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ORIGINAL PAPER

Quercetin-Loaded Lecithin/Chitosan Nanoparticles for Functional Food Applications

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Abstract This study aimed at the encapsulation of quercetin into lecithin/chitosan nanoparticles using the electrostatic self-assembly technique, followed by evaluation of their functionality (antioxidant activity) and stability at different environmental conditions. These nanoparticles were characterized in terms of: average size, morphology, zeta potential, encapsulation efficiency, loading, and spectroscopic characteristics. Quercetin has been successfully encapsulated in lecithin/chitosan nanoparticles with an efficiency of 96.13±0.44 %. Nanoparticles presented a spherical morphology with an average size of 168.58±20.94 nm and a zeta potential of 56.46±1.94 mV. Stability studies showed that nanoparticles are stable to temperatures ranging between 5 and 70 °C and a pH variation from 3.3 to 5.0. Moreover, encapsulated quercetin showed improved antioxidant properties when compared to free-quercetin. Our results suggest that quercetin-loaded lecithin/chitosan nanoparticles can be used in the manufacture of functional foods.

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Introduction

Nanotechnology has potential to produce new food ingredients and innovative products, with considerable benefits to human health (Magnuson et al. 2011). This can be achieved through the development of new structures for applications in functional foods (Huang et al. 2010). The consumption of foods providing health benefits as well as basic nutrition is very attractive to the consumer, and significant developments from the food industry have emerged in recent years.

Polyphenols typically used as part of the human diet are one of the most numerous and ubiquitous groups of plant metabolites that have a large spectrum of biological activities and may be considered chemopreventive agents in humans (Fang and Bhandari 2010). Quercetin [3,3′, 4′, 5,7-pentahydroxyflavone] is one of the most representative compounds of the flavonoid family, having been assigned a wide range of health benefits, including antioxidant activity, cancer prevention, protection to DNA damage, anti-inflammatory, and cardio-protective actions (An et al. 2010). However, quercetin is a molecule with a low bioavailability due to its low solubility, gastrointestinal instability, and low absorption in the gastrointestinal tract, which limits its biological effects in vivo (Chitkara et al. 2012). A good option to improve bioavailability of quercetin is nanoencapsulation that can increase the absorption in the gastrointestinal tract and enhance the molecule's stability against possible adverse conditions during processing, distribution, storage, and consumption of food products. Promising results, reported by Li et al. (2009), showed that when encapsulating quercetin in solid lipid nanoparticles, consisting of soy lecithin and glyceryl monostearate, the oral bioavailability in rats has doubled. An additional benefit is the

increase of quercetin solubility. Quercetin encapsulation will allow its use as antioxidant in food products where solubility is a problem.

The oxidation of proteins and lipids is responsible for the development of unpleasant odors and flavors that make food unfit for consumption. These changes are not restricted to nutritional quality, due to the degradation of fat-soluble vitamins and essential fatty acids, but they also affect the integrity and safety of food through the formation of potentially toxic compounds (Ramalho and Jorge 2006). In this context, encapsulation of quercetin emerges as an alternative for the food industry to improve the defense against free radicals in various environments in a controlled manner.

Lecithin is a mixture of phospholipids of vegetable origin comprised mainly of phosphatidylcholine and phosphatidylethanolamine, and it is considered a safe and biocompatible excipient, already used in many pharmaceutical formulations (Şenyiğit et al. 2010). Chitosan is a cationic and linear heteropolysaccharide composed of units of *N*-acetylglucosamine (GlcNAc) and glucosamine (GlcN) linked by β-1,4 bonds. The quantity and sequence of these units determines the physical, chemical, and biological properties of the polymer (Prashanth and Tharanathan 2007). Chitosan, commonly obtained from the alkaline or enzymatic deacetylation of chitin (Shahidi and Abuzaytoun 2005) showed multiple properties such as biocompatibility, biodegradability, bioadhesion, absence of allergenicity and toxicity, anti-hypercholesterolemic, and antimicrobial activity; and has been presented as having potential for application in the industries of food, cosmetics, and pharmaceutics (Gerelli et al. 2008; Chuah et al. 2009; Şenyiğit et al. 2010). In particular, studies have shown that when used in optimum conditions of preparation (i.e., concentration, pH, ionic strength, and electrophoretic mobility); chitosan provides structural stability to nanoemulsions and liposomes (Chuah et al. 2009; Panya et al. 2010).

Recently, lecithin and chitosan-based nanoparticles obtained from self-organized supramolecular electrostatic interactions between a positively charged polysaccharide and negatively charged lipids, have been described as a suitable carrier for hydrophobic molecules (Sonvico et al. 2006). Hafner et al. (2009) showed that melatonin-loaded lecithin/chitosan nanoparticles, when using Lipoid S45 as lecithin source, presented mucoadhesive properties, absence of cytotoxicity, and promoted the increase of permeability of melatonin against an in vitro model of epithelial barrier consisting of Caco-2 cells.

In the present study the encapsulation of quercetin into lecithin/chitosan nanoparticles is reported. Subsequently, their functionality is evaluated through antioxidant activity measurements and their stability is related to pH, temperature, and storage time as essential parameters for the use of quercetin-loaded lecithin/chitosan nanoparticles by the food industry, either as part of functional foods or controlling harmful free radicals effects in foods.

Material and Methods

Materials

Soya lecithin (Lipoid S45) was purchased from Lipoid (Ludwigshafen, Germany), chitosan (90 % deacetylation, MW=3.4×10^5 Da) from Aqua Premier Co. Ltd (Thailand) and 99 % ethanol from Panreac (Spain). The solvents used were of analytical grade and obtained from Merck (Darmstadt, Germany). All other reagents were purchased from Aldrich (Steinheim, Germany). Water was purified through a Millipore Q purification system (Millipore Corp., Bedford, MA).

