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Biochimica et Biophysica Acta

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

Nanoencapsulation of quercetin and resveratrol into elastic liposomes

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ARTICLE INFO

Article history:

Received 23 April 2012

Received in revised form 2 September 2012

Accepted 19 October 2012

Available online 25 October 2012

Keywords:

Quercetin

Resveratrol

Sodium deoxycholate

Elastic liposome

Experimental design

Nanotechnology

ABSTRACT

Based on the fact that quercetin (QUE) and resveratrol (RES) induce a synergic inhibition of the adipogenesis and increase apoptosis in adipocytes, and that sodium deoxycholate (SDC) has necrotic effects, the nanoencapsulation of QUE and RES into SDC-elastic liposomes is proposed as a new approach for dissolving the subcutaneous fat. The concentration of constituents and the effect of the drug incorporation into cyclodextrin inclusion complexes on the stability of QUE/RES-loaded liposomes were studied. The best liposomal formulation reduced the use of phosphatidylcholine and cholesterol in 17.7% and 68.4%, respectively. Liposomes presented a mean diameter of 149 nm with a polydispersion index of 0.3. The zeta potential of liposomes was slightly negative (−13.3 mV) due to the presence of SDC in the phospholipid bilayer. Encapsulation efficiency of QUE and RES into liposomes was almost 97%. To summarize, QUE/RES-loaded elastic liposomes are stable and suitable for subcutaneous injection, thereby providing a new strategy for reducing subcutaneous fat.

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1. Introduction

The natural compounds quercetin (QUE) and resveratrol (RES) are both flavonoids that have effects on the biochemical and metabolic functions of adipocytes, such as the inhibition of adipogenesis and the induction of apoptosis. QUE and RES in association caused a reduction in cell viability of more than 70% in primary human adipocytes and 3T3-L1 murine adipocytes [1,2]. The treatment of 3T3-L1 adipocytes with QUE attenuated adipogenesis and decreased the expression of adipogenesis-related factors and enzymes by up-regulating the levels of phosphorylated adenosine monophosphate-activated protein kinase and its substrate. Finally, QUE induced apoptosis by modulation of the ERK and JNK pathways, down-regulating the expression of anti-apoptotic bcl-2 and activation of caspase-3 [3]. RES caused the inhibition of cell proliferation during early preadipocyte development; the inhibition of differentiation and lipid accumulation during later preadipocyte development; and the induction of lipolysis and apoptosis of mature adipocytes. RES also mediated the down-regulation of adipocyte specific transcription factors and enzymes via genes that modulate mitochondrial function [4]. Moreover, RES activated the expression of Sirt1, reducing lipid accumulation by the repression of PPAR γ in differentiated adipocytes [2].

Despite the action of QUE and RES in reducing adipocyte viability, there are few *in vivo* studies in this subject and the use of both these flavonoids for improving dissolution of subcutaneous fat is not described in the literature. In addition, QUE and RES have low aqueous solubility and low bioavailability [5]. In this way, liposomes offered advantages in the attempt to overcome all these limitations. Liposomes are self-assembled colloidal vesicles consisting of one or more concentric phospholipid bilayers organized around an aqueous inner compartment, and are used as nanocarriers for drugs, biomolecules and diagnostic agents [6,7]. Cevc and Blume [8] introduced the first elastic liposomes with high deformability (called Transfersomes®), which are formed by the association of phosphatidylcholine (PC) with sodium cholate or sodium deoxycholate (SDC), thus improving drug delivery. The main difference between deformable and conventional liposomes is the high and stress-dependent adaptability of such deformable vesicles, which enables them to squeeze between the cells, despite their large average vesicle size [9,10]. As reported, SDC is one of the components of the subcutaneous “phosphatidylcholine injection” used to dissolve localised subcutaneous fat. SDC can also be used for treating lipomas and lipodystrophy [11,12]. In this formulation, phosphatidylcholine acts as a buffer to delay and diminish the unopposed dramatic effect of SDC. This leads to a massive fat necrosis by its detergent effect and changes in the skin overlying the treatment region [11,13].

In this framework, the present study proposes an innovative elastic liposomal formulation for the dissolution of subcutaneous fat. Two experimental designs were developed to identify the constraints and to study

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experimental conditions such as the concentration of phospholipids and SDC, the entrapment of QUE and RES in the phospholipid bilayer or their encapsulation in the aqueous phase of liposomes as cyclodextrin inclusion complexes. The stability of liposomes and the release kinetics of QUE and RES were also investigated.

2. Materials and methods

2.1. Materials

Soya phosphatidylcholine (PC) (Epikuron 200) was obtained from Lucas Meyer (Hamburg, Germany), 2-Hydroxypropyl- β -cyclodextrin (HP β -CD) were obtained from Fluka (Steinheim, Germany). Cholesterol (CH), stearylamine (SA), phenolphthalein (PHP), poly-L-lysine, osmium tetroxide, sodium deoxycholate (SDC), quercetin (QUE) and resveratrol (RES) were obtained from Sigma (St. Louis, MO, USA). Acetonitrile, chloroform, methanol and monobasic potassium phosphate were purchased from Merck (Darmstadt, Germany). Analytical grade chemicals without any further purification and deionised water were used in all the experiments.

2.2. Methods

2.2.1. Apparent solubility and dissolution rate of QUE and RES

Excess amounts of QUE and RES were added to water, 50 mM potassium phosphate buffer solution (PP) at pH 7.4 or 50 mM phosphate buffered saline (PBS) at pH 7.4 following which they were incubated at 37 ± 1 °C and shaken until reaching equilibrium. The content of QUE and RES was assessed by UV spectrophotometry (Pharmacia Ultraspec 3000 Pro spectrophotometer) at 370 and 307 nm, respectively. The solubility was determined using the QUE (0.31–20 μ g/ml) and RES (0.02–20 μ g/ml) standard curves, respectively. The dissolution constant was calculated from the cumulative amount of dissolved drug (μ g) versus time (h) plots according to USP [14]. Data were fitted by linear regression analysis and the slope of the linear equation corresponds to the dissolution rate.

2.2.2. Inclusion complexes of SDC, QUE and RES with HP β -CD

Drug inclusion complexes with hydroxypropyl- β -cyclodextrin (SDC:HP β -CD; RES:HP β -CD and QUE:HP β -CD) were prepared at a 1:1 molar ratio using the co-precipitation technique [15,16].

