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Review

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The amphiphilic nature of saponins and their effects on artificial and biological membranes and potential consequences for red blood and cancer cells

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Saponins, amphiphiles of natural origin with numerous biological activities, are widely used in the cosmetic and pharmaceutical industry. Some saponins exhibit relatively selective cytotoxic effects on cancer cells but the tendency of saponins to induce hemolysis limits their anticancer potential. This review focused on the effects of saponin activity on membranes and consequent implications for red blood and cancer cells. This activity seems to be strongly related to the amphiphilic character of saponins that gives them the ability to self-aggregate and interact with membrane components such as cholesterol and phospholipids. Membrane interactions of saponins with artificial membrane models, red blood and cancer cells are reviewed with respect to their molecular structures. The review considered the mechanisms of these membrane interactions and their consequences including the modulation of membrane dynamics, interaction with membrane rafts, and membrane lysis. We summarized current knowledge concerning the mechanisms involved in the interactions of saponins with membrane lipids and examined the structure activity relationship of saponins regarding hemolysis and cancer cell death. A critical analysis of these findings speculates on their potential to further development of new anticancer compounds.

Introduction

Several reviews have characterized the biological and pharmacological effects[36] of saponins, and some have specifically considered saponin effects on membranes[7], their hemolytic activity, and their activity on cancer cells[8,125]. Nevertheless, there is a lack of reviews linking the amphiphilic character and other molecular specificities of saponins with their effect on membranes and resulting pharmacological and pharmaceutical consequences.

In the present review we examined the amphiphilic character of saponins and their ability to self-aggregate and reviewed the capacities of saponins to interact specifically with membrane lipids. We further described their effects on different membrane models, including monolayers and bilayers. A brief section covers *in silico* models of saponin activity. Finally, we examined different aspects of saponin-induced hemolysis and cancer cell death, including cytolysis, apoptosis, and autophagy.

Definition and role of saponins in nature

Saponins, which are found in plants and certain other organisms, are known for their multiple pharmacological activities[173]. The name saponin originates from the Latin word "*sapo*" (soap) and describes the surfactant character of saponins and their ability to produce foam. Many saponin-containing plants have therefore been used traditionally as soaps[16]. Although the role of saponins in plants is not sufficiently understood, they seem to serve primarily as defensive molecules[119]. Many saponins are toxic to insects, fish, fungi, bacteria, plants, parasites, and mammals[24,36,112,116,155,162,168,173]. In holothurians and star fish, saponins are repulsive or toxic to predators[162,173].

The indiscriminate use of the word "saponin" in the literature is potentially confusing: The term is sometimes used to refer to a specific saponin (for example α-hederin) or it may be used to describe a mixture of saponins extracted from a plant. The commercial Merck saponin (saponin pure white, *Saponinum album*) is a crude saponin fraction obtained from roots and rhizomes of *Gypsophyla paniculata* L. Because different "saponin" manufacturers use non-identical plants to extract the saponin fraction, research results must be compared with caution. To avoid confusion, we use the trademarked term "saponin®" to designate crude extracts from different manufacturers and the general terms "saponin" and "saponins" to refer to any non-commercial molecule. Similarly, *Quillaja* saponins isolated from the *Quillaja saponaria* Molina. bark can be obtained in different degrees of purity. "Quil-A" is a purified aqueous extract of the bark[108,150]. Other fractions with higher saponin content exist. For

example, QS-21 is a fraction of Quil-A, purified by reverse phase chromatography[122]. We refer to all such extract types as "*Quillaja* saponins".

The amphiphilic structure of saponins

Structurally, saponins are amphiphilic compounds composed of one or more hydrophilic sugar parts and a lipophilic steroid or triterpenic part (sapogenin) (Figure 1). Other substances that are structurally closely related to saponins, such as cardiotonic heterosides or glycoalkaloids, are sometimes referred to as saponins. Because their structures and effects on membranes are similar, these substances are treated equally. Saponins are classified into monodesmosidic, bidesmosidic, and polydesmosidic saponins according to the number of sugar chains—one, two, or more, respectively. A wide structural variety of saponins can be found in nature due to the presence of different sugars, sugar branchings, and sapogenins. The most common sugars are D-glucose, L-rhamnose, D-galactose, D-glucuronic acid, L-arabinose, D-xylose, and D-fucose. Cardiotonic heterosides and glycoalkaloids also contain other types of sugars. Several books and reviews on naturally occurring saponin structures can be consulted[16,33].

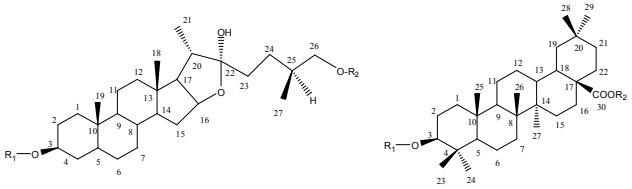


Fig. 1 Left: A steroid pentacyclic saponin (furostanol type). Right: A triterpenoid pentacyclic saponin (oleanane type). The saponin is either monodesmosidic or bidesmosidic depending on the number of sugar chains (one or two chains, respectively). R1 and R2 are usually ramified sugars, bound via an ether or ester link.

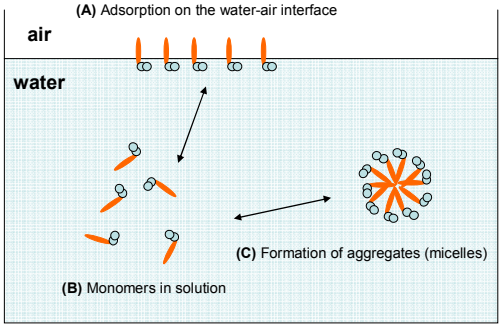
Saponin behavior at hydrophobic-hydrophilic interfaces

The behavior of saponin molecules at hydrophobic-hydrophilic interfaces and subsequent physical consequences are critical for their activity and will be discussed hereunder.

Accumulation at hydrophobic/hydrophilic interfaces

The air-water interface is the most common hydrophobic-hydrophilic interface. Most saponins are water-soluble thanks to their polar sugar component. Depending on their solubility, some saponins are dissolved as monomers whereas others accumulate at the air-water interface with their hydrophilic head oriented to the waterside and their hydrophobic tail oriented to the airside (Scheme 1, A) This behavior reduces the surface tension of the water by decreasing the number of hydrogen bonds per length [64]. Many saponins are known as water surfactants (Table 1)[14,67,102,122,128,174]. *Quillaja* saponins are able to build a highly elastic monolayer with their hydrophobic triterpenic tail pointing to the airside[147].

The effects of saponins on lipid monolayers adsorbed at the air-water interface are discussed in a later section.



Scheme 1 Behavior of saponins in aqueous solution.

Saponins as surface-active agents

Because of their "biosurfactant" ability, saponins are often used in the pharmaceutical, cosmetic, and food industry. Saponins are able to stabilize emulsions (emulsifiers) because of their ability to reduce interfacial energy between different phases (hydrophobic-hydrophilic) (Table 1). Many cosmetics (creams, lotions, and milks[147,159]) contain saponins as emulsifiers. In soaps and shampoos, saponins are used to reduce the surface tension of water to stabilize the formation of foam[9].

Saponins have the potential to stabilize nanosuspensions and nanoemulsions, which are biphasic systems containing very small droplets (<100 nm). They show numerous interesting pharmacological and pharmaceutical properties including a decrease of hemolysis[28] or an increase of the immune response to antigens[17].

Table 1 Effects of saponin at interfaces

Effect	Techniques	Type of interaction	Consequences	Saponin	Ref
Adsorption at air-water interface	Tensiometry	Saponin/air-water surface	Reduction in water surface tension , foam forming ability	Soyasaponins, Anchusosides, Glycyrrhizin, Digitonin, Hederacolchiside, α-Hederin, Hederacoside C β-Escin, β-Sitosterol, Ginsenoside Rg2, Glycyrrhizinic acid, Primulic acid	14,67,102,122,128,147,174
Adsorption at other interfaces	Tensiometry, viscosity measurements, miscibility tests, quasi-elastic light scattering	Saponin/interface	Reduction of interfacial tension, stabilization of (nano)emulsions or (nano)suspensions	*Quillaja* saponins, *Yucca* saponins, Ginsenosides, Acetylated aescin, Extract of *Sapindus mukorossi*	9,17,28,147,159

Formation of amphiphilic aggregates (micelles and other nanoscaled objects) by saponins

At the critical micelle concentration (CMC), saponins form aggregates in solution that remain in equilibrium with free monomers whose concentration does not exceed the CMC (Scheme 1, B, C). These molecular aggregates are "soft" or fluid-like structures because intermolecular forces are weak and limited to hydrogen bonds and Van der Waals, hydrophobic, or screened electrostatic interactions. Micelles are regarded as three-dimensional molecular aggregates. They are generally spherical in shape although other forms of aggregates can be produced by self-aggregation or inter-aggregate interaction. The presence of diverse amphiphilic species favors the complexity of these structures, which can be considered as nano-objects if they present a limited size of 1-100 nm in one or more dimensions. According to the British standard commission, micelles and ISCOMs® (see hereunder) are nanoparticles because they are nanoscaled in all three dimensions[15]. Nano-objects may form a visible precipitate that is sometimes regarded as an "insoluble complex" in the literature; nanoparticles with a defined aggregation number and size are not visible with the naked eye and can be considered "soluble"[64].

