Surface Chemistry Study of the Interactions of Benzalkonium Chloride with Films of Meibum, Corneal Cells Lipids, and Whole Tears

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Purpose. To perform a surface chemistry study of the interactions between benzalkonium chloride (BAC), a common preservative used in ophthalmic formulations, and tear film (TF) constituents.

Methods. The interactions between BAC and human tears, meibum, and rabbit corneal cell lipid extracts at the air-water interface were examined in vitro during controlled compression-expansion of the film area by a Langmuir surface balance, surface potential measurements, and pendant drop-axisymmetric drop shape analysis (PD-ADSA). Surface pressure-area isotherms and isocycles were used to assess the sample’s lateral elasticity and capability of compressing and spreading during dynamic area changes. Lipid film morphology was monitored by Brewster angle microscopy. The viability of BAC-treated Statens Serum Institut rabbit cornea (SIRC) cell cultures was also examined. The BAC concentration was kept within the clinical range of 0.001% to 0.02%.

Results. In the Langmuir balance and PD-ADSA experiments, the interactions between BAC and lipids or tears resulted in (1) impaired lipid spread and formation of discontinuous nonuniform surface layers, (2) increased surface pressure-area hysteresis during compression and expansion, and (3) displacement of the lipids by BAC from the surface. A decrease (>50%) in SIRC cell viability was observed. The effects occurred within seconds after BAC exposure, and their magnitude increased with BAC concentration.

Conclusions. The surface chemistry approach used in this study provided molecular-scale insights into the detrimental effect of BAC on TF, which well explain the TF instability and corneal epithelial barrier dysfunction after exposure to BAC in the in vivo human eye. (Invest Ophthalmol Vis Sci. 2011;52: 4645–4654) DOI:10.1167/iovs.10-6271

The cationic surfactant benzalkonium chloride (BAC) is a commonly used preservative, as it is one of the few ingredients that fulfills the European Pharmacopoeia challenge test for inhibition of bacterial growth during storage of ophthalmic formulations. However, in vivo studies have repeatedly reported that BAC decreases tear film (TF) noninvasive breakup time and induces dry eye symptoms. The molecular scale mechanisms of these adverse effects remain unclear.

The purpose of the present study was to gain insight into how the interactions with BAC modify the surface properties of individual TF compounds (meibomian lipids) and of whole human tears. In vitro, the samples were spread to form film over the air-water interface of a Langmuir trough or of a pendant drop. The subphase below the films consisted of physiological saline solution: with or without BAC applied in the clinical concentration range of 0.001% to 0.02%. The films were subjected to quasi-equilibrium and dynamic area compression and expansion (Figs. 1A, 1B), and the dependence of the film surface pressure on area was measured by Langmuir surface balance and by pendant drop-axisymmetric drop shape analysis (PD-ADSA).

The surface potentials and the morphology of the Langmuir films were monitored with a surface potential (ΔV) sensor and Brewster angle microscopy (BAM), respectively. As a detrimental effect of BAC on the corneal surface integrity has been reported frequently, Langmuir surface balance experiments were also performed, to study the interactions of BAC with the membrane lipid extract of Statens Serum Institut rabbit cornea (SIRC) cells. The lipid extract films mimic the corneal epithelium membrane’s outer half, which is oriented toward the aqueous tear and is exposed to interaction with eyedrop constituents. The surface chemistry data were compared with the changes of SIRC cell viability and capability of forming a confluent cellular monolayer after treatment with BAC. The in vitro surface chemistry results correlated with the recently published in vivo findings on impaired spreading of the tear film lipid layer (TFLL) and on the barrier dysfunction of the corneal epithelium after treatment of human eyes with BAC-preserved timolol.

Materials and Methods

Collection of Human Meibum and Whole Tears

The samples for the study were collected from healthy volunteers among the regular laboratory members: three women (23–40 years old) and one man (31 years old). Their surface properties were identical with the ones of meibomian and tear samples obtained from 20

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measurements were performed: slow quasi-equilibrium compression and expansion of the film area (A) during cycling. (C) Arrows: the direction of the area changes. The compression and expansion of \( \pi(A) \) isotherms can show significant hysteresis (C, loop 1) or can closely overlap (C, loop 2).

