Self-assembly in saponin mixtures: escin / tea, tea / glycyrrhizic acid, and escin / glycyrrhizic acid mixtures

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ABSTRACT

Saponins are plant based biosurfactants that are obtained from a wide variety of plant species. They are surface active glycosides with a hydrophobic group, which is commonly a triterpenoid or steroidal group, and saccharide hydrophilic units. Their structures are markedly different from conventional synthetic surfactants, and present interesting challenges in understanding their optimal packing in self-assembled structures such as micelles. Furthermore the wide variety of molecular structures available provide interesting opportunities to optimise the packing in mixed systems.

In the current literature there is limited information available on saponin self-assembly, and especially in saponin mixtures. The general view is that globular to more elongated micelle structures form at relatively low concentrations. Here small angle neutron scattering, SANS, is used to investigate and quantify the structure of escin, tea and glycyrrhizic acid micelles and particularly of escin / tea, tea / glycyrrhizic acid and escin / glycyrrhizic acid mixtures at relatively low concentrations. The focus is principally on how the different saccharide headgroup structures affect the self-assembly.

Tea saponins form relatively small globular micelles, whereas escin and glycyrrhizic acid form larger more elongated globular micelle structures. The micelle structure and changes observed reflect the packing constraints associated with the aglycone triterpenoid hydrophobic section and the differences in the saccharide headgroups. In the escin / tea and glycyrrhizic acid / tea mixtures the greater intrinsic curvature associated with the tea saponin dominates the micelle structure over much of the composition range explored. For the escin / glycyrrhizic acid mixture the micelle size and aggregation number go through a minimum at an approximately equimolar composition, and this implies an intrinsic difficulty in efficient packing of the two different saponin structures, which results in an increase in the preferred curvature.

1. INTRODUCTION

Saponins are surface active glycosides with a wide range of structural details, derived from a wide range of plant species (1-5). Their surface activity, as manifest in their surface adsorption (6-15) and self-assembly (15-22) properties, arise from a hydrophobic scaffold which comprises of a triterpenoid, steroid or steroid-alkaloid group and a hydrophilic group which consists mainly of different saccharide groups. The wide range of different molecular structures found within the different plant species gives rise to a range of physicochemical and biological properties (3, 4). Their intrinsic surface activity properties, adsorption at interfaces and self-assembly in solution, resulted in some traditional and well established applications, as foam stabilisers in beverages (23), and in emulsion stabilisation in foods (2, 23). Their use as natural medicines (1) derive from their anti-fungal, anti-inflammatory, anti-bacterial, anti-viral and anti-cancer properties; properties which make them increasingly important in cosmetics, shampoos and in modern medication (2, 6). In many products containing surfactants there is also now a strong drive towards using natural or bio-surfactants (24-28) that are bio-sustainable and bio-compatible, and are derived or synthesised from renewable and sustainable sources. This increasing environmental awareness and the attractions of the wider properties and benefits of saponins has resulted in a resurgence of interest.

The unusual structure of the saponins gives rise to some marked surface properties, resulting in surface layers which are highly rigid and exhibit strong surface viscoelastic properties (10-14). The surface rheological properties have been extensively characterised (10-14). In contrast, neutron reflectivity, NR, surface tension, ST, and other methods have been used to probe their adsorption properties (6-9, 15). Penfold et al (6) showed how the structure of the saponin hydrophilic portion affected the adsorption isotherm and the saturation adsorption values, and these measurements established the predominantly nonionic nature of the adsorption. Their measurements of the structure of the adsorbed layer reinforced an earlier supposition that the high surface viscoelasticity arises from a relatively densely packed headgroup region in which strong inter-molecular bonds exist. This interpretation was also supported by the recent molecular dynamics simulations of Tsibranska et al (7).

Given the unusual structure of the saponin molecule the nature of their self –assembly and micelle formation in solution is of particular interest, but only a limited number of studies exist (15-22). Following the early work of Oakenfull (21) and Mitra and Dungan (16-18) the formation of micelles by the Quillaja saponin and their properties under different circumstances

was established. Tykarska et al (19) and Matsuoka et al (20) focussed on the micelle structure of glycyrrhizic acid under different solution conditions, reporting rod-like structures. Saha et al (29), Wan et al (30) and Rala et al (31) reported fibril formation in glycyrrhizic acid solutions under certain circumstances. Dargel et al (22) used small angle x-ray scattering, SAXS, and fluorescence to study the micelle structure of escin over a range of temperatures and solution conditions. In a study of the adsorption and self-assembly of glycyrrhizic acid Tucker et al (32) used SANS to characterise the self-assembly of some different saponins, comparing the relative structures of the micelles formed from glycyrrhizic acid, escin, tea and Quillaja saponins, and investigating the impact of electrolyte. Importantly in the context of the results presented in this paper they highlighted the differences in micelle structure arising from the different molecular arrangements of the saponins studied.

The wider exploitation and application of surfactants usually involve mixtures, with other surfactants, proteins and polymers, in order to exploit synergistic effects and to manipulate and optimise particular solution properties; and this is also the pattern that the wider exploitation of saponins will also likely follow. Hence a number of studies have focussed on the mixing properties of saponins with conventional surfactants (33-38), other saponins (39), and proteins (40-44), and have focussed predominantly of their surface properties.