In the pharmaceutical and healthcare sector, research on nanoparticles and nano-objects is characterized by the wide field of possible applications in areas such as cardiovascular diseases, musculoskeletal, neuro-degenerative and psychiatric disorders, cancer, diabetes mellitus, and bacterial and viral infections[124]. The shape and chemical composition of nanoparticles and nano-objects can be purposely modified to enhance the pharmacokinetic and pharmacodynamic properties of drugs and imaging agents. Nanoparticles and nano-objects can be used to increase drug concentrations in targeted cells or tissues, thus improving efficacy[133] and reducing toxicity by inhibiting the interaction with sensitive tissues[21]. Additionally, an increased immunogenic response can be achieved[100]. The self-aggregating properties of saponins therefore constitute a promising research area that involves the formation of completely new nano-objects and nanoparticles.

Self-aggregation of saponins

The formation of micelles is observable with many saponins (Table 2)[9,14,25,103-105,131,174]. For example, micelles composed of a highly purified fraction of *Quillaja* saponins are spherical with an aggregation number of 65 and a diameter of 3-7 nm. The CMC of these micelles increases with temperature as well as with ionic strength. Micelle size also increases with temperature[103-105,131,145]. Other saponins such as ginsenoside Ro are able to form vesicles of 30-50 nm, and ginsenoside Rb_1 and Rg_1 interaggregate species of spherical micelles. Mixtures of saikosaponins and ginsenoside Rb_1 induce the formation of worm-like micelles[25].

Aggregation of saponins with sterols

Mixed molecular aggregates of finite size that are composed of more than one amphiphilic species (*i.e.,* saponins and sterols) are often mixed micelles (Table 2). *Quillaja* saponins are able to form mixed micelles with cholesterol and therefore enhance the solubility of the sterol by a factor of 1000. These micelles are

larger than pure saponin micelles (10 nm *versus* 7 nm) and have a higher aggregation number and CMC. Cholesterol is part of the lipid compartment of the micelle[103-105]. Demana et al. observed the formation of worm-like micelles for different saponin/cholesterol proportions[30]. Saponins of *Saponaria officinalis* L., *Quillaja saponaria* Molina., and *Glycine max* L. are also able to form micelles of rod-, worm-, or spherical shape with bile acids[143].

In addition to soluble mixed micelles, insoluble complexes composed of sterols and saponins have also been described (Table 2). Sterols are able to form water insoluble complexes with digitonin called digitonides[52,53,62]. α-Tomatine and the α-chaconine/α-solanine mixture are able to form insoluble complexes with sterols. α-Tomatine has the ability to form complexes that are quite similar to those of digitonides. The aglycone tomatidine lacks this ability[126]. Alfalfa saponins (extracted from *Medicago sativa* L.) form insoluble complexes with cholesterol that are dissociable in pyridine[6]. We showed that α-hederin is able to interact with cholesterol to form an insoluble precipitate in a buffer solution at pH=7.4[97].

The direct interaction of saponins with cholesterol and the subsequent complexation or formation of micelles has a potential application in the development of hypercholesterolemia drugs. (Hypercholesterolemia increases the risk of developing cardiovascular diseases.) Because of their amphiphilic character, saponins would likely able to influence micelle formation between sterols and bile acids, which is necessary for sterol absorption. Digitonin, alfalfa saponin, and *Quillaja* saponins[16] potentially form insoluble complexes with cholesterol in the intestinal lumen and could therefore reduce cholesterol absorption. *Karaya* root saponins are known to interact preferentially with bile salts, which are necessary for micelle formation, thereby decreasing cholesterol absorption[2]. Some saponins may have the ability to transform into phytosterols through hydrolytic enzymes in the lumen. They could therefore act as prodrugs for phytosterols, whose cholesterol absorbing properties are well known[70,92]. In contrast, tiqueside and pamaqueside, two synthetic saponins, inhibit the transport of cholesterol from the lumen through the enterocyte brush border membrane by acting on unknown protein targets[31,107].

Simultaneous aggregation of saponins with sterols and phospholipids and the formation of pharmacologically active nanoparticles

For some saponins, coincubation with phospholipids and sterols produces a wide variety of aggregates, including hexagonal and cubic phases, bilayers, rod-like, helical, and worm-like micelles, and the formation of increasingly complex structures such as immune stimulating complexes (ISCOMs®) (Figure 2) (Table 2)[64,64,65,108]. These structures have shown a huge adjuvant potential and could be used in the formulation of vaccines. Their formation depends primarily on the preparation mode used. Consequently, some structures seem to be metastable and transform into other structures[29,30,108]. Because a multitude of saponins and lipids are present in some extracts used to prepare the nanoparticles (especially in *Quillaja* saponins), it is often difficult to identify which molecules are present in these 3D structures and to determine the interactions between them[30,71,108,118,151]. ISCOMs® are cage-like complexes of 40 nm in diameter[56]. Electron microscopy observations revealed that ring-like micelles can aggregate into ISCOMs®. Based on this observation, Kersten and Crommelin proposed their model for the structure of ISCOMs® in which one building block is equal to one ring-like structure[29,71,108,118]. Under certain conditions, ISCOMs® can have a shelf-life of several years[56].

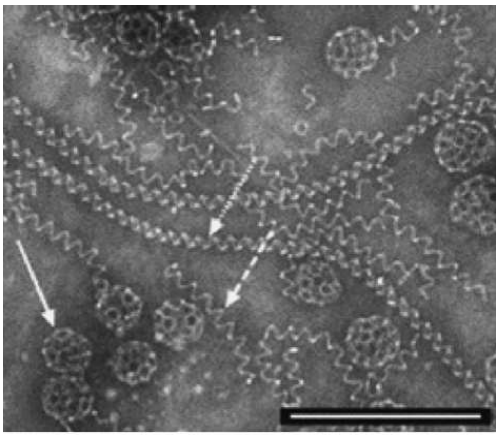


Fig. 2 Transmission electron microscopy of cage-like ISCOM® matrices (solid arrow), helices (dashed arrow), and double helices (dotted arrow) (bar = 100 nm)[108].

Some research groups prepared ISCOM®-like structures containing different types of lipids and saponins. Modified ISCOMs® (Posintro™), which contain DC-cholesterol (dimethylaminoethane-carbamoyl-cholesterol) instead of cholesterol, have a reduced negative particle charge and have been shown to pass through skin. These modified ISCOMs® could potentially be used to immunize the organism through a transdermal patch applied to the skin[99].

Cucumarioside A2 from marine macrophytes forms tubular nano-objects (called "tubular ISCOMs®"), which improve immunogenicity by a factor of four[75].

In addition to the formation of ISCOM®-like structures, other types of nano-objects can be prepared from mannosylated saponins based on oleanolic and glycyrrhizic acids. Transmission electron microscopy (TEM) has shown the formation of ring-like micelles, rod-like tubular structures, and helical and thread-like micelles[26]. An ethanol red ginseng root extract incubated with cholesterol and phosphatidylcholine produced ginsomes, spherical nanoparticles with a diameter of 70-107 nm. These ginsomes, which are mainly composed of ginsenoside Rb2, Rc, Rb1, and Rd, were able to stimulate the immune response[146,178]. Nanoparticles from *Quillaja* saponins preferentially induced apoptosis in cancer cells and were less hemolysis-inducing than pure extracts[56].

Table 2 Formation of amphiphilic aggregates

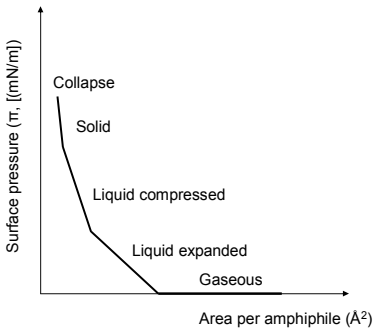
Effect	Techniques	Type of interaction	Cholesterol presence in model	Consequences	Cholesterol dependency	Saponin	Ref
Self-aggregation	DLS, tensiometry, solubilization of fluorescent probes, NMR, TEM, dielectric permittivity	Saponin/saponin	No	Formation of spherical micelles or other types of amphiphilic aggregates	No	*Quillaja* saponins, Digitonin, Hederacolchiside, α-Hederin, Hederacoside C, β-Escin, β-Sitosterol, Ginsenoside Ro, Rb1, Rg1 Glycyrrhizinic acid, Primulic acid	9,14,25,103-105,131,145,174
Aggregation with sterols	DLS, tensiometry, TEM	Saponin/sterol or Saponin/bile acid	Yes	Formation of soluble mixed micelles or aggregates (composed of cholesterol and saponins)	Yes	*Quillaja* saponins, *Saponaria officinalis, Glycine max*	30,103-105,143
	Observation, light microscopy, DHE fluorescence spectroscopy, TEM, solubility product, stoichiometric reaction	Saponin/sterol	Yes	Formation of insoluble sterol/saponin complexes	Yes	Digitonin, α-Tomatine, α-Chaconine, α-Solanine, Alfalfa saponins, α-Hederin	6,52,53,62,97,126
Aggregation with sterols and phospholipids	TEM	Saponin/phospholipid/ cholesterol	Yes	Formation of ISCOMs®	Yes	*Quillaja* saponins	29,30,56,71,108,118,151
	TEM	Saponin/phospholipid/ cholesterol		Formation of ginsomes	Yes	Ginsenosides Rb2, Rc, Rb1, Rd	146,178
	TEM	Saponin/phospholipid/ cholesterol		Formation of "tubular ISCOMs®"	Yes	Cucumarioside A2	75
	TEM	Saponin/phospholipid/ cholesterol		Ring-like micelles, rod-like tubular structures, helical and thread-like micelles	Yes	Mannosylated saponins of oleanolic and glycyrrhizic acid	26
Aggregation with DC-cholesterol and phospholipids	TEM	Saponin/phospholipid/ DC-cholesterol	No	Posintro™	No	*Quillaja* saponins	99

Effects and interactions of saponins with membrane models

Following our description of how saponins interact with different membrane components in a hydrophilic environment, this section emphasizes saponin interactions in artificial membrane models. Membranes provide an amphiphilic environment that can be described by a hydrophobic gradient increasing from the hydrophilic interfacial domain to the hydrophobic core. Studies on the effects of saponins using artificial membrane models have generated valuable data concerning the interactions of these molecules with different membrane components in an amphiphilic environment. These studies further provide insights into the mechanisms of membrane lysis.

