Effect of chitosan concentration on PLGA microcapsules for controlled release and stability of resveratrol

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A B S T R A C T

The polyphenols as nutraceutical and therapeutic agents are gaining growing interest for their beneficial effects and potential in human health. In order to protect their scaffolds and functionality, and to improve the bioavailability, the microencapsulation can represent a promising strategy.

This study reports on the formulation of the natural resveratrol (RSV) into microcapsules (MCs) prepared by using different concentrations of chitosan (CS) and poly(D,L-lactic-co-glycolic acid) (PLGA) as polymeric matrix. MCs were prepared by W/O/W double emulsion method and characterized in terms of morphology, size, encapsulation efficiency, physicochemical and thermal properties. RSV release behavior from MCs was evaluated under simulated gastrointestinal fluids, and the long term stability was monitored at different storage conditions.

MCs resulted to have spherical shape and different morphology, with size ranging from 11 to 20 μm, and encapsulation efficiencies of 40–52%, depending on the CS concentration. Moreover, MCs containing CS exhibited a significant lower release of RSV than those containing only PLGA. Furthermore, all tested formulations were able to ensure a good retention and stability of encapsulated RSV until 6 months.

In summary, CS/PLGA MCs can be proposed as an attractive delivery system to control the release and long term protection of RSV.

1. Introduction

The use of polyphenols in the field of functional foods and nutraceuticals is gaining growing interest in the recent years for their beneficial effects on health [1,2]. The functionality and bioavailability of polyphenols as nutraceuticals and preventive/therapeutic agents are strongly affected by their chemical properties, and can be reduced or compromised during processing, due to the instability to oxygen, temperature, light, as well as to the environmental conditions during its passage through the gastrointestinal tract [3–5]. To overcome these limitations, the microencapsulation can represent a promising option and could enable the enrichment of various products with important applications in foods and nutrition in the next future [6].

One of the most investigated polyphenols, the phytoalexin resveratrol (RSV) (3,5,4′-trans-trihydroxystilbene), largely detected in grapes and red wine [2,4], has shown interesting properties as a preventive agent of several important pathologies, such as neurodegenerative processes, viral infections, vascular diseases, and cancers [7–9]. Several studies have focused on the development of micro- and nano-particles to stabilize and protect RSV, to increase its water solubility, to achieve a controlled release, and to improve its effectiveness [10]. For example, (a) calcium-pectinate beads containing RSV were proposed for the site-specific delivery to the lower gastrointestinal tract [11], (b) lipid-core nanocapsules increased the RSV concentration in the brain tissue [12], and (c) nanosomal RSV formulations demonstrated an improved systemic bioavailability [13]. Moreover, controlled release and stabilization of RSV were achieved through incorporation of RSV into cross-linked chitosan microspheres of 53–311 μm by vanillin [14]. Again, polyester microparticles were

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found to delay the dissolution profile of RSV at pH 6.8, and were also endowed of radical scavenging activity [15].

In our previous studies, Chitosan (CS)- and Alginate (Alg)-coated Poly(D,L-lactic-co-glycolic acid) (PLGA) nanoparticles have been developed for controlled release and protection of RSV against light-exposure degradation [16]. Furthermore, we encapsulated RSV into nanoparticles composed of poly(epsilon-caprolactone) (PCL) and PLGA-poly(ethylene glycol) blend as a carrier for prostate cancer prevention and therapy [17].

Focusing on the nutraceutical application and, in particular, to obtain an oral controlled delivery system, in the present study the RSV encapsulation into novel polymeric microcapsules (MCs) was investigated.

In spite of this, PLGA and CS were selected as suitable materials because they are chemically inert, nontoxic, biodegradable and biocompatible [18,19]. Recent studies have revealed that PLGA micro- and nanoparticles provided effective and sustainable release of several polyphenols [18,20]. As far as the CS is concerned, nanoparticles based on this natural polymer have been shown to enhance the absorption of catechins found in green tea [21] as well as protection of insulin against pH and enzymatic degradation in the gastrointestinal tract [22].