Nanoparticles Preparation

Lecithin/chitosan nanoparticles were prepared according to the methodology described by Sonvico et al. (2006) with some modifications. The suspension of nanoparticles was obtained by dropwise injection (syringe having internal diameter of 0.38 mm, 1 mL min^{-1} injection rate) of 2 mL of ethanolic solution of Lipoid S45 (25 mg/mL) in 23 mL aqueous solution of chitosan (0.11 mg/mL) under mechanical stirring (24,000 rpm, TP 18/10-10N Ultraturrax, IKA Werke, Staufen, Germany). The chitosan aqueous solution was prepared by diluting a standard solution of 0.2 % chitosan (*w*/*v*) in lactic acid (1 %*v*/*v*). The incorporation of quercetin at different concentrations (50–800 μg/mL) was performed by its dissolution in ethanolic solution of lipoid S45. The lecithin/chitosan nanoparticles and quercetin-loaded lecithin/chitosan nanoparticles were called Np and Quercetin-Np, respectively.

Physical–Chemical Characterization

Particle Size and Zeta Potential Measurements

The hydrodynamic diameter (size-average) and polydispersity index (PDI) of the nanoparticles were determined by dynamic light scattering (Zetasizer Nano ZS, Malvern Instruments, UK). The samples were diluted (1:3) with Milli-Q water (Milli-Q synthesis, 18.2 MΩ cm) and analyzed in a folded capillary cell. Detection of the scattered light was carried out at 173° (NIBS=non-invasive backscatter detection) to reduce the path length of the scattered light from the samples and to minimize the risk of multi-scattering at 25 °C. The zeta potential was measured by laser Doppler microelectrophoresis using a Zetasizer ZS Nano (Zetasizer Nano ZS, Malvern Instruments, UK). All measurements were

carried out in triplicate with three readings for each of them. The results are given as mean ± standard deviation of the nine values obtained.

Quercetin Encapsulation Efficiency and Loading

The quercetin loading and encapsulation efficiency of the Np were conducted according to the modified procedures previously described by Li et al. (2009). Encapsulated and non-encapsulated portions of quercetin from the Quercetin-Np suspension were separated using centrifugal filter devices (Amicon Ultra-0.5, Millipore®) with centrifugation at 14,000 rpm for 30 min. The ultrafiltrate and nanoparticles resulting from centrifugation were diluted with absolute ethanol and quercetin content was measured by UV/Vis spectrophotometry (Jasco, Japan) at 373 nm. The encapsulation efficiency of quercetin (EE) and quercetin-loading (L) were calculated by the following equations:

$$\mathrm{EE}(\%) = (W_{\mathrm{total}} - W_{\mathrm{free}}) \times 100 / W_{\mathrm{total}}$$

$$L(\%) = (W_{\mathrm{total}} - W_{\mathrm{free}}) \times 100 / W_{\mathrm{np}}$$

where W_{total} was the total quercetin weight in nanoparticles suspension; W_{free} was the weight of free-quercetin in nanoparticles suspension; and W_{np} was the vehicle weight.

Nanoparticles Morphology

The morphology of Quercetin-Np was observed by transmission electron microscopy (EM 902nd, Zeiss, West Germany). The sample was diluted (1:3) with Milli-Q water and immobilized on copper grids for observation.

UV/Vis Spectroscopy

The UV–Vis absorption spectra of Quercetin-NP and free-quercetin were obtained in a spectrophotometer (Jasco, Japan) in the range of 200–700 nm at 1.0 nm intervals. Samples were measured at 25 °C in a rectangular quartz cuvette with 1 cm path length. All determinations were performed in triplicate.

Fluorescence Spectroscopy

The intrinsic fluorescence spectra of Quercetin-NP and free-quercetin between 350 and 650 nm were determined with a fluorescence spectrophotometer (JASCO FP-6300, Tokyo, Japan). The excitation and emission bands were set at 2.5 nm and excitation wavelength at 617 nm. All determinations were performed in triplicate.

Fourier Transform Infrared Spectroscopy

Fourier transform infrared (FTIR) spectra of Quercetin-Np and free-quercetin were obtained by an infrared spectrometer (PerkinElmer 16 PC spectrometer, Boston, USA). The pre-lyophilized samples were ground with spectroscopic grade potassium bromide (KBr) powder and then pressed into 1 mm pellet for FTIR measurement in the range of 450–4,000 cm^{-1} with 4 cm^{-1} resolution, using 16 scans.

Antioxidant Activities Assays

DPPH Scavenging Capacity

The free radical scavenging capacity of free-quercetin, Np, and Quercetin-Np was measured by 1,1-diphenyl-2-picrylhydrazyl (DPPH) assay reported by Kumari et al. (2010) with modifications. Briefly, 20 μL of sample solution was added into 200 μL of ethanolic solution of DPPH (200 μM) and the mixture was then vortexed vigorously and left for 30 min at room temperature in the dark. The absorbance of the reaction solution was measured at 515 nm (ELISA reader; Bio-Rad).

The percentage of scavenging of free radicals by the sample was calculated according to the following equation:

$$\text{Scavenging capacity}(\%) = \left(1 - \mathrm{Abs}_{515\ \text{sample}}\right) \times 100 / \mathrm{Abs}_{515\ \text{control}}$$

All determinations were performed in triplicate and results are given as the mean ± standard deviation.