Interaction with supported monolayers

One of the most common ways to investigate interactions between exogenous compounds and lipid membranes relies on supported monolayers (Langmuir-Blodget films), films of water-insoluble lipids floating on a water surface (Scheme 1). A two-dimensional monolayer is comparable to a three-dimensional gas system. Different phases of the monolayer can be observed by increasing the lipid density or lipid coverage (Scheme 2). Before its collapse, the monolayer successively passes through a gaseous state, a liquid expanded state, a liquid compressed state, and a solid state. Phase coexistence is sometimes detectable, for example the liquid expanded and the liquid compressed state. Monolayers serve as valuable models to demonstrate the insertion of saponins as well as their effect on phase separation and the formation of domains.



Scheme 2 Diagram of surface pressure *versus* area isotherm.

Insertion of saponins into monolayers

Some saponins (Table 3) are able to insert into different types of monolayers in the absence of cholesterol[4,46,113]. For α-tomatine, however, insertion into a monolayer has only been observed in the presence of cholesterol[148]. This insertion is effective only when the hydroxyl function in position 3 of the sterols present in the monolayers is in β[148]. In addition, insertion is pH-dependent because nitrogen protonation of the glycoalkaloid increases dissolution in the aqueous phase[166]. For glycyrrhizin, insertion is dependent on monolayer surface pressure. At concentrations higher than the CMC of saponin, the molecule accumulates mainly in the space just below the monolayer[130].

Induction and interaction with phase separation

Expanding on investigations of saponin insertion, Brewster angle microscopy has revealed the formation of domains in monolayers composed of DMPC and selected sterols incubated with α-tomatine[148,166]. The authors suggest that these domains are mainly composed of sterol-glycoalkaloid complexes. Although domains in monolayers can be considered 2D micelles, the CMC required for domain formation in monolayers is reduced by a factor of 10 compared with the CMC needed to form micelles in solution[64,129]. Consequently, the formation of saponin-sterol aggregates (or even of self-aggregation) could be facilitated in a lipid environment.

A ternary model composed of DOPC/palmitoylsphingomyelin/cholesterol (1:1:1, molar ratio) was used to investigate the effects of glycyrrhizin on lipid phase separation. At concentrations below its CMC, glycyrrhizin reduced the size of raft domains. Above the CMC, the appearance of striped regions devoid of phospholipids suggested the formation of membrane defects, which could be responsible for membrane permeabilization[130].

Interaction with bilayer models

Bilayer models, which are a better approximation of biological membranes than monolayers, have been used extensively to explore the effects of saponins on membranes. It is possible to monitor the effects on supported planar bilayers (SPB), black lipid membranes, liposomes (multi-lamellar vesicles [MLV], large unilamellar vesicles [LUV], giant unilamellar vesicles [GUV], and small unilamellar vesicles [SUV]).

Binding to membranes composed of phospholipids

Few studies have investigated the interaction between saponins and bilayers composed of phospholipids. Digitonin and desglucodigitonin can be bound by equilibrium binding (no full insertion) to membranes composed solely of egg yolk phosphatidylcholine[113]. In corresponding studies, α-Hederin was able to reduce the surface potential of membranes composed of DMPC, suggesting it binds to the membrane. This binding most probably occurred through the interaction between the negatively charged carboxylic function on the triterpenic ring structure and the positive charge of DMPC[97].

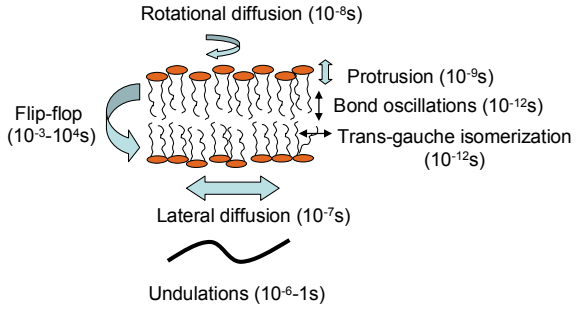
Binding to membranes containing phospholipids and cholesterol

In a model of egg yolk phosphatidylcholine and cholesterol, the formation of an equimolar complex induced a permanent insertion of digitonin into the membrane[113]. The study proposed three essential steps for binding of digitonin to membranes containing cholesterol and phospholipids. First, with increasing digitonin/cholesterol ratios, digitonin and cholesterol formed "aggregated" species in the membrane. Second, at higher molar ratios, an intermediate complex was composed of a mixture of equimolecular complexes and aggregated species. Third, an equimolecular complex formed in the bilayer [3]. Glycoalkaloids were found to bind to membranes exclusively in the presence of cholesterol. Results also suggested the formation of an

equimolecular complex of sterols and glycoalkaloids[72].

Effects on membrane lipid dynamics

Lipids in a membrane are in constant motion (flip-flop, rotation…) characterized by different correlation times (Scheme 3). A variety of techniques, such as ^{2}H-NMR, EPR, fluorescence spectroscopy, and fluorescence probes, can be used to obtain information concerning membrane order at different time scales and membrane levels.



Scheme 3 Approximate correlation times of lipid motion in membranes[42].

A lipid bilayer may be present in different states depending on environmental temperature and lipid composition: a fluid-like state, termed the liquid crystalline phase (L_α), is associated with high lipid mobility and low order. Conversely, a solid-like state or gel phase (L_β) shows reduced lipid motion and a high order. Cholesterol has a well-known influence on lipid dynamics because of its rigid ring structure; it reduces gauche-trans isomerization, the rotational and lateral diffusion of lipids, which results in an ordering effect on the liquid crystalline state[127,132]. The rigid ring structure of cholesterol possesses a planar side (α-side) and a "rough" side (β-side). The β-side forces lipids in the gel state to occupy a larger surface area. A fluidizing effect in the gel state is thus observed[93,96,127]. We anticipate that the possible interaction of saponins with cholesterol in membranes could considerably influence the dynamic parameters of a membrane. The rigid ring structure of saponin, which is very similar to that of sterols, should itself have a significant effect on membrane dynamics even when no interaction with cholesterol is present. The present review is, however, limited to studies performed at temperatures for which membranes are in the liquid crystalline (or liquid ordered) state because all mammal membranes must be considered "fluid"[42].

In the absence of cholesterol, the lateral diffusion of fluorescent phosphatidylethanolamine[4] was slightly reduced, anisotropy for fluorescently labeled lipids and DPH[97,98] generally increased, the EPR order parameter of phospholipids[3,37,113] increased, and the ^{2}H-NMR order parameter of labeled phospholipids[3] decreased.

In the presence of cholesterol, the anisotropy of fluorescently labeled lipids as well as the EPR and the ^{2}H-NMR order parameters of labeled phospholipids and cholesterol were generally reduced[3,97,113].

The different acquisition time scales (ns to μs) of these techniques explains the differing order parameters between results obtained by EPR or fluorescence spectroscopy and NMR. EPR and fluorescence spectroscopy work at 10^{-9}-10^{-8}s. On this time scale, the main observations are gauche-trans isomerization (10^{-10}s) and rotational diffusion (10^{-8}s) (Scheme 3), which are clearly reduced by saponins in the absence of cholesterol, or increased by saponins in the presence of cholesterol. The ordering effect of cholesterol seems to be inhibited by saponins. ^{2}H-NMR works at 10^{-5}s. A reduced order parameter suggests that lipid motions corresponding to correlation times of 10ns-10μs increase regardless of the cholesterol content.

Effect on lateral organization of membrane lipids

The lateral organization of lipids into domains has become a recognized concept in cell membrane biology[93]. At high cholesterol concentrations, saturated phospholipids, sphingolipids, and sterols are able to segregate from lipids presenting non-saturated acyl chains (L_d, L_α or liquid crystalline phase) and form domains of a new lipid phase: the L_o phase or liquid ordered phase. In addition to its ordering effect (see above), cholesterol has a condensing effect that reduces the lateral space occupied by lipids and increases membrane thickness of domains (L_o phase). The L_o phase can be considered intermediate between L_α and L_β.

