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Encapsulation of naturally occurring flavonoids into liposomes: physicochemical properties and biological activity against human cancer cell lines

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Abstract

Liposomes consisting of egg phosphatidylcholine were prepared by a thin-film hydration method followed by sonication and were used to investigate the percentage encapsulation of four flavonoids (quercetin, rutin, isoscutellarein and isoscutellarein diglycoside). The lipid recovery and the flavonoid-to-lipid molar ratio were measured using high-performance thin-layer chromatography/flame ionization detection and UV-vis spectroscopy. Differential scanning calorimetry was used to study the effect of the flavonoids on the phase transition temperature and on the enthalpy of the main phase transition of dipalmitoylphosphatidylcholine bilayers, and their ability to influence the membrane fluidity. The final liposomal formulation incorporating flavonoids, as well as free flavonoids, were tested for their activity against human cancer cell lines using the sulforhodamine B assay. The results showed that the encapsulation efficiency varied from 95% (0.21 flavonoid-to-lipid molar ratio) to 37.5% (0.09 flavonoid-to-lipid molar ratio) for isoscutellarein and its glycoside, respectively. The differential scanning calorimetry data showed close thermal and dynamic effects depending on the structure of the flavonoids, and suggest that there is a relationship between flavonoid molecular structure and the interaction with model membranes. Liposomal isoscutellarein showed improved growth inhibiting activity against all cell lines tested in comparison with that of its free form, which was inactive (>100 μM).

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Introduction

Many anticancer drugs are either natural compounds or have been developed from naturally occurring parent compounds. Much attention is currently being paid to flavonoids, which are found in fruit, vegetables, seeds, herbs, flowers, olive oil, tea and red wine (Middleton et al 2000). Flavonoids are one of the classes of heterocyclic natural compounds that are widely distributed in plants as glycosides or as free aglycones. They are subdivided according to their structure into flavonols, flavones, flavanones, chalcones and anthocyanidins. They exhibit several biological effects such as anti-inflammatory, antibacterial, antifungal, antiviral, antiulcer, hepatoprotective, antitumour and antioxidant activity (Narajana et al 2001). Many of these effects are the consequence of their ability to scavenge free radicals, to inhibit enzymes and to interact with biomembranes (Saija et al 1995a, b). The relationship between fruit and vegetables in the human diet and cancer prevention has been reported by Block et al (1992). Several flavonoids have been studied for their antitumour activity; a carbonyl group at C-4 of the flavone nucleus was found to be responsible for their activity. The flavone derivative, flavone-8-acetic acid, has been shown to have considerable antitumour effects (Thomsen et al 1991). Another flavone, genistein, has been proposed to be responsible for the lower rate of breast cancer in Asian women and this effect may be related to the high isoflavone-containing soy content of their diet (Shao et al 1998; Middleton et al 2000). Genistein has received much attention as a potent anticancer agent owing to its wide range of effects on a number of cellular processes (Middleton et al 2000). Polymethoxylated flavones isolated from citrus were examined for their antiproliferative activities against several human cancer cell lines. Their strong antiproliferative activities suggest that they may serve as anticancer agents in humans (Kawaii et al 1999; Manthey & Guthrie 2002). Quercetin, which belongs to the flavonols, has been reported as