Reducing Power

The test ferric reducing antioxidant power (FRAP) was performed according to Benzie and Strain (1996) with some modifications. Initially, 10 μL of sample (free-quercetin, Np, Quercetin-Np) were mixed with 290 μL of FRAP reagent. The mixture was subsequently incubated at 37 °C for 15 min and the absorbance determined at 593 nm (ELISA reader; Bio-Rad). A calibration curve was prepared with $FeSO_4 \cdot 7H_2O$ as standard, in the range of 50–1,500 μM. FRAP reagent was freshly prepared by mixing 10 mM 2,4,6-tris (1-pyridyl)-5-triazine in 40 mM HCl with a 20 mM $FeCl_3$ solution and 0.3 M acetate buffer (pH 3.6) in the proportion 1:1:10 (*v*/*v*/*v*). The results were expressed as micromoles equivalent Fe^{2+} (Fe^{2+} μM).

Anti-lipid Peroxidation Assay

The anti-lipid peroxidation effect of free-quercetin, Np, and Quercetin-Np on $FeCl_2$–ascorbic acid-induced lipid

peroxidation in rat liver homogenate was carried out according to Wu et al. (2008). The 20 % liver homogenate (*w*/*v*) was prepared with 150 mM Tris–HCl buffered saline solution (pH 7.2) and further centrifuged at 500×*g* for 10 min. Then 50 μL of the homogenate were mixed with 30 μL of the sample, 10 μL of 4 mM $FeCl_2$, and 10 μL of 0.2 mM ascorbic acid. The mixture was incubated at 37 °C for 1 h, followed by the addition of 100 μL of 0.1 M HCl, 40 μL of 9.8 % SDS, 180 μL of deionized water, and 400 μL of 0.6 % TBA to each tube. Tubes were vigorously shaken and placed at 100 °C for 30 min. After cooling, 1,000 μL n-butanol was added to the tubes and centrifuged at 1,000×*g* for 25 min; the supernatant was collected and the absorbance measured at 532 nm (ELISA reader; Bio-Rad). All determinations were performed in triplicate and results are expressed as mean ± standard deviation.

The percentage of lipid peroxidation was determined using the following equation:

$$\%\,\text{peroxidation} = \left(\text{Abs}_{532\ \text{induced}} - \text{Abs}_{532\ \text{sample}}\right) \times 100 / \left(\text{Abs}_{532\ \text{induced}} - \text{Abs}_{532\ \text{control}}\right)$$

where $\text{Abs}_{532\ \text{induced}}$ is the absorbance of a reaction in which 100 % of lipid peroxidation occurred (containing distilled water instead of test sample); $\text{Abs}_{532\ \text{sample}}$ is the absorbance of the samples (free-quercetin, Np, and Quercetin-Np); $\text{Abs}_{532\ \text{control}}$ is the absorbance of control reaction in the absence of rat liver homogenate.

Stability Studies of Quercetin-Np

Effect of pH

The effect of pH on the stability of Quercetin-Np was evaluated adjusting the pH of the samples from 3.5 to 8.0 (using 1 M NaOH). The samples were stored at room temperature (25 °C) and left overnight for analysis of hydrodynamic diameter and zeta potential.

Effect of Temperature

The temperature effect in Quercetin-Np stability was analyzed in the Zetasizer Nano ZS (Malvern Instruments, UK), which allows scanning the temperature while measuring particle size and polydispersity. The cell used for readings was a 12-mm square glass cell, with circular aperture and stopper and temperatures varied from 5 to 70 °C. The samples were cooled at 5 °C for 1 h and the reading was performed at that temperature. Subsequently, the samples were gradually heated up to 70 °C, being always maintained for 1 h at predetermined temperatures (5, 10, 15, 20, 25, 30, 35, 40, 45, 50, 55, 60, 65, and 70 °C) for analysis. Each measurement was performed in triplicate and the results expressed as mean ± standard deviation.

Effect of Storage Time

The Quercetin-Np was stored at 4 and 30 °C for 90 days in the dark. The stability was determined by analysis of the hydrodynamic diameter, quercetin concentration, and DPPH scavenging capacity. To determine quercetin concentration an aliquot of Quercetin-NP suspension was diluted with absolute ethanol and the absorbance measured spectrophotometrically at 373 nm using quercetin as standard, in the range of 1–50 μg/mL.

DPPH scavenging capacity assays were performed by adding 1 mL of a DPPH stock solution (100 μM in methanol) to 100 μL of Quercetin-Np suspension. The mixture was incubated in the dark under stirring for 30 min and absorbance was measured at 515 nm in a spectrophotometer, at 25 °C in a rectangular quartz cuvette with a 1-cm path length. DPPH concentration was expressed as μM (ε=11.240 mol L^{-1} cm^{-1} Portes et al. 2009). DPPH radical degrades spontaneously making it impossible to use the same initial solution when the experiment is performed for long periods of time (days). To reduce the fluctuations during preparation of the initial DPPH solution, we decided to calculate the concentration of the solution using the molar extinction coefficient on a specific solvent (in this case methanol), which is the reason why a different method for DPPH was used at this stage of the work.

All analyzes were carried out in triplicate and results were expressed as mean ± standard deviation.

Statistical Analysis

All results were expressed as mean ± standard deviation, and analyzed with one-way analysis of variance followed by a multi-parametric Tukey's post hoc test in GraphPrism® (GraphPad Software Inc., San Diego, CA). Statistical significance was established at $p<0.01$.

