I. Effect of HA at 35° C

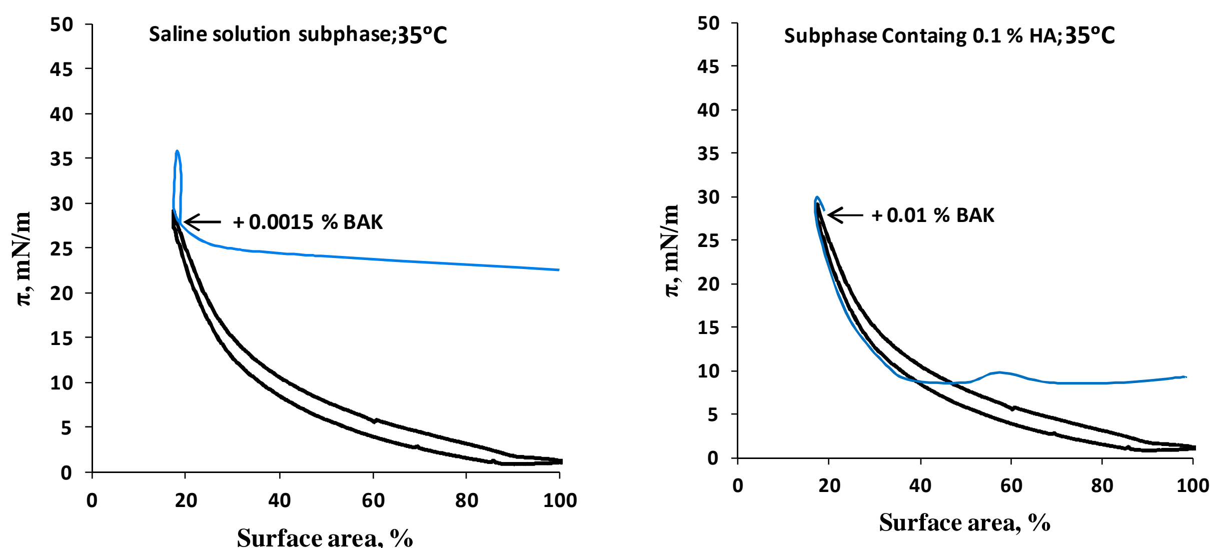

Fig. S1. HA suppresses the penetration of C12-BAC to meibomian lipid film at 35°C.

The figure illustrates the capability of HA to suppress to suppress the insertion, i.e. to decrease the value of π_{ins}, of BAK at 35°C. The right panel presents the efficiency of 0.1% HA in suppressing the penetration capability of C12-BAC at the maximum concentration of cationic surfactant. The presence of 0.1-0.3 % HA resulted in decrease of $\pi_{ins} \leq 16$ mN/m for the whole C12-BAC concentration range.

