methods

High-throughput evaluation of pulmonary surfactant adsorption and surface film formation

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Abstract The assessment of new therapeutic strategies to cure surfactant-associated lung disorders would greatly benefit from assay systems allowing routine evaluations of surfactant functions. We present a method to measure surfactant adsorption kinetics into interfacial air-liquid interfaces based on fluorescence microplate readers. The principle of measurement is simple, robust, and reproducible: Wells of a microtiter plate contain an aqueous solution of a light-absorbing agent. Fluorescence is excited and collected from the top of the wells so that fluorescently labeled surfactant injected into the bulk can be detected only once adsorbed into the air-liquid interface. Mass transfer from the bulk to the interface is achieved by orbital shaking implemented in the plate reader instrument. The method has been tested and validated by using phospholipids or surfactants of different origins, by using albumin as surfactant inhibitor, and by comparison of results with Wilhelmy balance measurements. The method is suited for implementation in high-throughput screening routines for conditions affecting, or improving, surfactant film formation. In contrast to surface tension measurements, our method gives a direct readout of the amount of surfactant adsorbing into the interface, including the functionally important amount of material firmly associating with the interfacial film.—Ravasio, A., A. Cruz, J. Pérez-Gil, and T. Haller. **High-throughput evaluation of pulmonary surfactant adsorption and surface film formation. *J. Lipid Res.* 2008.** 49: **2479–2488.**

Supplementary key words air-liquid interface • fluorescence • lamellar bodies • lung • microplate • surface tension

Lung surfactant is a complex mixture of phospholipids and proteins that acts as a surface-active material able to reduce the surface tension at the respiratory air-liquid interface of lungs, thus preventing collapse of the alveoli at the end of expiration (1). Functional lung surfactant quickly adsorbs into the interface from the first breath and efficiently replaces surfactant "spent" during subsequent respiratory excursions. The operative surfactant film is probably more complex than a simple monolayer, inasmuch as it seems to really consist of a stable surface-associated multilayer reservoir that, during the respiratory cycle, ensures a highly dynamic redistribution of phospholipids and proteins from the hypophase to the interface and vice versa (2).

In addition to constitutive disorders (3, 4) or immaturity of the lungs at birth (5), many environmental factors and pathological events can seriously impair the surfactant system and, consequently, lead to pulmonary dysfunction (6, 7). In particular, accidental aspirates (e.g., meconium, hydrochloric acid), airborne particles (e.g., bacteria, pollen, dust), toxins, vapors, organic molecules derived from combustion, and ozone and other environmental pollutants can reach the most distal parts of the respiratory tract and contribute, to a variable extent, to surfactant inactivation. Additionally, the surfactant system can be further damaged by substances leaking through the capillary-epithelial barrier [in particular plasma proteins such as albumin (8) or the C-reactive protein (9)] in response to acute lung injury, usually associated with pro-inflammatory processes (10, 11).

Today, supplementation of the lungs of premature babies with an exogenous surfactant preparation has decreased considerably the mortality and morbidity associated with the development of infant respiratory distress syndrome (12, 13). Most clinical surfactants currently in use, however, consist of relatively crude extracts from animal sources (14), and several attempts have been made toward the development of new, entirely synthetic human-like therapeutic surfactant preparations (15). On the other hand, the clinical surfactants available at present have proven to be ineffective in ameliorating the symptoms of patients with acute respiratory distress syndrome, the major cause of mortality

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Abbreviations: AT II, alveolar type II; BB, Brilliant Black; CBS, captive bubble surfactometer; LBP, lamellar body-like particle; LTG, LysoTracker Green; NS, native surfactant; PBS, pulsating bubble surfactometer; RFU-bg, background-corrected relative fluorescence units.

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in critical care units (10, 16). Finally, a growing number of pharmaceutical strategies will be considered in the future to deliver drugs directly through the lung epithelium. In this sense, use of pulmonary surfactant as a drug-delivery agent seems promising and has been tested to vehiculize antibiotics and anti-inflammatory or immunosupressive drugs (17–19). Alterations in surfactant performance resulting from such therapeutic interventions would reverse the positive aspects of these otherwise clever administration strategies (i.e., a large surface area for drug uptake combined with an ultra-thin barrier) and lead to an additional threat to an already compromised body.

Both the detailed evaluation of pathological factors affecting pulmonary surfactant function and the development of new and improved clinical surfactants are critically dependent on the availability of reliable methods to qualitatively and quantitatively estimate surfactant adsorption and surface film formation. At present, these experiments are labor intensive, costly, and technically demanding procedures. A good surfactant must show very rapid interfacial adsorption to form surface films with equilibrium surface pressures around 45–48 mN/m (24–27 mN/m surface tension) in a few seconds, but also must be able to reduce surface tension to values in the order of 1–2 mN/m (70–72 mN/m surface pressure) upon repetitive compression-expansion cycling (20). Such surface properties can be evaluated in traditional surface balances, in the so-called pulsating bubble surfactometer (PBS), or in the more sophisticated captive bubble surfactometer (CBS). Surfactant adsorption as measured in miniaturized King-Clements devices (21, 22) or the compression isotherms of surfactant as obtained in Langmuir balances (23) provide detailed measurements that can be correlated with potential configurations and interactions of lipid and protein molecules as organized at the air-liquid interface. The technical constraints of these experiments, however, are usually far from relevant physiological conditions regarding temperature, appropriate surfactant concentrations, and surface cycling. Surfactant can be assessed in a PBS under more physiological-like conditions (24), and this technique is probably the most used in clinical research. However, it has been shown that the PBS has problems in producing accurate data on the surface behavior of surfactants at very low tensions (high surface pressures) (25), the part of the compression-expansion isotherms where the difference between optimal and suboptimal surfactant preparations may be critical. Assessment of surfactant preparations in the CBS has produced the most reliable data under physiologically relevant conditions (26), but requires a substantial effort in terms of data processing. All these techniques have to be applied in a regime of sample-by-sample analysis, which precludes an extensive evaluation of the multiple conditions and parameters thought to govern surfactant function.

