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A sulfur-free peptide mimic of surfactant protein B (B-YL) exhibits high in vitro and in vivo surface activities.

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RESEARCH ARTICLE

REVISED A sulfur-free peptide mimic of surfactant protein B (B-YL) exhibits high in vitro and in vivo surface activities [version 2; referees: 2 approved]

Previously titled: An oxidation-resistant peptide mimic of surfactant protein B (B-YL) forms an amphipathic helix-hairpin in liposomes with high surface activity

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Abstract

Background: Animal-derived surfactants containing surfactant proteins B (SP-B) and C (SP-C) are used to treat respiratory distress syndrome (RDS) in preterm infants. SP-B (79 residues) plays a pivotal role in lung function and the design of synthetic lung surfactant. Super Mini-B (SMB), a 41-residue peptide based on the N- and C-domains of SP-B covalently joined with a turn and two disulfides, folds as an α-helix hairpin mimicking the properties of these domains in SP-B. Here, we studied 'B-YL', a 41-residue SMB variant that has its four cysteine and two methionine residues replaced by tyrosine and leucine, respectively, to test whether these hydrophobic substitutions produce a surface-active, α-helix hairpin.

Methods: Structure and function of B-YL and SMB in surfactant lipids were compared with CD and FTIR spectroscopy, and surface activity with captive bubble surfactometry and in lavaged, surfactant-deficient adult rabbits.

Results: CD and FTIR spectroscopy of B-YL in surfactant lipids showed secondary structures compatible with peptide folding as an α-helix hairpin, similar to SMB in lipids. B-YL in surfactant lipids demonstrated excellent *in vitro* surface activity and good oxygenation and dynamic compliance in lavaged, surfactant-deficient adult rabbits, suggesting that the four tyrosine substitutions are an effective replacement for the disulfide-reinforced helix-turn of SMB. Here, the B-YL fold may be stabilized by a core of clustered tyrosines linking the N- and C-helices through non-covalent interactions involving aromatic rings.

Conclusions: 'Sulfur-free' B-YL forms an amphipathic helix-hairpin in surfactant liposomes with high surface activity and is functionally similar to SMB and native SP-B. The removal of the cysteines makes B-YL more feasible to scale up production for clinical application. B-YL's possible resistance against free oxygen radical damage to methionines by substitutions with leucine provides an extra edge over SMB in the treatment of respiratory failure in preterm infants with RDS.

Keywords

Synthetic lung surfactant, Surfactant Protein B (SP-B), SP-B peptide mimics, Respiratory Distress Syndrome (RDS), Circular Dichroism (CD) spectroscopy, Fourier-Transform InfraRed (FTIR) spectrometry, Captive Bubble Surfactometry (CBS), lung lavage rabbit model

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## Introduction

Lung surfactant is a lipid-protein mixture that is synthesized by alveolar type II cells and secreted into the alveolus where it reduces surface tension at the air-liquid interface. Mammalian lung surfactant harvested by lavage consists of approximately 80% phospholipids, 10% neutral lipids and 10% protein[1]. Phospatidylcholine (PC), and particularly dipalmitoylphosphatidylcholine (DPPC), is the major phospholipid constituent of lung surfactant. DPPC enhances the formation of a rigid film at the air-liquid interface that reduces alveolar surface tension to low values during dynamic compression, whereas fluid phospholipids and neutral lipids are important because they significantly improve film spreading[2,3]. The highly hydrophobic surfactant protein B (SP-B) and, to a lesser extent, surfactant protein C (SP-C), facilitate the absorption of phospholipids into the air-liquid interface and thus play an important role in the reduction of alveolar surface tension. SP-B is pivotal for normal lung function, by hereditary SP-B deficiency being fatal in newborn infants[4] and also in SP-B knockout mice[5]. Human SP-B is a 79 amino-acid, lipid-associating monomer (MW ˜8.7 kDa) found in the lung as a covalently linked homodimer. Early theoretical studies based on homology comparisons indicated that the SP-B monomer consists of 4-5 α-helices[6–10] with three intramolecular disulfide bridges (i.e., Cys-8 to Cys-77, Cys-11 to Cys-71 and Cys-35 to Cys-46)[11], and belongs to the saposin protein superfamily[12]. The helical bundle for SP-B folds into two leaves, with one leaf having α-helices 1 (N-terminal helix), 5 (C-terminal helix) and 4 and the other composed of α-helices 2 and 3[13,14].

Intratracheal administration of animal-derived lung surfactants, which contain only polar lipids and native SP-B and SP-C, has greatly improved morbidity and mortality of premature infants with neonatal respiratory distress syndrome (RDS) as a result of surfactant-deficiency due to lung immaturity[15]. Existing clinically available formulations are extracted from lung lavages or homogenates from pigs (Curosurf®) and cows (Infasurf®, Survanta®), and contain small amounts of SP-B and SP-C (<< 2% of total weight) in a lipid extract with DPPC as its main component. Based on the predicted 3D-saposin motif for SP-B, we have developed minimal SP-B constructs that have desirable structural properties and maintain high activities in animal models of surfactant deficiencies[9,10]. For example, Super Mini-B (SMB) is a 41-residue, 'short-cut' peptide (Figure 1A), based on the primary sequence, secondary structure and tertiary folding of the known sequence of native SP-B (79-residues), that mimics the high surfactant activity of its parent protein[10,14,16]. SMB incorporates the N-terminal α-helix (˜residues 8-25) and C-terminal α-helix (˜residues 63-78) of native SP-B as a single linear peptide (Figure 1A), joined together with a customized turn to form a α-helix hairpin (α-helix/turn/α-helix, αtα)[17]. SMB
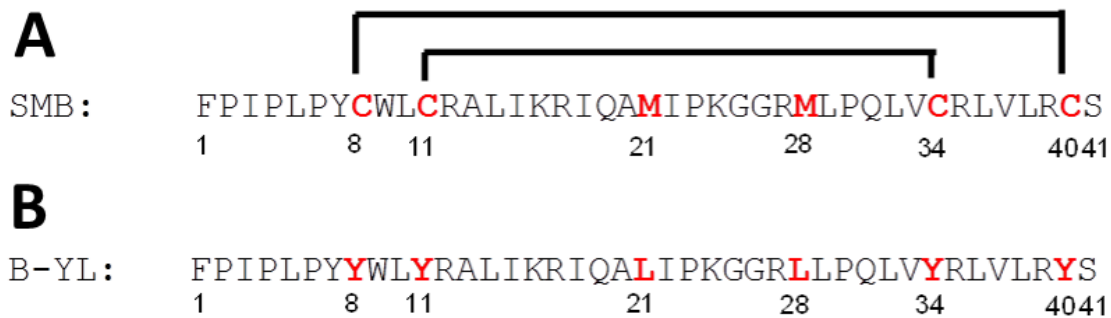
Figure 1. Sequences for Super Mini-B (SMB) and B-YL. (**A**) SMB (41 amino-acid residues; 1-letter amino-acid notation), with the N- and C-terminal Phe-1 and Ser-41 as indicated, as well as the sulfur-containing cysteines (Cys-8, Cys-11, Cys-34, Cys-40) and methionines (Met-21, Met-28) in red. The disulfide-linkages are shown between Cys-11 and Cys-34 and between Cys-8 and Cys-40. (**B**) B-YL (41 residues) shares the same sequence as its parent SMB, except that the cysteines and methionines are replaced by tyrosines (Tyr-8, Tyr-11, Tyr-34, Tyr-40) and leucines (Leu-21, Leu-28) in red, respectively.

has two vicinal disulfide bonds (i.e., Cys-8 to Cys-77 and Cys-11 to Cys-71) that further covalently link the N- and C-terminal α-helices, and also a hydrophobic N-terminal insertion sequence (i.e., residues 1-7; FPIPLPY). Experimental procedures validated the above structural model for SMB, including conventional ^{12}C-FTIR spectroscopy, mass spectroscopy, I-TASSER, ModWeb and SWISS MODEL homology modeling, and Molecular Dynamics (MD) simulations in lipid mimics and lipid bilayers[14,16,18]. When formulated with a lipid composition that mimics that of native lung surfactant, SMB has shown excellent surface activity with fresh and stored preparations, which was closely associated with the formation of an α-helix hairpin[10,14,18].

Because surfactant therapy is life-saving in preventing and treating RDS in preterm infants, on-going research is studying whether surfactant therapy may be efficaciously extended to pediatric and adult patients with clinical acute lung injury (ALI) or the acute respiratory distress syndrome (ARDS)[19]. ALI and ARDS may each be caused by direct exposure of lungs to pathogens, oxidative air pollutants, cigarette smoke and other irritants in the alveolar space, and by the presence of endogenous reactive oxygen species (ROS) in damaged lungs due to permeability edema or the inflammatory response. Subsequent oxidative alterations may produce dysfunctional and even inactive lung surfactant in these diseases[20,21]. SP-B is an important target for ROS-induced oxidative surfactant inactivation[22,23]. Specifically, oxidation of native SP-B involves alterations in the methionines (Met-29, Met-65) and tryptophan (Trp-9), which correlates well with the loss of *in vitro* surfactant activity[23]. Mimics based on the native SP-B sequence may be likewise sensitive to oxidation processes. Kim *et al.*[24] reported that ozone treatment of SP-B(1–25), an SP-B mimic whose sequence overlaps residues 1–25 of SMB (Figure 1A) and native SP-B, variably oxidized amino-acids known to react with ozone. In contrast to the nearly complete homogenous oxidation of the susceptible SP-B(1–25) residues (i.e., Cys-8, Cys-11, Trp-9, and Met-21) in the solvent phase, only a limited subset of residues (Trp-9 and Met-21) oxidized in the hydrophobic interfacial environment provided by the lipid surfactant layer[24]. In additional studies, Hemming *et al.*[25] showed that exposure of either SP-B $_{1-25}$ or SMB at the air-water interface to dilute ozone (~2 ppm) produced a rapid loss of surface activity (i.e., increase in surface tension). Because decreases in tryptophan fluorescence occurred concurrently with increasing surface tension for these two SP-B mimics[25], it is likely that oxidative disruption of the indole ring of Trp-9 can play a role in the diminished surface activity in the full-length protein, possibly due to a fraying of the N-terminal α-helix[25,26].

Synthetic lung surfactant with SP-B and SP-C peptide mimics offers substantial advantages over current animal-derived surfactants for treating surfactant deficiency in neonatal RDS. Current research on synthetic lung surfactant has focused on designing peptide mimics of natural surfactant proteins that are highly effective, stable, and easy to manufacture[9,10]. Here, we conducted structural and functional experiments on 'B-YL' (Figure 1B), a 41-residue SMB variant that has its four Cys and two Met residues replaced by Tyr (Tyr-8, Tyr-11, Tyr-34 and Tyr-40) and Leu (Leu-21 and Leu-28), respectively, and tested whether these hydrophobic substitutions produce a surface-active, α-helix hairpin. Tyrosine was substituted for cysteine because of its aromatic ring interactions that emulate disulfide formation[27–29] and methionine, that is easily oxidized, was replaced by leucine based on its similar molecular structure and polarity[30].

