Herein, we report a method for covalent linkage of phenol red to silk hydrogels via horseradish peroxidase (HRP)-mediated tyrosine crosslinking and provide a solution for optical pH sensing in phenol red free cell culture media. We show that these protease degradable silk tyrosine cross-linked hydrogels prepared with DMEM media have improved crosslinking kinetics due to the presence of phenol red and the reaction is concentration dependent. At low phenol red concentrations, release studies confirmed no dye leakage from the hydrogels post crosslinking and phenol red absorption spectra confirmed chemical interaction of the dye with the silk hydrogel network. Further, LC-MS was used to characterize the reaction products of phenol red and tyrosine crosslinking, confirming the covalent bonding of phenol red dye to N-acetyl-L-tyrosine. Live-Dead staining and alamar blue assays with primary lung fibroblasts

encapsulated within phenol red-silk hydrogels showed no toxicity and confirm the cytocompatibility of the hydrogels. Finally, we show that phenol red-silk hydrogels can function as fluorescent pH sensors, due to the di-tyrosine fluorescence quenching of phenol red, with a pKa of 6.8, suitable for cell culture applications.

2. Methods:

2.1. Preparation of aqueous silk solution: Aqueous silk solutions were prepared using previously established procedures [14, 16]. Briefly 5 g of *B. mori* silkworm cocoons were immersed in 2 L of boiling 0.02M Na₂CO₃ (Sigma-Aldrich, St. Louis, MO) solution for 60 mins. The degummed silk fibers, removed of sericin protein, were subsequently rinsed three times with distilled water and air-dried. To produce aqueous silk solution, the fibers were solubilized in a solution of 9.3M LiBr (Sigma-Aldrich, St. Louis, MO) at 60°C for 4 hrs, followed by dialysis against 2L of distilled water using a regenerated cellulose membrane (3.5kDa MWCO, Spectrum Laboratories, Rancho Dominguez, CA). The solubilized silk protein solution was then centrifuged twice (12,700g, 20 mins, 4°C) to remove insoluble particulates and stored in 4°C until the day of experimentation. All silk solutions were used within 30 days from the date of storage or discarded. For sterilization, silk solutions were filtered with a 0.2 µm polyethersulfone (PES) Nalgene bottle top filter (Thermo Scientific, Rockford, IL). Silk protein concentration was established by drying a known mass of silk solution and assessing the mass of the remaining solids.

2.2. Preparation of Phenol red-silk di-tyrosine hydrogels: Silk di-tyrosine cross-linked hydrogels were prepared using Horseradish peroxidase (HRP) type IV (Sigma-Aldrich, St. Louis, MO), and hydrogen peroxide (Sigma Aldrich, St. Louis, MO) at the same concentrations established previously [14]. Briefly, 10 µl of 1,000U/ml stock solution of HRP and 10 µl of 165mM hydrogen peroxide were added to 1 ml of 3% (w/v) silk solution (final concentration of 10U/ml of HRP and 1.65mM H₂O₂) at 37°C for di-tyrosine crosslinking (termed silk-H₂O hydrogels, as the solvent used for casting is water). DMEM media with high glucose, L-glutamine and phenol red (Life Technologies, Grand Island, NY) was used to prepare silk-0.5xDMEM+PR gels, where 1xDMEM media is diluted to a final concentration of 0.5x in the gels.

The same DMEM formulation without phenol red was used to prepare silk-0.5xDMEM-PR gels. Phenol red (Sigma-Aldrich, St. Louis, MO) was solubilized in 0.1N NaOH to produce a stock solution at 5 mg/ml. Silk-1xPBS gels were prepared by diluting 10xPBS to a final concentration of 1x in the formulation. To prepare phenol red-silk tyrosine hydrogels with 1xPBS as solvent, 7.5mg/L, 15mg/L or 30 mg/L phenol red was added, where 15mg/L is the concentration of phenol red found in commercial DMEM media (catalog number: 11965-092, Life Technologies, Grand Island, NY).

2.3. Rheology: Rheological measurements were conducted on di-tyrosine cross-linked silk hydrogels to determine gelation time and mechanical strength [14]. All experiments were carried out using a TA instruments ARES-LS2 rheometer (TA Instruments, New Castle, DE) using a 25 mm stainless steel conical plate (angle: 0.0994 rad) and a temperature controlled Peltier plate set to 37°C. Briefly, phenol red-silk di-tyrosine hydrogels were prepared by mixing

600 μ l of the different constituents, at the concentrations mentioned earlier and 420 μ l was dispensed on the tester. Prior to gelation, the cone geometry was lowered to the specified gap and low viscosity oil was placed around the outside edge of the cone to prevent water evaporation. Dynamic time sweeps were conducted at a frequency of 1 Hz (6.283 rad/s) and 1% strain for 10,800 s or until the sample had reached a plateau modulus.