**Figure 1.** Simplified depiction of a Langmuir trough (A) and of PD-ADSA (B). Both setups perform controlled area compression-expansion cycling of the lipid-whole tear sample (by the movable barriers of the trough or by suction in and out of the drop) and enable measuring the dependence of surface pressure (\( \pi \)) on the film area (A) during cycling. (C) Arrows: the direction of the area changes. The compression and expansion of \( \pi(A) \) isotherms can show significant hysteresis (C, loop 1) or can closely overlap (C, loop 2).

(12 women and 8 men) healthy individuals (data not shown). None of the volunteers used glasses or contact lenses and did not apply eye cosmetics before the sample collection. All volunteers gave written informed consent. The collection procedures were in accordance with the Declaration of Helsinki and were approved by the Sofia University Ethics Committee.

Human meibomian lipids were expressed from eyelids by applying pressure with two opposing cotton buds. The meibum was collected from the lid margin with a platinum spatula daily for a week, to minimize the effect of day-to-day intraindividual variations. The samples were collected, weighed, and dissolved in chloroform to a unified stock solution with a concentration of 1 mg lipid/mL. Before the experiments, the obtained donor-specific lipid solutions were kept at ~80°C. The composition of meibum was examined by thin-layer chromatography and revealed the presence mainly of wax and sterol esters, in agreement with a previous report. Isolation of the Membrane Fraction of SIRC Cells

The plasma membrane fraction was obtained by the procedure of Evans with modifications.

Control experiments tested the effect of BAC (Sigma-Aldrich, St. Louis, MO) on films by synthetic lipids (Avanti Polar Lipids, Alabaster, AL) such as dipalmitoylphosphatidylcholine (DPPC) and dipalmitoylphosphatidylglycerol (DPPG).

**Langmuir Surface Balance Experiments**

Surface pressure (\( \pi \))-area (A) isotherms were measured with a computer-controlled Langmuir surface balance (Kibron, Helsinki, Finland), equipped with a \( \mu \)-Trough XL, area 225 cm², volume 40 mL, by the Wilhelmy wire probe method.

Human meibum dissolved in chloroform was spread (43 \( \mu \)L of 1 mg/mL) over the air-saline solution interface with a microsyringe (Hamilton, Reno, NV). An acrylic cover was put over the trough to protect against dust contamination and to suppress subphase evaporation. After 15 minutes was allowed for chloroform evaporation, film area compression was started with two symmetrically moving barriers. For the experiments in which the effect of BAC on meibum film was tested, before the compression, up to 250 \( \mu \)L BAC solution was injected into the saline solution below the lipid film to the designated preservative concentration in the trough subphase. Two types of measurements were performed: slow quasi-equilibrium compression and fast dynamic compression-expansion isocycling. In the quasi-equilibrium experiments, the compression rate was 11 mm/min, which is known to be sufficiently slow to allow for the reorientation and relaxation of the lipid molecules at the surface. To examine the performance of meibum in making rapid area changes, dynamic isocycling was performed with the maximum possible barrier rate (140 mm/min) at which there was no leakage of the film. Ten consecutive cycles were performed with each sample. Normally, between the first and third cycles, the \( \pi(A) \) loops achieved a stationary shape, and the \( \pi(A) \) isocycles were then obtained and analyzed.

For certain experiments, the film surface potential \( \Delta V \) was registered by using a vibrating plate with a sensor (\( \mu \)-Spot; Kibron). All isotherms were repeated at least three times; the difference between the repetitions was up to 2%. The corneal temperature usually ranges between 30°C and 35°C and at normal to cold weather conditions TF temperature can be lower due to heat exchange. The presented experiments were performed at 28°C. The effects of BAC on the sample’s surface properties were reproducible at 37°C.

The morphology of the films was observed by BAM (MicroBAM2; Nima Technology Ltd., Coventry, UK). For experiments on the membrane lipid extract of SIRC cells the protocols were identical with those for the human extract.