In this study, RSV-loaded MCs were formulated by W/O/W double emulsion technique using various concentrations of CS and PLGA as polymeric matrix. The obtained MCs were characterized in terms of morphology, size, encapsulation efficiency, Raman spectroscopy, and differential scanning calorimetry. Subsequently, RSV release behaviors were evaluated under simulated gastrointestinal fluids, and the long term stability was monitored at different storage conditions (temperature and time).

2. Materials and methods

2.1. Materials

Poly-(d,l-lactide-co-glycolide) (lactide:glycolide 75:25 molar ratio, inherent viscosity 0.14–0.22 dL/g, acid terminated, molecular weight 4000–15,000), Chitosan (CS, low molecular weight, 75–85% deacetylated chitin, viscosity 200–800 cP), Resveratrol (RSV), and polyvinyl alcohol (PVA, Mw 31,000–50,000, 98–99%) were purchased from Sigma-Aldrich (Steinheim, Germany). All other chemicals were analytical grade and were used without further purification.

2.2. Encapsulation of RSV

RSV-loaded MCs were prepared by a W/O/W double emulsion method. PLGA and RSV were dissolved in ethyl acetate (1.0 mL) and then added dropwise to aqueous phase comprising 1.0 mL of PVA (1% w/v) and different concentration of CS (0.2%, w/v) in acetic acid solution (1% v/v). The mixture was homogenized to make the W/O, and then added to 45 mL of PVA aqueous solution (0.3% w/v). The resulted W/O/W emulsion was evaporated under vacuum to remove the organic solvent. MCs were then centrifuged (7000 rpm, 10 min) and washed with water to remove the unencapsulated RSV and residual CS. The obtained MCs were lyophilized for further characterization and utilization. The composition of prepared MCs is reported in Table 1.

2.3. Particle size and morphology

The size and morphology (shape and surface characteristics) of MCs were characterized by Scanning Electron Microscopy (SEM) (model DSM 962; Carl Zeiss Inc., Germany). The samples were analyzed at 20 kV acceleration voltage after gold sputtering, under argon atmosphere.

Table 1
Theoretical composition of microcapsules.

Batch	PLGA (% w/w)	CS (% w/w)	RSV (% w/w)
MC-0	99	–	1
MC-1	98	1	1
MC-2	97	2	1
MC-3	95	4	1

2.4. Drug loading content, encapsulation efficiency and yield of production

The amount of RSV encapsulated was determined by dissolving an aliquot of MCs (1.0 mg) in 0.1 mL of methylene chloride. The extraction solvent was evaporated and residue dissolved in 0.1 mL of acetonitrile/water solution (21/79, v/v). The obtained solution was analyzed by HPLC using the method previously reported [16].

The drug loading content (DLC %), drug entrapment efficiency (EE %), and yield of production (YP %) were presented by following equations, respectively:

$$\text{DLC}\,\% = \left(\frac{\text{Weight of drug in MCs}}{\text{Weight of MCs}}\right) \times 100$$

$$\text{EE}\% = \left(\frac{\text{Actual amount of RSV encapsulated in MCs}}{\text{Initial amount of RSV used}}\right) \times 100$$

$$\text{YP}\,\% = \left(\frac{\text{Weight of MCs recovered}}{\text{Weight of polymer and drug fed initially}}\right) \times 100.$$

2.5. Raman spectroscopy

The chemical composition of the RSV, CS, and PLGA (raw materials), and RSV-loaded MCs was analyzed by Raman spectroscopy (Senterra Raman microscope; Bruker Optics, Ettlingen, Germany), using an excitation wavelength of 532 nm at 5 mW. The spectra were acquired in the range 180–3200 cm^{-1}.

