Zein-based colloidal particles for encapsulation and delivery of epigallocatechin gallate

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Abstract

Zein, a water insoluble plant protein from a renewable natural source, has been recently exploited in the production of protein-based colloidal particles for the encapsulation of lipophilic compounds. However, the encapsulation of water-soluble bioactive molecules, such as epigallocatechin gallate (EGCG), is strongly limited by the weak physical interactions occurring with the zein matrix during the precipitation phase. We demonstrate that the use of sodium caseinate, as colloidal stabilizer, enables the development of novel structured colloidal particles with peculiar properties: (a) the encapsulation efficiency of EGCG is significantly increased with respect to zein alone; (b) EGCG is highly bioaccessible and can fully express its antioxidant activity, likely because of its localization prevalently on the outer layer of the structured particles; (c) a tunable release rate of EGCG is observed during in vitro digestion, which is controlled by the particle composition; (d) the Pickering stabilization effect on emulsions, combined with EGCG interaction with lipase enzyme, provides an interesting way to modulate the rate of fat digestion. Zein-based, structured colloidal particles constitute remarkable, naturally derived systems for the tunable and multi-functional delivery of EGCG.

Keywords: Zein; Epigallocatechin gallate; Sodium caseinate; Colloidal particles; Antioxidant activity; Fat digestion

1. Introduction

Biopolymeric particles, thanks to their excellent food compatibility and natural origin, have been widely investigated for the encapsulation and delivery of functional compounds in food products (Chen, Remondetto, & Subirade, 2006). However, being water soluble, most of the food or pharma-approved biopolymers require physical or chemical alterations to form colloidal particles that are stable in aqueous systems (Patel & Velikov, 2014). For example, ionic gelation has been exploited to induce the formation of calcium alginate or chitosan particles (Vauthier & Bouchemal, 2009), whereas hydrophobic modifications enable the synthesis of sodium alginate particles (Yao, Ni, Xiong, Zhu, & Huang, 2010). Such spontaneous complexation of macromolecules with phenolic compounds has been exploited not only to modulate polymer functionality (Patel, Seijen ten-Hoorn, Hazekamp, Blijdenstein, & Velikov, 2013), but also to drive the formation of colloidal particles.(Patel, Seijen-ten-Hoorn, & Velikov, 2011; Ashok R Patel, Nijsse, & Velikov, 2011).

Recently, zein, the main storage protein of corn, has become an extremely attractive delivery system for functional ingredients in food applications, not only because of its fully natural, biodegradable and food-grade origin, but also because it exhibits spontaneous molecular interactions with a large number of hydrophilic compounds (Davidov-Pardo, Joye, & McClements, 2015; Patel, Heussen, Hazekamp, Drost, & Velikov, 2012; Patel, Hu, Tiwari, & Velikov, 2010; Patel & Velikov, 2014; Wu, Luo, & Wang, 2012). Zein is one of the few hydrophobic, water-insoluble materials approved for food use by the FDA (Patel & Velikov, 2014). All these features have fundamental implications on the simplicity of generating colloidal particles, as well as on the ease of loading them with functional ingredients. Moreover, owing to its resistance to digestive enzymes, zein particles are in general characterized by slower digestibility in the gastrointestinal tract than other proteins, and consequently by enhanced capability of controlled release of the payload molecules (Patel & Velikov, 2014).

A well-established facile and versatile method of fabrication of zein colloidal particles is based on the co-dissolution of zein and of the non-polar hydrophobic payload compounds in an aqueous ethanol solution (60-90 wt. %), followed by rapid dilution with water. Dilution brings the system outside the solubility limits of both zein and of the payload compounds, which hence co-precipitate (Patel et al., 2012). In the case of polar, hydrophilic ingredients, however, which do not precipitate upon water dilution, it is not yet clear from published literature results how effectively they can be encapsulated in zein colloidal particles.

Among hydrophilic bioactive compounds, epigallocatechin gallate (EGCG) has attracted increasing attention in recent years, because of its significant health beneficial properties (Singh, Shankar, & Srivastava, 2011), associated with the specific capability to slow down fat digestion (Koo & Noh, 2007). Different studies have addressed the issues related to its encapsulation and controlled delivery and release in the gastrointestinal tract (Guri, Haratifar, & Corredig, 2014; Lestringant, Guri, Gülseren, Relkin, & Corredig, 2014). However, to date, not many studies have addressed EGCG encapsulation in zein-based carriers; the only available literature reference on the topic describes in fact the stabilization and release of EGCG from electrospun zein fibers (Li, Lim, & Kakuda, 2009).