Results and Discussion

Nanoparticles Characterization

Particles Size

The size and polydispersity index are essential analyses for characterization of nanoparticles, since they influence important parameters such as loading, release, and stability of the compound inside nanoparticles. It is known that the smaller the particle the greater is the exposed surface area, which leads to a faster release of encapsulated drugs. Smaller

particles also have an increased risk of aggregation during storage, being important the development of nanoparticles with a low PDI to achieve maximum stability by a better control (and lesser dispersion) of particles' size. It is worth mentioning that the reproducibility of parameters such as stability and release is directly connected to a low PDI (≤0.4), since a high PDI means that there is no uniformity in the size distribution of the sample. Figure 1a shows that increasing quercetin concentrations in Quercetin-Np formulations lead to higher values of average size and polydispersity index. The formulations containing 0, 50, 70, and 100 μg of quercetin/mL suspension were homogeneous with low polydispersity index (PDI≤0.34) and average sizes of 117.7±5.73, 133.25±8.91, 168.58±20.94, and 172.06±5, respectively. Results are in agreement with the works published by Ghosh et al. (2011) and Pool et al. (2012) that when encapsulating quercetin in poly(lactic-co-glycolic acid) (PLGA) nanoparticles obtained particle sizes of 13.28±7.8 nm and 399.67±10.86 nm, respectively. When the nanoparticles were prepared with values higher than 100 μg quercetin/mL nanoparticle aggregation was observed a few minutes after production. This behavior can be explained by the presence of high concentrations of quercetin that will interfere with the process of self-organization of nanoparticles by blocking the repulsive forces between the nanoparticles, due to the decrease of spacing between adjacent nanoparticles, thus leading to aggregation. A similar behavior was observed by Barbieri et al. (2013) for lecithin/chitosan nanoparticles with high loading of tamoxifen citrate. Based on these results only Quercetin-Np with concentrations lower than 100 μg of quercetin/mL were further evaluated.

Quercetin Loading and Encapsulation Efficiency

Loading and encapsulation efficiency depends largely on the compound solubility in the matrix material or polymer (dissolution or solid dispersal) (Kumari et al. 2010). The encapsulation efficiency of quercetin into lecithin/chitosan nanoparticles (Fig. 1b) presented values higher than 95 % for all used concentrations (50, 70 and 100 μg / mL) ($p<0.01$). These efficiencies are higher than those obtained by Ghosh et al. (2011) and Pool et al. (2012) for PLGA nanoparticles (encapsulation efficiency below 80 %). The high encapsulation efficiency obtained with lecithin/chitosan nanoparticles may be related to the fact that both lecithin and quercetin are hydrophobic molecules, which allows a strong affinity between them. Consequently, a larger amount of quercetin was preferably inserted into the lipid nuclei of the nanoparticles, and only a small amount of quercetin was lost in the aqueous phase during the preparation process. This loss is in agreement with the value of solubility for quercetin in water (0.514 μg/mL) (Saija et al. 2003). Other molecules with hydrophobic character such as progesterone, melatonin, and clobetasol-17-propionate were also successfully encapsulated in lecithin/chitosan nanoparticles (Hafner et al. 2009; Sonvico et al. 2006; Şenyiğit et al. 2010).

Figure 1b also shows that increasing the quercetin concentration in the formulation resulted in improved quercetin loading ($p<0.01$). However, formulations containing 100 μg/mL of quercetin showed signs of instability (precipitation) after 1 week; therefore, nanoparticles with 70 μg/mL of quercetin were chosen to be used in further analyses.

Zeta Potential

Zeta potential represents the surface charge that can influence the stability of suspended particles through electrostatic repulsion among them. Quercetin-Np showed zeta potential values of 56.46±1.94 mV. This value is suitable for forming a stable suspension of nanoparticles, since particles can be dispersed stably when the absolute value of zeta potential is above 30 mV (Liu et al. 2007). At the original pH of Quercetin-Np (pH 3.3), the phosphate groups (PO_3^-) of lecithin (pKa ~ 1.5) and the amino groups (NH_3^+) of chitosan

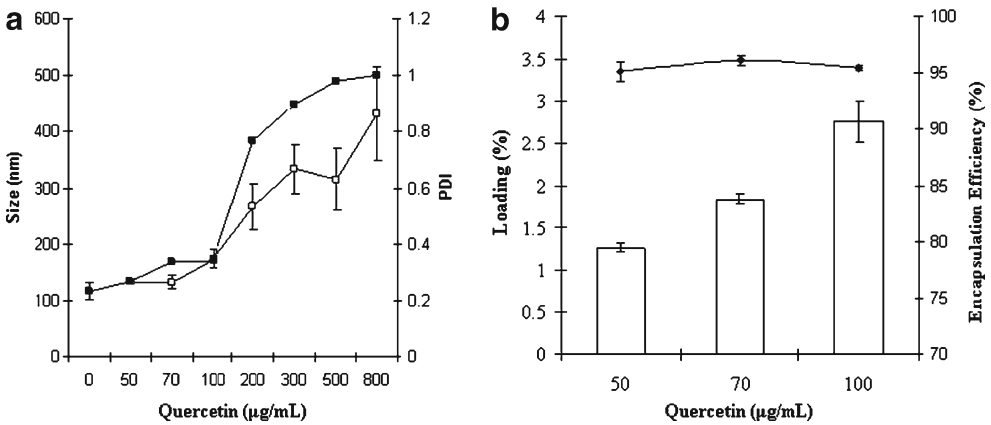

Fig. 1 **a** Average particle size (*black squares*) and polydispersity index (*white squares*) versus quercetin concentration. **b** Influence of quercetin concentration in the formulation, loading (*white squares*) and encapsulation efficiency (*black diamonds*) of Np. All determinations were performed in triplicate and the results expressed as mean ± standard deviation

(pKa ~ 6.2–7.0) have opposing charges; it is thus presumed that a protective chitosan layer around the particles of Quercetin-Np is formed through electrostatic interactions (Chuah et al. 2009). Thus, the amino groups of chitosan layer, protonated at pH 3.3, are responsible for the highly positive zeta potential of nanoparticles. When assessing chitosan solutions with pH values between 2.5 and 3.5, Carneiro-da-Cunha et al. (2011) showed that zeta potential can vary between 31.6 and 63.5 mV.

