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Novel colloidal associations of soyasaponins and lipid components (DPPC, cholesterol) as potential adjuvants for vaccines

Carolin de Groot [a], Mathias Müsken [b], Maren Bleckmann [c], Thomas Ebensen [d], Carlos A. Guzmán [d], Christel C. Müller-Goymann [a,e,*]

[a] Institut für Pharmazeutische Technologie, Technische Universität Braunschweig, Braunschweig, Germany
[b] Helmholtz-Centre for Infection Research, Central Facility for Microscopy, Braunschweig, Germany
[c] Institut für Biotechnologie, Technische Universität Braunschweig, Braunschweig, Germany
[d] Helmholtz Centre for Infection Research, Department of Vaccinology and Applied Microbiology, 38124 Braunschweig, Germany
[e] Center of Pharmaceutical Engineering (PVZ), Technische Universität Braunschweig, Braunschweig, Germany

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ABSTRACT

Soyasaponins from soybean (Glycine max) represent promising new potent adjuvants for vaccine research because of their immunostimulating properties and weak hemolytic activity. In the present study, saponin microstructures of soyasaponins (soyasaponin Bb, soyasaponin Ab) with lipid components (cholesterol, DPPC (dipalmitoylphosphatidylcholine)) were designed by the lipid film method. In interaction studies between soyasaponins (soyasaponin Ab/Bb) and Langmuir monolayers (model membranes), composed of cholesterol and DPPC, marked interactions between soyasaponins and a pure cholesterol monolayer were observed. No interaction was detected for soyasaponins with a pure DPPC monolayer. The intercalation of soyasaponins in a mixed DPPC/cholesterol (3:1, w/w) monolayer was only observed for the monodesmosidic soyasaponin Bb whereas the second sugar chain of the bidesmosidic soyasaponin Ab impaired the access to the monolayer. Transmission electron microscopy was used for visualizing particle formation of soyasaponins and lipid components. Pseudo-binary systems (soyasaponin Ab/Bb, cholesterol) formed colloidal associations built up from ring-like subunits in the nanometer size range. In pseudo-ternary systems (soyasaponin, cholesterol, DPPC) soyasaponin Bb attacked the liposomal membrane by forming colloidal associations. Colloidal associations in pseudo-ternary systems with soyasaponin Ab, cholesterol and a phospholipid were only observed in the presence of PE (phosphatidylethanolamine) instead of DPPC. In an MTT assay with a HaCaT cell line (keratinocyte cell line) the cell viability was neither affected by the soyasaponins nor by the corresponding formulations. Both the pure soyasaponin solution and the saponin formulations may be promising adjuvant systems for the intradermal vaccine application. Furthermore, interaction studies between the model antigen ovalbumin and colloidal associations of saponins and cholesterol using MST (Microscale Thermophoresis) gave first indications of an antigen binding to colloidal associations. Ex vivo T-cell proliferation in the presence of soyasaponin Ab was confirmed.

1. Introduction

Adjuvants are important components for achieving efficient immune responses of subunit vaccines. They modulate immune responses and contribute to a significant increase in effectiveness. The variety of adjuvants is limited, thus vaccine research is looking for new efficient and tolerable adjuvants.

Various saponins from different medicinal plants were found to have high immunomodulatory effects, e.g. quillaja saponins, soyasaponins, lablabosides, ginsenosides etc. [1–3]. However, some saponins show strong hemolytic activity which limits their use in

Abbreviations: Chol, cholesterol; DPPC, dipalmitoylphosphatidylcholine; ßE, ß-escin; HPLC, High-performance Liquid Chromatography; ISCOM, immunostimulating complex; MST, Microscale Thermophoresis; PC, phosphatidylcholine; PE, phosphatidylethanolamine; PVDF, polyvinylidine fluoride; Sab, soyasaponin Ab; SBb, soyasaponin Bb; TEM, transmission electron microscope.

* Corresponding author at: Institut für Pharmazeutische Technologie, Technische Universität Braunschweig, Mendelssohnstraße 1, D-38106 Braunschweig, Germany.
E-mail address: c.mueller-goymann@tu-braunschweig.de (C.C. Müller-Goymann).

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human, e.g. escin saponins [1,4]. The interaction of saponins with cholesterol in membranes is suggested to be responsible for the hemolytic activity [5].

The quillaja saponin (Quil A), a water-soluble triterpene saponin from the bark of *Quillaja saponaria* Molina has strong immunostimulatory properties regarding Th1 and Th2 immune responses but it has high hemolytic activity [6]. This saponin forms nanoparticular structural elements, i.e. ISCOMs (immunostimulating complexes), with cholesterol and phospholipids of about 40 nm as a promising adjuvant and antigen-presenting system [7]. Due to the hemolytic activity of the quillaja saponin ISCOMs were initially used in animal vaccines [8]. By higher purification of the quillaja saponin the hemolytic activity decreased. One of these purified fractions is QS-21. GlaxoSmithKline (GSK) developed an adjuvant system (AS01), which combined QS-21 with liposomes consisting of cholesterol and unsaturated DOPC (dioleoylphosphatidylcholine) [9]. Due to the high affinity of pure QS-21 to cholesterol, responsible for the hemolytic activity, liposomes were added to the adjuvant system. Utilizing the interaction of QS-21 with liposomal cholesterol, a stable formulation can be obtained together with less toxicity [10]. Recently, a novel adjuvant of ginsenoside-based nanoparticles, ginsomes, were developed [11]. Equally to the ISCOMs, ginsomes consist of phospholipids, cholesterol and the saponin. The ginsomes were proved to raise both the Th1 and Th2 immune response in mice and may therefore be used as possible adjuvants in the future.

Good adjuvant properties were also demonstrated for soyasaponins. In particular, the soyasaponins Ab and Bb indicate high adjuvant activity as different studies proved [12,13]. The complex molecular structure and the variety of soyasaponins have long been known [14–18]. Soyasaponins belong to the class of oleanane triterpenoid saponins and are found in soybeans (*Glycine* max) and other legumes [19–21]. Soyasaponins are amphiphilic molecules and are subdivided into the main groups A and B, differentiating the pentacyclic aglycon (soyasapogenol A, B) and the attached sugar moieties. Group A saponins are bidesmosidic with attached sugar chains at C-3 and C-22 via an ether linkage, e.g. soyasaponin Ab (SAb), and thus more polar than group B saponins which are monodesmosidic with a single sugar chain at C-3, e.g. soyasaponin Bb (SBb) (Fig. 1) [22–24]. In the same way as quillaja saponins and ginsenosides, soyasaponins stimulate both the Th1 and the Th2 immune response making them superior to the classical adjuvant aluminium hydroxide. It has been proved that soyasaponins are well tolerated due to their weak hemolytic activity [1]. Soyasaponin Ab and Bb are promising candidates as adjuvants for the development of new vaccines.

Based on previous studies of colloidal associations built from monodesmosidic triterpene saponin ß-escin and lipid components we hypothesized that further saponins should be able to form those colloidal structural elements [25]. The aim of this study was to investigate the interaction of SAb and SBb with lipid components including cholesterol and saturated DPPC using the Langmuir film method and to design saponin microstructures as adjuvant system. Furthermore, saponin microstructures should be investigated for their suitability as possible adjuvant systems. The visualization of microstructures in pseudo-binary (saponin:cholesterol) and pseudo-ternary systems (saponin:cholesterol:phospholipid) was conducted by transmission electron microscopy (TEM). To investigate the influence of different phospholipids on particle formation, the saturated DPPC was compared with unsaturated PE from soybean in pseudo-ternary systems of ß-escin and soyasaponin Ab. In addition, we studied the cell viability of HaCaT cells via an MTT assay in the presence of aqueous solutions of soyasaponins as well as colloidal associations of soyasaponins and lipid components to compare it with aqueous solutions of the saponin ß-escin from horse chestnut and its colloidal associations, to examine the effect of saponins on living cells. A keratinocyte cell line was used to consider new ways of vaccine application, such as the intradermal route. Vaccination via skin is attractive because the skin has immunological properties and protects the body from infections [26]. Finally, we investigated the binding of the model antigen ovalbumin to colloidal associations of saponins (SBb, SAb,