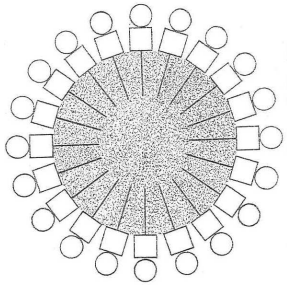
Because some saponins are able to form aggregates with cholesterol in 3D and 2D systems (see above), we expect these saponins to have an effect on the lateral organization of the membrane. The three following examples are in agreement with this assumption. The saponin®-enabled solubilization of alkaline phosphatase (a protein present in the L_o phase) by Triton X-100 that occurs in liposomes containing cholesterol but not in liposomes that only contain sphingolipids suggests cholesterol dependent domain disruption[134]. We demonstrated the ability of α-hederin to form worm-like domains with increased intrinsic curvatures in membranes containing cholesterol and partial segregation of cholesterol and phospholipids[98]. Lastly, the cofactor for the acrosome reaction-inducing substance (Co-ARIS), a steroidal monodesmosidic saponin, was able to co-localize and provoke the expansion of ganglioside-GM1 clusters[111].

Permeabilizing activity

Numerous studies have shown that cholesterol is a key factor in saponin-induced membrane permeabilization. For most saponins (Table 3) cholesterol was identified as an enhancing or necessary factor in permeabilization[4,46,72,89,97,98,113]. Nevertheless, for some saponins (Table 3), especially bidesmosides, cholesterol was an inhibiting or unnecessary factor in membrane permeabilization[57,89]. We therefore assume that several mechanisms may lead to permeabilization. Published data on the different modes of action of monodesmosidic and bidesmosidic saponins can be summarized as follows.

Mechanisms involved in saponin-induced membrane permeabilization

In 1962, Bangham et al. observed hexagonal structures exclusively in cholesterol-containing planar membranes incubated with saponin®. They proposed a micellar arrangement of saponins and cholesterol in the membrane that resulted in the formation of a pore and corresponded to the observed hexagonal structures (Scheme 4)[10]. Other mechanisms, summarized in the following, have since been suggested.



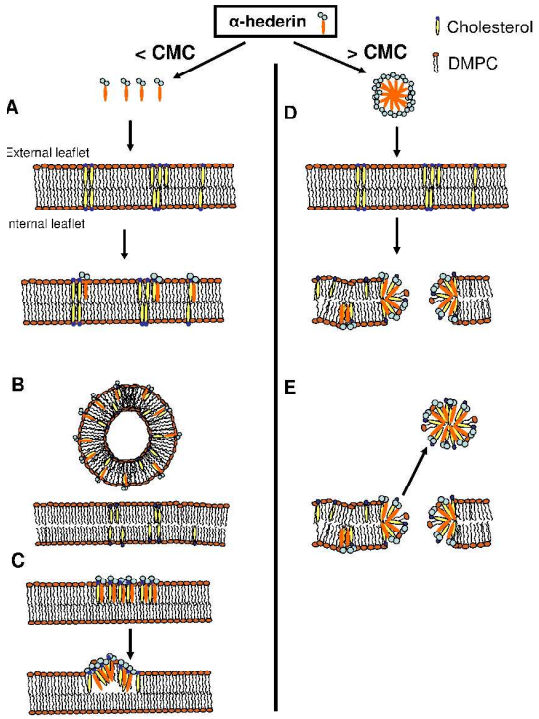
Scheme 4 *Micellar rearrangement of saponin with cholesterol in the membrane as proposed by Bangham et al.: Cholesterol (○), saponin (—□). The central speckled area represents the pore* [10].

Three principal mechanisms of monodesmosidic saponins have been described.

First, saponin interaction with sterols led to equimolecular complexes in the membrane as observed for monodesmosidic glycoalkaloids (presenting sugar residues at C_3). When these complexes reached a certain density, hydrophilic interactions between the sugar moieties induced the formation of a new lipid phase and the three-dimensional shape of the sterol-glycoalkaloid complexes then determined the formation of new spherical buds or tubules. Membrane disruption occurred as a consequence of membrane rearrangement[72]. Glycoalkaloid-induced tubular aggregates have also been observed by other teams[34].

Second, a mechanism based on the formation of toroidal pores was established in a POPC/DOPE/Chol model for avenacin A1, a monodesmosidic triterpenoid saponin. The hydrophilic interaction between the sugar moieties first led to an aggregation of saponins and cholesterol and further caused the formation of pores[4].

Third, for α-hederin, we discovered a concentration-dependent permeabilization mechanism that is based on the aggregation with sterols and phospholipids and an induced membrane curvature in GUVs (Scheme 5). A curvature-dependent permeabilization had been proposed for dioscin, as simulation suggests (see *in silico* models)[91]. At concentrations below their CMC, α-hederin monomers bound to the external monolayer (Scheme 5A). The created area difference and curvature between the outer and inner monolayer induced vesiculation (Scheme 5B). Further aggregation of saponins, cholesterol, and phospholipids led to the formation of worm-like aggregates in the membrane (Scheme 5C), which were responsible for transient defects and a gradual permeabilization. Domain formation and permeabilization speed increased with the size of the sugar chain attached at C3 of the triterpenoid ring[97,98]. At concentrations exceeding the CMC, α-hederin induced direct pore formation in the membrane (Scheme 5D) and caused the loss of membrane material (Scheme 5E), suggesting that micelles (or aggregates) were able to directly interact with the membrane and deliver high amounts of saponins close to the membrane.



Scheme 5 Model of membrane interaction for α-hederin, a monodesmosidic triterpenoid saponin. At concentrations lower than the CMC, α-hederin monomers bind to cholesterol (A) and induce vesiculation (B) and lateral phase separation (C). At concentrations higher than the CMC (D), α-hederin aggregates provoke pore formation and the loss of membrane material (E)[97,98].

Binding to cholesterol-enriched domains led to immediate membrane permeabilization and the formation of increasingly macroscopic pores. α-Hederin was more likely to accumulate at the rim of the formed pore and stabilize it by reducing line tension because of its amphiphilic character. This model supposes the induction of a positive curvature strain on the external monolayer. The two hydrophilic sugars gave an axe-like shape to the saponin and the molecule therefore induced positive curvature stress in a transbilayer direction, which led to the formation of macroscopic pores or worm-like aggregates. This model takes into account the concentration dependent self-aggregating properties of the saponin[14], its three-dimensional shape, its affinity for cholesterol, and its amphiphilic character[97,98]. Nevertheless, further investigation of the correlation between the permeabilizing effect and the self-aggregating properties is necessary.

In contrast to what was observed for monodesmosidic saponins, cholesterol was thought unnecessary for membrane permeabilization by avicin D, a bidesmosidic saponin. This saponin did not completely destabilize the membrane; it formed stable pores (~1.1 nm), which presented a certain selectivity towards the ion charge that depended on the phospholipid composition of the membranes[89], suggesting an interaction of avicin D with phospholipids. Other bidesmosidic saponins displayed a similar behavior[57].

Structure-activity relationship (SAR) studies

The differing modes of action (see previous paragraph) and the fact that most SAR studies describe hemolysis and the lytic effect

on living cells, not on artificial models, make it difficult to establish a structure-activity relationship of the membrane permeabilizing activity of saponins. Despite this difficulty some general rules can be formulated regarding the permeabilizing activity of monodesmosidic saponins.

A special polar sugar group in C3 is necessary to induce curvature and consequent pore formation. This prerequisite could favor interactions with sterols in membranes by shielding the hydrophobic sterol ring from water (umbrella effect)[97]. Other studies suggest that the formation of pores is accelerated by hydrophilic-hydrophilic sugar interactions between saponins[4,72].

In addition, some SAR studies compared important structural features of the membrane sterol regarding the permeabilization induced by monodesmosidic saponins. The hydroxyl function at position C3 in β, the alkene function in C5 and C6, and the side chain at C17 (for a limited number of carbons) increased the membrane permeabilizing activity[72,123].

Table 3 Effects of saponins on membrane models

Effect	Techniques	Type of interaction	Cholesterol presence in model	Consequences	Cholesterol dependency	Saponin	Ref
Insertion	Lateral pressure measurements in monolayers	Saponin/cholesterol	Yes	Increasing lateral pressure	Yes	α-Tomatine	148,166
	Lateral pressure and surface tension measurements, fluorescence of avenacin A1 in monolayers	Saponin/phospholipid	Yes/No	Increasing lateral pressure, reduction of surface tension, Avenacin A1 insertion	No	Digitonin, Merck saponin®, Avenacin A1	4,46,113
	Lateral pressure measurements of monolayers	Saponin/rafts	Yes	Insertion up to a certain lateral pressure. Above critical value → accumulation below monolayer (formation of stripes)	?	Glycyrrhizin	130
Aggregation of cholesterol and saponin	BAM in monolayers	Saponin/cholesterol	Yes	Domain formation	Yes	α-Tomatine	148,166
Interaction with raft models	BAM, fluorescence microscopy of monolayers	Saponin/cholesterol	Yes	Decrease of raft size below CMC and formation of striped regions above CMC	Yes	Glycyrrhizin	130
Binding	Binding to LUV, reduction of surface potential	Saponin/phospholipid	Yes/No	Reduction of surface potential/equilibrium binding	No	Digitonin, Desgluco-digitonin, α-Hederin	97,113