II. HA prevents the evaporation of aqueous solution pure and in presence of BAK

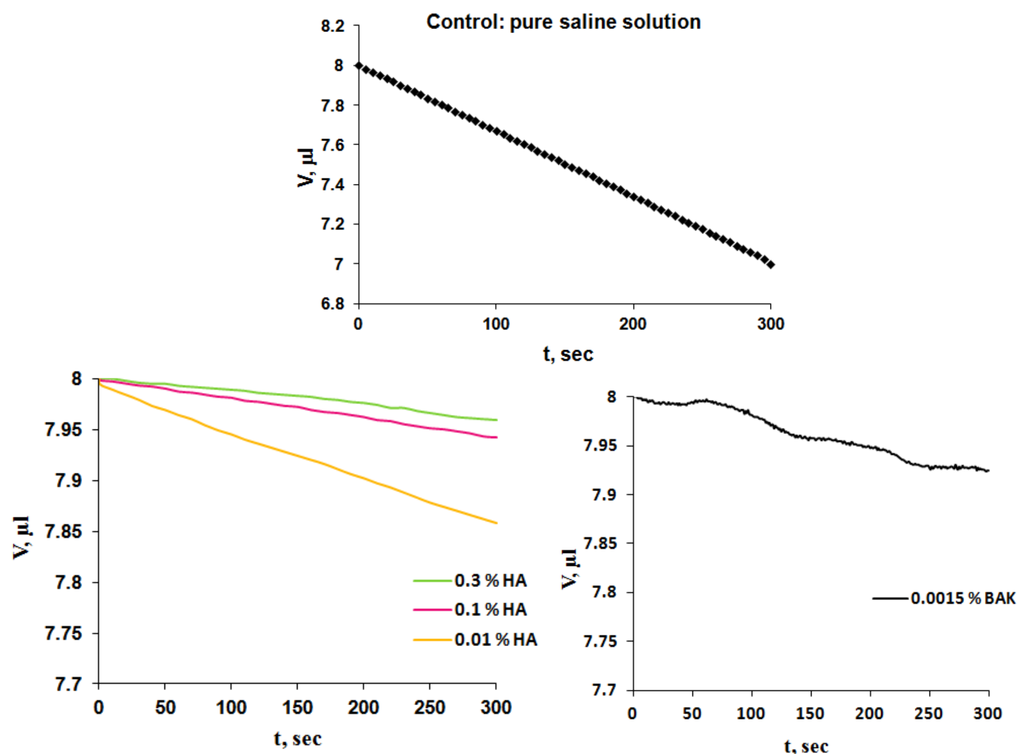

Fig. S2. HA and C12-BAC suppress the evaporation of saline solution drops. The ingredients, HA and BAK, were dissolved in phosphate buffered saline solution at the denoted concentrations The experiments were performed in CAM 101 contact angle meter (KSV, Finland) in pendant drop mode. The instrument was equipped

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with a humidity and temperature controlled chamber set at 28ºC. Polymer solution drops were formed at the end of a stainless steel needle attached to a motor-driven Hamilton syringe. The drop shape was recorded by a video camera. The two-dimensional images of the drop recorded by a video camera were processed and fitted to Gauss–Laplace equation by the built-in software to obtain the drop volume.The experimental SD is less than 5%.

Figures clearly show that:

- HA acid efficiently preserved the evaporation of aqueous solution. The effect is proportional to HA concentration. Probable explanation is the capability of HA to strongly hydrate via hydrogen bonding of water molecules.
- BAK is able to efficiently preserve aqueous evaporation too. The explanation is that BAK is polar molecule which gets strongly hydrated and thus suppresses evaporation. The results even for the lowest of the tested BAK concentrations, of 0.0015 % BAK, show that evaporation is essentially illegible within the period of the measurement.
- When HA (0.01/0.1/0.3 %) and BAK ($1.5x10^{-3}$-$1x10^{-2}$ M) are mixed together, the mixed solutions showed high resistance to evaporation and within the experimental accuracy the volume/time trends were identical to pure HA solutions (data not shown). Although part of the carboxylic –COOH groups in HA are engaged in electrostatic binding of BAK, the –COOH groups which remain free plus the multiple hydroxyl –OH groups and amide –NH groups of HA are known to participate in hydrogen bonds with water (Almond and Sheehan, Glycobiology, 2003, 13: 255–264) and thus maintain the water holding property of HA. It is possible also that the bulky HA/BAK complexes cover the drop surface and protect the drop vicinity from evaporation.

III. HA suppresses the detrimental effect of C12-BAC on the confluence and viability of SIRC cells in concentration dependant manner

The Statens Seruminstitut Rabbit Cornea (SIRC) cell line was validated by the European Centre for Validation of Alternative Methods (Adolphe M, Blein O. *SIRC cytotoxicity test* 1990; Invittox protocol: 40) for the purpose of ophthalmic cytotoxicity studies on corneal cells and SIRC cells were found to properly mimic the pharmacotoxicological properties of the corneal epithelium in toxicology studies, including the damage due to exposure to eyedrop preservatives (Goskonda et al., J Pharm Sci. 1999, 88:180-184; Goskonda et al., Pharm Dev Technol., 2000, 5: 409-416; Sakaguchi et al., Toxicol in Vitro., 2011, 25: 796-809).

SIRC cells were cultured in Dulbecco's Modified Eagle Medium (DMEM), supplemented with 10% fetal bovine serum until 95–100% confluence is reached. The confluent cellular monolayers consisted of $5.8x10^{+5}$ cells ($6x10^{+4}$ cells/cm^2). Then the medium was removed and the cells were incubated with desired concentrations of C12-BAC or C12-BAC/HA mixtures in phosphate-buffered saline for up to 5 minutes as described previously.[52] Then the preservative solution was swept out and cells were washed and reincubated in DMEM. The SIRC cultures confluence, before and after treatment, was monitored and pictures were taken regularly with Nikon ECLIPSE TS100-F microscope and photo documentation system. 20xmagnification was used.