2.4. UV/VIS spectra: UV/VIS measurements were made using a Hitachi F4500 Spectrofluorometer (Hitachi, Schaumburg, IL). Wavelength sweeps of free phenol red or phenol red immobilized within silk di-tyrosine hydrogels were carried out in a 96 well glass bottom Sensoplate (Greiner Bio-one, Monrow, NC). Briefly, 1xPBS (pH 7.2) solutions were prepared at pH 3.0, 5.0, 9.0 and 11.0 by the addition of 1M HCL or 1M NaOH (Sigma-Aldrich, St. Louis, MO) and a pH meter. Three microliters (3 μ l) of phenol red stock solution was added to 1ml of 1xPBS at variable pH, for a final concentration of 15mg/L phenol red. One hundred and fifty microliters (150 μ l) of phenol red solution was then carefully pipetted into the 96 well plate (n=5) and measurements of absorbance were made at 300 nm to 700 nm at 1 nm increments. To obtain UV/VIS measurements of immobilized phenol red, 1 ml of phenol red-silk di-tyrosine hydrogel was prepared with water as solvent and 15mg/L phenol red concentration. One hundred and fifty

microliters (150 μ l) of this solution was added to each well of the 96 well plate (n=5) and placed in a 37°C incubator for 30 minutes. To change the pH of the gels, 100 μ l of 1xPBS at variable pH, was added on top of the gels and allowed to incubate for 1 hr at room temperature. Visible color changes were observed in the hydrogels at the 1 hr time point and absorbance measurements were made shortly afterwards.

2.5. Phenol red release studies: Phenol red release studies were performed in a 96 well glass bottom Sensoplate (Greiner Bio-one, Monrow, NC). Briefly, 100 μ l of silk-H₂O gels or silk-H₂O + phenol red (silk-H₂O+PR) were cast in a 96 well plate. **Silk-H₂O served as negative controls.** Silk-H₂O+PR gels were prepared using phenol red concentrations of 7.5 mg/L, 15mg/L and 30 mg/L (silk-H₂O+7.5mg/ml PR, silk-H₂O+15mg/ml PR, silk-H₂O+30mg/ml PR). Following crosslinking, wells containing hydrogels were flooded with 100 μ l of 1xPBS and allowed to incubate for different times. At 2, 4, 6, 8, 17 and 48 hours, supernatant was collected and wells were replenished with fresh 1xPBS solution. Phenol red absorbance at 430 nm was measured in supernatant 1xPBS solutions using a Hitachi F4500 Spectrofluorometer and a phenol red standard curve was created to study the cumulative release of phenol red from silk-H₂O hydrogels. To study whether phenol red was immobilized within the hydrogel network, a separate set of silk-H₂O gels were cross-linked and allowed to soak in 1xPBS containing phenol red (7.5 mg/L PR, 15mg/L PR and 30 mg/L PR) for 3 hours. Following incubation, hydrogels were placed in fresh 1xPBS solution and supernatant samples were collected at 2, 4, 6, 8, 17 and 48 hours respectively. Absorbance values were measured and cumulative release profiles were obtained using phenol red standard curve.

2.6. Fluorescence spectroscopy: To study di-tyrosine reaction kinetics, intrinsic fluorescence was measured after reacting N-acetyl-L-tyrosine and phenol red in different ratios. Briefly, 1ml of reaction mixture was mixed with 10U/ml of HRP and 1.65mM H₂O₂. One hundred microliters (100 μ l) of this mixture was then added to a 96 well glass bottom Sensoplate (Greiner Bio-one, Monrow, NC) and fluorescence was measured every minute for 30 minutes using excitation and emission wavelengths 290 nm and 415 nm, respectively, at 37°C. N-acetyl-L-tyrosine was solubilized in water to produce a stock solution at 50mM. Due to low solubility in water, phenol red was solubilized in 0.1M NaOH to form a

stock solution at 5 mg/ml (14mM). In the first set of experiments, di-tyrosine fluorescence time sweeps were recorded with constant phenol red concentration of 15mg/L (0.04 mM) and increasing concentrations of N-acetyl-L-tyrosine (0.5 mM to 25mM). In the second set of experiments, N-acetyl-L-tyrosine concentration was kept constant at 0.5mM and increasing molar ratios of phenol red was added (0.04mM to 5mM).

2.7. Liquid chromatography and Mass Spectroscopy (LC-MS): Mass Spectrometry- an Agilent 6410 triple-quadrupole mass spectrometer equipped with in positive electrospray ionization mode was used in this study. The Agilent Mass Hunter software operated the spectrophotometer in scan mode between 200 m/z and 1000 m/z. The nitrogen gas temperature was set at 300⁰C and a flow rate of 11 L/min. The nebulizer pressure was 45 psi and the capillary voltage was set to 4000 V. Solutions of N-acetyl-L-tyrosine, phenol red and HRP-reacted N-acetyl-L-tyrosine were evaluated to confirm detection and m/z for the reactants and products. HRP was removed from the reaction mixture using a 3.4 kDa MWCO centrifugal filter (EMD Millipore, Billerica, MA). Solutions were directly injected into the mass spectrophotometer at 33 μ L/min. For LC-MS analysis, an Agilent 1200 series LC was used. Twenty microliters of sample was injected in a C18 analytical column (Zorbax Eclipse Plus, 4.6 mm $\times$ 75 mm, 3.5 μ m, Agilent, Santa Clara, CA) at 40 $^{\circ}$ C equilibrated with 95:5 water: acetonitrile containing 0.1% v/v formic acid. After 1.0 min, a gradient was started, ramping to 5:95 water: acetonitrile containing 0.1% formic acid over 6.0 min and held at high acetonitrile for 2.0 min.