Methods

Materials

HPLC grade chloroform, methanol, trifluoroethanol (TFE), and acetonitrile were purchased from Fisher Scientific (Pittsburgh, PA 15275), trifluoroacetic acid from Sigma Chemical Co (Saint Louis, MO 63103), NMR quality deuterated water was from Aldrich Chemical Co. (St. Louis, MO 63103), and Sephadex LH-20 chromatography gel from Pharmacia (Uppsala, Sweden). Phospholipids were supplied by Avanti Polar Lipids (Alabaster, AL 35007), and Sodium Dodecyl Sulfate (SDS) detergent was from Sigma Chemical Co (Saint Louis, MO 63103).

The Super Mini-B (SMB) peptide sequence (Figure 1A) was synthesized using a standard Fmoc protocol with a Symphony Multiple Peptide Synthesizer (Protein Technologies, Inc., Tucson, AZ 87514) or a CEM Liberty microwave synthesizer (CEM Corporation, Mathews, NC 28104), cleaved-deprotected and purified using reverse phase HPLC as described earlier[14,18]. This synthesis protocol included folding of the peptide in a structure-promoting TFE-buffer solvent system to promote oxygen-mediated disulfide linkages between Cys-8 and Cys-40 and between Cys-11 and Cys-34[10,14]. This covalently stabilized connectivity gave the peptide a helix-hairpin conformation, comparable to the topological organization seen for the N- and C-terminal helical domains of the saposin family of proteins[9,10,12]. The synthesis of B-YL was identical to that of SMB, except for replacing cysteines with tyrosines (Tyr-8, Tyr-11, Tyr-34, and Tyr-40) and methionines with leucines (Leu-21 and Leu-28), and also omitting the oxidation step. The purified SMB and B-YL peptides were each freeze-dried directly, and the masses were confirmed by MALDI TOF mass spectrometry as described previously[17]. Peptide concentrations were routinely quantitated using UV absorbance based on the assay procedure developed by Anthis and Clore[31].

Preparation of proteins and lipids in surfactant dispersions

Peptide and lipids were formulated as lipid-peptide dispersions to have a total of 3% by mole fraction of SMB or B-YL and 35 mg of total lipid [i.e., DPPC: POPC: POPG 5:3:2 mole:mole:mole] per mL of dispersion[18]. The peptide was dissolved in 10 mL of trifluoroethanol and co-solvated with the lipid in chloroform, followed by removal of the solvents with a stream of nitrogen gas and freeze drying of the resulting lipid-peptide film to remove residual solvent. The film was then dispersed with Phosphate Buffered Saline and the sample flask containing the hydrated film was rotated for 1 h at 60°C to produce a solution of multilamellar vesicles (MLVs)[14]. Lipid controls were similarly prepared but without peptide. These dispersions were then stored at 4°C prior to structural and functional measurements. To determine the molecular mass of peptides formulated with

lipids, the peptide was separated from lipid using normal phase chromatography with Sephadex LH-20[32]. Mass spectral analysis of the B-YL peptide indicated there was no change in the molecular weight due to oxidation of tyrosines or tryptophan for one year when formulated with surfactant lipids.

Circular dichroism (CD) spectroscopy of the secondary structure of the B-YL mimic

CD spectra (190–260 nm) of the B-YL peptide in various structure-promoting environments, including surfactant dispersions, were measured with a JASCO 715 spectropolarimeter (Jasco Inc., Easton MD 21601). The instrument was routinely calibrated for wavelength and optical rotation using 10-camphorsulphonic acid[33]. The sample solutions were scanned using 0.01 cm pathlength cells at a rate of 20 nm per minute, a sample interval of 1 nm, and a temperature of 37°C. Sample concentration was determined by UV absorbance at 280 nm[31]. Peptide concentration was 100 μM in sample solutions with either TFE:Phosphate buffer (10 mM, pH 7.0) having a volume ratio of 4:6 (v/v), SDS micelles (100 mM) in phosphate buffer (10 mM, pH 7.0), or Single Unilamellar Vesicles (SUVs) of simulated surfactant lipids (DPPC: POPC: POPG; 5:3:2, mole:mole:mole). Surfactant lipid SUVs were prepared at a concentration of 2.6 μM lipids/mL of phosphate buffer solution (10 mM, pH 7.0) by bath sonication for 10 minutes (https://avantilipids.com/tech-support/liposome-preparation/). Sample spectra were baseline corrected by subtracting spectra of protein-free solution from that of the protein-bound solution and expressed as the Mean Residue Ellipticity [θ]$_{MRE}$ as shown in equation (1):

$$[\theta]_{MRE} = ([\theta] \times 100)/(1 \times c \times N) \qquad (1)$$

The symbol θ is the measured ellipticity in millidegrees, l is the pathlength in cm, N is the number of residues in the peptide, and c is the concentration of the peptide in mM.

Quantitative estimates of the secondary structural contributions were also made with SELCON 3[34] using the spectral basis set for membrane proteins, option 4 implemented from the DichroWeb website[35,36].

Attenuated-Total-Reflectance Fourier-transform infrared (ATR-FTIR) spectrometry of the B-YL and SMB peptides

ATR-FTIR spectra were recorded at 37°C using a Bruker Vector 22 FTIR spectrometer (Pike Technologies, Fitchburg, WI 53719) with a deuterium triglyceride sulfate (DTGS) detector. The spectra were averaged over 256 scans at a gain of 4 and a resolution of 2 cm^{-1}[14]. For spectra of B-YL and SMB in TFE solutions, self-films were first prepared by air-drying peptide originally in 100% HFIP onto a 50 × 20 × 2 mm, 45° attenuated total reflectance (ATR) crystal for the Bruker spectrometer. The dried peptide self-films were then overlaid with solutions containing 40% TFE/60% deuterated-10 mM sodium phosphate buffer (pH 7.4), at a peptide concentration of 470 μM. Control solvent samples were similarly prepared for FTIR analysis, but without peptide. Spectra of peptides in solvent were obtained by subtraction of the solvent spectrum from that of peptide solvent. For FTIR spectra of B-YL and SMB in either SDS micelles or surfactant lipids, each lipid-peptide solution was transferred onto a germanium ATR crystal. The aqueous solvent was then removed by flowing nitrogen gas over the sample to produce a thick lipid-peptide (lipid:peptide ratios of 10:1, mole:mole)[14]. The multilayer film was then hydrated to ≥35% with deuterated water vapor in nitrogen for 1 h before acquiring the spectra[37]. The spectra for either the B-YL or SMB peptides in the film were obtained by subtracting the spectrum of a peptide-free control sample from that of the peptide-bound sample. The relative amounts of α-helix, β-turn, β-sheet, or random (disordered) structures in lipid-peptide films were estimated using Fourier deconvolution (GRAMS AI 8, version 8.0, Thermo Fisher Scientific, Waltham, MA 02451). The respective areas of component peaks were calculated using curve-fitting software (Igor Pro, version 1.6, Wavemetrics, Lake Oswego, OR 97035)[38]. FTIR frequency limits were: α-helix (1662-1650 cm^{-1}), β-sheet (1637-1613 cm^{-1}), turn/bend (1682-1662 cm^{-1}), and disordered or random (1650-1637 cm^{-1})[39].

Captive bubble surfactometry

Adsorption and surface tension lowering ability of surfactant preparations were measured with a captive bubble surfactometer at physiological cycling rate, area compression, temperature, and humidity[14]. The captive bubble surfactometer used here was a fully-computerized version of that described and built by Schürch and coworkers[40,41]. Quasi-static compression and expansion of the air bubble was performed in discrete steps at a rate of 5% of the bubble volume every 10 sec with continuous video recording of the bubble shape. Dynamic compression and expansion cycling was performed between 10 and 110% of the original bubble area at a cycling rate of 20 cycles/min. Both modalities show extreme flattening of the air bubble in active surfactant preparations. We used a B-YL surfactant mixture consisting of 3 mole% of B-YL peptide formulated in surfactant lipids (DPPC:POPC:POPG 5:3:2, mole:mole:mole) with a concentration of 35 mg/mL. Surfactant lipids alone were used as negative control and SMB surfactant (3 mole% of SMB in surfactant lipids) and the clinical surfactant Curosurf® (porcine lung extract containing both SP-B and SP-C and 80 mg/mL of lipids) as positive control. We routinely analyze surfactant preparations at an average surfactant lipid concentration of ~25 μg/mL in the bubble chamber, but as Curosurf® is more concentrated than synthetic surfactant, we applied 1 μL of synthetic surfactant at 35 mg/mL and 0.5 μL of Curosurf® at 80 mg/mL to the bubble chamber (~1.5 mL volume), and performed all measurements in quadruplicate.

In vivo experiments

Animal experiments were performed under established protocols reviewed and approved by the Institutional Animal Care and Use Committee of the Los Angeles Biomedical Research Institute at Harbor-UCLA Medical Center (LA BioMed protocol # 020645). All procedures and anesthesia were in accordance with the American Veterinary Medical Association (AMVA) guidelines. Any suffering of the rabbits was ameliorated by providing optimal anesthesia and sedation as outlined below.

The lung lavage rabbit model represents a relatively pure state of surfactant deficiency over at least 6–8 h and allows for serial measures of arterial blood gases and lung compliance in

ventilated, surfactant-deficient animals with a clinical picture of respiratory failure as seen in neonatal RDS and ALI/ARDS. Respiratory failure secondary to surfactant deficiency has a high mortality if not treated with a highly surface-active surfactant preparation. Thirty-three young adult, New Zealand white rabbits, weighing 1.0–1.4 kg, were purchased from IFPS Inc. (Norco, CA). The animals were housed as pairs for a minimum of 24 h in the C.W. Steers Biological Resources Center of LA BioMed, using large cages with non-traumatic and moisture absorbent bedding, and provided with rabbit toys and food and water *ad libitum*. Husbandry was provided by veterinary technicians under supervision of a veterinarian. The number of animals has been determined from a population correlation=0.6, α=0.05, tails=2, and power=0.8, which gives a sample size of 16 (2x8). Therefore, we generally use 8 animals to test clinical efficacy of an experimental surfactant preparation (here: 3 mole% B-YL in surfactant lipids, n=9) with groups of 8 animals as positive controls (here: Curosurf® and 3 mole% SMB in surfactant lipids, both n=8) and 8 animals for negative controls (here: surfactant lipids alone [DPPC:POPC:POPG 5:3:2 mole: mole:mole], n=8). These treatment group sizes allow significant differences to be found between rabbits receiving an optimal surfactant and positive and negative controls. Animals were assigned to a surfactant preparation using a randomized algorithm and experiments were performed in a special laboratory area set up for the provision of intensive care.

