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Experimental Modelling of Flavonoid-Biomembrane Interactions

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Flavonoid, Interaction, DOPC, Biomembrane Model, Rapid Cyclic Voltammetry, Langmuir trough, BAM, SAXS
ABSTRACT: Non-specific interactions of flavonoids with lipids can alter the membrane’s features (e.g. thickness and fluctuations) as well as influence their therapeutic potentials. However, relatively little is known about the details of how flavonoids interact with lipid components. Structure-dependent interactions of a variety of flavonoids with phospholipid monolayers on a mercury (Hg) film electrode were established by rapid cyclic voltammetry (RCV). The data revealed that flavonoids adopting a planar configuration altered the membrane properties more significantly than non-planar flavonoids. Quercetin, rutin and tiliroside were selected for follow-up experiments with Langmuir monolayers, Brewster Angle Microscopy (BAM) and small angle X-ray scattering (SAXS). Relaxation phenomena in DOPC monolayers and visualization of the surface with BAM revealed a pronounced monolayer stabilization effect with both quercetin and tiliroside, whereas rutin disrupted the monolayer structure rendering the surface entirely smooth. SAXS showed a monotonous membrane thinning for all compounds studied associated with an increase in the root mean square fluctuations of the membrane. Rutin, quercetin and tiliroside decreased the bilayer thickness of DOPC by ~0.45 Å, 0.8 Å, and 1.1 Å at 6 mol %, respectively. In addition to the novelty of using lipid monolayers to systematically characterize the structure activity relationship (SAR) of a variety of flavonoids; this is the first report investigating the effect of tiliroside with biomimetic membrane models. All the flavonoids studied are believed to be localized in the lipid/water interface region. Both this localization and the membrane perturbations have implications for their therapeutic activity.
Introduction

Flavonoids are of great importance due to their potential antioxidative, anti-inflammatory and immune regulatory actions and many epidemiologic studies show a relationship between flavonoid consumption in the diet and reduced risk of several chronic diseases, including cancer and diabetes.\textsuperscript{1,2,3} Quercetin, one of the most widely studied flavonoids, has been claimed to be a better mast cell stabilizer than Cromolyn which is the only related pharmaceutical available on the market.\textsuperscript{4} Quercetin was found to regulate allergic reactions by limiting the release of mediators that trigger allergy, such as histamine, prostaglandin DS (PGD2), tumor necrosis factor (TNF) and several cytokines like IL-8.\textsuperscript{4} Similarly, rutin, a glycoside of quercetin, was demonstrated to serve as an anti-inflammatory agent in the treatment of osteoarthritis, as monitored via the decrease in the serum level of a type II collagen biomarker, Coll2-1.\textsuperscript{5}

The molecular mechanism of action of flavonoids is a longstanding debate. Although their mechanism has been widely attributed to specific interactions involving protein-flavonoid binding, recent studies have intimated that flavonoids alter biomembrane organization which might then lead to modifications in membrane protein function.\textsuperscript{6,7,8} The simplest techniques which can be used to test this hypothesis involve the use of biological membrane models with associated biophysical techniques. The advantages of these systems arise from their reduced complexity and improved experimental control compared to cell cultures.\textsuperscript{9,10}

In spite of the work done looking at the biological activity of flavonoids, the interaction mechanism between flavonoids and model biological membranes has not yet been fully understood and the literature so far remains controversial. Flavonoid concentration and lipid
membrane composition as well as the diversity of techniques employed could contribute to the variation of the results.\textsuperscript{11}

One motivation of this study was to use a very well characterized system to avoid the influence of an additional variable involved in flavonoid-membrane interactions. For this reason, we have excluded cholesterol as many other authors have done\textsuperscript{6}. Hence, unlike many conventional membrane models of liposome bilayers, this study aimed to use monolayer membrane models in proof of concept experiments with one lipid type; 1,2 dioleoyl-sn-glycero-3-phosphocholine (DOPC) to screen flavonoid interactions. For this, a number of techniques including an on-line high-throughput sensing system, custom designed for biomembrane interactions, were applied.\textsuperscript{12}

This system is based on an electrochemical membrane model consisting of a DOPC monolayer coated mercury (Hg) electrode and rapid cyclic voltammetry (RCV), which allows rapid screening of large numbers of compounds and has been extensively used by other workers in the area.\textsuperscript{13,14,15}

Although this sensing device was previously used to screen a number of membrane active compounds from toxins to drugs, the current work aimed to reinforce the sensing system for food ingredients for the first time. A successful application of such a sensing system can rapidly establish a structure activity relationship of a variety of food compounds with membranes.