2.8. Cellular viability using fluorescence microscopy: Normal Human Lung Fibroblasts (NHLF) (Lonza, Walkersville, MD) were cultured using Fibroblast Growth Medium-2 (FGM-2) bulletkit (Lonza, Walkersville, MD), containing basal media, serum, human Fibroblast Growth Factor-B (hFGF-B), insulin and GA-1000. Cells between passage 2 and passage 6 were used for all experiments. To assess viability, cells were encapsulated within silk-H₂O, silk-1xPBS, silk-1xPBS+PR or silk-0.5xDMEM hydrogels at a concentration of 1x10⁶ cells/ml of hydrogel precursor solution. One hundred and ten microliters (110 μ L) of cell suspension was then added to each well of a 24 well glass bottom plate (MatTek Corporation, Ashland, MA) and allowed to sit at 37 $^{\circ}$ C until gelation. After gelation, FGM-2 media was flooded into

the wells to stop crosslinking. At 24 hours post encapsulation, media was aspirated from the wells and hydrogels were washed once with warm 1xHank's Balanced Salt Solution (HBSS) (Sigma-Aldrich, St. Louis, MO) before placing them in 1xHBSS with 5 μ M calcein AM (BD Biosciences, San Jose, CA) and 3 μ M propidium iodide (PI) (Sigma-Aldrich, St. Louis, MO) for 20 minutes in 37°C. Hydrogels were washed twice with 1xHBSS before imaging them using a Keyence fluorescence microscope (Model: BZ-X) with appropriate filters. Using the Z-stack feature, image stacks of 100 μ m depth were acquired using a 20x objective. Step size was set to 30 μ m for all groups, based on average cell size. Representative of three fields were imaged for every sample and a total of 4 samples/group was used for achieving statistical significance. Images obtained using different filters (Calcein and PI) were subsequently analyzed using ImageJ software. Images from each channel were separated and thresholding was applied to isolate only stained cells of interest. Once thresholding was complete, images from different channels were merged, with pseudo-coloring and subsequently counted to obtain live and dead cell counts.

2.9. Metabolic activity: The relative metabolic activity of cells within each hydrogel was determined by AlamarBlue assay (Life Technologies, Grand Island, NY) according to the manufacturer's directions. The different hydrogels were cast into a 48 well Costar cell culture plates (Sigma-Aldrich, St. Louis, MO) and allowed to sit in 37°C until gelation. All hydrogels were washed once with warm 1xHBSS and incubated in FGM-2 medium with 10% AlamarBlue reagent for 4h at 37°C. Care was taken to protect the plate from exposure to light. Following incubation, aliquots of 100 μ l were placed into black 96 well plates and fluorescence quantified using a spectrofluorometer with the peak excitation and emission wavelengths of 570 and 585nm respectively. Acellular hydrogels were used to adjust for background fluorescence.

2.10. Statistical Analysis: Data are expressed as mean and error bars denote standard deviation. One or two-way analysis of variance (ANOVA) and Tukey post-hoc analysis were used to determine statistically significant differences. Statistical significance was accepted at the $p < 0.05$ level and indicated in figures as * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$.

3. Results:

3.1 Phenol red in DMEM media accelerated di-tyrosine silk crosslinking in a concentration dependent reaction:

Recent work from our laboratory showed that silk di-tyrosine hydrogels can be formed using water or 0.5xDMEM as solvent [14]. To study the effect of phenol red (PR), identical DMEM media formulation, with and without phenol red was used for testing. **Figure 1A** shows the increase in storage modulus of 3% (w/v) silk solution during crosslinking with either water (silk-H₂O) or DMEM (silk-0.5xDMEM) as solvent. **Figures 1B and 1C** show the storage modulus and time at crosslinking completion, respectively. Although, crosslinking was initiated at the same time across all samples, completion of crosslinking, defined as the point where there was no corresponding increase in storage modulus with time, was different between the groups. DMEM gels with phenol red (DMEM+PR) crosslinked in an average time of 62 minutes (62±4 minutes), significantly shorter ($p<0.001$) compared to DMEM gels without phenol red (DMEM-PR) which completed crosslinking at an average of 89 minutes (89±8 minutes). Apart from reducing crosslinking time, addition of phenol red also resulted in a significant increase ($p<0.01$) in storage modulus from 0.56kPa (DMEM-PR) to 0.72kPa (DMEM+PR). Silk-H₂O gels were significantly stronger ($p<0.0001$) than DMEM+PR gels and recorded an average storage modulus of 4.8kPa (4.78±0.04 kPa). However, both silk-H₂O gels and DMEM+PR gels completed crosslinking at approximately the same time of 60 minutes.

To establish the role of phenol red in di-tyrosine silk crosslinking, the effect of phenol red concentration was studied. DMEM is a complex media that constitutes a mixture of salts, amino acids and vitamins. To isolate the function of phenol red, a simpler solvent of 1xPBS was chosen. The final concentration of phenol red found in commercial 1xDMEM media is 15mg/L (0.042 mM). Therefore, concentrations of 7.5mg/L (0.021 mM), 15mg/L (0.042 mM) and 30mg/L (0.085 mM) phenol red were tested for their role in silk di-tyrosine crosslinking. **Figure 2A** shows time sweeps and increase in storage moduli; **Figure 2B and 2C** show storage moduli and time at crosslinking completion respectively. Silk-