	Binding to LUV, HPLC	Saponin/cholesterol	Yes/No	Cholesterol dependent binding	Yes	α-Tomatine/ α-Chaconine	72
	^{2}H-NMR, scintillation counting	Saponin/cholesterol	Yes	Permanent binding and formation of complexes with cholesterol	Yes	Digitonin, Desgluco-digitonin	3,113
Effect on dynamic properties	EPR, fluorescence spectroscopy of order sensitive probes	Saponin/phospholipid	No	Reduction of trans-gauche isomerizations and rotational diffusion	No	Digitonin, Ginsenoside, Avenacin A1, α-Hederin	3,4,37,97, 113
	EPR, fluorescence spectroscopy of Laurdan and DPH	Saponin/cholesterol	Yes	Increase of trans-gauche isomerizations and rotational diffusion	Yes	Digitonin, α-Hederin	3,97,113
	^{2}H-NMR	Saponin/cholesterol	Yes/No	Increase of motions in the µs time scale	No	Ginsenoside, Digitonin	3,37
Effect on lateral organization	Triton X-100 extraction from liposomes	Saponin/cholesterol	Yes/No	Extraction of alkaline phosphatase from liposomes	Yes	Saponin®	134
	AFM	Saponin/cholesterol-Ganglioside	Yes/No	Co-localization with ganglioside GM1 clusters and expansion of clusters	Yes	Co-ARIS	111
	AFM, confocal microscopy, FRET, ^{31}P-NMR	Saponin/cholesterol/ phospholipids	Yes/No	Formation of worm-like aggregates with phospholipids and cholesterol	Yes	α-Hederin	97,98
Permeabilization Pore formation	Conductivity measurements, release of entrapped markers	Saponin/cholesterol	Yes/No	Higher permeabilization for K^+ than to Cl^-	Yes	Saponin®, Digitonin	46,113
	Electron microscopy	Saponin/cholesterol	Yes/No	Micellar arrangement of saponins and cholesterol in membrane	Yes	Saponin®	10
	Freeze-fracturing, release of fluorescent probes, SAR, ^{31}P-NMR, HPLC, molecular modeling	Saponin/sterol/ phospholipid	Yes/No	Permeabilization, spherical and tubular budding due to formation of irreversible glycoalkaloid/sterol matrix	Yes	α-Tomatine, α-Chaconine, (glycoalkaloids)	34,72
	Conductivity measurements, FRAP	Saponin/cholesterol	Yes/No	Pore formation due to hydrophilic sugar interactions	Yes	Avenacin A1	4
	^{31}P-NMR, release of fluorescent marker (calcein), effect on DHE, FRET, effect on GUV (budding, wrinkling, dextran release)	Saponin/cholesterol/ phospholipids	Yes/No	α-Hederin forms macroscopic pores by inducing membrane curvature and domains due to lipid aggregation	Yes	α-Hederin	97,98

	Calcein release, QSAR	Saponin/phospholipid	Yes/No	Cholesterol independent membrane disruption	No	Bidesmosidic triterpenoid saponins	57
	Conductance measurements, molecular modeling	Saponin/cholesterol/ phospholipid	Yes/No	Pore formation due to interaction with phospholipids and importance of the side chain of Avicin D and G	Yes (avicin G), No (Avicin D)	Bidesmosides (Avicin G, Avicin D)	89

In silico models of saponin-lipid and saponin-membrane interactions

In silico models allowed us to hypothesize how saponins could be able to interact with membrane constituents or membranes at a molecular level (Table 4). Although such studies can never replace experiments on membrane models, they can explain and underline experimental data.

Some studies have simulated the molecular interactions of saponins with sterols and phospholipids based on a minimum interaction energy model. Dioscin, a monodesmosidic saponin, preferentially binds to cholesterol in a hydrophobic environment. Therefore, cholesterol extraction from the membrane with dioscin seems unlikely. Although the interaction between the hydroxyl group in cholesterol and the sugar present in dioscin is most probable, however a "head to tail" interaction cannot be excluded[91]. Other saponins bind to sterols by superposing their hydrophobic rings[72,114].

Further studies have investigated the simultaneous interaction of saponins with sterols and phospholipids, proposing a ternary structure composed of phospholipids, saponins, and cholesterol for glycoalkaloids and α-hederin[72,98].

In parallel to these studies, valuable data have been obtained through *in silico* models that were able to simulate the activity of saponins on an entire membrane. A Monte-Carlo simulation (Big layer) that mimicked a 2D monolayer composed of α-hederin, cholesterol and DMPC showed that α-hederin preferentially partitions between phospholipids and cholesterol and favors large aggregates of cholesterol in the membrane[98]. A coarse-grained molecular dynamics simulation of a DPPC-POPC-PSM-Chol lipid bilayer showed that dioscin accumulates in membrane rafts and increases their membrane curvature. This curvature causes membrane disruption[91]. The proposed mechanism is very similar to observations made for α-hederin using membrane models (see above).

Table 4 *In silico* models of saponin/lipid and saponin/membrane interaction

Effect	Techniques	Type of interaction	Cholesterol presence in model	Consequences	Cholesterol dependency	Saponin	Ref
Cholesterol/ saponin/ phospholipid interaction	Molecular modeling	Saponin/ cholesterol	Yes	Superposing of hydrophobic rings	Yes	Dioscin	91
		Saponin/ cholesterol/ phospholipid	Yes	Possible ternary complex	Yes	Glycoalkaloids α-Hederin	72,98,114
Aggregation in monolayer	Monte Carlo simulation	Saponin/ cholesterol/ phospholipid	Yes	Partition between cholesterol and phospholipids and aggregation of cholesterol	Yes	α-Hederin	98
Pore formation in bilayer	Molecular dynamics simulation	Saponin/ cholesterol/ phospholipid	Yes	Induction of curvature in raft models	Yes	Dioscin	91

Effects of saponins on red blood cells

Considering the critical role of cholesterol in membrane permeabilization, red blood cells constitute an ideal model because they are characterized by a high cholesterol amount in the plasma membrane[5,55]. In addition, red blood cells lack a nucleus and several intracellular organelles and are therefore simpler models than other eukaryotic cells.

Many saponins are known for their hemolytic effect, and several studies investigating lysis of red blood cells have been performed. Presented here are selected examples (Table 5) of studies that analyzed the mechanisms of saponin-hemolysis.

Despite the number of studies available, certain issues regarding saponin-induced hemolysis remain controversial. In the following, we offer an extensive discussion of these issues.

Morphological description of hemolysis

Although frequently neglected, the morphological description of saponin-induced hemolysis could potentially contribute to valuable information regarding saponin activity. Levin and Korenstein reported that erythrocytes treated with saponin® transformed into "ghost" cells. These erythrocytes lost their biconcave shape and became spherical; a process that could not be reversed with adenosyl triphosphate (ATP)[88]. This irreversible transformation of shape, which was not accompanied by any important changes in membrane elasticity, could occur because of a disturbance in membrane cytoskeleton interactions[12,144].

At a nanoscopic level, transmission electron microscopy has revealed the presence of long-lasting holes or pits in red blood cells incubated with saponins and the formation of multi-lamellar stacks composed of crystallized lipids of the membrane[12,142]. Pits were uniformly distributed and had a diameter of 4-5 nm. The authors predicted the consequent development of bigger holes or larger defects[136], as was demonstrated with α- and δ-hederin for GUVs[97]. The development of larger pores would be consistent with the saponin-induced release of proteins from the cytoplasm[12,135].

Possible correlation between hemolytic and surfactant activity

Many studies suggested a correlation between surfactant and hemolytic activities for some saponins [14]. Nevertheless, no clear correlation has as yet been established[51,128,137,165]; we can thus dismiss the hypothesis that hemolysis is driven solely by a detergent-like mechanism.

The role of membrane cholesterol

The importance of membrane cholesterol for hemolysis is uncertain and remains subject to debate. Some studies indirectly indicate that saponins aggregate with cholesterol. Added to media, cholesterol was able to inhibit saponin hemolysis, suggesting that the saponin was "complexed" by the sterol[156]. Furthermore, several amphipaths were able to displace cholesterol from phospholipids and thus increased the hemolytic potency of *Quillaja* saponins[81,82]. This finding is, however, in disagreement with studies led by Segal et al. who suggested that cholesterol does not serve as a specific binding site for saponins because no clear relationship between cell cholesterol amounts and hemolysis was established[138,139].

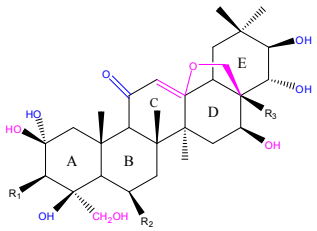
Importance of the sugar residue for hemolysis

For monodesmosidic saponins containing a glucose residue, Segal et al. found that the hemolytic activity of aglycones is similar to that of their corresponding saponin. They concluded that saponins, before becoming effective, are first cleaved into their sapogenin by glycosidases (glucosidases or galactosidases)[138,140]. This conclusion contradicts other results that showed that several sapogenins (oleanolic acid, gitogenin, hederagenin, and others) had no hemolytic effect. Surprisingly, preincubation of red blood cells with sapogenins even inhibited saponin-induced hemolysis. Inhibition of saponin hemolysis was also achieved when erythrocytes were preincubated with other non-hemolytic saponins[22,41,51,158,170].

It is therefore unlikely that glycosidases are necessary to "activate" the hemolytic potency of all saponins. Some genins may nevertheless possess their own permeabilizing activity. Interestingly, we were able to demonstrate the permeabilization of GUVs by hederagenin for very long incubation times (48h). This effect is consistent with observations of hemolysis for different aglycones and might be dependent on the interaction with phospholipids[98,138,140].