In this paper, we present a new method to evaluate surfactant adsorption into interfacial air-liquid interfaces and formation of efficient surface-active films based on a fluorescence plate reader, with the capability of simultaneously following dozens of samples under physiologically relevant conditions. The principle of measurement is simple, robust, and highly reproducible. The wells of a microtiter plate are filled with an aqueous solution containing a light-absorbing agent that blocks any light passing into or returning from the bulk phase. Fluorescence is excited and collected from the top of the plate so that fluorescently labeled surfactant injected into the bulk solution can only be detected and quantitated once adsorbed into an interfacial film. Using standard 96-well microtiter plates, a high sample throughput regime can be achieved, including continuous measurements under temperature-controlled conditions. The method has been tested and validated by using fluorescently labeled phospholipids (i.e., DPPC, DPPC-POPG mixture) or surfactants of different origins [i.e., native surfactant (NS) from lung lavages, lamellar body-like particles (LBPs) collected from rat alveolar type II (AT II) cells]. In contrast to methods based on pure surface tension measurement, this method gives a direct readout of the amount of surfactant reaching the interface and stably associating with the interfacial film. However, the method is not suitable for reporting about surface tension changes upon surface compression-expansion dynamics. Nevertheless, it easily, quickly, and quantitatively accesses surfactant interfacial film formation and can be used to implement high-throughput screening of substances, environmental conditions, and surfactant preparations.

MATERIALS AND METHODS

Reagents and solutions

Chloroform (Chl) and methanol (MeOH), both of spectrophotometric grade, were from Sigma (Sigma-Aldrich, Germany) and CarlRoth (CarlRoth GmbH, Germany), respectively; BODIPY-PC 2-(4,4-difluoro-5,7-dimethyl-4-bora-3a,4a-diaza-s-indacene-3-dodecanoyl)-1-hexadecanoyl-sn-glycero-3-phosphocholine, fluorescent polystyrene beads, and LysoTracker Green DND-26 (LTG) were from Invitrogen-Molecular Probes (Carlsbad, CA); DPPC (1,2-dipalmitoyl-*sn*-glycero-3-phosphocholine) was purchased from Avanti Polar Lipids (Birmingham, AL); POPG (1-palmitoyl-2-oleoyl-*sn*-glycero-3-[phospho-rac-(1-glycerol)]) was from Sigma. High-purity BSA (fraction V receptor-grade lyophilized) was purchased from SERVA (SERVA Electrophoresis, Germany). Brilliant Black (BB) and all other salts and chemicals were purchased from Sigma. The bulk solution used in all experiments was buffered salt solution containing, in mM: NaCl 140, KCl 5, $MgCl_2$ 1, $CaCl_2$ 2, HEPES 10. pH and osmolarity were adjusted to meet physiological conditions (pH 7.4; 309 mOsm).

Surfactant preparations

Multilamellar suspensions of synthetic phospholipids (DPPC or the binary mixture DPPC-POPG, 7:3, w/w) were prepared at a phospholipid concentration of 5 mg/ml by hydrating dry lipid films in bulk solution and allowing them to swell for 1 h at 45°C, which is above the phase transition temperature of DPPC (41°C). During hydration, multilamellar liposomes were formed by mechanical shaking using a vortex.

NS was purified from porcine lung lavages and separated from blood components by NaBr density-gradient centrifugation as previously described (8). NS presumably contained both large and small surfactant aggregates, due to the ultracentrifugation used. LBPs were collected from isolated and purified rat AT II cells cultured on 10 cm-diameter petri dishes, which were stimulated with

100 μM ATP and 10 nM phorbol 12-myristate 13-acetate. With this kind of stimulation, AT II cells release a considerable amount of their surfactant phospholipids, together with the surfactant proteins A, B, and C. Rat AT II cells were isolated as described elsewhere (27). Amounts of surfactants were estimated by phosphorous determination or choline determination using a coupled enzymatic reaction as previously described (28). Briefly, α-lecithin standards and samples were mixed with the reaction buffer containing 20 U/ml phospholipase D, 20 U/ml choline oxidase, 200 U/ml HRP, and 10 nM Amplex Red. All reagents were purchased from Invitrogen-Molecular Probes (Amplex Red Phospholipase D Assay Kit) except phospholipase D (Sigma-Aldrich). Disruption of the LBP native structure to obtain "interface-treated" LBPs was performed by strong shaking of a 3 cm petri dish containing 1 ml of the LBP solution for 2 h at 37°C.

Surfactant labeling

BODIPY-PC was dissolved in DMSO to yield a concentration of 1 mg/ml. Surfactant materials were adjusted to 0.5 mg/ml, except LBPs, which were used at 0.015 mg/ml (LBPs were not concentrated further, to preserve their structural integrity as much as possible). All surfactants were stained by incubation with BODIPY-PC at 37°C for 2 h to obtain a final molar ratio of 4% (dye/surfactant). We found that up to this concentration, BODIPY molecules are sufficiently separated from each other so that attenuation of fluorescence by homo-FRET will not occur to a significant extent (data not shown). After staining, the surfactants were diluted with the bulk solution to the final working concentration of labeled surfactant as indicated.

Multi-well plate measurements

Experiments were performed in 96-well microtiter plates (A. Hartenstein GmbH, Austria) made of transparent polystyrene. The plate reader used was a TECAN GENios Plus from TECAN GmbH Swiss (**Fig. 1A**). This instrument allows control of the temperature (37°C) and an orbital shaking of the entire plate in between single measurements during a kinetic cycle. The beam of light is aligned to the center of each well and has a measured diameter of ~2.2 mm. Therefore, the actual surface area monitored by the instrument is limited to the central part of the well and the signal near the meniscus is excluded. All experiments were performed in triplicate and results are expressed as mean ± SEM.

Wilhelmy balance measurements

Interfacial adsorption of surfactant samples was also followed on a Teflon-made King-Clements-type of surface balance (22), specially designed by Nima (Coventry, UK) (29). The total volume of subphase was 1.5 ml, thermostatized at the indicated temperature, and continuously stirred with a 2 mm-long magnetic bar. The experiments were started by injection of a small volume (typically 5–50 μl) of a given surfactant preparation into the subphase through an oblique lateral perforation, and pressure-time kinetics were followed through continuous acquisition of surface pressure data with a Whatman #1 paper Wilhelmy plate connected to a pressure sensor. All experiments were performed in triplicate and expressed as mean ± SEM.

RESULTS

Principle of measurement and assessment of optimal working conditions

The principle of measurement (Fig. 1B) is based on the simple idea of blocking any light going into and coming back from the bulk solution, and therefore exclusively detecting the fluorescence emitted by surface-associated fluorophores.