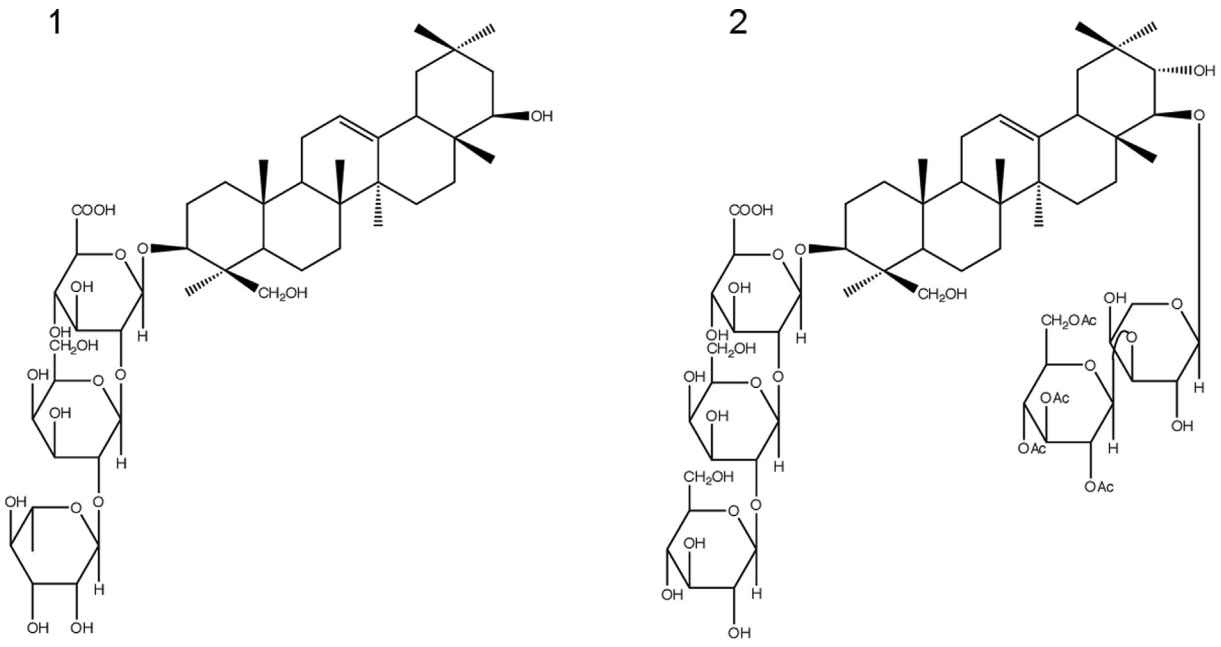

Fig. 1. Chemical structure of (1) monodesmosidic soyasaponin Bb and (2) bidesmosidic soyasaponin Ab. The aglycons (soyasapogenol B (left), A (right)) are linked with different sugar chains attached at C-3 and C-22. Sugar moieties of SBb (C-3): ß-D-glucuronopyranosyl, ß-D-galactopyranosyl, α-L-rhamnopyranosyl. Sugar moieties of SAb (C-3): ß-D-glucuronopyranosyl, ß-D-galactopyranosyl, ß-D-glucopyranosyl; (C-22): α-D-arabinopyranosyl, acetylated ß-D-glucopyranosyl. Nomenclature according to Zhang and Popovich, 2009 [22].

ß-escin) and cholesterol by MST (Microscale Thermophoresis) and the immunological activity of saponin formulations in an ex vivo DC – T cell stimulation assay.

2. Materials and methods

2.1. Materials

Cholesterol (Chol, ≥99% purity) and soyasaponin Bb were purchased from Sigma Aldrich (Taufkirchen/Seelze, Germany). 1,2-Dipalmitoyl-*sn*-glycero-3-phosphocholine (DPPC, 99% purity) and 1,2-Diacyl-*sn*-glycero-3-phosphoethanolamine (PE, 98% purity) from soybean was kindly donated by Lipoid (Ludwigshafen, Germany). Soyasaponin Ab was obtained from Fortopchem Technology (Hongkong, China). The triterpene saponin β-escin (98.7% purity) was obtained from MP Biomedicals (Ohio, USA). The model antigen ovalbumin EndoFit™, a chicken egg albumin, was purchased from InvivoGen (Toulouse, France). Organic solvents used for the preparation of saponin containing dispersions and for cleaning the Langmuir trough were of analytical grade. All substances were used as received. For all experiments, water was purified by ultrafiltration (Millipore, Eschborn, Germany).

HaCaT cells were kindly donated by Fusenig, DKFZ (Heidelberg, Germany). They were continuously cultivated at standard conditions (37 °C with 5% CO_2) in Dulbecco's Modified Eagle Medium (DMEM). For the experiments the cells were used from passage 93–116.

2.2. Methods

2.2.1. Langmuir film balance technique

The surface pressure (π) measurements were performed with a Nima 611 Langmuir through (Coventry, England) using the Wilhelmy plate method [25]. The Langmuir trough, coated with inert polytetrafluorethylene was equipped with a film balance on an antivibration table. The trough contained the aqueous subphase and held a total volume of 64 ml. The Wilhelmy plates were made from chromatography paper (Whatman CHR1 chromatography paper, perimeter 20.6 mm), 10 mm width and 20 mm length and were attached to an S-ring connected to a pressure sensor. The plates were partially immersed into the subphase. Two barriers were positioned onto the trough, a static barrier with the pressure sensor on top and a movable barrier that continuously compresses the monolayer.

The lipids (cholesterol, DPPC) were dissolved in chloroform and then dropped onto the subphase using a microliter syringe with a reproducibility adaptor (Hamilton, Bonaduz, Switzerland). The experimental setup was left for 15 min to allow evaporation of the solvent and formation of the lipid film. The temperature was kept constant at 20 °C for all measurements. The surface pressure (π)/area (A) isotherms were continuously recorded by constant compression of the monolayer up to the collapse point. The collapse point indicates the end of the measurement where the monolayer finally breaks and falls apart. All measurements were performed with a saponin (SBb, SAb) containing subphase in an appropriate concentration (10^{-3} mg/mL) compared to an aqueous subphase. These measurements were repeated three times.

For surface pressure (π)/time (t) measurements a pure cholesterol monolayer was first precompressed on an aqueous subphase to an appropriate surface pressure. The barrier speed was then set to zero. Subsequently, 5 ml of the subphase was replaced by 5 ml of a concentrated aqueous soyasaponin solution behind the barrier from beneath the monolayer. The resulting concentration of the soyasaponin in the subphase was 10^{-3} mg/mL. The surface pressure development was recorded over 10,000 s.

2.2.2. Preparation of saponin containing dispersions for transmission electron microscopy (TEM)

Liposomal dispersions containing the saponin (SBb, SAb) were prepared using the lipid film method by Bangham et al. with small modifications [27]. First, the lipid components (DPPC or PE, cholesterol) and the saponin were dissolved in methanol to get a clear solution. The organic solvent was then removed under vacuum at 45 °C using a rotary evaporator. The round bottom flask with the dried lipid film was stored in a desiccator over night to achieve a complete evaporation of the solvent residues. The dried lipid film was then rehydrated in NANOpure water and briefly treated with the ultrasonic bath to obtain a homogeneous dispersion. All dispersions had a total concentration of 0.67 mg/mL.

Sample preparation for TEM was performed as previously described [25]. In brief, samples were allowed to adhere to a carbon film (one minute), which was taken up by a copper grid. The grid was washed twice in distilled water and a drop of 4% (w/v) aqueous uranyl acetate was floated onto it. After another minute, the excessive liquid was removed and the grid was air-dried. Sample images were taken with a Zeiss Libra 120 Plus equipped with a 2 K slow scan CCD camera at various magnifications and an acceleration voltage of 120 kV.

2.2.3. In vitro viability of HaCaT keratinocytes by MTT assay in the presence of saponins

The MTT assay was conducted using an immortalized keratinocyte cell line (HaCaT) from human skin to determine the cytotoxicity of saponin formulations. The MTT assay was performed according to Mosmann with slight modification [28].

Two different monodesmosidic triterpene saponins, that is soyasaponin Bb and ß-escin, as well as the bidesmosidic triterpene saponin soyasaponin Ab were tested. The tested concentrations of pure aqueous saponin solutions were in the range of 0.001–0.5 mg/mL dissolved in DMEM-medium:H_2O (1:1, v/v). Additionally, saponin dispersions with different ratios of saponin and lipid components (cholesterol, DPPC) were investigated (Table 1, 2). HaCaT cells were plated in quadruple for each formulation using 96-well-plates (TPP, Trasadingen, Switzerland). Each plate contained positive control wells with DMEM-medium:H_2O (1:1, v/v) without saponin, negative control wells with DMEM-medium:

Table 1
Composition of different soyasaponin formulations (SBb, SAb) containing cholesterol and DPPC. The individual concentrations of the components and the total concentration of the formulation are shown. Each of the formulation was diluted 1:2 and 1:5 with DMEM-medium:H_2O (1:1, v/v). The dispersions were examined with regard to viability of HaCaT cell line with an MTT assay.