The main idea behind this work is the development of zein-based composite colloidal particles for EGCG encapsulation, which enable high EGCG bioaccessibility, to explicate its activity, and tunable EGCG release during in vitro digestion, with the final goal of assessing their potential use in the modulation of fat digestion rate.

2. Materials and Methods

2.1 Materials

Green tea extracts containing minimum 90% Epigallocatechin gallate (EGCG, Mol. Wt. 458.4 g/mol) as per supplier's claim (TEAVIGO™) were obtained from DSM, The Netherlands. Zein, sodium caseinate, gum arabic (food grade) and maltodextrins (dextrose equivalent 4.0-7.0), all food grade, were purchased from Sigma Aldrich Inc., USA. HCl (1 N) and NaOH (1 N) solutions were purchased from Merck Co., Germany. Absolute ethanol was obtained from VWRBDH Chemicals, U.K. Reagents and buffer salts for lipolysis studies including pepsin (600 U/mg, Sigma 77160), pancreatin (8× USP, Sigma P7545), bile extracts (Sigma B8631), Tris(hydroxymethyl)aminomethane hydrochloride (TRIS-HCl), Intralipid® 20% (Phospholipid stabilized soybean oil emulsion), NaCl, and $CaCl_2$ were purchased from Sigma Aldrich Inc., USA. 2,4,6-Tripyridyl-s-triazine, ferric chloride hexahydrate, ascorbic acid, Folin–Ciocalteu's phenol reagent, were also purchased from Sigma Aldrich Inc., USA. Sodium carbonate, sodium acetate and hydrochloric acid were purchased from Merck KGaA, Germany. Water purified by a Milli-Q system was used for all experiments.

2.2. Preparation of colloidal particles

Colloidal particles were prepared using a modified method based on zein antisolvent precipitation (Patel, Bouwens, & Velikov, 2010). In this work, three different stock solutions were prepared, based on an ethanol/water binary solvent (80:20 w/w). The first solution contained 25 mg/g zein, the second solution 2 mg/g EGCG, and the third solution consisted of the pure solvent. 10 mL of the first stock solution were mixed with 15 mL of a mixture in different proportions of the remaining stock solutions, to obtain EGCG/zein ratios ranging from 0.0 to 0.5 g/g, at fixed zein concentration (10 mg/g). Subsequently, the solutions were rapidly

poured in 75 mL of Milli-Q water, pure or containing sodium caseinate (NaCas, 2.5 mg/g final concentration), under continuous stirring (1000 rpm) using a magnetic stirrer (model EM3300T, Labotech Inc., Germany). The dispersions thus formed were subjected to ethanol removal under reduced pressure (50 ± 5 mbar, 35 °C) using a rotary evaporator (Rotavapor R-114, Buchi, Switzerland). By different combinations of the stock solutions and use of pure water or an aqueous NaCas solution as antisolvent, pure zein colloidal particles (ZP), colloidal particles of zein co-precipitated with EGCG (EGCG@ZP), composite particles of zein stabilized by NaCas (NC-ZP) and composite particles of zein co-precipitated with EGCG and stabilized by NaCas (EGCG@NC-ZP) were obtained. The final dispersions were then stored at 4 °C until used for further analytical characterization. The pH of the resulting dispersion (ZP and EGCG@ZP) was 4.0 ± 0.1, whereas dispersions NC-ZP and EGCG@NC-ZP had pH = 6.5, due to the buffering activity of NaCas.

