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On the structure of immune-stimulating saponin-lipid complexes (iscoms)

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Immune-stimulating complexes (iscoms) are stable complexes of cholesterol, phospholipid and Quil A, a triterpene saponin mixture in the size range from 40 to 100 nm. They can be used as antigen carriers in subunit vaccines. In this paper it is demonstrated that iscoms are rigid, negatively charged vesicles in which small water soluble molecules like carboxyfluorescein cannot be retained. The negative zeta-potential prevents iscoms from aggregation. The chemical composition of iscoms in one dispersion varied considerably. A typical example of the composition of iscoms is cholesterol/phospholipid/Quil A = 1.0:1.2:6.2 by weight for the iscom matrix, that is iscoms without antigen, and 1.0:1.3:5.1 for antigen-containing iscoms. A hypothetical model for the structure of the iscom matrix and related structures is presented, based on analytical chemical, physico-chemical and electronmicroscopic data. In this model iscoms are considered to be multi-micellar structures, shaped and stabilized by hydrophobic interactions, electrostatic repulsion, steric factors and possibly hydrogen bonds. The individual micelles are relatively flat, ring-shaped structures, the center offering space for one of the two bulky sugar chains of the saponins.

Introduction

There is a trend to use purified antigens instead of whole pathogens for vaccination in order to prevent negative side effects. Often this results in a loss of immunogenicity. Therefore, potent adjuvants are needed to boost the immune response. The triterpene saponin mixture Quil A (QA) is such an adjuvant. It is used in veterinary vaccines for many years [1]. QA is able to form complexes with lipids and amphiphilic antigens. The complex formation probably increases the adjuvanticity/toxicity ratio. The formed structures are called iscoms [2]. The iscom matrix is a promising carrier for protein antigens. Iscoms have been shown to stimulate the humoral as well as the cellular immune response to many – mainly viral – membrane proteins [3]. Also protection has been achieved after immunization with iscom based vaccines against viruses like Epstein-Barr virus [4], feline leukemia virus [5], measles virus [6] and bovine herpes virus type 1 [7].

Not much is known about the structure of iscoms. In negative staining electron microscopy they appear as disks or spheres with a honeycomb motive. It was shown before that iscoms are spherical structures, consisting of subunits. Besides, their chemical composition was determined [8].

Important features of many saponins are their hemolytic activity and their ability to form complexes with sterols. It is thought that these two characteristics are related. A number of publications about the effect of saponins on lipid structures appeared in the literature [9–12], but there still is no clear understanding about the precise mechanism and the molecular requirements that determine the preference of saponins for sterols and, to a lesser extent, phospholipids. Complex formation with sterols is not the only mechanism of action in the process of lysis. The steroid saponin digitonin is a strong complex former with sterols and is hemolytic. The hydrophilic moiety of digitonin (the sugar part) [13] as well as one of the hydrophobic moieties of the sterol (the length of the side chain) [14] play a role. But it was also demonstrated that digitonin is able to form pores

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in cholesterol-free model membranes, although at much higher concentrations than with cholesterol-containing membranes [15]. For other saponins (triterpene as well as steroid types) it was shown that the relation between sterol complex formation and hemolytic activity is unclear [16–19]. Soybean saponin, for instance, is strongly hemolytic but this hemolytic activity cannot be prevented by the addition of cholesterol [17,20]. These data indicate that more than one mechanism plays a role in the interaction of saponin and lipids.

In this paper more details about the structure of iscoms are given and a possible model for these saponin-lipid complexes as well as other observed saponin-lipid complexes is presented. As a model antigen outer membrane pore protein I from *Neisseria gonorrhoeae* (PIC3) was used [21].

Materials and Methods

Protein purification. Gonococcal pore protein IB, serotype C3 was purified as described [22]. The procedure includes lysis with Zwittergent 3-14, ion-exchange chromatography and gel permeation chromatography.