an antineoplastic compound, exerting growth inhibitory activity against several cancer cell lines in-vitro and exhibiting a synergistic cytotoxic effect with cisplatin against drug-resistant leukaemia cells in-vitro (Middleton et al 2000). Catechin, quercetin and resveratrol, which are the main polyphenols in red wine, were shown to inhibit the growth of human breast cancer cells (Damianaki et al 2000). In-vitro studies have revealed the cytotoxic activity of flavonoids belonging to the group of kaempferol glycosides (Dimas et al 1999a, b). In-vitro and animal studies have demonstrated that flavonoids may inhibit cancer cell growth by binding to type II receptors, which are overexpressed in a wide range of tumour tissues such as breast, ovarian, colon and lung (Cipak et al 2003). Interest in flavonoids has also increased because of their antioxidant activity and their ability to prevent heart disease (Gordon & Roedig-Penman 1998). Many biological properties of flavonoids may be related to their capacity to penetrate into cell membranes and to affect their biological activity (Demetzos et al 2001). Several flavonoids are water-insoluble compounds with a slow dissolution rate and this is a major drawback for in-vivo administration of such lipophilic compounds (Lauro et al 2002). Liposomes are a non-toxic carrier system for intravenous delivery of lipophilic drugs among others. The surface charge and size of liposomes can modulate their in-vivo stability and improve the pharmacokinetic properties of the encapsulated drugs (Allen & Stuart 1999; Drummond et al 1999). Liposomes are also considered as an effective model membrane, very close to the structure of biological membranes owing to their phospholipid bilayer structure. In most of the studies concerning liposomes and flavonoids published to date, liposomes served mainly as model membranes on which the biological activity of the flavonoids was assessed (Ioku et al 1995; Bonina et al 1996). A few studies have focused on encapsulating flavonoids in liposomes and reported the localization of flavonoids in the liposomal membranes as well as the effects of flavonoids on the physicochemical characteristics of the liposomal formulations (Saija et al 1995b; Arora et al 1998). Flavonoids, quercetin, rutin and a glycoside of isoscutellarein, recently isolated from the plant *Stachys spruneri* (family *Lamiaceae*) (Goniotaki 2003) were encapsulated in liposomes composed of egg phosphatidylcholine (EPC) (Figure 1). The aglycone of isoscutellarein glycoside, isoscutellarein, which was obtained after hydrolysis of the isoscutellarein diglycoside, was also studied (Goniotaki 2003). The encapsulation efficiency of glycosides and of the corresponding aglycones, as well as the physicochemical characteristics of the final liposomal formulations, were compared. Differential scanning calorimetry (DSC) was used to explore the relationship between flavonoid interactions with dipalmitoylphosphatidylcholine (DPPC) membranes and to explain the variation in the encapsulation efficiency of the four flavonoids as well as the differences on the physicochemical properties of the four liposomal formulations. This thermodynamic technique is suitable for studying the thermal effects of additives in membrane bilayers and has been used to assess the effect of flavonoids on membrane bilayers (Saija et al 1995a). Parameters used in a DSC thermogram are the phase transition temperature (T_m) and the enthalpy of the main phase transition (ΔH).

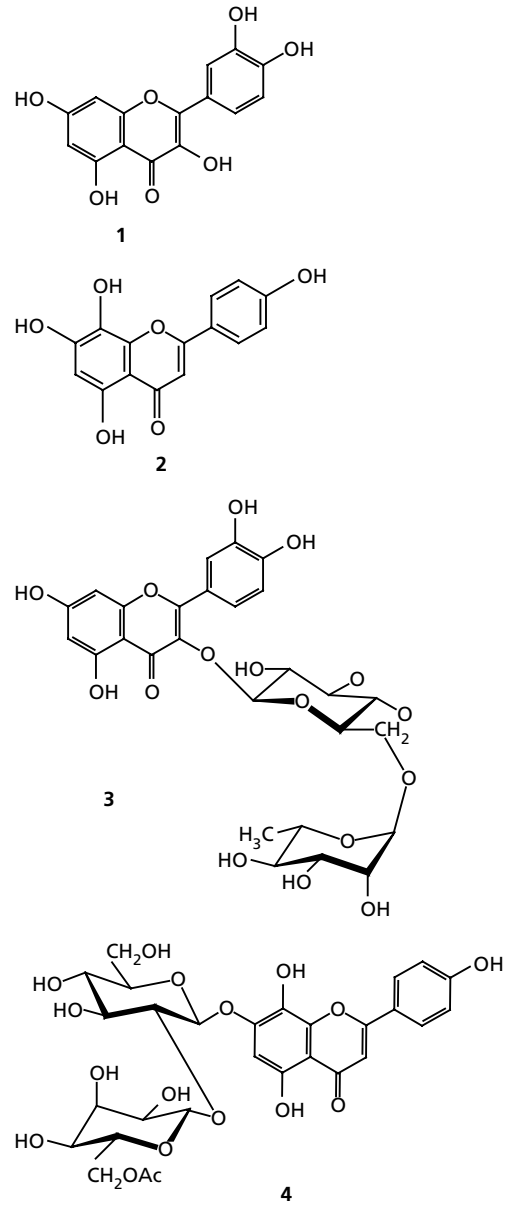

Figure 1 Structures of flavonoids: **1**, quercetin; **2**, isoscutellarein; **3**, rutin; and **4**, isoscutellarein glycoside.

