International Journal of Pharmaceutics 423 (2012) 69–76

Contents lists available at ScienceDirect

International Journal of Pharmaceutics

journal homepage: www.elsevier.com/locate/ijpharm

Development of a liposomal formulation of the natural flavonoid fisetin

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ARTICLE INFO

Article history:
Received 2 February 2011
Received in revised form 8 April 2011
Accepted 28 April 2011
Available online 6 May 2011

Keywords:
Liposomes
Liposome optimization
Fisetin
Flavonoid
Cytotoxicity

ABSTRACT

The natural flavonoid fisetin (3,3′,4′,7-tetrahydroxyflavone) has been shown to possess antiangiogenic and anticancer properties. Because of the limited water solubility of fisetin, our aim was to design and optimize a liposomal formulation that could facilitate its *in vivo* administration, taking into account the availability and cost of the various components. Several methods were evaluated such as probe sonication, homogeneization, film hydration and lipid cake formation. A selection of lipid and lipid-PEG was also performed via their incorporation in different formulations based on the size of the liposomes, their polydispersity index (PDI) and the fisetin encapsulation yield. An optimal liposomal formulation was developed with P90G and DODA-GLY-PEG2000, possessing a diameter in the nanometer scale (175 nm), a high homogeneity (PDI 0.12) and a high fisetin encapsulation (73%). Fisetin liposomes were stable over 59 days for their particle diameter and still retained 80% of their original fisetin content on day 32. Moreover, liposomal fisetin retained the cytotoxicity and typical morphological effect of free fisetin in different tumour and endothelial cell lines. In conclusion, based on its physico-chemical properties and retention of fisetin biological effects, the developed liposomal fisetin preparation is therefore suitable for *in vivo* administration.

1. Introduction

Several edible plant-derived compounds have been linked to the chemoprevention and treatment of cancer (Surh, 2003). Among these natural compounds, the flavonoids have shown several pharmacological properties of interest in the prevention and treatment of cancer (Havsteen, 2002; Lopez-Lazaro, 2002; Middleton et al., 2000).

In a program aimed at finding novel antiangiogenic agents, we have recently identified the natural flavonoid fisetin (3,3′,4′,7-tetrahydroxyflavone) as an interesting lead that can stabilize endothelial cells *in vitro* at non cytotoxic concentrations (Touil et al., 2009). Fisetin is present in several fruits, vegetables, nuts and wine (Arai et al., 2000; Kimira et al., 1998), and displays a variety of biological effects including antioxidant, anti-inflammatory (Park et al., 2007; Woodman and Chan, 2004), anti-carcinogenic and *in vitro* antiangiogenesis (Fotsis et al., 1997). Fisetin has been shown to inhibit several molecular targets, including cyclin-dependent kinases (Lu et al., 2005a,b; Sung et al., 2007), DNA topoisomerases I and II (Constantinou et al., 1995; Olaharski et al., 2005), urokinase (Jankun et al., 2006), actin polymerization (Böhl et al., 2007), and androgen receptor signalling (Khan et al., 2008a).

Although fisetin has recently been shown to possess interesting anticancer activity *in vivo* in mice bearing lung carcinoma (Touil et al., 2010) and prostate tumours (Khan et al., 2008a), its *in vivo* administration remains problematic due to the relative water insolubility of this compound (Guzzo et al., 2006). To facilitate its *in vivo* administration, we therefore chose to formulate fisetin into liposomes and to evaluate if the new formulation retains the biological properties of fisetin.

Liposomes are artificial vesicles, composed of lipidic amphiphiles, usually phospholipids, which organise themselves in water to form an aqueous core surrounded by a lipidic bilayer. This structure allows liposomes to transport both hydrophilic and lipophilic compounds and have led to their clinical use as drug carriers of several drug classes including antibiotics, antifungals and anticancer agents (Allen and Cullis, 2004; Langer, 1998).

Abbreviations: Fisetin, 3,3′,4′,7-tetrahydroxyflavone; Chol, cholesterol; DODA, dioctadecyldimethylammonium chloride; DOPC, 1,2-dioleoyl-*sn*-glycero-3- phosphocholine; DOPE-rhodamine, 1,2-dioleoyl-*sn*-glycero-3-phosphoethanolamine-rhodamine; DSPE-PEG_{2000}, 1,2-distearoyl-*sn*-glycero-3-phosphoethanolamine-N-[amino(polyethylene glycol)-2000]; GLY, glycine; P90G, Phospholipon® 90 G; PDI, polydispersity index; PEG_{2000}, poly(ethylene glycol)$_{45}$; SDS, sodium dodecyl sulphate; TEM, transmission electron microscopy; CT26, colon tumour 26; 3LL, Lewis lung carcinoma; EAhy926, immortalized human umbilical vein endothelial cells.

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doi:10.1016/j.ijpharm.2011.04.066

Concerning the anticancer agents, liposomes have been shown to allow a higher tumour accumulation of the drug (Gabizon, 1992). This tumour retention effect is apparently due to the liposomal drug extravasation through the tumour porous capillary endothelium, which appears to be a consequence of the rapid angiogenesis occurring in tumours and not in normal tissues (Yuan et al., 1995). Several liposomal forms of anthracyclines are currently employed in the clinic and these formulations have contributed to significantly reduce toxicity while maintaining their anticancer activity in breast (Batist et al., 2001; O'Brien et al., 2004) and soft tissue carcinoma (Siehl et al., 2005).