Preparation of iscoms and related structures. QA was supplied by Iscotec (Lulea, Sweden), phosphatidylethanolamine type III A (PE), cholesterol (chol) and octylglucoside (OG) were purchased from Sigma (St. Louis, MO). Iscoms were prepared as described previously [8]. Mixed micelles in Tris buffered (10 mM) saline (140 mM) pH 7.4 (buffer A) were prepared consisting of QA/PE/chol/OG/PIC3 = 4 : 1 : 1 : 40 : 0 by weight for the iscom matrix (i.e., without antigen) and 4 : 1 : 1 : 40 : 0.4 for protein-containing iscoms. The OG concentration was 40 mg/ml. The micelles (1 to 5 ml) were dialysed against two changes of 1 l buffer A for 24 h at 4 °C to remove OG. The formed iscoms were purified on a sucrose gradient (10 to 60% sucrose in buffer A. 18 h at 50 000 × *g*, 10°C). The iscoms, visible as a turbid band at about 35% sucrose, were recovered either as a complete band (routinely) or fractionated for determining the heterogeneity. The iscoms were dialysed against buffer A and kept at 4°C for short periods or at −20°C for prolonged storage. QA-chol dispersions were prepared by dialysis of QA-chol-OG micelles. QA/chol ratios were 4 : 1 and 2 : 1 (by mass).

Chemical analysis. Cholesterol content was assessed by gas chromatography of the silylated derivative [23]. Phospholipid concentrations were determined by the phosphorus assay of Bartlett [24]. QA amounts were determined with reversed phase HPLC as described before [8], with some modifications. The iscoms were analysed on a Hypersil ODS 5 μm analytical column (Shandon, Runcorn, UK). The mobile phase consisted of an acetonitrile (Baker Chemicals BV, Deventer, The Netherlands) in water gradient (from 32% to 40% acetonitrile in 13 min) buffered with 0.01 M ammonium acetate (Baker) (pH 6.0). Peaks were detected with a UV spectrophotometer operating at 208 nm. Quantification was achieved by measuring the peak height of the three major peaks in the chromatogram using a standard curve of Quil A. The Bradford assay was used to determine protein content [25]. PIC3-iscom samples were precipitated with ethanol and resolubilized in 2% OG before the assay was done.

Hemolytic activity. V-shaped cups of a microtiter plate were filled with 100 μl 0.5% (v/v) erythrocytes from *Cercopithecus aureus* (monkey) in McIlvain buffer (pH 7.2) (13.1 mM citric acid, 173.8 mM Na_2HPO_4). 100 μl of sample or QA standard (concentration ranging from 0.5 to 8.0 μg/ml) was added. After incubation for 3 h at 37°C the plate was centrifuged for 5 min at 2000 rpm (Hettich Rotixa IKS, Tuttlingen, F.R.G.) and 100 μl supernatant was transferred to a flat-bottom microtiter plate. The extinction at 405 nm was measured with a microtiter plate reader (Titertek Multiskan MCC, Flow Laboratories, Herts, U.K.).

Size. The hydrodynamic radius of iscoms was determined by dynamic light scattering with a System 4600 size analyser (Malvern Instruments Ltd., Worcestershire, U.K.).

Fluorescence polarization. Fluorescence polarization experiments were performed as described before [26]. 1,6-Diphenyl-1,3,5-hexatriene (Sigma) was used as hydrophobic fluorescence probe. Steady-state fluorescence polarization was measured with a fluorescence spectrophotometer (Elscint MV-1a, Elscint Ltd., Haifa, Israel).

The zeta-potential. The surface potential was determined by measuring the electrophoretic mobility with a Zetasizer II C (Malvern Instr. Ltd.) at 25°C.

Determination of internal volume. One ml of iscom matrix was prepared in the presence of 1 mM 5(6)-carboxyfluorescein (CF) (Eastman Kodak Company, Rochester, NY). After dialysis of the mixed micelles against buffer A with 1 mM CF for 48 h, the iscoms were washed two times with buffer A by pelleting in an ultracentrifuge for 4 h at 75 000 × *g*. Fluorescence of the resuspended iscom matrices was measured in a Perkin-Elmer LS 5 Luminescence Spectrometer (Perkin-Elmer Ltd., Beaconsfield, U.K.). The excitation wavelength was 490 nm and the emission wavelength was 520 nm.

Preparation of liposomes. Small unilamellar vesicles were prepared with the dilution method as previously described [26].