Reichart el al (37) studied the impact of a range of cosurfactants on the emulsifying properties of Quillaja saponin. Jian et al (36) reported synergistic effects in foam formation of a saponin derived from *Camellia oleifera Abel* tea saponin with a range of synthetic surfactants. Tucker et al (33, 34) used NR and ST to explore the surface and micelle mixing of escin with nonionic surfactants and with the anionic surfactant, SDS. A detailed evaluation of the mixing using the pseudo phase approximation, PPA, was made; and which further illustrated the predominantly nonionic nature of escin. For escin / nonionic surfactant mixtures the mixing was close to ideal, and the interaction was stronger in the micelles than at the surface. The change from attractive to repulsive interaction as the length of the ethylene oxide group, EO, of the nonionic alkyl polyoxyethylene ether surfactants changed from EO_5 to EO_8 was assumed to be due to the incompatibility between the saponin sugar groups and the EO groups of the nonionic surfactant. In contrast, the mixing for escin / SDS was more strongly synergistic, attractive, and again with a stronger micelle than surface interaction. This was consistent with that generally observed in ionic / nonionic mixtures, and also indicated a strong steric component to the excess free energy of mixing. Tucker et al (35) also explored the evolution in micelle structure in escin / SDS mixtures using SANS. They reported the formation of globular micelle structures

which increased in size as solutions became richer in escin. The micelle size went through a minimum at relatively SDS rich composition, reflecting the impact of the disparity in the molecular structures and changes in the packing.

There is only limited information about the behaviour of saponin mixtures. Dai et al (39) investigated the interaction between the ginsenoside biosurfactant and saikosaponin a. They showed how the interaction of the different ginsenoside structures with saikosaponin a resulted in different microstructures, which included spherical and wormlike micelles and vesicles.

In saponin / protein mixing Kezwon et al (38) demonstrated synergistic interactions between Quillaja saponin and the proteins lysozyme, β-lactoglobulin, and β-caesin. Piotriowski et al (40) attributed the synergistic adsorption in Quillaja saponin and β-lactoglobulin mixtures to the adsorption of saponin / protein complexes at the interface. Reichart et al (37) reported synergies in the formation of oil in water emulsions for the Quillaja saponin with different food grade surfactants, which included lecithin and sodium caseinate. Wojciechowski et al (41) showed synergies in ST and foamability in Quillaja saponin / lysozyme mixtures. Bottcher et al (42) demonstrated synergistic interactions in foam formation in Quillaja saponin / β-lactoglobulin mixtures, and illustrated how the strong surface interaction differs from those observed in protein / surfactant systems.

This paper builds substantially upon the recent SANS study of Tucker et al (35) on the self-assembly of escin / SDS mixtures, and explores the evolution in the self-assembly of some saponin mixtures. In particular the micelle structure of escin / tea, tea / glycyrrhizic zcid and escin / glycyrrhizic acid saponin mixtures are determined using SANS at a fixed relatively low surfactant concentration and a range of solution compositions. The study focusses on how varying the saccharide structure but retaining a relatively similar triterpenoid hydrophobic group affects the micelle packing and hence structure. In broader terms the study provides the opportunity to explore how synergistic mixing of the biosustainable and biocompatible saponin based surfactants with different structures can be used to manipulate self-assembly.

2. EXPERIMENTAL DETAILS

(i) Materials and measurements made.

The escin was obtained from Sigma (Cas no 6805-41-0, batch no BLV8469V-2, 96% purity) and was used as supplied. The escin batch used was the same as used in references 6, and 33-35. The glycyrrhizic acid was obtained from Sigma in the form of the ammonium salt (>97% purity). It was used as supplied, and was the same source material as used in reference 32. The

tea saponin, *Camelia Oleifera Abel* tea saponin, obtained from Nanjing Zelang Medical Technology Co. Ltd., China (supplied through Zhejiang Yuhong Import Export Co. Ltd.), with 96% purity was used as supplied, and was from the same source as used in references 6, 32. The molecular structure of the escin, glycyrrhizic acid and tea saponins are shown in figure 1. The key molecular parameters, MW etc., are summarised in table S1 in the Supporting Information.

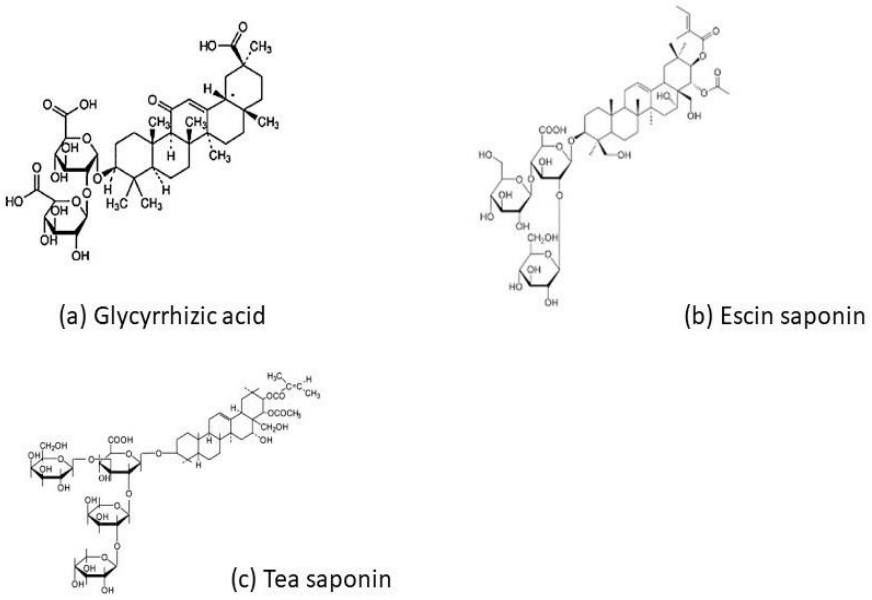


Figure 1. *Molecular structure of glycyrrhizic acid, escin, and tea saponins*

The NaCl and D_2O used in the sample preparation were obtained from Sigma, and high purity water (Elga Ultrapure) was used. All glassware and quartz sample cells used in the sample preparation and the SANS measurements were cleaned in alkali detergent (Decon90) and extensively rinsed in high purity water.

No adjustments to the solution pH were made, and dissolution in aqueous solution resulted in a measured pH in the range 4 to 5. In this pH range the carboxyl groups are mostly protonated and the saponins behave predominantly as a nonionic surfactants.