The aim of the present study was to design and prepare liposomal formulations encapsulating flavonoids in order to overcome their insolubility in water and prepare suitable formulations for in-vivo administration. These preparations could be adequate for therapeutic and cosmetological applications. Isoscutellarein and its glycoside have not been tested before for their cytotoxicity. In addition, these compounds belong to the same flavone group as genistein and it is interesting to study their effects against cancer cell lines. We report the results that concern the efficiency of the flavonoid encapsulation and the physicochemical properties of the final liposomal formulations, as well as the assessment of the cytotoxic/cytostatic activity of these formulations against three human cancer cell lines in-vitro.

Materials and Methods

Materials

EPC was obtained from Avanti Polar Lipids Inc., (Alabaster, AL, USA). Rutin and quercetin were commercially available (rutin trihydrate from Merck, Darmstadt, Germany, and quercetin dihydrate from Fluka Biochemica, Buchs, Switzerland). Isoscutellarein glycoside was isolated from the plant *S. spruneri* (family *Lamiaceae*), while its aglycone was obtained after hydrolysis of the isoscutellarein glycoside. All chemicals were of analytical grade. High-performance liquid chromatography (HPLC)-grade water was obtained after filtration by the PRO PS Labconco System (Labconco, Kansas City, MO, USA). Sephadex G-75 was purchased from Fluka Biochemica. Cell culture reagents were purchased from Euroclone Life Sciences Division (Milano, Italy), sulforhodamine B and trizma-base were from Sigma (St Louis, MO, USA), and acetic acid was from Fluka Biochemica. The cell lines were obtained from the National Cancer Institute (National Institutes of Health, USA).

Liposome preparation and flavonoid incorporation

Liposomes were prepared by the thin-film hydration method (Mayer et al 1990). Briefly, the lipid film was prepared by dissolving EPC (0.090 μmol) in chloroform. Quercetin (0.020 μmol) was dissolved in ethanol, while rutin (0.020 μmol), isoscutellarein (0.020 μmol) and the isoscutellarein glycoside (0.020 μmol) were dissolved in methanol. Each flavonoid solution was then added to the lipid solution and the organic solvents were removed under reduced pressure. The samples were left to dry overnight in a desiccator. The addition of 3 mL of HPLC-grade water (pH = 5.6) performed the hydration of the lipidic film, and multilamellar vesicles were formed by vigorous shaking of the suspensions in a water bath above the gel-to-crystalline phase transition of the lipid (41°C) and stirring for 75 min. The resultant liposomal suspension composed of multilamellar vesicles was subjected to 15 successive freeze–thaw cycles (Mayer et al 1990) by dipping the samples in a dry-ice (solid CO_2)/*n*-butanol bath and in a water bath at 41°C. The resultant large unilamellar vesicles were sonicated in an ice bath for two 10-min periods, interrupted by a 5-min resting period in an ice bath using a probe sonicator (amplitude 100, cycle 0.7-UP 200S; Dr. Hielscher GmbH, Berlin, Germany). The resultant vesicles were allowed to anneal for 30 min at room temperature. In order to prepare a liposome suspension that could be successfully reconstituted after lyophilization, large unilamellar vesicle liposomes, free or incorporating flavonoid, were prepared, hydrating the lipid film by adding 0.30 M sucrose instead of water. The sucrose-to-lipid ratio was 9.5 mg mg^{-1}. The un-entrapped flavonoid was removed at room temperature by gel filtration chromatography through a Sephadex G-75 column, equilibrated with HPLC-grade water (pH = 5.6).

Determination of lipids and flavonoids

EPC was determined by high-performance thin-layer chromatography coupled with a flame ionization detector (Iatroscan MK-5; Iatron Laboratories, Inc., Tokyo, Japan). The hydrogen flow rate was 160 mL min^{-1}, the air flow rate was 1900 mL min^{-1}, and the scan speed was 30 s/scan. As stationary phase, Chromarods-SII (Iatron Laboratories, Inc.) in a set of 10 rods was used (De Schrijver & Vermeulen 1991; Hatziantoniou & Demetzos 2003). The flavonoid concentration was measured at 25°C in a Perkin Elmer Lambda 6 UV-vis spectrometer (Perkin Elmer, Boston, MA, USA) by using a calibration curve at: $\lambda = 255$ nm for quercetin, $\lambda = 307$ nm for isoscutellarein, $\lambda = 263$ nm for rutin, and $\lambda = 280$ nm for the isoscutellarein diglycoside.