H₂O gels were stronger with an average storage modulus of 4.76 kPa (4.76 ± 0.03 kPa) and addition of 1xPBS (silk-1xPBS) resulted in gels with significantly lower ($p < 0.0001$) storage moduli of 0.74 kPa (0.74 ± 0.09 kPa). Silk-1xPBS gels also recorded significantly longer ($p < 0.0001$) crosslinking times at 95 minutes (95 ± 3 mins) compared to silk-H₂O gels which cross-linked in 65 minutes (65 ± 1 mins). Interestingly, addition of phenol red to silk-1xPBS resulted in reducing crosslinking time and was concentration dependent. Gels prepared with 7.5mg/L, 15mg/L and 30mg/L phenol red cross-linked on average in 26, 15 and 6 minutes, respectively, the latter representing a 93% decrease in crosslinking time compared to silk-1xPBS gels. Crosslinking times of gels prepared with phenol red at all three concentrations were significantly ($p < 0.0001$) lower compared to silk-1xPBS gels. Addition of phenol red also resulted in an increase in storage modulus compared to silk-1xPBS gels. Phenol red-silk gels prepared with 7.5mg/L, 15mg/L and 30mg/L phenol red recorded an average storage modulus of 1.10, 1.22 and 1.33 kPa, respectively. Storage moduli of silk gels prepared with 30 mg/L phenol red were significantly higher ($p < 0.0001$) than silk-1xPBS gels, an 80% increase in storage modulus. However, no significant differences in storage moduli was noted between 15mg/L and 30mg/L phenol red-silk gels or between 7.5mg/L and 15mg/L phenol red-silk gels.

To confirm that changes in crosslinking were independent of pH, the pH of hydrogel precursor solutions were recorded prior crosslinking. Silk-1xPBS and silk-0.5xDMEM solutions with or without phenol red recorded pH of 7.1-7.2, while silk-H₂O was slightly acidic at a pH of 6.5. To confirm that the concentration-dependent effect of phenol red was independent of the solvent, in this case 1xPBS, the same experiment was repeated with water as solvent, where different concentrations of phenol red were added to silk-H₂O gels. The results from this experiment again confirmed that increasing phenol red concentration decreases crosslinking time. However, the increase in storage modulus noticed with silk-1xPBS hydrogels was not observed in silk-H₂O hydrogels (**Supplementary Figure 1**).

3.2 Diffusion studies and UV-VIS absorbance spectra indicate that phenol red is immobilized within silk hydrogel matrix during di-tyrosine crosslinking:

To confirm that phenol red was immobilized within the hydrogel network during crosslinking, phenol red release studies were performed. Briefly, silk-H₂O gels were prepared with phenol red added at the time of di-tyrosine crosslinking (**Figure 3A**). Following crosslinking, all gels were placed in 1xPBS (pH 7.2) to facilitate phenol red diffusion out of the gel. Absorbance values were noted at 2, 4, 6, 8, 17 and 48 hours and a phenol red standard curve (**Supplementary Figure 2**) was used to monitor cumulative release of phenol red. **Figure 3C** shows the cumulative release of phenol red into 1xPBS during the 48-hour time period. Silk-H₂O gels prepared with 7.5mg/L phenol red (0.021 mM) showed no release of phenol red. In comparison, silk gels prepared with 15mg/L (0.042 mM) phenol red and 30mg/L (0.085 mM) phenol red showed a burst release at 2 hours and reached a maximum of 3% and 19%, respectively. Therefore, little or no phenol red was released when dye was added at the time of di-tyrosine crosslinking. When the same experiment was repeated, but with phenol red added to silk-H₂O gels following di-tyrosine crosslinking completion (**Figure 3B**), different results were obtained. Cumulative release profiles showed a similar burst release within the first 6 hours, but >90% of phenol red was released from the gels within the first 48 hours (**Figure 3C**). Silk gels prepared with 7.5mg/L, 15mg/L and 30mg/L phenol red added following di-tyrosine crosslinking completion, showed maximum releases of 92%, 97% and 98%, respectively. Therefore, almost all the phenol red was released when added following di-tyrosine crosslinking completion.

Phenol red is used as a pH indicator because of its ability to undergo resonance transformation, during which the electron distribution in the bonds within the molecule changes between acidic and basic state (pH) of the solvent (**Figure 4C**) [17]. To test whether the molecule retained its ability to undergo resonance transformation following immobilization, silk-H₂O gels were prepared with 15mg/L phenol red and placed in 1xPBS at different pHs (pH 3.0, 5.0, 7.2, 9.0 and 11.0). Absorbance UV/Vis spectra of immobilized phenol red were noted using a spectrophotometer and free phenol red was used for controls.

The spectrum of immobilized phenol red (**Figure 4B**) showed different absorption bands compared to its solution counterpart (**Figure 4A**). In **Figure 4A**, the acidic form (pH 3.0) of free phenol red had an absorption maximum at 432 nm and this peak decreased as solution pH increased. Interestingly, there was a blue shift in absorption maximum in phenol red immobilized silk gels seen at 415 nm (**Figure 4B**). The absorption maxima follow the same trend as with free phenol red and decreased with increased pH. The basic form (pH 11.0) of free phenol red had absorption maxima at 560 nm (**Figure 4A**) and was red shifted to 570 nm (**Figure 4B**) in phenol red immobilized silk gels. The isobestic point at 495 nm (**Figure 4B**) for pH 5-9 was red shifted from the isobestic point of free phenol red at 481 nm (**Figure 4A**). These variations in absorption spectra indicate that phenol red was chemically interacting with the silk hydrogel network and was able to undergo resonance transformation, albeit with differences to the free, unbound form. To further confirm that phenol red underwent resonance transformation after immobilization within the hydrogel network, we used time lapse imaging. The online version of article includes video confirming resonance transformation and reversibility of pH sensing in phenol red-silk hydrogels, showing color change from pink (basic) to yellow (acidic) and back to pink (basic).