All the measurements were made at 25C and all the prepared solutions were clear and fluid.

The measurements were made in 0.1M NaCl for compatibility with previous studies (30-33), to ensue any departure from ideal mixing was not too extreme, to minimise electrostatic interactions and to replicate solution conditions for possible formulations.

All the SANS measurements were made in D_2O and 0.1M NaCl, at a surfactant concentration of 5 mM, a concentration that ensured good quality SANS data from relatively weak scatterers and compatibility with potential future applications. A range of solution compositions for escin / tea, tea / glycyrrhizic zcid and escin / glycyrrhizic acid saponin mixtures were measured.

(ii) Small angle neutron scattering, SANS

The SANS measurements were made on the SANS2D diffractometer (43) at the ISIS neutron facility. The scattered intensity, I(Q), was measured over a Q (where Q is defined as $Q=4\pi\sin\theta/\lambda$, θ is the scattering half angle, and λ is the neutron wavelength) range ~ 0.006 to 0.5 $Å^{-1}$ using the 'white beam time of flight' method. The Q range was determined using a sample to detector of 4 m and a neutron wavelength range from 2 to 12 Å, separated by time of flight. The sample scattering was normalised to the detector response and the spectral distribution of the incident beam and established on an absolute scattering cross section scale, in cm^{-1}, using standard procedures (44). The data are presented have the solvent and sample cell background scattering subtracted. This contribution to the scattering is ~ the sample scattering for Q values ≥ 0.25 $Å^{-1}$, and this limits the range of Q for which the modelling can be reliable.

3. RESULTS and DISCUSSION

(a) Escin, Tea, and Glycyrrhizic acid saponins

The SANS data were all measured at a surfactant concentration of 5 mM and in the presence of 0.1 M NaCl, for the escin, tea and glycyrrhizic acid saponins, and for the tea / glycyrrhizic acid, tea / escin, and escin / glycyrrhizic acid mixtures. Figure 2 shows the SANS data for the tea, escin and glycyrrhizic acid saponins.

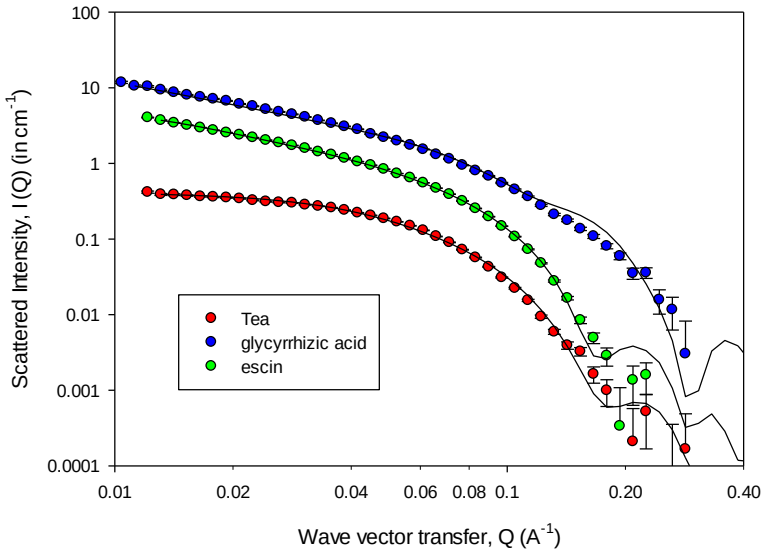


Figure 2. *SANS data for tea, escin and glycyrrhizic acid saponins at 5 mM and in the presence of 0.1 M NaCl. The data for escin and glycyrrhizic acid are shifted vertically by x8 and x16 compared to the data for the tea saponin; see legend for details. The solid lines are model fits, as discussed below and in the Supporting Information, and for the key model parameters summarised in tables S2 and S3 in the Supporting Information. Error bars are included, and at low Q values are ≤ size of the plotting symbol*

By inspection the SANS data in figure 2 for all three saponins are consistent with the scattering from relatively dilute globular micellar structures, in which the contribution from the structure factor, S (Q), is minimal. As such the scattering reflects mainly the form-factor, F(Q), of the micelles. The shallower slope at low Q (see data for the Tea saponin) is consistent with smaller more globular structures. The steeper slope (for escin and glycyrrhizic acid) which is tending towards a Q^{-1} dependence is indicative of more elongated structures. Hence the data for the tea saponin is consistent with relatively small globular micelles, and the data for escin and glycyrrhizic acid are consistent with relatively larger globular more elongated structures. The form of the scattering at high Q indicates that the short dimension of the elongated structures is similar for the tea and escin saponins, but noticeably smaller for glycyrrhizic acid. The data are analysed quantitatively using an established core-shell model (45-48) described in detail in the Supporting Information, and used extensively in the analysis of micelle and mixed micellar systems (45, 49). More recently it has been adapted to accommodate the different structure and packing associated with the saponins, as described in the Supporting Information, and elsewhere (32, 30) in detail. Importantly, with this adaptation, the same relatively simple model

is applied to all the data and provides a consistent approach to follow the evolution in the micelle structure with composition for the different saponin mixtures. The key model parameters are summarised in table S2 in the Supporting Information, and the key parameters refined in the modelling are the micelle aggregation number, ν, and the parameter ext, as described below.