Nanoparticle Morphology

Transmission electron micrographs of Quercetin-Np are shown in Fig. 2. Figure 2a shows that the nanoparticles are spherical and polydisperse, with sizes ranging from 60 to 500 nm. Figure 2b shows a compact lipid nuclei surrounded by a contrasted layer of chitosan which confirms its presence as the outmost layer surrounding the quercetin–lecithin complex, as the result of expected electrostatic interaction based on oppositely charges (zeta potential) of solutions previously measured, in order to support the assembly.

Spectroscopy Evaluation

Figure 3a shows UV–Vis spectra of free-quercetin (spectrum a) and-Quercetin-Np (spectrum b). The spectrum of free-quercetin shows two absorption bands, at 257 and 373 nm, related to conjugations in the B-ring and A-ring, respectively, similar to those described by Timbola et al. (2006). Quercetin-Np showed a characteristic peak of turbidity, which is indicative of nanoparticles formation. A similar profile was obtained by Kumari et al. (2010) for PLA (Poly-D, L-lactide) nanoparticles. As well as for free-quercetin, the spectrum of Quercetin-Np also showed absorption bands at 257 and 373 nm, confirming the presence of quercetin in the Np's.

Quercetin is slightly fluorescent and extremely sensitive to changes that may occur in the medium, such as polarity and hydrogen bonding effects. Thus, its encapsulation in Np caused increased fluorescence emission intensity (Fig. 3b) compared with free-quercetin, indicating changes in the microenvironment. This is probably because the Np offers a less polar and more rigid protective microenvironment shielding the excited species from quenching and non-radioactive decay processes. Similar results were obtained with the encapsulation of quercetin into chitosan nanoparticles (Zhang et al. 2008).

FTIR analyses were performed to investigate a possible reaction between quercetin and Np. The FTIR spectrum obtained for free quercetin (Fig. 3c) showed bands and typical molecular peaks of its structure: 1,381 cm^{-1} (C–OH), 1,610 cm^{-1} (C=C), 1,264 cm^{-1} (C–O–C), 1,662 cm^{-1} (C=O), and 3,403 cm^{-1} (O–H stretch). The Np spectrum (Fig. 3c) showed characteristic chitosan bands with peaks at 1,650 and 1,590 cm^{-1} attributed to NH-bending units of glucosamine. The absorption bands at 1,051 cm^{-1} (C–O–C) and 1,080 cm^{-1} (skeletal vibration involving the C–O stretching) are characteristic of its saccharide structure. The presence of an absorption band at 1,217 cm^{-1} corresponding to P=O stretching indicates an ionic interaction between the phosphate groups of lecithin and the amino group of chitosan. No characteristic peaks of quercetin were observed in the spectrum of Quercetin-Np, which may be indicative of quercetin encapsulation in the Np, and this interaction probably occurred by hydrophobic interactions or hydrogen bonds. The disappearance of characteristic peaks of quercetin after encapsulation was also observed in chitosan nanoparticles by Zhang et al. (2008).

Antioxidant Activity Evaluation

The DPPH scavenging capacity assay was used to evaluate the ability of free-quercetin, Quercetin-Np, and Np to donate protons. Figure 4a shows that the capacity of the phenolic group of quercetin to donate hydrogen in order to stabilize free radicals was maintained after encapsulation. Quercetin-Np were capable to reduce higher numbers of DPPH molecules when compared with free-quercetin ($p<0.01$), which can be related with improved dissolution properties of quercetin after encapsulation. This phenomenon was also

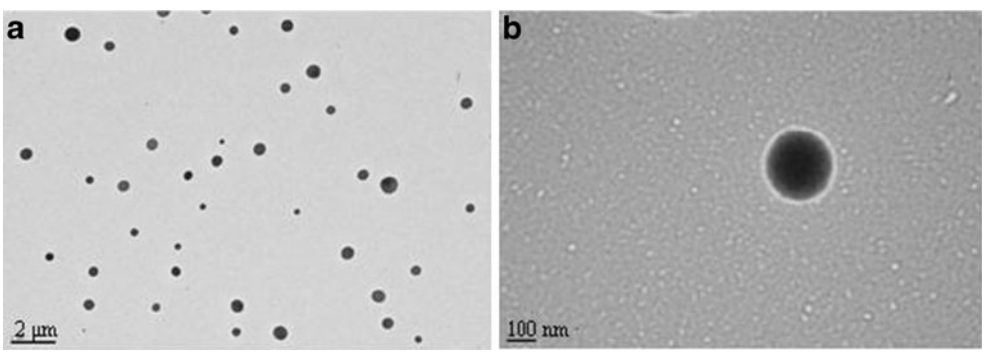

Fig. 2 Transmission electron microscopy images of: **a** morphology and dispersivity of Quercetin-Np; **b** compact core of quercetin–lecithin complex surrounded by a transparent layer of chitosan