Subsequent to the initial RCV screening, selected flavonoids were studied via classic spread Langmuir monolayers at the air-water (A-W) interface. The aim of using such a conventional method with free-standing monolayers was to correlate the interactions observed on the DOPC adsorbed Hg electrode. Moreover, Langmuir monolayers are excellent systems to monitor film stability. Such information is especially useful in investigation of the therapeutic effects of flavonoids. As far as we are aware, the interaction of only one flavonoid, quercetin, with
phospholipid monolayers has been studied in detail previously, whereas in this study a range of flavonoids have been studied. Surface pressure ($\pi$) versus area per molecule ($A$) isotherms facilitated study of the adsorption and penetration of the flavonoids on and into DOPC monolayers. In addition, a direct observation of the monolayer stability in the absence and presence of the test compounds was made by measuring the desorption rate of the spread monolayers at constant surface pressure.\textsuperscript{16} The adsorption and stability behavior of the monolayers was also studied by Brewster Angle Microscopy (BAM).

Finally, characterization of flavonoid lipid interactions was achieved using phospholipid vesicles, a step up in complexity from monolayers. Small angle X-ray scattering (SAXS) was performed to quantitatively analyze the interactions between flavonoids and lipid multilamellar vesicles (MLVs) in terms of alterations in (i) lattice spacing, (ii) membrane thickness, (iii) water layer thickness, and (iv) positional membrane fluctuations.

All three model systems above used DOPC as the lipid component for several reasons. The fluid nature of DOPC is highly compatible with fluid Hg, which allows the spread monolayer to be defect-free and self-sealing.\textsuperscript{17} Furthermore, DOPC MLVs are the most preferred systems for SAXS studies since they produce a stable lamellar (L\textsubscript{α}) phase which is ubiquitous in real biological membranes, unlike non-lamellar lipids.\textsuperscript{18,19}

To the best of our knowledge, this is the first systematic study investigating both monolayers and bilayers for interactions of a wide range of flavonoids with biomembrane models. A consensual view was derived from the data using a `cross platform` approach via distinctly varying techniques which give complementary information on the physical, chemical mechanism of interactions. A range of flavonoids (Figure 1) with significantly different structures was
selected for study based on: the existence of double bond in the ring C, the degree of hydroxylation and the presence of sugar group moiety.

**Experimental Methods**

**Materials**

DOPC with the purity of > 99% was purchased from Avanti Polar Lipids (USA). The flavonoids quercetin dihydrate, rutin trihydrate (quercetin-3-β-D-rutinoside), naringenin, hesperetin, (+) - catechin hydrate and naringin were supplied by Sigma-Aldrich (Germany). Kaempferol and tiliroside were obtained from Extrasynthese (France). Phosphate buffered saline (PBS) powder was used to prepare the pH 7.4 buffer solution, and was obtained from Sigma-Aldrich. For monolayer studies, one batch of PBS was dissolved in one liter of Milli-Q water (Millipore Inc., Ω = 18.2 MΩ.cm) to give 0.01 M phosphate, 0.138 mol dm⁻³ NaCl + 0.0027 mol dm⁻³ KCl. Ethanol (absolute, AR, Merck) and dichloromethane (anhydrous, ≥ 99.8%, Sigma-Aldrich) were used as received without further purification. A flavonoid concentration of 10 µmol dm⁻³ was chosen for monolayer studies as it is in the range of physiological uptake concentrations in body²⁰ and a dose-dependent response was investigated in this range via RCV.

**Electrochemical measurements**

A stock solution of flavonoids dissolved in ethanol and a stock solution of DOPC (2 mg ml⁻¹) in PBS buffer were freshly prepared prior to experiments.

The microfabricated platinum (Pt) electrodes (MPE) (Tyndall National Institute, Ireland) were composed of eight 0.48 mm radius discs and used as working electrodes. Their cleaning procedure has been previously described in detail.¹² Electrodeposition of Hg on Pt electrode was performed in a standard three electrode cell that contains Ag/AgCl, 3.5 mol dm⁻³ KCl as reference electrode and platinum rod as a counter electrode. Electrodes were connected to an
Autolab PGSTAT 30 potentiostat (Eco Chemie, Utrecht, Netherlands) interfaced to a PowerLab 4/25 signal generator (AD Instruments Ltd.) monitored by Scope™ software. Current vs. potential RCV scans were recorded via the software at a scan rate of 40 V s⁻¹. Prior to lipid deposition, the surface of Hg is cleaned in situ by flushing the system with PBS buffer at the voltage of ~ -3.0 V and 100-200 µL of the lipid solution was injected into the system at the same voltage, as described previously. After obtaining a stable DOPC monolayer on Hg, the flavonoids from the stock solutions were introduced into the flow cell at the predetermined concentrations and alterations in the monolayer were monitored by RCV with a total sampling time of 5 minutes by cycling the potential from -0.4 to -1.2 V. The ethanol concentration did not exceed 1 % in the final solution and all controls in this study refer to the control with exposed ethanol alone.