3.3 Phenol red forms di-tyrosine covalent bonds with N-acetyl-tyrosine in the presence of HRP and hydrogen peroxide:

To further elucidate the mechanistic function of phenol red in silk tyrosine crosslinking and to confirm covalent bond formation with phenol red and tyrosine, di-tyrosine formation from N-acetyl-L-tyrosine (Tyr) was studied with water as solvent, using fluorescence spectroscopy and LC-MS (Liquid Chromatography-Mass Spectroscopy). **Figure 5A, B** show di-tyrosine formation from N-acetyl-L-tyrosine and phenol red in the presence of HRP and H_2O_2 via fluorescence time sweeps (ex: 290 nm; em: 415 nm). **Figure 5A** shows fluorescence time sweeps of phenol red reacted with increasing molar ratios of N-acetyl-L-tyrosine. Phenol red (15mg/L; 0.042 mM) alone did not result in di-tyrosine fluorescence (**Figure 5A**). But, phenol red with increasing amounts of tyrosine resulted in proportional increases in di-

tyrosine fluorescence. Also, time taken to reach maximum fluorescence decreased with increasing tyrosine concentration (**Figure 5A**). Phenol red reacted with 2.5mM tyrosine (PR + 2.4mM Tyr) recorded higher di-tyrosine fluorescence and maximal fluorescence was reached within 1 minute compared to 4 minutes in the case of phenol red reacted with 0.5mM tyrosine (PR + 0.5mM Tyr). The fluorescence maxima of groups with Tyr concentrations >2.5 mM are beyond the detection level of this assay. **Figure 5B** shows fluorescence time sweeps of N-acetyl-L-tyrosine reacted with increasing molar ratios of phenol red. Tyrosine (0.5mM) without phenol red recorded maximum di-tyrosine fluorescence, with fluorescence maxima at 3 minutes. When 0.04 mM of phenol red is reacted with tyrosine (Tyr +0.04mM PR), a decrease in di-tyrosine fluorescence was noticed, with no change in fluorescence maxima (**Figure 5B**). With increasing concentrations of phenol red, time taken to reach maximal fluorescence increased, accompanied by a decrease in di-tyrosine fluorescence. These results are in contrast to the accelerating potential of phenol red in silk tyrosine crosslinking recorded via rheology.

A mass spectrophotometry study was performed to confirm the oxidation reactive of tyrosine with phenol red. For the oxidation reaction of N-acetyl-L-tyrosine with phenol red using HRP, m/z values of 224.1, 355.0, 445.2, 447.1 and 576.1 corresponding to N-acetyl-tyrosine, phenol red, di-N-acetyl-tyrosine, doublet of N-acetyl-tyrosine and reacted N-acetyl-L-tyrosine with phenol red (**Figure 6C**). The observed m/z values were confirmed using stock or reacted solutions of N-acetyl-L-tyrosine, phenol red and HRP-reacted N-acetyl-L-tyrosine (**Supplementary Figure 3**). Further evaluation of the oxidation reactive of N-acetyl-L-tyrosine with phenol red was performed by LC-MS. The observed retention times were 3.2, 3.548 (3.599), 3.778 and 3.875 minutes for N-acetyl-L-tyrosine, phenol red, doublet of N-acetyl-L-tyrosine and reacted N-acetyl-L-tyrosine with phenol red, respectively (**Supplementary figure 3**). These retention times corresponded to expected hydrophathy of the reactants and produces.

3.4 Cellular viability and toxicity associated with Phenol red-silk hydrogels:

To assess the cytocompatibiltiy of phenol red, human pulmonary fibroblasts were encapsulated within phenol red di-tyrosine silk hydrogels. At 24 hours post encapsulation, fluorescence microscopy

was used to assess viability via calcein and PI staining. **Figure 7A** shows the ratio of live cells at 24 hours within the different groups. Cells within silk-H₂O were the least viable and had the lowest ratio of live cells. Fibroblasts encapsulated within 0.5xDMEM silk gels and 1xPBS gels had significantly greater ($p < 0.0001$) cell viability compared to the silk-H₂O gels. Phenol red di-tyrosine silk gels prepared with 7.5 mg/L (0.021 mM), 15 mg/L (0.042 mM) and 30 mg/L (0.085 mM) phenol red had significantly greater ($p < 0.0001$) viability compared to the silk-1xPBS gels. Also, no differences in viability were found between silk gels fabricated with different concentrations of phenol red. **Figure 7C (a) to (f)** are representative images taken from the Z-stacks of different groups showing calcein (green) and PI positive cells (red). No viable cells were found within silk-H₂O and **Figure 7C (a)** shows only PI stained cells. Silk-1xPBS gels and silk-0.5xDMEM gels (**Figure 7 (b)-(f)**) had both calcein and PI positive cells, with greater number of calcein positive cells.