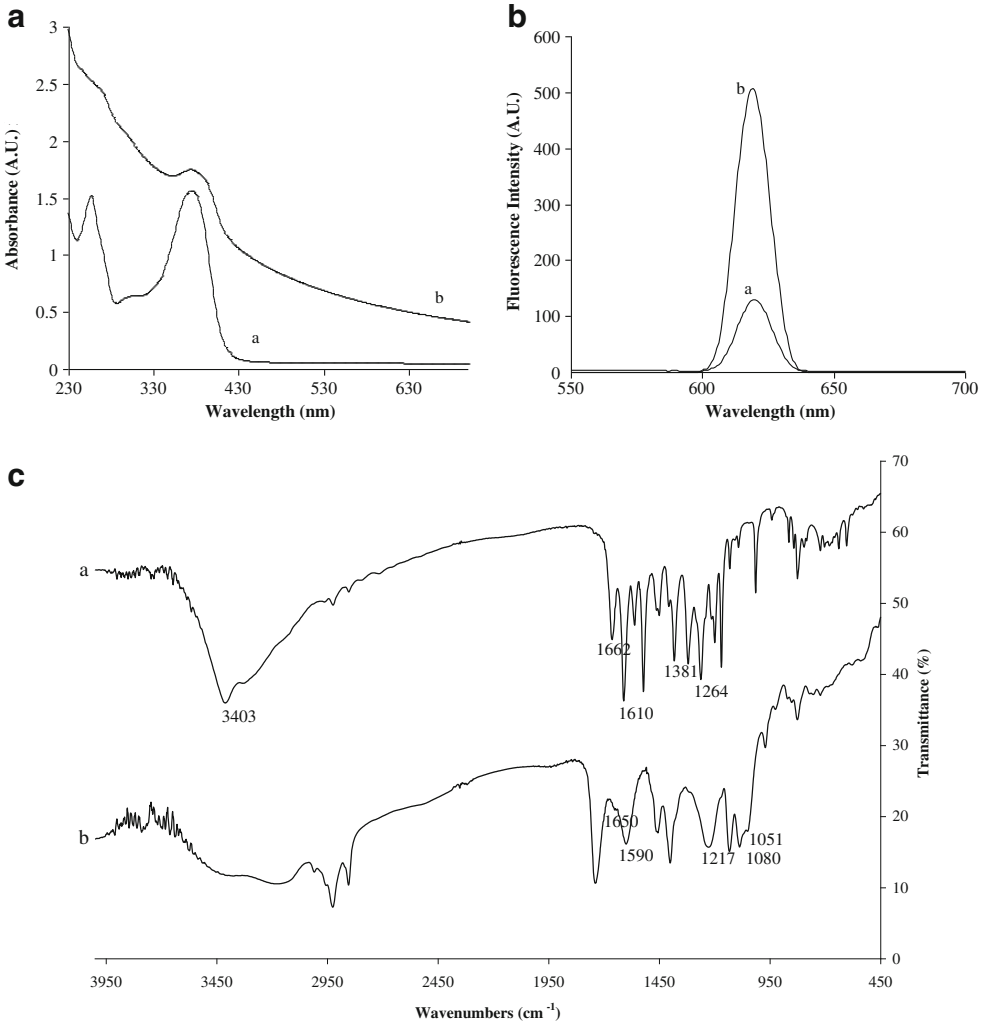

Fig. 3 **a** UV–Vis spectra of free-quercetin (*a*) and Quercetin-Np (*b*). **b** Fluorescence emission spectra of equimolar *a* encapsulated and *b* free-quercetin molecule. **c** FTIR spectra of free-quercetin (*a*) and Quercetin-Np (*b*)

observed by Wu et al. (2008) when encapsulating quercetin in nanoparticles based on polyvinyl alcohol and Eudragit®.

The reducing power of a compound can serve as a significant indicator of its antioxidant capacity (Firuzi et al. 2005). Figure 4b shows the reducing power of free quercetin, Quercetin-Np, and Np. Quercetin-Np were more effective in reducing Fe^{3+} to Fe^{2+} in comparison with free-quercetin ($p<0.01$). This property of nanoparticles is entrusted exclusively to the ability to donate protons of the phenolic groups of quercetin, since the Np were not able to reduce Fe^{3+}. The increased reducing power of quercetin after encapsulation again can be probably explained by an improvement in the dissolution properties of quercetin in aqueous medium.

The oxidation process of food products during production and storage causes a sequence of particularly unfavorable changes in the sensory properties of the product (appearance of rancidity, changes in color and texture) responsible for quality downgrading and economic losses (Gramza and Korczar 2005). An assay based on inhibition of stimulated lipid peroxidation in rat liver homogenate by a system of ferrous ion–vitamin C was used to evaluate the protective effect of Quercetin-Np against lipid peroxidation. Figure 4c shows the percentage of peroxidation using as standard a reaction where there is 100 % peroxidation of lipids present. It is observed that systems containing free-quercetin and Quercetin-Np showed only 8.23 and 17.62 % of lipid peroxidation, respectively, leading to protection levels of approximately 91 % (free-quercetin) and 83 % (Quercetin-Np), demonstrating the efficiency of the system of Quercetin-Np. A rate of 235 % for peroxidated lipids in the system containing Np can be explained by the fact that nanoparticles are mainly composed of lecithin which is also subject to peroxidation by the ferrous ion–vitamin C system used.

Evaluation of Quercetin-Np Stability

Effect of pH Changes

The foods' pH may range from acidic (e.g., from 2.5 to 4, in some soft drinks) to slightly alkaline (e.g., from 7 to 7.4, in