To achieve this configuration, we added to the bulk solution a light-absorbing agent (BB) with a high molar extinction coefficient (ε) for both the emission and excitation wavelengths used to monitor BODIPY (ε_{485nm} = 1,117.2 ± 18.4 and ε_{540nm} = 3,577.3 ± 60.2 l*mol^{-1}*cm^{-1}; **Fig. 2A**). In addition, BB does not show any affinity for the interface, and even the highest concentrations of BB tested

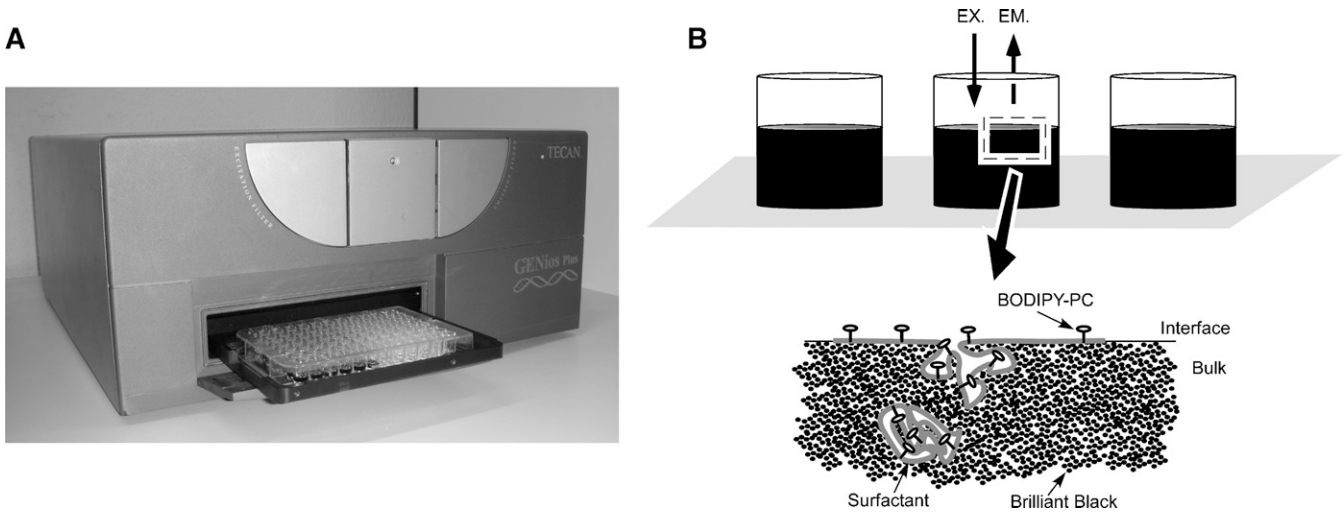

Fig. 1. A: Photo of plate reader instrument (TECAN GENios Plus) containing a 96-well microtiter plate partially filled with experimental solution. B: Illustration of the basic concepts of the adsorption assay. Examples of 3 wells out of a 96-well microtiter plate are shown. Each well is filled with 80 μl solution containing 5 mg/ml of the strongly light-absorbing agent Brilliant Black (BB) (absorption maximum at ~580 nm). After injecting 20 μl of BODIPY-PC-stained surfactant sample into the bulk solution and applying orbital shaking, fluorescence strictly coming from surface-adsorbed material is measured by the instrument by top reading mode, while nonadsorbed bulk fluorescence is quenched by BB. EX and EM indicate "Excitation" and "Emission", respectively.