2.6. Differential scanning calorimetry (DSC)

Thermal transition properties of empty and drug-loaded MCs were measured using a DSC Q100 V 9.0 calorimeter (TA Instrument, New Castle, USA). The thermograms were obtained at a scanning rate of 10 °C/min in 30–500 °C temperature range and performed under an Ar purge (50 mL/min). The thermal measurements were carried out on pure RSV, PLGA, CS, on drug-loaded MCs, and on the physical mixture of PLGA/RSV, and PLGA/CS/RSV.

2.7. In vitro release studies

The in vitro release tests of RSV formulations were carried out at pH 1.2 (0.1 M HCl) for 2 h, followed by PBS at pH 7.4 for 5 h, to simulate gastric and intestinal fluids, respectively. About 2.0 mg of RSV-loaded MCs were suspended in 200 μL of water and placed into dialysis bags, suspended in 20 mL of release media under sink conditions, then incubated at 37 °C and stirred at 200 rpm. At predetermined time intervals, 1 mL of sample was withdrawn and replaced with equal volume of the corresponding fresh medium to maintain a constant volume. Samples were filtered and RSV concentration was assayed by HPLC method [16].

2.8. Determination of microcapsules stability

The stability of RSV-loaded MCs was evaluated by monitoring RSV retention and chemical stability during storage. MCs in aqueous suspensions were incubated under static conditions at 4 and 25 °C for 6 months. At determined time intervals (3 and 6 months), the suspensions were centrifuged (10,000 rpm, 10 min), supernatant was removed, and MCs were dissolved in methylene chloride. The amount of RSV was detected by HPLC analysis, as described above.

2.9. Statistical analysis

All data are expressed as mean ± Standard Deviation (S.D.) from three replicates. The significance of differences was assessed by one-way analysis of variance (ANOVA) (Origin®, version 7.0 SR0, OriginLab Corporation, USA) followed by Tukey's multiple comparison test and considered significant when $p < 0.05$.

3. Results and discussion

3.1. Formulation of MCs

RSV-loaded MCs were successfully prepared in a stepwise fashion by the W/O/W double emulsion technique [23] using PLGA and CS as polymeric materials and PVA as stabilizer.

In this study, we selected the ethyl acetate as organic solvent instead of the most often used methylene chloride, because the fast diffusion of ethyl acetate from oil phase into outer aqueous phase would benefit rapid solidification of MCs, with consequent improvement of the entrapment efficiency [24].