**PD-ADSA of Dynamic Isocycles of Whole Human Tears**

The experiments were performed with a contact angle meter (CAM 101; KSV, Helsinki, Finland) in pendant-drop mode. The instrument was equipped with a humidity- and temperature-controlled chamber set at 28°C. Whole human tears (volume: 30 \( \mu \)L) were used to form drops (25 mm² initial area) at the end of a stainless steel needle attached to a motor-driven syringe (Hamilton, Reno, NV). A dynamic compression-expansion cycle of the drop was completed at a constant rate within 30 seconds. The drop shape was recorded with a video camera. The images were processed and fitted to the Gauss-Laplace equation with the built-in software, to obtain the tear’s area and the surface pressure. The accuracy of surface pressure measurements was \( \pm 0.5 \) mN/m.

Ten consecutive cycles were performed with each sample. The \( \pi(A) \) loops achieved stationary shape between the first and third cycles, and the \( \pi(A) \) isocycles were obtained.

**Analysis of the Quasi-equilibrium Compression Isotherms**

The TF’s reciprocal compressibility, \( C^{-1} \), at a given surface pressure was calculated from \( \pi(A) \) quasi-equilibrium compression isotherms using the equation
\[ C_s^{-1} = -A_s \left( \frac{d\sigma}{dA} \right) \]  

(1)

where \( A_s \) is the area at the indicated \( \pi \). The higher the value of \( C_s^{-1} \), the lower the lateral elasticity of the surface films and vice versa.\(^\text{20}\)

Analysis of the Dynamic Compression–Expansion Isocycles

The surface-pressure–area curves obtained at compression and expansion of the film surface can strongly differ (Fig. 1C, loop 1) or closely overlap (Fig. 1C, loop 2) in their course. The discussed effects can be quantitatively evaluated by the integration of the \( \pi(A) \) expansion–compression curves and calculation of the isotherm reversibility (RV):

\[ RV = 100 \left( \frac{\int_{A_0}^{A_e} \pi dA}{\int_{A_0}^{A_c} \pi dA} \right) \]  

(2)

For films of hydrophobic molecules like the meibomian lipids, the magnitude of the hysteresis is determined by the kinetics with which the compressed surface films restore their structure and spread at the interface during area expansion.\(^\text{21}\) Highly reversible films (Fig. 1C, loop 2) restore their structure and spread at the interface with a rate commensurate to that of area expansion (achieved by the barriers of the Langmuir trough or by the dilation of the pendant drop). Lower reversibility of the films (Fig. 1C, loop 1) reflects slower reorganization and spreading of the surface films compared with the rate of area expansion.

Evaluation of the Adsorption of BAC at the Pure Air–Water Interface and at the Interface with Predeposited Meibomian Film

A multiwell plate trough (Kibron) consisting of 15 individual circular wells, 500 \( \mu \)L volume each, was used in these experiments.\(^\text{22,23}\)

For measuring the adsorption of BAC at the pure air-water interface, a preservative solution with the desired concentrations was loaded into the wells, and the equilibrium surface pressures were recorded.

For probing the adsorption of BAC to the surface with predeposited meibomian film, lipids dissolved in chloroform were spread at the air-water interface and 5 minutes were allowed, for the chloroform to evaporate. Various amounts of meibum were deposited so that different initial surface pressure (\( \pi_0 \)) values were achieved. Then 3 \( \mu \)L of BAC solution were injected into the subphase to give 0.001% bulk concentration of preservative. The equilibrium increase in the surface pressure (\( \Delta \pi \)) after the injection of BAC was measured. The \( \Delta \pi \) increments were plotted versus \( \pi_0 \), to measure the critical surface pressure \( \pi_{cr} \), the surface pressure at and above which BAC will no longer be capable of penetrating the lipid film.\(^\text{8}\)

Cultivation of SIRC Cells, Treatment with BAC, and Measurements of Cell Culture Confluence and of Cell Viability

SIRC cells were cultured in Dulbecco’s modified Eagle’s medium (DMEM), supplemented with 10% fetal bovine serum, until 95% to 100% confluence was reached. The confluent cellular monolayers consisted of \( 5.8 \times 10^4 \) cells (\( 6 \times 10^3 \) cells/cm\(^2\)). Then, the medium was removed, and the cells were incubated with 0.001%, 0.005%, 0.01%, and 0.02% BAC in phosphate-buffered saline for up to 5 minutes, as described previously.\(^\text{7,8}\) The BAC solution was then swept out, and the cells were washed and reincubated in DMEM. The SIRC cultures’ confluence, before and after BAC treatment, was monitored, and images were captured regularly with an inverted microscope (Eclipse TS100-F; Nikon, Tokyo, Japan), with a photo documentation system, at 20X magnification.