Hydrophobic compounds like flavonoids are not frequently formulated as liposomes, because a rapid exchange of the compounds may occur between the liposomal membrane and the cellular membrane (Fahr et al., 2006). However, this pharmaceutical formulation has recently been shown to improve the solubility of the flavonoid quercetin while maintaining its cytotoxicity *in vitro*, increase its blood residence time and its *in vivo* anticancer activity in mice (Yuan et al., 2006).

In the present study, our aim was therefore to develop and optimize a liposomal formulation of the flavonoid fisetin to facilitate its *in vivo* administration. We report here the different steps investigated to efficiently and easily encapsulate fisetin, while reducing the costs of the preparation by the assessment of the lipid/fisetin ratio, the choice of the liposomal method of preparation, and the excipient composition. Stability and *in vitro* cytotoxicity studies are also presented.

2. Experimental methods

2.1. Chemicals

Fisetin, cholesterol, DMSO, Hepes and phosphate buffers have been purchased at Sigma–Aldrich. Phospholipids such as dioleoyl-phosphatidylcholine, distearylphosphatidylethanolamine-poly(ethyleneglycol)$_{2000}$ have been purchased from Aventi-Polar, Inc., and P90G has been obtained from Lipoid. Dichloromethane, triethylamine, chloroform, methanol, absolute ethanol and silica gel were provided by Carlo Erba Reactif, SDS. BOP was purchased at Advanced ChemTech. CHOL-PEG$_{2000}$(OH) was synthesized as previously described (Masson et al., 2004) and DODA-GLY-PEG$_{2000}$ has been synthesized as described in Section 2.2.

2.2. Synthesis of DODA-GLY-PEG$_{2000}$ (2-dioctadecylcarbamoyl-methoxyacetylamino) acetic acid-(ω-methoxy)-polyethylene glycol 2000 ester)

mPEG$_{2000}$-glycine and DODA glycolic acid were obtained as described (Bhat et al., 2007; Huetz et al., 1997). DODA glycolic acid (1.41 g; 2.2 mmol) and mPEG-glycine (4.82 g; 2.2 mmol) were dissolved in dichloromethane (100 mL), triethylamine (1.55 mL; 11 mmol) and BOP (0.98 g; 2.2 mmol) were added and the mixture was stirred for 2 h at room temperature. The solution was concentrated and the residue chromatographed on silica gel eluted with dichloromethane/methanol (95/5%, v/v). The DODA-GLY-PEG$_{2000}$ was obtained after evaporation of the solvent and crystallized in 2-propanol (3.18 g; 54%). mp: 49.2 °C; ^{1}H NMR ($CDCl_3$): δ (ppm) 8.15 (s, 1H, N–H); 4.28 (m, 4H, CH_2); 4.15 (m, 2H, CH_2); 4.09 (m, 2H, CH_2); 3.64 (m, 180H, CH_2); 3.37 (s, 3H, CH_3); 3.28 (m, 2H, CH_2–N); 3.08 (m, 2H, CH_2–N); 1.51 (m, 4H, CH_2); 1.25 (m, 60H, CH_2); 0.87 (t, 6H, CH_3). ^{13}C NMR ($CDCl_3$): δ (ppm) 170.29; 169.35; 168.18; 71.58; 70.02; 68.67; 64.15; 58.81; 31.74; 29.50; 29.17; 22.50; 13.98; *m/z* 2669.8.

2.3. Solubility tests

Increasing amount of fisetin was mixed in different solvents. Solubilisation value is given for solutions where a plateau was reached as determined by reading the absorbance at 340 nm by UV spectrophotometry. Fisetin solubility was found to be 4.35 mg/mL in ethanol, 4.65 mg/mL in methanol and 9.75 mg/mL in a mixture methanol/chloroform (50/50%, v/v).

2.4. Liposome preparation

2.4.1. Probe sonication method

The aim of this method was to directly obtain an acceptable liposome size by avoiding the extrusion process. Components (600 mg total lipids) were weighed and added directly in Hepes buffer (15 mL, 20 mM; pH 7.4) which were then submitted to discontinuous sonication for 2–5 cycles (duty cycle of 90%) with a Branson Sonifier 450 (Danbury, US) displaying an output power of 400 W. A disrupter horn of 6 mm adapted to the 15 mL volume was used and the flask was plunged into ice to avoid aggregation. Liposome diameter and polydispersity index (PDI) were determined after each cycle. The data reported in Table 3 correspond to the best result obtained after two cycles.

2.4.2. Homogeneization method

The aim of this method was to obtain directly an acceptable liposome size by avoiding the extrusion process. Components (600 mg total lipids) were weighed and added directly in Hepes buffer (20 mM; pH 7.4) at a concentration of 40 mg/mL, which were then submitted to homogeneization during 1–50 min at different speeds (6500–9500 rpm) with an UltraTurrax T25 (IKA, Staufen, Germany), while keeping the flask into ice. Liposome size and PDI were determined on fractions harvested every 2 min and the data presented in Table 3 correspond to the results obtained after 20 min because there was no change beyond that time.