2.2.3. HPLC validation method for determining QUE and RES in elastic liposomes

Samples of elastic QUE and RES-loaded liposomes were diluted in methanol and injected into the HPLC system. Two different methods for determining QUE and RES were validated. The chromatographic system (FPLC ÅKTA purifier) operated by the software UNICORN 4.1 (Amersham Biosciences) consisted of a quaternary pump (model P-900), an auto sampler and a UV-900 detector (Amersham Biosciences). The chromatographic run was performed using a reverse phase C₁₈ column (125 Å, 3.9 × 300 mm i.d. particle 5 μ m, Bondapack®) with 50 μ l of the sample injection volume at 25 °C. HPLC analysis of QUE (Method 1) was carried out using a mobile phase consisting of acetonitrile and methanol (4:6 v/v) at a flow rate of 1.2 ml/min and detection at 370 nm, while the assay of RES (Method 2) was performed using a mobile phase consisting of acetonitrile and methanol (6:4 v/v) at a flow rate of 0.3 ml/min and detection at 307 nm.

The method for quantifying QUE and RES in elastic liposomes was validated following the procedures presented by the European Medicines Agency (EMA, CPMP/ICH, 381, 95). Absorbance vs. concentrations curves were plotted using standard solutions of QUE or RES at concentrations ranging from 2 to 80 μ g/ml and 1 to 50 μ g/ml, respectively. Assays were performed in triplicate. The linearity of the standard curves was validated for each drug by the least square method

and one-way analysis of variance (ANOVA), by assuming $p < 0.05$. The limits of detection and quantification were also determined.

2.2.4. Preparation of elastic QUE/RES-loaded liposomes

SDC elastic liposomes containing QUE and RES entrapped in their phospholipid bilayer or SDC:HP β -CD, RES:HP β -CD or QUE:HP β -CD inclusion complexes encapsulated in their aqueous inner cavity were prepared using the thin lipid film method [17,18]. Briefly, a thin lipid film, named as organic phase (OP), consisting of PC and CH with or without SA was produced from a mixture of CHCl₃:MeOH (3:1 v/v) under magnetic stirring. The solvents were removed under pressure at 37 ± 1 °C, 80 rpm for 60 min. This film was then hydrated with 10 ml of an aqueous phase (AP) containing 50 mM potassium phosphate buffer (PP) at pH 7.4 or 50 mM phosphate buffered saline (PBS) at pH 7.4, resulting in the formation of multilamellar liposomes. This liposomal suspension was then sonicated (Vibra Cell, BRANSON, USA) at 200 W and 40 Hz for 300 s under low temperature (4 °C) to form small unilamellar liposomes. SDC, QUE or RES were placed in the organic phase for the lipid film formation; and SDC:HP β -CD, RES:HP β -CD and QUE:HP β -CD were placed in the aqueous phase during the lipid film redispersion.

Lyophilised liposomes were obtained using threosulose (10%) as cryoprotectant. Samples were frozen at -80 °C and lyophilised (EZ-DRY, FTSS System, New York, USA) at 200 bars for 48 h.

2.2.5. Experimental designs

At first, a two-level 2^{5-1} fractional experimental design was carried out to optimize the formulation of liposomes. Factors of formulation that mainly influenced the physicochemical characteristics of liposomes (such as constituent concentrations (PC and CH) and drug inclusion complexes with HP β -CD) were evaluated at two levels and central point. The factors were defined as follows: Factor A = PC (69.95, 82.32 and 94.69 mM); Factor B = CH (18.74, 23.52 and 28.30 mM); Factor C = SDC in the organic phase (OP = 1.45–0.725 mM) or in the aqueous phase (AP = 1.45–0.725 mM); Factor D = QUE (OP = 0.66–0.33 mM and AP = 0.66–0.33 mM); Factor E = RES (OP = 0.88–0.44 mM and AP = 0.88–0.44 mM). The mean size, polydispersity index (PDI), zeta potential (ζ mV) and drug encapsulation efficiency (EE%) of liposomes were used as the response variables of the design study. Next, a two-level 2^3 - full experimental design was employed for studying the drug concentration effects and the co-encapsulation of QUE and RES (1:1 molar ratio) on the liposomal physicochemical parameters evaluated at two levels and central point as mentioned above. The factors were defined as follows: Factor A = CH (13.96, 18.74 and 23.52 mM); Factor B = SDC (1.45, 3.625 and 5.80 mM); Factor C = QUE and RES (1.54, 3.08 and 4.62 mM).

2.2.6. Physicochemical characterisation of elastic QUE/RES-loaded liposomes

Drug encapsulation efficiency (EE%) was determined through the ultrafiltration/ultracentrifugation technique using Ultrafree® units (Millipore, USA). After centrifugation of the samples (Ultracentrifuge KT-20000, Kubota, Japan) at 8776g for 1 h at 4 °C, the contents of QUE and RES in the supernatant were measured by a validated HPLC method as described above. The EE% was determined as follow:

$$EE\%_{Drug} = \frac{[\text{measured}_{Drug}] - [\text{unloaded}_{Drug}]}{[\text{measured}_{Drug}]} \quad (1)$$

The total EE% was defined in terms of the partial contents of QUE and RES co-encapsulated in liposomes:

$$EE\% = \frac{1}{2} (EE\%_{QUE} + EE\%_{RES}) \quad (2)$$

The particle size and polydispersity index (PDI) of elastic liposomes were determined by standard photon correlation spectroscopy

(Beckman-Coulter Delsa™ NanoS Particle analyser, Germany) at a fixed angle of 90°. The zeta potential (ζ mV) of liposomes was measured at 25 °C using the electrophoresis technique (Zetatrac NC-148, Microtrac, USA). The measurements were carried out for dispersed and lyophilised forms of liposomes.

2.2.7. Scanning electron microscopy of elastic QUE/RES-loaded liposomes

Samples for scanning electron microscopy (SEM) evaluation were obtained by dispersing a drop (20 μ l) of the liposomal formulation onto a coverslip containing poly-L-lysine solution (0.01% w/v), dried at room temperature (25 °C) and fixed with 2% osmium tetroxide vapour for 1 h at 4 °C. The coverslip was then fixed on an SEM-stub using conductive double-sided tape, coated with a thin layer (100 nm) of gold/palladium in a vacuum sputtering, and examined (JSM-5600 microscope, Jeol, Japan) at 5 and 10 kV.

2.2.8. Stability of liposomal formulations

The stability of elastic QUE/RES-loaded liposomes was evaluated using both the standard accelerated and the long-term stability testing. Samples of conventional (QUE-Lipo and RES-Lipo) and SDC elastic QUE/RES-loaded liposomes were submitted to centrifugation (3510g for 1 hour at 4 °C) and horizontal mechanical stirring (100 strokes/min for 48 h at 37 °C). For long-term stability testing, samples of liposomes in the dispersed forms were stored at 4 °C for 45 days. The mean size, polydispersity index (PDI) and pH of formulations were analysed in the assays.

