



## Methyl- $\beta$ -cyclodextrin restores the structure and function of pulmonary surfactant films impaired by cholesterol

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### ABSTRACT

Pulmonary surfactant, a defined mixture of lipids and proteins, imparts very low surface tension to the lung–air interface by forming an incompressible film. In acute respiratory distress syndrome and other respiratory conditions, this function is impaired by a number of factors, among which is an increase of cholesterol in surfactant. The current study shows *in vitro* that cholesterol can be extracted from surfactant and function subsequently restored to dysfunctional surfactant films in a dose-dependent manner by methyl- $\beta$ -cyclodextrin (M $\beta$ CD). Bovine lipid extract surfactant was supplemented with cholesterol to serve as a model of dysfunctional surfactant. Likewise, when cholesterol in a complex with M $\beta$ CD (“water-soluble cholesterol”) was added in aqueous solution, surfactant films were rendered dysfunctional. Atomic force microscopy showed recovery of function by M $\beta$ CD is accompanied by the re-establishment of the native film structure of a lipid monolayer with scattered areas of lipid bilayer stacks, whereas dysfunctional films lacked bilayers. The current study expands upon a recent perspective of surfactant inactivation in disease and suggests a potential treatment.

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### 1. Introduction

Pulmonary surfactant forms a molecular film at the hydrated air–alveolar interface and reduces surface tension, from 70 mN/m at a free air–water interface to an equilibrium value of  $\sim$ 23 mN/m. As the area of the alveolar interface decreases upon expiration, the molecular film becomes compressed and the surface tension drops further, a critical function first established *in vitro* using lung extracts [1,2]. Direct measurements in healthy lungs reveal a surface tension of almost zero at functional reserve capacity [3].

Avery [4] established impaired lung function in respiratory distress syndrome (RDS) in premature infants is rooted in a lack of pulmonary surfactant. RDS is now successfully treated by intratracheal administration of exogenous surfactant [5–12]. Acute respiratory distress syndrome (ARDS), a common (incidence 1.5 to 5.3/10<sup>5</sup> population/year) and deadly (mortality rate of 36% to 60%) [13–17] spectrum of disease is associated with dysfunctional surfactant rather than a lack thereof. Abnormally high surface tension caused by surfactant dysfunction results in diminished lung compliance, a decreased lung volume, reduced airway patency and potentially

severe hypoxemia. Pulmonary surfactant dysfunction has also been implicated with the reduced patency of small airways in cystic fibrosis (CF) (e.g. [18,19]), with the development of acute lung injury (ALI) via ventilator induced lung injury (VILI), and other diseases of the lung. Surfactant dysfunction in ARDS/ALI has been targeted for treatment with replacement surfactant therapy. However, unlike the situation with RDS, success has been inconsistent outside of paediatric and possibly direct lung injury-induced ARDS/ALI (for a review, see [20]). It is now generally accepted that successful treatment for ARDS/ALI will depend on an in-depth understanding of relevant impairment mechanisms.

Proportionally increased cholesterol exerts a strong inhibitory effect on surfactant function [21,22]. Cholesterol, which makes up about half of the neutral lipids in surfactant [23,24], is elevated from a physiological level of 5% to 8% w/w with respect to phospholipids to about 20% w/w in animal models of lung injury [25] and 16% to 40% w/w in human ARDS [26]. In an animal model of VILI, cholesterol in surfactant was elevated almost two fold over controls [27] and was directly responsible for surfactant inhibition [28]. A similar relationship has yet to be thoroughly evaluated in ARDS, however.

The relatively hydrophobic interior of the toroid-shaped methyl- $\beta$ -cyclodextrin (M $\beta$ CD) molecule is able to host various hydrophobic molecules, including cholesterol. High concentrations of M $\beta$ CD can extract substantial amounts of cholesterol from cell membranes [29], unilamellar phospholipid/cholesterol vesicles [30], and interfacial

Abbreviations: Chol-BLES, bovine lipid extract surfactant with cholesterol added; MST, minimum surface tension; IA, initial adsorption; QS, quasi-static cycle; D, dynamic cycle

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surfactant films [30] into a water-soluble cholesterol–cyclodextrin complex. In a related application, M $\beta$ CD cholesterol-complexes (“water-soluble cholesterol”) can be used to deliver cholesterol to plasma membranes in cell cultures. In the current study, we investigated whether impairment of surfactant films could be reversed *in vitro* by exposure of spread films to M $\beta$ CD and whether normal surfactant could be rendered dysfunction by exposure to water-soluble cholesterol.

We investigated films formed from bovine lipid extract surfactant (BLES), used clinically in the treatment of surfactant deficiencies [31]. BLES contains all of the lipid and protein components of natural surfactant, with the exceptions of the hydrophilic surfactant proteins SP-A and SP-D. Moreover, cholesterol content is reduced in BLES to approximately 1.5% w/w with respect to phospholipids [31]. Here, native BLES was used to model normal surfactant, and the range of surfactants in the diseased lung was mimicked by the addition of 15%, 20% or 30% w/w cholesterol to BLES with respect to phospholipids (chol-BLES). To determine if physiological function could be restored, chol-BLES films containing various proportions of cholesterol were spread on buffer containing M $\beta$ CD at concentrations of 5, 10 or 40 mg/mL. Lastly, the molecular structures of native BLES, 20% chol-BLES, and M $\beta$ CD-treated 20% chol-BLES films were imaged using atomic force microscopy (AFM).

A captive bubble surfactometer (CBS) was used for measuring surfactant function, since this technique comes closest to mimicking lung function as determined *in vivo* from pressure-volume studies [32]. Near-physiological conditions modelled in the CBS include temperature (37 °C), cycling rates (20 cycles/min), and interfacial adsorption from minute volumes of concentrated (27 mg/mL) surfactant containing dense aggregates [22]. Functional assessment of surfactant began with measurements of film formation, as indicated by the drop in surface tension of an air–buffer interface upon surfactant spreading. Subsequently, surface tension upon slow (quasi-static, QS) and rapid (dynamic, D) surface area reduction and expansion was measured. The minimal surface tension (MST) reached during compression was the primary indicator of surfactant function in this test.