2.4.3. Film hydration method

Fisetin (12 mg, 42 μmol), lipids with the ratio indicated in Table 1 (365 mg, 438 μmol) and organic solvent (9 mL) were introduced into a clean and dry round-bottom flask. Solvent evaporation was performed on a Buchi evaporator (Buchi, Switzerland), equipped with a diaphragm pump (Vacuubrand, Germany) and a cooler immersion (Tc40e, Huber), at decreasing pressure starting from 200 to 100 mbar at room temperature to form a regular film on the bottom of the flask, then reducing the pressure by steps of 20 mbar every 10 min to reach 6 mbar which was maintained during 5 h. The hydration process was then carried on for 24 h with Hepes buffer at room temperature (15 mL, 20 mM; pH 7.4), followed by successive extrusion on 0.8, 0.4 and 0.2 μm membranes under nitrogen pressure (Lipex Biomembranes, Vancouver, Canada). Finally, the liposomes were purified by passage on a Sephadex G-25 column. Liposome size and PDI were determined as described below.

2.4.4. Lipid cake formation method

Fisetin (12 mg, 42 μmol), organic solvent (9 mL), and lipids (365 mg, 438 μmol) were both introduced in a clean and dry 500 mL flask equipped with a magnetic stirrer. Solvent evaporation was performed on a Buchi evaporator (Buchi, Switzerland), equipped with a diaphragm pump (vacuubrand, Germany) with magnetic stirring during 3 h until a dry extract was obtained. The hydration process was then carried on for 6 h at room temperature with Hepes buffer (15 mL, 20 mM; pH 7.4) under stirring conditions. Liposomes were then extruded successively on 0.8, 0.4 and 0.2 μm membranes, under nitrogen pressure (Lipex Biomembranes, Vancouver,

Table 1
Proportions of the liposomal components used in the various formulations tested (wt%).

Constituents	1	2	3	4	5	6	7	8
DOPC	79.5	78	74	79.6		79.6		
P90G					79.6		79.6	79.6
Cholesterol	6	6	6	4	4	4	4	4
DSPE-PEG_{2000}	13	13	13	13.2	13.2			
Chol-PEG_{2000}-OH						13.2	13.2	
DODA-GLY-PEG_{2000}								13.2
Fisetin	1.5	3	6	3.2	3.2	3.2	3.2	3.2

Canada) and finally purified on a Sephadex G-25 column. Liposome size and PDI were determined as described below.

2.5. Fisetin liposomal formulations tested

The different fisetin liposomal formulations evaluated in this study are described in Table 1.

2.6. Determination of the liposome size by dynamic light scattering

Dynamic light scattering measures the motion of particles in a medium of known viscosity and refractive index. The Stokes–Einstein equation ($D = kT/6\pi R\eta$ where D is the particle scattering coefficient, T the temperature, k the Boltzmann constant, R the particle radius and η the viscosity of the solvent) links the correlation function to the hydrodynamic radius allowing to access to the hydrodynamic particle diameter. Particle diameter was determined by dynamic light scattering on a Zeta Sizer NanoSeries Malvern (Malvern Instruments, Venissieux, France). The concentration of the samples was approximately 0.5 mg/mL in Hepes buffer. PDI reflects the polydispersity of the suspension ranging from 0 to 1, with the lower values indicating a more monodispersed suspension. Measurements have been performed in triplicate before and after purification. Ten measures have been performed at each time point for the evaluation of the colloidal stability.

2.7. Determination of fisetin encapsulation

The encapsulation yield has been determined according to the following formula:

Percent encapsulation (%, w/w) = (encapsulated fisetin concentration/total amount of fisetin in the formulation) × 100. The total amount of fisetin in the formulation was determined before purification. The concentration of encapsulated fisetin was evaluated after separation of liposomal and free fisetin on a Sephadex G-25 column, by two different methods, high-performance liquid chromatography (described hereafter in Section 2.7.1) and UV spectrophotometry which was optimised in 96 well plates for the stability studies. All data were double checked by the two methods, with the exception of the stability studies performed by UV spectrophotometry. The amount of fisetin (mg) per total lipid amount (g) is also given in Tables 2 and 4.

2.7.1. High performance liquid chromatography

Liposomes were diluted (1/100) in methanol, vortexed, and 100 μL were injected onto a reversed-phase HPLC system (Shimadzu CLASS-VP®, version 5.3), equipped with an octadecylsilane column (Beckman Ultrasphere ODS, 5 μm; 4.6 mm × 250 mm) thermostated at 20 °C, and a UV detector set at 360 nm. The mobile phase was composed of 52% methanol and 48% acidified water (2%, v/v glacial acetic acid), at a flow rate of 1 mL/min. In these conditions the retention time of fisetin was 6.6 min and the calibration curve was linear with R^2 near unity (0.9972) with the following equation $y = 206{,}196x$, where the slope is expressed in μV × s.