The changes in cell viability due to treatment with BAC were evaluated by MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide) colorimetric assay.\(^\text{25}\)

RESULTS

Adsorption of BAC at the Pure Air–Water Interface and at Interface with Predeposited Meibomian Film

The adsorption of BAC at the pure air-water interface was estimated, to determine the critical micelle concentration (CMC) of the preservative (Fig. 2A). The CMC corresponded to 0.01% BAC, at which a plateau of \( \pi \) at 39 mN/m was realized. The obtained value concurred with the one determined in another study.\(^\text{7}\) Thus, the range of clinically used BAC concentrations (0.001%–0.02%) spans more than two concentration regions: below and above the CMC of the preservative.

The adsorption of BAC to the air-water interface covered with predeposited meibum film with initial surface pressure \( \pi_0 \) was also measured (Fig. 2B). After BAC was injected into the subphase, if the preservative penetrated the surface film, it was registered as an increase in surface pressure (\( \Delta \pi \)). The pene-

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tration of BAC into the meibum film decreased with the increase in $\pi_0$. This result is easy to explain, because a higher value of $\pi_0$ means a higher surface concentration of lipid and less free area available for the incorporation of BAC molecules. By the linear extrapolation of $\Delta \pi(\pi_0)$ dependence, the so-called critical surface pressure $\pi_{cr}$ was obtained: the minimum surface pressure of the predeposited lipid film at which and above which BAC is not able to enter the interfacial film. It can be seen that $\pi_{cr}$ was identical with the surface pressure of BAC at CMC (39 mN/m). The results presented in Figure 2B are from experiments with the meibum of one person. The value of $\pi_{cr} = 39 \pm 0.7$ mN/m was reproduced also with meibum from each of the other three volunteers and with "mixed" meibum (an equiweight mixture of the samples collected from each volunteer) which suggests reproducibility of $\pi_{cr}$ despite individual differences. Synonymously, $\pi_{cr}$ exceeding 35 mN/m was registered for synthetic lipids such as DPPC or corneal cell lipid extract (data not shown). These values were significantly higher than the surface pressure of the tears in an open eye ($\sim 30$ mN/m) and of the cellular biomembranes ($30-33$ mN/m). Therefore, it can be expected that under physiological conditions, BAC penetrates the corneal epithelial cells.

### Compression Isotherms and Dynamic Isocycles of Human Meibum and Tears, with and without BAC

The surface pressure-area compression isotherms of human meibum across the subphase of saline solution are shown in Figure 3A. The shape of the isotherms was similar to the ones reported by other authors. The $\pi(A)$ isotherm was converted by equation 1 to the dependence of the reciprocal compressibility ($C_A^\pi$) on $\pi$ (Fig. 3A, bold line). Across the entire $\pi$ scale, $C_A^\pi$ remained below 100 mN/m, and the relation between $C_A^\pi$ and $\pi$ was not linear. The $\pi(C_A^\pi)$ trend showed the lowest ($30$ mN/m) $C_A^\pi$ at $\pi$ of $11$ mN/m, indicating that structural changes were taking place in the lipid film at the given $\pi$. BAM (Figs. 3C, 5D) revealed that, even at low surface pressures (between 0.5 and 10 mN/m), meibum formed continuous but rough films, which is characteristic of lipid multilayers. The films were composed of bright lipid aggregates that contain some areas of lower lipid amounts, resembling black stripes. When the surface pressure was increased above 10 to 11 mN/m (i.e., the $\pi$ value at which inflection in $C_A^\pi$ was observed), the black stripes closed, and the lipid film became more dense and uniform. A similar correlation between the inflection of the $\pi(C_A^\pi)$ dependence and the closing of the black stripes is already reported in bovine meibum films.

When the film was subjected to dynamic compression and expansion (Fig. 3B), the shape of the compression $\pi(A)$ isotherm remained identical with the one observed at the quasi-equilibrium compression rate. Both $\pi(A)$ trends, at compression and at expansion, closely overlapped, as shown in other studies, and as reflected by the very high value of the isotherm reversibility, $R_v$, of 98%.