## 2. Materials and methods

### 2.1. Surfactant preparation

BLES (a kind gift from BLES Biochemicals Inc, London, Ontario; see Yu et al. [31] for the detailed composition of BLES) suspended at a phospholipid concentration of 27 mg/mL in non-buffered (pH 5–6) saline with 2.5 mM calcium was used with or without the addition of 15, 20 or 30% (w/w) cholesterol (Sigma Chemicals, St. Louis, MO). In order to add cholesterol quantitatively to BLES, a lipid extraction procedure was performed as described in [21]. The phospholipid/cholesterol solution was dried under N<sub>2</sub> and re-suspended in buffer (140 mM NaCl, 10 mM HEPES and 2.5 mM CaCl<sub>2</sub>; pH 6.9) to obtain an aqueous suspension of BLES and cholesterol at a concentration of 27 mg phospholipids/mL. Native BLES (i.e. without cholesterol added) underwent the same extraction procedure to ensure differences in the surface activity between normal BLES and chol-BLES were solely due to the difference in cholesterol content.

### 2.2. Surface activity assessment

Surface activity of surfactant was determined using a computer controlled CBS as described in detail in refs. [21,33], with the following modification: a transparent capillary was used to deposit a ~0.5  $\mu$ L volume of surfactant near the air–buffer interface within the CBS under precise visual control. Following the introduction of surfactant into the chamber, a 5-min film formation period (initial adsorption, IA) elapsed, during which time the bubble was not

manipulated. Changes in surface tension and interfacial area were monitored as described in ref. [34]. Experiments in which the bubble contacted the microsyringe capillary were excluded from the IA analysis. Quasi-static cycling commenced 5 min after rapid expansion of the bubble, during which the bubble volume was first reduced and then enlarged by altering the internal volume of the chamber in a stepwise fashion over 4 compression–expansion cycles. In the dynamic cycling portion of the experiment, bubble volume was smoothly and rapidly changed over the same range at a rate of 20 cycles/min. Surface tension values are reported as mean  $\pm$  SEM. The temperature of the subphase was maintained at a constant value of 37 °C throughout the experiments using an automated heated fan and water bath [33].

To evaluate the effect of M $\beta$ CD, powdered M $\beta$ CD (Sigma Aldrich, Catalogue-Nr. C4555) was dissolved in buffer to a final concentration of 40, 10, or 5 mg/mL and added to the CBS chamber prior to the addition of surfactant. Assessment of surface activity as described above was conducted after spreading chol-BLES, immediately and again after a 0.5 h or 1–2 h waiting period. To probe the effect of water-soluble cholesterol on BLES, an M $\beta$ CD–cholesterol complex (“water-soluble cholesterol”, Sigma Aldrich, Catalogue-Nr. C4951) was added to the buffer to a final concentration of 40 mg/mL before spreading BLES.

### 2.3. AFM imaging

For visualization of the structure of surfactant films, a Langmuir surface balance (surface area = 750 cm<sup>2</sup>) (Nima Technology, Coventry, Great Britain) was employed. Surface pressure and area were measured with a PS4 pressure sensor and IU4 micro-processor interface (Nima, Coventry, Great Britain). The trough was thoroughly cleaned and calibrated using standard buffer. Surfactant films were spread at the air–aqueous interface from a 27-mg phospholipid/mL chloroform solution until the surface pressure reached 10–20 mN/m ( $\gamma$  ~55–65 mN/m). The system was allowed to equilibrate for 10 min to allow the chloroform to evaporate and the surfactant to diffuse. Concentrated M $\beta$ CD was injected beneath the surface of the film to an appropriate final concentration when indicated, and the system was allowed to equilibrate for a further 30 min. Next, the film area was reduced at a rate of 100 cm<sup>2</sup>/min until  $\gamma$  ~26 mN/m was reached at which time a sample of each film was collected on a mica support by the Langmuir–Blodgett transfer for AFM imaging. The samples were collected upon area reduction at the end of a shoulder in the area–surface tension isotherm. For deposition, a mica support was first lowered across the interface at a speed of 45 mm/min and then retracted at 25 mm/min. A film was deposited upon the upstroke while the surface tension was kept constant. To obtain AFM images of the topography of these samples, a Nanowizard AFM (JPK Instruments, Berlin, Germany) was used with intermittent contact mode silicon cantilevers (NCH-20, Nanoworld, Neuchatel, Switzerland).

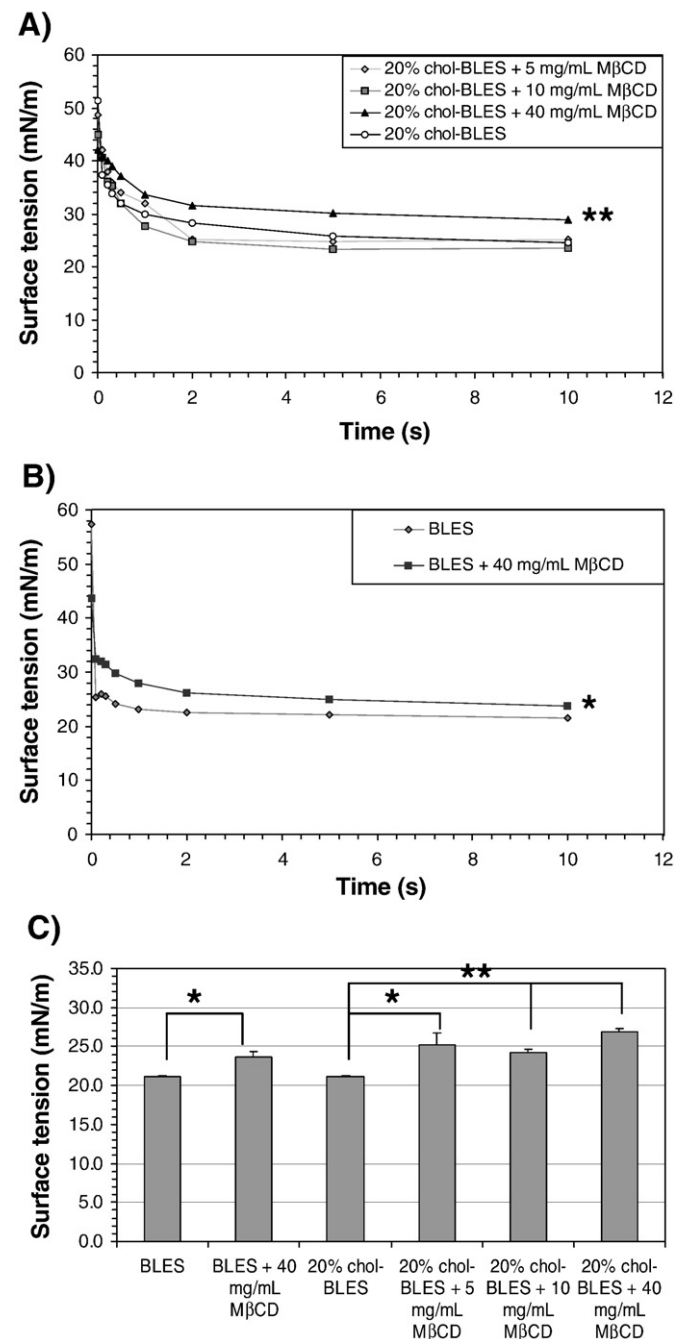
### 2.4. Statistical analysis of CBS data