2.2.9. In vitro release kinetics of QUE and RES from elastic liposomes

The release kinetics of QUE and RES from elastic liposomes was performed using the dialysis sac technique (cellulose membrane, cut-off = 12,400 MW, Sartorius, Germany). For this purpose, sink conditions were established according to apparent solubility studies (item 2.2.1). An aliquot of 0.4 ml elastic liposomes was transferred to the dialysis sac, which was sealed and immersed in dark flasks containing 250 ml of 100 mM potassium phosphate buffer solution (pH 7.4). The release system was maintained under magnetic stirring (100 rpm) at 37 ± 1 °C. At a predetermined sampling time, 1 ml aliquot of medium was drawn and replaced with the same volume of fresh release medium. QUE and RES content were measured by HPLC as described above. Results are expressed as the percentage of released drug as a function of time and values represent the mean \pm standard deviation (SD) of three assay replicates.

2.2.10. Statistical analysis

The statistical analyses of data were carried out using the Statistica 6.0® software (StatSoft Inc., Tulsa, OK, USA). Differences were considered significant at $p < 0.05$.

3. Results

3.1. Apparent solubility and dissolution rate of QUE and RES

The results of the apparent solubility of QUE and RES in water, 50 mM PP buffer solution and 50 mM PBS at pH 7.4 are shown in Table 1. The solubility of QUE increased tenfold in PP buffer solution at 7.4 pH (3.2 μ g/ml) with respect to its solubility in water (0.3 μ g/ml). This result is explained by the slight ionization of QUE that occurred at pH 7.4 ($pK_a = 6.74$) [19]. These results corroborate those previously reported by Priprem et al. [20], where a QUE solubility of 0.7 μ g/ml in water and 4.3 μ g/ml in 7.4 pH buffer solutions at 37 °C, respectively, was determined. On the other hand, no change in the solubility of RES was found in pH 7.4 buffer solutions because no ionization of the RES molecule that occurred at this pH ($pK_a = 9.3$) [21]. These results were not unexpected bearing in mind the pK_a values of these flavonoids. Further, as the QUE molecule has the potential to ionize phenolic hydroxyl groups, a pH-dependent solubility is to be expected. As previously described for a QUE dissolution study [22] at

Table 1

The apparent solubility and dissolution rate of QUE and RES at 37 °C.

Drug/solvent	Solubility (μ g/ml)	Dissolution rate $\times 10^2$ (μ g h ⁻¹)
QUE		
Water	0.3	0.09
PP	3.2	2.00
PBS	2.2	1.00
RES		
Water	21.0	8.00
PP	20.0	7.00
PBS	21.0	9.00

PP = 50 mM phosphate potassium buffer (pH = 7.4); PBS = 50 mM phosphate buffer saline (pH = 7.4).

lower a pH, the free H⁺ ion tends to keep QUE in its molecular state and its ionization is avoided. On the other hand, the concentration of H⁺ ion decreases gradually with an increase in pH. Therefore, at high pH QUE behaves as a weak acid, which means that QUE is being ionized, leading to an increase in its solubility.

3.2. Inclusion complexes of SDC, QUE and RES with HP β -CD

The $K_{C1:1}$ of SDC:HP β -CD was determined according to Cadena et al. [15] with value of 2.12×10^4 M⁻¹. The $K_{C1:1}$ of QUE:HP β -CD and RES:HP β -CD inclusion complexes were previously determined by Jullian et al. [23] and Lu et al. [24] with values of 1.42×10^3 M⁻¹ and 1.82×10^3 M⁻¹, respectively.

3.3. HPLC validation method for determining QUE and RES in elastic liposomes

Two independent HPLC methods were validated to quantify QUE and RES in liposomes without drug extraction. The detection was carried out at different wavelengths, 370 nm for QUE and 307 nm for RES. The retention time of QUE was 3.56 min (Method 1) and 11.13 min for RES detection (Method 2). The standard curves for the linearity assays were plotted with five concentrations allowing the derivation of the following regression linear equations: $A = 3.86 (\pm 0.17) \times [QUE_{\mu\text{g/ml}}] - 13.31 (\pm 7.93)$ and $A = 23.31 (\pm 0.33) \times [RES_{\mu\text{g/ml}}] - 24.29 (\pm 9.6)$. The standard curves were validated by the least squares method showing correlation coefficients higher as 0.9975 and the one-way analysis of variance (ANOVA) showing F and p values of 57,133.04 and 0.0002 for QUE and 4,884.57 and less than 6×10^{-6} for RES, respectively. The limits of detection were 0.78 μ g/ml for QUE and 0.13 μ g/ml for RES, respectively. The corresponding limits of quantification were 2.59 and 0.43 μ g/ml, respectively.

3.4. Properties of elastic QUE/RES-loaded liposomes

After the formulation, the macroscopic appearance of the liposomal dispersions was observed. Liposomal formulations exhibited a milky appearance with a typically bluish reflection characteristic of nanometric dispersed vesicles [18]. Table 2 shows the effect of the PP or PBS buffers on the properties of liposomes. No significant differences in mean size between PP-liposomes with or without SA and PBS-liposomes with SA were observed using the Tukey test ($p < 0.05$). However, PBS-liposome without SA showed the largest mean size. A slight increase in the mean diameter of the liposomes without SA occurred, probably due to an increase of the vesicle aggregation, given the low zeta potential of liposomes. Suitable PDI values were obtained in all formulations. Based on these results, PP-liposomes were thus chosen for nanoencapsulation of drugs.

The physicochemical characteristics of elastic QUE/RES-loaded liposomes are shown in Table 2. Elastic liposomes with SA showed smaller sizes than those without SA. With reference to the PDI value, no significant differences were observed. QUE-loaded liposomes

Table 2
Pre-formulation study of SDC elastic liposomes containing QUE, RES or inclusion complexes using different buffer solutions.