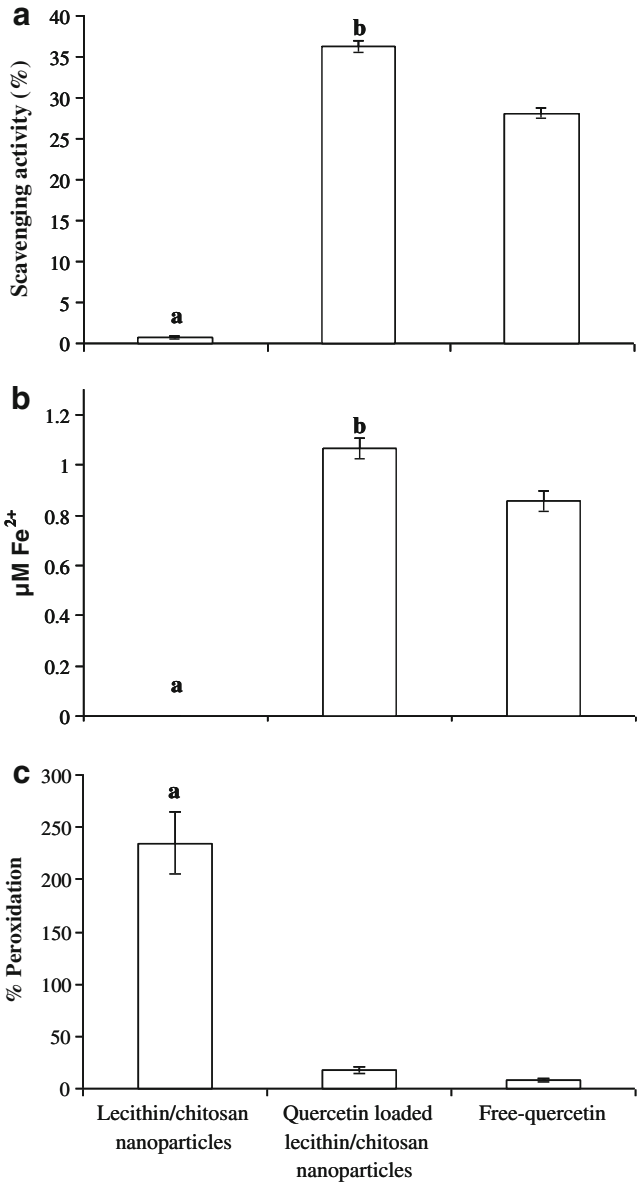

Fig. 4 Antioxidant activity of Np, Quercetin-Np, and free-quercetin evaluated by: DPPH scavenging capacity (**a**), reducing power (FRAP) (**b**), and anti-lipid peroxidation assay (**c**). All determinations were performed in triplicate and the results expressed as mean ± standard deviation. *a* Significantly different from the Quercetin-Np and free-quercetin at $p<0.01$; *b* significantly different from free-quercetin at $p<0.01$

some dairy products), depending on the nature of the product. In addition, pH can vary during production, storage, and consumption. Medium pH determines the ionization of groups present on the surfaces of nanoparticles and, consequently, their surface final loading density (Heurtault et al. 2003). Thus, it is very important to ensure that Quercetin-Np remain stable when exposed to pH changes.

Figure 5a shows the effect of pH variation on zeta potential of Quercetin-Np. As pH was raised from 3.3 to 6.5, the electrical charge of the particles was decreasing and became negative for values above pH 7.0. The density reduction of positive charges in Quercetin-Np with pH increase is possibly the result from deprotonation of amino groups present in chitosan, whose pKa is about 6.2–7.0. As chitosan loses its positive charge, electrostatic attraction between the anionic lecithin molecules and the cationic chitosan molecules decreases. Therefore, it is possible that chitosan molecules have dissociated from the surface of Quercetin-Np, which would explain the observed negative zeta potential at alkaline pH values.

Variations in surface charge of Quercetin-Np, resulting from the increase of pH values, significantly influence their stability in solution (Fig. 5b). The average size of Quercetin-Np remained constant up to pH 5.0 with signs of aggregation at pH 5.5 and later flocculation above pH 6.5. For pH values between 3.3 and 5.0 the Quercetin-Np is highly positive which generates a strong electrostatic repulsion. As already mentioned, as the solution pH was increased, chitosan molecules responsible for the high density of positive charges in Quercetin-Np are deprotonated and consequently the repulsion between the Quercetin-Np also decreased leading to an aggregation (pH 5.5–6.0). The pH range where a higher degree of flocculation of Quercetin-Np was observed occurred between 6.5 and 7.0, which corresponds to the pKa region of chitosan. This behavior of flocculation and aggregation in the pH range close to the pKa of chitosan suggests that Quercetin-Np stability is predominantly governed by the presence of this polysaccharide. The flocculation observed may be explained by partial desorption of chitosan molecules from the surface of Quercetin-Np, leading to formation of polymeric bonds between chains. Quercetin-Np at pH 8.0 became more stable because chitosan molecules lost most of their positive charge and are therefore not effective to induce flocculation. Moreover, lecithin zeta potential is constant above pH 2.0 and contributes to the overall charge of Np's. Above pH 8.0 chitosan chains are deprotonated and Np's charge will be dominated by lecithin charge. Similar behavior was observed by Chuah et al. (2009) in emulsions of soy lecithin coated with chitosan.

Effect of Temperature Variation

The resistance of Np's to temperature changes is of great importance for food applications, once some foods undergo some type of thermal processing during production, storage, or consumption, such as pasteurization, sterilization, or cooking (Guzey and McClements 2006).

Quercetin-Np showed to be stable under thermal treatment in the range 5–70 °C (Fig. 6a). The coalescence phenomenon that normally occurs in lipid systems was not observed, probably due to the protective layer of chitosan, with high density of positive charges, which generates electrostatic repulsion between particles. Chuah et al. (2009)