The values of the micelle aggregation numbers, ν, for tea, escin and glycyrrhizic acid saponins reflect the different micelle sizes. For the tea saponin ν is ~ 80, and is similar to that observed in most globular micelles formed by conventional surfactants. The aggregation number for the escin and glycyrrhizic acid saponins is much larger, ~270, and is representative of a larger more elongated structure. The modification to the core-shell micelle model, used extensively for conventional surfactants (45, 49), includes an additional parameter, ext, which accommodates changes in the packing requirements of the triterpenoid hydrophobic group of the saponin compared to the alkyl chain of a conventional surfactant, as described in more detail in the Supporting Information. The inclusion of ext also allows for some mixing between the hydrophobic and hydrophilic parts of the saponin. For glycyrrhizic acid ext is ~ 1.0 and the short dimensions of the elongated structure, R_1 and R_2, are relatively small; corresponding to ~ 14 and 19 Å. For the aggregation number ~ 280 this gives rise to a relatively large ellipticity, ee, ~ 30, and hence the micelle is highly elongated. For the tea and escin saponins a larger value for ext is required to accommodate the form of the scattering at high Q, and ext is ~ 1.4 to 1.45. This results in larger R_1 and R_2 values, which are 20 and 25 Å respectively. For the tea saponin with a relatively small aggregation number results in a modest ellipticity with ee ~ 3.0. For escin the larger aggregation number, ~ 270, results in a significant increase in the ellipticity, ee~10.0, and the escin micelles are more elongated than the tea micelles.

The three saponins all have different molecular structures. Although triterpenoid hydrophobic groups are in detail different they are broadly similar. The significant differences are in the number of hydrophilic saccharide groups and different number of carboxyl groups, see table S1 in the Supporting Information, and in their conformation. Glycyrrhizic acid, escin and tea saponins have an increasing number of saccharide groups; 2, 3 and 4 respectively. In conventional surfactants the larger headgroup results in smaller more globular micelle structures, and favour a greater curvature. Here the tea saponin has the largest headgroup, and forms the smallest more globular micelle structure. Both glycyrrhizic acid and escin, which have 2 and 3 saccharide group respectively form more elongated structures. The other notable difference in the micelle structures is the difference in the value of ext, and its corresponding

impact upon R_1 and R_2. Despite the aglycone group being similar for all three saponins, ext is ~ 1.4 to 1.45 for the tea and escin saponins, and ~ 1.0 for glycyrrhizic acid. Although the micelle structures with the higher value for ext also have the larger number of saccharide groups the other significant difference is that they also have only one carboxyl group, whereas glycyrrhizic acid has three carboxyl groups. Although increasing the headgroup size from ~2-3 to 4 saccharide groups results in a transition from elongated to globular structures, both the headgroup structure, as reflected by the number of saccharide groups and number of carboxyl groups, can also affect the packing associated with the hydrophobic core of the micelle. As characterised by the value of the parameter ext, there is a marked difference in the inner dimension, R_1, of the micelle when the number of carboxyl groups increases from one to three. This implies that the headgroup structure and inter-headgroup interactions impact upon the packing of the hydrophobic part of the saponin. This also implies that associated with the larger value of ext for the escin and tea saponins, with the larger number of saccharide groups and the lower number of carboxyl groups, there is likely to be some intermixing between the hydrophobic and hydrophilic regions.

The variation in the saponin structures reported here broadly follow the well-established packing criteria of Israelachivili et al (50). That is, increasing the effective area / molecule and hence the preferred curvature associated with the larger tea saponin headgroup results in smaller more globular micelles. However, in detail, as shown by the variation in structural dimensions for the different saponins, the packing requirements are more complex and involve the specific interactions within the headgroup region which impact upon the structure.

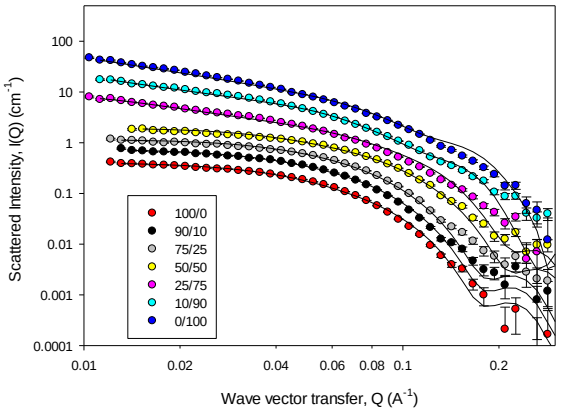
Although the packing constraints at the planar air-water interface are different to those in the micellar phase, it is interesting to compare how the saturated or limiting adsorption values vary with saponin headgroup structure. The limiting area / molecule values for escin, tea and glycyrrhizic acid saponins are 69, 79 and 87 $Å^2$ respectively (6, 32), see also table S1 in the Supporting Information. Comparing the escin and tea values it is clear that the additional saccharide group results in a larger area / molecule for the tea saponin. Furthermore Penfold et al (6) were able to show that the structure of the escin and tea saponin adsorbed layers was slightly different, as reflected in the extent and density of the hydrophobic layer adjacent to the air phase. Glycyrrhizic acid, which has the smallest number of saccharide groups but a greater number of carboxyl groups, has a limiting area / molecule larger than the escin and tea values. This implies that both the number of saccharide groups and number of carboxyl groups both have an impact upon the packing in the adsorbed layer. The role of the carboxyl group might

imply that the adsorption will depend upon pH. However although the cmc is pH dependent, it has been shown (6, 32) that for both escin and glycyrrhizic acid the saturation adsorption is largely independent of pH and added electrolyte, and that the saponins are only weakly anionic. In the case of glycyrrhizic acid the lower saturation adsorption is then likely to be due to intra and inter headgroup interactions between carboxyl groups associated with partial deprotonation.