Figure 7B shows fluorescence and alamar blue metabolic activity for 20 days following encapsulation within the different gels. Fibroblasts encapsulated within silk-H₂O gels had the least metabolic activity and were maintained over the 20 day culture period. All other groups show similar metabolic activity for the duration of culture, except for silk-1xPBS and silk-1xPBS-7.5mg/L phenol red, which showed decreased metabolic activity at the 20 day time point. Cells encapsulated within all silk gels with phenol red showed similar metabolic activity, confirming the absence of cytotoxicity due to the presence of the phenol red in the gels.

3.5 Phenol red-silk gels provide fluorescence based pH sensing and are ideal for cell culture applications:

Although phenol red by itself has very little or no fluorescence, phenol red coupled with tyrosine cross-linked hydrogels can serve as a fluorescent pH sensor. To test the ability of phenol red-silk gels to serve as intelligent environmental pH sensing gels, the fluorescence emission spectrum of the hydrogels was recorded between 350-500 nm. **Figure 8A** shows the fluorescence emission spectrum of phenol red-silk hydrogels at pH 5.0 – 9.0. The pH range of 5.0-9.0 was chosen due to its relevance for mammalian

cell culture, where pH fluctuations between pH 6.0-8.0 are common using sodium bicarbonate or HEPES buffered media. Di-tyrosine fluorescence maximum at 417 nm was quenched by phenol red absorbance maximum at 415 nm, resulting in pH-dependent fluorescence maxima for phenol red-silk hydrogels. **Figure 8B** shows sigmoidal curves obtained after plotting fluorescence maxima as a function of pH in HEPES and DPBS buffers. The sigmoidal curve mirrors the pH dependent absorption curves of phenol red with pKa 7.92 [6] and pH dependent fluorescence emission of di-tyrosine which has a phenolic pKa of 7.0 [18]. The average pKa of phenol red-silk hydrogel system was calculated to be 6.8.

4. Discussion:

4.1. Mechanistic action of phenol red on silk tyrosine crosslinking:

Cross-linked HRP silk hydrogels were prepared by the addition of HRP and H_2O_2 to aqueous silk solution [14]. HRP silk hydrogels have unique mechanical properties and were studied for their ability to serve as biomaterial substrates to support two and three dimensional cell culture. Since water is the solvent for silk protein, silk- H_2O hydrogels are not isotonic. To overcome this limitation, DMEM media was diluted to 0.5x final concentration before cell seeding. Phenol red in DMEM culture media accelerated the enzymatic crosslinking reactions of silk. To quantitate the effect of phenol red, DMEM media with and without phenol red were used to form silk hydrogels and dynamic time sweeps were conducted using a rheometer, where the progression of crosslinking was followed as an increase in storage modulus of the gels. Crosslinking time was significantly lower in 3% (w/v) silk gels prepared with 0.5xDMEM+PR compared to 0.5xDMEM-PR (**Figure1**). We confirmed the accelerating effect of phenol red on silk tyrosine crosslinking in silk-1xPBS hydrogels (**Figure 2**), to avoid the confounding effects of amino acids and vitamins in DMEM media. Results from these studies confirmed that phenol red reduced silk tyrosine crosslinking time in a concentration dependent reaction.

When HRP and H_2O_2 are added to silk, tyrosyl radicals are generated from the oxidation of phenolic amino acid L-tyrosine and subsequently converted to N,N-di-tyrosine, an intensely fluorescent product [19, 20]. However, di-tyrosine is not the only product formed by oxidizing free L-

tyrosines. Previous studies have shown that L-tyrosine can undergo oxidation to form reaction products including di-tyrosine, iso-dityrosine, pulcherosine, tri-tyrosine and di-isodityrosine [21, 22]. Also, phenol red can undergo oxidation due to phenolic groups, in the presence of HRP and H_2O_2 [23]. Therefore, mixing phenol red and silk in the presence of with HRP and H_2O_2 should result in an increase in the total number of substrate tyrosyl radicals participating in tyrosine crosslinking and subsequently accelerate crosslinking of phenol red-silk hydrogels. Results from phenol red release studies (**Figure 3**) support this hypothesis and suggest chemical immobilization within the hydrogel network. When silk hydrogels were prepared with phenol red added during silk tyrosine crosslinking, little or no release of phenol red was noticed for 48 hours post crosslinking. However, when phenol red was added after silk tyrosine crosslinking, almost all of the phenol red diffused out of the gel within 4 hours. Therefore, phenol red participates in the chemical reaction along with silk and is immobilized within the hydrogel network. Additionally, the UV-VIS spectra (**Figure 4**) of phenol red-silk gels confirmed chemical immobilization via altered absorbance bands compared to free phenol red. The strong absorption peak of basic phenol red observed at 560 nm and the absorbance maximum of the acidic phenol red at 432 nm were shifted to 570 nm and 415 nm, respectively.