Structure-activity relationships

Although the hemolytic activity of saponins has been investigated by several SAR studies, their differences in protocols and types of erythrocyte make it difficult to compare results. We summarized the results of studies that tested a large number of saponins under identical conditions (Figure 3). Some studies comparing the activities of steroid *versus* triterpenoid saponins showed that steroid saponins induce faster hemolysis[157].

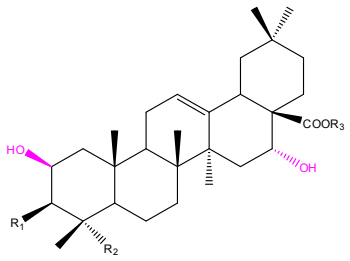


(Note that certain chemical functions exclude the presence of others.)

Magenta: structural features that enhance hemolytic activity.
Blue: structural features that inhibit hemolytic activity.
R_1: Sugars are necessary for hemolytic activity.
The residue (α-L-Rha(1→2)-α-L-Ara) results in high hemolytic activity.
For some genins, activity increases with the number of sugars.
The activity changes when sugar branching changes.
If the number of sugars is constant, α-L-Rha → β-D-Glc (1→2), (1→4), and (1→6) are more active than (1→3)
R_2: Triterpenoid saponins (-OH enhances activity)
Steroid saponins, diosgenin (-OH and alkane chains reduce activity, except -COC_5).
R_3: Triterpenoid saponins (-COOH and esterification of COOH increase activity).

Fig. 3 SAR studies on the hemolytic activity of monodesmosidic saponins[22,41,158,165,170].

Even if their surfactant activity increased, bidesmosidic (sugar residue at C3 and C28) triterpenoid or steroid saponins were in most cases less hemolytic than monodesmosidic saponins [51,128,165,170]. Some general enhancing properties are summarized in Figure 4.



Magenta: structural features that enhance hemolytic activity.
- R_1 = at least 1 sugar,
- R_2 = COOH, CH_2OH (needed),
- R_3 = if 1 sugar is present there must be at least 3 sugars in R_1. Highest hemolytic activity is obtained with 4 sugars and R_1 = 1 sugar.

Fig. 4 SAR studies on hemolytic activity of bidesmosidic saponins[165,170].

Excessive saponin-induced hemolysis can lead to anemia culminating in death[149]. The pharmacological use of saponins is only feasible if they can be excluded from causing significant hemolysis or if they do not pass into the blood stream. Research and synthesis of hemolysis-free saponins is therefore crucial. Understanding the mechanisms involved in saponin hemolysis and the implications of molecular features could facilitate the synthesis of high-activity and low-toxicity compounds. Gauthier et al. showed that, in comparison with oleanane type saponins, lupane-type saponins have a very low tendency to induce hemolysis combined with an increased ability to induce apoptosis in cancer cells[41]. As previously stated, the formation of saponin nanoparticles may offer an interesting solution to reducing the hemolytic activity of saponins by maintaining or increasing their activity towards cancer cells. The following section examines the effect of saponins on cancer cells.

Toxicological drawbacks due to saponin hemolysis

Table 5 Effect of saponins on red blood cells

Techniques	Type of interaction	Consequences	Cholesterol dependency	Saponin	Ref
TEM, freeze-fracture EM, Ferritin labeling, Hemoglobin release	Saponin/ ?	Increasing defects (holes), protein release	?	Merck pure saponin, Alfalfa saponin	12,135,136, 142
Microscopy, measurements of membrane fluctuation	Saponin/cytoskeleton-membrane	ATP independent shape transformation into ghosts	?	Saponin® (pure white)	12,88,144
Hemoglobin release, Tensiometry	Surfactant activity	No clear correlation between surfactant and hemolytic activity	?	Monodesmosides and bidesmosides	14,51,128,137,165
Amphipath cholesterol activation, variation of cholesterol content of erythrocytes or media	Saponin/Cholesterol	Aggregation with cholesterol leads to hemolytic activity	Yes	*Quillaja* saponins, α-Hederin, Dioscin, Timosaponin A-III, β-escin, Saikosaponin d, Holotoxin A	81,82,156
Cholesterol depletion of erythrocytes	Saponin/Cholesterol	No clear correlation between cholesterol amount and hemolytic activity	No	Digitonin, Styrax saponin A Aescin, Smilagenyl-β-maltoside, Tigogenyl-β-maltoside, Styrax sapogenin-A	138,139
Inhibitors of glycosidases, saponin extraction from lysed cells	Saponin/membrane glycosidases	Hydrolysis of saponin into sapogenin by contact with erythrocytes	No	Digitonin, Tomatine, Solanine, Styrax saponin-B, Glycyrrhizin	138,140

Effects of saponins on cancer cells

In contrast to red blood cells, eukaryotic cells possess a nucleus and intracellular organelles. These subcellular compartments are separated from the cytoplasm by membranes that have different compositions in lipids and proteins. The cholesterol content of membranes of different organelles is very variable[163,171], which could explain the specificity of some saponins towards a certain type of organelle. As we have previously shown, saponin effects are not restricted to membrane lysis because saponins can influence the dynamics or lateral organization of membranes. All these effects can lead to the activation or inhibition of membrane proteins or even induce signaling pathways causing programmed cell death[19,43,85,90,120,175,177].

Effect on dynamic properties of the lipid membrane

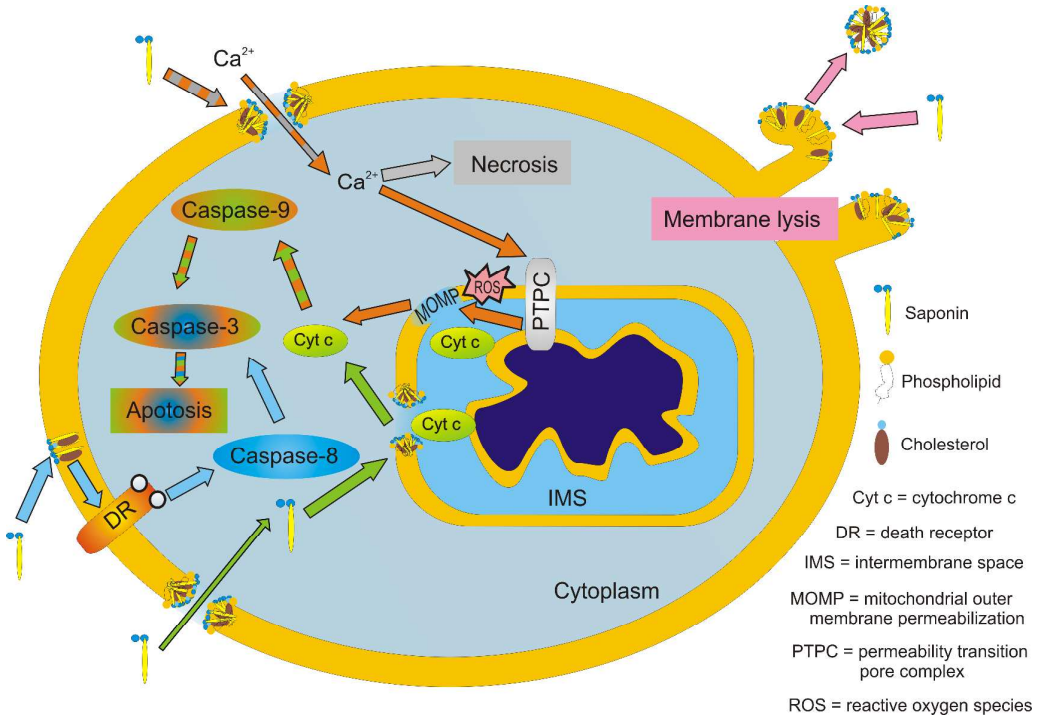
In physiological conditions, mammalian cellular membranes are always in a fluid state[42]. It should therefore be possible to compare results concerning membrane dynamics with the effect of saponins on artificial membranes in the liquid crystalline (or liquid ordered) state. The modulation of dynamic membrane properties in cells can have multiple effects on membrane proteins and cell metabolism[49,63,80].

In many cases, the effects of saponins on the dynamic properties of artificial membranes are dependent on cholesterol content (see above). Because cholesterol content varies considerably between cell types and organelles, the modulation of order parameters may vary (Table 6) accordingly. For example, ginsenoside Rg3 reduced the fluorescence anisotropy of DPH and TMA-DPH in multidrug resistant cells only. This decrease correlated with a decrease in resistance towards adriamycin[80]. Other saponins increased or decreased different order parameters in different cell types independent of their lytic potential[49,63,68,117]. Ginsenoside Re significantly reduced the micro viscosity of DPH in mitochondria isolated from rat brains. This reduction might explain the protective effect of ginsenoside against cerebral-ischemia injury because mitochondria play an important role in ROS production and subsequent lipid peroxidation[179].

Effect on lateral membrane organization (interaction with rafts)

Cell membrane rafts are very heterogenous, functional lateral domains of 10-200 nm enriched with cholesterol and sphingolipids and unstable in time. These domains also contain glycosphingolipids and GPI-anchored proteins and are platforms for protein signal transduction. Their disruption or aggregation may induce pathways leading to programmed cell death and may produce other effects[66,93]. In some types of cancer cells (especially prostate cancer), lipid rafts have higher amounts of cholesterol compared with non-malignant cells[121,180], which could make them interesting targets for saponin activity.