Liposomal formulations*	Mean size (nm)	PDI	ζ (mv)	EE (%)
PC-CH-SA-PP	117.1 ± 10.1 ^a	0.291 ^{ab}	21.87 ± 1.55	ND
PC-CH-SA-PBS	130.6 ± 10.3 ^a	0.346 ^b	32.42 ± 0.74	ND
PC-CH-PP	133.3 ± 11.1 ^a	0.327 ^{ab}	-5.17 ± 0.96	ND
PC-CH-PBS	166.7 ± 20.6 ^b	0.280 ^a	0.47 ± 0.01	ND
PC-CH-SA-SDC	110.8 ± 13.4 ^a	0.324 ^a	21.68 ± 2.72	ND
PC-CH-SDC	134.7 ± 3.6 ^b	0.301 ^a	-13.3 ± 3.5	ND
PC-CH-SA-SDC:HPβCD	106.5 ± 10.8 ^a	0.320 ^a	20.99 ± 2.64	ND
PC-CH-SDC:HPβCD	123.6 ± 8.5 ^{ab}	0.314 ^a	-8.94 ± 0.24	ND
PC-CH-SA-QUE	110.1 ± 9.3 ^a	0.347 ^a	25.93 ± 1.87	86.0 ± 5.20 ^a
PC-CH-QUE	124.9 ± 2.1 ^b	0.298 ^a	-3.72 ± 1.37	97.3 ± 1.30 ^b
PC-CH-SA-QUE:HPβCD	146.1 ± 5.6 ^c	0.298 ^a	22.86 ± 1.67	93.9 ± 1.76 ^b
PC-CH-QUE:HPβCD	126.2 ± 1.4 ^b	0.272 ^a	-10.01 ± 2.76	95.5 ± 0.58 ^b
PC-CH-SA-RES	118.8 ± 9.5 ^a	0.321 ^a	22.91 ± 1.46	96.4 ± 2.64 ^a
PC-CH-RES	142.5 ± 5.1 ^b	0.327 ^a	-0.47 ± 0	98.3 ± 0.86 ^a
PC-CH-SA-RES:HPβCD	120.7 ± 1.7 ^a	0.272 ^a	21.02 ± 1.40	93.8 ± 0.86 ^a
PC-CH-RES:HPβCD	115.0 ± 14.3 ^a	0.337 ^a	-6.95 ± 1.96	95.5 ± 1.92 ^a

*Molar ratio (mol/mol%): PC:CH (3.5:1), PC:CH:SA (7:2:1); [SDC = 1.45]; [QUE = 0.66]; [RES = 0.88]; ^{a-c}Mean values from three replicates. Average with different letters differs statistically by Tukey test ($p < 0.05$); ND = not determined.

showed a statistically significant difference in mean size ($p < 0.05$), but no significant differences were found in the PDI. QUE-loaded liposomes with SA presented the lowest average diameter. In addition, It was also observed that the SA influenced the UV absorptivity of QUE (data not shown), resulting in a reduction of more than 10% in EE%. Among the formulations produced with QUE, liposomes without SA were chosen for further experiments because they showed a higher EE%, as well as maintaining drug stability during the manufacturing process. Furthermore, the mean size of liposomes was compatible with the subcutaneous injection (> 100 nm), allowing them to remain at the site of injection [25]. In relation to the mean size, EE% and PDI, RES-loaded liposomes showed no statistical significant differences ($p < 0.05$). RES-loaded liposomes without SA were thus chosen, due to their higher EE% and also the co-encapsulation of QUE without being affected by SA.

Table 3
Two-level 2^{5-1} fractional experimental design of elastic liposomes containing QUE and RES or inclusion complexes.

Runs	PC (mM)	CH (mM)	SDC (mM)	QUE* (mM)	RES* (mM)	Size (nm)	PDI	ζ (mV)	EE (%)
1	69.95	18.74	OP - 1.45	OP - 0.66	AP - 0.88	128.3 ± 1.78	0.257	-7.73 ± 0.68	92.26 ± 0.52
2	94.69	18.74	OP - 1.45	OP - 0.66	OP - 0.88	139.5 ± 6.04	0.305	-8.29 ± 0.30	93.82 ± 0.18
3	69.95	28.30	OP - 1.45	OP - 0.66	OP - 0.88	133.6 ± 3.24	0.285	-13.34 ± 1.36	93.92 ± 0.14
4	94.69	28.30	OP - 1.45	OP - 0.66	AP - 0.88	122.5 ± 4.82	0.284	-5.68 ± 0.92	94.66 ± 0.08
5	69.95	18.74	AP - 1.45	OP - 0.66	OP - 0.88	131.5 ± 3.95	0.333	-5.72 ± 0.50	93.34 ± 0.25
6	94.69	18.74	AP - 1.45	OP - 0.66	AP - 0.88	164.5 ± 3.52	0.342	-3.38 ± 1.20	92.56 ± 0.21
7	69.95	28.30	AP - 1.45	OP - 0.66	AP - 0.88	129.1 ± 1.89	0.274	-7.35 ± 1.23	89.62 ± 0.75
8	94.69	28.30	AP - 1.45	OP - 0.66	OP - 0.88	133.3 ± 2.48	0.262	-7.38 ± 1.15	91.18 ± 0.21
9	69.95	18.74	OP - 1.45	AP - 0.66	OP - 0.88	129.7 ± 2.48	0.251	-16.69 ± 0.85	90.84 ± 0.70
10	94.69	18.74	OP - 1.45	AP - 0.66	AP - 0.88	129.4 ± 2.71	0.264	-8.44 ± 1.29	88.17 ± 0.15
11	69.95	28.30	OP - 1.45	AP - 0.66	AP - 0.88	139.5 ± 3.42	0.268	-8.54 ± 0.20	85.37 ± 0.41
12	94.69	28.30	OP - 1.45	AP - 0.66	OP - 0.88	135.9 ± 2.03	0.272	-6.64 ± 0.69	95.59 ± 0.06
13	69.95	18.74	AP - 1.45	AP - 0.66	AP - 0.88	133.3 ± 1.78	0.248	-6.28 ± 1.59	87.30 ± 0.82
14	94.69	18.74	AP - 1.45	AP - 0.66	OP - 0.88	138.2 ± 1.89	0.485	-4.51 ± 0.90	90.45 ± 0.25
15	69.95	28.30	AP - 1.45	AP - 0.66	OP - 0.88	145.7 ± 0.59	0.293	-6.03 ± 0.23	87.29 ± 1.15
16	94.69	28.30	AP - 1.45	AP - 0.66	AP - 0.88	123.6 ± 3.79	0.374	-11.28 ± 0.99	77.41 ± 0.39
17	82.32	23.52	OP - 0.725	OP - 0.33	OP - 0.44	130.0 ± 6.33	0.283	-9.98 ± 0.93	89.41 ± 0.11
			AP - 0.725	AP - 0.33	AP - 0.44				
18	82.32	23.52	OP - 0.725	OP - 0.33	OP - 0.44	133.3 ± 5.05	0.284	-7.47 ± 0.80	93.58 ± 0.56
			AP - 0.725	AP - 0.33	AP - 0.44				
19	82.32	23.52	OP - 0.725	OP - 0.33	OP - 0.44	130.1 ± 2.26	0.272	-10.28 ± 0.98	92.85 ± 0.19
			AP - 0.725	AP - 0.33	AP - 0.44				

*Co-encapsulation of QUE and RES in the phospholipid bilayer or aqueous phase.