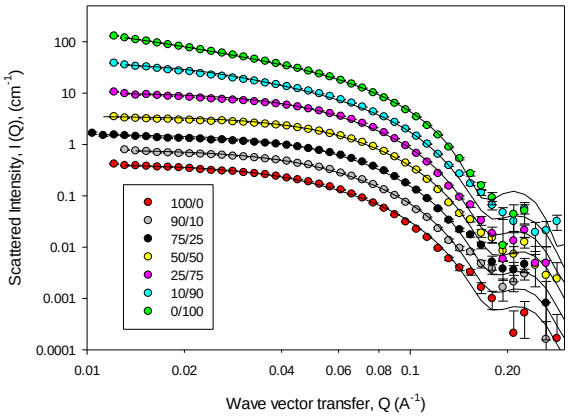
The results presented here are broadly consistent with other studies in the recent literature for the structure of escin and glycyrrhizic acid micelles (20, 22), and also as discussed recently (32). Matsuoka et al (20) used SAXS to characterise the micelle structure of glycyrrhizic acid and at a solution concentration of 5 mM and pH 5 observed rodlike structures, which were interpreted as rods with a radius of 15 Å and a length of 210 Å. Dargel et al (22) used SAXS and Fluorescence measurements to study escin self-assembly as a function of temperature, in the range 10 to 40 ºC. At low temperatures rodlike structures, with a radius of 18Å and a length of 91Å, were reported, whereas ellipsoids were observed at higher temperatures. In contrast the formation of fibril structures associated with gelation has been reported and exploited in particular circumstances and conditions for glycyrrhizic acid (29-31). In the data presented here there is no evidence for fibril formation, and the solutions involving glycyrrhizic acid remained in a fluid ungelled state throughout. This is consistent with the static and sheared SANS data in a previous study (32). In that study the sheared and static SANS data showed relatively modest elongation and suggests that in these conditions nanofibrillar network are a transient state.

(b) Saponin mixtures

The significant differences in the micellar structures for escin, tea and glycyrrhizic acid imply that some interesting effects would be observed in the self-assembly of saponin mixtures. SANS measurements were made for tea / glycyrrhizic acid, tea / escin, and escin / glycyrrhizic acid mixtures at a solution concentration of 5 mM and in the presence of 0.1 M NaCl. The results are shown in figure 3, and the key model parameters are summarised in table S3 in the Supporting Information.



(a)



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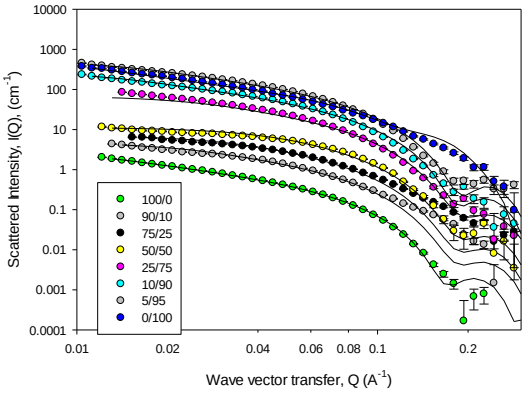


(c)

Figure 3. *SANS data for 5 mM saponin mixtures in 0.1 M NaCl, (a) tea / glycyrrhizic acid, (b) tea / escin, and (c) escin / glycyrrhizic acid, see legend for details. Each curve is shifted vertically with respect to the previous curve by x2. The solid lines are model calculations as*

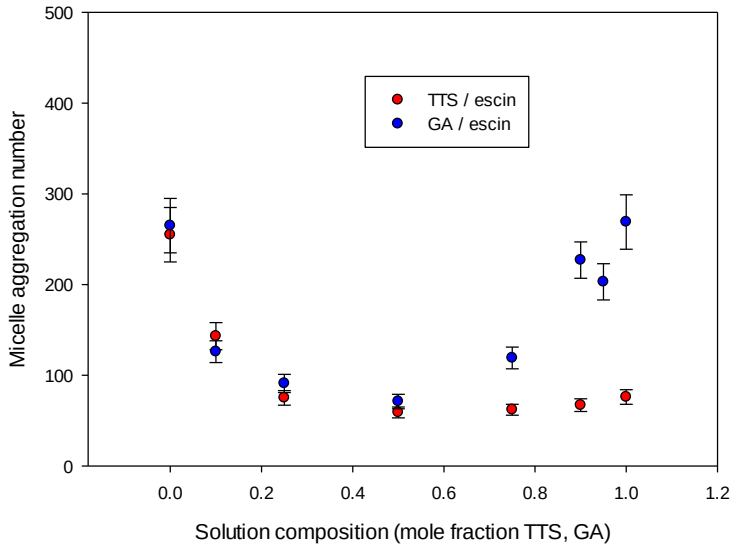
described earlier and in the Supporting Information, and for the key model parameters in tables S2 and S3. Error bars are included, but at low Q values they are ≤ the size of the plotting symbols.

The trends in the data in figures 3a and b for the mixtures involving the tea saponin show clearly the transition from the globular structures of the tea saponin to the more elongated structures of glycyrrhizic acid and escin. In figure 3c, for the escin / glycyrrhizic acid mixture, the evolution in the form of the scattering is more complex. The scattering from the escin and glycyrrhizic acid rich compositions are consistent with elongated structures, whereas at the intermediate compositions the scattering is consistent with the more globular structures.

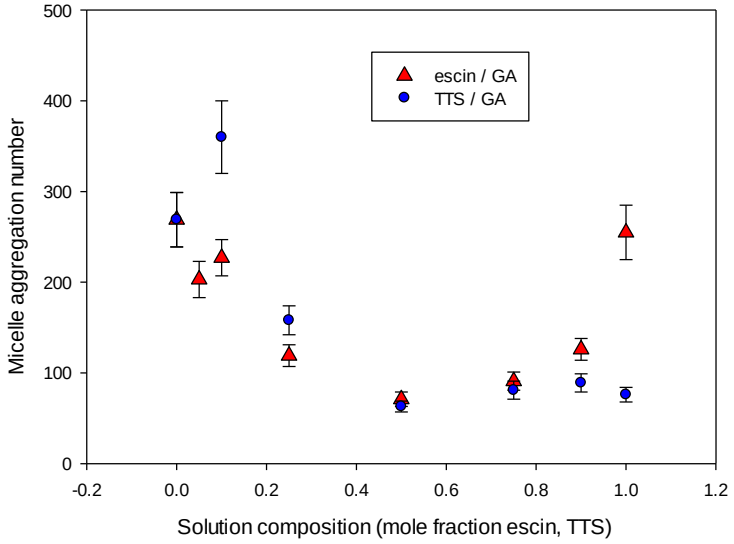
These trends are reflected in the variation in the key model parameters which are summarised in tables S2 and S3 in the Supporting Information; where the same modified core-shell model has been applied to all the data, with variations in the parameter ext and assuming the micelle compositions is the same as the solution composition. The Supporting Information contains a more detailed description of the model and its constraints.