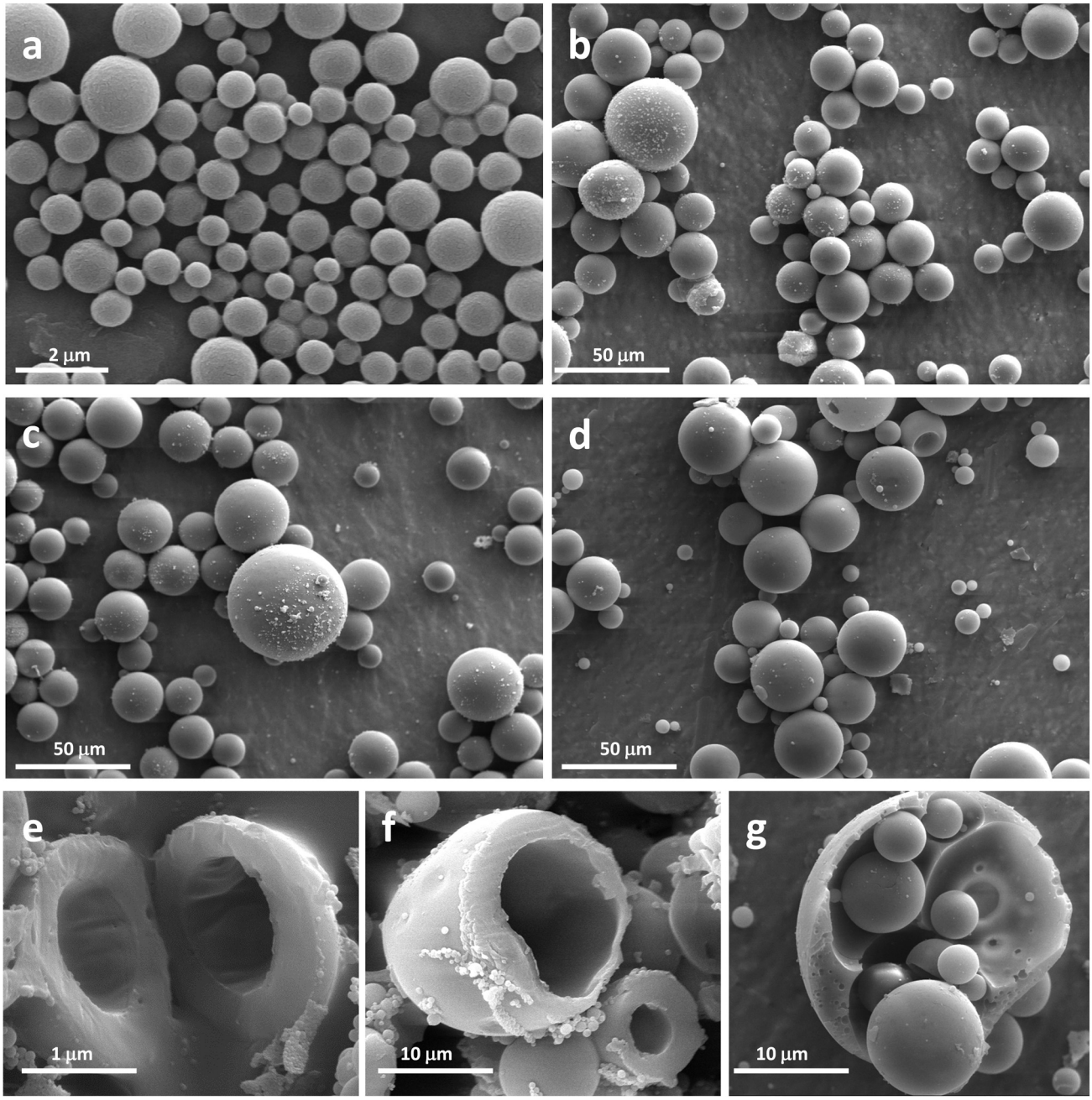

Fig. 1. SEM images of MC-0 (a), MC-1 (b), MC-2 (c), and MC-3 (d), and SEM of cross-section of MC-0 (e), MC-1 (f), and MC-3 (g) at high magnification.

3.2. Size and morphology

As reported in Table 2, MC-0, containing only PLGA, are characterized by the lowest diameter, while the MCs formulated in the presence of CS show a significant increase in size that resulted strongly influenced by CS concentration. Although the mean particle size did not change with CS solution at 1% and 2%, it significantly increased with the CS at 4%. These differences can be mainly attributed to the higher CS concentration in the primary emulsion that determined a higher viscosity of the solution and thus a more difficult dispersion of PLGA with a larger drop size formation [25].

As shown in Fig. 1a–d, all MCs present spherical shape but different morphologies. More specifically, as displayed in Fig. 1e–g, the MCs containing only PLGA are characterized by a single-core and a nonporous shell (Fig. 1e). The formulation in the presence of CS at 1% and 2% did not produce significant changes on the MCs morphology (Fig. 1f), suggesting that possible electrostatic interactions between the protonated amino groups of CS and carboxylic end groups of PLGA could be established and, after removal of the solvent, the polymeric mixture precipitate forming the shell of microcapsules [26]. It is worth noting that a multi-cores structure with some internal capsules into a bigger one's characterizes the MC-3 batches (Fig. 1g). We suppose that, in this case, the viscosity inside the emulsion droplets should increase faster during the solvent evaporation, thus promoting an aggregation of MCs that, after complete removal of organic solvent, would remain embedded in the shell of the final multiple core-shell microcapsule [25,27].

