Encapsulation of rutin and naringenin in multilamellar vesicles for optimum antioxidant activity

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ABSTRACT

Rutin and naringenin, two phenolic compounds with antioxidant properties were encapsulated in lipid-based onion-type multilamellar vesicles (MLVs). After vesicles formation, the free, adsorbed/encapsulated analytes were well separated with size exclusion chromatography (SEC), and rutin and naringenin were quantified with UV-HPLC at 258 nm and 290 nm. A mathematical model was developed to separately calculate the encapsulation and the adsorption yields of both phenols. Naringenin was shown to be poorly encapsulated (<10%) but highly adsorbed on MLVs surface (>60%) whatever MLVs composition. Conversely, rutin showed high encapsulation efficiency (>60%). Entrapment of rutin was proved to be efficient since no leak was observed within 30 days in concentrated MLVs phase, while 16.0 ± 0.3% of rutin was still encapsulated after 30 days when MLVs were diluted in water. Free rutin broke up into quercetin while the encapsulated one remained stable. DPPH assay confirmed that only free and adsorbed rutin participated in antioxidant activity.

1. Introduction

The human body suffers from oxidative stress due to high exposure to sunlight, pollution, daily stress, unbalanced diet or smoke (Rovito & Oblong, 2013). It is commonly accepted that oxidative stress causes excessive production of reactive oxygen species (ROS) (Cheng et al., 2013) and is associated with the origin of some human diseases including cancer and aging (Chen, Lin, & Hsieh, 2007). Some studies have shown that a diet rich in polyphenolic compounds can help to fight against ROS production due to their antioxidant activity (Yen et al., 2013). Such compounds are also used for their antioxidant properties to enhance the shelf life of food products that can suffer from UV radiation, temperature or oxygen contact (Myers, Fuller, & Yang, 2013). This use of antioxidants assumes a decrease of their concentration before adsorption and thus to preserve the antioxidant activity of molecules until consumption, to insure an extended life-span, or to avoid unpleasant taste of high concentration of some molecules (astringency, bitterness) (McClements, 2010).

Two flavonoids were chosen here to develop an encapsulation system: naringenin and rutin. These molecules were chosen because of their different polarity (See Supplemental information, S1); quercetin, which is the aglycon form of rutin was not used as it is not stable in our conditions. Naringenin is the predominant flavanone in grapefruit (Sansone et al., 2011) whereas rutin is a common flavonol glycoside of vegetables (Jacopini, Baldi, Storchi, & Sebastiani, 2008). Both display antioxidant and antimicrobial activities. Rutin was successfully encapsulated using ionotrophic gelation (Jantrawut, Assifaoui, & Chambin, 2013), in chitosan...
As protecting and delivery systems, liposomes (lipid-based vesicles) display many advantages; biocompatibility, non-toxicity, adjustable size, enhancement of molecules chemical and physical stability (Müller, Petersen, Hommoss, & Pardeike, 2007), ability to encapsulate hydrophobic and hydrophilic molecules (Charrois & Allen, 2003), and controlled delivery (Maherani, Arab-Tehrany, Kheirolomoom, Geny, & Linder, 2013). However, liposomes are usually produced by the hydration of a dried lipid film with an aqueous phase in excess. This process requires the use of organic solvents. Moreover, encapsulation yields are often limited. Onion-type multilamellar vesicles are obtained with an easier process, consisting in shearing a swollen lamellar phase leading to the formation of controlled, reproducible, multilamellar vesicles, the so-called onions (Diat & Roux, 1993). Onions display the same advantages as unilamellar liposomes but their production does not require organic solvents (Roux, Degert, & Laversonne, 1996). Moreover, they were shown to encapsulate biomolecules with high encapsulation efficiency (Mignet, 2000; Olea & Faure, 2003; Dhanikula, Lafleur, & Leroux, 2006; Prévot et al. & Faure, 2012).

This study investigates the encapsulation of naringenin and rutin in onion-type MLVs. The aim of this work is to optimise a protective and delivery system with model molecules before the encapsulation of a natural extract.