As the cmc's are all relatively low, see table S1, and are ~ 0.1 mM, it is assumed that at the concentration of the measurements, 5 mM, the micelle composition is similar to the solution composition. On the basis of the interaction of saponins with a range of conventional surfactants (33, 34) the saponin-saponin interaction will be relatively weak, and so the micelle mixing will be close to ideal. The measurements were made at a solution concentration of 5 mM, and this is ~ 50 times the cmc. Both these factors justify the assumptions that the micelle composition is similar to the solution composition.

The values of ext vary across the composition range from their pure component values, and correspondingly the values of R_1 and R_2 vary (see table S3 in the Supporting Information). In figure 4 the variation in the micelle aggregation number with composition is shown.



(a)



(b)

Figure 4. *Variation in micelle aggregation number with solution composition for (a) glycyrrhizic acid / escin and tea / escin, and (b) escin / glycyrrhizic acid and tea / glycyrrhizic acid mixtures; see legend for details.*

For the tea / escin and tea / glycyrrhizic acid mixtures the micelle aggregation number is ~ 70 to 80 for the tea rich compositions, and at escin or glycyrrhizic acid rich compositions the aggregation number increases towards values associated with the more elongated structures of escin and glycyrrhizic acid, ~ 250 to 300. For the tea /glycyrrhizic acid mixture the onset of

growth occurs only once the composition is ~ 75 mole % glycyrrhizic acid (see figure 4b). For the tea / escin mixtures the transition occurs at even richer escin compositions, ~ 90 mole % escin (see figure 4a). For both mixtures there is a shallow minimum in the variation in aggregation number which is more pronounced for the tea / escin mixture, and in both case occurs at an equimolar composition. For both mixtures involving the tea saponin the structure and constraints associated with the tea saponin dominate the structure over much of the composition range. For the tea / escin mixture the model parameters ext is ~ 1.4 to 1.45 over the entire composition range, whereas for the tea / glycyrrhizic acid mixture it varies from 1.4 to 1.0.

The most striking variation in the aggregation number with composition is seen for the glycyrrhizic acid / escin mixture (see figure 4b). Although the aggregation number for the pure components, escin and glycyrrhizic acid, are both large, ~ 250 and 270 respectively, the aggregation number goes through a pronounced minimum at an equimolar solution composition, with a mean aggregation number ~ 70. Although the micelle aggregation number varies significantly across the composition range the structure is dominated by the constraints imposed by the escin component and the model parameter ext is ~ 1.4 over the entire composition range apart from the pure glycyrrrhizic acid data. The significant decrease in the micelle aggregation number as escin and glycyrrhizic acid form mixed micelles suggests that the packing associated with the two different headgroups results in an effective increase in the mean area / molecule which promotes the greater curvature associated with the smaller more globular micelle structures. Indeed it results in micelle structures similar to that encountered in the micelles formed by the tea saponin and tea rich saponin mixtures. This may be due to the additional carboxyl groups in the glycyrrhizic acid headgroup and due to the packing difficulties associated with the different number of saccharide groups in the escin and glycyrrhizic acid headgroups. In the tea / saponin (escin or glycyrrhizic acid) mixtures such contributions are not evident, as the micelle structure is already dominated by the larger headgroup of the tea saponin.

Although recent studies on the adsorption of escin and other saponins (6) and of the escin / surfactant mixtures (33, 34) imply that the saponins behave predominantly as nonionic surfactants, there is also evidence of the role of specific interactions and the involvement of the impact of steric constraints. This was particularly evident in the surface rheological properties (10-14). The surface and micelle mixing in escin / nonionic (polyethylene glycol, C_nEO_m) surfactant mixtures was reported to be close to ideal mixing (33). However the mixing

properties change as the ethylene oxide group size increased from EO_5 to EO_8, associated with the increasing constraints and the increasing impact of the incompatibility between the sugar and EO groups. Penfold et al (34), in studying the surface and micelle mixing in SDS / escin mixtures, showed that the synergistic mixing had both electrostatic and steric contributions, but was dominated by the steric component. In saponin / protein (40-42) it was shown that specific interactions were responsible for the surface adsorption properties, as illustrated particularly in their unusual surface rheological properties.

Of particular relevance to the data presented here is the work of Dai et al (39) who investigated the interaction of three ginsenosides, R0, Rb1 and Rg1, with different structures, with saikosaponin a, SSA. The different ginsenoside structures were shown to result in different self-assembled structures, where R0 formed vesicles, and Rg1 and Rb1 formed spherical micelles. Rg1 contained the least number of sugar groups and R0 contained a charged glucuronic group; and these differences impacted not only on the ginsenoside structure but also with their interaction with SSA and the resulting mixed structures. The greater number of sugar groups in Rb1 resulted in a stronger interaction with SSA, and both Rb1 and Rg1 formed spherical and wormlike micelles with SSA. The presence of the glucuronic acid group in R0 also resulted in vesicle structures in R0 / SSA mixtures.

These results on the mixing of saponins and of saponins with proteins and conventional surfactants reinforce the significance of the results presented here for the variation in the self-assembly of saponin mixtures. Furthermore they illustrate the importance of specific interactions and steric constraints associated with the different headgroup structures on the form of the self-assembly.