Liposome size and ζ-potential measurements

The size and ζ-potential of liposomes are the parameters that indicate their physical stability. The liposome dispersion was stored in glass vials at 4°C. The first measurements of size and ζ-potential were performed immediately after preparation of the liposomes, and thereafter at fixed time intervals over a 2-month period. A-100 μL liposome dispersion was 10-fold diluted in HPLC-grade water (pH 5.6), and the z-average mean and ζ-potential of the empty and loaded large unilamellar vesicles were measured in order to determine the effect of flavonoid loading on liposomal ζ-potential. Samples were scattered (633 nm) at an angle of 90° and measurements were made at 25°C in a photon correlation spectrometer (Zetasizer 3000; Malvern Instruments, Malvern, Worcs, UK) and analysed by the CONTIN method (Malvern Instruments).

Reconstitution of lyophilized liposomes

Freeze-dried liposomal preparations that were prepared with 0.30 M sucrose were reconstituted by adding HPLC-grade water. The physicochemical properties of the reconstituted liposomes were examined at room temperature. Water was added to each vial followed by gentle agitation until all the material was suspended. Each sample was allowed to anneal for a period of 15 min followed by vortexing, and then allowed to rest for 15 min before determining the physicochemical parameters.

Statistical analysis

Statistical analysis of the effects of flavonoids on their incorporation efficiency, on the size and ζ-potential, as well as on the thermal properties of DPPC membranes (T_m, T_{onset} and ΔH), was performed using one-way analysis of variance where applicable. In these cases, means of the individual groups were compared post-hoc using Tukey's Honestly Significantly Different test. Where analysis of variance criteria were not fulfilled, the Kruskal-Wallis test was used followed by Games-Howell post-hoc analysis using SPSS for Windows release 11.0.0 (SPSS Inc., Chicago, IL, USA). All results were from three independent experiments, each one run in triplicate.

In-vitro cytotoxicity studies

The cytotoxicity of free flavonoids, liposomes incorporating flavonoids and empty liposomes was tested against the following cancer cell lines: SF268 (central nervous system), H460 (non-small cell lung) and MCF7 (breast). The cell lines were purchased from the National Cancer Institute and were grown in RPMI-1640 medium supplemented with 10% heat-inactivated fetal calf serum, 2 mM L-glutamine and 50 μg mL^{-1} gentamycin in a 37°C humidified incubator and 5% CO_2 atmosphere. Cell viability was assessed by the trypan blue dye exclusion method at the beginning of the experiment and was always greater than 98%. Cells were seeded into 96-well plates (100 μL/well) at densities ranging from 5000 to 40 000 cells. The plates were incubated for 24 h to allow adaptation of cells before addition of the test agents. At the beginning of the experiment, a plate of each cell line was fixed in-situ with trichloroacetic acid and sulforhodamine B staining as described previously (Monks et al 1991; Pluquet et al 2003), to provide a measurement of the cell population for each cell line at the time of drug addition. To determine the activity, free or liposomal flavonoids were added at the same time to each cell line in decreasing concentrations (five 10-fold dilutions) starting from 100 μM, and the cultures were incubated for an additional 48 h. Cultures containing a buffer comprising 100 mM TES + 100 mM NaCl (pH = 7.5, 300 mOs) served as negative controls. Following addition of free flavonoid or flavonoid-loaded liposomes and empty liposomes, cells were cultured for 48 h. The assay was terminated by the addition of cold trichloroacetic acid, sulforhodamine B staining was performed and absorbance was measured at 530 nm on an EL-311 Biotek MicroELISA reader (Biotek, Winooski, VT, USA) (Monks et al 1991; Pluquet et al 2003).

The data represent the mean of experiments performed in triplicate. The parameters GI50, total growth inhibition and LC50 were determined using our own customized software (Pluquet et al 2003). Briefly, the GI50 parameter indicates the growth inhibition action of the test compound and was calculated by the equation: $100 \times (T - T_z)/(C - T_z) = 50$. Total growth inhibition indicates the cytostatic effect of the test compound and was calculated by the equation: $100 \times (T - T_z)/(C - T_z) = 0$. LC50 indicates the test compound concentration that induces cytotoxicity in 50% of the cells and was calculated by the equation: $100 \times (T - T_z)/T_z = -50$. T and T_z indicate the absorbance values at the time cells received the test compound (T_z) and after a period of treatment (T); C indicates the absorbance value measured in untreated cells (control) after an incubation period equal to the treatment period (Monks et al 1991; Pluquet et al 2003).