2. Materials and methods

2.1. Materials

Rutin hydrate (≥94%) and naringenin (≥98%) were purchased from Sigma-Aldrich (Saint-Quentin Fallavier, France). Emulmetik 930 (E930, a 95% purified phosphatidylcholine isolated from soy-bean lecithin, containing less than 1% of lyso phosphatidylcholine) was obtained from Unipex (Saint-Ouen L’Aumone, France). Glycerin 99.5% and propylene glycol (PG) were purchased from Intermichim (Bobigny, France). Solvents (water, acetonitrile and 2-propanol) were from HPLC grade, used with 0.1% of formic acid (Sigma-Aldrich, Saint-Quentin Fallavier, France) for HPLC study. Triton X100 was purchased from Sodipro (Echirolles, France).

2.2. Preparation of onion-type multi-lamellar vesicles

The general preparation of onion-type multilamellar vesicles (MLVs) was described in details elsewhere (Roux et al., 1996). Flavonoids were first dissolved in 2–4 mL of 60:40 v/v glycerin: propylene glycol mixture (PG). For rutin and naringenin concentrations below 30 mg/mL of GPG and 8 mg/mL of GPG, respectively, dissolution was performed in mild conditions (magnetic mixing at 40 °C). For higher concentrations, dissolution required the use of an ultra-sound bath (from 30 to 90 min) (Fisher Scientific FB15049, Germany). A lamellar phase was prepared by mixing Emulmetik 930 (ca. 250 mg) with the flavonoid-GPG solution in a given weight proportion (from 35/45 to 15/85 (w%/w%)). Mixing was performed with a micro-spatula for 3–4 min in a 4 mL vial. The tube was centrifuged at 4000 rpm for 5 min (Sigma 2–6, Germany). Shearing was then resumed followed by further centrifugation. The cycle was repeated 3 times until the resultant yellow/brown lamellar phase appeared homogeneous. “Empty” onions (blank onions) were prepared with lipid and GPG containing no flavonoid. Once sheared, onions were in close contact forming a compact phase. They were then dispersed in distilled water (25 mg onions/mL, unless specified) by gentle mixing with a mechanical stirrer (500 rpm) for 3 h.

2.3. Optical microscopy

MLVs were observed with an Optech microscope using 40X and 100X objectives. The former objective was supplied with a phase contrast filter.

2.4. Particle size analysis

Vesicle size was measured using a Mastersizer 2000S (Malvern Instruments), with a measure scale from 20 nm to 2 mm. Four measurements were performed per sample.

2.5. Size exclusion chromatography (SEC)

SEC was performed to separate MLVs from free flavonoids in order to measure both the encapsulation and adsorption percentages. An open microcolumn (1 × 8 cm) filled with a Sephacryl S300HR gel (Sigma Adrich, Saint-Quentin Fallavier, France) was used. The column flow, which was about 1 mL/min, was controlled and kept constant by fixing the vertical position of the eluent pool (distilled water).

In order to determine the elution profile of free (non-entrapped) flavonoid, a mixture (400 μL) of rutin and naringenin was deposited onto the column gel. To do this mixture, rutin and naringenin were first both diluted in GPG at 30 mg/mL and 8 mg/mL, respectively, and then diluted in water (*100). 0.5–1 mL fractions were collected and a total volume of 60 mL was eluted. Each collected fraction was analysed using UV-HPLC at 258 and 290 nm for rutin and naringenin, respectively. The elution volume (Ve) ranged from 6 to 25 mL, and 15 to 40 mL for rutin and naringenin, respectively. Total amount of analytes was eluted.

The MLVs elution volume was determined using MLVs containing both rutin (30 mg/mL of GPG) and naringenin (8 mg/mL of GPG). SEC column was initially saturated with 400 μL of blank MLVs and washed with distilled water. MLVs (25 mg/mL of water) were found to elute between 1.5 and 5 mL.

To determine the amount of adsorbed and encapsulated flavonoids, flavonoid-containing MLVs were separated from the dispersing medium by SEC, and the MLVs-containing fractions (10 mg) were treated with 20 w% Triton X-100 (80 μL) to destroy MLVs for HPLC convenience. The measured flavonoid concentration corresponds to the fraction of flavonoid adsorbed on and encapsulated in MLVs.