2.7.2. Determination of fisetin encapsulation by UV spectrophotometry

A calibration curve was prepared with a range of fisetin concentrations from 10 to 100 μg/mL in methanol. Purified liposomes were diluted 100 times in methanol to dissolve lipids and fisetin. All samples were loaded on a 96-well plate and absorbance values were read at 340 nm on a Berthold spectrophotometer. The amount of fisetin prior and post liposome purification was calculated according to the calibration curve, with the following equation $y = 0.232x - 0.0075$, $R^2 = 0.9975$.

2.8. Stability studies

Liposomal fisetin suspensions were stored at 4 °C and samples were regularly withdrawn to perform size measurements as described in Section 2.6 and fisetin encapsulation assessment as described in Section 2.7. Percentage of fisetin release was calculated using the following formula: % fisetin release = 100 − (encapsulated fisetin concentration/fisetin concentration in the liposome at the beginning of the stability study) × 100.

2.9. Cytotoxicity tests

The murine Lewis lung carcinoma cell line (3LL) and the colon 26 (CT26) carcinoma cell line were purchased from the American Type Culture Collection (ATCC). The human endothelial cell line (EAhy 926) was obtained from Dr. Edgell (Edgell et al., 1983) and used with permission. The cell lines were grown in DMEM containing 2 mM L-glutamine, 10% foetal bovine serum, 100 U/mL penicillin and 100 μg/mL streptomycin (37 °C, 5% CO_2). Cells were plated onto 96-well plates at 5000 cells per well in 100 μL of culture medium. Twenty-four hours after plating, 100 μL of medium containing the compound of interest (final concentrations ranging from 100 to 0.8 μg/mL, in 2-fold dilutions) was added to the wells (in triplicate) containing the cells and incubated for 24 h at 37 °C and 5% CO_2. After the 24 h exposure period to the test compounds, cell viability was evaluated using the MTT test and absorbance was read at 562 nm in a microplate reader (BioKinetics Reader, EL340). Results are expressed in percent of viability compared to the same concentration of solvent (DMSO for the fisetin, and empty liposomes for the fisetin liposome).

2.10. Electron microscopy

2.10.1. Transmission electron microscopy

Liposomal fisetin prepared as described were loaded on a Formvar/carbon copper grid 200 mesh from Agar Scientific. Uranyl acetate was used to stain the samples. Analyses were performed on a microscope JEOL, JEM 100S.

2.10.2. Cryo electron microscopy

Liposomal fisetin prepared as described were loaded on a Formvar/carbon copper grid 200 mesh (Quantifoil®). After a quick blotting with a filter paper, the grid is immersed in liquid ethane to vitrify the suspension. The grid was then transferred in a microscope JEOL 2100, equipped with a CRYO objective under the cryotransfer station Gatan 626 maintained in liquid nitrogen. Observations were performed at −180 °C, images were registered in phase contrast at minimal electron dose (10 electrons/Å/s), and at ×40,000 magnification with a Gatan Ultrascan 1000 CCD camera.

Table 2
Characteristics of liposomal fisetin preparations **1**–**3**. Components are expressed in weight for 100 mg of powder. The diameter was determined by dynamic light scattering (DLS). Diameter (nm) of the two populations is indicated. PDI, polydispersity index, represents the homogeneity of the preparation. % (w/w) represents the percentage of fisetin encapsulated versus fisetin in the formulation. The ratio fisetin per total lipid is expressed in mg/g.

Preparation no.	DOPC/DSPE-PEG$_{2000}$/ Chol/Fisetin	Diameter (nm)	Peaks (nm)		PDI	% (w/w)	Fisetin/lipid (mg/g)
1	79.5/13/6/1.5	148	57	170	0.32	35	7
2	78/13/6/3	125	35	127	0.35	62	15
3	74/13/6/6	330	45	260	0.43	54	11

2.11. Immunofluorescence microscopy

EAhy 926 endothelial cells were plated on a 24 well plate (150,000 cells/mL) and incubated overnight at 37 °C in 5% CO_2 humidified atmosphere. The medium was discarded and liposomal fisetin (200 μL, 11 mg/mL with 1% of DOPE-rhodamine) or free fisetin were added for 2 h at 37 °C onto the cells. The cells were fixed (4% paraformaldehyde), permeabilized, and saturated with a 3% BSA solution containing 1% Triton × 100 (1 h at room temperature). Cells were processed for indirect immunofluorescence as follows: cells were incubated 1 h at 37 °C with the mouse anti-α-tubulin monoclonal antibody (Sigma) at 1/2000 dilution and further incubated with an anti-mouse fluorescein isothiocyanate (FITC) secondary antibody (Sigma) at 1/400 dilution for 45 min in the dark at room temperature. Photographs were taken on a Zeiss fluorescence microscope and a Zeiss LSM-510 confocal microscope (FITC: excitation 488 nm, emission 530 nm; DOPE-rhodamine: excitation 541 nm, emission 572 nm) (Carl Zeiss France, Le Pecq, France).