The cell viability in presence of C12-BAC or C12-BAC/HA mixtures was evaluated by MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide) colorimetric assay (Mosmann, J ImmunolMethods., 1983, 65: 55-63).

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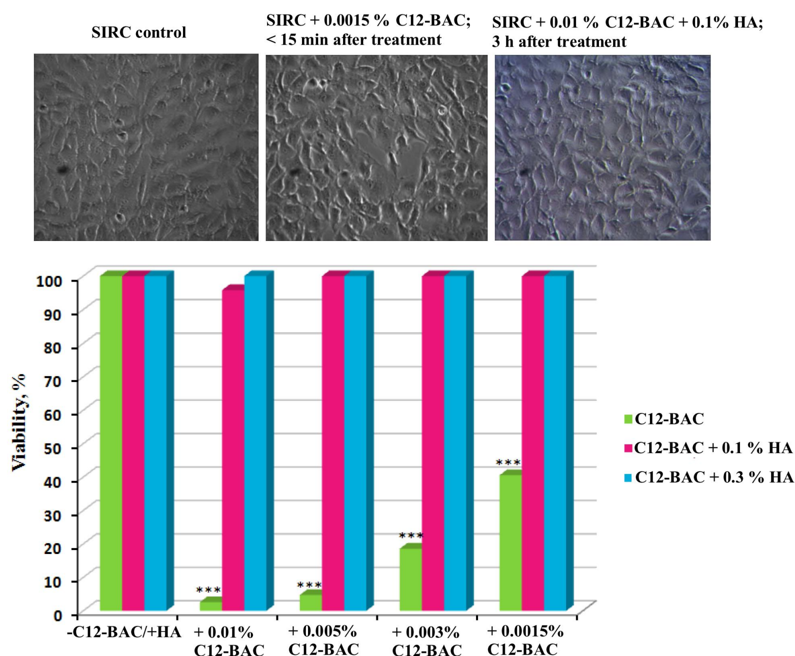

Fig. S3. Evaluation of changes in viability of SIRC cells as determined by MTT test between groups, control group treated with C12-BAC in absence of HA and groups in which together with C12-BAC, HA in the denoted concentration is implemented. The statistical analysis is performed by two tailed Student's t-test analysis and GraphPad Prism 4.0. A p-value below 0.05 was considered to be statistically significant; * = $P < 0.05$, ** = $P < 0.01$ and *** = $P < 0.001$. The data are averaged from four repetitions with SD less than 5 %. First column represents control data in the first column (- C12-BAC/+HA) are on the viability of SIRC in C12-BAC – controls: phosphate buffered saline pure (green column), plus 0.1 % HA (pink column) and plus 0.3 % HA (blue columns) respectively. The upper row presents the appearance of treated and control SIRC cell monolayers.

As reported in previous studies BAC decreases the viability and confluence of SIRC cell cultures. The presence of 0.1-0.3% HA (Fig. 12) suppressed the preservative' effect in the whole concentration range. The effect of HA in cell culture experiments and in Langmuir surface balance studies with lipid films was dependant on the concentration of the polymer. If HA was applied at 0.001% limited polymer efficiency in the neutralization of the C12-BAC adverse effects at high 12-BAC concentrations was observed (see Table 2 and Fig. S4).

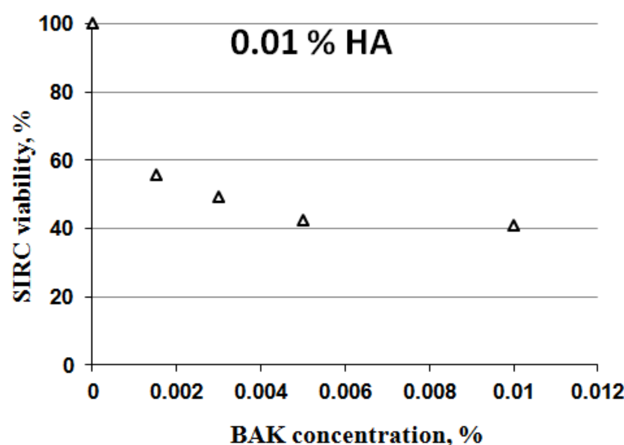

Fig. S4. 0.01% HA have limited efficiency in the neutralization of C12-BAC detrimental effect on cell viability.