Formulation	SBb/SAb concentration [mg/mL]	Chol concentration [mg/mL]	DPPC concentration [mg/mL]	Total concentration [mg/mL]
SBb/SAb:Chol (3:1)	0.2513	0.0838	–	0.335
SBb/SAb :Chol (3:1)_1:2	0.1256	0.0419		0.168
SBb/SAb:Chol (3:1)_1:5	0.0503	0.0168		0.067
DPPC:SBb/SAb:Chol (1:3:1)	0.2010	0.0670	0.0670	0.335
DPPC:SBb/SAb:Chol (1:3:1)_1:2	0.1005	0.0335	0.0335	0.168
DPPC:SBb/SAb:Chol (1:3:1)_1:5	0.0402	0.0134	0.0134	0.067

Table 2
Composition of different ß-escin (ßE) formulations containing cholesterol and DPPC. The individual concentrations of the components and the total concentration of the formulation is shown. Each of the formulation was diluted 1:2, 1:3, 1:10, 1:50 and 1:100 with DMEM-medium:H_2O (1:1, v/v). The dispersions were examined with regard to viability of HaCaT cell line with an MTT assay.

Formulation	ßE concentration [mg/mL]	Chol concentration [mg/mL]	DPPC concentration [mg/mL]	Total concentration [mg/mL]
ßE:Chol (3:1)	0.5025	0.1675	–	0.670
ßE:Chol (3:1)_1:2	0.2513	0.0838		0.335
ßE:Chol (3:1)_1:3	0.1675	0.0558		0.223
ßE:Chol (3:1)_1:10	0.0503	0.0168		0.067
ßE:Chol (3:1)_1:50	0.0101	0.0034		0.013
ßE:Chol (3:1)_1:100	0.0050	0.0017		0.007
DPPC:ßE:Chol (1:3:1)	0.4020	0.1340	0.1340	0.670
DPPC:ßE:Chol (1:3:1)_1:2	0.2010	0.0670	0.0670	0.335
DPPC:ßE:Chol (1:3:1)_1:3	0.1340	0.0447	0.0447	0.223
DPPC:ßE:Chol (1:3:1)_1:10	0.0402	0.0134	0.0134	0.067
DPPC:ßE:Chol (1:3:1)_1:50	0.0080	0.0027	0.0027	0.013
DPPC:ßE:Chol (1:3:1)_1:100	0.0040	0.0013	0.0013	0.007

H_2O (1:1, v/v) + 1%TritonX-100 and blank wells without cells. For all experiments aqua ad injectabilia Ph. Eur. was used.

The cells were incubated for 24 h in DMEM-medium (200 μL/well) under standard conditions at 37 °C with 5% CO_2. After 24 h the medium was removed and 100 μL of the test sample was added to the well. Subsequently, the cells were incubated for 2 h/24 h under standard conditions. After the incubation time the samples were replaced by 100 μL/well of DMEM-medium with 0.05% of MTT-solution with a subsequent incubation time of 2 h. Then, the MTT-solution was replaced by a lysis solution (isopropanol, doubled distilled water, hydrochloric acid, sodium dodecyl sulfate) to release the resulting colored salt from the cells. After an incubation time of 2 h the absorption was measured at a wavelength of 570 nm with an infinite M Plex multiplate reader (Tecan, Männedorf, Switzerland). The positive control with DMEM-medium:H_2O (1:1, v/v) was set to 100% cell viability.

2.2.4. Microscale Thermophoresis (MST) – binding of ovalbumin to colloidal saponin associations

Ovalbumin was labeled in PBS (0.01 M Na_2HPO_4, 0.0018 M KH_2PO_4, 0.137 M NaCl, 0.0027 M KCl, pH 7.4) using the Amine Reactive Monolith Protein Labeling Kit RED-NHS 2nd Generation (NanoTemper) following the manufacturer's protocol. The concentration determined in the Nanodrop (Peqlab) of the labeled ovalbumin was 8.05 μM and the labeling efficacy 0.21. Aliquots were stored at −80 °C. Before the experiments labeled ovalbumin was diluted in PBS with 0.1% Tween to 30 nM and centrifuged for 15 min at 15.000 rpm. For the binding check the labeled ovalbumin and different colloidal associations of saponins (SBb, SAb, ß-escin) and cholesterol were tested in the Monolith NT.Automated (NanoTemper) in Monolith NT.Automated Capillary Chips (NanoTemper) and compared with the signal of ovalbumin diluted in water. An excitation power of 90% and a high MST power was applied.

The used saponin dispersions were made by the lipid film method as described in 2.2.2. The soyasaponin dispersions had a total concentration of 0.67 mg/mL. The ß-escin formulation was filtered through a PVDF (polyvinylidene fluoride) membrane followed by a quantification of ß-escin and cholesterol with HPLC (High-performance Liquid Chromatography) as previously described [25]. The concentration of the ß-escin formulation was 0.22 mg/mL within the total volume. The saponin dispersions were diluted 1:1 v/v with the 30 nM ovalbumin solution mentioned above.

2.2.5. Measurement of cellular proliferation

Dendritic cells (DCs) derived from bone marrow of Balb/c mice were maturated for 5 days using complete RPMI medium (containing 10% fetal bovine serum, 100 U/mL penicillin and 100 μg/mL streptomycin; Gibco, UK) supplemented with GM-CSF (5×104 U/ml). After 5 days, matured DCs were stimulated for 24 h in the presence of 1 μg/mL HA (H1N1 California, #13/164) to determine the antigen-specific basal proliferation and with 100 μg/well of saponin formulations alone to determine the unspecific proliferation. In addition, stimulation took place in the presence of 1 μg/mL HA along with 100 μg/well of a saponin formulation of ßE:Chol:DPPC 3:1:1, w/w (Table 2) with a total concentration of 0.67 mg/mL, a liposomal formulation of Chol:DPPC 1:1, w/w with a total concentration of 0.268 mg/mL or with pure aqueous saponin solutions of SAb or ß-escin with a concentration of 0.4020 mg/mL, respectively. To study the T cell proliferation, CD4+, CD8+ T cells and CD19+ B cells from TCR HA mice were used. Immune cells of these mice express rearranged T cell receptor α/β chains specific for the MHC Class II I-Ed restricted determinant site from influenza virus A/PR/8/34 hemagglutinin (PR8 HA) [29]. $2–3 \times 10^6$ CFSE-labeled in vitro enriched CD4+, CD8 + T cells and CD19+ B cells from TCR HA transgenic animals were co-incubated with DCs restimulated with antigen co-administered with different saponin formulations or liposomes. After 8 days, the T cells were stained for CD3, CD4, CD8, CD19, live/dead marker (LIVE/DEAD Fixable Dead Cell Stain Kit from Invitrogen™) as a marker of viability and the loss of CFSE. Readout was performed by flow cytometry using a FACS Canto instrument (BD Biosciences, Germany) as described in Ebensen et al. [30].

3. Results and discussion

3.1. Langmuir film balance

The Langmuir film balance experiments were performed to investigate the interaction between soyasaponins (SBb, SAb) and lipid components (cholesterol, DPPC) by intercalation of the saponin from the subphase into the lipid monolayer. The Langmuir film balance technique is a sensitive method and enables the elucidation of interaction studies on a molecular level in a two-dimensional (2D) model system. 2D monolayers of pure cholesterol, pure DPPC and a mixture of DPPC:cholesterol (mass ratio 3:1) were investigated on an aqueous subphase compared to a saponin containing subphase. The surface pressure π was recorded during continuous compression of the monolayer. The π/A-isotherm is defined as the change in surface pressure π as a function of the area per molecule A.

During compression the monolayer passes several phases. In the gaseous phase (G) the lipid molecules do not interact with each other due to high distances between them. Thus, no surface pressure is measured. At further compression the molecules straighten

up and the area per molecule decreases, which results in a denser alignment (liquid condensed phase (LC)). Thus, the surface pressure increases. Finally, the monolayer collapses when the molecules are too densely packed. The collapse point is a relevant parameter in interaction studies indicating the minimum required area per molecule in the monolayer. All transition points (G-LC) and the collapse points of the different monolayers are summarized in Table 3A.