Surface pressure-area isotherms of Langmuir monolayers

A specialist in-house Langmuir film balance featuring a rhomboidal shape PTFE barrier whose set up was described in detail previously, was used throughout this work. The surface pressure-area (π-A) isotherms were produced by a Wilhelmy plate of roughened mica (~3 cm) which was dipped into the subphase at the center of the trough.

A lipid solution of DOPC was prepared in an ethanol : dichloromethane mixture at the ratio of 1:9. A monolayer was obtained by spreading DOPC solution (0.2 mg/mL) over the subphase of PBS buffer (pH, 7.4). Following solvent evaporation for 15 minutes, the monolayer was slowly compressed at the rate of 8.6 mm² s⁻¹ (corresponding to 3.6 Å² min⁻¹), starting from an initial (maximum) through area of 22 500 mm². This compression rate is considered to be slow enough to represent the “true equilibrium” isotherm since decreasing the compression rate further created no difference in the π-A isotherms. The monolayer was compressed to π = 30 mN m⁻¹, which
corresponds to a typical biological membrane pressure\(^2\), and flavonoids were injected beneath the monolayer to give a final subphase concentration of 10 µmol dm\(^{-3}\). The system was then allowed to stabilize for around 20 minutes after flavonoid injection and the film stability was monitored as a function of the change in the trough area by maintaining the pressure constant at 30 mN m\(^{-1}\) for one hour. Measurement of area loss at \(\pi = 30\) mN m\(^{-1}\) was detected for DOPC monolayer alone and in the presence of flavonoids by extrapolating the trough area \((A)\) at time \(t\), relative to the initial starting area \((A_o)\), i.e. \(A/A_o\) as a function of time.

At the end of 1 h, the monolayer was expanded to its maximum area and recompressed to the collapse point. Recompression was done to observe any changes in the \(\pi-A\) isotherm indicative of flavonoid-monomonolayer interactions.

**Brewster Angle Microscopy (BAM)**

Brewster Angle Microscopy is a widely used technique for studying thin film structure on liquid surfaces. The BAM system and its operation described in detail elsewhere\(^2\) and only brief details are presented here. A BAM2plus Brewster Angle Microscope (NFT, Gottingen, Germany), combined with the Langmuir trough above was employed to visualize the morphology of the DOPC monolayers before and after flavonoid addition. The laser output power was kept constant at 18% for all experiments and the shutter speed for the camera was fixed at 1/50 s at the Brewster angle of 53.15\(^\circ\) for the pure A-W interface. The analyzer and polarizer angles were set to zero. Since there is no p-polarized reflection from pure water at the Brewster angle, in principle BAM only records the reflected intensity arising from the surface film at the interface. Although the lateral resolution of the BAM2 images is considerably less (~2 µm) compared to fluorescence microscopy, its advantage arises from being a completely non-invasive technique without requiring sample labelling.\(^2\)
Small Angle X-ray Scattering (SAXS) Experiments

For the X-ray experiments, MLVs were prepared according to standard protocols.\textsuperscript{25} Briefly, a DOPC stock solution was made by dissolving 10 wt.% of lipid in ethanol. Similar ethanol stock solutions of flavonoids (quercetin, rutin or tiliroside) were also prepared, then each mixed with the DOPC solution resulting in a final concentration of 6 mol % of flavonoid. Each mixture was vortexed for 2 minutes and the solvent was evaporated in a vacuum oven for 24 h. After obtaining dry thin films at the bottom of the sample tube, 90 µL of water of approximately 0.1 mM ionic strength, pH: 7.1 (PBS buffer diluted 100x to prevent osmotically induced inhomogeneity of MLV bilayer stacking) was added into the sample tube to fully hydrate the films.

The operated SAXS camera set-up (SAXSpace, Anton Paar, Austria) is described in great detail elsewhere.\textsuperscript{26} Briefly, a collimation block unit vertically focuses a line shaped beam of Cu-Kα radiation with a wavelength, $\lambda = 0.154$ nm on to the detector plane. For the SAXS experiments, the high resolution mode was chosen, which permits detection of a minimum scattering vector, $q_{\text{min}}$, of 0.04 nm\(^{-1}\) ($q = (4\pi/\lambda) \sin\theta$, where $2\theta$ is the scattering angle). All studied samples were filled into the same vacuum-tight, reusable 1 mm quartz capillary to guarantee exactly the same scattering volume. The experiments were performed at 25 °C with a temperature stability of 0.1 °C. A Mythen X-ray detector (Dectris Ltd, Baden, Switzerland) system was used to record the 1D scattering patterns.