To obtain powder samples, dispersions were spray dried upon mixing with maltodextrins (MD) alone and in combination with gum arabic (GA), which were used as carrier materials, as previously described in the preparation of fully water-soluble particles to deliver catechins (Peres et al., 2011). When used alone, MD was added to the zein particles dispersion up to a weight ratio MD:zein = 4:1, which corresponds to a final MD concentration of 1.3 mg/g (ZP+MD). When used in combination, GA and MD were added to the zein particles at the same final concentration of 1.3 mg/g and were dosed in a weight proportion of MD:GA = 6:4 (ZP+GA/MD). In the case of zein particles stabilized with NaCas, spray dried particles were stabilized with MD alone (NC-ZP+MD). As a reference, free EGCG was mixed with a combination of GA and MD (GA/MD), at the same EGCG, GA and MD concentration of ZP+GA/MD. Drying was carried out using a Buchi B-290 Mini Spray Dryer, operating with a solvent trap at T = -22 °C. A peristaltic pump at 5 mL/min fed the 1.5 mm two-fluid nozzle having an atomizing pressure of 2 bars. The inlet (controlled) and outlet (measured) air

temperatures were T_{in} = 165 °C and T_{out} = 90-81 °C, respectively. The spray-dried powder was collected in the cyclone (typical yield of 20-35%) and stored at ambient conditions until further analysis.

2.3. Analysis of Particle Size and Surface Potential

The mean particle size or z-average diameter d_H, the polydispersity index PdI and ζ-potential of undiluted dispersions were measured by dynamic light scattering (DLS) and electrophoretic mobility using a Zetasizer Nano (Malvern Instruments Ltd., UK). All measurements were carried out at 25 °C, and the results reported are the average of three readings.

2.4. Transmission Electron Microscopy (TEM)

The shape of formed particles was analyzed by taking TEM photographs using a Tecnai20 transmission electron microscope (FEI Co., The Netherlands). The particles were dispersed in medium (i.e., Milli-Q water at appropriate pH), and one drop of the diluted dispersion was placed on a 200-mesh carbon-coated copper grid and left to dry. The surface of the grid was made hydrophilic by glow discharge to assure complete wetting. The photographs were taken at various magnifications and 100 kV voltage.

2.5. Encapsulation efficiency

The encapsulation efficiency (EE) of EGCG in zein colloidal particles was determined using equation 1:

$$EE = 100 \times (1 - EGCG_F/EGCG_E) \quad (1)$$

In which $EGCG_E$ is the total amount of EGCG contained in the suspension and $EGCG_F$ is the amount of free EGCG. The free EGCG was assessed by determining the amount of EGCG that was collected in the filtrate receiver after being centrifuged at 10,000 g for 10 min (Micro Centrifuge 5415C, Eppendorf, US) using centrifugal filters (Ultrafree®-M 10,000 NMWL Filter Unit, Millipore, Cork Ireland) (Davidov-Pardo et al., 2015; Luo, Zhang, Whent, Yu, & Wang, 2011). The recovery yield of EGCG after passing through the filter (88.2%) was taken into account to correct the actual amount of EGCG in the filtrate. The EGCG contained in the particles, and the filtrate was assessed by diluting the suspension in ethanol during ultrasonication (Patel et al., 2011) before quantification as described in Section 2.6. The suspensions without EGCG were used as blank.

2.6. EGCG quantification

When encapsulated in zein particles, EGCG was preliminarily extracted by dispersing the colloidal particles in ethanol and sonicating for 10 min. The suspension was then centrifuged at 10,000 g for 10 min (Micro Centrifuge 5415C, Eppendorf, US). For free EGCG, this step was skipped. The concentration of EGCG in the supernatant was then measured using the modified method developed in-house based on Folin and Denis (Patel et al., 2011). A reaction mixture was prepared with 0.5 mL sample solution, 2.0 mL deionized water, and 0.5 mL Folin–Ciocalteu's phenol reagent (2.5% sodium tungstate and 0.5% phosphomolybdate in 1.25% phosphoric acid), and was allowed to stand for 10 min at room temperature. The reaction mixture was then mixed with 2.0 mL of 20% w/v sodium carbonate and allowed to stand for 60 min at room temperature. The absorbance of the green–blue solution thus formed was read at 765 nm using UVIKON XL UV–Vis spectrophotometer (SECOMAM, France). A standard curve of EGCG prepared at the concentration of 20–180 µg/mL was used to quantify the amount of EGCG.