To determine the amount of adsorbed flavonoids on MLVs, blank MLVs were dispersed in an “aqueous” solution containing either naringenin or rutin, or both flavonoids. This solution was prepared as described previously: flavonoid-GPG solution was diluted in water to get a final concentration in flavonoid similar to the dispersions of flavonoid-loaded MLVs. SEC was performed to separate MLVs from the dispersing medium. MLVs fractions were then treated with Triton X-100. The measured flavonoid concentration corresponds to the fraction of flavonoid adsorbed on MLVs surface.

2.6. High performance liquid chromatography (HPLC)

HPLC analyses were performed using an Agilent 1200 system (Courtaboeuf, France), equipped with a quaternary solvent delivery system, an autosampler, a degasser and a DAD (Diode-Array
Detection) detector. Separation was performed using a Luna C18 column (Phenomenex, 250 × 4.6 mm; 5 µm). Rutin was detected at 258 nm with a retention time ($T_R$) of 13.1 min while naringenin was detected at 290 nm with a $T_R$ of 18.3 min. Calibration curves for both molecules were obtained after dissolving each one in 2–3 mL GPG to get a 30 mg/mL and 8 mg/mL concentration in rutin and naringenin, respectively. Subsequent dilutions in water were then performed to get concentrations ranging from 0.2 to 100 µg/mL, and 0.05 to 26.46 µg/mL for rutin and naringenin, respectively. Response factors were determined by linear regression for each standard with $R^2$ coefficients all deemed acceptable above 0.99.

The column was eluted with a flow rate of 1.0 mL/min, and the composition of the mobile phase consisted of 0.1% acid formic acidified water (A), acetonitrile (B) and 2-propanol (C). Gradient conditions were as follows: 0–5 min, 5% B; 5–20 min, 5–62% B; 20–21 min, 100 % B; 21–23 min, 100–5% B. To assay rutin and naringenin adsorbed or/and encapsulated into MLVs, SEC fractions containing MLVs (10 mg) were treated with 20 w/% Triton X100 (80 µL) to destroy them. For samples containing E930 and/or Triton X100, an additional gradient was applied as follows: 23–28 min, 60 % B and 40% C; 28–33 min, 60 % B and 40% C; 33–35 min, 5% B. Injection volume was 30 µL.

2.7. DPPH radical-scavenging activity

The antioxidant activity of the samples was determined using the known scavenging effect of DPPH free radical. Classically, DPPH is dissolved in methanol and its assay is performed at 517 nm (Sharma & Bhat, 2009; Manjunath, Lavanya, Sivayothi, & Reddy, 2011). In our case, a 0.6 mM DPPH solution was first prepared in MeOH/water 75:25 v/v. Rutin was dissolved in GPG (25 mg/mL GPG) and then diluted in water to get a rutin final concentration ranging from 0.5 µg/mL to 16 µg/mL. 0.25 mL DPPH solution was then added to 1.750 mL of rutin solution. Two peaks were visible in UV spectra located at 517 nm and 700 nm with these solvents. The DPPH activity was measured at 700 nm for both systems (rutin solution and rutin-containing MLVs) since sensitivity was better at this wavelength. For studies on MLVs, 0.25 mL DPPH (0.6 mM) was mixed with 1.750 mL of MLVs dispersion. MLVs were prepared using rutin dissolved in GPG (25 mg rutin/mL GPG). The rutin concentration was then varied by changing the MLVs concentration. The absorbance at 700 nm was determined after 40 min incubation at room temperature in the dark. Inhibition activity was calculated in the following way:

\[ I(\%) = \left( \frac{A_0 - A_1}{A_0} \right) \times 100 \]  

where $A_0$ is the absorbance of the control (samples with DPPH but no rutin), $A_1$ is the absorbance of rutin. For each measurement, a blank with solvents only was used. All tests were carried out in triplicate.

3. Results and discussion

3.1. Vesicles characterisation

The validation of onion-type multilamellar vesicles formation was performed by optical microscopy. Onions were imaged while some unilamellar liposomes were also present but in negligible amount. The onions’ sizes were smaller than 0.5 µm as estimated by this method (see Supplemental information, SI2).