The rabbits received anesthesia with 50 mg/kg of ketamine and 5 mg/kg of acepromazine intramuscularly prior to placement of a venous line via a marginal ear vein. After intravenous administration of 2 mg/kg of propofol and 2 mg/kg of midazolam for anesthesia and sedation, a small incision in the skin of the anterior neck allowed for placement of an endotracheal tube and a carotid arterial line. After insertion of the endotracheal tube, mechanical ventilation was initiated and muscle paralysis induced with intravenous vecuronium (0.1 mg/kg) to prevent spontaneous breathing. During the ensuing duration of mechanical ventilation, anesthesia consisted of continuous intravenous administration of 30 mg/kg/h of propofol and, as needed, additional intravenous dosages of 2 mg/kg of midazolam for sedation, whereas muscle paralysis was maintained with hourly intravenous administration of 0.1 mg/kg of vecuronium. Maintenance fluid was provided with a continuous infusion of lactated Ringer's solution at a rate of 10 mL/kg/h. Heart rate, arterial blood pressures and rectal temperature were monitored continuously (Labchart® Pro, ADInstruments Inc., Colorado Springs, CO).

The rabbits were ventilated with a volume-controlled rodent ventilator (Harvard Apparatus, South Natick, MA) using a tidal volume 7.5 mL/kg, a positive end-expiratory pressure of 3 cm H_2O, an inspiratory/expiratory ratio of 1:2, 100% oxygen, and a respiratory rate sufficient to maintain the partial pressure of carbon dioxide ($PaCO_2$) at ˜40 mmHg. Airway flow and pressures and tidal volume were monitored with a pneumotachograph connected to the endotracheal tube (Hans Rudolph Inc., Kansas City, MO). When the partial pressure of oxygen in arterial blood (PaO_2) was >500 mmHg at a peak inspiratory pressure <15 cm H_2O in 100% oxygen, surfactant deficiency

was induced with repeated intratracheal instillation and removal of 30 mL/kg of warmed normal saline. When the PaO_2 was stable at <100 mmHg (average 4 lavages), B-YL surfactant or a surfactant control (SMB, Curosurf® or surfactant lipids alone) was then instilled intratracheally at a dose of 100 mg/kg body weight, similar to dosages given to premature infants with RDS. Curosurf® is more concentrated (80 mg/mL) than SMB and B-YL surfactant and lipids alone (35 mg/mL), so Curosurf® was given at 1.25 mL/kg and SMB and B-YL surfactant and lipids alone at 2.9 mL/kg. Oxygenation was followed by measuring arterial pH and blood gases and lung compliance at 15 min intervals over a 2 h period. Dynamic lung compliance was calculated by dividing tidal volume/kg body weight by changes in airway pressure (peak inspiratory pressure minus positive end-expiratory pressure) (mL/kg/cm H_2O).

Animals were sacrificed 2 h after surfactant administration with an overdose (200 mg/kg) of intravenous pentobarbital. End-points were oxygenation and dynamic lung compliance at 120 min after surfactant administration.

Statistical analysis
All data are expressed as mean ± SEM. Statistical analyses (IBM Statistical Package for the Social Sciences (SPSS) 23.0) used Student's t-test for comparisons of discrete data points, and functional data were analyzed by one-way analysis of variance (ANOVA) with Scheffe's post hoc analysis to adjust for multiple comparisons. Differences were considered statistically significant if the P value was <0.05.

Results
Spectroscopic analysis of B-YL and SMB in lipid mimic and surfactant lipid environments
The secondary structures for B-YL in either lipid mimetics (i.e., 40% TFE/60% deuterated-sodium phosphate buffer, pH 7.4 and deuterated aqueous SDS micelles) or surfactant lipids [i.e., deuterated aqueous DPPC: POPC: POPG 5:3:2 (mole:mole: mole) multilayers] were studied with conventional ^{12}C-FTIR spectroscopy. Representative FTIR spectra of the amide I band for B-YL in these environments were all similar (Figure 2), each showing a principal component centered at ˜1654–1655 cm⁻¹ with a small low-field shoulder at ˜1619–1626 cm⁻¹. Because earlier FTIR investigations of proteins and peptides[39,42] have assigned bands in the range of 1650–1659 cm⁻¹ as α-helical, while those at ˜1613–1637 cm⁻¹ are characteristic of β-sheet, B-YL probably assumes α-helical and β-sheet structures and possibly other conformations in these environments. Self-deconvolutions of the Figure 2 spectra confirmed that B-YL is polymorphic, primarily adopting α-helix but with significant contributions from β-sheet, loop-turn and disordered components (Table 1). Interestingly, the relative proportions of secondary conformations determined from FTIR spectra of B-YL (i.e., α-helix > loop-turn ˜ disordered ˜ β-sheet) in both lipid-mimetics and surfactant lipids of varying polarity are all comparable, suggesting an overall stability of the B-YL structure that is remarkably conserved. It is also important to note that the proportions of these secondary conformations are all compatible with B-YL principally assuming an α-helix hairpin[10,14,18].
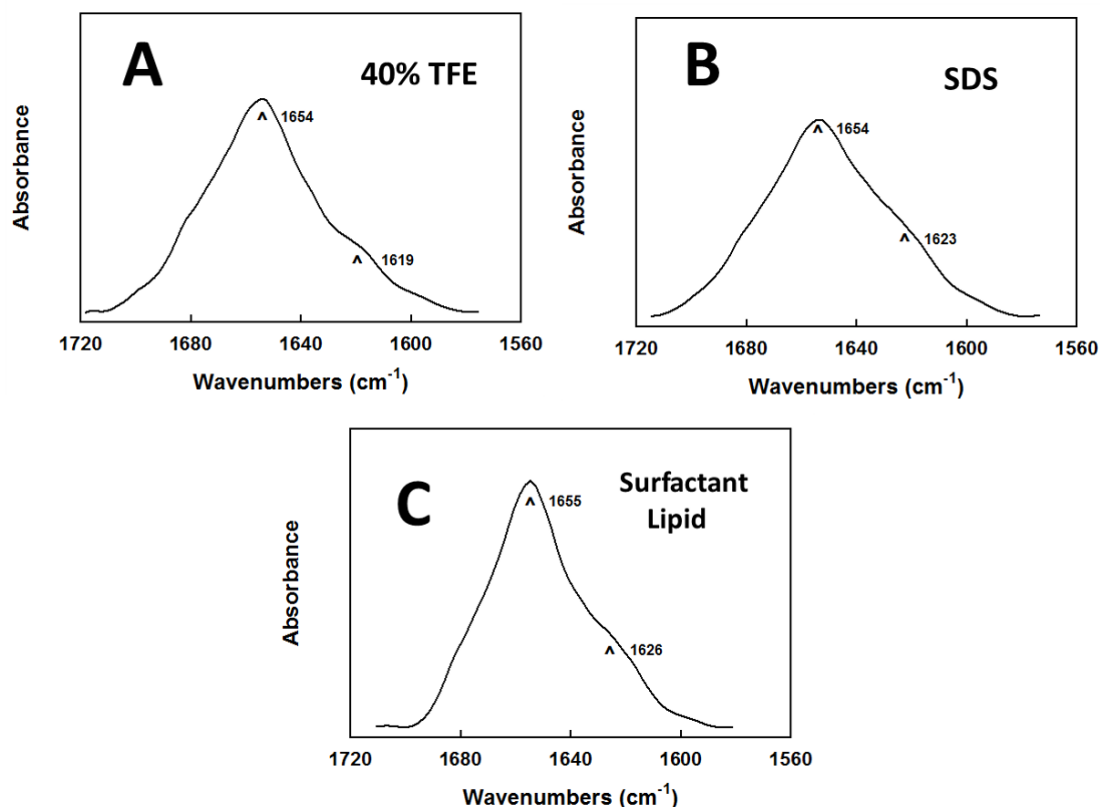
Figure 2. FTIR spectra of the B-YL mimic in several lipid-mimetics and surfactant lipids. Attenuated total reflectance Fourier transform infrared (ATR-FTIR) spectral plots show the absorbance (in arbitrary units) as a function of wavenumbers (cm^{-1}) (See **Methods** and **Results**). (**A**) Deuterated aqueous 40% TFE. (**B**) Deuterated aqueous SDS. (**C**) Deuterated aqueous surfactant lipid. In (**A**), (**B**) and (**C**), the IR spectra each show dominant α-helical components centered at 1654–1655 cm^{-1} (arrows), with minor bands at ˜1619–1626 cm^{-1} (arrows) due to β-sheet, ˜1682–1662 cm^{-1} due to loop-turn/bend, and ˜1650–1637 cm^{-1} due to disordered or random conformations. Peptide concentrations were 470 µM for the TFE solvent spectra, and 10:1 lipid:peptide (mole:mole) for the SDS detergent and surfactant lipid spectra. The areas under each absorbance curve are normalized.

The secondary conformations for B-YL in lipid mimics (i.e., 40% TFE, aqueous SDS) or synthetic surfactant lipids [aqueous (DPPC:POPC:POPG 5:3:2 mole:mole:mole] were also studied with Circular Dichroism (CD) spectroscopy, to validate the above FTIR results. CD spectra for B-YL in these environments (Figure 3) were all similar, each indicating a major α-helical component characterized by a double minimum at 208 and 222 nm[43–45]. Deconvolutions of the Figure 3 spectra showed that B-YL is polymorphic, principally adopting α-helix (˜45–52%), but with significant contributions (i.e., ˜10–26% each) from loop-turn, disordered/random and β-sheet components (Table 1). Interestingly, the secondary conformation proportions determined from CD analysis for B-YL (i.e., α-helix > random ˜loop-turn ˜β-sheet) in both surfactant lipids and lipid mimetics are all compatible with B-YL folding as an α-helix hairpin[18]. Moreover, the overall maintenance of these secondary conformations from CD spectra in Table 1 additionally supports our FTIR

findings that B-YL assumes a stable 3D-structure in environments of varying polarity.