3.3. Encapsulation efficiency

As shown in Table 2, the encapsulation efficiency of MC-0 resulted only 9%, suggesting that PLGA did not ensure a good encapsulation of RSV. On the other hand, the formulation of CS/PLGA MCs determined a significant increase of RSV loading capacity, with encapsulation efficiency values that ranged from 41% for MC-1 to 52.5% for MC-3, respectively.

As observed in our previous study, the improvement of RSV encapsulation efficiencies can be related to the presence of hydrophilic phenolic hydroxyl groups of RSV that can establish extensive hydrogen bonding, promoting the interaction with CS [16]. Moreover, this behavior is consistent with the enhanced RSV loading capacity observed with increasing CS concentration. Interestingly, the formulation of RSV into MCs significantly improved the encapsulation efficiencies with respect to maximum 32% previously observed with nanoparticles produced by nanoprecipitation method [16].

Finally, the W/O/W double emulsion method provided good yield of productions that ranged from 51 to 57%.

3.4. Raman spectroscopy

Raman spectra of pure PLGA, CS, and RSV, of drug-loaded PLGA, and CS/PLGA MCs, and of the physical mixture of PLGA/RSV, and PLGA/CS/RSV are presented in Fig. 2. The PLGA spectrum shows peaks at 1770 cm^{-1}, 1130 cm^{-1} and 1044 cm^{-1} due to the C=O stretching vibrations. Furthermore, the C–H bending and stretching vibrations are observed at 1453 cm^{-1} and 870 cm^{-1}, respectively. In the spectrum of CS, the strong band corresponding to stretching vibrations of amide bond is confirmed at 1380 cm^{-1}. The peaks between 1090 and 1200 cm^{-1} are characteristic of C–O stretch of –CH–OH in cyclic alcohols and of $-CH_2-OH$ in primary alcohols, respectively [28]. The spectrum of RSV shows the characteristic peaks of olefinic band at 996 cm^{-1}, C–O stretching at 1160 cm^{-1}, and C–C aromatic double-bond stretching at 1600–1630 cm^{-1}. The intense peaks of RSV at 1600–1630 cm^{-1} are evident in MC-0 and MC-3, and support the loading of RSV into MCs.

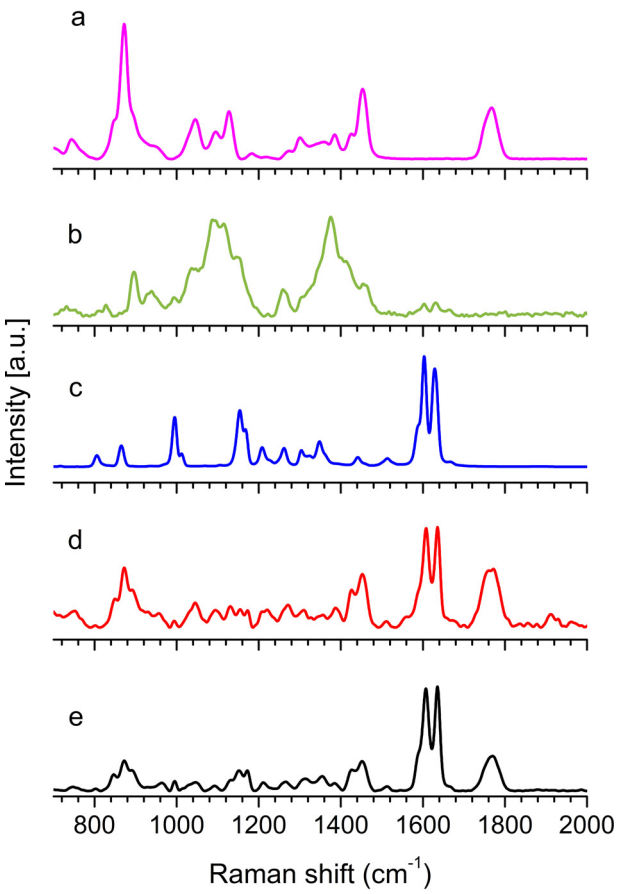

Fig. 2. Raman spectra of PLGA (a), CS (b), RSV (c) raw materials, and MC-3 (d), and MC-0 (e), reported as examples.