The transition point (G-LC) from the gaseous phase (G) to a liquid condensed phase (LC) of a pure cholesterol monolayer on an aqueous subphase occurred at 47.56 ± 0.47 Å^2 (Fig. 2A). The replacement of an aqueous phase with a SBb subphase resulted in a shift of the isotherm (grey curve). The required area per molecule increased, which is shown by a higher area at G-LC (58.0 ± 0.61 Å^2) and at the collapse point (39.32 ± 0.05 Å^2). The area per molecule at collapse pressure increased by 3.26 Å^2, indicating an intercalation of saponin molecules from the subphase into the monolayer.

Similar results were observed with a cholesterol monolayer on a SAb subphase (Fig. 2D). The collapse point shifted by 2.52 Å^2 from 39.67 ± 0.034 Å^2 to 42.19 ± 0.21 Å^2. The shift was slightly weaker indicating that less SAb molecules intercalated into the cholesterol monolayer compared to SBb. When comparing the results of soyasaponins with those of ß-escin from previous studies, ß-escin showed an even stronger affinity to cholesterol [25]. The collapse point of a cholesterol monolayer on a ß-escin containing subphase increased by 5.87 Å^2.

The affinity of soyasaponins to cholesterol was also observed in π/t-measurements. The initial and final surface pressure of a pure cholesterol monolayer on a soyasaponin subphase are given in Table 3B. Soyasaponin molecules migrated into the cholesterol monolayer over a period of 10,000 s along with an increase in surface pressure. The increase in surface pressure was higher in the presence of SBb molecules compared to SAb. At a moderate precompression of the monolayer of about 5 mN/m (lower curve) the surface pressure increased by 8.38 mN/m from 5.42 mN/m up to 13.80 mN/m (Fig. 2G). Even at higher precompression (upper curve), with a denser packed cholesterol layer, SBb molecules migrated into the monolayer, increasing the surface pressure by 7.65 mN/m. The initial slope of the curve indicates a rapid movement of the SBb molecules into the monolayer. The amount of SAb molecules migrating into the monolayer was lower. The surface pressure increased by 3.27 mN/m with a moderately precompressed monolayer (lower curve) and by 1.73 mN/m for a highly precompressed monolayer (upper curve) (Fig. 2H). The movement of SAb molecules into the monolayer was much slower compared to SBb molecules reflecting a continuous increase of the curve during the entire period. In accordance with the results of the π/A isotherms, the amount of the ß-escin molecules intercalated into the cholesterol monolayer was highest over a period of 10,000 s [25]. The surface pressure increase of a moderately pre-compressed monolayer on a ß-escin subphase was 23.26 mN/m and that of a highly pre-compressed monolayer was 12.0 mN/m.

Different results were observed with a pure DPPC monolayer. The π/A-isotherms of a pure DPPC monolayer on an aqueous subpase and on a soyasaponin subphase are given in Fig. 2B/E. The transition from the gaseous into the liquid condensed phase occurred at a higher area per molecule compared to a cholesterol monolayer. This is related to the larger molar mass of the DPPC molecules resulting in a higher space requirement per molecule. The compression isotherms of both subphases overlapped implying that the soyasaponin molecules remained in the subphase and were not interacting with the DPPC molecules at the surface.

The isotherms of a mixed DPPC:cholesterol monolayer with a mass ratio of 3:1 showed slight differences between the two soyasaponins. The transition point of the monolayer on an aqueous subphase and on a SAb subphase was at the same molecular area (63.21 Å^2), see Fig. 3F. Neither a shift in collapse point was detected. The addition of SBb molecules into the subphase resulted in a slight shift (0.9 Å^2) of the collapse point (Fig. 2C). The entire isotherm shifted to larger molecular areas for each surface pressure. This result suggests a slight intercalation of the SBb molecules into the monolayer whereas SAb remained in the subphase.

Both soyasaponins interacted with cholesterol from a pure cholesterol monolayer and were therefore suggested to have a high affinity for cholesterol. The interactions might be due to van der Waals forces between the hydrophobic backbones. Furthermore, SBb intercalated into a mixed DPPC:cholesterol monolayer. This might be due to the high affinity for cholesterol, because no interaction was observed between soyasaponins and DPPC. Overall, the interactions are weaker than the interactions with a pure cholesterol monolayer. The slightly weaker intercalation of SAb into the monolayers might be attributed to the second sugar chain of the saponin molecule, which might impair access to the monolayer.

When comparing the results of the Langmuir study with those of the monodesmosidic triterpene saponin ß-escin from horse chestnut, ß-escin showed a much stronger interaction with cholesterol in pure cholesterol monolayers as well as in mixed monolayers of cholesterol and DPPC.

3.2. Transmission electron microscopy (TEM)

In order to elucidate the effects of soyasaponin Ab and Bb on cholesterol and DPPC in aqueous dispersions, transmission electron microscopy (TEM) was used. The TEM images of different dispersions are shown in Fig. 3. A pseudo-binary system of SBb and cholesterol with a mass ratio of 3:1 formed colloidal associations built up from ring-like subunits (Fig. 3A). These subunits have a mean diameter of 21.5 ± 2.81 nm. The size and shape of these new colloidal associations were quite heterogeneous. Variation of

Table 3A
Required area per molecule from different monolayer composition (pure cholesterol, pure DPPC, mixed DPPC:cholesterol) on a soyasaponin Bb/Ab subphase compared to a pure aqueous subphase at transition point (gaseous (G) – liquid expanded (LC)) and at collapse point. [n = 3], mean ± s.d.

	Transition point [Å^2] (G– LC)		Collapse point [Å^2]	
	H_2O	SBb	H_2O	SBb
Cholesterol	47.56 ± 0.47	58.0 ± 0.61	36.06 ± 0.29	39.32 ± 0.05
DPPC	57.31	57.31	42.94 ± 0.27	42.71 ± 0.03
DPPC:Cholesterol (3:1)	59.69	62.35	40.75 ± 0.05	41.65 ± 0.49
	H_2O	SAb	H_2O	SAb
Cholesterol	49.78 ± 0.18	60.38	39.67 ± 0.34	42.19 ± 0.21
DPPC	57.31	57.31	45.29 ± 0.22	45.47 ± 0.24
DPPC:Cholesterol (3:1)	63.21	63.21	43.92 ± 0.42	43.87 ± 0.48