Comparative FTIR spectroscopic studies were next performed on Super Mini-B (SMB) in lipid mimics and surfactant lipids to assess whether the B-YL substitutions in Figure 1 perturb the structure of the parent SMB. The secondary structures for SMB in lipid mimetics (i.e., deuterated 40 % TFE, deuterated aqueous SDS) and surfactant lipids (deuterated aqueous DPPC:POPC:POPG 5:3:2 mole:mole:mole) were investigated with conventional ^{12}C-FTIR spectroscopy. Figure 4 shows that representative FTIR spectra of the amide I band for SMB in these environments were similar, each indicating a dominant α-helical component centered at ˜1654–1655 cm^{-1} with a small low-field shoulder due to β-sheet at ˜1618–1620 cm^{-1}[40,43]. Self-deconvolution of the FTIR spectra in Figure 4 demonstrated that SMB in either TFE, SDS or surfactant lipids folds with secondary

Table 1. Spectroscopic proportions of secondary structure[a] for B-YL and SMB in lipid-mimetics and surfactant lipid.

System	% Conformation[a]			
	α-Helix	Loop-Turn	β-Sheet	Disordered
FTIR Analysis of B-YL[b]				
40% TFE	44.9	20.1	14.0	21.0
SDS	43.5	21.5	15.4	19.6
Surfactant Lipid	44.3	18.9	15.1	21.7
CD Analysis of B-YL[c]				
40% TFE	46.9	11.8	15.1	26.2
SDS	52.0	13.9	9.9	24.2
Surfactant Lipid	44.6	16.3	13.3	25.8
FTIR Analysis of SMB[d]				
40% TFE	47.1	19.4	20.2	13.3
SDS	44.9	22.2	12.2	20.7
Surfactant Lipid	42.3	27.5	11.7	18.5

[a]Tabulated results are means from four closely-reproduced separate determinations for each condition and spectral type.

[b]See Figure 2. ATR-FTIR spectra were estimated for proportions of the secondary structure for B-YL in 40% trifluoroethanol (TFE), sodium dodecyl sulfate SDS micelles and surfactant lipid-MLV films using self-deconvolution of the peptide amide I band (see **Methods** and **Results**).

[c]See Figure 3. Circular dichroism (CD) spectra were analyzed for proportions of the secondary structure for the B-YL mimic in 40% TFE, SDS micelles or surfactant lipid using spectral deconvolution (see **Methods** and **Results**).

[d]See Figure 4. ATR-FTIR spectra were estimated for proportions of the secondary structure for Super Mini-B (SMB) in SDS micelles and surfactant lipid-MLV films using self-deconvolution of the peptide amide I band (see **Methods** and **Results**).
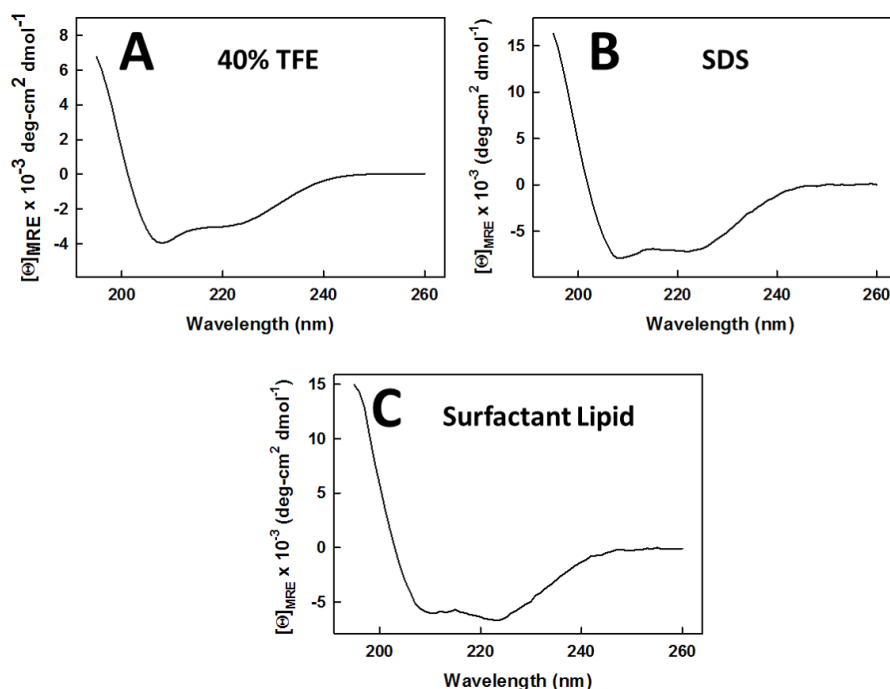
Figure 3. CD spectra of the B-YL mimic for SP-B in several lipid-mimetics and surfactant lipid. Circular Dichroism (CD) spectral plots show the mean residue ellipticities ($[\theta]_{MRE}$ x 10^{-3} deg-cm^2 dmol^{-1}) as a function of wavelength (nm). (**A**) 40% TFE. (**B**) SDS. (**C**) Surfactant Lipid. The double minimum at 208 and 222 nm in each plot indicates that α-helix is the dominant secondary structure for B-YL in these environments. Peptide concentrations were 100 μM. The optical pathlength was 0.01 cm and the temperature was 37°C. Spectra represent the average of 8 scans.
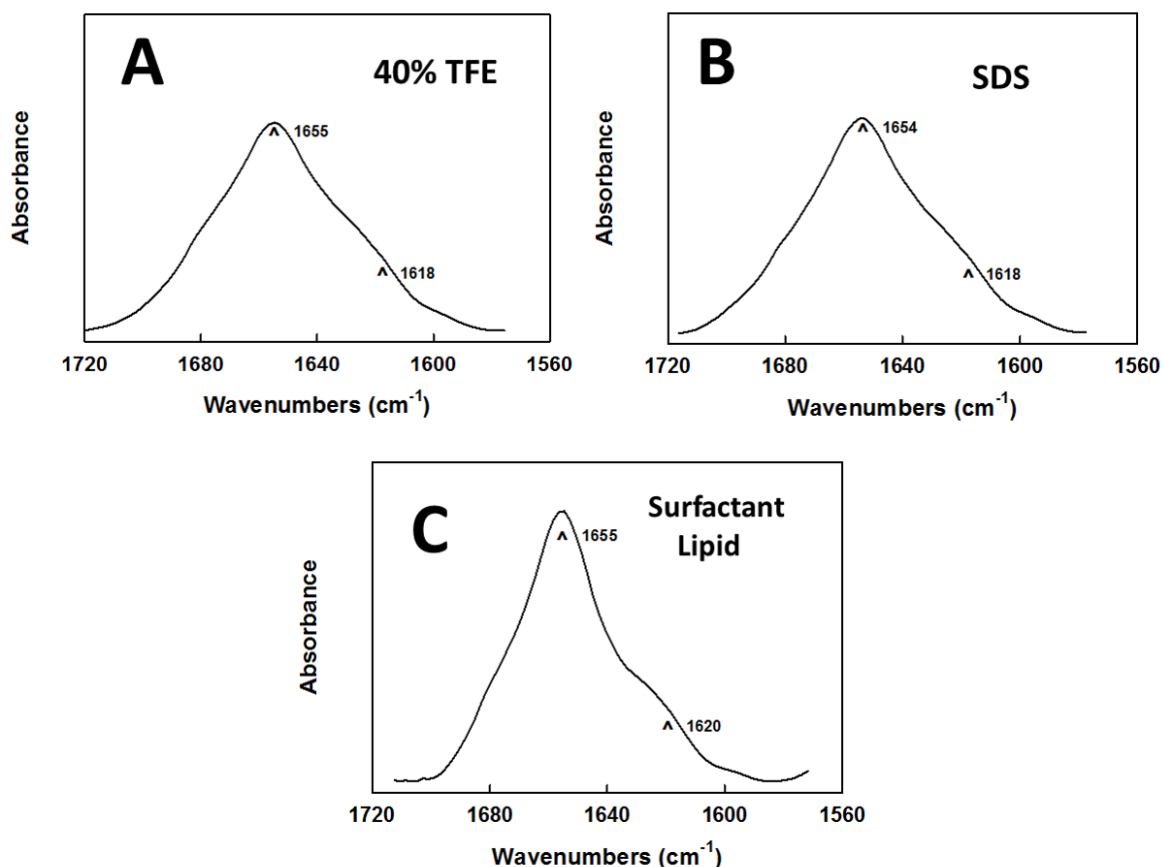
Figure 4. FTIR spectra of Super Mini-B (SMB) in several lipid-mimetics and surfactant lipids. Attenuated total reflectance Fourier transform infrared (ATR-FTIR) spectral plots show the absorbance (in arbitrary units) as a function of wavenumbers (cm⁻¹) (See **Methods** and **Results**). (**A**) Deuterated aqueous 40% TFE. (**B**) Deuterated aqueous SDS. (**C**) Deuterated aqueous surfactant lipid. In (**A**), (**B**) and (**C**), the IR spectra each show dominant α-helical components centered at 1654–1655 cm⁻¹ (arrows), with minor bands at ˜1618–1620 cm⁻¹ (arrows) due to β-sheet, ˜1682–1662 cm⁻¹ due to loop-turn/bend, and ˜1650–1637 cm⁻¹ due to disordered or random conformations. Peptide concentrations were 470 µM for the TFE solvent spectra, and 10:1 lipid:peptide (mole:mole) for the SDS detergent and surfactant lipid spectra. The areas under each absorbance curve are normalized.

structures that are characteristic of the α-helix hairpin (Table 1). Our finding that the respective secondary structure profiles for B-YL and SMB are similar in Table 1 suggests that the amino-acid substitutions in B-YL (e.g., four Cys residues replaced by Tyr) do not disrupt the α-helix hairpin conformation.

Captive bubble surfactometry

Captive bubble surfactometry of B-YL and SMB surfactants (3 mole% peptide in DPPC:POPC:POPG 5:3:2 mole:mole:mole), Curosurf®, and surfactant lipids alone demonstrated excellent surface activity of B-YL (Figure 5). Surface activity of BYL and SMB surfactant and Curosurf® were consistently and equally low during quasi-static cycling with values ≤1 mN/m, indicating that the modifications in the B-YL peptide did not lead to a loss in *in vitro* surface activity compared to its parent peptide SMB. In contrast, minimum surface tension values

of surfactant lipids alone far exceeded those of B-YL, SMB and Curosurf® surfactant and amounted to ˜18 mN/m (p<0.001).