3.5. Differential scanning calorimetry (DSC)

DSC analysis is a powerful technique that provides information regarding the physical properties like crystalline or amorphous nature of the samples [29]. The DSC thermograms of pure RSV, PLGA, CS, on drug-loaded PLGA, and CS/PLGA MCs, and on the physical mixture of PLGA/RSV, and PLGA/CS/RSV are presented in Fig. 3. The DSC scans of pure polymers exhibited the endothermic decomposition peak centered at 373 °C and the exothermic peak at 320 °C, corresponding to PLGA and CS thermal decomposition, respectively [16,30].

The DSC curve of RSV showed the characteristic endothermic peak at 270 °C, corresponding to its melting temperature, that disappeared in the DSC scans of RSV-loaded MCs (curves d and f), suggesting that it is present in the amorphous phase and may have been homogeneously dispersed in the polymer matrix [31]. According to previous observations, the scans of MC-3 and PLGA/CS/RSV mixture (curves f and g, respectively) shared a considerable shifting of the endothermic decomposition peak of PLGA from 373 °C to 318 °C that is indicative of intermolecular interactions formed between CS and PLGA [32].

3.6. In vitro release studies

The development of delivery systems able to protect RSV during its transit inside the organism after oral administration is important to preserve its pharmacological properties [33].

Table 2
Mean diameter (μm), encapsulation efficiencies (EE), RSV loading content (DLC), and yields of production (YP) of prepared MCs.

Formulation	Size (μm)	EE (%)	DLC (%)	YP (%)
MC-0	0.84 ± 0.29	9.22 ± 1.25	0.09 ± 0.01	56.63 ± 1.90
MC-1	12.56 ± 2.43*	40.99 ± 0.57*,§	0.41 ± 0.01*,§	52.16 ± 1.62*
MC-2	11.80 ± 2.39*	46.96 ± 1.24*,§	0.47 ± 0.01*,§	53.81 ± 1.94
MC-3	19.79 ± 4.36*,§	52.51 ± 1.83*,§	0.53 ± 0.02*,§	51.06 ± 1.06*

* Significantly different from the MC-0.
§ Significantly different from other MCs containing CS.

The potential capability of prepared MCs to control the RSV release as useful strategy to improve its bioavailability was evaluated in simulated gastrointestinal fluids. The cumulative percentage of RSV released from MCs as a function of time is reported in Fig. 4. It can be seen that the MC-0 batch showed the fastest dissolution rate, with about 70% of RSV released within the first 2 h and 100% over a period of 4 h. On the other hand, a prolonged RSV release was found for all the CS/PLGA MCs, with a different dissolution rate that was mostly dependent on the amount of CS used for the formulation. The enhanced release rate of MC-0 with respect to CS/PLGA batches could be mainly attributed to the increased specific surface area caused by great reduction of particle size [34]. Regarding MCs formulated with CS, a nearly superimposed release profile was obtained from MC-1 and MC-2 containing 1% and 2% of CS, respectively, with about 55% of RSV released in acidic medium, and their total RSV content within the following 5 h at pH 7.4. Conversely, the larger amount of CS in the MC-3 leads to a significant decrease in the amount of RSV released, with only 40% dissolved within the first two hours and a retarded dissolution rate in the intestinal simulated fluid. This finding could be related to a multicore structure of MC-3 that slow down the diffusion rate of the encapsulated RSV molecules.