The disruption of rafts has various effects on cell membranes such as receptor activation or changes in ion channel permeability (Table 6)[13,18,54,60,110,134,141,160,181]. The translocation of some receptors or membrane proteins to rafts and the disruption of rafts upon treatment with different saponins was confirmed by confocal or biphoton microscopy. Ginsenoside Rh2 and avicin D both induced such an effect, leading to apoptosis activation through the extrinsic pathway (Scheme 6, blue pathway)[68,120,175,177]. Co-ARIS, which is a saponin cofactor for the acrosome reaction inducing substance, was able to alter the lateral cholesterol distribution in sperm and disrupted the caveola system in CHO-K1 cells[111].



Pink pathway: pore formation and direct membrane lysis[41,44,45,47,102,115,164,172].
Grey pathway: necrosis induced by pore formation and increased Ca^{2+} influx[161].
Green pathway: apoptosis induced by direct permeabilization of the outer mitochondrial membrane[50,86,89].
Orange pathway: apoptosis induced by the increase of intracellular calcium, reactive oxygen species production (ROS), activation of the permeability transition pore complex (PTPC), and mitochondrial outer membrane permeabilization (MOMP)[20,153].
Blue pathway: apoptosis induced through raft activity and activation of death receptors[68,120,175,177].

Scheme 6 Pathways of known saponin-induced cancer cell membrane lysis, necrosis, and apoptosis and their connections to membrane activity.

Cell deaths induced by saponins

Different kinds of saponin-induced cell death including cell lysis, necrosis, apoptosis, and autophagy (Scheme 6 and Table 6) have been observed. Cell lysis and necrosis both include the destruction of the plasma membrane. Apoptosis and autophagy are programmed cell deaths; they are induced via various stimuli and executed through specific pathways. To classify these saponin-induced cell deaths, it is particularly important to describe the occurring morphological changes and provide biochemical evidence [39,78].

Morphological features of saponin-induced cell death

Treatment with different saikosaponins resulted in various manifestations in Ehrlich ascites tumor cells (Figure 5). Minor structural changes can induce major changes in morphological appearence[1]. The main morphological features induced by saponins are 1) the formation of "blebs" (Figure 5b)[1,40], which are classical hallmarks of necrosis and apoptosis[11,78] but may also be direct consequences of saponin membrane interactions, as shown for GUVs[97,98], 2) the size increase or disappearence of microvilli (Figure 5c-f) and other changes in membrane topology such as the formation of a granular surface (Figure 5d-e)[40,68,102,152], and 3) the formation of intracellular vesicles[152]. These vesicles could correspond to autophagic vacuoles, or to a direct effect on the membrane[35,73].

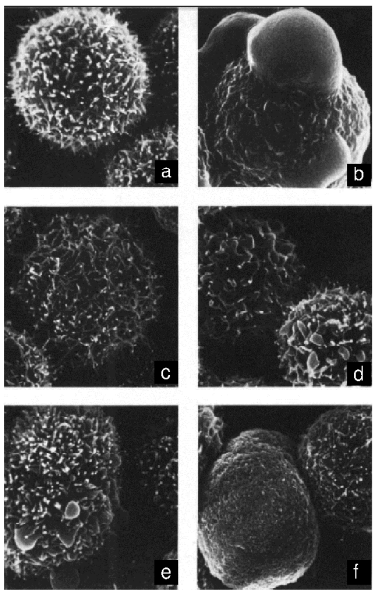


Fig. 5 Scanning electron microscopy of Ehrlich ascites tumor cells.

(a) Control. (b) Cells treated with saikosaponin a (large protrusions: blebs). (c) Saikosaponin b1 (longer microvilli than in control). (d) Saikosaponin b2 (coral reef-like surface). (e) Saikosaponin c (blebs and microvilli). (f) Saikosaponin d (disappearance of microvilli)[1].

Saponin induced necrosis and membrane lysiss

Characteristics of necrosis are an increase in cellular volume, the formation of blebs, the destruction of the plasma membrane, and the release of cytoplasm into the surrounding environment. These changes are accompanied by the activation of Ca^{2+}-dependent enzymes that are able to lyse the cytoskeleton (Scheme 6, grey pathway)[11,78,161].

Saponin membrane lysis and saponin-induced pore formation produce osmotic swelling terminating in membrane rupture (Scheme 6, pink pathway). Both cell deaths are interconnected because saponin-induced pore formation may increase intracytosolic Ca^{2+}, activate Ca^{2+}-dependent enzymes and necrosis (Scheme 6, grey pathway). It is unclear whether regulated necrosis or necroptosis is induced by saponins because data on RIP1 and RIP2 (receptor interacting protein 1 and 2) have not yet been published[39].

Direct membrane lysis provoked by saponins can occur very rapidly at high saponin concentrations[97,102]. Electron microscopy revealed the formation of holes larger than 1 µm after only 2 minutes of incubation with 10 µM hederacolchiside A1 (Figure 6).

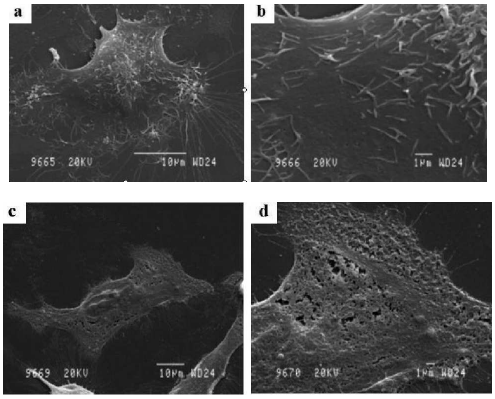


Fig. 6 Scanning electron microscopy: Control MEL-5 cells (a, b), appearance of holes after 2 min of treatment with hederacolchiside A1 (c, d)[102].

Similarly, oleanane-type saponins rapidly induced the permeation of small hydrophilic molecules such as calcein and propidium iodide in cancer cells [41,45]. Saponins either induced pores whose size increased with concentration and time[171] or pores large enough to produce a fast release of LDH and other proteins[44,47,102,172]. It is possible to visualize saponin®-induced pores in fibroblasts[84] through AFM. The capacity of saponins to rapidly induce large holes in plasma membranes makes them useful tools for the immunohistochemistry of intracellular proteins[102,115,164].

Interestingly, tetrandrine (a bisbenzylisoquinoline) increased the size of *Quillaja* saponin-induced pores . This ability was neither observed with digitonin nor with ginseng saponins; we therefore suppose that tetrandrine interacts specifically with the pore formed by *Quillaja* saponin[87].

Some studies investigated the cholesterol dependence of membrane permeabilization. Membrane lysis and cell death of monocytic cells induced by α-hederin decreased when membrane cholesterol was depleted[97]. For MEL-5 cells, Tof-SIMS analysis revealed no colocalization of cholesterol and hederacolchiside A1 when pores formed after 2 min of treatment. After 30 min of incubation, hederacolchiside A1, phospholipids, and cholesterol seemed to aggregate in the same areas[102]. This behavior is similar to observations of GUVs incubated with α-hederin, where cholesterol and phospholipids aggregated in the same domains[98].

Lysis of cell organelles

Saponins showed a certain degree of specificity regarding lysis of different organelles (Table 6). Brain microsomes derived from plasma membrane and treated with saponin® showed ring-like micellar structures. This was not observed in cardiac sarcoplasmic reticulum. Microsomes derived from endoplasmic reticulum were more resistant to saponin® lysis than vesicles derived from plasma membranes[61]. In muscle cells, β-escin and saponin® were able to permeabilize the traverse tubular system[83]. Avicins were able to perforate the outer mitochondrial membrane and thus induced the release of cytochrome c, which led to inhibited respiration and the induction of apoptosis[86].

The susceptibility of different organelles to *Gypsophila* saponins correlated with the cholesterol/phospholipid ratio of their membranes (plasma membrane > lysosomal membrane > Golgi membrane > outer mitochondrial membrane > inner mitochondrial membrane > endoplasmic reticulum)[171].

Apoptosis

Morphologically, apoptosis leads to the condensation of chromatin, the fragmentation of the nucleus, the formation of membrane blebs, and the existence of apoptotic bodies[11,78]. Apoptosis is mediated by two major pathways, the intrinsic and the extrinsic pathway[69].

Although numerous papers have investigated saponin-induced apoptosis, our review concentrated on studies examining apoptosis in direct relationship with membrane interaction (Table 6). An elucidation of every discovered pathway is beyond the scope of this review.

The intrinsic pathway

The intrinsic pathway depends primarily on the disruption of the external mitochondrial membrane and the release of proapoptotic proteins such as cytochrome c from the intermembrane space to the cytosol, which can be achieved by permeabilizing the outer mitochondrial membrane[77]. In contrast to the internal membrane, the external mitochondrial membrane presents a high content in cholesterol.

The intrinsic pathway induced by some avicins most probably results from direct pore formation in the outer mitochondrial membrane and the release of cytochrome c into the cytosol (Scheme 6, green pathway)[50,86,89].

Both α-Hederin and macranthoside B provoked an increase in reactive oxygen species (ROS) and an extracellular Ca^{2+} influx, leading to the opening of the permeability transition pore complex (PTPC)[58,59,76] and to apoptosis (Scheme 6, orange pathway)[20,27,47,97,153]. The depletion of membrane cholesterol inhibited α-hederin-induced apoptosis (data not published). ROS production might also be a consequence of direct mitochondrial membrane activity and act as an apoptosis amplifier[169].