Nonparametric tests (SPSS 14.0) of differences in mean MST attained during QS and D cycling, and at various times during IA were performed with  $p < 0.05$  used as the standard for significance in all cases. Mean MST values for QS1 and D20 were considered to represent the initial effect of treatment and the stability of this effect, respectively. The Friedman test for multiple related measures was used first to test for main within treatment effects and the Wilcoxon paired related measures test was used for subsequent orthogonal comparisons after overall significance was determined. The Kruskal–Wallis and the Mann–Whitney  $U$  tests for independent samples were similarly used to determine overall between treatment effects and to make subsequent orthogonal comparisons.

### 3. Results

#### 3.1. Untreated controls

Upon spreading, BLES lowered the surface tension of the air–buffer interface from  $\sim 70$  mN/m to an equilibrium value of  $\sim 21$  mN/m, often within a fraction of a second (Fig. 1). The surface tension of these films readily neared zero during each of the quasi-static and the



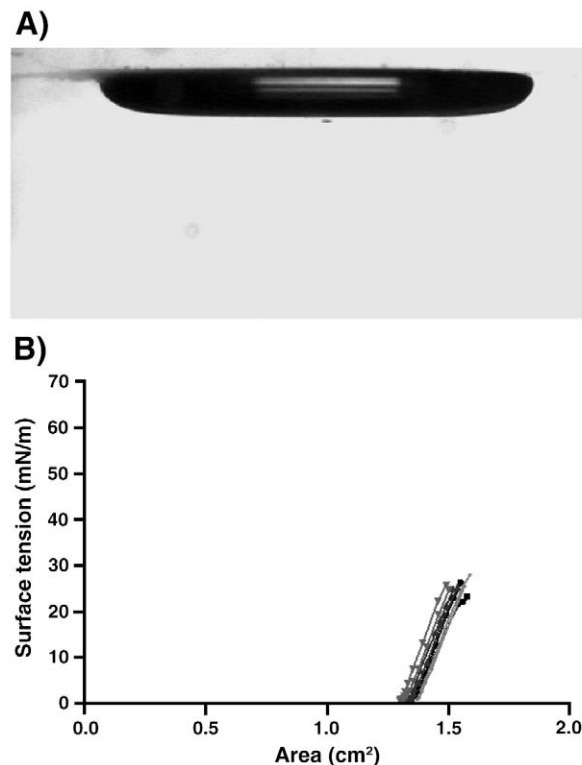
**Fig. 1.** Initial adsorption (film formation) at 37 °C of 20% chol-BLES and native BLES (27 mg/mL) in the presence of MβCD. (A) Mean surface tension over the first 10 s after injection of 20% chol-BLES in 0 ( $n=9$ ), 5 ( $n=3$ ), 10 ( $n=13$ ), or 40 mg/mL ( $n=12$ ) of MβCD. (\*\* $p<0.01$  compared to mean surface tension of 20% chol-BLES at all time points measured, except 0.1 s). Error bars have been omitted for clarity. (B) Mean surface tension over the first 10 s after injection of native BLES in 0 ( $n=5$ ) or 40 mg/mL ( $n=4$ ) of MβCD. (\* $p<0.05$  compared to mean surface tension of native BLES at all time points measured). (C) Mean surface tension 300 s after injection of native BLES or 20% chol-BLES into subphases containing different concentrations of MβCD. Error bars: positive standard error of the mean (SEM). (\*\* $p<0.01$ , \* $p<0.05$ ).

dynamic compression and expansion cycles (Fig. 2, see also [21]). BLES films required minimal area reduction (10–15%) to bring about low surface tension as indicated by the slope of the surface tension over area curve. This behaviour may be regarded as the effect of a layer of already tightly packed surfactant phospholipids becoming even more compacted at the air–water interface [35].

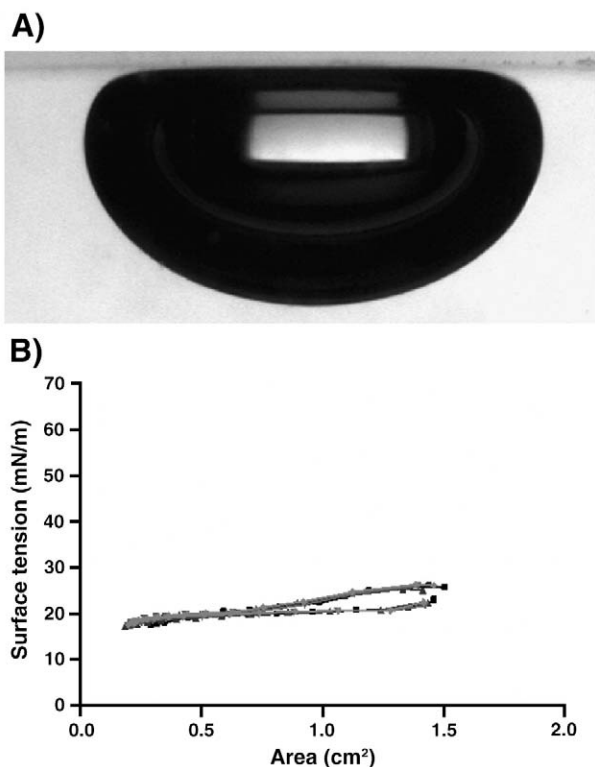
Much the same as BLES, untreated 20% chol-BLES rapidly lowered surface tension upon spreading to an equilibrium value  $\sim 23$  mN/m (Fig. 1). There was no significant difference in mean surface tension between BLES and 20% chol-BLES at any time point measured during film formation ( $p>0.05$ ). Upon compression, however, 20% and 30% chol-BLES films did not lower surface tension below  $\sim 16$  mN/m irrespective of the extent of area reduction, giving rise to an extended plateau in the area–surface tension isotherm (Fig. 3, see also [21]). This plateau indicates continuous film collapse from the interface into the buffer [35]. Compression of 15% chol-BLES films lowered surface tension to  $<5$  mN/m with substantial area reduction ( $\sim 70\%$ ) in D1–5 (data not shown), but these low surface tensions were not reached by D20 (Fig. 4), indicating functional deterioration.