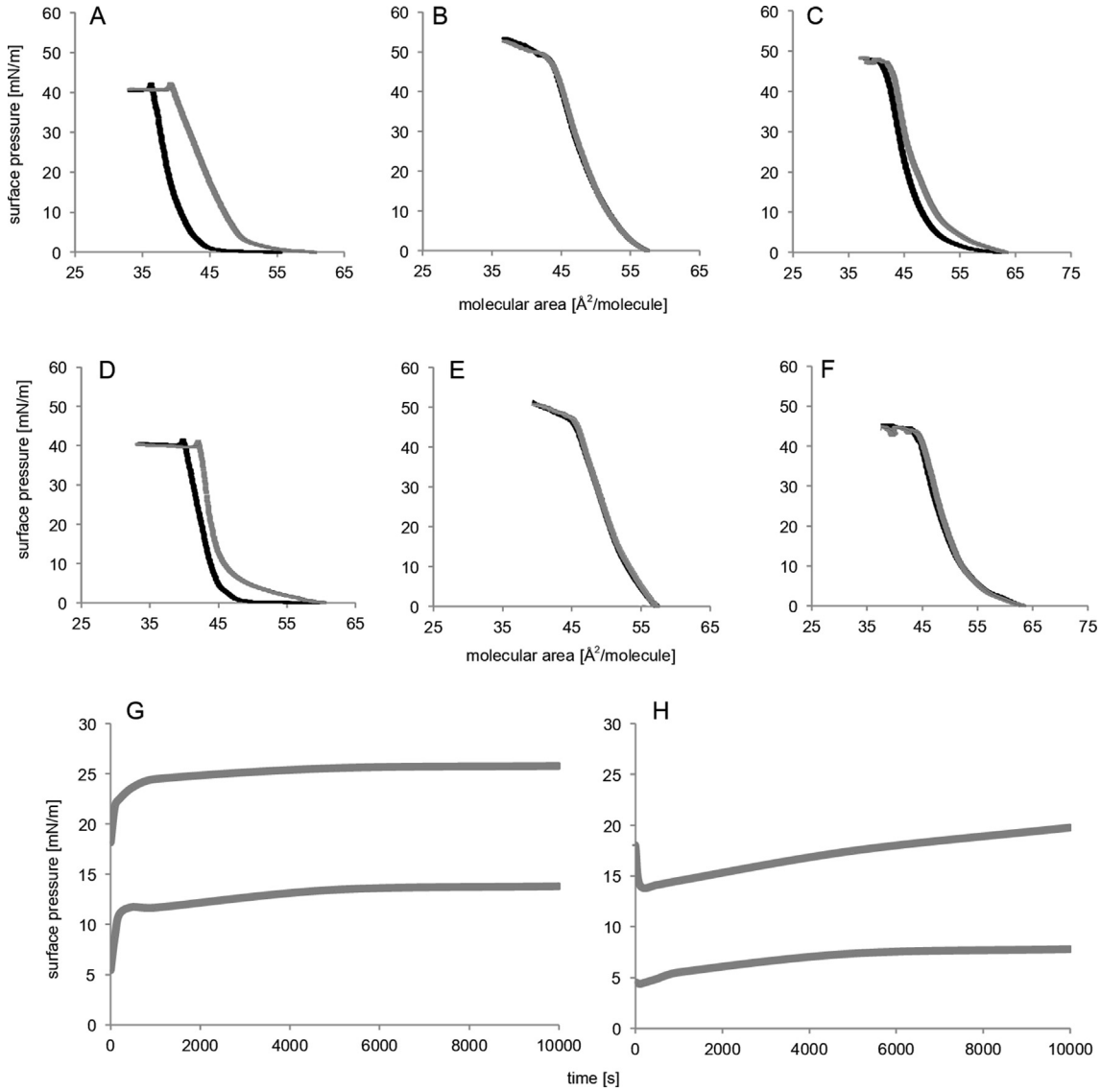

Fig. 2. Langmuir π/A-isotherms of a (A/D) cholesterol, (B/E) DPPC, and (C/F) DPPC:cholesterol (mass ratio 3:1) monolayer on a (A-C) soyasaponin Bb subphase (1×10^{-3} mg/mL) and (D-F) soyasaponin Ab subphase (1×10^{-3} mg/mL) compared to a pure aqueous subphase. aqueous subphase = black isotherms; soyasaponin subphase = grey isotherms. Langmuir π/t-measurements of a pure cholesterol monolayer on a (G) soyasaponin Bb and (H) soyasaponin Ab subphase. The surface pressure development was followed over 10,000 s.

Table 3B
Initial and final surface pressure of a pure cholesterol monolayer on a soyasaponin Bb and Ab subphase.

Subphase	Initial surface pressure [mN/m]	Final surface pressure [mN/m]
SBb	5.42	13.80
	18.13	25.78
SAb	4.54	7.81
	18.04	19.77

the mass ratio of SBb and cholesterol as to 4:1 and 5:1 also led to the same colloidal associations (data not shown). A further increase in saponin mass ratio to 5.7:1 led to associations built up from worm-like subunits (Fig. 3B). With decreasing cholesterol content the shape of the subunits was changing. Fig. 3C shows different structures compared to the previous ones. The saponin proportion was further increased to a mass ratio of 10:1, resulting in quite small circular subunits. The circular subunits partly aggregated to larger structures. Only a few isolated ring-like subunits were visualized in the surrounding area (indicated by arrows). The addition of DPPC to the dispersion led to the formation of liposomes with attached colloidal structures built up from ring like subunits, mentioned before in Fig. 3A (Fig. 3D). It is suggested that SBb forms colloidal associations by attacking the liposomal membrane based on the recognition of cholesterol in the membrane. Similar observations were also made for the quillaja saponin by Paepenmüller and Müller-Goymann, who used unsaturated egg yolk phosphatidylcholine instead of DPPC [31].

Analogue dispersions with varying mass ratios of cholesterol and saponin were prepared with SAb. A pseudo-binary system of SAb and cholesterol (3:1 w/w) resulted in circular colloidal associations (Fig. 3E), similar to the ISCOM structures [32]. These new colloidal structures are in the size range of 100–200 nm and are made of ring-like subunits with a mean diameter of about 19.21 ± 2.57 nm. In Fig. 3F thread-like structures aggregating into knots are represented. These structures were mainly observed in a pseudo-binary system of SAb and cholesterol (5.7:1 w/w). Only a few structures, which were similar to the one in Fig. 3E but with

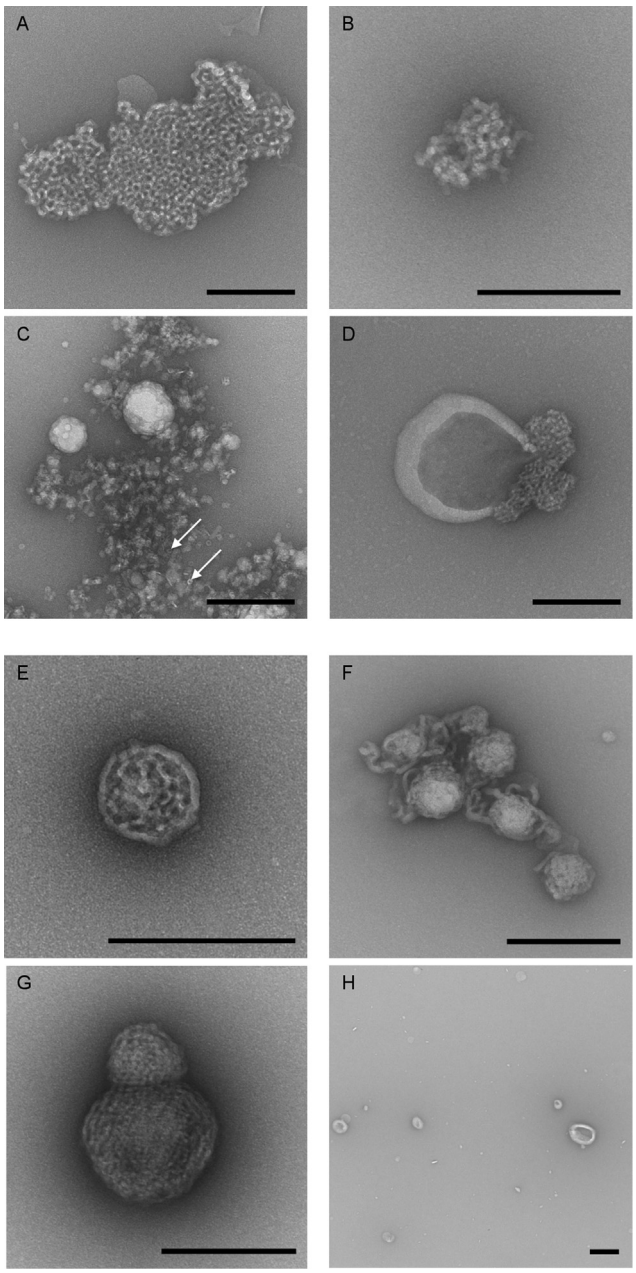

Fig. 3. TEM images of pseudo-binary and pseudo-ternary systems of soyasaponins, prepared by the lipid film method: (A) SBb:cholesterol mass ratio 3:1, (B) SBb:cholesterol mass ratio 5.7:1, (C) SBb:cholesterol mass ratio 10:1, (D) DPPC: SBb:cholesterol mass ratio 1:3:1, (E) SAb:cholesterol mass ratio 3:1, (F) SAb:cholesterol mass ratio 5.7:1, (G) SAb:cholesterol mass ratio 10:1, (H) DPPC: SAb:cholesterol mass ratio 1:3:1. Bar 200 nm.

a more heterogeneous shape, were found. Pseudo-binary systems with a further increase in SAb content (10:1 w/w) resulted in tightly packed structures with small subunits (Fig. 3G). The subunits are close together and form larger spherical associations of heterogenous size. Some differences were observed in a pseudo-ternary system containing SAb instead of SBb which is shown in Fig. 3H. In the presence of DPPC, cholesterol, and SAb, liposomes were observed exclusively. It is suggested that the second sugar chain at the aglycone of SAb hinders sterically the interaction with the liposomal membrane and thus the access to the cholesterol molecules to form novel colloidal associations.