In the next set of experiments, BAC was injected in concentrations from 0.001% (below CMC) to 0.02% (above CMC) into the subphase below the meibum films that were expanded to their initial area and to 0 surface pressure. We found that the effects of 0.001% and 0.002% BAC and the effects of 0.01% and 0.02% on the surface properties of the tested samples (meibum, corneal cells lipid extracts, and whole tears) were identical. The results described were chosen to illustrate the impact of the preservative in the two regions: (1) below the CMC of BAC and (2) at the CMC of BAC (as the preservative effects above CMC are identical with the ones at CMC).

For the entire concentration range, the preservative rapidly adsorbed to the surface film, which was registered as an increase in $\pi$ and in the surface potential immediately after the injection of BAC into the subphase (Figs. 4A, 4B).

Notable is the difference in the kinetics of $\pi$ and $\Delta V$ changes after injection of BAC in concentrations below CMC (Fig. 4A). It can be seen that up to 400 seconds after the inclusion of BAC, $\pi$ increased steeply to 33 mN/m, whereas $\Delta V$ rose smoothly from 180 to 250 mV. After this moment $\pi$ slowly (for the next 900 seconds) decreased up to a plateau value of 23 mN/m, whereas $\Delta V$ continued to increase up to 520 mV. The opposite trends in the transients of $\pi$ and $\Delta V$ point to the complex structural reorganization taking place at the interface. When BAC was injected into the subphase at (and above) CMC, both $\pi$ and $\Delta V$ rose rapidly and simultaneously and soon reached stable plateau values (Fig. 4B).

![Figure 3](http://iovs.arvojournals.org/pdfaccess.ashx?url=/data/journals/iovs/933461/)

**Figure 3.** Langmuir surface balance studies of human meibum films over pure saline solution subphase. (A) Quasi-equilibrium $\pi(A)$ isotherm processed by equation 1 to derive the presented $\pi(C_A^\pi)$ dependence. (B) Dynamic $\pi(A)$ isocycle with an isotherm $R_v$ of 98%. (C-F) BAM images (1700 x 1700 µm) of meibum film representative of the designated $\pi$ range. The dark, less-reflective black stripes tended to close at $\pi > 10$ mN/m.
The dynamic isocycling of the mixed BAC–meibum films revealed π(A) loops different from those of the pure meibum (Fig. 5).

Below CMC of the preservative, the compression and expansion π(A) trends showed increased hysteresis and decreased isotherm reversibility (Rv = 80% at 0.001% BAC and Rv = 75 at 0.005% BAC), compared with the pure lipid films (Rv = 98%). At (and above) CMC (0.01% BAC), the isotherm reversibility recovered (Rv = 98%) but the shape of π(A) dependence was almost flat in contrast to the “steep” isotherm observed when only lipid was present at the surface (Fig. 3A). “Flat” isotherms are characteristic of water-soluble surfactants, whereas for water-insoluble substances (like lipids), a steady increase of π when the area is compressed is to be expected.35

The alterations in the film texture caused by the injection of BAC at concentrations below CMC were visualized by BAM (Figs. 5A–D). It can be seen that at preservative concentrations below CMC (0.001% and 0.005% BAC; Fig. 5A) when compressed to 80–40% of the initial area, the mixed film was discontinuous and nonuniform, in contrast to the morphology displayed by the lipid alone (Figs. 3C, 3D). The two surface film patterns separated at the interface: (1) condensed lipid-like aggregates, with appearance different from the pattern characteristic of the meibum alone and (2) fluidlike, dark, noncontrast regions. These nonreflective, dark regions represent areas enriched with BAC molecules, which, owing to their high solubility in water, do not form visible aggregates at the surface. The formation of the two types of regions (the reflective bright islands and dark liquid areas) may explain the opposite trends of π and ΔV between 300 and 1300 seconds on Figure 4A. Although the restructuring of the surface film may result in the gradual decrease of the surface pressure, the condensation of the lipid molecules should increase the normal component of their dipole moments, which in turn will increase ΔV. At further compression to 20% of the initial area (Fig. 5B), the lipid condensates consolidated, but the surface layer texture remained heterogeneous and did not recover the continuous morphology of the lipid film in absence of preservative (Figs. 3E, 3F). At 0.01% preservative (Figs. 5C, 5D) for the entire range of surface pressures, the films looked dark and fluidlike (i.e., the surface was enriched with BAC molecules), and some isolated bright lipid islands were observable only at a high degree of compression.