#### 3.2. The effect of MβCD on chol-BLES function

Surface tension was assessed during IA at set time points over the first 5 min of film formation using 20% chol-BLES and various concentrations of MβCD (Fig. 1). Surface tension decreased to near equilibrium values very rapidly ( $<1$  s), which was likely the result of our injection technique [22]. Increasing concentrations of MβCD up to 10 mg/mL did not significantly affect surface tension over the first 10 s compared to untreated chol-BLES. However, mean surface tensions in the presence of 40 mg/mL MβCD were significantly higher at all time points (except 0.1 s) compared to untreated chol-



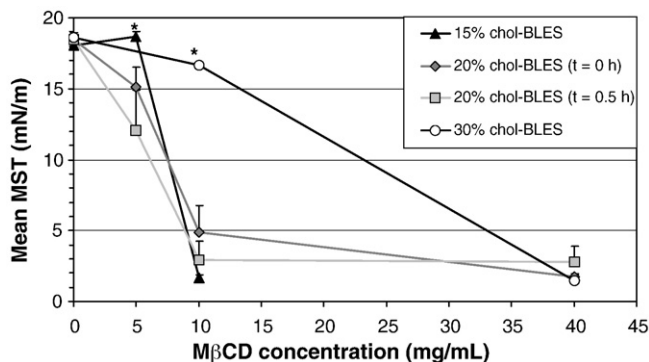
**Fig. 2.** Functional surfactant behaviour during CBS cycling. (A) An image of a bubble with functional surfactant (BLES) being compressed as the surface tension nears zero. (B) Surface tension over area graph corresponding to the bubble shown in (A). Black lines indicate the first quasi-static cycle with subsequent quasi-static cycles shown in increasingly lighter shades of gray. The compression part for each cycle (i.e. area reduction) is to the right and below the respective expansion part of each cycle.



**Fig. 3.** Dysfunctional surfactant behaviour during CBS cycling. (A) The image of a bubble with inhibited surfactant (20% chol-BLES) being compressed to a surface tension near 19 mN/m. (B) Surface tension over area graph corresponding to the bubble shown in (A). Black lines indicate the first quasi-static cycle with subsequent quasi-static cycles shown in increasingly lighter shades of gray.

BLES (Fig. 1). Also, there was a significant trend towards increased equilibrium surface tensions as the subphase concentration of M $\beta$ CD was increased, an effect which was observed previously [30] to a greater extent.

Surfactant function was rapidly and stably restored for 20% and 30% chol-BLES at 40 mg/mL M $\beta$ CD (Fig. 4). Upon treatment, the mean MST at QS1 of 20% chol-BLES ( $10.3 \pm 2.0$  mN/m,  $n = 11$ ) was not significantly different from the mean MST of treated 30% chol-BLES ( $7.5 \pm 3.3$  mN/m,  $n = 3$ ) ( $p = 0.815$ ). Likewise, the mean MST of 20% chol-BLES and 30% chol-BLES treated with 40 mg/mL M $\beta$ CD at D20 ( $1.8 \pm 0.2$  and  $1.5 \pm 0.4$ , respectively) were not statistically different



**Fig. 4.** Mean MST of 15%, 20%, and 30% chol-BLES films at D20 as functions of M $\beta$ CD concentration. 15% chol-BLES was tested at 0 ( $n = 4$ ), 5 ( $n = 3$ ), and 10 mg/mL ( $n = 3$ ) of M $\beta$ CD. 20% chol-BLES was tested at 0 ( $n = 9/4$ ), 5 ( $n = 8/8$ ), 10 ( $n = 15/11$ ), and 40 mg/mL ( $n = 11/6$ ) of M $\beta$ CD were tested at 0 h and 0.5 h ( $n$  values refer to the respective time intervals). 30% chol-BLES was tested at 0 ( $n = 3$ ), 10 ( $n = 3$ ), and 40 mg/mL ( $n = 3$ ) of M $\beta$ CD. Error bars: positive SEM. (\* $p < 0.05$  compared to mean MST of 20% chol-BLES at the same concentration of M $\beta$ CD).

( $p = 0.612$ ). The ability of a film to reach a near-zero MST after repeated rapid cycling (i.e. D20) indicates the ability of surfactant to functionally re-adsorb to the interface from surface-associated reservoirs formed during compression [33]. However, at 10 mg/mL M $\beta$ CD, 20% chol-BLES reached a significantly lower mean MST at D20 ( $4.9 \pm 1.9$  mN/m,  $n = 15$ ) than 30% chol-BLES ( $16.6 \pm 0.1$  mN/m,  $n = 3$ ;  $p = 0.010$ ), suggesting that the ability of M $\beta$ CD to restore surfactant function is affected by both surfactant cholesterol content and M $\beta$ CD concentration. Similarly, 10 mg/mL M $\beta$ CD lowered mean MST of 15% chol-BLES at D20 ( $1.7 \pm 0.2$  mN/m,  $n = 3$ ) to a greater extent than in 20% chol-BLES, although this difference did not achieve significance ( $p = 0.484$ ). Interestingly, at 5 mg/mL M $\beta$ CD, mean MST at D20 was slightly but significantly higher in 15% chol-BLES ( $18.7 \pm 0.3$  mN/m,  $n = 3$ ) than in 20% chol-BLES ( $15.1 \pm 1.4$  mN/m,  $n = 8$ ;  $p = 0.040$ ).