To better characterise their size, laser light scattering was performed and the influence of replacing water by GPG (60:40 v/v) in onion composition was also evaluated. As detailed in Section 2, naringenin and rutin were solubilised in GPG since they were poorly soluble in water. The substitution of water by GPG (without any flavonoid) leads to both a decrease of vesicles size and of size dispersion (see Supplemental information, SI3). The diameter of vesicles ranged from 0.4 to 20 µm, and from 0.1 to 2 µm when vesicles were prepared with water and GPG, respectively. The addition of rutin and naringenin in GPG did not have any significant impact on MLVs size (maximum volume distribution at 0.48 µm).

Another MLVs composition was also studied since it revealed to be optimised for rutin encapsulation (see Section 3.3.3): 33/67 (w%/w%) (E930/rutin-GPG) instead of 55/45 (w%/w%). For this optimised composition, MLVs were smaller: their diameter ranged from 60 to 500 nm with a mean size at 160 nm (see SI3). It must be noted that only the biggest ones could then be imaged by optical microscopy (300 nm is the resolution limit).

3.2. Adsorption of rutin and naringenin on vesicle surface

Onion-type MLVs have been largely studied for their encapsulation ability. In some cases, such as proteins (Prévoteau & Faure, 2012), adsorption of non-encapsulated molecules on onion surface was reported. Moreover, Van Dijk et al. demonstrated that flavonoids have a strong affinity for vesicle surface (Van Dijk, Driessen, & Recourt, 2000). We first evaluated whether adsorption of naringenin and rutin on MLVs surface could occur using the method described in Section 2.5. The yield of adsorbed actives is defined as:

\[ \%A = \frac{m_A}{m_f + m_d} \times 100 \]  

where $m_a$ is the mass of rutin or naringenin adsorbed on MLVs surface and measured in the MLVs fractions after Triton X100 disruption, and $m_f$ is the mass of free (i.e. non-adsorbed) rutin or naringenin. $m_f + m_d$ is measured in all SEC fractions.

Blank MLVs were dispersed in an aqueous solution of rutin (289 µg/mL) and naringenin (77 µg/mL). HPLC revealed adsorption of both flavonoids from 10 min of incubation with MLVs: 17.9 ± 6% and 65.6 ± 1.9% for rutin and naringenin respectively (Fig. 1). Rutin adsorption increased during the two first hours and then stabilized at 28%. Naringenin adsorption was almost maximal from 10 min dispersion. The MLVs dispersion time was then fixed to 3 h to ensure a complete and stable adsorption of flavonoids on MLVs before measuring encapsulation and adsorption yields.

3.3. Encapsulation of rutin and naringenin in MLVs

3.3.1. Expression of the encapsulation yield

As significant adsorption was measured on MLVs surface, experiments were performed to distinguish adsorption onto the
MLVs surface from encapsulation inside MLVs, and to calculate the corresponding percentage of encapsulated and adsorbed flavonoids.

The percentage of encapsulated and adsorbed rutin or naringenin is defined by the following equation:

\[
\%_{E+A} = \frac{m_{E+A}}{m_E + m_A + m_{E+A}} \times 100 = \frac{m_{E+A}}{m_E + m_A} \times 100
\]

where \(m_{E+A}\) is the mass of rutin or naringenin encapsulated and adsorbed (measured in the MLVs fractions after MLVs destruction), \(m_E\) is the mass of rutin or naringenin encapsulated in MLVs, \(m_A\) is the mass of free rutin or naringenin.

Calculation efficiency is defined by:

\[
\%_E = \frac{m_E}{m_E + m_{E+A}} \times 100 = \frac{m_{E+A} - m_A}{m_E + m_{E+A}} \times 100
\]

One can express \(\%_E\) as a function of \(\%_A\) and \(\%_{E+A}\) as shown below. Using Eq. (3) in Eq. (4) gives:

\[
\%_E = \%_{E+A} - \frac{\%_A}{100} \times 100
\]

Equation (2) and Eq. (3) give:

\[
\frac{m_A}{m_E + m_{E+A}} = \frac{\%_A}{100} \times \frac{m_E}{m_E + m_{E+A}} = \frac{\%_A}{\%_E - \%_A} \times \frac{100}{\%_E}
\]