Results

The morphology of the prepared QA-complexes was determined by negative staining electron microscopy. If a mass ratio of QA/PE/chol = 4 : 1 : 1 is used, iscoms are formed as defined by Morein et al. [2], i.e., spherical structures with ring-like subunits (Fig. 1). A QA/chol ratio of 4 : 1 in the absence of PE reveals ring shaped

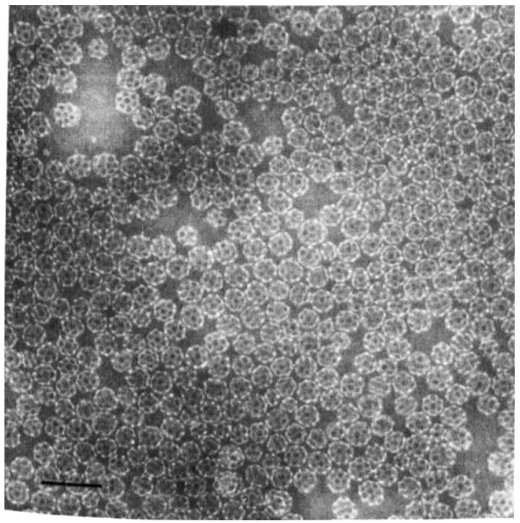

Fig. 1. Negative staining electronmicrograph of iscom matrices. The initial mass ratio was QA/PE/chol = 4 : 1 : 1. Bar is 100 nm.

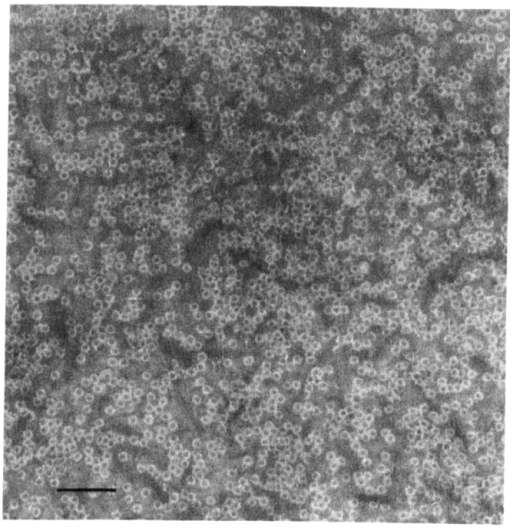

Fig. 2. Negative staining electronmicrograph of QA-chol micelles. The initial mass ratio was QA/chol = 4 : 1. Bar is 100 nm.

subunits (Fig. 2), whereas a ratio of 2 : 1 forms a precipitate of large lamellae (Fig. 3). The ring shaped subunits and the lamellae were not further investigated.

The heterogeneity of iscoms in one dispersion was established by fractionation of the slightly turbid iscom band in the sucrose gradient. The sucrose concentration in the band of iscom matrices amounted from approx. 27% to 34%. The PIC3-iscoms covered a density range corresponding to sucrose solutions with concentrations from approx. 29% to 39%. In Table I the composition of the iscoms as well as the total mass in the different fractions is shown. The hydrodynamic size of the iscom matrices increased further down the gradient, but not as much as PIC3-iscoms, showing a 2-fold size increase (Table I). The hemolytic activity of QA was reduced about 17-fold by complexation with lipids (Table II).

A measure of the fluidity of bilayers and micelles can be obtained from fluorescence polarization data of a lipophilic probe incorporated in the structure to be investigated [27,28]. Rotation of the probe causes the emitted light to depolarize after excitation with polarized light. The polarization P is defined as $(I_{\|} - I_{\perp})/(I_{\|} + I_{\perp})$ were $I_{\|}$ is the intensity of the emitted light parallel to the direction of the polarized excitation light, and $I_{\perp}$ is the intensity of the light perpendicular to the excitation light. The maximal value for P is 0.5. Regions of high rigidity prevent rotation of the probe. This limits depolarization and a high P value is obtained. The

TABLE I

Composition of two iscom populations separated by gradient centrifugation

Sample	QA (mass%)	Chol. (mass%)	PE (mass%)	PIC3 protein (mass%)	Total mass (mg/ml)	Size (nm)
Iscom matrix, fraction 1 [a]	78	9	13	– [b]	2.8	43
3	74	12	14	–	3.9	48
5	71	13	15	–	2.9	52
7	64	14	22	–	0.7	55
PIC3 iscoms, fraction 1	69	11	19	1	1.0	41
3	66	13	17	4	2.8	48
5	65	13	17	5	1.9	59
7	62	12	19	6	1.2	92

[a] Fraction 1: top fraction.
[b] Not done.