The ability of BAC to alter the surface pressure and the structure of meibomian lipid films agrees with in vitro findings51,52 showing perturbations of the lipid films at ≤0.001% of BAC and with in vivo observations2,4 of the instability of TF in human eyes (after treatment with BAC-preserved timolol) and rabbit eyes (after instillation of 0.001% BAC).

The effects of BAC on whole tear isocycles measured by PD-ADSA were similar to those discussed above (Fig. 6). Although the results of tears of only one person are shown, experiments using the samples of all volunteers revealed similar trends. Pure tear π(A) isocycles displayed high reversibility (Rv = 98%). The pure tears’ surface pressure at the initial area, 32 to 33 mN/m (i.e., surface tension, 40.9–39.9 mN/m), agreed very well with the surface tension (43.6 ± 2.7 mN/m) of uncompressed tears previously reported.11,26 BAC readily adsorbed at the air-tear interface, which is registered as the increase in tear surface pressure in the initial area. Below CMC (0.001%–0.005% preservative) the isotherms’ reversibility decreased (Rv = 80% compared with Rv = 98% for pure tears), whereas at and above CMC (0.01%–0.02% preservative), the reversibility increased but the isotherm shape became almost flat and practically coincided with the π(A) isocycles of the pendant drop, by pure BAC solution.

**Interactions of BAC with Membrane Lipid Extract of SIRC Cells and with Cell Cultures**

The influence of BAC (below and above CMC) on the surface properties is identical with the data discussed above (Fig. 7): (1) decrease in Rv and formation of heterogeneous films at 0.001% to 0.005% BAC and (2) flat isotherms and dark fluidlike films at and above CMC.

BAC strongly decreased the viability of SIRC cells and their capability of forming confluent cellular monolayers (Fig. 8). The damage in the cellular layer appeared rapidly, within 5 to 10 minutes after treatment with BAC, despite the brief exposure of the cells to the preservative. The SIRC cell line was validated by the European Centre for Validation of Alternative Methods (1990; Invitotox protocol 40; Invitotox Database for In Vitro Toxicology; http://www.invitotox.com) for use in ophthalmic cytotoxicity studies of corneal cells, and the SIRC cells were found to properly mimic the properties of the corneal epithelium in pharmacotoxicologic studies.53–56 including the damage due to exposure to BAC.55 Although SIRC cells are commonly referred to as an epithelial cell line,53–56 they actually may be of fibroblast origin.59 Thus, it is most appropriate to describe them simply as a corneal cell line.

**Discussion**

**Surface Behavior of Pure Human Meibum and Whole Tears**

Our BAM images and compressibility analysis (Fig. 3) agree with the literature data40 that meibum spontaneously forms a multilayer (instead of a monolayer) film when spread at the air-water interface. Normally, lipid monolayers at low surface pressure have high lateral elasticity (low C_{er} values) and a
liquid-expansion–like phase that does not significantly modify the Brewster angle of the surface. Only at further compression when high surface pressures are achieved are condensed lipid phases with low lateral elasticity ($C_s^{-1}$) observed. In contrast, human meibum, even at low $\pi$ ($0.5–10$ mN/m), forms a rough, continuous film at the air–water interface, becoming more uniform when $\pi$ increases and preserves high lateral elasticity ($C_s^{-1} < 100$ mN/m) for the whole $\pi$ range.

The Langmuir films of meibum displayed nonlinear $\pi(C_s^{-1})$ dependence, with a minimum $C_s^{-1}$ (at $\pi = 11$ mN/m) and subsequent reversal of the slope toward higher $C_s^{-1}$ values—a trend typical of multicomponent viscoelastic lipid multilayers. The structural rearrangements at this point (closure of

Figure 5. Top: Dynamic $\pi(\Lambda)$ isocycles of meibum films after injection into the subphase of the denoted concentrations of BAC. The isotherm $R_v$ is shown for each isocycle. (A–D) Representative BAM images ($1700 \mu m \times 1700 \mu m$) are shown of meibum films in the presence of BAC at a low to intermediate (80–40% of the initial area) and a high (20% of the initial area) degree of area compression.