In vivo experiments

Animal experiments directly examined the *in vivo* pulmonary activity of B-YL surfactant when instilled intratracheally in ventilated young adult rabbits with surfactant deficiency and impaired lung function induced by repeated saline lung lavages (Figure 6). Surfactant was administered by intratracheal instillation after the PaO₂ was reduced to stable levels <100 mmHg when breathing 100% oxygen. For this timeframe of study, this model reflects a relatively pure state of surfactant deficiency in animals with mature lungs. Rabbits receiving B-YL, SMB and Curosurf® surfactant had significantly improved arterial oxygenation over the 2 h period of study post-instillation compared to control rabbits instilled with surfactant lipids alone (Figure 6,
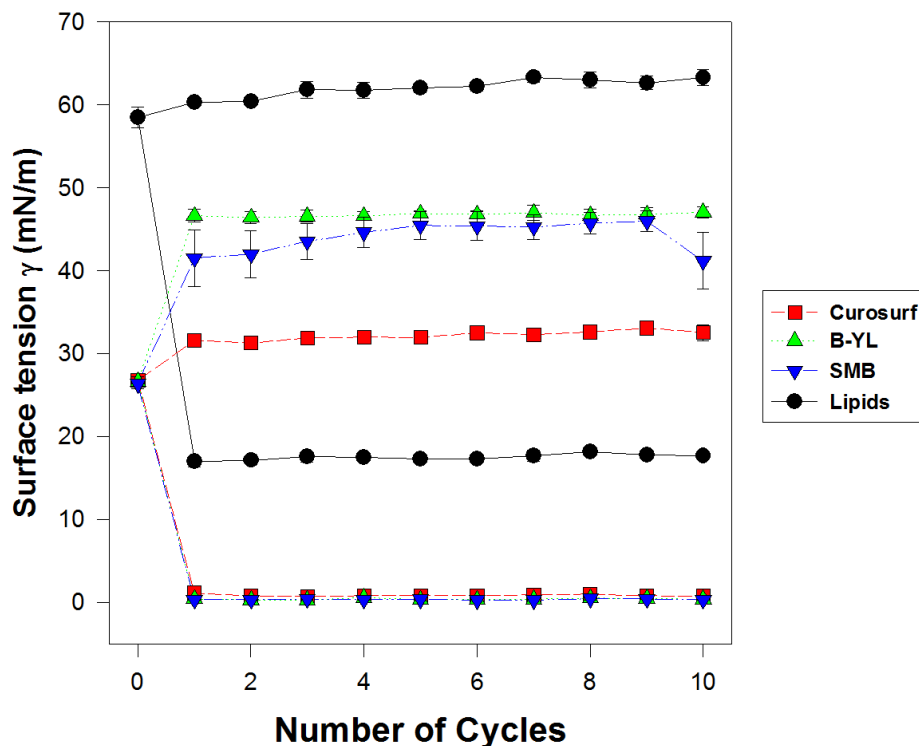
Figure 5. Surface tension reduction activity measured with captive bubble surfactometry. Surface activity of 3 mole% B-YL and Super Mini-B (SMB) in surfactant lipids were compared with a clinical surfactant (Curosurf®) as positive control and surfactant lipids alone (DPPC: POPC:POPG 5:3:2 mole:mole:mole) as negative control. The lower part of each curve indicates minimum surface tension and the upper part indicates maximum surface tension during 10 compression-expansion cycles. Minimum surface tension values of B-YL and SMB surfactant were similar to those of Curosurf®. Values are mean ± SEM of N=4-5.

p<0.001). Dynamic lung compliance also significantly improved over the same period of post-instillation study in rabbits treated with B-YL and SMB surfactants and Curosurf® compared to lipid-only controls (Figure 6). During the first 75 min after surfactant administration, mean PaO$_2$ values of the B-YL group were slightly lower than in the Curosurf® group (p<0.03), but were not different from those in the SMB group. Thereafter, mean PaO$_2$ values were similar among the three active surfactant preparations. Dynamic lung compliance of B-YL and SMB surfactants and Curosurf® was not statistically significant different throughout the study period. These data indicate that the modifications in the SP-B peptide mimic B-YL did not lead to a loss in *in vivo* surface activity, despite a difference in initial kinetics compared to Curosurf®.

Discussion

The basic premise tested here was whether the 'sulfur-free' B-YL peptide, a 41-residue Super Mini-B (SMB) variant that has its four cysteine residues replaced by tyrosine and its two methionine residues replaced by leucine, would fold with the same α-helix hairpin conformation earlier shown by SMB and Mini-B to correlate with high *in vitro* and *in vivo* surfactant activities[8,10,14,18].

In the present studies, circular dichroism (CD) and FTIR spectroscopy of 'B-YL' in surfactant lipids showed secondary

structures compatible with the peptide folding as an α-helix hairpin, similar to that of SMB in lipids (Figure 2–Figure 4; Table 1). Moreover, captive bubble surfactometry indicated excellent surface activity for B-YL surfactant (Figure 5), while also showing good oxygenation and dynamic compliance in lavaged, surfactant-deficient adult rabbits, an animal model of surfactant deficiency (Figure 6). One possible explanation of this correlation between α-helix turn and surfactant activity in B-YL is that the disulfide bonds of the parent SMB have been replaced by a core of clustered Tyr that instead crosslinks the N- and C- helices through noncovalent interactions involving aromatic rings. In this context, it is worthwhile to compare these B-YL results with an earlier functional and NMR structural investigation of Tachyplesin I (TP-I)[46]. TP-I is a 17-residue peptide that exhibits high antimicrobial activity and forms a β-hairpin (i.e., antiparallel β-sheet) stabilized by two disulfide cross-links. Interestingly, replacement of the disulfides with four tyrosines produced a TP-I analog (TP-1Y) with a stable β-hairpin conformation in solution and high antimicrobial activity, while the corresponding TP-I analog with four alanines (TP-IA) was unstructured in solution and inactive. Because of the proximity of the tyrosine side chains, Laederach *et al.*[46] proposed that the β-hairpin conformation and high antimicrobial activity of TP-IY were stabilized by aromatic ring stacking interactions (i.e., "π-stacking" interactions)[27–29]. It is tempting to speculate that tyrosines may be playing a similar role in maintaining the
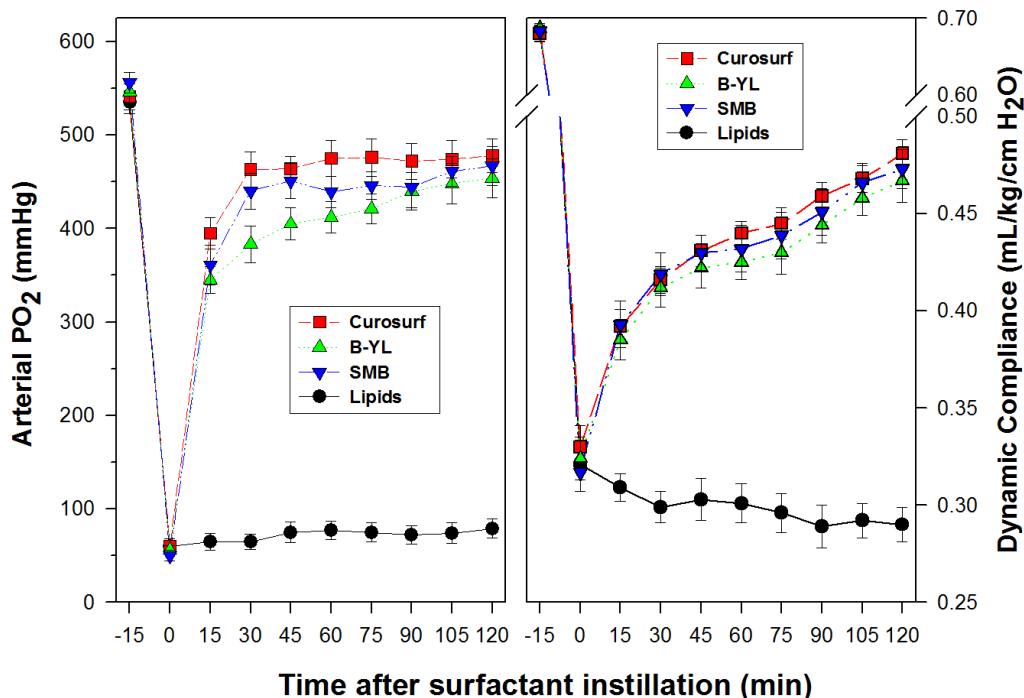
Figure 6. Arterial oxygenation and dynamic lung compliance in lavaged, surfactant-deficient, ventilated young adult rabbits treated with surfactant. Oxygenation (arterial PO_2 in mmHg) and dynamic lung compliance (mL/kg/cm H_2O) of 3 mole% B-YL (n=9) and SMB (n=8) in surfactant lipids were compared with a clinical surfactant (Curosurf®) (n=8) or lipids alone (DPPC:POPC:POPG 5:3:2 mole:mole:mole) (n=8). Surfactant (100 mg/kg) was administered intratracheally as a bolus at time 0. Values are mean ± SEM. Differences in oxygenation and dynamic compliance between B-YL, SMB and Curosurf surfactants were not statistically significant, except for slightly reduced PaO_2 values for B-YL surfactant compared to Curosurf®) (p<0.03), but not to SMB, during the first 75 min post-instillation. Differences between B-YL, SMB and Curosurf®) surfactant with lipids alone were significant (p<0.001).

α-helix hairpin structure and high surfactant activity of B-YL in lipid environments. To address remaining questions, we are planning long-time (≥ 1.0 μsec) production runs for all-atom MD simulations of B-YL to ensure that our final models are fully equilibrated in hydrated surfactant lipid bilayers.

Our 'sulfur-free' B-YL mimic may prove to be superior to its parent SMB for treating premature infants with RDS for several reasons. First, B-YL is less expensive and faster to synthesize than SMB because it omits an oxidation step and is self-folding. Second, the substitution of Cys and Met residues with Tyr and Leu, respectively, may render the B-YL mimic less susceptible to inactivation by reactive oxygen species.

Lung immaturity and surfactant deficiency are the main cause of RDS in very preterm infants. Mechanical ventilation and exposure to high oxygen concentrations may lead to an inflammatory lung process resulting in bronchopulmonary dysplasia (BPD), a chronic lung disease of preterm infants. Use of antenatal steroids, administration of exogenous surfactant, and advanced modes of ventilation have shown only limited benefits in preventing BPD. In a neonatal rat model of hyperoxia-induced lung injury, we found that nebulized PPARγ agonist pioglitazone (PGZ) with B-YL surfactant accelerates lung maturation and prevents neonatal hyperoxia-induced lung injury more than with either modality alone, thereby potentially preventing BPD more

effectively[47]. These findings suggest a potential role for B-YL surfactant as a vehicle for intrapulmonary drug therapy.

Conclusion
The 'sulfur-free' B-YL forms an amphipathic helix-hairpin in surfactant liposomes with high surface activity, and is functionally similar to its parent (SMB) and native SP-B. This self-folding peptide is easier to synthesize and may provide an extra edge over SMB in the treatment of respiratory failure in premature infants with RDS.