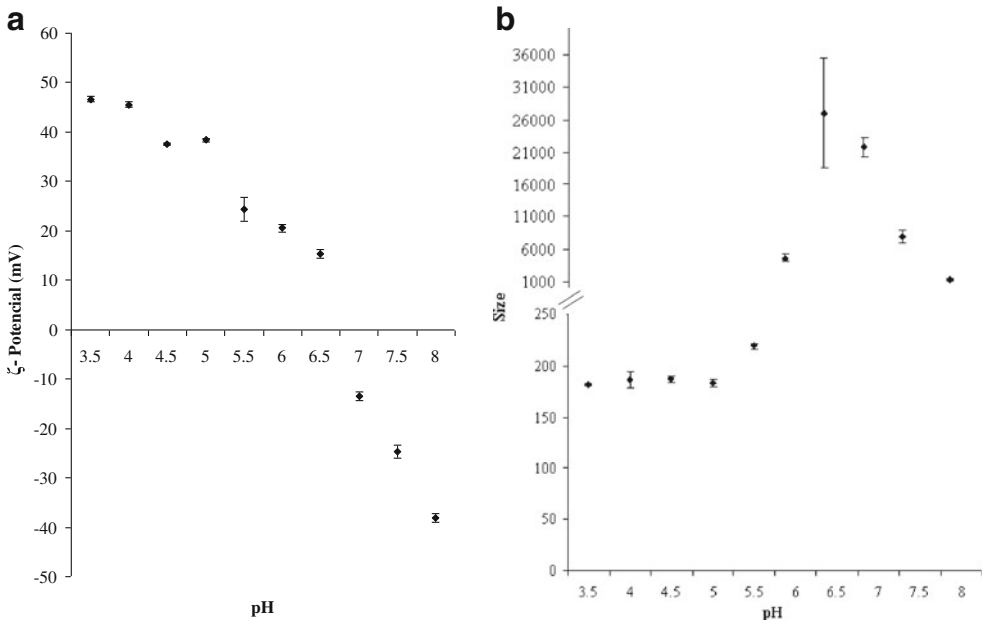

Fig. 5 **a** Effect of pH variation on zeta potential of Quercetin-Np; **b** effect of pH variation on the average size of Quercetin-Np. All determinations were performed in triplicate and the results expressed as mean ± standard deviation

when assessing the thermal treatment effect (30–90 °C) in lecithin nanoemulsions found that addition of chitosan to these systems makes them more stable to temperature variations. The slight reductions in average size and PDI observed in Quercetin-Np are probably a result of disaggregation of particles caused by agitation of molecules with the energy input into the system when temperature is raised.

Stability During Storage Time

Np's can be considered stable when there is no perceptible change in the particle size distribution, in the state of aggregation or in the spatial arrangement within the colloidal system over a period of observation (Dickinson 2003). Quercetin-Np stability was evaluated during 90 days at 4 °C and 30 °C. No statistically significant differences ($p<0.01$) were observed in particle size, PDI, and zeta potential (data not shown). The same behavior was reported for melatonin-loaded lecithin/chitosan nanoparticles stored at 4 °C for 60 days, for average size and zeta potential (Hafner et al. 2009).

Figure 6b shows the variation of quercetin concentration and antioxidant activity (DPPH scavenging capacity) during Quercetin-Np storage at 4 and 30 °C. The Quercetin-Np stored at 30 °C showed gradual and linear reduction in DPPH consumption at the sixth day of storage combined with the

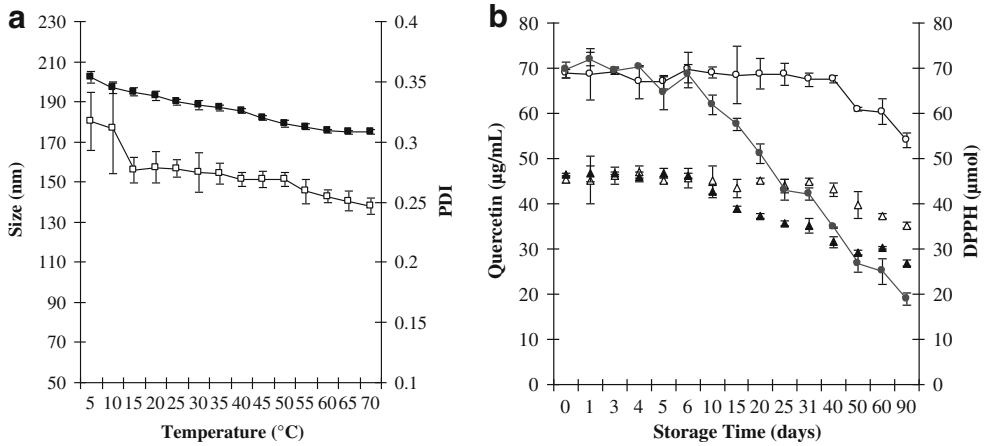

Fig. 6 **a** Effect of temperature variation in the average size (*black squares*) and polydispersity index (*white squares*) of Quercetin-Np. **b** Effect of storage time in quercetin concentration (μg/mL) and DPPH scavenging capacity (μmol) of Quercetin-Np stored at 4 and 30 °C. Key: *whites circles* concentration of Quercetin-Np stored at 4 °C, *black circles* concentration of Quercetin-Np stored at 30 °C, *white triangles* DPPH scavenging capacity by Quercetin-Np stored at 4 °C, and *black triangles* DPPH scavenging capacity by Quercetin-Np stored at 30 °C. All determinations were performed in triplicate and the results expressed as mean ± standard deviation

degradation (oxidation) of quercetin present in the Np's. This phenomenon can be explained by the fact that lecithin, the main constituent of Np's, consists of unsaturated phospholipids susceptible to spontaneous oxidation by molecular oxygen dissolved in the suspension. This hypothesis was confirmed by detection of peroxides in Np after 10 days of storage at 30 °C (data not shown). Storage at 4 °C reduced the rate of oxidation of Quercetin-Np and quercetin degradation started to be observed after the 50th day of storage.

Conclusion

Quercetin has been successfully encapsulated in lecithin/chitosan Np's using the self-assembly technique. This study showed that Quercetin-Np is stable to temperature (5 to 70 °C) and pH variations in values ranging between 3.3 and 5.0. NP's stored at 4 °C showed resistance to aggregation for 90 days and stability to retention of quercetin concentration for 40 days. The results suggest that Quercetin-Np can be used as an ingredient of functional foods or as an alternative in the defense against free radicals damaging foods in hydrophilic environments. The features presented by Quercetin-Np make them attractive not only for the food industry, but also for cosmetic and pharmaceutical industries.

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