The extrinsic pathway

The extrinsic pathway is activated by membrane death receptors present in lipid rafts[32,43]. Disorganization of these rafts can thus

lead to an activation or inhibition of membrane death receptors, as was established for avicin D and ginsenoside Rh2 (Scheme 6, blue pathway)[68,120,175,177].

Autophagy

Autophagic cell death is accompanied by cytoplasmic vacuolization, causing the cell to auto digest. Major proteins involved in this process are beclin 1, ATG5, and LC3. Unfortunately, saponin® can cause the formation of structures that resemble GFP-LC3 puncta (a hallmark of autophagy) in HeLa cells. It has been shown that these structures are a consequence of non-specific protein aggregation induced by the saponin and can therefore not be considered as a hallmark of autophagy[23].

However, some saponins were able to induce autophagy as a protective mechanism against apoptosis (listed in Table 6)[74,154]. In contrast, Avicin D induced autophagic cell death when apoptosis was inhibited[176].

Cancer treatment potential of saponins

The described effects on cell lysis, necrosis, apoptosis, and autophagy suggest that saponins could be potential candidates for cancer treatment. Furthermore, ginsenosides and other saponins were able to reduce cell growth by inhibiting proteins involved in the cell cycle (cyclins or cyclin-dependent kinases) and also inhibited other important cancer promoting pathways[48,79,94,95,109,167].

Moreover, several saponins induced cell death via multiple mechanisms (apoptosis, necrosis, and autophagic cell death) and pathways (ROS, activity on organelles, permeabilization of the outer mitochondrial membrane), which could potentially prevent resistance development and increase treatment efficacy.

In addition, saponins have shown specific cytotoxicity towards cancer cells[73,101,153] and the formation of saponin-containing nanoparticles could enhance the selectivity towards cancer cells and reduce their hemolytic potential, which could increase their therapeutic index[56].

Finally, saponins could be used to overcome chemotherapeutic resistance to other therapeutic agents. The involvement of cholesterol in cancer progression as well as cancer resistance[106,121] is well known. Cholesterol-enriched rafts are known to promote cancer, and an accumulation of cholesterol in mitochondria leads to chemotherapeutic resistance[106]. The specific interaction of some saponins with cholesterol and the disruption of lipid rafts led to apoptosis in cancer cells[38,121,177].

Table 6 Effects of saponins on cancer cells

Effect	Techniques	Type of interaction	Consequences	Cholesterol dependency	Saponin	Ref
Effect on dynamic properties	FRAP of 1,1'-Dioctadecyl-3,3,3',3'-tetramethylindo-dicarbocyanine	Saponin/?	Reduction of the diffusion coefficient of 1,1'-Dioctadecyl-3,3,3',3'-tetramethylindo-dicarbocyanine	?	Digitonin	63
	EPR	Saponin/?	Increase of EPR order parameter, Decrease of infection by HIV, influenza A virus, vesicular stomatitis virus	?	Glycyrrhizin	49
	Fluorescence anisotropy DPH, TMA-DPH	Saponin/?	Decrease of anisotropy, decrease of resistance toward adriamycin in multidrug resistant cells	?	Ginsenoside Rg3	80
	DPH fluorescence anisotropy	Saponin/?	Reduced micro viscosity in mitochondria	?	Ginsenoside Re	179
		Saponin/?	Reduced micro viscosity in plasma membrane	?	Ginsenoside Rh, Rh2	68,117
Effect on lateral organization	Sensibility of rafts to Triton X-100 extraction	Saponin/cholesterol	Disruption of domains containing cholesterol/redistribution of raft-associated proteins	Yes	Saponin®, Digitonin, Saponin® (Sigma)	13,18,54, 60,110,1 34,141,1 60,181
	Confocal microscopy	Domain (raft) interaction	Caspase-8 Activation	Yes	Ginsenoside Rh2, Avicin D	68,120,1 75,177
	Fluorescence microscopy, radiolabeled saponin	Co-ARIS/Cholesterol	Change in domain (raft) structure and composition, induction of acrosome receptor	Yes	Co-ARIS (monodesmosidic steroid)	111

Necrosis/Lysis	Cell death assays, Calcein-AM release, PI influx, light and electron microscopy, flow cytometry, LDH release, AFM	Saponin/plasmatic membrane	Necrosis-like cell death	Yes	Oleanane type monodesmosidic saponins, Macranthoside B, Digitonin, *Quillaja* saponins, *Gypsophila* saponins Saikosaponins	1,40,41,44,45,47,84,87,97,102,115,164,171,172
	Detection of mitochondrial permeabilization, Isolation of organelles	Saponin/ outer mitochondrial membrane, ER, traverse tubular system	Outer mitochondrial membrane, ER, traverse tubular system	Yes	Avicins, Saponin®, β-Escin	61,83,86
	Differential centrifugation, enzyme markers	Saponin/organelles	Lysis of organelles increases with cholesterol content	Yes	*Gypsophila* saponin	171
Apoptosis	Markers intrinsic apoptotic pathway (release proapoptotic proteins, etc.)	Saponin/ mitochondria	Activation of mitochondrial pathway (intrinsic)	?	Avicins	50,86,89
	Extracellular calcium influx (Ca^{2+}), ROS activation	Saponin/ plasmatic membrane	Permeabilization of plasmatic membrane, activation of mitochondrial pathway (intrinsic)	Yes	α-Hederin, Macranthoside B	20,27,47,97,153
	Markers extrinsic apoptotic pathway, caspase-8 activation, Fas activation, raft disorganization	Saponin/rafts	Activation of death receptor pathway (extrinsic)	Yes/No	Ginsenoside Rh2, Avicin D	68,120,175,177
Autophagy	LC3-I→LC3-II transformation autophagic vacuoles	Saponin / ?	Protection against apoptosis	?	Timosaponin AIII, Ginsenoside Rk1	74,154
	LC3-I→LC3-II transformation Autophagic vacuoles, Atg5, Atg 7 activation	Saponin / ?	Activation of autophagy when apoptosis is inhibited	?	Avicin D	176

Conclusions

This review summarized the results of studies investigating the chemico-physical properties of saponins and their effects on membrane components, artificial membrane models, erythrocytes, and cancer cells.

Their molecular structure composed of osidic polar parts and apolar parts gives saponins an amphiphilic character. Saponins are able to reduce the interfacial (or surface) tension between phases of different polarity and stabilize emulsions or foams. Some saponins also possess the ability to self-aggregate into different types of aggregates.

The molecular structure of several saponins allows them to interact with lipid membrane components like phospholipids and cholesterol. Interaction and mutual aggregation can lead to the formation of several types of aggregates, such as nanoparticles and other nano-objects, which could be used in vaccination or cancer therapy.

The interaction with membranes has been studied *in silico* and in artificial models as well as in erythrocytes and cancer cells.

The ability of saponins to modulate the dynamic properties of bilayers on different time scales is primarily sterol-dependent. In cancer cells, this can lead to a decreased resistance to chemotherapeutic agents or prevent viral infections.

Moreover, saponins show the ability to change the lateral organization of bilayers and the disruption of lipid rafts,

provoking the activation of death receptors in cancer cells and other raft dependent proteins.

The mechanism by which saponins are able to permeabilize membranes is to a large extent structure-dependent. Although the permeabilizing activity of monodesmosidic saponins relies on the presence of cholesterol, as was demonstrated in both artificial models and cancer cells, some bidesmosidic saponins do not seem to require sterols to exert their permeabilizing effect. *In silico* studies and artificial models have shown that the three-dimensional structure and the presence of sugars at C3 in monodesmosidic saponins favors sterol interaction and induces membrane curvature, leading to pore formation and the transformation of the bilayer into non-bilayer structures. In artificial models and cells, saponin induced the aggregation of both cholesterol and phospholipids. The *in silico* formation of a ternary complex composed of saponins, phospholipids, and cholesterol also predicted this behavior. The critical micellar concentration of a saponin influences further its permeabilizing ability.

Hemolysis of saponins has been studied extensively. Nevertheless, the subject remains controversial, and further investigation is especially needed to clarify the role of membrane cholesterol and the importance of the sugar chain. It is possible that some aglycones exhibit hemolytic activity. This possibility is reinforced by the fact that hederagenin showed permeabilizing activity on GUVs.

Cell death induced by saponins can in some cases be correlated with their permeabilizing activity, but in addition to causing direct membrane lysis, many saponins induce apoptosis and autophagy or inhibit the cell cycle and the proliferation of cells. Apoptosis is in some cases a direct consequence of the activity of saponins on membranes. The extrinsic pathway is induced subsequent to the activation of death receptors and the reorganization of lipid rafts; the intrinsic pathway is induced via the release of proapoptotic proteins from the intermembrane space of mitochondria.

However, the ability of saponins to directly target proteins involved in cell death must be taken into account. The multitudes of mechanisms by which saponins act on cancer cells and the ability of saponins to overcome chemotherapeutic resistances makes them interesting candidates for cancer research.

We provided an overview of the complexity of saponin activity, which is strongly dependent on their molecular structure and physicochemical properties. As our understanding of the numerous interactions of saponins with membranes and their resulting consequences improves continually—in particular thanks to studies on membrane models and the integration of biophysical concepts—further investigation of these fascinating compounds will certainly contribute additional valuable data, expanding their potential to act on cancer cells and other targets.

Notes and references

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