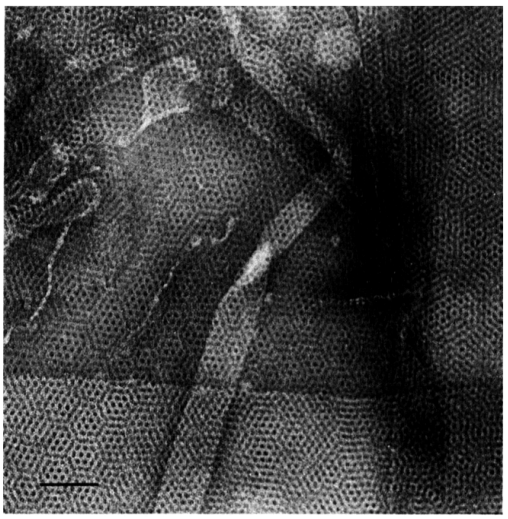

Fig. 3. Negative staining electronmicrograph of QA-chol lamellae. The initial mass ratio was QA/chol = 2:1. Bar is 100 nm.

fluorescence polarization of DPH in iscoms was compared with that of DPH in fluid state and gel state unilamellar liposomes (Table III). The rigidity of iscoms is comparable with that of the most rigid liposomes investigated in an earlier study [26].

The charge of iscoms was determined for iscoms in buffer A and in 10 mM Tris (pH 7.4). The zeta-potential in buffer A was -20 ± 1 mV ($n = 3$) for iscom matrices and -21 ± 2 mV ($n = 3$) for PIC3-iscoms. The zeta-potential in Tris-buffer was -32 ± 3 mV for iscom matrices and -26 ± 1 mV for PIC3-iscoms ($n = 3$). The role of this charge on the stability of iscoms was investigated by acid-base titration of iscoms in Tris-buffer with HCl or NaOH (Fig. 4). When the pH decreased below 3.0 for iscom matrices or below 3.5 for PIC3-iscoms aggregation occurred. The zèta-potential also

TABLE II

QA amount and hemolytic activity of two iscom populations separated by gradient centrifugation

Sample		QA (μg/ml)	Hemolytic activity (corresponding to x μg/ml free QA)
Iscom matrix, fraction	1	2188	134
	3	2855	160
	5	2080	136
	7	464	37
PIC3 iscoms, fraction	1	711	35
	3	1881	150
	5	1232	98
	7	737	69

TABLE III

Fluorescence polarization measurements

Preparation	Fluorescence polarization P
Iscom matrix	0.381
PIC3 iscom	0.397
Empty liposome ('fluid' state) [a]	0.183
PIC3 liposome ('fluid' state)	0.191
Empty liposome ('gel' state) [b]	0.420
PIC3 liposome ('gel' state)	0.421

[a] Molar composition: egg phosphatidylcholine/chol/egg phosphatidylglycerol = 7:2:1. Size 118 nm.
[b] Molar composition: dipalmitoylphosphatidylcholine/chol/dipalmitoylphosphatidylglycerol = 7:2:1. Size = 285 nm.

decreased at decreasing pH, although the potential of aggregated iscoms becomes zero only at very low pH values. It seems as if there is a critical potential at which aggregation starts. The aggregation process was reversible upon increase of the pH. NaCl concentrations of 140 mM and 600 mM at pH 7.4 did not influence the particle size (not shown). The aggregation behaviour of iscoms in buffer A was similar to the situation in Tris-buffer (not shown). These observations indicate that screening of the electrostatic potential in high ionic