Data availability
Raw data are available on OSF: http://doi.org/10.17605/OSF.IO/6295P[48]

Data are available under the terms of the Creative Commons Zero "No rights reserved" data waiver (CC0 1.0 Public domain dedication).

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Data Source

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Current Referee Status: ✓ ✓

✓   **Jesús Pérez-Gil** [iD]

Department of Biochemistry, Faculty of Biology and Research Institute Hospital 12 de Octubre, Complutense University, Madrid, Spain

All the questions raised have been properly addressed in my opinion.

Competing Interests: No competing interests were disclosed.

I have read this submission. I believe that I have an appropriate level of expertise to confirm that it is of an acceptable scientific standard.

✓   **Valerie Booth** [iD]

Department of Biochemistry , Memorial University of Newfoundland, St. John's, NL, Canada

All concerns have been addressed.

Competing Interests: No competing interests were disclosed.

I have read this submission. I believe that I have an appropriate level of expertise to confirm that it is of an acceptable scientific standard.

?   **Jesús Pérez-Gil** [iD]

Department of Biochemistry, Faculty of Biology and Research Institute Hospital 12 de Octubre, Complutense University, Madrid, Spain

This study summarizes data on the design, synthesis, structure, lipid-protein interactions and surface activity -in vitro and in vivo- of a synthetic peptide analog that mimics certain functional segments of pulmonary surfactant protein SP-B. The team responsible has a large expertise in the design and optimization of this type of peptide mimics of surfactant proteins, and the current work is a natural and smart extension of previous work.

In the current study, an analog to a well-know synthetic surrogate of SP-B, the so-called "mini-B", has been produced and analyzed in detail. This 'B-YL' peptide is a mini-B version in which cysteines forming disulphide bonds, in both wild-type SP-B ad mini-B, have been substituted by tyrosines. The rational is that tyrosines could also contribute to stabilize peptide-peptide interactions via pi-stacking and so yielding the typical alpha-helical hairpin bridging N-terminal and C-terminal helical segments of SP-B together. The substitution of Cys by Tyr would on the other hand supposedly render a peptide surrogate with lower propensity to oxidation, according to the authors, thanks to the elimination of cysteine residues. The authors essentially conclude that the B-YL analog is entirely comparable, structurally and functionally, to the mini-B construct, and therefore that it would be equally useful to produce synthetic surfactants for therapeutic applications.

In general term, the experiments have been properly carried out, and they provide extensive proofs that the peptide behaves "in general terms" as it was expected. In this sense, it is interesting to see how the substitution of two disulphides by tyrosine clustering could also serve as a basis to build a membrane active peptide fold such as the one of miniB. This is certainly an advantage from the point of view of its synthesis and production. It would be for instance a large advantage to produce inexpensive large amounts of this type of peptides by overexpression in bacteria, where formation of disulphides would certainly be a limitation.

What I am not so sure is whether this peptide would actually be less susceptible to oxidation, and whether this peptide is actually a good mimic of SP-B, or at least as good as miniB.

As mentioned by the authors, one of the targets of oxidation-promoted inactivation of surfactant proteins are the aromatic residues. Actually, oxidation of the single tryptophan of SP-B (as shown by the group of Possmayer), located at its N-terminal helical segment, is associated with complete inactivation of the protein. It had been previously demonstrated (see for instance Ryan et al.(2005)[1] or Serrano et al.(2006)[2]) that this tryptophan is essential at the N-terminal end of SP-B, because it takes part of a highly hydrophobic-at-interface segment which defines very high affinity for insertion into amphipathic environments such as the air-liquid interface. Now this tryptophan has been substituted in the whole peptide by 4 tyrosines. Tyrosines may maintain a high affinity for the interface, but at the cost of being also highly susceptible to oxidation. The authors could have shown if it is true that exposure of B-YL to oxidant reactions preserve its activity better than in the case of mini-B.

Another major problem in my opinion of the substitution of disulphides by aromatic residues is that it may change dramatically the way the peptide perturb surfactant membranes and films, which is crucial for a good surface activity. Disulphides surely impose a very strong constraint to the way the two helical segments orientate into the membrane. As a matter of fact, the tilting of the amphipathic N-terminal and C-terminal helical segments of SP-B, which is crucial for perturbing lipid packing to the point of facilitating the transfer of surface active lipid molecules between different bilayers or between the bilayers and the interfacial film, may be subtly different in the case of miniB, and even more in the B-YL analog. I may agree that tyrosine clustering could roughly join the C-terminal and N-terminal segments together, but they would do that in a much more flexible, dynamic and fluctuating way than the disulphides, and that may reduce the efficiency for the protein to induce membrane perturbation. This could be the reason why

the maximum surface tension during cycling of lipid-peptide films at the CBS is higher in the case of B-YL than in mini-B, and much more than in the case of Curosurf containing true SP-B, where the full protein structure may impose a much more defined geometry to the N-terminal/C-terminal helical cluster. The limited MD simulations in the presence of lipids also seem to show differences in tilting of the helical segments with respect to the lipid surface. The authors have neglected these differences, but it would be important to show if they do really exist and whether they are reproducible.

Other problem related with the final geometry of the lipid-protein complexes comes from the high hydrophobicity-at interface of the aromatic residues. The introduction of the 4 tyrosines may force a orientation of the mini-B construct which is much more anchored to the surface of the membrane, where aromatic residues tend to partittion, than it is the case for the tyrosine-free miniB analog.

The authors may argue that the MD simulations do not reflect substantial differences in this respect, but a 500 nsec simulation may or may not be enough to get the fully equilibrated state. Have the authors data to evaluate whether longer simulation times could reflect further differences?

There is an additional important issue that may arise from the substitution of disulphides by tyrosines that in my opinion has not been properly addressed. If tyrosine clustering is enough to sustain intramolecular peptide-peptide interactions stabilizing the miniB hairpin, it may also sustain intermolecular peptide-peptide interactions, which could end in peptide aggregation and clustering, potentially reducing lipid dispersion and surface activity. The simulations run do not contain multiple copies of the peptide, which could assess whether intramolecular interactions are or not preferred over intermolecular interactions, provided that long enough simulation times are applied. This problem does not exist, obviously, when the intramolecular interactions are maintained through covalent disulphide bonds.

Other questions that would require some discussion include:

The use of surfactant therapy on therapies to treat ALI and ARDS is under strong debate. It is doubtful that a simple synthetic surfactant such as those containing mini-B-like peptides as surrogates of SP-B would be ever useful to treat ARDS patients.

The way captive bubble experiments have been performed has been poorly explained and produce some confusion. In page 6, it is stated that CBS has been operated at "physiological cycling rate". However, in page 14, it is stated that "Surface activity of … were consistently and equally low during quasi-static cycling…" What has been the compression-expansion cycling rate used? There is also some confusion with respect to the concentration at which the different surfactants have been tested in the CBS. In page 6, it is stated that "We routinely analyze surfactant preparations at an average surfactant lipid concentration of 25 microgram/mL". Later it is stated that "We used a B-YL surfactant mixture consisting on… with a concentration of 35 mg/mL". Could you, please, clarify what is the lipid concentration used to obtain each of the CBS isotherms? How this concentration is applied (in the bulk hypophase in which the bubble is formed, or directly at the air-water interface)?

The results of MD simulations have been taken as a proof that the cys-by-tyr substitutions can also sustain the intramolecular peptide-peptide interactions defining the formation of the miniB-like helical hairpin in surfactant lipids. The question is what were the initial points of at which those simulations were started? Where the aromatic sidechains already clustered or where the peptides initially embedded into the membranes in an extended conformation, to see whether the propensity for the hairpin to be formed could be entirely sustained by the affinity of tyrosines to pile? This second type of experiments could be crucial. Even better if the simulation cell could contain not one but several copies of the peptides.

The experiments in vivo seems to indicate that the B-YL analog is less active than miniB or Curosurf to produce good oxygenation indexes, at least at the shortest times. The authors state that there is no statistical difference between the activity in vivo of the two different analogs. Have they tested statistical significance of the means only at the end times or have they checked whether differences at shorter times could indicate that there may be a significant difference in kinetics (which can also functionally important)?

In summary, I think the study can be of interest for the scientists in the field, and may open alternatives in the design of other peptides or proteins potentially mimicking the structure-function determinants of surfactant proteins. However, instead of merely summarizing the main parallelisms found, the Discussion should be completed by addressing all the questions raised in this report, which could add substantially to the structural and functional features that may be important to test when designing future new peptide surfactant additives for therapeutic purposes.

References
1. Ryan MA, Qi X, Serrano AG, Ikegami M, Perez-Gil J, Johansson J, Weaver TE: Mapping and analysis of the lytic and fusogenic domains of surfactant protein B.*Biochemistry*. 2005; **44** (3): 861-72 PubMed Abstract | Publisher Full Text
2. Serrano AG, Ryan M, Weaver TE, Pérez-Gil J: Critical structure-function determinants within the N-terminal region of pulmonary surfactant protein SP-B.*Biophys J*. 2006; **90** (1): 238-49 PubMed Abstract | Publisher Full Text

Is the work clearly and accurately presented and does it cite the current literature?
Partly

Is the study design appropriate and is the work technically sound?
Yes

Are sufficient details of methods and analysis provided to allow replication by others?
Partly

If applicable, is the statistical analysis and its interpretation appropriate?
Partly

Are all the source data underlying the results available to ensure full reproducibility?
Partly

Are the conclusions drawn adequately supported by the results?
Partly

Competing Interests: No competing interests were disclosed.

I have read this submission. I believe that I have an appropriate level of expertise to confirm that it is of an acceptable scientific standard, however I have significant reservations, as outlined above.

Frans Walther, LA BioMed at Harbor-UCLA Medical Center, USA

Response to Reviewer Dr. Pérez-Gil:

1. What I am not so sure is whether this peptide would actually be less susceptible to oxidation, and whether this peptide is actually a good mimic of SP-B, or at least as good as miniB.

As mentioned by the authors, one of the targets of oxidation-promoted inactivation of surfactant proteins are the aromatic residues. Actually, oxidation of the single tryptophan of SP-B (as shown by the group of Possmayer), located at its N-terminal helical segment, is associated with complete inactivation of the protein. It had been previously demonstrated (see for instance Ryan et al.(2005)[1] or Serrano et al.(2006)[2]) that this tryptophan is essential at the N-terminal end of SP-B, because it takes part of a highly hydrophobic-at-interface segment which defines very high affinity for insertion into amphipathic environments such as the air-liquid interface. Now this tryptophan has been substituted in the whole peptide by 4 tyrosines. Tyrosines may maintain a high affinity for the interface, but at the cost of being also highly susceptible to oxidation. The authors could have shown if it is true that exposure of B-YL to oxidant reactions preserve its activity better than in the case of mini-B.