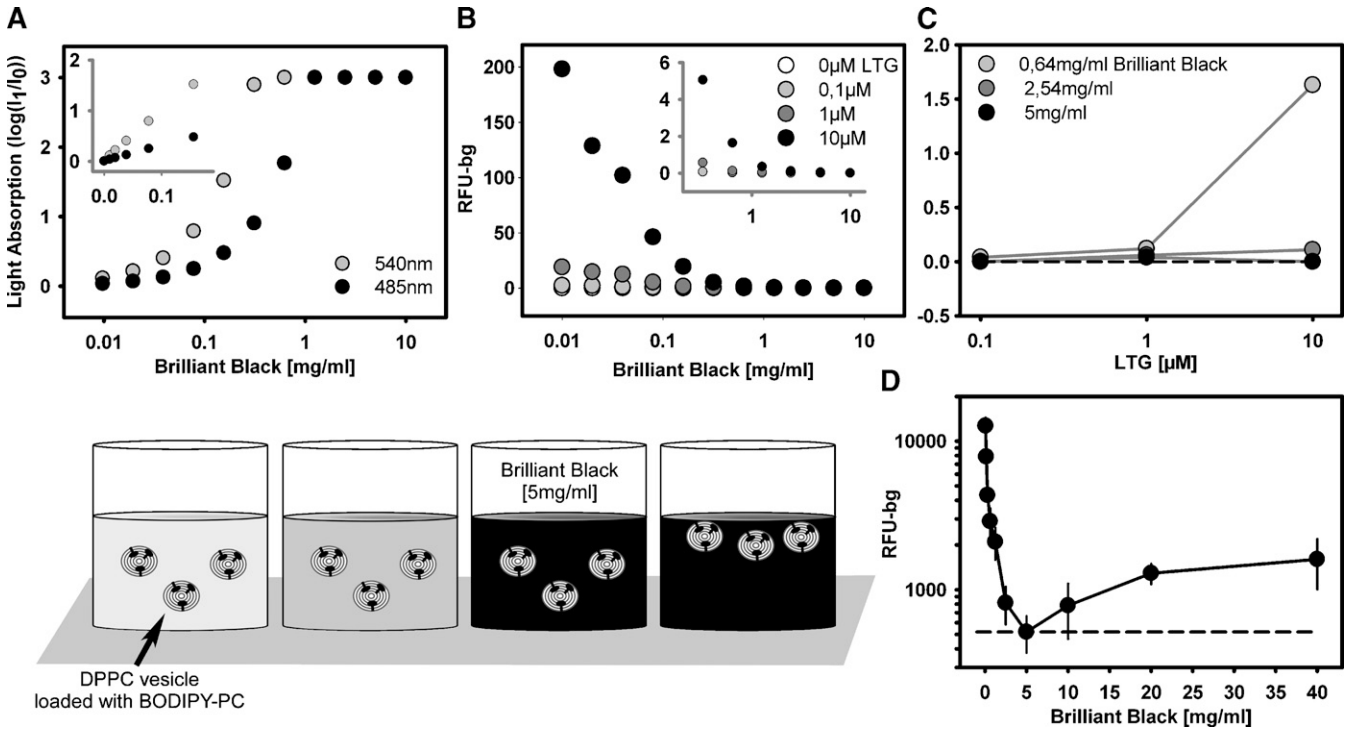

Fig. 2. Quench of light/fluorescence from the bulk solution. In A, a serial dilution of BB in buffered solution shows how light is totally absorbed by BB at concentrations higher than 1 mg/ml at the excitation and emission wavelengths used for BODIPY-PC. Inset in A shows, in nonlogarithmic scale, the linear part of the plots used to calculate the extinction coefficient, ε, of BB at the wavelengths used to measure BODIPY-PC fluorescence. In B, the ability of BB to quench fluorescence of the water-soluble dye LTG is shown at four different probe concentrations. Inset in B (enlarged scale) and C (replot of same data as in B) show that 5 mg/ml BB is sufficient to fully quench bulk fluorescence of LTG even at the highest concentrations of LTG tested. In D, the fluorescence of a DPPC suspension (1 μg) stained with 4% BODIPY-PC is measured in the presence of various amounts of BB. Increasing the BB concentration rapidly blocks the fluorescence of DPPC bilayers in the bulk solution. However, above 5 mg/ml BB, the increasing density of the solution forces labeled DPPC toward the interface (shaking was not applied in this experiment). Illustration shows the distribution of suspended DPPC vesicles at increasing BB concentrations.

(10 mg/ml) did not significantly change the surface pressure measured at the air-liquid interface in a Wilhelmy balance (surface pressure of bulk solution = 0.89 ± 0.13 mN/m + BB = 0.76 ± 0.29 mN/m). To test this principle, we aimed at using a highly fluorescent hydrophilic dye (LTG) in combination with BB. At a concentration of 5 mg/ml, BB is able to completely block the strong fluorescence of LTG coming from the bulk compartment (Fig. 2B, C). The optimal concentration for BB to block bulk fluorescence was found to be 5 mg/ml, because at higher concentrations of this quencher, the increased density of the liquid presumably forces surfactant to move toward the interface (Fig. 2D). Using 5 mg/ml of BB, the thickness of space inspected by the beam of light (i.e., the depth below the surface that is actually sampled) was estimated to be only ~100 nm. This value was determined experimentally according to the relation of the fluorescence intensity of a pure LTG solution to the length of the light path through this solution. Because this relation was linear (data not shown), we could use it to convert the difference in relative fluorescence units (RFUs) measured between background (a pure 5 mg/ml BB solution) and signal (10 μM LTG dissolved in 5 mg/ml BB) into a distance. Because this difference was extremely small and close to zero (compare also with Fig. 2B, C), instrumental gain had to be set to a maximum, and measurements were compared among 12 wells under each condition. The sensitivity of the method to detect the association of fluorescent material with the interface has been assessed by depositing fluorescently labeled DPPC, dissolved in Chl-MeOH (1:1), directly on top of 100 μl of BB-containing solution. After evaporation of the organic solvents, the fluorescence originating at the interface was measured (**Fig. 3**). The background-corrected relative fluorescence units (RFU-bg) start to rise at DPPC amounts >0.02 μg, indicating that fluorescence can be measured at one-fifth of the calculated amount needed to form a monolayer covering the surface of the well at the equilibrium surface pressure (48 $Å^2$/molecule). Below this amount, the sensitivity of the method is limited by a low signal-to-noise ratio. Therefore, in the particular case that the experiments require a higher sensitivity in this range, it is advisable to use 96-well plates made of black plastic, which produce less scattered light than transparent ones. RFU-bg steeply increased further until the full monolayer was formed (0.1 μg). Unexpectedly, the fluorescence continued to rise beyond this value, probably because it was generated by collapsed structures still connected to the monolayer (shaking was not applied). However, this increase slowed down, probably due to the deepening of the collapsed structures

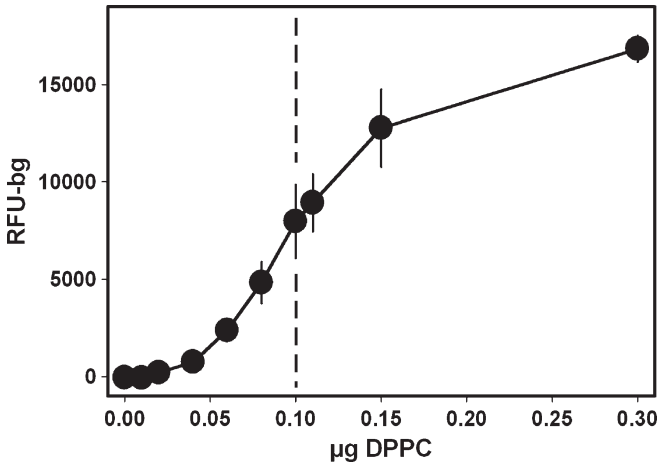

Fig. 3. Titration curve of BODIPY-PC fluorescence associated with the air-liquid interface. DPPC stained with 4% BODIPY-PC (in 1:1 Chl-MeOH solution) was spread onto the interface of a well using a Hamilton syringe. Signal was different from background at ≥0.02 µg and declined above 0.1 µg, the amount required to form a monomolecular phospholipid film (dashed line).

underneath the space inspected by the beam of light, or to the complete loss of material into the bulk where fluorescence is quenched. Additionally, some of the material could have been spread onto the plastic, where it was not detectable by the instrument.

In all methods previously used for adsorption measurement (e.g., Wilhelmy balance, CBS), the surfactant is injected into the bulk solution and forced by stirring to move toward the interface. In our system, this can be achieved by orbital shaking of the entire plate between every single measurement. The effect of stirring was tested by using fluorescent polystyrene beads (diameter ~2 µm; d ~1.1 g/l according to the manufacturer's instructions) in solutions of higher or lower density, respectively. As shown in **Fig. 4A**, beads floating at the interface (i.e., only weakly surface associated) are easily removed from it even at a short duration of shaking. In contrast, beads that tend to sediment due to their higher density than that of the solution are moved up by orbital shaking. From these measurements, we conclude that orbital shaking is able to force even dense surfactant material from the bulk toward the interface and to remove only weakly associated material from it. This indicates that our method is selectively detecting fluorescence coming from firmly surface-associated material only. Furthermore, the amount of beads reaching the interface seems to be related to the duration of shaking. Confirmation of these results can be seen in the experiment shown in Fig. 4B, where NS adsorbed better into the interface when the shaking was prolonged from 3 s to the maximum time limited by the instrument (30 s).