### 3.3. Cycling-dependent effects of M $\beta$ CD on 20% chol-BLES

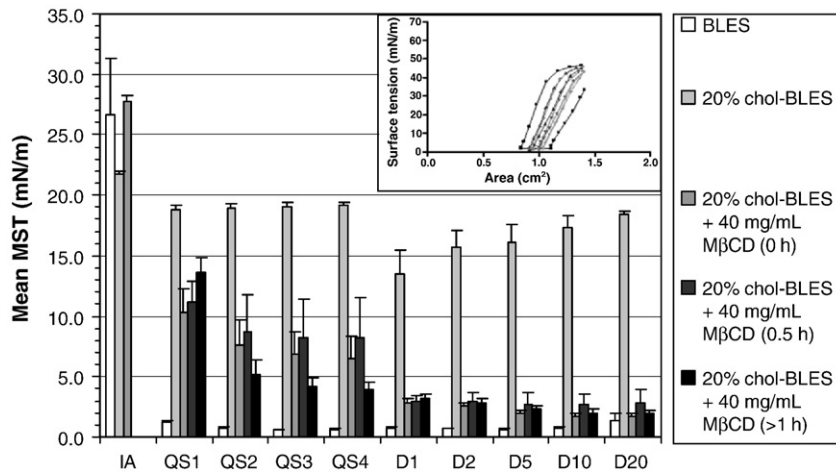
During QS1 of 20% chol-BLES samples treated with 40 mg/mL M $\beta$ CD, the mean MST value ( $10.3 \pm 2.0$  mN/m,  $n = 11$ ) was intermediate between the values for untreated chol-BLES ( $18.8 \pm 0.3$  mN/m,  $n = 9$ ,  $p = 0.006$ ) and native BLES ( $1.2 \pm 0.1$  mN/m,  $n = 5$ ,  $p < 0.0005$ ) (Fig. 5). The function of treated chol-BLES further improved over consecutive cycles, as there was a significant difference between mean MST at QS1 and D20 ( $p = 0.005$ ). At D20, the mean MST of treated chol-BLES films was not significantly different from the equivalent measurement for native BLES films ( $1.8 \pm 0.2$  mN/m and  $1.4 \pm 0.6$  mN/m respectively,  $p = 0.206$ ). Thirty minutes following treatment, mean MST of chol-BLES films ( $n = 6$ ) at QS1 was  $11.2 \pm 1.8$  mN/m and  $2.8 \pm 1.1$  mN/m at the D20 stage (Fig. 5). Neither value was significantly different from the corresponding value initially after M $\beta$ CD exposure ( $p = 0.208$  and  $p = 0.600$ , respectively). One to 2 h following the initial round of cycling, mean MST of treated chol-BLES ( $n = 6$ ) at QS1 was  $13.6 \pm 1.2$  mN/m and  $2.0 \pm 0.3$  mN/m by D20. Again, neither value was significantly different from the corresponding value immediately after M $\beta$ CD treatment ( $p = 0.138$  and  $p = 0.498$ , respectively). In summary, M $\beta$ CD restores the ability of a cholesterol-inhibited pulmonary surfactant to reach low MST in a cycling- but not time- dependent pattern.

### 3.4. The effect of M $\beta$ CD on native BLES function

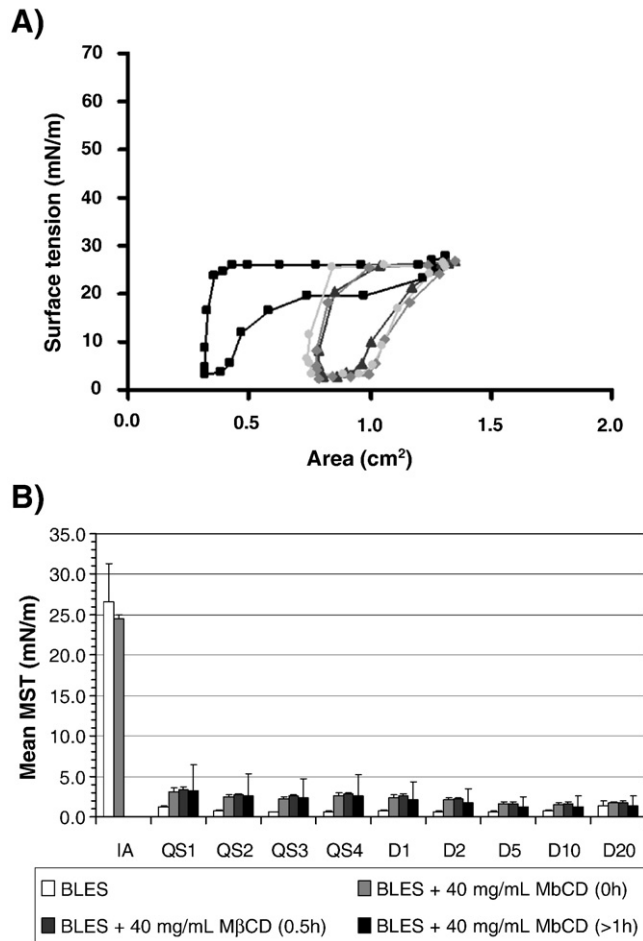
To establish whether M $\beta$ CD negatively affects normal surfactant function, native BLES was treated with 40 mg/mL M $\beta$ CD ( $n = 4$ ). Firstly, the equilibrium surface tension was increased ( $p = 0.014$ ) after adsorption of native BLES in 40 mg/mL M $\beta$ CD ( $23.6 \pm 0.6$  mN/m,  $n = 4$ ) compared to BLES adsorbed without M $\beta$ CD present ( $21.2 \pm 0.2$  mN/m,  $n = 5$ ) (Fig. 1). During QS1, the area reduction required to achieve MST in the presence of 40 mg/mL of M $\beta$ CD was  $\sim 70\%$  (Fig. 6A, cf. Fig. 2). This may have been because M $\beta$ CD molecules had formed a film at the interface that was eventually driven off the interface by surfactant adsorption. Consistent with this interpretation, we observed surface tensions of  $\sim 40$ – $50$  mN/m with bubbles in the presence of M $\beta$ CD before any surfactant was injected (Fig. 1) suggesting M $\beta$ CD is weakly surface active. From the second quasi-static cycle onwards, the function of BLES was unaffected by the presence of M $\beta$ CD (Fig. 6) and appeared indistinguishable from native BLES ( $n = 5$ ) in terms of mean MST at D20 ( $1.7 \pm 0.2$  mN/m and  $1.4 \pm 0.6$  mN/m, respectively,  $p = 0.140$ ). Hence, M $\beta$ CD did not have a lasting effect on the surface activity of native BLES.