3.5. Experimental designs

The nineteen experimental runs of the two-level 2^{5-1} fractional design and their responses are shown in Table 3. The experiments were randomly evaluated in order to nullify the effect of inappropriate nuisance variables. The preliminary runs (Table 2) were conducted initially to establish the feasible range of each formulation parameter. All liposomes had a mean size compatible with subcutaneous injection (> 100 nm), where the formulations prepared with the lower concentration of PC (69.65 mM) and SDC placed in the organic phase showed the lowest PDI values (Table 3). In addition, the high values of zeta potential were observed when SDC and RES were placed in the organic phase and the smallest PC concentration (69.65 mM) was used. Finally, the greatest EE% was observed when SDC and RES were used in the organic phase ($p < 0.05$). The mean values of pH and absolute viscosity values of all liposomal formulations were $7.64 ± 0.07$ and $0.372 ± 0.001$ mPa.s, respectively.

Fig. 1 shows the scatter plots indicating the best liposomal formulations (runs 9 and 3), which have in common the lowest PC concentration (69.65 mM), and SDC and RES placed in the organic phase. QUE high encapsulation efficiency was found regardless of its location in the organic or aqueous phase, allowing the choice of the organic phase in order to design a simple and less expensive liposomal formulation.

Based on these findings, a new experimental design was proposed, taking into account the variables CH concentration and QUE and RES co-encapsulation in the presence of SDC. The SDC concentration was also studied as it can lead to an improvement in the zeta potential of liposomes. The experimental runs of the two-level 2^3 full experimental design and their responses are summarized in Table 4. The concentration of CH was not statistically significant for all physico-chemical characteristics and was used in a minor concentration (13.96 mM) for further experiments. These results are shown in Fig. 2. The formulation prepared with CH, SDC, QUE and RES at 13.96, 5.8 and 4.62 mM (run 7) had the best parameters: high EE% ($97.5 ± 0.06%$), satisfactory values of mean size ($149.4 ± 2.47$ nm) and PDI ($0.295 ± 0.011$). These elastic liposomes were morphologically analysed by SEM, exhibiting vesicles well dispersed without aggregation (Fig. 3A) and spherical-shaped particles (Fig. 3B). In

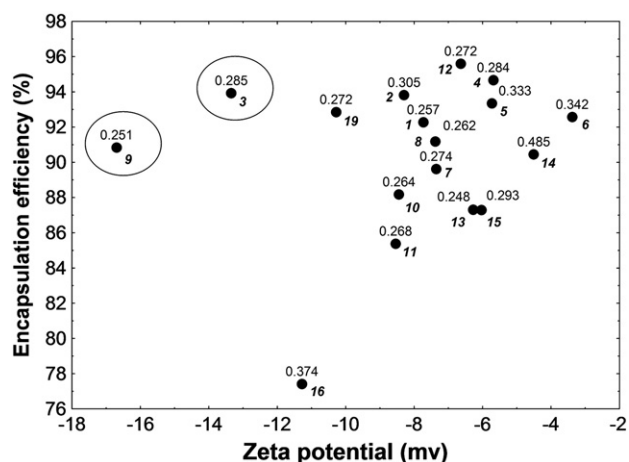


Fig. 1. Scatter plot of the formulations produced using a 2^{5-1} fractional experimental design and effects of response variables zeta potential, encapsulation efficiency and polydispersity index (formulation number indicated above).

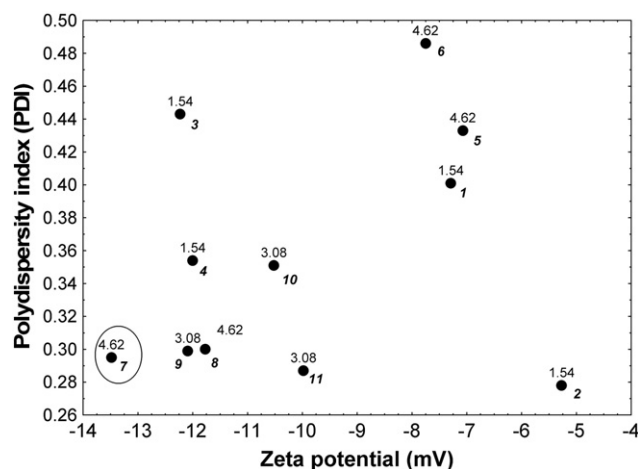


Fig. 2. Scatter plot of the formulations produced using a 2^3 full experimental design and effects of response variables zeta potential, polydispersity index, quercetin and resveratrol concentrations (formulation number indicated above).

addition, light fringes were observed, which could be misinterpreted as the lamellar structure of liposomes. But this phenomenon is due to the fixation with osmium tetroxide vapour [26]. The best formulation had the mean size and PDI measured before and after its use through 1 ml insulin syringes. The results were not statically significant by Tukey test ($p < 0.05$). The diameter of needle did not affect the liposomal characteristics.

3.6. Stability of liposomal formulations

Samples of conventional QUE-liposomes and RES-liposomes, and SDC elastic QUE/RES-loaded liposomes were submitted to accelerated stability testing (Table 5). No statistically significant differences were found ($p < 0.05$) in the mean size and PDI for both conventional and elastic liposomes assayed before and after centrifugation and horizontal mechanical stirring. Moreover, the samples had the same macroscopic appearance before and after standard accelerated stability tests.

Liposomes were also submitted to long-term stability testing with measurement of mean size and polydispersity index (Fig. 4). The SDC elastic liposome had the lowest variation in the mean size ($t_0 = 134.7 \pm 2.8$ nm, $t_{45} = 135.6 \pm 7.8$ nm) and PDI ($t_0 = 0.300$, $t_{45} = 0.308$) when compared with QUE-Lipo ($t_0 = 146.6 \pm 1.4$ nm, $t_{45} = 150.4 \pm 3.2$ nm and $t_0 = 0.327$, $t_{45} = 0.347$) and RES-Lipo ($t_0 = 140.9 \pm 2.2$ nm, $t_{45} = 144.8 \pm 1.0$ nm; $t_0 = 0.319$, $t_{45} = 0.328$) after storage at 4 °C for 45 days. The influence of SDC on the stability of elastic compared to

conventional liposomes was observed in Fig. 5. According to Gillet et al. [9], the addition of SDC may reflect a deformation increase in elastic liposomes, which can reduce stability, but this fact was not observed in the present study.