SAXStreat software (Anton Paar, Graz, Austria) was used to fine tune the primary beam position, before subtracting the background from water, capillary and air using the SAXSQuant software (Anton Paar, Graz, Austria). Background subtracted SAXS patterns have been analyzed according to the modified Caillé theory. The technique and underlying premises have been
described previously in depth\textsuperscript{27,28} – for reviews see Rappolt et al.\textsuperscript{29} and Pabst et al.\textsuperscript{30} The bilayer model used to interpret the data and its applications have also been described previously.\textsuperscript{31} Lamellar repeat distance $d$ and the head-to-head group thickness, $d_{\text{HH}}$ were directly obtained from the fits to the scattered intensities $I = S(q)|F(q)|^2/q^2$, ($S(q) =$ structure factor; $F(q) =$ form factor). Mean square fluctuations of the membrane position, $\sigma$ were derived from the Caillé parameter, $\eta$,

$$\sigma = \sqrt{\frac{d}{\pi}}$$

(1)

where $d_i$ is the lamellar repeat distance.
Results and Discussion

Electrochemical Screening of flavonoid-DOPC interactions

RCV plots for the DOPC monolayers with and without flavonoid at the studied concentrations are presented in Figure 2. It can clearly be seen that introduction of some compounds leads to a significant change in the current versus voltage plot. An alteration in the peak height, position and shape of the current peaks results in a “finger-print” RCV profile for each class of compounds. In RCV measurements with a DOPC coated electrode, suppression of the capacitance current peaks with little effect on the capacitance current baseline is representative of species adsorbing on the monolayer surface. A clear increase in the baseline capacitance current with depression of the capacitance peaks shows a penetration or disruption of the layer by compounds since the low dielectric constant of the monolayer's apolar core is disturbed.32

At the concentrations of 10 µmol dm$^{-3}$ (red line in Figure 2), the most significant changes in DOPC properties following introduction of flavonoids were seen with quercetin and kaempferol represented as a total capacitance peak current suppression and an increase in capacitance baseline height. Tiliroside showed a smaller effect; naringenin, hesperetin and catechin displayed an apparently even weaker interaction with DOPC – displayed as an intermediate capacitance peak current suppression and rutin and naringin apparently displayed no significant interaction with DOPC at all. The increase in baseline capacitance current following quercetin and kaempferol addition is strong evidence that these compounds penetrated the DOPC layer whereas the remaining compounds probably remained adsorbed on the surface.

The effect of flavonoids was found to be concentration dependent; at increased concentrations of 35 µmol dm$^{-3}$ (blue line-Figure 2), majority of the compounds revealed a more pronounced
effect. A slight response was even observed with rutin at this concentration along with an
indistinct hump formation on the baseline, but naringin still displayed no interaction.

The results above suggest that the interactions are directly related to the compound structure,
especially to their conformations. Quercetin and kaempferol show the strongest interaction with
significant penetration of the DOPC monolayer and they possess two coplanar rings whereas the
third ring is oriented at right angles. Presumably the planar two rings facilitate the interaction and
penetration of the compound within the membrane. However, even such strong interactions were
found to be reversible and DOPC membrane could be recovered, indicating flavonoids do not
replace or destabilize the membrane.

The insignificant interactions of rutin and naringin with DOPC could be related to the two
glycoside groups attached to quercetin and naringenin, respectively, which sterically hinder any
significant interaction. Compounds showing intermediate interaction with and no penetration of
the DOPC monolayer include naringenin, hesperetin and catechin. Each of these compounds has
the common structural characteristic where the second ring of the two-ringed structure is kinked,
which might hinder interaction. Tiliroside is a more bulky molecule and exhibits a stronger
interaction than the other three molecules. Tiliroside has one glycoside group positioned between
the flavonoid moiety and a further ring but the flavonoid group contains the two ringed planar
moiety which could account for the stronger interaction as with quercetin and kaempferol.

Detection limits in terms of the minimum concentrations of the compounds which exerted a
significant effect on the monolayer structural properties were calculated based on the RCV
measurements. The first current peak was used in estimation of the limit of detection (LOD) and
a detailed procedure for LOD calculation has been described previously. A rank order of the
LOD values is displayed in Table 1. These follow the general order of compound interaction as
described in the paragraph above. Furthermore the estimated LOD values are plotted against the corresponding log octanol-water partition confections (log $P$) are shown in Figure 3. The log $P$ value has been widely used to characterize the hydrophobicity of molecules and the tendency for flavonoids to interact with bio-mimetic membranes.$^{11}$ However, it can clearly be seen from Figure 3 that there is no systematic correlation between the LOD value and log $P$. The finding that flavonoid structure is the critical factor in determining its interaction with phospholipid membranes is commensurate with other studies, suggesting that in the presence of molecules of comparable hydrophobicity, the detailed structural properties of the molecules need to be carefully taken into consideration.$^{33,11,34}$