Response: As suggested by the other reviewer, Dr. Valerie Booth, we decided to remove all claims of "oxidation-resistance" in the title, abstract, conclusion and other parts of the text. The B-YL peptide is somewhat less sensitive to oxidation by Leu substitution for Met and storage for one year showed no Tyrosine or Tryptophan oxidation by mass spec. B-YL is actually as good a mimic of SP-B as Super Mini-B at captive bubble surfactometry and in the lavaged, surfactant-deficient rabbits.

2. Another major problem in my opinion of the substitution of disulphides by aromatic residues is that it may change dramatically the way the peptide perturb surfactant membranes and films, which is crucial for a good surface activity. Disulphides surely impose a very strong constraint to the way the two helical segments orientate into the membrane. As a matter of fact, the tilting of the amphipathic N-terminal and C-terminal helical segments of SP-B, which is crucial for perturbing lipid packing to the point of facilitating the transfer of surface active lipid molecules between different bilayers or between the bilayers and the interfacial film, may be subtly different in the case of miniB, and even more in the B-YL analog. I may agree that tyrosine clustering could roughly join the C-terminal and N-terminal segments together, but they would do that in a much more flexible, dynamic and fluctuating way than the disulphides, and that may reduce the efficiency for the protein to induce membrane perturbation. This could be the reason why the maximum surface tension during cycling of lipid-peptide films at the CBS is higher in the case of B-YL than in mini-B, and much more than in the case of Curosurf® containing true SP-B, where the full protein structure may impose a much more defined geometry to the N-terminal/C-terminal helical cluster. The limited MD simulations in the presence of lipids also seem to show differences in tilting of the helical segments with respect to the lipid surface. The authors have neglected these differences, but it would be important to show if they do really exist and whether they are reproducible.

Other problem related with the final geometry of the lipid-protein complexes comes from the high hydrophobicity-at interface of the aromatic residues. The introduction of the 4 tyrosines may force a orientation of the mini-B construct which is much more anchored to the surface of the membrane, where aromatic residues tend to partitition, than it is the case for the tyrosine-free miniB analog.

Response: Simulations show no difference in surface orientation. However, we decided to remove the homology models, molecular dynamic (MD) simulation and Membrane Protein Explorer (MPEx) from the manuscript as modeling was only a secondary focus and we plan to publish a more extensive data set in a follow-up paper.

3. The authors may argue that the MD simulations do not reflect substantial differences in this respect, but a 500 nsec simulation may or may not be enough to get the fully equilibrated state. Have the authors data to evaluate whether longer simulation times could reflect further differences?

Response: We extended simulations to one microsecond. However, we have decided to remove the homology models, molecular dynamic (MD) simulation and Membrane Protein Explorer (MPEx) from the manuscript as modeling was only a secondary focus and we plan to publish a more extensive data set in a follow-up paper.

4.   There is an additional important issue that may arise from the substitution of disulphides by tyrosines that in my opinion has not been properly addressed. If tyrosine clustering is enough to sustain intramolecular peptide-peptide interactions stabilizing the miniB hairpin, it may also sustain intermolecular peptide-peptide interactions, which could end in peptide aggregation and clustering, potentially reducing lipid dispersion and surface activity. The simulations run do not contain multiple copies of the peptide, which could assess whether intramolecular interactions are or not preferred over intermolecular interactions, provided that long enough simulation times are applied. This problem does not exist, obviously, when the intramolecular interactions are maintained through covalent disulphide bonds.

Response: Insertion sequence keeps this from happening. However, we decided to remove the homology models, molecular dynamic (MD) simulation and Membrane Protein Explorer (MPEx) paragraphs from the manuscript as modeling was only a secondary focus and plan to publish a more extensive data set in a follow-up paper.

5. The use of surfactant therapy on therapies to treat ALI and ARDS is under strong debate. It is doubtful that a simple synthetic surfactant such as those containing mini-B-like peptides as surrogates of SP-B would be ever useful to treat ARDS patients.

Response: We agree with the reviewer and removed the statements about surfactant therapy for ALI/ARDS from the abstract, the last paragraph of page 3, discussion and conclusion.

6. The way captive bubble experiments have been performed has been poorly explained and produce some confusion. In page 6, it is stated that CBS has been operated at "physiological cycling rate". However, in page 14, it is stated that "Surface activity of … were consistently and equally low during quasi-static cycling…" What has been the compression-expansion cycling rate used? There is also some confusion with respect to the concentration at which the different surfactants have been tested in the CBS. In page 6, it is stated that "We routinely analyze surfactant preparations at an average surfactant lipid concentration of 25 microgram/mL". Later it is stated that "We used a B-YL surfactant mixture consisting on… with a concentration of 35 mg/mL". Could you, please, clarify what is the lipid concentration used to obtain each of the CBS isotherms? How this concentration is applied (in the bulk hypophase in which the bubble is formed, or directly at the air-water interface)?

Response: We actually do both quasi-static and dynamic cycling on the CBS. Quasi-static compression and expansion of the air bubble is performed in discrete steps at a rate of 5% of the bubble volume every 10 sec with continuous video recording of the bubble shape. Dynamic compression and expansion cycling is performed between 10 and 110% of the original bubble area at a cycling rate of 20 cycles/min. Both modalities show extreme flattening of the bubble in active surfactant preparations. In figure 9 we present the data from quasi-static cycling. We routinely analyze surfactant preparations at an average surfactant lipid concentration of 25 μg/mL in the bubble chamber, but as Curosurf® is more concentrated than synthetic surfactant, we applied 1 μL of synthetic surfactant at 35 mg/mL and 0.5 μL of Curosurf® at 80 mg/mL to the bubble chamber (1.5 mL volume). After introducing surfactant and the air bubble into the CBS chamber, both move upwards to the interface with the agarose plug. This information was added to the methods section.

7. The results of MD simulations have been taken as a proof that the cys-by-tyr substitutions can also sustain the intramolecular peptide-peptide interactions defining the formation of the miniB-like helical hairpin in surfactant lipids. The question is what were the initial points of at which those simulations were started? Where the aromatic sidechains already clustered or where the peptides initially embedded into the membranes in an extended conformation, to see whether the propensity for the hairpin to be formed could be entirely sustained by the affinity of tyrosines to pile? This second type of experiments could be crucial. Even better if the simulation cell could contain not one but several copies of the peptides.

Response: See above, we decided to remove the homology models, molecular dynamic (MD) simulation and Membrane Protein Explorer (MPEx) from the manuscript as modeling was only a secondary focus and we plan to publish a more extensive data set in a follow-up paper.

8. The experiments in vivo seems to indicate that the B-YL analog is less active than miniB or Curosurf to produce good oxygenation indexes, at least at the shortest times. The authors state that there is no statistical difference between the activity in vivo of the two different analogs. Have they tested statistical significance of the means only at the end times or have they checked whether differences at shorter times could indicate that there may be a significant difference in kinetics (which can also functionally important)?

Response: In response to the reviewer, we calculated the statistical significance of differences in means at every 15 min time-point after surfactant instillation and found no significant differences in dynamic compliance for the 3 surface-active surfactant preparations throughout the experiments. During the first 75 min after surfactant administration, mean oxygenation values of the B-YL surfactant group were slightly lower than in the Curosurf® group ($p<0.03$), but not different from those in the Super Mini-B (SMB) group, suggesting initial differences in kinetics. Thereafter oxygenation was similar in B-YL, SMB and Curosurf® surfactants. This info was added to the results section.

Competing Interests: No competing interests were disclosed.

Valerie Booth 🆔

Department of Biochemistry , Memorial University of Newfoundland, St. John's, NL, Canada

Walther, Gupta, Gordon and Waring present studies of a peptide called "B-YL" which they propose may be of utility as a component of exogenous lung surfactant replacement therapy for conditions such as ARDS and ALI. In these conditions, the lung environment is "hostile" to lung surfactant proteins and a variety of inactivating components including reactive oxygen species are present. The experimental work is comprehensive and complementary, as well as expertly done and clearly described (with a few exceptions as detailed below). I appreciate the effort to include the *in vitro* and *in vivo* functional work, as well as the structural studies. I also believe that the general thrust of the work – creating peptide components that can act in place of full length lung surfactant proteins – is very worthwhile.

However, I do have trouble with the premise for the paper – that removing the cysteines and methionines will make the peptide "oxidation-resistant". The authors present no justification that cysteines are a target of ROS damage. In the lung surfactant literature, it's been shown that tryptophan 9 is the main target of oxidation, with methionine residues as a secondary target[1]. The peptide B-YL leaves this critical tryptophan unchanged and so may well be just as susceptible to damage as the parent peptide, super Mini-B. Furthermore, it's been proposed that one of the roles of methionines may be to protect the tryptophan by competing for ROS. So, removing the methionines may actually make the protein MORE susceptible to damage. Thus, I think the options are to either back up the assertion that the new peptide is "oxidation-resistant" with convincing experiments or references OR to remove all claims of "oxidation-resistant" from the title, abstract, conclusion and other parts of the text. I think the work stands up perfectly well without the oxidation-resistant angle, because as the authors point out, the removal of the cysteines makes it more feasible to scale up production to the scale needed for clinical work.

Here are the rest of my comments, roughly in the same order as the manuscript:
1. Page 3 bottom left. Could you add a sentence to describe what an alpha-helix hairpin is. It's referred to several times later on so it would be good to start with a clear picture of what it is.

2. Page 3, right hand column, 15 lines from the bottom reads "Trp-9 plays a role in the diminished surface activity possibly due to fraying of the N-terminal alpha-helix 24,25". Reference 25 actually suggests the activity may be changed because the oxidizing Trp to kynurenine causes the peptide to change its depth within the lipid layer.

3. Page 3, right hand column, 1st sentence of the last paragraph. I'm not sure we can say that "ALI/ARDS… REQUIRES the use of exogenous surfactants" since I'm not sure that it's ever been convincingly shown that exogenous surfactant treatment substantially helps patients with ALI/ARDS – can you re-phrase or back-up this statement.

4. Page 3, last sentence. It would be good to explain why tyrosine (instead of some other amino acid) was chosen to replace the Cys and Met.

5. Methods paragraph 2 and throughout. It is very (very!) confusing that Super Mini-B is referred to throughout as "oxidized" SMB. The "oxidation" is just the normal formation of disulfide bonds and since the paper is supposedly about making a "oxidation-resistant" version of SMB the two meanings are bound to be confounded by readers. I would just call it "SMB with disulfides" or something else along those lines.

6. Page 5, last paragraph. Instead of referring to a "recent homology modeling program" and then naming the actual program 5 lines later, I would put the name of the program right into this first sentence.