Eventually, injecting Eq. (6) into Eq. (5) leads to the wanted expression:

\[
\%_E = \frac{\%_{E+A} - \%_A}{100 - \%_A} \times 100
\]

3.3.2. Influence of flavonoid concentration on the encapsulation yield

A 55/45 (w%/w%) ratio (E930/flavonoid-GPG) was chosen for MLVs composition since this lipid/active ratio led usually to the highest encapsulation yield for MLVs composed of phosphatidylcholine (Oleà & Faure, 2003; Faure, Meyre, Trépout, Lambert, & Lebraud, 2009). Rutin and naringenin were encapsulated altogether, keeping a constant weight ratio between both molecules of 3.75. This ratio was chosen because 30 mg of rutin/mL and 8 mg of naringenin/mL are the limits of solubility in GPG in mild conditions (i.e. simple agitation at 40°C without any ultrasounds, see Section 2). Rutin concentration in GPG was varied from 30 to 3.75 mg/mL and naringenin, then from 8 to 1 mg/mL. The encapsulation efficiency was deduced from Eq. (7) after measuring \(\%_E\) and \(\%_A\), from Fig. 2. A high rutin encapsulation efficiency was measured for all tested concentrations. The best encapsulation yield, \(\%_{E_{\text{rutin}}} = 66.0 \pm 3.3\%\), was obtained for a solution containing both rutin and naringenin concentrated at 20 mg/mL of GPG and 5.3 mg/mL of GPG, respectively. The worse rutin encapsulation yield, \(\%_{E_{\text{rutin}}} = 54.0 \pm 5.7\%\), was obtained for the smallest rutin and naringenin tested concentrations: 3.75 and 1 mg/mL respectively (Fig. 2a). Naringenin was mostly adsorbed in comparison with rutin. A percentage of naringenin adsorption larger than 61.7 ± 2.1% was indeed measured for all experiments except for the two intermedialy concentrations where c.a. 12% was encapsulated (Fig. 2b).

Using the same lipid/GPG weight ratio (55/45 (w%/w%)), the encapsulation efficiencies were measured for systems containing fixed concentration of naringenin (8 mg naringenin/mL of GPG) and varying concentrations of rutin (from 30 to 3.75 mg rutin/mL of GPG). As previously reported, rutin showed high and rather constant encapsulation efficiency, c.a. 60%, for all the tested concentrations (see Supplemental information SI4). Maximum encapsulation yield was obtained for the weakest rutin concentration:

\[
65.4 \pm 3.0\%.
\]

Naringenin was not encapsulated in most of the cases except for one of the intermediaries’ concentration where the encapsulation yield was much weaker than rutin: c.a. 12%. Here again the adsorption of naringenin was predominant since \(\%_A\) reached almost 70%. Similar results were obtained when naringenin (8 mg/mL) alone was encapsulated in MLVs: c.a. 13% were encapsulated vs. 59% adsorbed.

In summary, c.a. 80% of both molecules were immobilized in MLVs but rutin was mainly encapsulated whereas naringenin was mainly adsorbed on MLV surfaces. This difference could be explained by the smaller molar weight of naringenin (Mw = 272 g/mol) compared to rutin (Mw = 610 g/mol). Naringenin being smaller than rutin may more easily leak from MLVs. Furthermore, molecules configuration may influence their affinity with phospholipidic membranes as assumed by Van Dijk et al. They showed by fluorescence quenching that the glycosylation of naringenin and eriodictyol (a flavanone as naringenin) results in a higher affinity for the vesicle membrane compared to the corresponding aglycons. Glycosylation of flavonoids would favour a more planar configuration and then an easier intercalation in vesicle membranes (Van Dijk et al., 2000). Eventually, Pawlikowska et al. (Pawlikowska-Pawlega et al., 2013) concluded from FTIR, EPR and NMR analyses that small flavonoids such as apigenin are preferentially located in the upper part of the phosphatidylcholine membrane interacting with headgroups through hydrogen bonds. All these reasons could explain the difference in naringenin and rutin behaviours.
3.3.3. Influence of lamellar composition on rutin encapsulation yield