Apart from accelerating silk tyrosine crosslinking, phenol red also increased the storage modulus of silk-1xPBS gels in a concentration dependent manner (**Figure 2**). The increase in storage modulus suggested an increase in crosslinks formed in silk hydrogel. Fluorescence spectroscopy could not confirm an increase in crosslinks due to spectral overlap between di-tyrosine fluorescence and phenol red absorbance. Further, the increase in storage modulus noticed with phenol red addition to silk-1xPBS gels was not observed in silk- H_2O gels (**Supplementary Figure 1**). Silk-1xPBS gels had significantly lower moduli and crosslinks compared to silk- H_2O gels. When inorganic salts were introduced into the chemical reaction in the form of DMEM or 1xPBS, the resulting hydrogels were much weaker, suggesting incomplete tyrosine crosslinking due to ionic interactions. In fact, tyrosine crosslinking experiments conducted with different concentrations of PBS showed that storage moduli and time of crosslinking were largely dependent on the ionic strength of the solvent (**Supplementary Figure 4**). A better understanding

of silk tyrosine crosslinking with different inorganic salts is required to understand the modulus promoting effect of phenol red.

4.2: Covalent bonding and resonance transformation of phenol red in phenol red-silk hydrogels:

The addition of phenol red resulted in accelerating silk tyrosine crosslinking (**Figure 1**), but fluorescence time sweeps revealed slower reactions with increasing molar ratios of phenol red (**Figure 5**). Fluorescence spectroscopy could not confirm covalent bonding between phenol red and N-acetyl-tyrosines, complicated by spectral overlap between di-tyrosine fluorescence and phenol red absorbance in the 400 nm region. However, mass Spectroscopy confirmed covalent bond formation between phenol red and N-acetyl-L-tyrosines. Oxidation products of N-acetyl-L-tyrosine and phenol red revealed the formation of a di-tyrosine product, suggesting a reaction of a single phenolic hydroxyl group in phenol red. An important characteristic of free phenol red is its ability to undergo resonance transformation between acidic and basic pH (**Figure 4B**). When phenol red (pK_a - 7.92) is taken from acidic to basic pH, one of the two phenol groups is de-protonated to form a phenoxide ion, which is then resonance stabilized to form a ketone, by the re-distribution of electrons [17]. UV-VIS absorbance spectra confirmed that phenol red underwent resonance transformation between the acidic and basic isoforms after chemical immobilization, suggesting that one of the two phenol groups remains free to exchange electrons even after immobilization. In further support of this conclusion, time lapse imaging of phenol red-silk hydrogels showed dynamic and reversible color changes in hydrogels from pink (pH-11.0) to yellow (pH-3.0). A reaction of both phenolic hydroxyls will disable the pH indicating properties of phenol red [11] and the results confirmed that phenol red has free phenolic hydroxyls for resonance transformation after covalent bonding.

4.3: Cytocompatibility, fluorescent pH sensing and application of phenol red-silk hydrogels:

To confirm the cytocompatibility of phenol red-silk hydrogels, primary lung fibroblasts were encapsulated in silk tyrosine cross-linked hydrogels prepared with 1xPBS and different concentrations of phenol red. Alamar blue was used to quantify metabolic activity and Calcein-PI staining assessed cellular

viability at 24 hours post encapsulation (**Figure 7**). Silk-H₂O gels are hypotonic and had the lowest ratio of live cells and alamar blue fluorescence. Cellular encapsulation in silk-0.5xDMEM gels and silk-1xPBS gels resulted in an increased ratio of live cells with respect to silk-H₂O gels, with no statistical significance between the groups. When phenol red was added to silk-1xPBS gels, a significant increase in the ratio of live cells was noted. However, there was no statistically significant difference in viability between different phenol red groups, indicating no phenol red toxicity at the tested concentrations. The moderate but significant increase in the ratio of live cells noticed in phenol red-silk gels can be attributed to increased crosslinking speed of the gels, which permitted the cells to be flooded with media immediately, hence improving their viability. Alamar blue fluorescence values of cells encapsulated in phenol red-silk gels showed comparable metabolic activity to silk-0.5xDMEM and silk-1xPBS gels.

A review of literature shows that phenol red could act as a weak estrogen mimic. Phenol red in cell culture media has been shown to bind the estrogen receptor in human breast cancer cell line MCF-7 and induce proliferation in a dose dependent fashion [24]. Estrogenic activity was also noted when primary rat pituitary cells, immature rat uterine cells, human ovarian surface epithelial cells and TD-47 human mammary gland cells were cultured in phenol red containing cell culture media [2, 4]. Phenol red-silk hydrogels present a suitable system for overcoming the limitations associated with free phenol red dye in cell culture media. Silk tyrosine cross-linked hydrogels covalently link phenol red to the hydrogel matrix and limit exposure of cells during culture, albeit maintaining pH sensing capacity, with no leakage. Results from our current study conclude that leak proof pH sensing hydrogels can be fabricated by reacting 7.5 mg/L phenol red with silk tyrosines (**Figure 3**).

Sol-gel sensors have been constructed using pH-dependent phenol red absorbance [6, 8-10]. Absorbance based pH sensors do not require expensive instrumentation, but fluorescence sensors offer improved sensitivity. Phenol red has been used to generate fluorescent-based pH sensors, based on resonance energy transfer (RET). Phenol red covalently bound to eosin was mounted on a fiber optic cable and provided pH dependent fluorescence emission, due to spectral overlap between eosin fluorescence emission and phenol red absorbance [25]. Although, phenol red is covalently bound to silk

tyrosine hydrogels, di-tyrosine fluorescence quenching seems to be the dominant mechanism for pH sensing, as silk hydrogels soaked in phenol red solution also showed a decrease in fluorescence.