DSC measurements

DSC was used for the study of multilamellar vesicles samples using a Mettler Toledo DSC822^e (Mettler Toledo, Schwerzenbach, Switzerland) with Julabo (Seelbach, Germany) intracooler cooling device. DPPC/flavonoid mixtures were prepared in a 9:2 molar ratio and were transferred as dry powder into 40-μL aluminium crucibles. They were hydrated with de-ionized water in a 1:2 w/w lipid-to-water ratio and the crucibles were hermetically closed. An empty aluminium crucible with lid was put on the reference side. The following temperature program was applied: isothermal segment at 0°C for 5 min, heating to 70°C with 5 K min^{-1}, cooling to 0°C with 5 K min^{-1}, and another three heating and two cooling segments with the same parameters. The instrument was calibrated regarding temperature and transition enthalpies using 4-nitro toluene and indium as reference substances. Enthalpies and characteristic temperatures were calculated using the Mettler Toledo STAR^e software.

Results and Discussion

Stability studies and flavonoid incorporation

All flavonoids were incorporated in liposomes at a final composition of EPC/flavonoid 9:2 (molar ratio). The incorporation efficiency was 86% for quercetin, 95% for isoscutellarein, 71% for rutin, and 37.5% for the isoscutellarein diglycoside, and the corresponding flavonoid-to-lipid molar ratio was 0.17, 0.21, 0.16 and 0.09 (initial 0.22) (Table 1). Statistical analysis showed that these differences were significant for rutin and isoscutellarein diglycoside ($P < 0.05$). Interactions between aglycones and EPC acyl chains are strong and, thus, these molecules interacted with the lipophilic region of liposomes, resulting in greater

Table 1 Recovery of phospholipids, flavonoid incorporation and flavonoid/phospholipid molar ratio in the final lyophilized preparations

Liposome composition	Phospholipid recovery			Flavonoid incorporation			Flavonoid/lipid	
	(mg mL^{-1})		%	(mg mL^{-1})		%	(mol/mol)	
	Initial	Final		Initial	Final		Initial	Final
EPC/quercetin (9:2)	24.1	23.2	96 ± 13.5	2.4	2.1	86.0 ± 12.1	0.22	0.17 ± 0.02
EPC/isoscutellarein (9:2)	24.9	24.6	99 ± 12.8	2.1	2.0	95.0 ± 13.2	0.22	0.21 ± 0.03
EPC/rutin (9:2)	23.3	22.7	97 ± 13.6	4.5	3.2	71.0 ± 10.0*	0.22	0.16 ± 0.16*
EPC/isoscutellarein glycoside (9:2)	23.6	21.5	91 ± 10.8	4.5	1.7	37.5 ± 4.0*	0.22	0.09 ± 0.001*

EPC, egg phosphatidylcholine. *The mean difference is significant at the 0.05 level.

Table 2 Physical stability of free liposomes and liposomes incorporating flavonoids during 2 months storage at 4°C

Time (weeks)		Free EPC	EPC/quercetin (9:2)	EPC/isoscutellarein (9:2)	EPC/rutin (9:2)	EPC/isoscutellarein glycoside (9:2)
0	Size	241.3 ± 8.4*	414.1 ± 12.6	190.4 ± 2.0	424.4 ± 34.0	287.6 ± 6.1
	PI	0.46	0.53	0.46	0.45	0.49
	ζ-Potential	−35.6 ± 8.8	−54.4 ± 7.8	−48.7 ± 5.9	−44.3 ± 3.6	−48.6 ± 4.8
2	Size	253.1 ± 7.6	407.0 ± 12.0	193.4 ± 1.9	466.5 ± 27.0	284.1 ± 5.7
	PI	0.31	0.50	0.55	0.95	0.62
	ζ-Potential	−38.0 ± 7.7	−50.0 ± 7.3	−35.4 ± 5.3	−43.4 ± 2.8	−37.2 ± 6.4
4	Size	256.3 ± 8.0	400.5 ± 13.0	195.1 ± 3.1	400.6 ± 15.0	298.3 ± 6.4
	PI	0.41	0.57	0.52	0.46	0.47
	ζ-Potential	−45.4 ± 8.9	−39.4 ± 7.3	−37.6 ± 3.9	−38.6 ± 4.1	−43.7 ± 3.6
8	Size	239.4 ± 7.0	384.5 ± 12.5	192.4 ± 7.0	388.0 ± 12.9	289.4 ± 6.0
	PI	0.47	0.68	0.56	0.65	0.50
	ζ-Potential	−55.2 ± 7.9	−57.3 ± 7.6	−42.6 ± 5.0	−47.1 ± 11.6	−45.5 ± 3.8