Experimental procedures

Experiments were conducted as shown in Fig. 4B: 6 wells out of 96 were filled with 80 µl bulk solution containing 6.25 mg/ml of BB. The plate was inserted into the microplate reader, and after reaching 37°C, the obtained signals

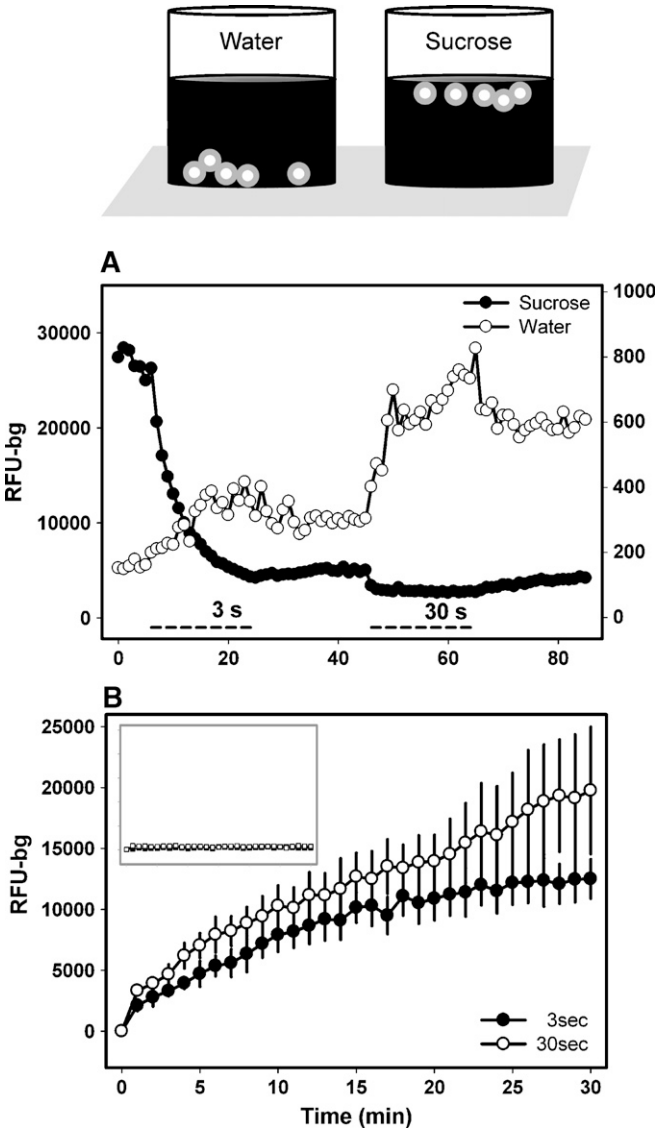

Fig. 4. Effects of orbital shaking on the distribution of fluorescent polystyrene beads (A) and on the adsorption of native surfactant (NS) at the interface (B). In A, fluorescent polystyrene beads (d ≈ 1.1 g/l) were placed in either pure water (d ≈ 1.0 g/l; empty circles) or in a sucrose solution (d ≈ 1.3 g/l; full circles), both in the presence of BB (5 mg/ml). Upon shaking (indicated by the dashed lines) between the single data points, beads on top of the sucrose solution were removed from the interface while beads that sedimented in low-density solution appeared on top. Two different shaking durations (3 s and 30 s) were tested. Illustration shows the distribution of the fluorescent beads prior to shaking. In B, NS stained with BODIPY-PC adsorbs into the interfacial surface upon orbital shaking. Higher increase in fluorescence signal with 30 s shaking denotes a more efficient adsorption rate. Inset in B shows that multilamellar vesicles made of pure DPPC (at any shaking intensity) were not able to adsorb into the interface at all.

were taken as background values. Measurements were performed in the top reading mode (see Fig. 1B) using the appropriate filter set (band-pass excitation and emission filters, 485 ± 10 nm and 540 ± 10 nm, respectively). Afterwards, the plate was taken out of the instrument, and 20 µl of solution containing the fluorescently labeled DPPC was injected into the bulk solution of 3 wells (yielding a final

concentration of BB = 5 mg/ml). In the same manner, 3 other wells were filled with fluorescently labeled NS. Thereafter, a kinetic cycle of fluorescence measurements was started (cycle time = 1 min, orbital shaking = 30 s, repeated during every 1 min cycle, shaking intensity set to "normal," precise rpm not specified by the manufacturer). Immediately afterwards, the same procedure was repeated with the only difference of 3 s (instead of 30) of orbital shaking during every 1 min cycle. Values of fluorescence have been corrected for background and plotted as the mean ± SEM of three replicates. Importantly, all adsorption experiments were performed using precisely this procedure, including the temperature (37°C) and the instrument parameters (e.g., light intensity, gain). Therefore, consistency of the method can be verified by comparing the kinetics obtained in different experiments (e.g., Figs. 4B, 5, 6).

Surface adsorption of various surfactant materials and inhibitory effect of plasma proteins

The described procedure to evaluate surface adsorption of surfactant has been tested by using three different surfactant materials: DPPC and DPPC-POPG (7:3, w/w) suspensions and NS purified from lung lavages. DPPC, which is the major constituent of lung surfactant, showed no or only a very weak adsorption into the air-liquid interface (**Fig. 5A, D**). However, when mixed with POPG, some of the phospholipids redistributed from the bulk into the interface (Fig. 5B, E). Finally, NS showed a rapid