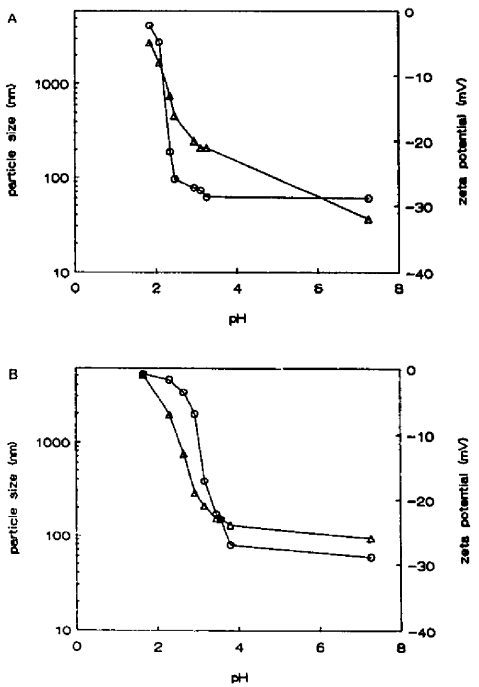

Fig. 4. Hydrodynamic size (○) and zeta-potential (△) of iscoms vs. pH. Iscom matrices (A) and PIC3-iscoms in 10 mM Tris-HCl (B) were titrated with 0.1 M HCl.

strength solutions does not cause aggregation, which suggests non-electrostatic stabilization. On the other hand, ionization of acid groups is probably essential to keep the iscoms in a deaggregated state.

Preparation of iscoms in the presence of 1 mM CF did not result in retention of the hydrophilic marker. The residual fluorescence was $6.6 \cdot 10^{-4}$%. If an isolated internal volume would be present a retention between 10^{-2} and 10^{-1}% is expected. This estimation is based on the inner size of iscoms as shown on freeze-fracture micrographs (31 nm), the number of iscoms per μg cholesterol (EM micrograph counting of iscoms with known numbers of latex particles added to the sample) and the amount of cholesterol present in the counted samples (calculation not shown). A residual fluorescence of $6.6 \cdot 10^{-4}$% means that at most 7% of the estimated maximal amount possible is still present after washing. Assuming that CF cannot readily pass through lipid bilayers, this means that it is unlikely that the internal aqueous phase is fully separated from the external medium.

Discussion

Iscom matrices show a considerable heterogeneity in composition; the relative amount of QA reduces further down the gradient. This change in composition also causes changes in size. Loss of QA results in bigger iscoms. This trend is also observed for PIC3-iscoms. The relative loss of QA down the gradient is less than that of iscom matrices. But a second factor is involved, i.e., the amount of protein, which increases. The QA/PIC3 ratio ranges from 17 in fraction 3 to 10 in fraction 7. The growth in size from fraction 1 to 7 is in agreement with earlier findings that decreasing the initial QA/PIC3 ratio causes a dramatic increase in size [26]. This size increase is possibly caused by the presence of multiple membrane spanning regions in PIC3 (Van der Ley, P., Institute for Public Health, personal communication). In this respect, the bacterial antigen might behave different from viral membrane proteins. In the case of viral membrane proteins increase in size at high protein concentrations is usually not observed. Instead a mixture of iscoms and protein micelles are formed, due to overload of the iscom matrices.

The fluorescence polarization measurements demonstrate the rigid character of iscoms. In principle, the fluorescence polarization technique as used in this study does not allow for discrimination between domains with different rigidity in the iscoms. A homogeneous distribution of DPH over these domains is supposed. It was shown that DPH partitions equally well between solid and fluid lipid domains [27,29]. Because the fluorescence polarization approaches its maximal value it might be speculated that large 'intra-iscom' differences in fluidity are unlikely.

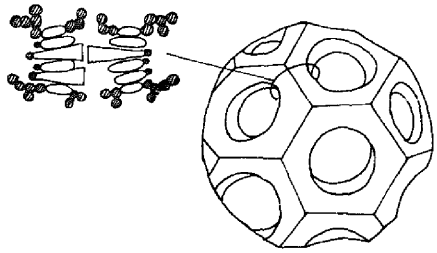

Fig. 5. A possible model of the iscom matrix as shown in Fig. 1 with cross section. The model shows the hydrophobic core, the cross section shows individual molecules (not to scale). The hydrophilic moieties are shaded: 8 sugars per QA molecule, the hydroxyl group in chol and the ethanolamine phosphate in the cone shaped PE.