The average pKa of phenol red-silk di-tyrosine cross-linked hydrogels was 6.8, implying increased sensitivity near neutral pH, useful for cell culture applications. The geometry of hydrogels and their pore size, dictated by silk concentration in the reaction, control diffusion rates of analytes into the hydrogels. Therefore, pH response times for phenol red-silk hydrogels can be tuned by altering silk concentration and by casting thin hydrogels (~100 μm). In this study, phenol red-silk hydrogels were tested in the pH range of 4.0-9.0, with no observed hydrogel degradation or phenol red leakage. However, it is important to note that silk hydrogels undergo acid hydrolysis at $\text{pH} < 2$ and in the presence of strong alkali, precluding the use of phenol red-silk hydrogel sensors under these conditions.

5. Conclusions:

The goal of this study was to understand phenol red-silk tyrosine interactions towards functionalized silk protein-based biomaterials to develop self-reporting pH systems for *in vitro* environments. Rheology time sweeps confirmed that phenol red in DMEM media accelerated silk tyrosine crosslinking in a concentration dependent reaction. Phenol red release studies and UV-Vis spectra confirmed phenol red entrapment within the hydrogel network, when added during di-tyrosine crosslinking. LC-MS analysis of HRP reacted N-acetyl-L-tyrosine and phenol red reaction products confirmed covalent bond formation between phenol red and tyrosine, with an ability to undergo resonance transformation, forming leak-proof hydrogels. Both live-dead staining and alamar blue fluorescence showed good cytocompatibility of the hydrogels for cell culture applications. Due to the spectral overlap between phenol red absorbance at 415 nm and di-tyrosine fluorescence emission maximum at 417 nm, phenol red-silk hydrogels provided absorbance and fluorescence-based pH sensing. With an average pKa of 6.8, phenol red-silk hydrogels are highly sensitive near neutral pH and are useful for pH sensing in cellular microenvironments, phenol red free systems and bioreactors.

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Figure Captions:

Figure 1: Effect of Phenol red in DMEM media on silk tyrosine crosslinking (A) Rheological time sweeps showing silk tyrosine gelation kinetics for 3% (w/v) silk gels made with H₂O and 0.5xDMEM with or without phenol red (B) Storage moduli of silk gels recorded at the end of time sweeps (C) Crosslinking time recorded in different silk gels. n = 3 per group.

Figure 2: Effect of Phenol red concentration on silk tyrosine crosslinking (A) Rheological time sweeps showing gelation kinetics for 3% (w/v) silk gels made with H₂O and 1xPBS with different phenol red concentrations (B) Storage moduli of silk gels recorded at the end of time sweeps (C) Crosslinking time recorded in different silk gels. n = 3 for silk- H₂O and silk-1xPBS; n = 4 for silk-1xPBS+PR per group.

Figure 3: Phenol red is immobilized within silk hydrogel matrix during di-tyrosine cross-linking (A) Silk-H₂O hydrogels made with or without phenol red during di-tyrosine cross-linking and then immersed in 1xPBS (B) Silk-H₂O hydrogels soaked in phenol red following di-tyrosine cross-linking and immersed in 1xPBS (C) Cumulative release profiles of phenol red from silk-H₂O gels prepared using two different schematics over 48 hours. n = 6 per group.

Figure 4: Phenol red retains its ability to undergo resonance transformation following immobilization within silk hydrogel matrix (A) UV-Vis absorbance spectra of free phenol red (B) UV-Vis absorbance spectra of phenol red immobilized in silk hydrogel (D) Resonance isoforms of phenol red at acidic and basic pH.

Figure 5: Di-tyrosine formation from HRP, H₂O₂ mediated oxidation of N-acetyl-L-tyrosine and phenol red (A) Di-tyrosine fluorescence time sweeps with phenol red (0.04 mM) and increasing molar ratios of tyrosine (B) Di-tyrosine fluorescence time sweeps with N-acetyl-L-tyrosine (0.5 mM) and increasing molar ratios of phenol red.

Figure 6: ESI-MS analysis of reaction products. (A) Mass to charge ratio of N-acetyl-tyrosine (M+H: 224.1), phenol red (M+H: 355.1), di-N-acetyl-tyrosine (M+H: 445.1) and product (M+H: 576.1) (B) Mass to charge ratio around the di-N-acetyl-tyrosine with a peak at 445.1 and 447.1. The inlay depicts analysis of di-N-acetyl-tyrosine with a peak at 447.1 indicative of 2M+H. (C) Mass to charge ratio around product.

Figure 7: Viability of pulmonary fibroblasts encapsulated in silk di-tyrosine hydrogels with or without phenol red (A) Live-dead assay showing viability of pulmonary fibroblasts at 24 hours post encapsulation in di-tyrosine silk hydrogels (B) Alamar blue data showing metabolic activity of pulmonary fibroblasts embedded within silk hydrogels (C(a)-(f)) Representative fluorescence images showing calcein (green) and PI (red). n = 6 per group for alamar blue assay; n = 3 per group for live-dead assay.

Figure 8: Fluorescence pH sensing capacity of phenol red-silk hydrogels (A) Fluorescence emission spectrum of phenol red-silk hydrogels shows pH dependent maximal fluorescence (B) pH response curves plotted using absolute fluorescence at 430 nm.

FIG2

FIG3

FIG4

FIG5

FIG6

FIG7

FIG8

Silk fibroin tyrosine crosslinking

Phenol red covalently linked to
silk tyrosines