It is interesting to put these results in the context of those from other studies. Previous studies confirm that the existence of a C2-C3 double bond in the structure of quercetin and kaempferol renders these compounds more planar$^{35}$ and consequently results in stronger interactions with membrane components, compared to the non-planar configurations of naringenin and hesperetin.$^{34}$ One of the very first studies comparing membrane interactions of quercetin, rutin, naringenin and hesperetin with DPPC liposomes, using differential scanning calorimetry (DSC), revealed that the strongest flavonoid-DPPC interaction were observed with quercetin.$^{36}$ These findings were supported by further studies,$^{34}$ investigating the affinity of a set of flavonoids, including quercetin and naringenin, towards artificial vesicles via fluorescence quenching of the membrane probe 1,6-diphenyl-1,3,5-hexa-triene (DPH). Quercetin was found to have a higher membrane affinity compared to naringenin. Both studies attributed such a response to quercetin’s planar structure, and proposed that the tilted configuration of naringenin and hesperetin rendered them less likely intercalate into the ordered structures of the packed phospholipid layers.$^{34}$
The effect of glycoside moieties in hindering flavonoid/lipid membrane interaction has been observed elsewhere. The disaccharide moiety of rutin hinders its ability to interact with DPPC bilayers by rendering the molecule less hydrophobic.\textsuperscript{36} Similarly, hesperetin (HT) was found to be more interactive with DMPC liposomes than its glucoside hesperidin (HD).\textsuperscript{37} Biophysical studies in conjunction with human studies reported that absorption of aglycones was not only faster but also in five times greater than their glucoside forms.\textsuperscript{38}

**Interactions of selected flavonoids with air-water monolayers of DOPC**

On the basis of the RCV results, the aglycone (i.e., without the sugar) quercetin, and two glycosides - tiliroside (one sugar moiety in the structure) and rutin (two sugar moieties in the structure), were selected for follow-up experiments with Langmuir monolayers, since they appeared to exhibit particularly representative effects of varying degrees of interaction.

As seen in Figure 4a, the introduction of quercetin (10 µmol dm\(^{-3}\)) into the subphase did not provoke an immediate increase in the monolayer area at low surface pressures (below 5 mN m\(^{-1}\)). However, the monolayer exhibited a more expanded isotherm when the pressure was increased further. The isotherm shifted towards a larger area per (DOPC) molecule (~ 67 Å\(^2\)) compared to that of the pure DOPC monolayer (~ 60 Å\(^2\)) at a surface pressure of 30 mN m\(^{-1}\). It is assumed this shift occurred due to the area occupied by quercetin molecules inserted into the monolayer, and is observed all along the isotherm, indicating that the molecule remained in the monolayer at even higher surface pressures. This result is in agreement with the only other study of quercetin interacting with Langmuir monolayers of phospholipid (DPPC), published recently.\textsuperscript{39} Similar to the present findings, the authors reported an increase in surface pressure due to the effective incorporation of molecule into the model lipid membrane.\textsuperscript{39} In the same study, a \(\pi-A\) isotherm for pure quercetin spread on the air/water interface was also produced with a maximum
surface pressure of just above 20 mN m\(^{-1}\) before the collapse.\(^{39}\) Based on the area per quercetin molecule \((A_{\text{flav}} = 89 \, \text{Å}^2)\) at \(\pi = 20 \, \text{mN m}^{-1}\), taken from this study, we attempted to quantify the amount of adsorption of quercetin into the DOPC monolayers, assuming ideal mixing. In other words, the mole fraction of quercetin \((f_{\text{flav}})\) in the mixture was calculated via:

\[
A_{\text{total}} = (1-f_{\text{flav}}) \, A_{\text{lipid}} + f_{\text{flav}} \, A_{\text{flav}} \quad (2)
\]

where, \(A_{\text{total}} (78 \, \text{Å}^2)\), \(A_{\text{lipid}} (72 \, \text{Å}^2)\) and \(A_{\text{flav}} (89 \, \text{Å}^2)\) signify the area per molecule in the mixed monolayer, area per lipid molecule and area per quercetin molecule, respectively. In the current study, \(f_{\text{flav}}\) was found as around 35\% which shows a high passive adsorption of quercetin molecule into DOPC monolayers.

**Figure 4b** shows the compression isotherm of DOPC monolayer in the presence of tiliroside (10 \(\mu\)mol dm\(^{-3}\)). Unlike quercetin, tiliroside injection at a surface pressure of 30 mN m\(^{-1}\) caused a very distinct increase in the molecular area of the DOPC monolayer at low surface pressures only. On compression to higher surface pressures, a decrease in the molecular area is observed, suggesting molecules being expelled from the film.