The results of the Langmuir monolayer studies were confirmed by the TEM images. The high affinity of soyasaponins to cholesterol is reflected in particle formation in aqueous solution. The content of cholesterol seems to influence the particle shape and structure. The pseudo-binary systems with a mass ratio of 3:1 yielded structural elements of ring-like shape similar to the one observed for ß-escin [25]. The difference between the two soyasaponins is the shape of the associations. In the presence of SAb and cholesterol circular associations were formed while more heterogeneous associations were formed with SBb. Furthermore, the intercalation of SBb into a mixed DPPC/cholesterol Langmuir monolayer is also reflected in TEM images showing the attack of SBb on liposomal membrane with cholesterol by the formation of ring-like subunits.

Pseudo-ternary systems of soyasaponin Ab and ß-escin, which had already been studied in previous studies [25], were also prepared with unsaturated PE instead of DPPC. TEM images are shown in Fig. 4. Both saponins, the monodesmosidic saponin ß-escin and the bidesmosidic saponin SAb formed colloidal associations built up from ring-like subunits in the presence of PE and cholesterol. The shape of the colloidal associations formed in pseudo-ternary systems of ß-escin, cholesterol and PE (Fig. 4A) is more heterogeneous compared to the structures which were formed with DPPC instead of PE. Surprisingly, colloidal associations were found in pseudo-ternary systems with SAb and PE (Fig. 4B), whereas formulations with DPPC (Fig. 3H) only led to the formation of liposomes. Phospholipids from different origin (natural, synthetic) and with different structural properties seem to have an influence on the formation of colloidal associations in pseudo-ternary systems with saponins, as could be shown in formulations with the saponin SAb. DPPC is a synthetic phospholipid containing saturated fatty acids that allows a rigid, ordered structure in membranes. On the other hand, PE is a natural unsaturated phospholipid that is more flexible. This flexibility of the PE molecules might facilitate the intercalation of saponin molecules between the lipids for the interaction with cholesterol. Furthermore, the polar head group of PE (ethanolamine) is smaller than the head group in the DPPC molecule (choline), which might be crucial for the intercalation of saponin molecules between the lipids. ISCOM structures were also formed in a pseudo-ternary system with PE, which was observed by Kersten et al. who showed that not only PC (phosphatidylcholine) forms ISCOM structures with Quil A and cholesterol [33].

3.3. In vitro viability of HaCaT keratinocytes by MTT assay in the presence of saponins

The viability of a keratinocyte cell line (HaCaT cell line) pretreated with saponin solutions was tested to examine the compatibility of saponins with skin cells for a future intradermal vaccination pathway.

Fig. 5A and B show that the HaCaT cells were not affected by aqueous soyasaponin (SBb, SAb) solutions in the range of

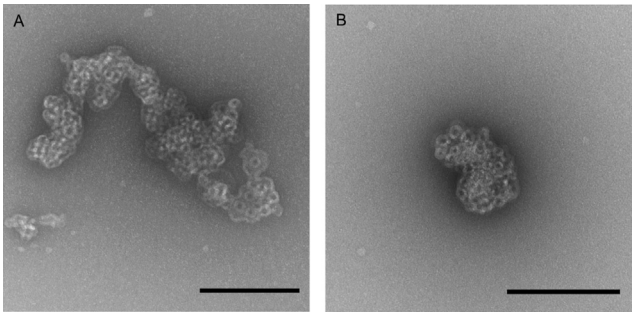

Fig. 4. TEM images of pseudo-ternary systems of ß-escin and soyasaponin Ab, prepared by the lipid film method: (A) PE:ß-escin:cholesterol mass ratio 1:3:1, (B) PE:SAb:cholesterol mass ratio 1:3:1. Bar 200 nm.

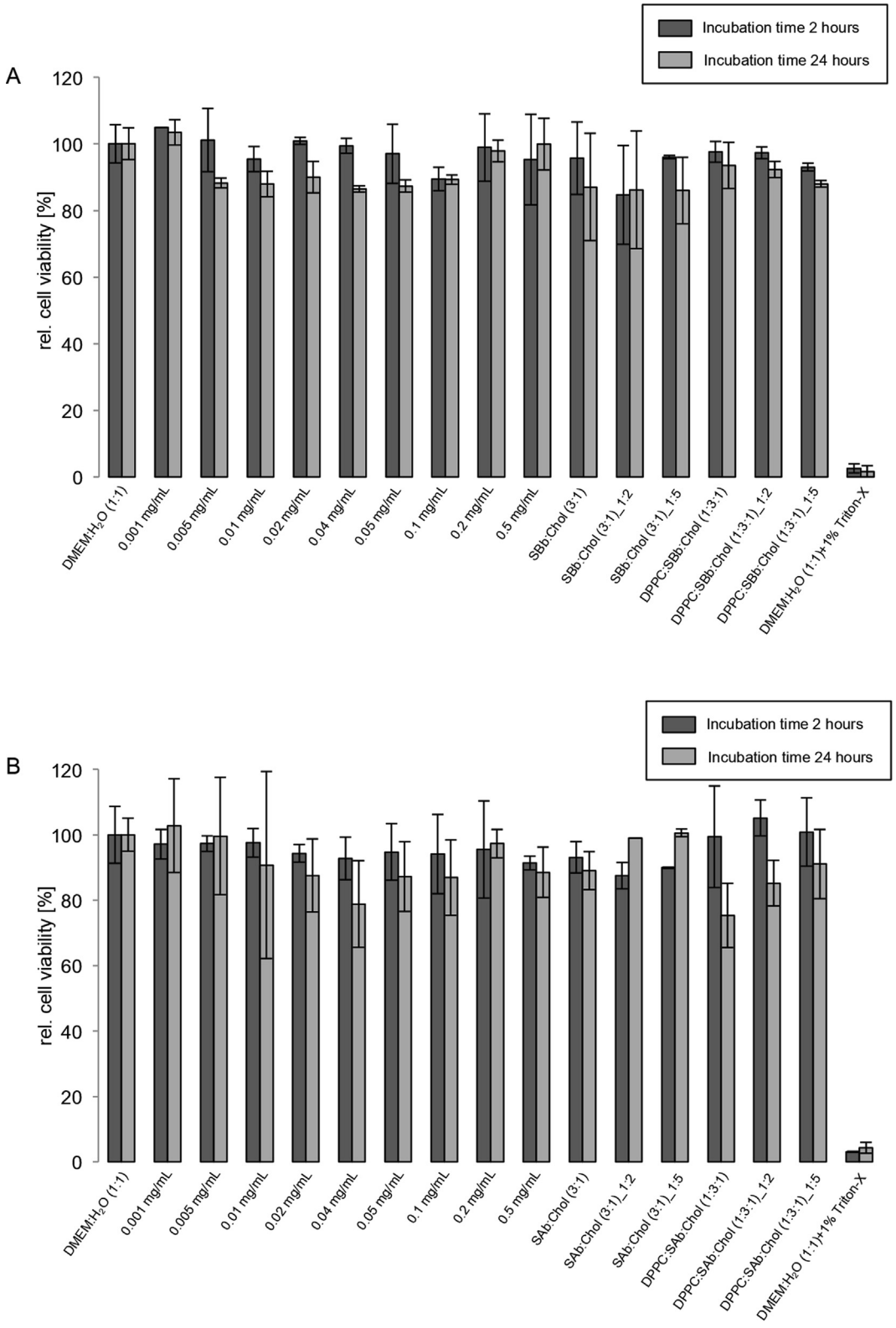

Fig. 5. MTT assay of an immortalized HaCaT cell line cultivated for 2 or 24 h in DMEM-medium with increasing concentration of (A) aqueous SBb solutions and (B) aqueous SAb solutions in the range of 0.001–0.5 mg/mL and with different saponin formulations (compare Table 1). Saponin formulations were produced using the film method. Data are expressed as mean ± s.d., n = 8 (out of two independent experiments).

0.001–0.5 mg/mL after a treatment of two and 24 h, respectively. Furthermore, formulations composed of soyasaponins (SBb, SAb) and lipid components (cholesterol, DPPC) in various dilutions were tested. The concentration of each compound and the total concentration of the formulation are listed in Table 1. Neither the soyasaponin formulations nor the pure aqueous soyasaponin solutions

had cytotoxic effects on HaCaT cells. Both after two and 24 h of incubation time with saponin formulations the viability of the HaCaT cells was between 80% and 100%.