EPC, egg phosphatidylcholine. Size, z-average mean in nm; PI, polydispersity index; ζ-potential in mV. *The mean difference is significant at the 0.05 level (not applied for quercetin and rutin).

incorporation efficiency. Results of the incorporation efficiency of flavonoid glycosides in liposomes showed a weaker interaction with the membranes, which strongly depend on the position and composition of the sugar moiety attached to the aglycone. The percentage recovery of phospholipids varied from 99% to 91% for isoscutellarein and its glycoside, respectively (Table 1). Table 1 shows that rutin was incorporated in greater amounts in liposomes than isoscutellarein glycoside.

The physical stability (4°C, HPLC-grade water pH 5.6) of large unilamellar vesicle liposomes, free and incorporating flavonoids, was monitored over a period of 2 months and the results are presented in Table 2. Free EPC liposomes were stable over a period of 2 months. Liposomes containing flavonoids were found to retain their stability (differences were not statistically significant) with particle size ranging from 414.1 nm (polydispersity index (PI): 0.53) to 384.5 nm (PI: 0.68) for formulations incorporating quercetin, 195.1 nm (PI: 0.52) to 190.4 nm (PI: 0.46) for formulations incorporating isoscutellarein, 466.5 nm (PI: 0.95) to 390.0 nm (PI: 0.65) for formulations incorporating rutin, and 298.3 nm (PI: 0.47) to 284.1 nm (PI: 0.62) for formulations incorporating isoscutellarein diglycoside.

Liposomal formulations incorporating quercetin and its glycoside rutin had similar sizes and were significantly larger than free EPC liposomes and formulations incorporating isoscutellarein and isoscutellarein diglycoside ($P < 0.05$). This led us to speculate that the conformational properties of the flavonoids quercetin and rutin, which belong to the flavonols, are responsible for this phenomenon. All liposomal formulations retained their initial size for at least 8 weeks (Table 2).

The initial ζ-potential of four liposomal formulations was measured and found to be –35.6 mV for free liposomes, −54.4 mV for liposomes incorporating quercetin, −48.7 mV for liposomes incorporating isoscutellarein, −44.3 mV for liposomes incorporating rutin, and −48.6 mV for liposomes incorporating isoscutellarein glycoside (Table 2). The differences were not statistically significant. Studies concerning the ζ-potential values of free liposomes and of liposomes incorporating flavonoids for 2 months showed stability values that were very close to the values of the initial preparations (Table 2). Liposomes incorporating flavonoids were freeze-dried and after rehydration the physicochemical properties of the reconstituted vesicles were assessed. After reconstitution, the size and the ζ-potential values were very close to those before freeze-drying (Table 3); the differences were not statistically significant. These results indicated that, after reconstitution, the freeze-dried formulation could be a stable product for further in-vivo studies.

Thermal effects of the flavonoids in phospholipid bilayers

DSC was used to compare the thermotropic properties of quercetin, rutin, isoscutellarein and isoscutellarein diglycoside in DPPC membrane bilayers. DPPC bilayers exist in the gel phase for temperatures less than 35°C and in the liquid crystalline phase for temperatures greater than 42°C. Between 35–42°C, the phospholipid bilayer exists in the ripple phase. The transition is followed by several structural changes in the lipid molecules as well as alterations in the geometry of the bilayers. The most prominent feature is the trans-gauche isomerization, taking place in the acyl chain conformation. The average number of gauche conformers influences the fluidity, which depends not only on the temperature, but also on perturbations owing to the presence of a drug molecule intercalating on the lipidic region. DPPC bilayers show the characteristic pre-transition with a low ΔH and a sharp T_m, both at the expected transition temperatures of 35.1 and 41.3°C. Thermal analysis results by using DSC are based on T_{onset}, T_m and ΔH. Fully hydrated DPPC bilayers incorporating flavonoids showed thermograms consisting of broad enthalpy transitions between 42.5

Table 3 Size (z-average mean) and ζ-potential of EPC/flavonoid (9:2) liposomes before freeze-drying and after reconstitution