At first glance, **Figure 4c** suggests no significant effect of rutin injected beneath DOPC monolayers, implying there is no definitive interaction. However, further surface characterization via BAM indicated significant effects of this compound on the monolayer (see later). A recent study pointed to a similar behavior of pure DOPC monolayers in the presence of the cationic photosensitizer methylene blue (MB)\(^{40}\), that has almost the same log \(P\)\(^{41}\) as rutin (\(\sim 0.9\)). Although, the surface pressure of DOPC remained unaltered with MB incorporation, further analysis using polarization-modulated infrared reflection-absorption spectroscopy (PM-IRRAS) revealed the adsorption of MB onto the polar surface which then further leads its binding close to the double bonds of the hydrophobic chains.
Film loss and hence contraction of the spread monolayer films is inevitable when monolayers are maintained at a constant surface pressure lower than the equilibrium spreading pressure ($\pi < \pi_e$) over a certain period of time.\textsuperscript{42} $\pi_e$ is the maximum surface pressure ($\pi_{eDOPC} = 46.1 \text{ mN m}^{-1}$) which monolayers can be compressed to before they collapse. Relaxation of the spread films arises from desorption of molecules into the bulk phase at $\pi$ values lower than $\pi_e$.\textsuperscript{43} This phenomenon gives information about the film stability. The effect of flavonoids on the molecular area relaxation for DOPC monolayers is demonstrated in Figure 5 at a constant $\pi$ of 30 mN m$^{-1}$. The change in area $A$, relative to the maximum area $A_0$, was monitored as a function of time, $t$. Here, note that the saw-tooth appearance on Figure 5 is due to the discrete time step between alterations of the barrier position to maintain constant $\pi = 30 \text{ mN m}^{-1}$. The striking membrane-stabilizing action of quercetin, suggesting lack of phospholipid desorption from the monolayer, is observed in Figure 5a. A similar stabilizing effect was observed also with tiliroside Figure 5b.

Membrane stabilization is an important phenomenon since it is believed to be a key mechanism by which many drugs exert their beneficial effects. A recent study indicated quercetin as a better mast cell `stabilizer` than Cromolyn, which is the only drug available on the market.\textsuperscript{4} Moreover, molecular dynamics simulations, NMR and imaging techniques were used to probe the membrane stabilizing effect of quercetin on DPPC bilayers\textsuperscript{44} and quercetin was found to bind to the membrane surface via hydrogen bonds at the lipid/water interface.\textsuperscript{44} Apart from quercetin, some other flavonoids have also been reported to exhibit stabilizing or destabilizing effects. Such observations were made with milk thistle flavonoids: silybin and dihydroxyquercetin (taxifolin). Dihydroxyquercetin stabilized the membranes whilst silybin destabilized the lipid layers.\textsuperscript{45}
Topographical Characteristics of DOPC monolayers

In agreement with previous studies, pure DOPC monolayers formed 2D foam-like structures with circular shapes and small domains within their interior at very low surface pressures (data is not shown). All these structures disappeared during monolayer compression and no visible features were seen up to the collapse point ($\pi \approx 40$), giving a homogeneous liquid expanded phase only. A few bright features were formed and flickered at the collapse point, which is assumed to be an indication of multilayer formation. These low density of bright ‘crystallites’ were observed with quercetin and tiliroside throughout the compression. Rutin, however, did not alter the observed morphology of the DOPC monolayer.

The images in Figure 6 illustrate the monolayer structure after the monolayer had been fully compressed to the collapse pressure ($\pi \approx 41$ mN m$^{-1}$). Figure 6a shows the formation of large lipid domains with pure DOPC. Although the molecular mechanism behind monolayer collapse is not completely understood, it is well known that compressing monolayers beyond the collapse pressure causes 2D to 3D transitions by the formation of the lipid aggregates. However, these aggregates, which appear as small, moving islands, were completely stabilized in the presence of quercetin and tiliroside (Figure 6b,c) and the monolayer appearance was still unaltered even after several hours (data not shown). In the presence of rutin (Figure 6d), the monolayer structure was completely disrupted and all typical DOPC domain structures disappeared. In other words, introduction of rutin rendered the monolayer appearance entirely smooth and devoid of lipid islands. One might think that the smooth appearance of the film in the presence of rutin could be due to the adsorption of the flavonoid. However, if this was the case, a different trend should have been observed in the RCV plots. Adsorption in RCV traces is characterised by the suppression of the peaks, such as in the case of silica nanoparticles, but this was not observed.
Rutin adsorption is therefore an unlikely explanation and the smoothness more probably due to modification of the lipid organisation in the monolayers.