As rutin was successfully encapsulated, MLVs composition was optimised with this flavonoid alone. In order to optimise the amount of encapsulated rutin, the E930/rutin-GPG ratio was changed, keeping constant rutin concentration (30 mg/mL of GPG). Previous studies (Prévoteau & Faure, 2012) showed that the maximal encapsulation efficiency in onion-type MLVs is reached when the lamellar phase is at maximal swelling, i.e. just before the diphasic composition when the rutin-loaded lamellar phase coexists with the rutin-GPG phase. Rutin-GPG is then in excess. Several lipid/rutin-GPG compositions were then studied from 55/45 to 15/85 (w%/w%) (Fig. 3a). The rutin encapsulation yield (%Erutin) first increases, from 53.7 ± 2.0 to 62.4 ± 0.6%, with the rutin-GPG weight percentage (%rutin), and then linearly decreases for rutin-GPG percentage higher than 63 w%.

These data can be modelled assuming that increasing the rutin-GPG proportion in the lamellar phase led to the passage from a monophasic lamellar domain (point D in the inset of Fig. 3a) to a diphasic domain where a fraction of rutin-GPG solution forms an excess phase (point B in the inset of Fig. 3a). With these assumptions, plotting %rutin vs. %Erutin should give two straight lines with their intercept being the composition of the swollen lamellar phase, i.e. the boundary between both domains (point A in the inset of Fig. 3a). The slope of the increasing line gives the encapsulation yield while that of the decreasing line allows the determination of the maximum swelling composition (see additional material, SI5). Fig. 3b displays the corresponding plots.

Using the mathematical model, the encapsulation yield is found to be 61 % and the optimal composition is 33/67 (w%/w%) of lipid/rutin-GPG solution. This optimum ratio is different from the 55/45 (w%/w%) usually found in phosphatidylcholine lamellar phase (Olea & Faure, 2003; Faure et al., 2009; Prévoteau & Faure, 2012). This difference is likely to be due to the nature of the lamellar hydrating solution; GPG instead of water.

3.3.4. Optimisation of rutin concentration

The lipid/rutin-GPG proportion was fixed to 33/67 (w%/w%), and the rutin concentration in GPG was increased. If our prediction on the phase diagram was correct, plotting the reverse of %Erutin as a function of the rutin concentration in GPG should give two separate behaviours: a constant value for the low concentrations corresponding to the monophasic lamellar domain and a straight line in the biphasic domain where rutin-GPG solution is in excess (see inset Fig. 4 point E, and additional material SI6). Results shown in Fig. 4 are in accordance with these predictions. The optimal rutin concentration was indeed 30 mg rutin/mL (49.1 μmol/mL) as shown in the previous section: %Erutin was found to be 62.5 ± 2.2%, i.e. 16.54 μmol of rutin per gram of MLVs. This value is higher than that reported by Park et al. who encapsulated rutin in ceramide liposomes with an optimal encapsulation efficiency of 58 % (Park, Lee, Kim, & Yu, 2013). Goniotaki et al. measured that 71 ± 10% was immobilized in liposomes. However, Goniotaki et al. did not distinguish adsorption from encapsulation (Goniotaki, Hatziantoniou, Dimas, Wagner, & Demetzos, 2004). Doing so, our optimal %E+Arutin value is 66.9 ± 1.3% corresponding to %Erutin 62.5 ± 2.2%, which is comparable to that from M. Goniotaki et al. taking into consideration their standard deviation.

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**Fig. 3.** (a) Encapsulation percentage of rutin (%Erutin) in function of rutin-GPG weight proportion (%rutin) in MLVs composition. Rutin concentration was fixed at 30 mg/mL of GPG. (Inset) – Schematic representation of the phase diagram of lipid/rutin-GPG system. (b) Determination of the optimal rutin-GPG weight proportion using the model described in SI5. The optimal MLVs composition for rutin encapsulation is 33/67 (w%/w%) of lipid/rutin-GPG solution with an encapsulation yield of 61%. 