Liposome composition	Size (nm)		ζ-Potential (mV)	
	Before freeze-drying	After reconstitution	Before freeze-drying	After reconstitution
EPC/quercetin (9:2)	414.1 ± 12.6 PI 0.53	410.8 ± 13.0 PI 0.47	−54.4 ± 7.8	−50.2 ± 5.9
EPC/isoscutellarein (9:2)	190.4 ± 2.0 PI 0.46	193.2 ± 1.9 PI 0.42	−48.7 ± 5.9	−42.3 ± 5.0
EPC/rutin (9:2)	424.4 ± 34.0 PI 0.45	430.2 ± 13.0 PI 0.52	−44.3 ± 3.6	−40.0 ± 7.7
EPC/isoscutellarein glycoside (9:2)	287.6 ± 6.1 PI 0.49	290.3 ± 7.0 PI 0.55	−48.6 ± 4.8	−43.2 ± 6.7

EPC, egg phosphatidylcholine; PI, polydispersity index.

($J\,g^{-1}$) (isoscutellarein glycoside) and 35.1 ($J\,g^{-1}$) (rutin) and of a T_m between 41.9°C (quercetin) and 40.2°C (isoscutellarein glycoside). Table 4 shows the characteristic values of T_{onset}, T_m and ΔH of the flavonoid interactions with the DPPC model bilayers. The results showed that all preparations have similar T_m values. ΔH values show that rutin exerts the maximum effect in the lowering of ΔH. This is probably owing to the fact that the lipophilic part of rutin is extended deep into the lipidic bilayer, in contrast to isoscutellarein diglycoside, which interacts less than rutin with the phospholipids as revealed by molecular modelling data and computer calculations using a Silicon Graphics O2 workstation using QUANTA (Molecular Simulation Inc., Cambridge, UK) (data not shown). These observations are in accordance with the liposome incorporation results of the two glycosides, which showed greater incorporation efficiency for rutin (71%) in comparison with that of isoscutellarein diglycoside (37.5%). An interesting result is the membrane perturbing effect observed with quercetin, isoscutellarein and rutin. All these flavonoids lowered ΔH up to 35.1 ($J\,g^{-1}$) whereas isoscutellarein diglycoside affected it only marginally (42.5 ($J\,g^{-1}$)) in comparison with that of DPPC (ΔH 45 $J\,g^{-1}$) ($P < 0.05$; Table 4).

From the above results, we speculate that the DPPC/flavonoid preparation showed the ability of the flavonoids to modify the ordered lipidic structure of DPPC bilayers. This modification depends on several factors, such as the amphiphilic or lipophilic character of the flavonoid, the presence of different substituents (i.e. glycoside group in rutin and isoscutellarein diglycoside) or different positions of hydroxyl groups (i.e. quercetin and isoscutellarein). These factors influence the flavonoid positioning between the acyl chains of lipids, causing modifications in the transition temperature and changes in enthalpy that indicate a direct effect on the membrane fluidity. Thus, a structure-dependent relationship in flavonoid thermotropic behaviour may be suggested (Saija et al 1995a).

Table 4 Transition temperature (T_m), onset temperature (T_{onset}) and enthalpy change (ΔH) of the studied multilamellar vesicle preparations

Sample	T_{onset} (°C)	T_m (°C)	ΔH ($J\,g^{-1}$)
DPPC	40.8 ± 1	41.3 ± 0.7	45.0 ± 0.1*
DPPC/quercetin (9:2)	39.1 ± 0.8	41.9 ± 0.8	36.4 ± 1.3*
DPPC/isoscutellarein (9:2)	33.9 ± 1.2	41.0 ± 0.6	38.1 ± 1.1*
DPPC/rutin (9:2)	38.3 ± 1.2	40.5 ± 0.7	35.1 ± 0.7*
DPPC/isoscutellarein glycoside (9:2)	38.6 ± 1.3	40.2 ± 0.7	42.5 ± 0.1*

DPPC, dipalmitoylphosphatidylcholine. *The mean difference is significant at the 0.05 level.

Table 5 Growth inhibiting concentration 50 (GI50), total growth inhibition (TGI) and lethal concentration 50 (LC50) of flavonoids against cancer cell lines.

Cancer cell line	Quercetin (μM)	Isoscutellarein (μM)	Rutin (μM)	Isoscutellarein glycoside (μM)
MCF7 (breast)				
GI50	24.19	> 100	> 100	> 100
TGI	>100	>100	>100	>100
LC50	>100	>100	>100	>100
H460 (non-small cell lung)				
GI50	8.00	>100	>100	>100
TGI	>100	>100	>100	>100
LC50	>100	>100	>100	>100
SF268 (central nervous system)				
GI50	31.75	>100	>100	>100
TGI	>100	>100	>100	>100
LC50	>100	>100	>100	>100

Flavonoids did not exhibit any activity against normal cells (peripheral blood mononuclear cells: resting or activated) at concentrations as high as 100 μM.