### 3.5. The effect of water-soluble cholesterol on the function of surfactant

To further evaluate the action of M $\beta$ CD and cholesterol on surfactant, we exposed native BLES to buffer containing 40 mg/mL of “water-soluble cholesterol” (equivalent to 1.6 mg/mL cholesterol solubilized with M $\beta$ CD, molar ratio: cholesterol/M $\beta$ CD  $\approx 1/7$ ). The

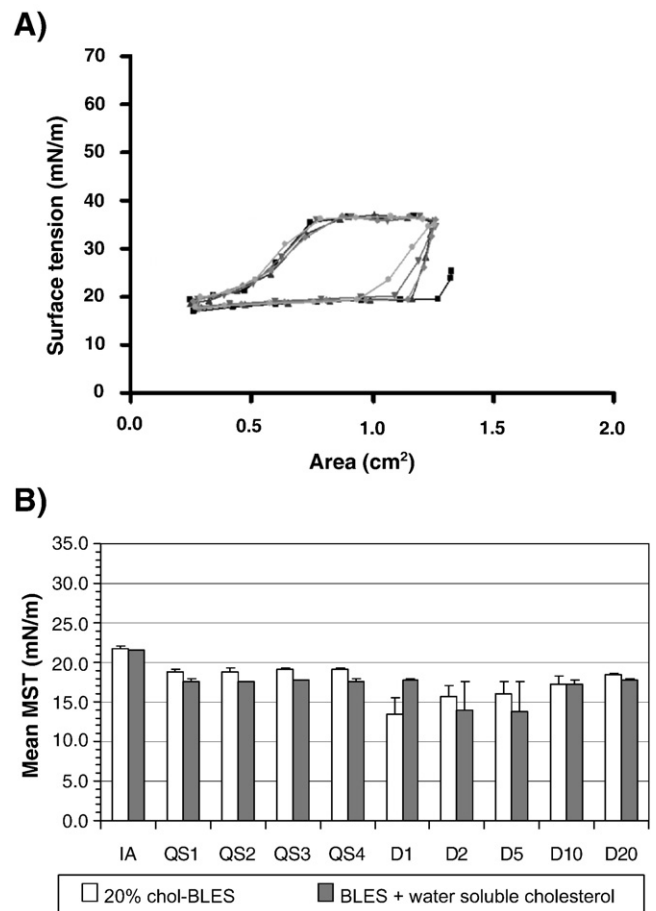


**Fig. 5.** Surface activity of 20% chol-BLES treated with 40 mg/mL M $\beta$ CD (A) A representative example of dynamic surface tension-area isotherms for M $\beta$ CD-treated 20% chol-BLES. Black lines indicate the first cycle with subsequent cycles shown in increasingly lighter shades of gray. (B) Mean MST during CBS cycling stages (initial adsorption, IA; quasi static cycles 1 to 4, QS1–4; and dynamic cycles 1, 2, 5, 10 and 20, D1–20) with native BLES ( $n=5$ ), 20% chol-BLES ( $n=9$ ), and 20% chol-BLES treated with 40 mg/mL M $\beta$ CD (0 h:  $n=11$ ; 0.5 h:  $n=6$ ; >1 h:  $n=6$ ). Error bars: positive SEM.



**Fig. 6.** Surface activity of native BLES (without cholesterol added) after treatment with M $\beta$ CD. (A) A representative example of quasi-static isotherms for native BLES in the presence of 40 mg/mL M $\beta$ CD ( $t=0$  h). Black lines indicate the first cycle with subsequent cycles shown in increasingly lighter shades of gray. (B) Mean MST during CBS cycling stages (initial adsorption, IA; quasi static cycles 1 to 4, QS1–4; and dynamic cycles 1, 2, 5, 10 and 20, D1–20) for native BLES with ( $n=4$ ) or without ( $n=5$ ) 40 mg/mL M $\beta$ CD. Testing was carried out immediately after injection of BLES into M $\beta$ CD (“0 h”), 0.5 h after the first test (“0.5 h”), and 1–2 h after the first test (“>1 h”) for trials with M $\beta$ CD. Error bars: positive SEM.

mean MST of BLES exposed to water-soluble cholesterol ( $n=4$ ) during QS1 ( $17.6 \pm 0.3$  mN/m) or D20 ( $17.7 \pm 0.2$  mN/m) was not significantly lower than the corresponding mean MSTs of 20%



**Fig. 7.** Surface activity of native BLES treated with “water-soluble cholesterol”. (A) Dynamic surface tension-area isotherms of BLES exposed to 40 mg/mL “water-soluble cholesterol” (equivalent to 1.6 mg/mL cholesterol) in the buffer. Black lines indicate the first cycle with consecutive cycles shown in increasingly lighter shades of gray. (B) Mean MST during CBS cycling stages (initial adsorption, IA; quasi static cycles 1 to 4, QS1–4; and dynamic cycles 1, 2, 5, 10 and 20, D1–20) for 20% chol-BLES ( $n=9$ ) and BLES with 1.6 mg/mL water-soluble cholesterol ( $n=4$ ) at  $t=0$  h.

chol-BLES ( $p=0.053$  and  $p=0.089$ , respectively). Therefore, water-soluble cholesterol immediately and stably rendered BLES dysfunctional, such that it was indistinguishable from 20% chol-BLES (Fig. 7).