3. Results and discussion

3.1. Determination of the maximum amount of fisetin inserted in the lipid bilayer

Fisetin is a lipophilic compound that solubilises into the lipidic bilayer because its log *P* octanol–water is estimated to be about 3.2 (Miteva et al., 2006). We initially evaluated the interaction of fisetin with dimyristoylphosphatidylcholine as solubilizer (Guharay et al., 2001), then evolved to more sophisticated liposome formulation to increase fisetin stability into the bilayer. Obviously, inserting a too large amount of fisetin would induce a destabilisation of the bilayer, therefore reducing the encapsulation yield. We first evaluated the maximal amount to be inserted into the lipid bilayer by increasing the amount of fisetin (1.5–6%, w/w) in a DOPC/Chol/DSPE-PEG$_{2000}$-based liposome by using the film hydration method (Table 1) (Fattal et al., 1993; Olson et al., 1979). Thereafter, we evaluated the size and observed the aspect of the liposomes by transmission electron microscopy (TEM).

As can be seen from the data in Table 2, the liposome size and the PDI increased with the amount of fisetin introduced in the preparation. A possible explanation to the increased PDI would be that the insertion of fisetin in the bilayer could reduce the lipid cohesion leading to polydispersed preparations. As a matter of fact, microscopic observations indeed showed that preparations **1** and **2** were homogeneous (Fig. 1A and B). The insertion of higher amount of fisetin in preparation **3** resulted in an increased liposome size and exclusion of the lipid components from the liposomes, as observed in Fig. 1C. These results are compatible with dynamic light scattering data presented in Table 1 which indicate a high diameter value of 330 nm. Consequently, due to its high PDI and diameter values, preparation **3** was not considered further.

The choice between formulation **1** or **2** was based on the fisetin encapsulation yield. Formulation **2** gave a 62% fisetin encapsulation compared to preparation **1** which presented only a 35% encapsulation value, by taking into account the initial amount of fisetin involved in the preparation, or 15 mg fisetin/g versus 7 mg fisetin/g total lipid, respectively (cf. Table 2). Preparation **2** was therefore chosen for further optimization, as described hereafter.

3.2. Liposomal fisetin optimization

Preclinical *in vivo* tests in mice require a higher liposomal volume than the ones usually required for *in vitro* testing. Those larger volumes also involved increased costs and hampered the formulation process, leading to irreproducible batches. We therefore aimed at improving the fisetin liposomal preparation by changing the various components and optimize the process as described below.

3.2.1. Choice of the method for optimization of formulation *2*

Several methods have been reported to prepare liposomes. In a research laboratory scale, the two main methods used for liposomal preparation are ethanol injection and film formation (New, 1990). Although the ethanol injection method appears to be the least expensive (Rodriguez and Moraes, 2010) and easy to perform (Thompson et al., 2005), injection of an ethanolic solution of fisetin and lipids in an aqueous medium induced precipitation of the flavonoid, due to its poor ethanol solubility.

Consequently, the film formation method was chosen to encapsulate fisetin. Liposome preparation by film formation involves three steps: film formation, hydration and size reduction. Of those steps, homogeneity of the film and volume of hydration appear difficult to scale-up. Thus, to avoid the film formation step, we evaluated a similar technique of solvent evaporation to dryness, in order to form a dry extract (lipid cake), followed by hydration and size reduction. Lipid cake formation consisted of simply drying the lipids under vacuum with constant stirring. To avoid the problems inherent to the film formation method, we also evaluated ultrasound-based sonication and homogeneization, because they advantageously avoid organic solvent use (Brandl et al., 1990). A comparison of the four techniques employed to prepare liposomal fisetin are presented in Table 3.

Table 3
Comparison of different methods of liposomal fisetin preparation. The values presented are for a batch of 50 mM of total lipid. PDI is the polydispersity index. Percent encapsulation is the percentage of encapsulation, as compared to the amount of fisetin initially weighed. ND, not determined because of high polydispersity.

Methods	Solvent	Diameter (nm)	PDI	% Fisetin encapsulation.	Observations
Probe sonication	–	300	0.51	ND	Non homogeneous
Homogeneization	–	1000	0.96	ND	Non homogeneous
Film hydration	+	140	0.13	56	Film adequate
Lipid cake formation	+	170	0.08	55	Highly viscous foam formed

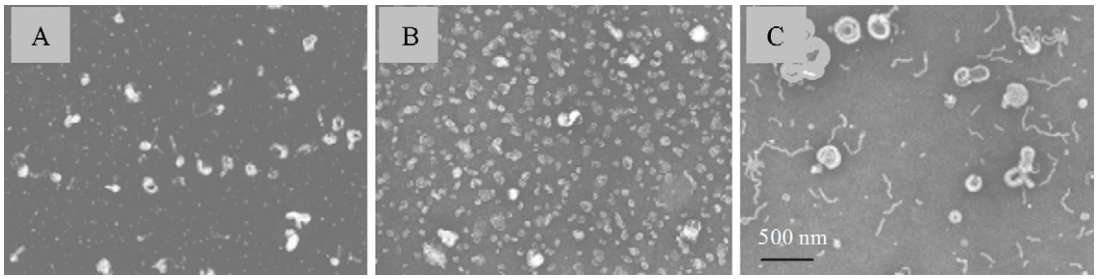

Fig. 1. Transmission electron microscopy photographs depicting formulations **1**, **2**, and **3**, labeled as A, B, and C, respectively (original magnification ×30,000).