4. CONCLUSIONS

The surface activity of saponins is manifest in their adsorption and self-assembly properties. The adsorption of saponins at different interfaces has now been extensively studied by a variety of methods (6-15, 32), but the form of their self-assembly less so (16-36). Of particular importance in their potential for wider applications are their mixing properties with conventional surfactants, proteins and other saponins, and a range of studies have been reported (35-42). In the results presented in this paper SANS has been used to characterise the self-assembly of the saponin mixtures of tea / escin, tea / glycyrrhizic acid and escin / glycyrrhizic acid. A standard micelle core-shell model (45) is used to analyse quantitatively the data, in which the model constraints are adapted and adjusted to accommodate the different molecular

structures of the saponins compared to conventional surfactants (32, 35). The model and the data for the first time provide a relatively detailed description of the structure of tea, escin and glycyrrhizic acid micelles, and the way in which the structure changes on mixing. In particular it shows how the packing constraints associated with the different headgroup structures, through the different number of saccharide and carboxyl groups, impacts upon the micelle size and shape. For the saponin mixtures presented here the situation is reminiscent of that encountered in the conventional surfactant mixing (55). That is, the steric interaction which dominates the mixing arises predominantly from the disparity between the headgroup structures of the different saponins. However, what is different here is that disparity also has an impact upon the packing in the hydrophobic region, the inner core of the micelle. The results and interpretation provide the opportunity to develop, using complementary techniques such as molecular dynamics simulations, a more detailed understanding of the nature of the interactions and packing which drive the evolution in the micelle structure in such mixtures. In many of the future applications involving saponins, for example, in many of the possible medical application such as drug delivery (51-54), the nature of the self-assembly and how it can be manipulated are important factors in their potential. The mixing of different saponins with different structures provides a particularly attractive opportunity in the exploitation of biosustainable and biocompatible surface active components.

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AUTHOR CONTRIBUTIONS

All the authors have contributed to the different aspects of the paper, which include experimental design and measurements, analysis and interpretation of the data, preparation and editing of the manuscript, and management of resources: and specifically IMT, AB, REP,

RKT, JP, PXL, JRPW, KM, JD have been involved in the measurements, interpretation of the data and experimental design. JRPW, SLH in the management of resources, and IMT, RKT, JP, PXL, JRPW, SLH in the editing of the manuscript.

DECLARATION OF COMPETING INTERESTS

The authors declare that they have no competing financial interests or personal relationship that could have appeared to have influenced the work reported in this paper.

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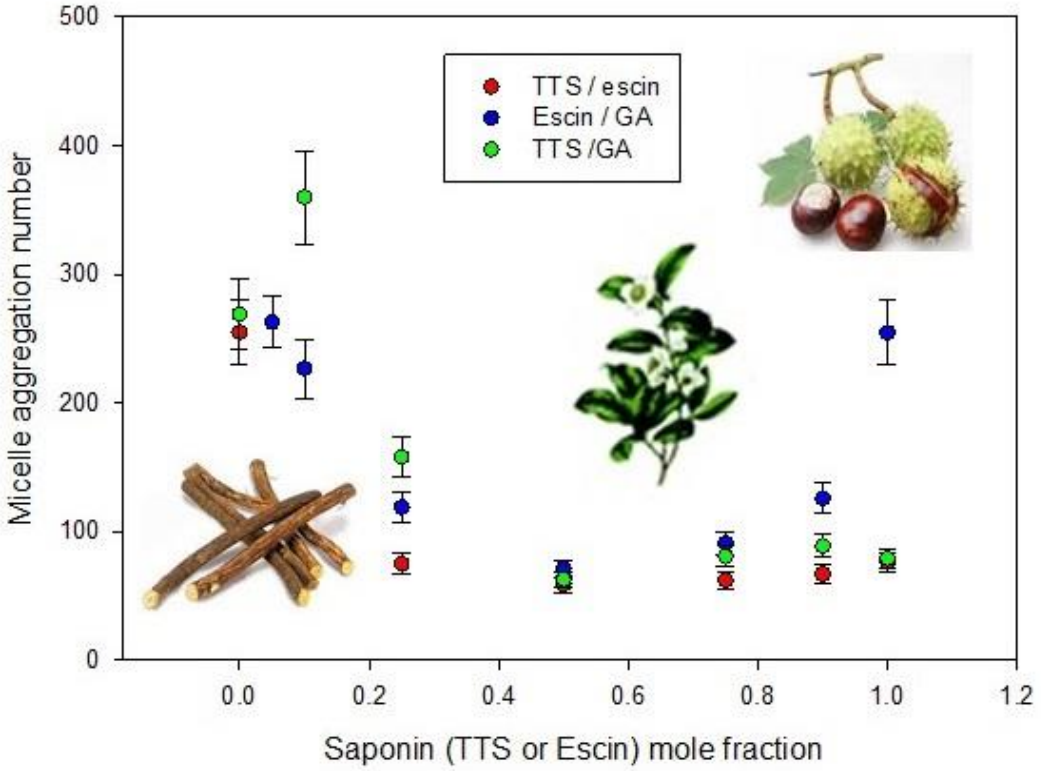
SUPPLEMENTARY INFORMATION

Some supplementary data, in the form of tables, and information relating to the SANS measurements and analysis, can be found online under Supplementary Information.

GRAPHICAL ABSTRACT



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SUPPORTING INFORMATION

Self-assembly of saponin mixtures: escin / tea, tea / glycyrrhizic acid, and escin / glycyrrhizic acid mixtures

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SANS ANALYSIS and MICELLE MODEL

The SANS scattered intensity is expressed in the decoupling approximation for solutions of globular interacting micelles as (45),

$$I(Q) = n\left[S(Q)\left|\langle F(Q)\rangle_Q\right|^2 + \left\langle \left|F(Q)\right|^2 \right\rangle_Q - \left|\langle F(Q)\rangle_Q\right|^2 \right] \qquad (1)$$

where <Q> denotes averages over all micelle sizes and orientation, and in the decoupling approximation it is assumed that there are no correlations between size and orientation, n is the micelle number density, S(Q) the inter-micelle structure factor, and F(Q) the micelle form factor. Where required S(Q) is calculated using the rescaled mean spherical approximation, RMSA, for a screened coulombic interaction between micelles (46, 47); which is determined by the micelle charge, z, micelle diameter, number density of micelles and the Debye-Huckel inverse screening length, κ. In the absence of charge S(Q) is simply the hard sphere S(Q) (48).