In-vitro cytotoxicity

Regarding the cytotoxicity of free flavonoids, quercetin, rutin, isoscutellarein and isoscutellarein diglycoside (Table 5),

Table 6 Growth inhibiting concentration 50 (GI50), total growth inhibition (TGI) and lethal concentration 50 (LC50) of liposomes incorporating flavonoids against cancer cell lines

Cancer cell line	EPC/quercetin (9:2)	EPC/isoscutellarein (9:2)	EPC/rutin (9:2)	EPC/isoscutellarein glycoside (9:2)
MCF7 (breast)				
GI50	93.1	82.6	100	NT
TGI	>100	>100	>100	NT
LC50	>100	>100	>100	NT
H460 (non-small cell lung)				
GI50	>100	45.1	90.9	NT
TGI	>100	>100	>100	NT
LC50	>100	>100	>100	NT
SF268 (central nervous system)				
GI50	41.9	42.6	66.9	NT
TGI	>100	>100	>100	NT
LC50	>100	>100	>100	NT

EPC, egg phosphatidylcholine; NT: not tested. Concentrations are in μM. Free liposomes did not exhibit any cytotoxic/cytostatic activity at the concentrations tested (highest tested concentration 100 μM); liposomes incorporating flavonoids did not exhibit any activity against normal cells (peripheral blood mononuclear cells: resting or activated) at concentrations as high as 100 μM.

the results showed that only quercetin had growth-inhibiting (GI50) activity against the cancer cell lines SF268 (31.75 μM), MCF7 (24.19 μM) and H460 (80.0 μM). All flavonoids were inactive against normal cells (peripheral blood mononuclear cells: resting or activated) at concentrations as high as 100 μM. The liposomal formulation of quercetin was less active in comparison with its free form (Tables 5 and 6) against the three cell lines. The liposomal formulation of rutin proved to be more active than its free form and showed remarkable growth inhibiting activity against two of the three cell lines (H460 and SF268) (Tables 5 and 6). Liposomal isoscutellarein showed considerable growth inhibiting activity against all cell lines, and was the best among the tested liposomal flavonoids (Tables 5 and 6). Free liposomes were inactive against all cell lines (highest tested concentration 100 μM). In the case of liposomal isoscutellarein, the improvement of its growth inhibiting activity in comparison with its free form could be attributed to its ability to reach the cancer cells in an effective concentration when incorporated into liposomes. Isoscutellarein is not soluble in water when dispersed in an aqueous environment. Thus, its activity is inhibited owing to its insolubility in the aqueous medium. This is reversed when the flavonoid is formulated in liposomes. Furthermore, this formulation should improve the interaction of the compound with cell membranes and enhance uptake from the cell, leading to enhanced activity. As far as the activity of liposomal rutin is concerned, the improvement of its activity when incorporated in liposomes could be attributed to modification of its interaction with the cell membranes caused by its formulation into liposomes. Studies aimed at improving the pharmacological activity of the incorporated flavonoids into liposomes could focus on achieving a better lipid-to-flavonoid molar ratio by using different phospholipids alone or in combination with cholesterol in order to prepare more effective liposomal formulations.

Conclusion

We report data underlining the differences between the encapsulation efficiency, the physicochemical properties and pharmacological action of four flavonoids in liposomes. The physicochemical properties and the stability of the final liposomal formulations as well as the encapsulation efficiency were studied. The interaction of the flavonoids with model membranes (DPPC) was studied and the flavonoid structure relationship to the lipidic environment was investigated using DSC. The results showed that the encapsulation efficiency as well as the interaction of flavonoids with the model membranes (DPPC) was structure dependent. The existence of a sugar moiety or hydroxyl groups in different positions on the flavonoid structure may play a role in the loading of flavonoids in liposomes and in their interaction with the DPPC membranes. The in-vitro cytotoxicity of the flavonoids as free compounds and incorporated in liposomes was studied and the results indicated an improvement in the case of liposomal isoscutellarein regarding its growth inhibiting activity (GI50) against cancer cell lines, in contrast to the activity of liposomal quercetin, which was reduced.

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