CF could not be incorporated into iscoms. This means that they are either massive or hollow with pores connecting the in- and outside medium. Freeze-fracture electron microscopy has revealed that iscoms are hollow spherical vesicles formed by subunits [8]. Therefore, pores are likely to be present.

Another feature of iscoms is that they have a negative surface potential at pH 7.4 and that this potential prevents iscoms from aggregation, although aggregated iscoms still can have a relatively high surface potential. It is known that most QA components contain glucuronic acid [30,31]. To induce a negative surface potential at least a part of the sugar chain containing the glucuronic acid should point outward.

With these data about iscoms in mind, it is possible to propose a model for their structure. The main characteristic of this model is the stable, non-bilayer organization of the constituents (for comprehensive reviews on lipid polymorfism, see Refs. 33, 34 and 35). Iscoms are considered to be vesicular structures composed of micelles that interact via hydrophobic forces (Fig. 5). The micelles have a hydrophilic core. Small water-soluble molecules can diffuse into and out of the iscom interior through these holes in the micelles. QA saponins are of the bidesmosidic type, i.e., they possess two sugar chains (Fig. 6) [31,32]: one at the hydroxyl group at carbon atom 3 of the aglycone and one attached to the quillaic acid via an ester bond with the carboxylic group at carbon atom 28. In the pore is space for one sugar chain. The other chain shields the hydrophobic upper or bottom side of the micelle containing chol and PE. The remaining hydrophobic parts of the molecules are prevented from exposure to the aqueous phase by aggregation with other micelles, eventually forming spherical structures analogous to the liposome formation model of Lasic [36]. Freeze-fracture electron micrographs of iscoms reveal spherical structures with surfaces that show small intrusions, possibly pores. Cross sections show the micellar subunits. Sometimes pore-like interruptions are visible [8].

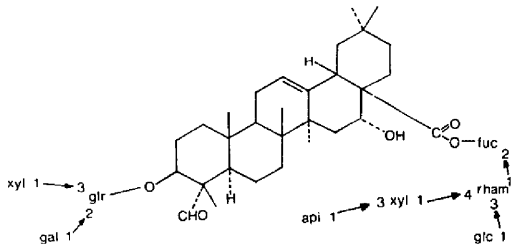

Fig. 6. Proposed structure of one of the main components of quillajasaponin [32]. xyl, xylose; glr, glucuronic acid; gal, galactose; api, apiose; fuc, fucose; rham, rhamnose; glc, glucose.

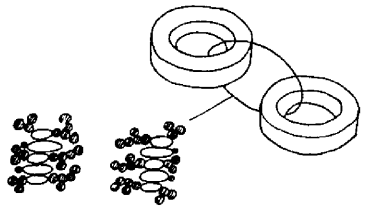

Fig. 7. A possible model of QA-chol micelles as shown in Fig. 2 with cross section. The model shows the hydrophobic core, the cross section shows individual molecules (not to scale). The hydrophilic moieties are shaded: eight sugars per QA molecule, the hydroxyl group in chol and the ethanolamine phosphate in the cone shaped PE.

A charged sugar like glucuronic acid is necessary to prevent aggregation of iscoms. If the glucuronic acid is at least partially protonated, aggregation occurs. Decreasing the electrostatic interactions by protonation is, on the other hand not fatal for the iscom structure: the aggregation is completely reversible upon restoring the electrostatic interactions and electron microscopic pictures of iscoms at low pH indeed showed only aggregated iscoms (not shown). The iscoms do not collapse or fuse at low pH. A subtle balance between hydrophilic and hydrophobic functional groups of the iscom components is required. In this paper only three variations in composition are described. Many other variations were also tested (not shown and Ref. 8). The ratio chol/PE/QA = 1 : 1 : 4 was the optimal ratio for the formation of iscoms. In all other ratios tested (more than 25 with egg-PE as phospholipid and others with different phospholipids, sterols and saponins), the regular iscoms structure was a minor component, if observed at all. The subtle balance mentioned above, is also demonstrated by the fact that following a regular iscom formation procedure with purified QA components often results in the appearance of large lamellae, aggregates or even helices. The lamellae and aggregates show the same subunit arrangement as iscoms (manuscript in preparation). Because of the reversible aggregation it might be speculated that most Quil A molecules are oriented in such a way that the sugar chain containing the glucuronic acid, which is the C-3 sugar chain, shields the upper and lower side of the micelle. Besides, strong repulsion forces would occur in the center of the micelle if many C-3 sugar chains were present there. This implies that the sugars at C-28 are likely to form or partially fill the pore. Inter-molecular hydrogen bonds between sugars may play a role in stabilizing the individual micelles as well as inducing aggregation of iscoms at low pH.