R² = 0.9982

R² = 0.9842
3.4. Kinetic release of rutin

We have demonstrated in previous sections the ability of MLVs to encapsulate a large quantity of rutin. It was then important to evaluate the permeability of these capsules, in other words, the kinetics of rutin leakage from the multilamellar vesicles composed of 33 w% lipid and 67 w% GPG containing 25 mg rutin/mL, kept at room temperature during 1 month. Above 25 mg rutin/mL of GPG, non-encapsulated rutin precipitated in water after few days. All dispersions were dispersed for 3 h the first day and shacked again for few seconds before fractionation the day of analysis. Fig. 5 indicates that rutin gradually leaked from MLVs. This leakage can be explained by the conditioning conditions (room temperature) and by the instability of onion-type MLVs when dispersed in water: onions are known to be thermodynamically unstable and to return to lamellar structure when dispersed, in c.a. 7 weeks (Olea & Faure, 2003), which is comparable to liposomes stability (Goniotaki et al., 2004). This was evidenced by optical microscopy. Only onion-type multilamellar vesicles were observed the first day, whereas unilamellar vesicles appeared after 25 days. After 31 days, 16.0 ± 0.3% of rutin was still encapsulated. An interesting point is the instability of free rutin. After 9 days, a high percentage of quercetin was observed by HPLC on fractions containing free rutin, showing that rutin lost its sugar groups. Quercetin formation was much less pronounced in MLVs fractions (after their destruction), indicating that only the adsorbed rutin molecules were degraded (See additional information, SI7). This observation demonstrates the ability of MLVs to protect rutin and preserve its integrity.

When onion-type MLVs were stored without being diluted/dispersed in water, almost no rutin release was measured (less than 10% in 31 days). This was expected since after mixing lipid with rutin-GPG solution, the resulting onions are in close compact without any external liquid phase; this drastically limits molecules release.

3.5. Antioxidant activity: DPPH assay

The ability of rutin to reduce DPPH radicals was determined by UV absorbance at 700 nm (see Section 2). Fig. 6 shows the DPPH radical scavenger activity as a function of the rutin concentration for rutin-loaded MLVs and for rutin solution. Without MLVs (full squares), DPPH radical scavenger activity stabilized around 60% for rutin concentrations higher than 8 µg/mL. When assays were realised on the optimised rutin-loaded MLVs (empty circles), a diminution of DPPH radical scavenger activity was observed at...
equivalent rutin concentration, showing the protection of encapsulated rutin.

Knowing that 62.5 ± 2.2% of rutin was encapsulated (see previous section), we calculated from free rutin radical scavenger activity the theoretical activity of MLVs, assuming that only free and adsorbed rutin was responsible for their scavenging activity. Fig. 6 shows that for almost all assays, the theoretical radical scavenger activity (empty triangles) corresponded with the measured data. Only two measurements gave weaker data than those expected when the rutin concentration was 4 and 5 μg/mL. Firstly, this confirms the validity of our models to differentiate adsorption from encapsulation, and secondly, this evidences that anti-oxidant activity was thus provided by adsorbed and free rutin, showing that adsorbed rutin molecules are actives contrarily to encapsulated ones.

4. Conclusions

The immobilization of rutin and naringenin was successfully performed in phosphatidylcholine-based MLVs: ca. 80% was either encapsulated or adsorbed on MLVs surface. A mathematical model was developed to allow the distinction between encapsulated and adsorbed molecules. Naringenin was mainly adsorbed on MLVs surface (>60%), whereas rutin was mainly encapsulated (>60%).

A maximum of 16.5 μmol of rutin was encapsulated per gram of MLVs, when composed of 33 w% of E930 and 67 w% of rutin-GPG (30 mg/mL of GPG), corresponding to an encapsulation yield of 62.5%. This value is higher or comparable to those published on the encapsulation of rutin in liposomes.

Concerning leakage, 16.0% ± 0.3 of rutin was still encapsulated in onion-type MLVs dispersion after 31 days whereas no leakage was measured when MLVs were kept in their concentrated form within a month. Moreover, MLVs were shown to protect rutin from degradation in quercetin. Eventually, the DPPH test revealed that this confirms the validity of our models to differentiate adsorption from encapsulation, and secondly, this evidences that anti-oxidant activity was thus provided by adsorbed and free rutin, showing that adsorbed rutin molecules are actives contrarily to encapsulated ones.

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