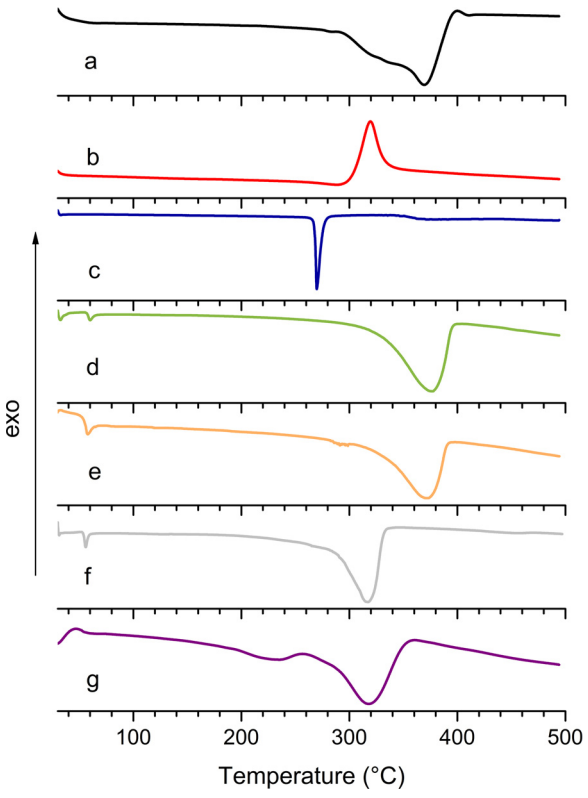

Fig. 3. DSC scans of PLGA (a), CS (b), RSV (c) raw materials, MC-0 (d), PLGA/RSV mixture (e), MC-3 (f), and PLGA/CS/RSV mixture (g).

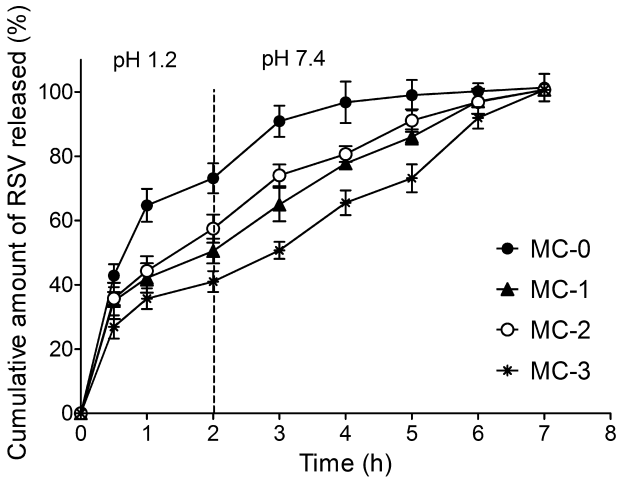

Fig. 4. In vitro release profiles of RSV from MCs performed at 37 °C for 2 h in 0.1N HCl (pH 1.2), followed by PBS at pH 7.4. Data are means ± SD, $n = 3$.

3.7. *Stability test*

In order to investigate the retention and chemical stability of microencapsulated RSV, the effects of storage time (3 and 6 months) and storage temperature (4 and 25 °C) were evaluated.

As reported in Fig. S1a, all MCs showed a good stability for a period of 3 months both at 4 and 25 °C, without any significant change in the RSV retention. As indicated in Fig. S1b, as the storage time increases the percentage of RSV retained at 25 °C was found to be much more decreased (88%) for MC-0 compared to other formulations. The major cause of RSV loss from MC-0 could be due to its dissolution into supernatant that was subsequently removed during the test. Our results suggested that the CS provides greater protection to the MCs during long term storage. It is worth noting that the UV–visible spectra analysis of all samples revealed the presence of biologically more active RSV *trans* isoform, without the appearance of the peak absorption of *cis* isomer (data not shown), thus showing that MCs are able to prevent the RSV degradation.

4. Conclusions

In conclusion, the nutraceutical RSV can be successfully encapsulated by W/O/W double emulsion technique into CS/PLGA microcapsules. Interestingly, the CS concentration influences the size and morphology of MCs, as well as the encapsulation efficiencies and the RSV release rate in simulated gastrointestinal conditions. Furthermore, all the tested MCs were able to ensure a good long term storage stability of RSV. Among the tested MCs, those obtained using 4% w/w of CS are emerged as promising formulations for further development, because of the better encapsulation capacity and the most effective controlled release of RSV.

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