Figure 6. Dynamic $\pi(\Lambda)$ isocycles of whole human tears, with or without BAC (at the designated concentrations). The isotherm $R_v$ is shown for each isocycle.
the black stripes and formation of a more continuous and thicker film) may be due to the squeezing out of film-forming molecules from the interface during compression, which then forms a multilayered structure, as observed in a synthetic meibum replica. The analysis of TFLL kinetics of spreading performed by the Voigt model and by the “spring-sloppy pistons” model synonymously reveal the viscoelastic behavior of the TF lipid layer.

The multilayer structure may well be responsible for the characteristic surface properties (low \( C_s \) and high \( R_v \)) of meibomian lipids and whole tears. Triglyceride–cholesterol ester mixtures (i.e., compositions partially similar to meibum) are reported to form highly compressible and reversible multilayers. The very high isotherm reversibility, \( R_v \), of 98% of meibum and tears indicates rapid spread and recovery of the film structure during dynamic area changes.
The data agree with in vivo studies, showing rapid TFLL spreading that closely follows the upward movement of the eyelid in “healthy” eyes. In contrast to multilayers, monolayers of disaturated phosphatidylcholines with acyl chains longer than C14 (the major phospholipids of the biomembranes) and of tissue lipid extracts (like pulmonary surfactant lipids) are much less reversible, and RV can reach values as low as 45%.46

Surface Behavior of BAC-Treated Human Meibum and Whole Tears

In a detailed clinical study, Ishibashi et al. demonstrated a significant decrease of TF noninvasive breakup time in eyes treated with BAC-preserved timolol, whereas TF in eyes exposed to timolol without BAC remained stable.

This instability of TF produced by BAC is presumably at least partly due to the perturbation of the lipid layer by the preservative. BAC is a highly surface active, cationic compound that readily adsorbs at the pure air–water interface (Fig. 2A) and penetrates the preformed meibum film (Fig. 2B) at surface pressures (39–40 mN/m) significantly higher than the surface pressure of the tears in an open eye (~30 mN/m).11,26

BAC exerted similar effects on the dynamic π(A) isocycles of meibum (Fig. 5) and whole tears (Fig. 6). It penetrated the surface film and decreased isotherm reversibility (below CMC) or displaced the meibum–tear constituents from the interface, resulting in flat π(A) trends (above the preservative’s CMC). Such plateau-like π(A) isotherm is characteristic of adsorption layers formed from water-soluble surfactants and is identical with the isotherm obtained for pure BAC solutions. The lack of overlapping between the π(A) isocycles of meibum/whole tears, with and without BAC, indicates that the ones that penetrated the surface film strongly modify its structure, remain inside it, and do not desorb to the subphase, even at high degrees of compression. In vivo BAC molecules that manage to enter the TFLL may be efficiently “screened” from the rapid aqueous tear turnover and can cause long-term adverse effects. Such assumption correlates well with clinical findings—a detrimental effect on TF stability observed even 30 minutes after instillation of BAC-preserved timolol (i.e., when the aqueous tear turnover should already be fully completed).

BAM observations permitted correlation of the changes in π(A) isocycles with the BAC-induced effects on the structure of the meibum films. Polar penetrant molecules, like BAC, are known to spontaneously repel and separate in distinguished film regions when brought in contact with hydrophobic lipids like meibum48 at the interface. This explains why penetration of BAC molecules (applied below CMC at 0.001%–0.005%), disturbs the integrity of the meibum. Heterogeneities (Figs. 5A, 5B) similar to the breakup of the TFLL observed in vivo,4 are formed in the resultant film, which is composed of condensed meibum “islands” and of fluid BAC-enriched regions.

At and above CMC, the injected BAC adsorbs to the meibomian film, not as individual monomers but as micelles that solubilize the lipids, and dark, fluid-like BAC-enriched layers (Figs. 5C, 5D) are formed with very few lipid aggregates left on the surface.

Concerning the rapid dilution of BAC in aqueous tears, the preservative effects at submicellar concentrations look more physiologically relevant. The interactions of BAC, below and above CMC, with meibum films are illustrated in Figure 9.