**SAXS measurements of bilayer flavonoid interactions**

Small angle X-ray scattering (SAXS) experiments were performed to structurally characterize both fully hydrated pure DOPC MLVs and flavonoid loaded vesicles. 6 mol % of flavonoid was chosen for the SAXS study because this concentration was high enough to differentiate between the effects of the different flavonoids but low enough so as not to exceed their solubility limits in the systems. All scattering patterns and their best fits and refined electron density profiles (EDPs) are displayed by solid lines in Figure 7a and Figure 7b, respectively. EDPs derived from the applied global fitting procedure allowed the determination of structural bilayer parameters summarized in Figure 8. These parameters are the (i) lattice spacing, \( d \), (ii) phosphate-to-phosphate distance within a bilayer, which is also known as head-to-head group thickness, \( d_{HH} \), (iii) water layer thickness \( (d_w = d - d_{HH}) \) and (iv) the Caillé parameter, \( \eta \), from which the mean fluctuations, \( \sigma \), of the membrane position can deduced according to Equation 1.

The observed structural behavior of pure DOPC bilayers agrees well with literature findings. While we observed a \( d \)-spacing of 62.5 Å and \( d_{HH} \) of 36.2 Å at room temperature, Nagle et al., determined values of 63.1 Å and 36.7 Å, respectively, at 30 °C.

Remarkably, Figure 8a shows that the lattice spacing, \( d \), do not change significantly by the addition of flavonoids (less than 1% variation), but a closer look at the membrane thickness, \( d_{HH} \) (Figure 8b), reveals that all compounds studied lead to a membrane thinning effect (up to 5%). Rutin, quercetin and tiliroside decrease the bilayer thickness of DOPC by ~0.45 Å, 0.8 Å and 1.1 Å, respectively. Although the thinning in the presence of rutin is not as large, it clearly still
requires some interaction of rutin with the bilayers. The only existing study in literature, characterizing flavonoid-DOPC systems via SAXS was conducted by Raghunathan et al., which focused on membrane thickness and for the flavonoids genistein and daidzein only: a similar membrane thinning effect was revealed in their combined experimental and simulation study. As shown with Figure 8c and 8d, the membrane thinning is accompanied by an increase in the water layer thickness, \( d_w \), and a concomitant increase also in the fluctuation parameter, \( \sigma \), with tiliroside displaying the largest (6.6 Å; +25%) and rutin the smallest (6.1 Å; +15%) values, which compare to positional membrane fluctuations of 5.3 Å in the pure bilayer system. We note that the fluctuation parameter depends on both the bulk compression modulus and the bending modulus of the membrane itself. \(^{27}\) It is necessary to interpret any flavonoid induced membrane thinning being caused at least in part by an increase in the bilayer fluidity (increased number of gauche conformers in the hydrocarbon chains), since it is well known that this leads to both an increase in bilayer fluctuations and an increase in bilayer repulsion.\(^{29}\) Although at 6 mol % tiliroside displayed the most significant changes when incorporated into bilayers, at lower concentrations of 1 mol% (data not shown) quercetin was slightly more interactive than tiliroside, which correlated well with the RCV results discussed in the previous section. The observed structural changes via the X-ray data analysis suggests different penetration depths of the studied flavonoids. Since rutin increases the membrane fluidity the least (see Figure 8d), a greater molecular overlap with the hydrocarbon chains can be excluded, and we speculate hydrogen bonding to water and/or polar head groups of the bilayer to be the prominent interactions, placing rutin mainly in the polar interface of the membrane. In contrast, quercetin and tiliroside display a more significant increase in the gauche-trans conformer ratio in the
bilayer (note, the membrane fluctuation are enhanced by about 25%; Figure 8d), and hence might penetrate further into the bilayer. The current view for the localization of the three compounds correlates well with most other existing studies, which suggest localization of flavonoids between the boundary of the lipid/water interface and the upper half of the hydrocarbon chain region.\textsuperscript{52,53,54,55,56,57} Notwithstanding this view, some studies suggest that the flavonoids partition into the hydrophobic region of the membrane.\textsuperscript{58,59} An NMR study has proposed flavonoid localization at the lipid/water interface: the hydroxyl groups have a tendency to hydrogen bond to the polar head group zone.\textsuperscript{54} A FTIR study suggested the same explanation for quercetin.\textsuperscript{55} Movileanu et al.,\textsuperscript{53} proposed a pH-dependent insertion of quercetin within membranes. Quercetin molecules inserted between the polar head groups at alkaline pH, with deeper localization at acidic pH, due to intercalation of quercetin molecules between the acyl chains. Rutin-membrane interactions have also been studied via Infrared (IR) spectroscopy. The frequency shift of hydroxyl and keto groups indicated rutin associated with the polar end of the phospholipids.\textsuperscript{60} In contrast to these findings, Arora et al.,\textsuperscript{58} proposed flavonoids and isoflavonoids tended to insert into the hydrophobic core of membranes, as evidenced by a significant decrease in membrane fluidity.