The micelle structure, defined by the form factor F(Q) is modelled using the well established core-shell model which has been extensively applied to a wide range of micellar systems formed from conventional surfactants (45). F(Q) is defined as,

$$F(Q)=V_1(\rho_1-\rho_2)F_0(Qr_1) + V_2(\rho_2-\rho_s)F_0(Qr_2) \qquad (2)$$

$$F_0(Qr_i) = 3j_i(Qr_i)/(Qr_i) \qquad (3)$$

where $j_i(Qr_i)$ is a first order spherical Bessel function, ρ_1, ρ_2, and ρ_s are the scattering length densities of the micelle core, shell, and solvent, and R_1 and R_2 are the radii of the core and shell.

The model is usually constrained such that the inner radius containing the hydrophobic alkyl chains (in the case of conventional surfactants) with a maximum dimension of the fully extended chain length, l_c. For aggregation numbers, ν, larger than can be accommodated by that constraint it is assumed that the micelle becomes elliptical, prolate ellipse, with core dimensions of R_1 and R_1.ee; where ee is the ellipticity. The outer radius, R_2, is defined by the space filling requirements of the headgroup and associated hydration. From known molecular volumes, dimensions and scattering lengths, F(Q) can be calculated. Within those constraints the key model parameters are then the aggregation number, ν, and the micelle charge, z. This approach generally works well for micelles from conventional surfactants (45) and for many mixed surfactant micelles (49).

More recently the same general approach has been applied to the analysis of saponin SANS data (32), and the SANS data for escin / SDS mixtures (33). The different structure of the saponins compared to conventional surfactants requires some modification to the relatively simple constraints of the model outlined above. The molecular dimensions, volumes and packing constraints are less well defined and in particular the maximum dimension of the core is less clearly definable. To accommodate these issues an additional factor, ext, is included; such that the inner core dimension is modified to l_c. Ext. Ext typically varies in the range 1.0 to 1.45. The key model parameters are then, ν, ext and z (if required). The key molecular dimensions and scattering lengths of the different saponin components used in the modelling are summarised in table S4.

TABLES

Table S1. *Key Molecular parameters for saponins*

Saponin	**MW (g/mol)**	**CMC (mM at natural pH)**	**Main structural features**	**Limiting area/molecule (±2 Å²)**
escin	1101	0.11	3 saccharide groups 1 carboxyl group	69
glycyrrhizic acid	822	~0.10	2 saccharide groups 3 carboxyl groups	87
tea	1259	0.30	4 saccharide group 1 carboxyl group	79

Table S2. *Key model parameters from analysis of SANS data for 5 mM tea, escin and glycyrrhizic acid in 0.1 M NaCl*

Saponin	**Aggregation number, ν**	**ext (±0.05)**	**R_1 (±1Å)**	**R_2 (±1Å)**	**Ellipticity, ee**
tea	76±8	1.40	20	25	3.0±0.5
escin	255±30	1.45	20	25	9.0±1
glycyrrhizic acid	269±30	1.00	14	19	32±1

Table S3. *Key model parameters from analysis of SANS data for 5 mM saponin mixtures in 0.1 M NaCl.*

(a) Tea / glycyrrhizic acid

Solution composition (mol ratio)	**Aggregation number, ν**	**ext (±0.05)**	**R_1 (±1Å)**	**R_2 (±1Å)**	**Ellipticity, ee**
100/0	76±8	1.4	20	25	3.0±0.05
90/10	89	1.4	20	25	3.5
75/25	81	1.4	20	25	3.2
50/50	63	1.2	17	21	4.0
25/75	158±16	1.1	15	19	13.0±1
10/90	360±40	1.1	15	19	29.0
0/100	269±30	1.0	14	19	32.0

(b) Tea / escin

Solution composition (mol ratio)	**Aggregation number, ν**	**ext (±0.05)**	**R_1 (±1Å)**	**R_2 (±1Å)**	**Ellipticity, ee**
100/0	76±8	1.4	20	25	3.0±0.05
90/10	67	1.4	20	25	2.6
75/25	62	1.4	20	25	2.4
50/50	59	1.4	20	25	2.3
25/75	75	1.45	20	25	2.7
10/90	145±15	1.45	20	25	5.0
0/100	255±30	1.45	20	25	9.0±0.1

(c) Escin / glycyrrhizic acid

Solution composition (mol ratio)	Aggregation number, ν	ext (±0.05)	R_1 (±1Å)	R_2 (±1Å)	Ellipticity, ee
100/0	255±30	1.45	20	25	9.0±0.1
90/10	126±15	1.4	20	24	5.0±0.05
75/25	91±10	1.4	20	24	3.6
50/50	71±8	1.4	20	24	2.8
25/75	119±10	1.4	20	24	4.7
10/90	227±25	1.4	20	24	8.9±0.1
5/95	203±20	1.4	20	24	8.0
0/100	269±30	1.0	14	17	32.0±0.1

Table S4. *Molecular dimensions and scattering lengths for the different saponin components*

Saponin	Headgroup volume ($Å^3$)	Headgroup scattering length ($x10^{-3}$ Å)	Aglycone volume ($Å^3$)	Aglycone scattering length ($x10^{-3}$ Å)	l_c (Å)
escin	744	5.22	1240	10.62	14
Glycyrrhizic acid	496	4.81	1240	10.62	14
tea	992	9.62	1240	10.62	14