Furthermore, the lowest pH variation of SDC elastic liposomes ($t_0 = 7.42$, $t_{45} = 7.49$) was found in comparison with RES-Lipo ($t_0 = 7.46$, $t_{45} = 7.39$) and QUE-Lipo ($t_0 = 7.58$ and $t_{45} = 7.17$). A decrease in the pH might indicate lipid degradation with fatty acids formation [27], but this was not observed in the SDC elastic liposomes, suggesting thus lower lipid peroxidation occurred. Further, the use of 100 mM phosphate buffer seems to be suitable to prepare these liposomal formulations.

The elastic liposome had mean size of 179.04 ± 2.90 nm, PDI of 0.290 and EE% of $95.45 \pm 0.12\%$ before lyophilisation. The lyophilised formulation was hydrated with water to the original volume and the mean size of vesicles was measured (197.07 ± 7.25 nm, PDI of 0.300). EE% was assayed as $95.26 \pm 0.15\%$ and pH was 7.49. As expected, it was observed a slight increase in the vesicle mean size after hydration of the lyophilised liposomal formulation. In addition, no statically significant differences in PDI and EE% values ($p < 0.05$) were found after hydration of lyophilised liposomes.

3.7. In vitro release kinetics of QUE and RES from elastic liposomes

As shown in Fig. 5, the *in vitro* release profiles of elastic QUE- and RES-loaded liposomes were compared. QUE was released from

Table 4

Two-level 2^3 full experimental design and physicochemical properties of elastic QUE/RES-loaded liposomes.

Runs	CH (mM)	SDC (mM)	QUE + RES* (1:1, mM)	Size (nm)	PDI	ζ (mV)	EE (%)
1	13.96	1.45	1.54	153.11 ± 9.86	0.401 ± 0.079	-7.29 ± 0.97	98.53 ± 0.27
2	23.52	1.45	1.54	143.48 ± 4.87	0.278 ± 0.027	-5.27 ± 0.52	98.42 ± 0.12
3	13.96	5.80	1.54	124.93 ± 8.41	0.443 ± 0.093	-12.23 ± 0.84	97.55 ± 0.07
4	23.52	5.80	1.54	123.40 ± 3.85	0.354 ± 0.038	-12.00 ± 0.31	98.60 ± 0.06
5	13.96	1.45	4.62	142.00 ± 7.95	0.433 ± 0.089	-7.07 ± 0.37	99.50 ± 0.04
6	23.52	1.45	4.62	146.62 ± 9.82	0.486 ± 0.083	-7.75 ± 0.44	97.89 ± 0.02
7	13.96	5.80	4.62	149.43 ± 2.47	0.295 ± 0.011	-13.48 ± 0.25	97.49 ± 0.06
8	23.52	5.80	4.62	125.50 ± 2.57	0.300 ± 0.011	-11.77 ± 0.93	99.02 ± 0.05
9	18.74	3.625	3.08	141.07 ± 1.44	0.299 ± 0.014	-12.09 ± 0.53	98.73 ± 0.04
10	18.74	3.625	3.08	129.33 ± 6.14	0.351 ± 0.051	-10.52 ± 1.34	99.34 ± 0.00
11	18.74	3.625	3.08	120.99 ± 2.54	0.287 ± 0.016	-9.98 ± 0.82	98.42 ± 0.09

*Co-encapsulation of QUE and RES in the phospholipid bilayer.

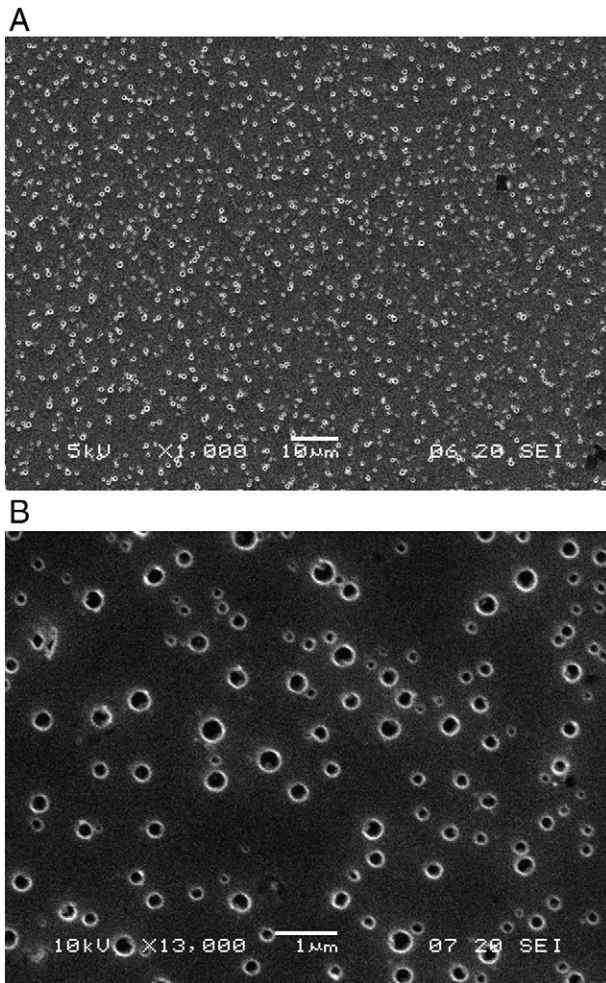


Fig. 3. SEM micrographs of elastic QUE/RES-loaded liposomes.

liposomes (56.3%) at 1 h, which corresponds to the burst effect. Next, a controlled release was verified from 56.3% to 98.3% at 57 h. For RES, the burst effect corresponded to 53.3% at 1 h, followed by a controlled release from 53.3% to 96.4%, reaching the maximum release at 54.5 h. The *in vitro* kinetic behaviour of QUE/RES-loaded liposomes seems to be suitable bearing in mind that an initial burst effect is required to quickly achieve levels as high as 100 µM of QUE and RES, which are essential for the enhanced inhibition of adipogenesis and induction of apoptosis [2].

Table 5

Accelerated stability testing of liposomal formulations.