The probe sonication and homogeneization methods were attractive because they did not require solvent use (Table 3). We have worked on different conditions in order to optimize the liposome preparation, taking into account the volume, the temperature, the output power applied and the duration of the process application, as described in the experimental part. For ultrasound application, liposome size and PDI were measured after each cycle in order to determine the evolution of the liposomes under the conditions used. As for the homogeneization process, liposome size and PDI were determined every 2 min. Despite the efforts to optimize the methods on the fisetin liposomal formulation, these two methods did not yield homogeneous preparations and fisetin aggregates were observable. It thus appears that the three steps are required for liposomal fisetin preparation, i.e., film formation, hydration and size reduction.

For the two other methods (film hydration and lipid cake formation), only the first step differed. Either a lipid film was formed by evaporation under slight rotation and controlled reduced pressure, or a lipid cake was formed under reduced pressure, stirring the lipids with fisetin. In this last process, the consistence of the dry extract formed and its apparent inhomogeneous hydration did not appear to be a controlled process, but it nonetheless allowed obtaining highly homogeneous liposomes after extrusion (PDI = 0.08), as shown in Table 3. This lipid cake formation process was therefore chosen for the following optimization experiments.

3.2.2. Optimization of the liposomal formulation

Liposomes prepared with the above synthetic phospholipids of very high quality were relatively expensive. To reduce these costs, we decided to replace the lipid and lipid-PEG components. The 99% pure DOPC was replaced by another phosphatidylcholine P90G of slightly lower purity (94%) which is approved by the FDA in pharmaceutical formulations. The DSPE-PEG_{2000} was replaced by different lipid-PEG of the same PEG length differing by their lipid moiety. Thus, distearoyl-phosphatidylethanolamine (DSPE) which is mainly responsible for the high cost of this lipid, was replaced by a cholesterol or a dioctadecyl-glycine (DODA-GLY) moiety. The ratios of the different components, based on the previous results, are presented in Table 1.

Five fisetin liposomal formulations (reported in Table 4) were all prepared following the above described lipid cake formation protocol. In these experiments, the scale was based on a lipid concentration of 100 mM and a final volume of 100 mL. No practical difficulties have been encountered with the newly tested components. The size of the unpurified and purified steps, and percent encapsulation of the new liposomal formulations were assessed and are presented in Table 4.

Because changes in the liposome composition can affect liposome properties (Khan et al., 2008b), we then evaluated the characteristics of the new formulations to choose the alternative components. Replacing DOPC by P90G did not improve liposome homogeneity or percent encapsulation by considering samples **4** and **5** (Table 4). However, in presence of Chol-PEG_{2000}-OH (samples **6** and **7**) instead of DSPE-PEG_{2000} (samples **4** and **5**), this modification appeared advantageous for both DOPC and P90G lipids in terms of liposome size, but the percent encapsulation was reduced. Because fisetin is solubilized in the lipid bilayer, we inferred that a bicatenar lipid bearing PEG could better stabilize the bilayer compared to a cholesterol lipid bearing PEG. We therefore synthesized a DODA-GLY-PEG compound to evaluate if DSPE-PEG_{2000} could be replaced.

A straightforward synthesis of DODA-GLY-PEG was achieved by the BOP mediated condensation of the low cost and easily prepared mPEG-GLY-NH2 (Bhat et al., 2007) and DODA-glycolic acid (Huetz et al., 1997). The choice of the linker between the PEG and the lipid was first based on previous studies showing higher stability of cationic lipoplexes by PEG-linker-lipids, compared to PEG–lipid, probably by a better exposition of the PEG outside the lipid bilayer (Nicolazzi et al., 2003), and second for the biodegradability brought by the ester function in this particular linker. DODA-GLY-PEG was then inserted into formulation **8**.

From the data in Table 4, preparation **8** exhibited the best characteristics in terms of polydispersity before (PDI = 0.16) and after column purification (PDI = 0.12). The liposome size and aspect was further characterized by cryo-transmission electronic microscopy. One representative image depicted in Fig. 2 confirmed the homogeneity of the suspension and showed that the liposomes formed where mostly unilamellar vesicles with a mean diameter around 100 nm.

Moreover, preparation **8** was also the best in terms of percent fisetin encapsulation (73%). Because it has previously been reported that there is a close relationship between flavonoid molecular structure and its interaction with model membranes (Goniotaki et al., 2004), one can not exclude that the molecular structure of

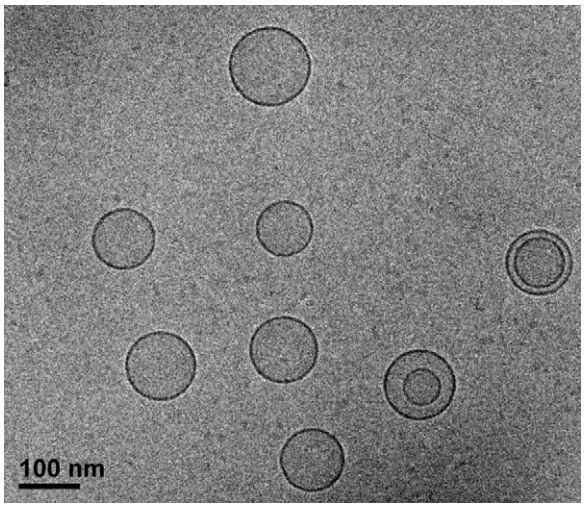

Fig. 2. Representative photograph of liposomal fisetin formulation **8** obtained by cryo transmission electron microscopy.