An essential prerequisite for stable TF to be observed is the presence of a continuous insoluble lipid multilayer as it enhances the formation of tangentially immobile surfaces that increase the TF resistance to thinning (the so-called Gibbs-Marangoni effect) and suppresses aqueous tear evaporation.50,51 Thus BAC-induced heterogeneity and solubilization of meibomian film obstructs these key functions of the TFLL.

**FIGURE 9.** A simplified presentation of the molecular mechanisms of the interactions between BAC and meibum. Tg: continuous meibum multilayer composed of cholesteryl esters (sterol ring shown as eclipse), waxes, triglycerides, and minor fraction of polar lipids. When experiments are performed below the CMC of the preservative (bottom left), BAC molecules adsorbed like individual monomers to the meibomian film. As BAC molecules are charged and polar whereas meibomian lipids are hydrophobic, both compounds spontaneously separate at the interface in fluid BAC-enriched regions and thick lipid islands; the heterogeneous film structure results in decreased isotherm reversibility (i.e., capability of spreading during area expansion) of the surface film. When experiments are performed at and above CMC of the preservative (bottom right), BAC micelles (open circles) adsorb at the interface. The micelles solubilize most of the lipids (filled circles) and BAC molecules displace meibum from the interface.
Effect of BAC on the Surface Behavior of Corneal Membrane Extracts and on SIRC Cell Viability and Capability of Forming Confluent Monolayers

In this study, BAC perturbed the integrity of membrane extract films and decreased the viability and the confluence of SIRC cell cultures. BAM observations confirm that below the CMC, the surfactant induced heterogeneity in the film structure, whereas at and above the CMC, it caused solubilization of the films. These effects of BAC were also successfully reproduced when the lipid film was composed of bovine meibum and a variety of synthetic lipids with long acyl chains: DPPC, DPPG, and a mixture of cholesterylmyristate and ethyl oleate (i.e., simplistic artificial mimics of meibum; results not shown). This reproducibility indicates that the influence of BAC on lipid films is practically independent of the nature of the lipid and is determined by the intrinsic surfactant properties of BAC itself.

General Comments on the Effects of BAC on the Surface Properties of Lipid Films: Possible Relation with BAC Adverse Effects In Vivo

We found that to partially suppress the capability of BAC to perturb the membrane integrity, it was necessary to decrease the preservative concentration by at least two orders (up to $<10^{-5}\%$), when compared with the lowest clinical concentration (0.001%). Key features of the BAC-induced alterations of lipid film structure and surface properties in vitro are that:

- BAC penetrates the lipid films (meibum, tear surface film, or cell biomembrane extract), modifies their structure and properties, and stably resides in the films during dynamic area changes.
- The BAC-induced effects occur rapidly (i.e., they are realized immediately after its injection).

The in vitro results provide a clue to the nature of the adverse effects of BAC in vivo. The concentration of BAC in aqueous tears decreases exponentially with each blink. However, it is highly probable that rapid changes in the integrity of the TFLL and of corneal cell membranes induced by BAC penetration take place in vivo. Once inserted in the lipid films at the ocular surface, the preservative molecules will be “excluded” from the aqueous tear compartment and thus “screened” from the aqueous tear turnover. As the TFLL turnover extends for hours, the preservative molecules inserted in the lipid film stay longer than those in the aqueous tears and can be responsible for the long-term adverse effects associated with BAC: TF instability and barrier dysfunction and damage of corneal epithelium. The preservative incorporation in the lipid layers after instillation of BAC-preserved eyedrops will be facilitated in dry eyes where the integrity of the TFLL and corneal cells membranes is compromised and the rate of aqueous tear turnover (i.e., the rate of preservative clearance) is frequently decreased.

The detrimental effect of BAC on TF stability and corneal surface integrity may be caused, not only by direct interaction of the preservative with the lipid bilayer, but also by electrostatic aggregation between the cationic BAC molecules and the anionic polysaccharide moieties of the glycosalloy. Apart from straight interactions with TF compounds, BAC can exert toxicity by inducing inflammation at the ocular surface. Once damaged, by one or by a combination of more mechanisms, the corneal surface loses its wettability, which in turn contributes to the destabilization of the TF.

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