The treatment of HaCaT cells with pure aqueous ß-escin solutions in the range of 0.001–0.5 mg/mL as well as formulations with lipid components is shown in Fig. 6. The concentration of each compound and the total concentration of the formulation is listed in Table 2. The cell viability of HaCaT cells decreased with increasing ß-escin concentration. Up to a concentration of 0.03 mg/mL the cell viability was close to 100% after an incubation time of two hours. A concentration of 0.04 mg/mL led to a decrease in viability below 50%. An incubation time of 24 h with pure ß-escin solutions in the tested concentration range led to a further decrease in viability of the cells. The ß-escin formulation with lipid components seems to be more tolerable. The two-component formulations ßE:Chol(3:1)_1:3 and ßE:Chol(3:1)_1:10 with ß-escin concentrations of 0.1675 mg/mL and 0.0503 mg/mL had less cytotoxic effects compared to pure ß-escin solutions in the same concentration range. The same observations were made with three-component formulations DPPC:ßE:Chol(1:3:1)_1:3 and DPPC:ßE:Chol(1:3:1)_1:10 with ß-escin concentrations of 0.1340 mg/mL and 0.0402 mg/mL, respectively.

The tested saponins have shown different effects on a HaCaT cell line. In higher concentrations pure ß-escin solutions had cytotoxic effects on a keratinocyte cell line. We suggest that the tolerability is related to the extent of the interaction of the saponin with cholesterol in the plasma membrane. In aqueous solution, soyasaponins had no negative influence on cell viability, nor as formulations with liposomal components. In ß-escin formulations with cholesterol and DPPC or cholesterol alone the cytotoxic effect of ß-escin was lower. Colloidal associations built from ß-escin, cholesterol and DPPC or ß-escin and cholesterol in aqueous solution seem to be more tolerable towards viable cells of the epidermis than pure ß-escin. Saponin bound in colloidal associations may have a higher affinity for cholesterol in the particle than for cholesterol in the plasma membrane.

3.4. Microscale Thermophoresis (MST) – binding of ovalbumin to colloidal saponin associations

Microscale Thermophoresis was applied to perform interaction studies between the model antigen ovalbumin and novel colloidal associations. The binding between ovalbumin and two-component systems consisting of the saponin (SBb, SAb, ß-escin) and cholesterol was examined. The MST traces and the signal-to-noise as well as the response of the different formulations are shown in Fig. 7.

In all cases (Fig. 7A/B/C) the labeled ovalbumin (target) together with the two-component systems (ligand) led to a higher relative fluorescence than the target alone after the onset of the temperature gradient. This change in fluorescence is caused by an altered thermophoresis behavior of the labeled ovalbumin, when bound by the ligand. These results indicate a binding between the model antigen and the two-component systems.

3.5. Cellular proliferation of dendritic cells

We explored the immunological potentiality of pure aqueous saponin solutions (SAb, ßE), a pseudo-ternary system containing ß-escin and a liposomal formulation (see Section 2.2.5) by using an *ex vivo* DC – T cell stimulation assay. Thereby, we measured the induction of splenocytes-derived HA-specific $CD4^+$ (Fig. 8A), $CD8^+$ T cell (Fig. 8B) and $CD19^+$ B cell (Fig. 8C) proliferation in response to HA co-administered with different saponin

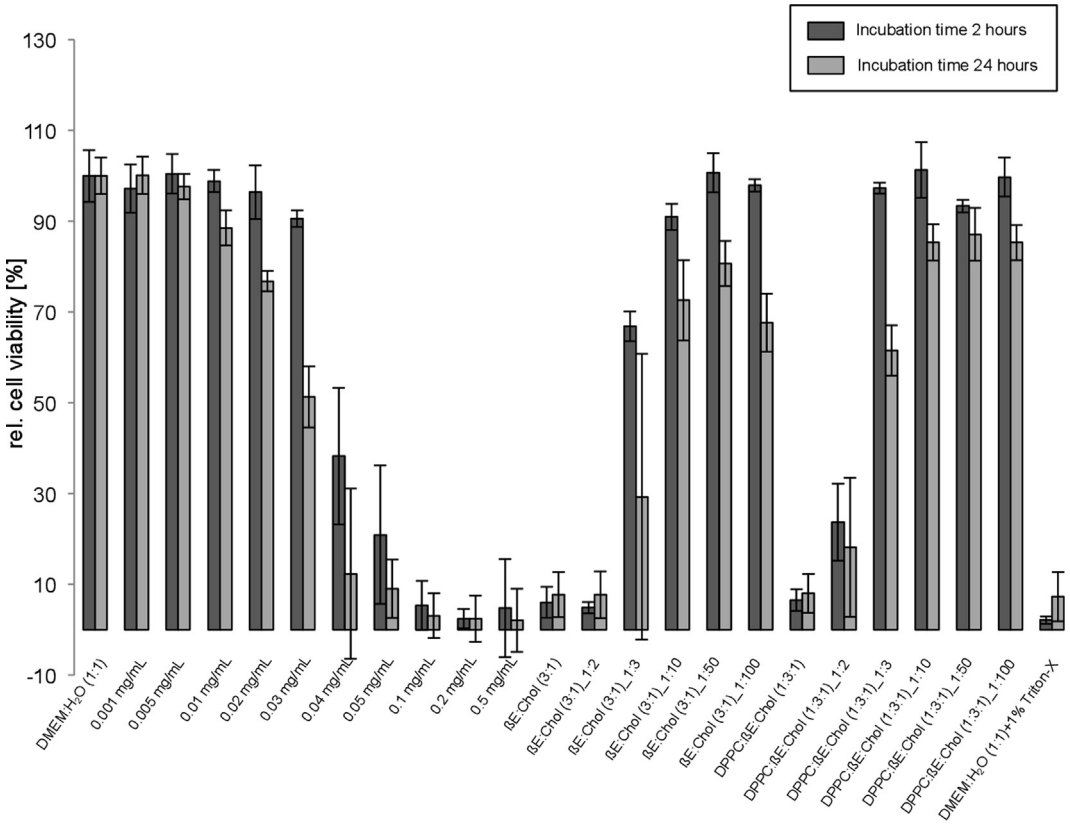

Fig. 6. MTT assay of an immortalized HaCaT cell line cultivated for 2 or 24 h in DMEM-medium with increasing concentrations of aqueous ß-escin solutions in the range of 0.001–0.5 mg/mL and with different saponin formulations (compare Table 2). Saponin formulations were produced using the film method. Data are expressed as mean ± s.d., n = 8 (out of two independent experiments).