Table 4
Diameter (nm) and polydispersity index (PDI) of fisetin liposomal formulations **4–8** before and post purification by size exclusion chromatography. Values given correspond to the means within a range of 1.5%.

Preparation number	Composition	Unpurified		Purified		% Fis encaps	Ratio Fis/lipid (mg/g)
		Diameter (nm)	PDI	Diameter (nm)	PDI		
4	DOPC, DSPE-PEG	260	0.35	190	0.15	52	12
5	P90G, DSPE-PEG	330	0.36	300	0.28	44	9
6	DOPC, Chol-PEG	180	0.26	155	0.17	47	10
7	P90G, Chol-PEG	145	0.26	140	0.13	30	6
8	P90G, DODA-PEG	195	0.16	175	0.12	73	18

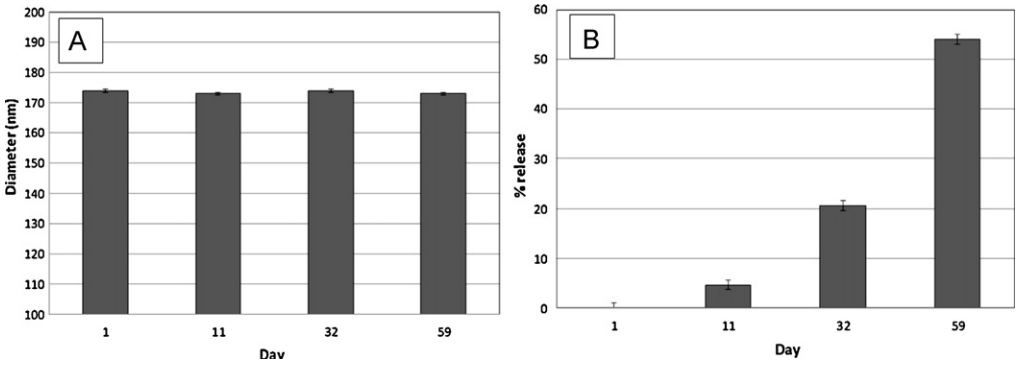

Fig. 3. Fisetin liposome diameter (nm) (A) and fisetin release (%), (B) after storage at 4 °C over a period of 59 days.

fisetin would interact differently with lipids of different nature, as seen in preparation **8**. An alternative hypothesis for this important gain in percent encapsulation could be that the DODA moiety stabilises the lipid packing around the fisetin molecules.

This preparation has been repeated 6 times, the mean diameter of the liposome obtained was 169 ± 15 nm with a mean polydispersity of 0.141 ± 0.05 showing the reproducibility of this formulation. Fisetin liposomes formulation **8** was therefore chosen for the stability studies and cellular cytotoxicity tests.

3.3. Stability of the liposomal fisetin

Colloidal stability of the liposomal fisetin and fisetin release from the liposomes are two main parameters related to liposomal stability. These two parameters were evaluated over a period of time by following the evolution of the liposome diameter by dynamic light scattering and the fisetin encapsulation into the liposomes after purification of samples at each time point.

As presented in Fig. 3A, liposome size did not vary significantly over 59 days. Fisetin was released slowly from the liposomes leading to an encapsulation of 50% after 59 days which means that it was still usable after this time period (Fig. 3B). Those results indicated that fisetin liposomes were stable and injectable after 59 days and therefore could be used in tumour regression studies performed usually on a 15 day time schedule in tumour bearing animals. However, after 10 days, the dose should be adapted or a new batch should be prepared to avoid any risk of fisetin aggregation post injection. Lyophilized batches could also be evaluated if the *in vivo* experiments would need to be pursued for longer periods of time.

3.4. Cytotoxicity and cell morphology of liposomal fisetin

To evaluate if fisetin cytotoxicity was maintained when encapsulated into liposomes, viability tests were performed on three different cell lines including the endothelial EAhy 926 cells, and two cancer cell lines (Lewis lung carcinoma (3LL) and colon tumour 26 (CT26)). The survival curve for 3LL cells presented in Fig. 4 shows that there was no difference between the free fisetin and the liposomal fisetin in the fisetin concentration required to kill 50% of cells (IC_{50}) which was 15.5 μg/mL for the former and 15.0 μg/mL for the latter. We also observed similar IC_{50} concentrations for the CT26 colon cells (liposome = 16.7 μg/mL; free 15.7 μg/mL) and EAhy 926 endothelial cells (liposome = 17.9 μg/mL; free 17.3 μg/mL) (data not shown).