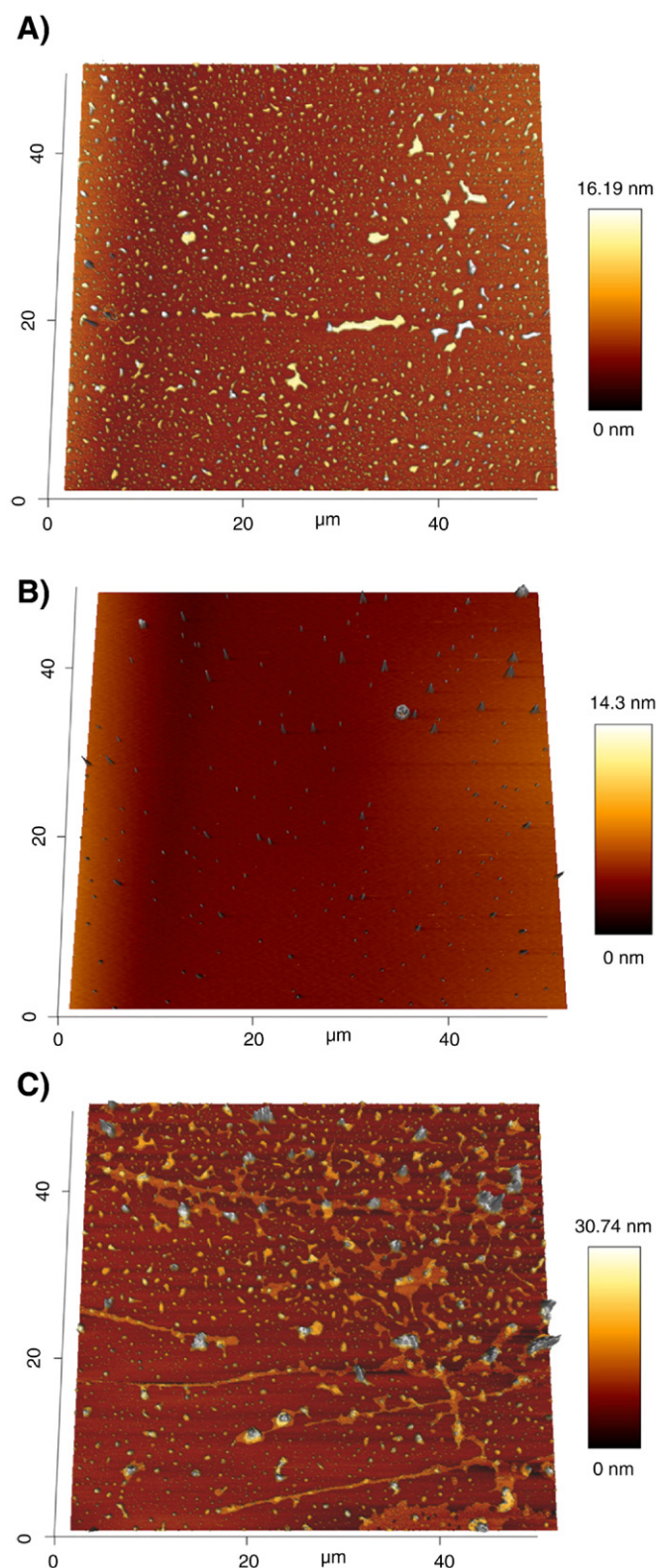
### 3.6. The effect of M $\beta$ CD treatment on the structure of surfactant

The structures of BLES and chol-BLES as revealed by AFM are shown in Fig. 8A and B, respectively. Fig. 8C shows the structure of a film of 20% chol-BLES treated with 40 mg/mL M $\beta$ CD. Films of native BLES and M $\beta$ CD-treated 20% chol-BLES films both showed lamellar structures scattered over the monolayer surface. Each step of a stack is 5 nm high, indicating a single lipid bilayer [36]. In contrast, untreated 20% chol-BLES films formed negligible lamellar structures upon compression, in agreement with our previous observations [35]. We showed earlier that bilayer stacks form from the monolayer upon film compression [35]. Dispersed multilayer patches, as well as the specific film architecture of a monolayer, have been associated with the unique mechanical property of functional pulmonary surfactant to sustain a high film pressure (i.e. low surface tension) without collapsing [36,37]. Therefore, the disrupted native structure of surfactant in 20% chol-BLES is reversed by M $\beta$ CD treatment, and these structural observations correspond well with *in vitro* surfactant function.

## 4. Discussion

We conclude that M $\beta$ CD can extract substantial amounts of cholesterol from interfacial surfactant films by forming water-soluble cholesterol-cyclodextrin complexes, similar to its effect on cell membranes [29]. In general, M $\beta$ CD has been shown to efficiently remove most of the cholesterol from other surfactant structures, including large aggregates [28] and unilamellar vesicle bilayers [30]. For pulmonary surfactant in suspension, M $\beta$ CD has been shown to remove 80–85% of cholesterol from large aggregates [28]. Our observation in the current study that surfactant function was restored in a cycling-dependent manner may be an indication that M $\beta$ CD is able to solubilise cholesterol from multilayered structures formed during compression. Since such structures were negligible in 20% chol-BLES films, which were eventually restored by M $\beta$ CD, it is likely that M $\beta$ CD removes cholesterol from both monolayer and multilayer surfactant. Conversely, an M $\beta$ CD cholesterol-complex can deliver cholesterol to cholesterol-free surfactant films. This indicates that cholesterol establishes equilibrium between the lipid environment of surfactant and an aqueous “sink” of M $\beta$ CD by distributing towards the cholesterol-depleted environment.

While M $\beta$ CD treatment efficiently restores both the structure and function of surfactant films inhibited by excess cholesterol, it might on the other hand abolish any beneficial function of physiological cholesterol levels within surfactant. However, with respect to minimal surface tension and low film compressibility, a beneficial effect of cholesterol in surfactant has not been reported, except in heterothermic mammals such as bats and dunnarts. Here, the level of cholesterol optimises surface activity of surfactant for the highly variable body temperature, which may reach a minimum of approximately 15 °C [38,39,40]. With respect to the efficiency of film formation, Bernardino de la Serna et al. [30] found that cholesterol depleted native surfactant displayed delayed surfactant adsorption to an elevated equilibrium value. In addition, increasing concentrations of M $\beta$ CD resulted in increasingly elevated equilibrium adsorption surface tensions [30]. While these results indicate a potential role for physiological amounts of cholesterol in promoting film formation, BLES, a natural surfactant which contains a minimal amount of cholesterol (~1.5%) [41], exhibits rapid adsorption to a normal equilibrium value (~21 mN/m). In the current study the highest concentration of M $\beta$ CD (40 mg/mL) resulted in slightly higher equilibrium surface tensions (~25 mN/m), whereas lower



**Fig. 8.** AFM topographical images of 2500  $\mu\text{m}^2$  regions of films formed from (A) native BLES, (B) untreated 20% chol-BLES, and (C) 20% chol-BLES treated with 40 mg/mL M $\beta$ CD for 30 min. Scale bars refer to height (nm) above the supporting mica plate. BLES films and treated chol-BLES films share a similar structure of a lipid monolayer (darkest shade) and interspersed elevated areas consisting of stacks of lipid bilayers. Untreated chol-BLES films lack the bilayer stacks [35].

concentrations of M $\beta$ CD had no statistically significant effect on adsorption. We found that removal of cholesterol from surfactant films by M $\beta$ CD did not affect surfactant function during subsequent compression and expansion cycling in the CBS. This is surprising in the light of recent [30] and earlier studies [42,43] that demonstrate extensive effects of physiological amounts of cholesterol on surfactant film architecture and lipid fluidity. Possibly, the treated films in the current study still contained sufficient cholesterol to support a physiological role.