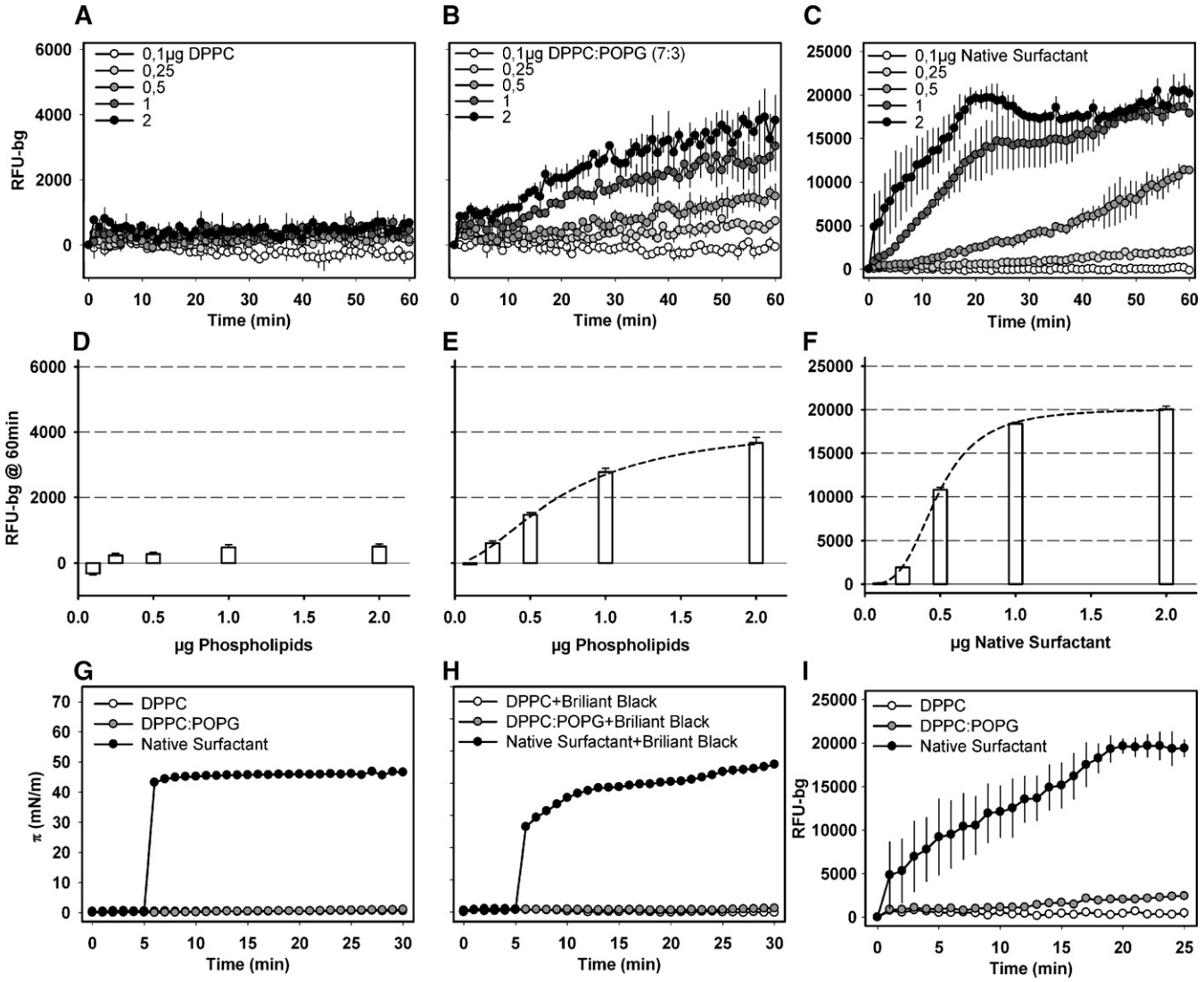

Fig. 5. Surface adsorption kinetics of various surfactant materials. A: Pure DPPC; B: DPPC-POPG (7:3, w/w) lipid mixture reconstituted as multilamellar suspensions; C: NS (note the different scaling in C compared with A and B). DPPC had extremely slow or nonexistent adsorption kinetics. Suspensions made of DPPC-POPG adsorbed into the air-liquid interface and formed a surface film. Finally, NS showed dramatic and rapid adsorption into the interfacial surface to a much larger extent than a simple monolayer. In D, E, and F, fluorescence values taken at 60 min (data from experiments A to C) have been plotted as a function of surfactant phospholipid concentration. Adsorbing surfactants (i.e., DPPC-POPG and NS) showed time- and dose-dependency. G: Wilhemy balance evaluation of surface pressure increase due to surfactant adsorption (surfactant injection at time 0). H: Presence of BB in the subphase changed the adsorption kinetics slightly, but not the principal behavior of all three materials studied or their endpoints reached. I: Replot of data from A, B, and C (2 µg) for a direct comparison between fluorescence and surface pressure measurements under comparable conditions.

adsorption into the air-liquid interface (Fig. 5C, F) that even exceeded the RFU-bg readings for a monolayer about double-fold (Fig. 3; note the difference in scale of the fluorescence detected upon adsorption of surfactant as compared with that detected upon adsorption of pure lipid preparations). Surface adsorption of surfactants is a time- and dose-dependent process, and these features can be followed either by their kinetics (Fig. 5A, B, C) or by the endpoints reached (Fig. 5D, E, F). BB, which does not show surface properties per se, i.e., it does not adsorb onto a clean interface, produces a slight reduction of the speed of adsorption of NS (Fig. 5G, H). Nevertheless, the kinetics of surfactant adsorption in the presence of BB are very similar when measured by fluorescence or when detected under similar conditions by the change in surface pressure (Fig. 5H, I). Importantly, the surface pressure measured as an endpoint when the adsorption kinetics reached a plateau was not affected by the presence of BB.

The surfactant system can be damaged by substances leaking through the epithelial barrier [in particular, plasma proteins such as albumin (8)] e.g., in response to inflammatory processes. For the purpose of validating our system, the effect of BSA was tested on the adsorption of surfactant. Complete inhibition of adsorption of DPPC-POPG suspensions was observed at all amounts of albumin tested, whereas a major, but not total, and dose-dependent inhibition was detected with respect to the activity of NS (**Fig. 6**).