Because fisetin has been reported to induce a typical morphological modification of endothelial cells (Touil et al., 2009), we also evaluated the effect of liposomal fisetin on the morphology of endothelial cells during a short incubation time of 2 h. As expected, free fisetin elicited the typical formation of pseudopodes (Fig. 5A). Liposomal fisetin could also cause the formation of pseudopodes as depicted in Fig. 5B, indicating that encapsulated fisetin retained the free fisetin activity on endothelial cell morphology. Also of note, liposomes were seen to be internalized, as shown by the presence of red dots inside the cells (Fig. 5B).

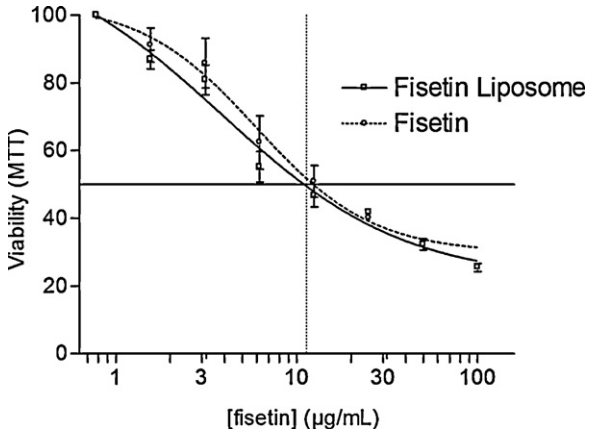

Fig. 4. Comparison of the cytotoxicity of free fisetin with liposomal fisetin on Lewis lung carcinoma cells (3LL) after 24 h exposure time.

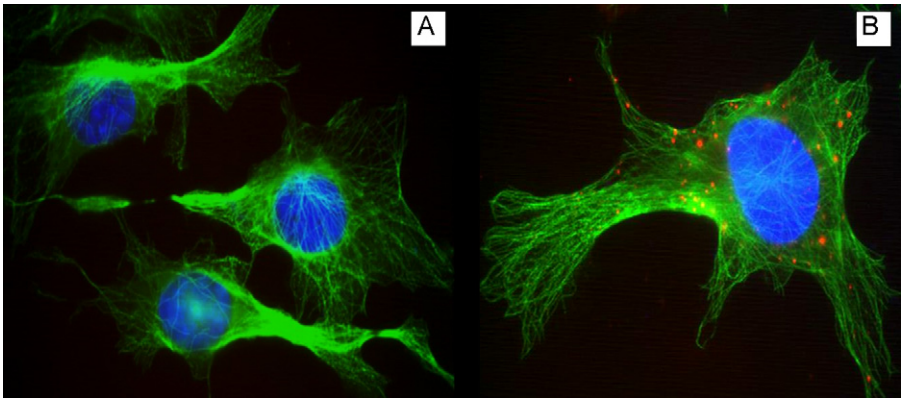

Fig. 5. Representative photographs depicting endothelial cells (EAhy 926) treated with free fisetin at a concentration of 11 μg/mL (A), and liposomal fisetin at 11 μg/mL (B), for a 2 h incubation time. Green represents the tubulin and the red dots in B are the DOPE-rhodamine labeled liposomes (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.).

Therefore, the encapsulation of fisetin into liposomes was shown to retain its biological activities, as substantiated by similar cytotoxicity values compared to the free fisetin, and also by its morphological activity observed on endothelial cells. The fact that similar level of cytotoxicity and morphological effects were observed could indicate that fisetin is rapidly released from the liposomes post membrane interaction as already reported for other lipophilic compounds (Fahr et al., 2006). We did not expect any improvement of the *in vitro* cytotoxicity at this point, eventually a reduced cytotoxicity if slow release would occur into the cells. However, for the *in vivo* situation, the liposomal fisetin formulation could be advantageous because it could prolong the residence time of this compound and allow for a better anticancer effect *in vivo*, as recently reported for another flavonoid (Yuan et al., 2006).

In conclusion, we have developed and optimized a liposomal formulation of fisetin, for the first time to our knowledge, that was shown to retain the biological properties of free fisetin. Because few PEG–lipids are readily available and are also expensive, we prepared DODA-GLY-PEG_{2000} which allowed the formation of highly homogeneous liposomes. In addition, substitution of DOPC by P90G lecithin and DSPE-PEG_{2000} by DODA-GLY-PEG_{2000} led to liposomal form of fisetin which solubilized fisetin and did not alter its biological properties. Based on these promising results, we plan to further test this liposomal fisetin preparation in tumour bearing mice to fully develop the antiangiogenic and anticancer activities of this natural flavonoid.

Acknowledgements

This research was supported by the Institut National de la Santé et de la Recherche Médicale (INSERM), by the Centre National de la Recherche Scientifique (CNRS) and by a grant from the Institut National du Cancer (INCa, Boulogne-Billancourt, France). We also thank René Lai-Kuen and Bruno Saubamea from Technical Platform of the IFR71/IMTCE–Cellular & Molecular Imaging–Faculty of Pharmacy–Paris Descartes University, and Eric Larquet from the IMPMC, CNRS-UMR7590, Université Pierre & Marie Curie, for the cryoelectronic transmission microscopy expertise and experiments.

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