The current study shows the pivotal role of excess cholesterol on surfactant function. However, aside from cholesterol, unsaturated membrane phospholipids; free fatty acids; mono-, di- and triglycerides; lysophospholipids; meconium [24,41,44,45]; surfactant protein and lipid degradation products; inflammatory agents [46–48]; reactive oxygen species [49, 50]; and plasma proteins [51–53] have all been implicated in surfactant inhibition. It has been argued that surfactant films can squeeze-out and thus rid themselves of unfavourable elements upon compression [36,54–57]. This indeed appears to be the case for surfactant exposed to plasma proteins, where an initially impaired surfactant regained function over subsequent compression and expansion cycles [22,58]. Though squeeze-out appears to protect pulmonary surfactant against most inhibitory effectors, this mechanism is ineffective for two types of inhibition. In one, dilute surfactant does not adsorb to the air interface in the presence of high concentrations of plasma proteins *in vitro* [52,53,58]. While surfactant secreted into the alveoli is normally highly concentrated [59], it might become sufficiently diluted by oedema fluid in diseased lungs to allow this inhibition mechanism to come into play [60]. In the second situation, loss of function caused by cholesterol persists throughout cycling, irrespective of surfactant concentration. Squeeze-out may depend on the ability of surfactant films to undergo monolayer–bilayer transitions, which is disrupted by excess cholesterol [35].

Our findings suggest the novel use of M $\beta$ CD to clarify the role of cholesterol-induced surfactant inhibition among the other possible inhibitory surfactant alterations. In a recent collaboration with Vokeroth et al. [28], applying M $\beta$ CD treatment outside of a defined surfactant system attributed a pivotal role to cholesterol in VILI. Rats ventilated with high tidal volumes showed lower oxygenation compared to a more gently ventilated control group. Subsequent analysis confirmed significant impairment of surfactant within the high-tidal volume group. Treatment with M $\beta$ CD *in vitro* greatly improved surfactant function [28], similar to the results of the present study. In summary, recent and emerging studies involving treatment of surfactant *in vitro* with M $\beta$ CD expose elevated levels of cholesterol as a generic surfactant inhibition mechanism.

Excess cholesterol in surfactant may be a consequence of inflammation. In the healthy lung, both Type II pneumocytes and macrophages rely on ATP-binding cassette transporters (ABCA1 and ABCG1) to efflux excess intracellular cholesterol [61,62]. Expression of ABCA1 and ABCG1 was reduced in macrophages following exposure to endotoxin [63]. Excess intracellular cholesterol leads to apoptosis [64] or necrosis [65] and thus cell membrane debris may be an additional source of cholesterol in alveolar fluid [63]. In addition to impaired reverse cholesterol transport, studies of oleic acid-induced lung injury in rats suggest *de novo* cholesterol synthesis in type II pneumocytes is increased under inflammatory conditions [66], potentially rendering surfactant dysfunctional from the outset [67].

Treating surfactant dysfunction promises increased survival in ARDS and the prevention or reduction of VILI. Instilling exogenous surfactant has been tested as a treatment for ARDS, but did not consistently decrease morbidity and mortality [20,68] (with the exception of paediatric ARDS [69]). The current study shows that chol-BLES and BLES adsorb equally well to the interface. As a consequence, exogenous surfactant may not preferentially adsorb to the air–lung interface to replace dysfunctional endogenous surfactant, potentially explaining the

lack of success with surfactant replacement therapy. Moreover, exogenous surfactant may rapidly become impaired by a substantial excess of cholesterol present in the diseased lung. In addition to or in place of removing excess cholesterol from the lung, reducing the production of excessive levels of cholesterol may be required to successfully counteract surfactant inhibition due to cholesterol.

The demonstrated efficacy of M $\beta$ CD in restoring surfactant function, combined with the low overall toxicity of M $\beta$ CD [70–72] and the capability to deliver cyclodextrins either as aerosols [73–75] or along with exogenous surfactant, makes this substance an attractive candidate for the treatment of surfactant dysfunction. When various  $\beta$ -cyclodextrins ( $\beta$ CDs) were instilled into the lungs of rabbits via an intratracheal bolus, dimethyl- $\beta$ CD (DM- $\beta$ CD) was efficiently cleared at a rate approximately equal to the glomerular filtration rate, indicating the absence of major pulmonary barriers to cyclodextrin absorption [76]. Methylated  $\beta$ CDs may further enhance transcellular pulmonary epithelial permeability by loosening apical tight junctions associated with lipid rafts [77]. Likely, cyclodextrin–cholesterol complexes would be cleared from the lung separately; the cyclodextrins via a paracellular route through the pulmonary epithelium and the hydrophobic sterol transcellularly [78,79]. We are currently exploring the effectiveness and initial safety of pulmonary M $\beta$ CD delivery in an animal model of ALI/ARDS.

## 5. Conclusions

Upon treatment with M $\beta$ CD, pulmonary surfactant rendered dysfunctional by an excess level of cholesterol rapidly regains the ability to consistently reach near-zero surface tensions in a cycling-dependent manner. By demonstrating that the native structure and function of cholesterol-inhibited surfactant can be fully restored by M $\beta$ CD, the current study introduces a new method for evaluating the role of cholesterol in surfactant dysfunction. Applied parallel studies by us and our collaborators validate this approach in VILI by demonstrating an important role for cholesterol in surfactant dysfunction. We propose further investigation into M $\beta$ CD treatment of surfactant dysfunction in such patients.

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