Formulation	Mean size (nm)		PDI	
	Before	After	Before	After
<i>Centrifugation (3510g for 1 h at 4 °C)</i>				
SDC elastic QUE/RES-liposomes	135.9 ± 10.1 ^a	121.1 ± 4.5 ^a	0.325 ± 0.02 ^a	0.322 ± 0.03 ^a
QUE-liposomes	141.5 ± 4.8 ^a	131.9 ± 5.6 ^a	0.336 ± 0.03 ^a	0.318 ± 0.03 ^a
RES-liposomes	145.5 ± 3.8 ^a	140.1 ± 13.1 ^a	0.328 ± 0.02 ^a	0.387 ± 0.08 ^a
<i>Horizontal mechanical stirring (100 strokes/min for 48 h at 37 °C)</i>				
SDC elastic QUE/RES-liposomes	141.9 ± 2.7 ^a	141.2 ± 8.2 ^a	0.325 ± 0.02 ^a	0.331 ± 0.02 ^a
QUE-liposomes	141.5 ± 4.8 ^a	141.7 ± 3.9 ^a	0.336 ± 0.03 ^a	0.327 ± 0.05 ^a
RES-liposomes	145.5 ± 3.8 ^a	149.8 ± 5.8 ^a	0.328 ± 0.02 ^a	0.358 ± 0.06 ^a

Mean data and standard deviation. Different letters differs statistically by Tukey test ($p < 0.05$).

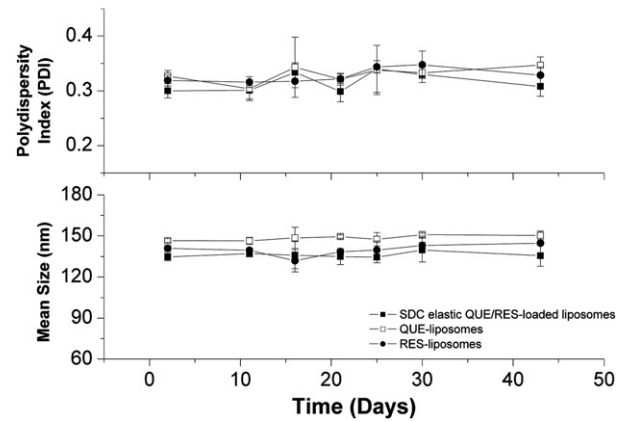


Fig. 4. Long-term stability testing of conventional QUE-liposomes and RES-liposomes, and SDC elastic QUE/RES-loaded liposomes after hydration of the lyophilised formulation with storage at 4 °C.

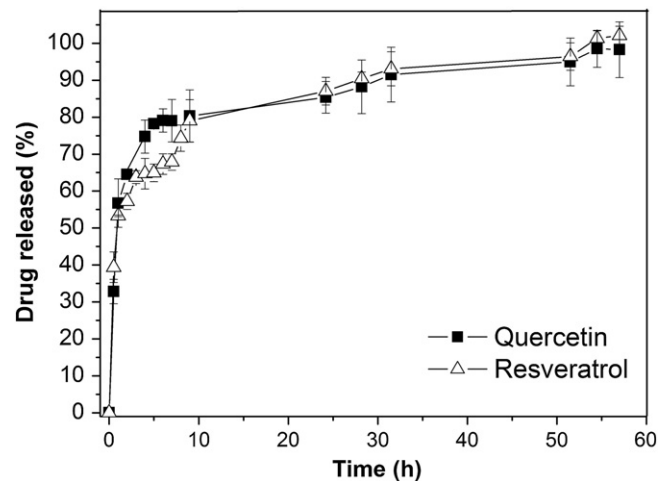


Fig. 5. *In vitro* kinetic profile of QUE/RES-loaded liposomes in 7.4 pH phosphate buffer solution at 37 °C.

4. Discussion

Treatments to dissolve subcutaneous fat based on the detergent action of SDC in lipid structures with high concentrations of phospholipid have been developed and commercialized, despite presenting several adverse effects [12]. As a result, there has been a renewed

interest in developing new products to be used as an adjuvant in treatment with SDC. Keeping this in mind, this study focused on the development and characterization of an innovative product based on elastic SDC liposomes containing QUE and RES co-encapsulated for subcutaneous administration.

It is well known that the stability of liposomes is dependent on both formulation and manufacturing method parameters. In the present study, the pre-formulation of liposomes varying the concentration of constituents and the phase of incorporation was carried out. The encapsulation of QUE or RES into liposomes altered the mean size, PDI and surface charge in relation to unloaded liposomes (Table 1). The presence of stearylamine, adds positive charged character to phospholipid bilayers of liposomes, caused a decrease of about 10% in the encapsulation efficiency of quercetin ($p < 0.05$). However, high encapsulation efficiencies of QUE in liposomes prepared with SA (PC:CH:SA, 7:2:1) or without SA (PC:CH, 7:2) were attained ($86 \pm 5.2\%$ and $97.3 \pm 1.3\%$, respectively). Indeed, it has been shown that QUE exhibits a high affinity for liposomes as a result of its planar configuration, which can be easily intercalated into the organized bilayer structure of the phospholipid molecules forming the liposomes [28]. As previously reported, QUE-loaded liposomes prepared with a mixture of egg phosphatidylcholine and cholesterol at a 1:1 molar ratio and quercetin (2:1:1) presented encapsulation efficiencies ranging from 62% to 81% [20].

On the other hand, no effect of SA on the entrapment efficiency of RES in the phospholipid bilayer of liposomes was found. Furthermore, the entrapment of QUE or RES did not squeeze out stearylamine molecules from the phospholipid bilayer, since a positive surface charge (zeta potential varying from 21 to 26 mV) of liposomes was measured (Table 2). The liposomes prepared with SA containing drug:HP β -CD inclusion complexes showed the same zeta potential than uncomplexed drugs, but the liposomes prepared without SA showed smaller zeta potential values than those containing drug:HP β -CD inclusion complexes. Moreover, drug penetration into the phospholipid bilayer can modify the zeta potential of liposomes. As previously reported, neutral and positively charged liposomes were more localized in the regional lymph nodes than negatively charged liposomes after subcutaneous administration [29]. Based on these findings, the liposomes prepared without SA (negatively charged liposomes) were thus chosen for further experimental designs.

From the results, the co-encapsulation of QUE and RES in elastic SDC-liposomes was considered using a full 2^3 experimental design. In this study, the influence of liposome constituents and the incorporation of drugs into cyclodextrin inclusion complexes were evaluated. All liposomal formulations presented a particle size greater than 100 nm (Table 3), being therefore suitable for subcutaneous administration. The absorption of liposomes from the injection site after subcutaneous administration is size-dependent and governed by the transport of vesicles through the interstitial organized structure [25]. In fact, it was found that large liposomes (larger than 100 nm) will have more difficulty in passing through the interstitial structure and will remain at the site of injection. On the other hand, smaller vesicles can migrate through the aqueous channels in the interstitial structure and reach the lymphatic system.