The presence of the cone shaped PE-molecules [37] increases the hydrophobic area substantially. In combination with proper QA/chol mixtures PE, therefore, provides the driving force for the transition of the micellar form to spherical structures. Omission of PE results in the formation of micelles, which do not aggregate (Figs. 2 and 7). If the PE is replaced by chol, no spheres are formed, but large, flat lamellae (Figs. 3 and 8). The aggregates that are presumably very rigid, might have such a high elastic curvature energy that small vesicular structures cannot be formed. The relative amount of QA in the complexes determines their size. High contents of QA will produce small structures and low contents cause aggregation. This is in agreement with the analytical data of the iscom fractions: relative loss of QA, i.e., loss of shielding sugar chains results in larger vesicles. A seemingly conflicting finding is the fact that without PE no iscoms are formed. It was shown by others that only QA and chol are necessary to prepare iscom matrices [38]. A comparison of the experimental details of the preparation methods (Table IV) shows that it may be possible that not all the MEGA-10 has been removed from the sample because it will take long to fully remove the detergent by dialysis. Therefore, residual MEGA-10 might replace the phospholipid.

In this paper a new model for lipid-saponin complexes is presented. A model proposed by Lucy and Glauert [12] describes the ring structure as an array of small (4 nm) globular micelles with a hydrophobic core.

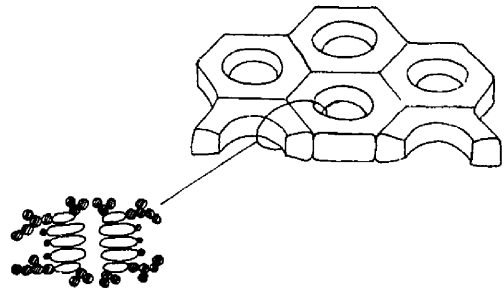

Fig. 8. A possible model of QA-chol lamellae as shown in Fig. 3 with cross section. The model shows the hydrophobic core, the cross section shows individual molecules (not to scale). The hydrophilic moieties are shaded: eight sugars per QA molecule and the hydroxyl group in chol.

TABLE IV

Differences between two iscom matrix preparation techniques based on detergent removal

	This study	Swedish study [38]
Detergent	octylglucoside	MEGA-10 [a]
CMC (mM)	23.2	6.2
Molecular weight	292.4	349.5
Initial concentration (%)	4	20
Molecular weight cut off dialysis tube	10000	1000

[a] Decanoyl-*N*-methylglucamide.

More recently, Özel et al. [39] described the morphology of iscoms, as determined by electron microscopy. They propose a model also composed of globular micelles. The size of these micelles is 12–15 nm (i.e., the size of one ring-like micelle described in this paper). The model described by us speculates on the structure of the micelle itself. A globular micelle, as proposed by Lucy and Glauert and Özel et al., raises the question where the two oppositely oriented sugar groups (in the case of QA) are located. The model of Özel et al. does not account for the existence of one, isolated ring-like structure because a pore is formed by five large (12–15 nm) micelles and the micelles themselves are not filled with stain, as can be observed by the electron micrographs of iscoms. We feel that the 'pore containing micelle' model shows a better fit with our experimental observations than the 'pore forming micelle' model.

Up to now no iscoms containing other saponins than QA have been described. The existing literature on saponin-lipid complexes shows a wide variety in structures, often of colloidal dimensions [9–12,40]. The ring-like structure as the basic subunit, however, is often observed (as shown in Figs. 2 and 3). The typical iscom structures as shown in Fig. 1, however, have only been described for QA-containing preparations.

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