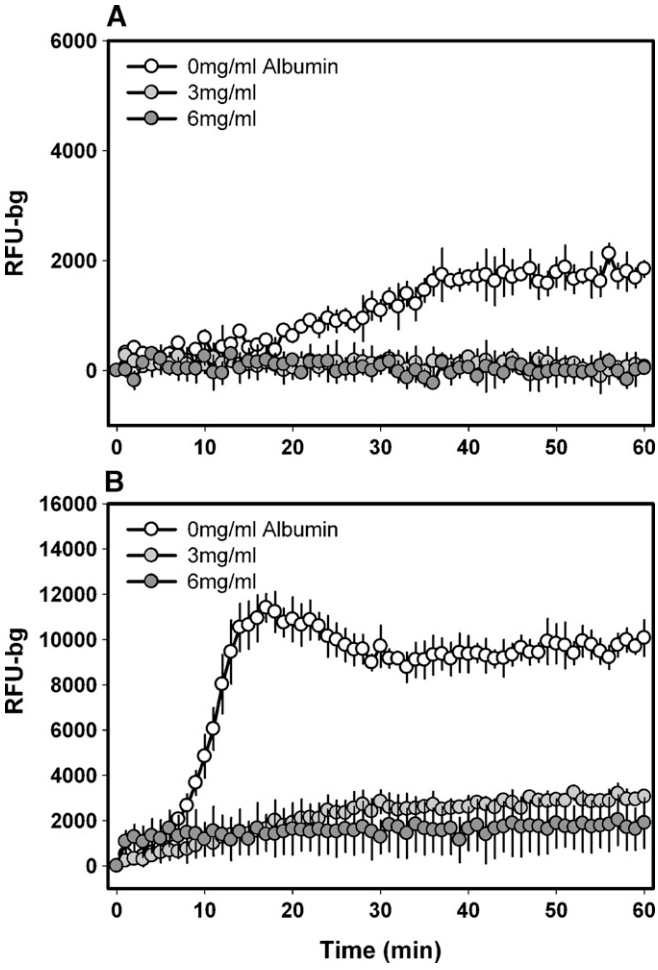

Fig. 6. Sensitivity of the fluorescence plate reader assay to evaluate inhibition of surfactant adsorption by serum albumin (BSA). BSA strongly inhibited the adsorption of DPPC-POPG lipid suspensions (A) or NS (B) onto the interfacial surface.

Adsorption of LBPs

In previous publications, it has been demonstrated that LBPs, the form in which surfactant is secreted by the cells, spontaneously and rapidly adsorb into a clean air-liquid interface (30, 31). Quantitative confirmation of these observations can be seen in **Fig. 7A**, where three different amounts of LBPs showed fast and dose-dependent adsorption kinetics. After the native structure of LBPs was disrupted by prolonged shaking in the presence of a large air-liquid interface (i.e., "interface-treated" LBPs), they still maintained similar kinetics although exhibiting a significant decrease in the amount of surface-adsorbed material (Fig. 7B). A comparison of adsorption kinetics of LBPs with NS at equal amounts (Fig. 7C) demonstrates that freshly secreted pulmonary surfactant (i.e., LBPs) adsorbed much faster than purified surfactant from lung lavages (i.e., NS). Interestingly, after a first very fast adsorption, LBPs seem to lack the ability to form large surface aggregates as detected by NS.

DISCUSSION

A qualitative and quantitative evaluation of the performance of pulmonary surfactant preparations has traditionally required setting up specialized equipment typical of surface chemistry laboratories. Determination of the abilities of surfactant samples to form surface films rapidly and efficiently with equilibrium surface tensions below 30 mN/m, and at the same time to be capable of reaching extremely low tensions under compression, is crucial to assessing structure-function correlations in normal and altered surfactants (1, 4, 20) and to optimizing clinical preparations with therapeutic potentials (14–16). Most of the techniques traditionally used to evaluate pulmonary surfactant are based on the direct use of different types of surface balances to monitor the changes in surface tension that surfactant promotes. Laboratories pursuing basic surfactant research make use of specially designed surface balances, with the potential to accurately measure surface pressure or surface tension variations with time or compression ratio, under the various quasi-static and dynamic regimes that permit the complete characterization of the surface behavior of lipid-protein complexes. Frequently, detailed physico-chemical characterization is preferred, under experimental conditions that may be far from physiologically significant. Exploration of the effects of temperature, humidity, rapid enough compression-expansion dynamics, or physiologically meaningful surfactant concentrations on pulmonary surfactant activity is difficult and, in many cases, almost impossible when using the most traditional surface balance approaches. Alternatively, characterization of pulmonary surfactant action in clinically

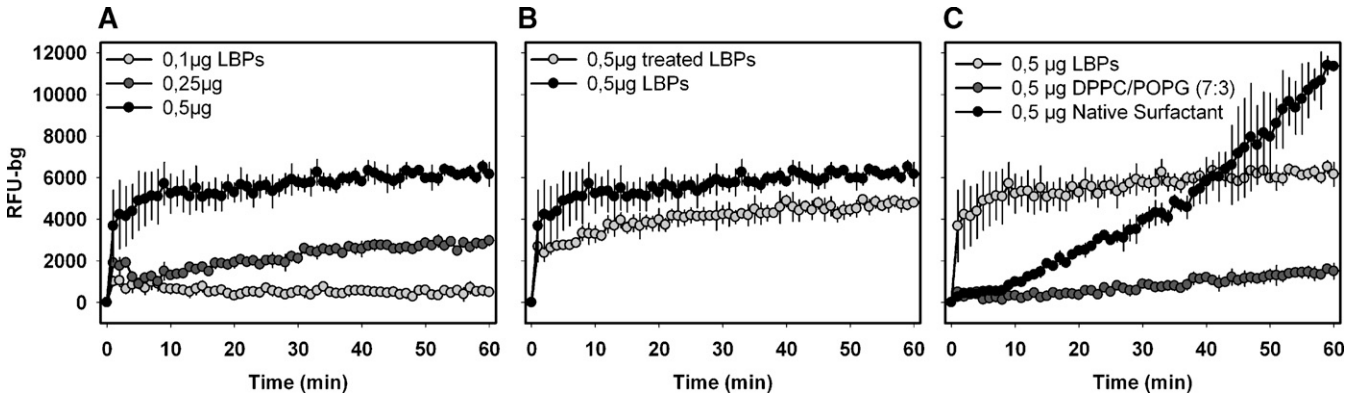

Fig. 7. Adsorption of lamellar body-like particles (LBPs) collected from stimulated alveolar type II cells as detected by the fluorescent plate reader. A: Kinetics of adsorption at different amounts of LBPs. B: Adsorption kinetics of LBPs (black) and interface-treated LBPs (gray; see text) were similar. Interestingly, intact LBPs adsorbed better than treated LBPs, although a loss of LBPs during treatment cannot be excluded. In C, adsorption kinetics of LBPs (data from A) compared with the kinetics of DPPC-POPG lipid suspension and NS at equal amounts (data from Fig. 5B, C). Freshly secreted surfactant (LBPs) revealed a faster adsorption than NS, but quickly reached saturation, which was not seen with NS, despite the use of the same concentrations.

relevant contexts has progressed through the development of clever experimental setups such as the PBS (24, 25), the capillary surfactometer (24), or the CBS (26), able to offer more physiological-like conditions for surfactant performance tests. In most cases, these approaches permit a closer approximation to the supposed in vivo performance of surfactant at the expense of a loss of accuracy in the determination of quantitative surface parameters. The use of the CBS is becoming in this sense the preferred tool for pulmonary surfactant biophysicists, because it is the only technique able to produce accurate enough data under the most physiological-like conditions, thanks to the seminal work of Samuel Schurch over the last decades (26, 32).