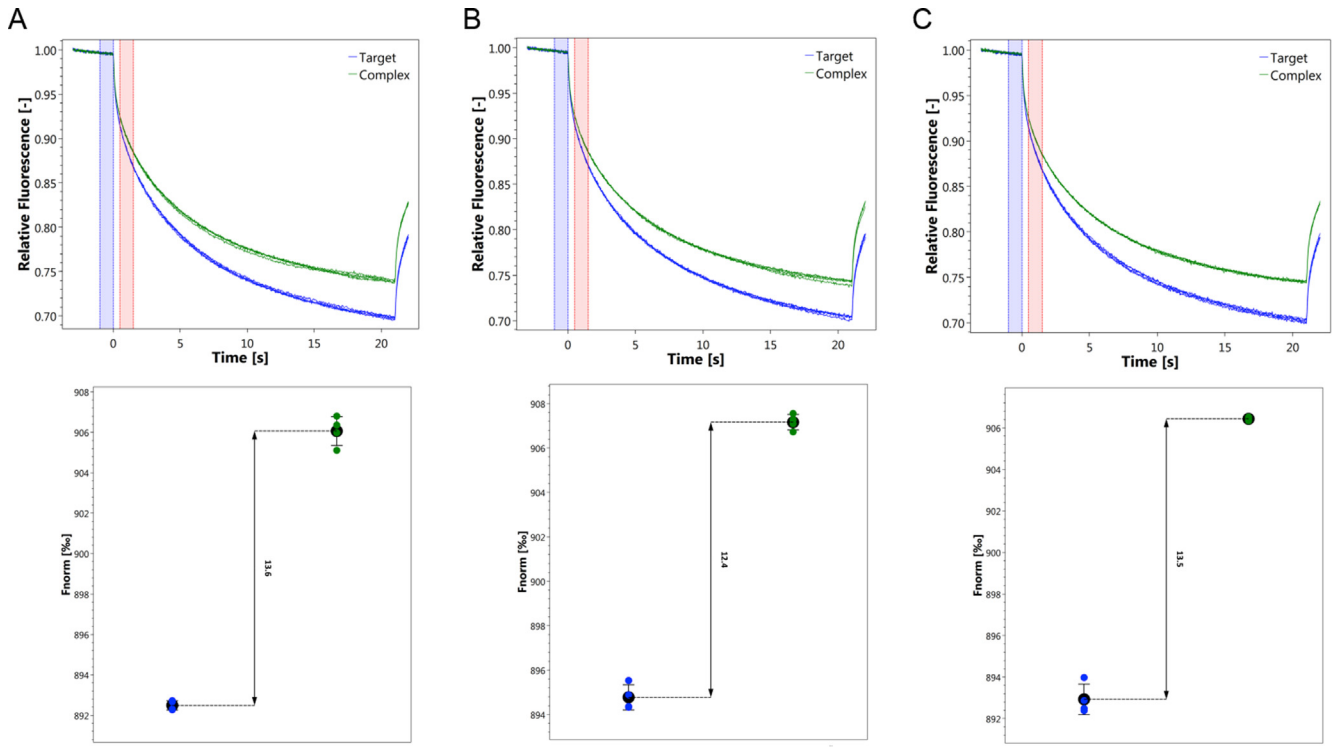

Fig. 7. MST traces and signal-to-noise ratio of labeled ovalbumin with (A) SBb:Chol mass ratio 3:1, (B) SAb:Chol mass ratio 3:1, (C) ß-escin:Chol 9.64:0.36. Traces. Corresponding to ligand-bound ovalbumin (green) and unbound ovalbumin diluted in water (blue), respectively. The response evaluation was taken at 1.5 s after start of the temperature gradient. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

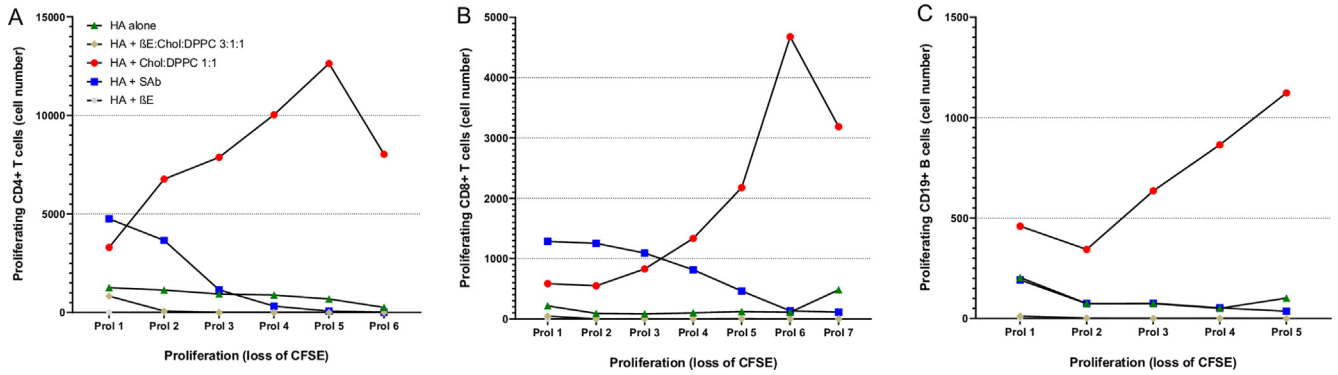

Fig. 8. Immunomodulatory effects of pure aqueous saponin solutions (SAb, ßE), a pseudo-ternary system containing ß-escin and a liposomal formulation on HA specific CD4+, CD8+ T and B cells. CFSE-labeled CD4+, CD8+ T cells and CD19+ B cells from TCR HA transgenic animals were co-incubated with antigen restimulated DCs for 5 to 8 days. The induction of HA-specific and unspecific proliferation of T cells, both CD4+ (A) and CD8+ (B), and B cells (C) was shown by reduction of CFSE labeling (loss of CFSE) and analyzed by flow cytometry. Cell numbers of proliferating splenocytes in response to HA alone, HA + ßE:Chol:DPPC (3:1:1), HA + Chol:DPPC (1:1), HA + SAb and HA + ßE on day 8 are presented.

formulations or liposomes shown by the loss of CFSE staining on the surface of CFSE labeled immune cells. The induction of unspecific T cell and B cell proliferation in response to HA alone was also evaluated to determine the antigen-specific basal proliferation level. Furthermore, possible toxicity of aqueous saponin solutions was also determined to differentiate from immunostimulatory activity. After 8 days, DC stimulated T and B cells were stained for the expression of CD3, CD4, CD8, CD19, live/dead marker and the ratio of $CFSE^{high}$ vs. $CFSE^{low}$ labeled cells was determined by flow cytometry. In Fig. 8 ABC, the proliferation potential of T and B cells in response to HA alone, HA + ßE:Chol:DPPC (3:1:1), HA + Chol:DPPC (1:1), HA + SAb and HA + ßE is presented. HA alone resulted in weak basal proliferation, whereas the co-administration with Chol:DPPC (1:1) resulted in a prolonged proliferation in T and B cells. In contrast, HA + ßE and HA + ßE:Chol:DPPC (3:1:1) were very toxic and harmful to the immune cells. The use of HA + SAb induced strong proliferation in $CD4^+$ and CD8 + T cells based on saponin activity which indicated the immunostimulatory activity of SAb saponins.

Taken together, from different saponin based formulations as well as from a liposomal formulation (without saponin) tested in primary DCs and HA specific T and B cells by the cell proliferation assay, ßE showed strong toxicity on both antigen presenting cells (DCs) and T and B cells. The most promising saponin in this preliminary *ex vivo* system was SAb, which was able to stimulate efficiently $CD4^+$ and $CD8^+$ T cells in combination with HA antigen. Nevertheless, these results need to be further validated in an animal model, which will help to define the best vaccination strategy,

such as route and dosages to identify the most efficient saponin based antigen delivery system.

4. Conclusion

In this study, we proved a high affinity of soyasaponin Ab and Bb to cholesterol. This observation was also confirmed by visualization of saponin-cholesterol associations using transmission electron microscopy. In a mass ratio of 3:1, soyasaponins and cholesterol form colloidal associations of ring-like subunits of about 20 nm in size similar to those of ß-escin as demonstrated in previous studies. The associations with SBb have a more heterogeneous shape whereas the associations with soyasaponin Ab are circular. With decreasing cholesterol content, the shape of the colloidal association changes.

With regard to different chemistry of soyasaponin Bb and Ab a second sugar chain at the saponin aglycone impairs the access to cholesterol in membranes as well as in DPPC/Chol monolayers, which was observed for soyasaponin Ab. This was confirmed in TEM images of a pseudo-ternary system with DPPC, cholesterol and SAb where only liposomes were observed. The exchange of DPPC with PE led to the formation of colloidal associations of ring-like subunits. We suggested that the higher flexibility and smaller head group of PE led to an increased intercalation of saponin molecules between the lipids facilitating an interaction with cholesterol.

Regarding new application routes of vaccines, e.g., via the intradermal route, HaCaT cells (keratinocyte cell line) were tested and found to well tolerate both soyasaponins in aqueous solutions and in formulations with cholesterol alone as well as with cholesterol and DPPC. We suggest that either the interaction of soyasaponins with cholesterol in plasma membranes does not affect the cell viability or soyasaponins do not interact with cholesterol in plasma membranes. Therefore, soyasaponins are promising adjuvant systems in vaccines for future intradermal application. In contrast, pure ß-escin solutions strongly reduced viability of HaCaT cells and are therefore not suitable for intradermal application. A lower tolerability of ß-escin might be related to its higher affinity for cholesterol compared to the soyasaponins, as confirmed in Langmuir monolayer studies. However, the colloidal associations of ß-escin, cholesterol and DPPC or of ß-escin and cholesterol are less toxic on HaCaT cells and also represent promising systems for the intradermal route. Formulated in colloidal associations, ß-escin interacts predominantly with cholesterol in colloidal structures than with cholesterol in the plasma membrane of epidermal cells. The extent of interaction between membrane cholesterol and saponin may be related to the tolerance of epidermal cells to saponins.

In addition, MST measurements gave an indication of the binding of antigens to colloidal saponin associations. The *ex vivo* DC – T cell stimulation assay offers the opportunity to identify immunomodulatory active moieties in a short-term experiment (15 days) using a disease-related antigen, such as HA (H1N1). The identification of SAb as the most promising saponin in this set of experiments indicated the high potential of saponin-based adjuvants. High toxicities of pure ß-escin solution (0.4020 mg/mL) and pseudo-ternary system with ß-escin of the same concentration are in accordance with the results from the MTT-assay on HaCaT cells.

Future animal vaccination studies have to show the immune response of antigen-loaded saponin formulations.

Declaration of Competing Interest

The authors declare no conflict of interest.

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