**Conclusions**

This study aimed to (i) screen eight flavonoids of systematically varying structure on lipid monolayers and (ii) identify the structure activity relationship (SAR) based on RCV (iii) link the SAR to the mechanisms of interactions involved and (iv) employ a widely varying techniques to support the RCV results and to obtain complementary information on the membrane behavior in the presence of flavonoids.
Electrochemical screening using a DOPC coated Pt/Hg electrode shows a clear structure-
activity relationship in the interaction between flavonoids and DOPC monolayers. Flavonoids
with two coplanar rings were more interactive. On the other hand, flavonoids with two linked
glycoside moieties interacted with DOPC less since these sugar moieties abolish the
hydrophobicity and also render these molecules larger in size. The extent of interactions can be
ranked in the order of quercetin > kaempferol > naringenin > hesperetin > catechin for flavonoid
aglycones and tiliroside > rutin > naringin for flavonoid glycosides.

Quercetin and tiliroside interacted to varying degrees with free-standing Langmuir
monolayers at the A-W interface, revealing a clear membrane stabilizing effect on the DOPC
monolayer, whereas rutin showed a different molecular mechanism of action, rendering the
monolayer surface completely smooth and devoid of islands. SAXS analysis of flavonoid loaded
DOPC bilayers showed a clear membrane thinning effect together with an increase in membrane
undulations. Rutin, quercetin and tiliroside thin DOPC by ~0.45, 0.8 Å, and 1.1 Å at 6 mol %,
respectively. X-ray analysis indicated the location of flavonoids in the bilayer as associated with
polar head groups. The use of a pure DOPC system, one of the most common unsaturated lipids
of the eukaryotic biomembranes, has led to a more detailed and profound analysis of the data
which provides a firm basis for the study of more complex and complete membrane systems in
the future that could include, for example, cholesterol and selected membrane proteins. These
additions, plus the extension to range of different added flavonoid concentrations and
temperatures, is the focus of ongoing work.
Figure 1. Two dimensional structure of compounds used in the current study (a) quercetin dihydrate and numbering pattern for flavonoids (b) kaempferol (c) tiliroside (d) naringenin (e) hesperetin (f) (+)-catechin (g) rutin trihydrate (h) naringin.
Figure 2. RCVs at 40 V s\(^{-1}\) of a pure DOPC-coated Pt/Hg electrode (black line) in the presence of flavonoids studied at concentrations of 10 µmol dm\(^{-3}\) (red line) and 35 µmol dm\(^{-3}\) (blue line) in PBS at pH 7.4.
Figure 3. Scatter plot to show the compounds’ LOD at DOPC-coated Hg electrode in PBS at pH 7.4 vs. their respective log octanol-water partition coefficient ($\log_{10}P$) for following compounds: quercetin (open square), kaempferol (filled triangle), tiliroside (filled circle), naringenin (inverted triangle), hesperetin (open triangle) and (+) - catechin (open circle).
Figure 4. $\pi$-$A$ isotherms of DOPC monolayer at the air-water interface in the absence (black line) and in the presence of 10 $\mu$mol dm$^{-3}$ (a) quercetin, (b) tiliroside and (c) rutin in the subphase (red line).
Figure 5. Desorption phenomena at constant pressure of 30 mN m\(^{-1}\) in DOPC monolayers (black line) in the presence of 10 \(\mu\)mol dm\(^{-3}\) (a) quercetin, (b) tiliroside and (c) rutin in the subphase (red line).
**Figure 6.** BAM images of DOPC monolayers after an hour monolayer collapse (a) pure DOPC monolayer and in the presence of 10 µmol dm$^{-3}$ (b) quercetin (c) tiliroside and (d) rutin in the subphase. (Please note that the numerous closely spaced lines the images are due to optical interference effects that have not been subtracted).
Figure 7. (A) Background subtracted SAXS patterns and corresponding fitted curves (solid lines). Data for pure DOPC (gray line) and DOPC with 6 mol. % of rutin (blue line), quercetin (red line) and tiliroside (green line) (B) EDPs of pure DOPC and DOPC-6 mol. % rutin (blue line), DOPC-6 mol. % quercetin (red line), DOPC-6 mol. % tiliroside (green line).
Figure 8. Membrane parameters in the presence of 6 mol. % of rutin, quercetin and tiliroside.
Table 1. $\log_{10}P$ values of flavonoids and their LODs using sensing device.

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<th>Flavonoids</th>
<th>Experimental $\log_{10}P$</th>
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</table>

$^a$ Experimental $\log_{10}P$ from Rothwell et al$^{61}$. $^b$ $\log_{10}P$ from DrugBank$^{62}$. $^c$ Experimental $\log_{10}P$ from Luo et al$^{63}$. $^d$ Experimental $\log_{10}P$ from Cooper et al$^{64}$. $^e$ Experimental $\log_{10}P$ from Shibusawa et al$^{65}$. 
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Table of Contents Graphic:
Table of Contents Graphic: Solubilization of flavonoids into DOPC bilayers