However, all of the methodologies currently available to evaluate pulmonary surfactant activity require a one-by-one sample examination, in experiments that usually take time in the range of an hour, with a prolonged delay between samples due to required cleaning and calibration routines. Such technical complexity usually permits only a limited evaluation of the reproducibility of the behavior of surfactant samples and prevents an extensive characterization of the effect of parameters widely recognized as affecting surfactant surface behavior. Another technical limitation is the amount of material required for the samples to be properly assessed, which is a major problem when samples are obtained from patients or small animal models. And not least, surface methods require proper technical qualification of the personnel in charge, which is not always available, and which prevents setting up pulmonary surfactant evaluation routines in every laboratory or clinical service working with surfactant preparations.

Although the spectrofluorimetric method developed here is not suited to reporting surface tension changes or to allowing dynamic surface compressions, it improves significantly on some of the limitations described above. We have shown that a direct time-course monitoring of the amount of a fluorescently labeled surfactant reaching the interface, under conditions eliminating the background signal coming from nonadsorbed material, successfully reproduces the outcome of classical methods that follow surfactant adsorption by measuring changes in surface tension (8, 33–35). The fluorimetric determination, however, can be taken to a multi-well plate reader, permitting carrying out dozens or even hundreds of simultaneous assays, meaning a change in scale with respect to the possibilities of evaluating surfactant performance with desired repetitivity and a proper analysis of statistical significance. The possibilities to assess the combined effect of physiologically relevant effects, including temperature, the ionic environment, surfactant concentration, or the presence of surface inhibitors or protectors, are largely enhanced, in an experimental setup that can be easily implemented in any laboratory.

Our fluorimetric assay offers, on the other hand, data complementary to the parameters obtained when examining pulmonary surfactant activity in classical surface balances. We demonstrate in the experiments shown here that segregation of fluorescently labeled surfactant out from the bulk phase goes beyond the formation of a mere surface-active monolayer, which is the only responsible parameter for the decrease in surface tension/increase in surface pressure. The fluorimetric signal that we can detect and monitor is indicative of the total amount of surfactant associated with the surface. It has been proposed that a good surfactant promotes not only formation of a simple surface layer of surface-active phospholipids, but also an interfacial film composed of several layers, probably including the monolayer directly exposed to air, responsible for the reduction in tension, and one or several associated surfactant bilayers (26, 36, 37). These associated bilayers constitute what has been called the surfactant surface reservoir, favoring rapid movement of surface-active species from and toward the interface during the different stages of the breathing cycle. Formation by surfactant of this multilayer surface reservoir has been recently documented by different physical techniques (38, 39). Hydrophobic surfactant proteins SP-B (36, 40, 41) and SP-C (36, 39, 42) have been proposed to play crucial roles in promoting for-

mation and stabilization of the surface-associated reservoir. Our experiments show that only protein-containing surfactant material is able to show accumulation of fluorescent material at the interface beyond the amount required to form a single monolayer, confirming that our assay not only correlates with surface tension reduction but also evaluates the capabilities of any given surfactant preparation to rapidly accumulate and sustain association of active material with the surface. The total amount of surfactant material accumulated at the surface was much higher when testing whole natural surfactant as isolated from lung lavages (NS) than in the case of LBPs obtained from type II cells. We speculate that the higher concentration of SP-A in surfactant from lavages than in LBPs, probably forming more complex or more polymorphic structures in NS, could be the factor promoting formation of multilamellar surface reservoirs. Certainly, the complementary analysis of results obtained by using surface balance measurements (strictly informing about the air-exposed side of the film) and fluorescence determinations (evaluating about formation of the whole film) will provide substantially better information when looking for surfactant structure-function correlations.

The major limitation of the assay proposed here is that it only assesses the capability of a surfactant to rapidly form a surface-associated layer, without providing direct clues to the potential of such a film to produce low enough surface tensions when subjected to compression (14, 20). In this sense, the complementary analysis of surfactant performance in specialized surface approaches such as those offered by the PBS or the CBS will still be required. However, exactly those analyses could be drastically facilitated by using our assay system to rigorously prescreen and subsequently preselect the desired experimental conditions and parameters. Such complementary studies would also be helpful to fully confirm and validate the adsorption data from our assay system, which necessarily have to be made in the presence of a high concentration of a quencher substance that eventually may interact with some molecules of interest and thus create a non-physiological condition under some circumstances.

A definitive and so far unavailable advantage of the method proposed here to evaluate surfactant film formation in a multi-sample regime is, in our opinion, its potential to be used as a base to develop high-throughput surfactant screening strategies. The search for new drugs of potential clinical interest directed to specific pathologically relevant targets is usually conducted through the analysis of the effect of hundreds to thousands of potential compounds, under many different possible conditions, in tests carried out under automated or semi-automated regimes (43–45). Our fluorescent plate reader assay has demonstrated its utility to evaluate surfactant inhibition effects such as those produced by serum proteins like albumin (8, 46). Deactivation of surfactant by serum components and by inflammatory mediators leaked into the alveolar spaces is thought to be a major determinant of the respiratory failure in acute respiratory distress associated with lung injury (16, 47). The surface-inhibitory activity of proteins such as albumin (8, 48), fibrinogen (49), C-reactive protein (9, 50), or lipases (51) toward surfactant has been well documented, as well as the deactivating effect of metabolites such as neutral lipids (46, 52), FFAs (53), bile salts (54), heme derivatives (55), or lysophospholipids (33, 53). Lung injury with different sources and to different extents is probably associated with particular complex and possibly multifactorial profiles of surfactant deactivation that have been only preliminarly characterized. A high-throughput massive analysis of surfactant surface activity in the presence of different compounds or fractions derived from pathologically conditioned media might allow a proper definition of surfactant-related pathophysiological phenotypes, which would presumably improve the chances of finding effective therapeutic interventions. A high-throughput screening strategy such as the one made possible with the method developed here might also contribute to finding new compounds with potential activity to prevent, reverse, or ameliorate surfactant impairment. Compounds of this kind would offer new tools to look for the molecular mechanisms of surfactant-related pathologies and at the same time would be the basis of new inhibition-resistant surfactants specifically optimized for particular respiratory diseases.

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