7. Page 5, last paragraph – which pdb files went into the homology model? I think it's important to know this as there's not that much sequence homology between SP-B and known structures so whichever ones I-TASSER is choosing will have a big influence on how the structures look.

8. Page 5 under the MD section… what was the initial position of the peptide within the bilayer and how did it change during the simulation? Note that Khatami et al.[2] found that the secondary structure of full length SP-B in MD simulations varied a lot with the position with respect to the bilayer. This is probably less important for the smaller peptide, but is still worth mentioning the initial position and the rationale for it.

9. Page 6 under Captive bubble surfactometry. I'm guessing 3% of B-YL peptide is by mole? Can you add this info (here and later on) so we're clear?

10. Page 7 top left. At some point (not necessarily here) it would be good to compare the dose of surfactant (in mg/kg body weight) to what is given to preemies. Also could you comment on why the dose is lower for Curosurf than for SMB?

11. Page 7. First paragraph under results and Table 1. I'm not sure its appropriate to quote "Beta-sheet" content since I don't think you are proposing there is an actual sheet involved. Could you choose something more appropriate, like "beta-strand" or "beta-hairpin" or just "beta-structure".

12. Figure 3. Since estimating secondary structure is so dependent on protein concentration, it would be good to know what method was used to determine the peptide concentration (desalted dry weight, UV, colorimeteric assay ?).

13. Page 10, and later. The "core of hydrophobic residues" should be compared to that of Mini-B's experimental structure. How much of this core is preserved in your predictions for the SMB and B-YL structures?

14. Page 10, bottom left and elsewhere. Can you replace all instances of "largely agrees with" with something more quantitative – like backbone RMSD.

15. Page 11, left hand column. In addition to the pi stacking interactions, I'm wondering what the hydroxyl on the tyrosines are doing – are they finding anything to hydrogen bond to? What?

16. Page 11, 2nd sentence of the 2nd paragraph: as for 14 above, please replace "generally in good agreement" with something more precise.

17. Page 11 – How do the starting and final structures and hydrophobic core compare to the experimental structure for Mini-B in SDS micelles?

18. Page 14 – right hand side, please replace "good overlap" and "remarkably analogous" with something more precise like RMSD.

References

1. Manzanares D, Rodriguez-Capote K, Liu S, Haines T, Ramos Y, Zhao L, Doherty-Kirby A, Lajoie G, Possmayer F: Modification of tryptophan and methionine residues is implicated in the oxidative inactivation of surfactant protein B.*Biochemistry*. 2007; **46** (18): 5604-15 PubMed Abstract | Publisher Full Text

2. Khatami MH, Saika-Voivod I, Booth V: All-atom molecular dynamics simulations of lung surfactant protein B: Structural features of SP-B promote lipid reorganization.*Biochim Biophys Acta*. **1858** (12): 3082-3092 PubMed Abstract | Publisher Full Text

Is the work clearly and accurately presented and does it cite the current literature?
Partly

Is the study design appropriate and is the work technically sound?
Partly

Are sufficient details of methods and analysis provided to allow replication by others?
Yes

If applicable, is the statistical analysis and its interpretation appropriate?
Yes

Are all the source data underlying the results available to ensure full reproducibility?
No

Are the conclusions drawn adequately supported by the results?
Partly

Competing Interests: No competing interests were disclosed.

I have read this submission. I believe that I have an appropriate level of expertise to state that I do not consider it to be of an acceptable scientific standard, for reasons outlined above.

Author Response 04 Jul 2018
Frans Walther, LA BioMed at Harbor-UCLA Medical Center, USA

Response to Reviewer Dr. Valerie Booth:

General response

Suggestion to remove all claims of "oxidation-resistance" from the title, abstract, conclusion, and other parts of the text.

Response: We removed all claims of "oxidation-resistance" from the title, abstract, conclusion, and text. As B-YL peptide is sulfur-free, the title was changed to: "A sulfur-free peptide mimic of surfactant protein B (B-YL) exhibits high in vitro and in vivo surface activities".

Specific comments:

1. Page 3, bottom left. Could you add a sentence to describe what an alpha-helix hairpin is. It's referred to several times later on so it would be good to start with a clear picture of what it is.

Response: We have added to Page 3 bottom left a definition of an alpha helix hairpin structure as well as provided a literature reference for it: "SMB incorporates the N-terminal α-helix (~residues 8-25) and C-terminal α-helix (~residues 63-78) of native SP-B as a single linear peptide (Figure 1A), joined together with a customized turn to form a α-helix hairpin (a-helix/turn/a-helix, ata) [Fezoui Y et al., 1994]."

2. Page 3, right hand column, 15 lines from the bottom reads "Trp-9 plays a role in diminished surface activity possibly due to fraying of the N-terminal alpha helix 24-25". Reference 25 actually suggest the activity may be changed because the oxidizing Trp to kynurenine causes the peptide to change its depth within the lipid layer.

Response: We have added here that this is in the full-length protein and in addition we have incorporated mass spec findings in the results section that indicate that the B-YL peptide does not undergo oxidation of its aromatic amino acids when stored in surfactant lipids for one year:
"Trp-9 can play a role in diminished surface activity in the full length native protein possibly due to fraying of the N-terminal alpha helix 24-25".
"Mass spectral analysis of the B-YL peptide indicated there was no change in the molecular weight due to oxidation of tyrosines or tryptophan for one year when formulated with surfactant lipids."

3. Page 3, right hand column, 1st sentence of the last paragraph. I'm not sure we can say that "ALI/ARDS… REQUIRES the use of exogenous surfactants" since I'm not sure that it's ever been convincingly shown that exogenous surfactant treatment substantially helps patients with ALI/ARDS – can you re-phrase or back-up this statement.

Response: We agree with the reviewer and removed this sentence and "surfactant dysfunction in ALI/ARDS" in the next sentence.

4. Page 3, last sentence. It would be good to explain why tyrosine (instead of some other amino acid) was chosen to replace the Cys and Met.

Response: We have added an additional sentence (line 2, page 4) to explain our rational in the selection of tyrosine and leu for cysteine and methionine. We opted for leucine rather than the commonly used norleucine because we felt that the use of non-natural amino acids may cause longer term metabolic problems *in vivo*.
"Tyrosine was substituted for cysteine because of its aromatic ring interactions that emulate disulfide formation [55, 56, 57] and methionine, that is easily oxidized, was replaced by leucine based on its similar molecular structure and polarity [44]."

5. Methods paragraph 2 and throughout. It is very (very!) confusing that Super Mini-B is referred to throughout as "oxidized" SMB. The "oxidation" is just the normal formation of disulfide bonds and since the paper is supposedly about making a "oxidation-resistant" version of SMB the two meanings are bound to be confounded by readers. I would just call it "SMB with disulfides" or something else along those lines.

Response: We have modified the text using "SMB" instead of "oxidized SMB" in accordance with the reviewer's comments, so that there is no confusion with oxidation of the peptide per se.

6. Page 5, last paragraph. Instead of referring to a "recent homology modeling program" and then naming the actual program 5 lines later, I would put the name of the program right into this first sentence.

Response: We decided to remove the homology models, molecular dynamic (MD) simulation and Membrane Protein Explorer (MPEx) from the manuscript as modeling was only a secondary focus and we plan to publish a more extensive data set in a follow-up paper.

7. Page 5, last paragraph – which pdb files went into the homology model? I think it's important to know this as there's not that much sequence homology between SP-B and known structures so whichever ones I-TASSER is choosing will have a big influence on how the structures look.

Response: Please see our response to reviewer's comment #6.

8. Page 5 under the MD section… what was the initial position of the peptide within the bilayer and how did it change during the simulation? Note that Khatami et al.[2] found that the secondary structure of full length SP-B in MD simulations varied a lot with the position with respect to the bilayer. This is probably less important for the smaller peptide, but is still worth mentioning the initial position and the rationale for it.

Response: Please see our response to reviewer's comment #6.

9. Page 6 under Captive bubble surfactometry. I'm guessing 3% of B-YL peptide is by mole? Can you add this info (here and later on) so we're clear?

Response: We used 3 mole % of B-YL or SMB peptide. We have clarified the concentration by including a reference [17] detailing the formulation used for SMB and B-YL peptides.

10. Page 7 top left. At some point (not necessarily here) it would be good to compare the dose of surfactant (in mg/kg body weight) to what is given to preemies. Also could you comment on why the dose is lower for Curosurf than for SMB?

Response: All animals received 100 mg lipids/kg bodyweight of one of the 4 surfactant preparations. The clinical surfactant Curosurf® is formulated by the manufacturer at 80 mg/mL and SMB and B-YL surfactant and lipids alone were formulated at 35 mg/mL, so Curosurf® was dosed at 1.25 mL/kg and the synthetic surfactants at 2.9 mL/kg. Clinical surfactant is routinely dosed at 100 mg/kg in premature infants with RDS, so the dosages used here were similar to those used clinically. This info was added to the methods on page 7.

11. Page 7. First paragraph under results and Table 1. I'm not sure its appropriate to quote "Beta-sheet" content since I don't think you are proposing there is an actual sheet involved. Could you choose something more appropriate, like "beta-strand" or "beta-hairpin" or just "beta-structure".

Response: We have replaced "Beta-sheet" with "beta-structure".

12. Figure 3. Since estimating secondary structure is so dependent on protein concentration, it would be good to know what method was used to determine the peptide concentration (desalted

dry weight, UV, colorimeteric assay ?).

Response: We have added the following description and reference with regard to peptide quantitation: "Peptides were routinely quantitated using UV absorbance based on the assay procedure developed by Anthis and Clore (Protein Science, 2013)".

13. Page 10, and later. The "core of hydrophobic residues" should be compared to that of Mini-B's experimental structure. How much of this core is preserved in your predictions for the SMB and B-YL structures?

Response: Please see our response to reviewer's comment #6.

14. Page 10, bottom left and elsewhere. Can you replace all instances of "largely agrees with" with something more quantitative – like backbone RMSD.

Response: Please see our response to reviewer's comment #6.

15. Page 11, left hand column. In addition to the pi stacking interactions, I'm wondering what the hydroxyl on the tyrosines are doing – are they finding anything to hydrogen bond to? What?

Response: Please see our response to reviewer's comment #6.

16. Page 11, 2nd sentence of the 2nd paragraph: as for 14 above, please replace "generally in good agreement" with something more precise.

Response: Please see our response to reviewer's comment #6.

17. Page 11 – How do the starting and final structures and hydrophobic core compare to the experimental structure for Mini-B in SDS micelles?

Response: Please see our response to reviewer's comment #6.

18. Page 14 – right hand side, please replace "good overlap" and "remarkably analogous" with something more precise like RMSD.

Response: Please see our response to reviewer's comment #6.

Competing Interests: No competing interests were disclosed.