No statistically significant differences in the physicochemical parameters of elastic SDC-liposomes with QUE and RES entrapped or encapsulated as cyclodextrin inclusion complexes were found. Similar results were obtained by Gillet et al. [9] for betametasone in HP γ CD and Crysmeb inclusion complexes encapsulated into elastic SDC-liposomes. The encapsulation of drug inclusion complexes was subsequently removed for further design experiments in order to obtain more simple formulations with reduced costs.

The liposomal formulations run 9 and run 3 had optimal physicochemical parameters (Fig. 1), but differed in relation to CH concentrations, which influence the rigidity of the phospholipid bilayer [30] and, as a result, the stability of liposomes. In contrast, SDC provides

a more fluid bilayer as described by Chen et al. [31]. As the results of the influence of CH and SDC concentrations on the physicochemical parameters of liposomes were inconclusive, a full 2^3 experimental design was developed with the aim of encapsulating a large amount of drug. Again, the mean size of all liposomal formulations was higher than 100 nm (Table 4). The PDI of the selected liposomes formulations varied between 0.2 and 0.3 (run 7), which is similar to liposomal formulations with encapsulated flavonoids obtained by Caddeo et al. [5] with values of 0.237 and 0.270 and Mignet et al. [32] with values of 0.320 and 0.350. Moreover, liposomes prepared with high SDC concentrations exhibited a smaller size than those with small SDC concentrations. This reduction of the particle size diameter of elastic SDC-liposomes may be attributed to the increased flexibility and reduced surface tension of these vesicles. In addition, the final pH of all formulations of elastic SDC-liposomes was 7.4. Indeed, it has been reported the hydration of elastic SDC-liposomes prepared with pH 7.4 buffer solutions reduces the vesicle size and improves drug loading [31].

No significant effect of CH concentrations on the physicochemical parameters was found (Fig. 2). On the other hand, the increase in SDC concentration caused a slight increase in the zeta potential (a statistically significant positive effect), due to the presence of SDC molecules negatively charged at the surface of liposomes.

The typical elastic SDC-liposomes obtained in this study were able to entrap 23 times more quercetin and resveratrol ($4620 \mu\text{M}$) than the formulations reported in the literature ($100 \mu\text{M}$). Yang et al. [2] studied the combined effects of QUE and RES treatment (both at $100 \mu\text{M}$) on 3T3-L1 cells and found a decrease in their viability ($73.5 \pm 0.9\%$) and an increase in apoptosis ($310.3 \pm 9.6\%$), which was more pronounced than the treatment with QUE or RES alone. In addition, Park et al. [1] studied the effects of QUE and RES ($100 \mu\text{M}$) associated with genistein ($50 \mu\text{M}$) on early- and mid-phase maturing, and lipid-filled mature 3T3-L1 cells. A decrease in cell viability and an induction of apoptosis were observed. Based on these findings, the association of QUE and RES in liposomes is justified by the enhancement of their synergistic effects.

In relation to SDC, the concentration used to dissolve localised fat is 42 mg/ml , which has necrotic effects and causes various adverse events [11]. In the present study the SDC concentration entrapped in liposomes was 2.4 mg/ml , which is less than that used to dissolve localised fat. However, a possible necrotic effect of the elastic liposomal formulations produced cannot be ruled out.

Experimental designs showed that a reduction in the PC and CH concentrations of 17.68% and 68.36%, respectively, can be achieved when manufacturing liposomes containing three times more nanoencapsulated SDC, QUE and RES (run 7). This formulation presented a mean size and PDI values of 150 nm and 0.29, respectively, a zeta potential slightly negative (-14 mV) and EE% higher than 97%. One of the major problems limiting the widespread use of liposomes is both physical and chemical stability [33]. Elastic liposomes with entrapped QUE and RES were examined repeatedly over time and compared with conventional liposomes; it was not observed variations in the physicochemical properties after stability tests. Even though their low zeta potential, the elastic liposomes (dispersion form), no vesicle aggregation was observed during 45 days of storage (Fig. 4) without variation of mean size and PDI. In addition, elastic liposome had similar EE% values before and after lyophilisation ($> 95\%$). On this basis, the liposomes prepared with PC, CH and SDC (69.65 , 13.96 and 5.8 mM , respectively) containing QUE and RES at 4.62 mM were chosen for the *in vitro* release kinetics.

In vitro release kinetic studies have been used as a surrogate indicator of *in vivo* drug availability [31], especially for poorly water-insoluble drugs such as QUE and RES. In this study, the kinetics showed an initial drug burst effect, which was considered important for attaining higher levels of QUE and RES ($> 100 \mu\text{M}$). The complete release of drugs was attained at 60 h (Fig. 5). QUE and RES were

entrapped in the phospholipid bilayer of liposomes and the presence of SDC probably enhanced lipid fluidity thus permitting enhanced drug leakage, as previously observed [34]. Our results corroborate those of Chen et al. [31] for the faster kinetic profile of fenofibrate from elastic (PC/SDC) than conventional (PC/CH) liposomes.

In spite of the satisfactory stability results of the SDC elastic QUE/RES-loaded liposomes, their use in the injectable form need further studies to ensure the safety and efficacy of the treatment of subcutaneous fat reduction.

5. Conclusion

This study offered a new elastic SDC-liposomal formulation containing co-encapsulated QUE and RES. The experimental design permitted to the optimization of elastic QUE/RES-loaded liposomes with suitable physicochemical properties and kinetic drug profiles for subcutaneous injection. This elastic SDC-liposome formulation thus displayed promising features for upcoming experiments. Currently, *in vitro* and *in vivo* studies are being undertaken in our laboratory to evaluate the potential effectiveness of the liposomal formulation in dissolving localized subcutaneous fat.

Abbreviations

AP	Aqueous phase with HP β -CD
CH	Cholesterol
EE%	Encapsulation efficiency
OP	Organic phase
PBS	Phosphate buffered saline
PC	Soya phosphatidylcholine
PDI	Polydispersity index
PP	Potassium phosphate buffer
QUE	Quercetin
RES	Resveratrol
SA	Stearylamine
SDC	Sodium deoxycholate

Greek Letters

ζ	Zeta potential
HP β -CD	2-Hydroxypropyl-beta-cyclodextrin

Conflict of interest statement

The research reported in this manuscript is part of a patent in the course of process.

Acknowledgments

The first author is grateful to the Foundation for Science and Technology Development of the Pernambuco State of Brazil – FACEPE for a PhD scholarship. The authors thank the Brazilian Council for Research and Technological Development, CNPq (grant# 477463) and FACEPE (grant # APQ0676-4.03/10) for financial support. Also much appreciated was the collaboration with the Institute of Technologies of the Northeast Brazil, CETENE. The technical assistance of Mr. Rafael Padilha in the SEM analysis was fully appreciated.

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