2.7. Antioxidant activity

Ferric reducing antioxidant power (FRAP) was used for determining the antioxidant properties of polyphenols (Patel et al., 2011). The procedure followed for FRAP assay was as follows: the working FRAP reagent was freshly prepared by mixing 2.5 mL of 20 mM $FeCl_3 \cdot 6H_2O$, 2.5 mL of 10 mM 2,4,6-tripyridyl-s-triazine in 40 mM HCl, and 25 mL of 300 mM acetate buffer (pH = 3.6), and warmed to 37 °C prior to use. A sample (50 µL) was mixed with 1 mL of working FRAP reagent and the absorbance (read at 593 nm) was measured after 20 min of incubation at 37 °C and centrifugation at 14,000 g for 10 min (Micro Centrifuge 5415C, Eppendorf, US) to remove the interference of suspended zein particles, using a UVIKON XL UV–Vis spectrophotometer (SECOMAM, France). A similar procedure was followed for the EGCG filtrated by centrifugal filters, as described in Section 2.5, skipping the centrifugation part and ensuring the same incubation time and temperature. Ascorbic acid standards (100–1000 µM) were processed in the same way, and antioxidant activity was reported as FRAP value (µM of ascorbic acid equivalent).

Antioxidant activity was also monitored over time when dispersing EGCG pure or encapsulated in zein particles, in simulated gastric fluid (SGF, 0.1 M HCl at pH = 1.2) and simulated intestinal fluid (SIF, phosphate buffer at pH = 7.4), and incubating at 37 °C in a multipoint stirrer (VARIOMAG, The Netherlands) equipped with a water bath (Janke and Kunkel Ika Labortechnik, The Netherlands).

2.8. EGCG release upon simulated digestion

Simulated digestion conditions consisted of gastric and intestinal digestion while mouth conditions were skipped because of the finely dispersed nature of the EGCG carriers. For

simulated gastric digestion, a carefully weighted sample of EGCG dried powder was mixed with 10 mL pepsin-HCl solution (2.5 mg/mL pepsin in 0.05M HCl) in a 50 mL centrifuge tube, containing five 1 cm^3-glass beads. The samples were incubated in a shaking (100 rpm) water bath at 37 °C for 120 min. Aliquots of the digestion mixture were collected for analysis at 15, 30, 60, 90 and 120 min.

After 120 min, the solution was neutralized with 1 mL 0.5 M NaOH, and 5 mL of bile solution (30 mg/mL bile extract in 0.1 M PBS buffer at pH = 6.5) and 5 mL of pancreatin solution (2.5 mg/mL pancreatin in 0.1 M buffer) were added. The samples were hence incubated in a shaking (100 rpm) water bath at 37 °C for 120 min. Aliquots of the digestion mixture were collected for analysis at 30, 60, 90 and 120 min. The two different stages of digestion (gastric and intestinal) were stopped by snap freezing with dry ice.

2.9. Simulated fat digestion

A 10× TRIS-HCl buffer solution at pH=7.5 was initially prepared by mixing 0.005 M TRIS-HCl, 0.02 M $CaCl_2 \cdot 2H_2O$ and 0.04 M NaCl in Milli-Q water (electrolyte solution). Subsequently, 250 mg bile extracts were dissolved in 5 mL 1× TRIS-HCl buffer by continuously magnetic stirring for 20-30 minutes in a water bath at 37 °C. The jacketed agitated vessel of the auto-titration system was then loaded with 3 mL of 20% Intralipid emulsion, 2.5 mL of 10× TRIS-HCl, 4.5 mL of bile extract solution, 15 mL of EGCG-containing sample (or Milli-Q water for reference), and 3 mL of Milli-Q water. The titration solution consisted of 0.1 M NaOH. The system was pre-titrated to 7.4, and then the experiments were started by adding 11.3 mg of pancreatin in 2 mL of 1× TRIS-HCl, mixing well with a pipette. The auto-titration system registered the volume of NaOH injected into the vessel over time to regulate the pH to 7.4, which was reduced by the progressive emulsion fat lipolysis.

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223 **2.10. Data analysis**

224 All experiments were performed in triplicate, and the results are given as mean values ±
225 standard deviation. EzANOVA software was used to perform one-way Analysis of Variance
226 (ANOVA) tests followed by the Tukey method, with the overall significance level set at 0.05.

227

3. Results and discussion

3.1. Morphology of zein particles loaded with EGCG

EGCG was co-precipitated with zein by an anti-solvent method based on the fast addition of the binary solvent to water (ZP) or an aqueous solution containing NaCas (NC-ZP). The mean particle size of pure zein colloidal particles was ~100 nm, with a positive surface ζ-potential = +30 mV measured at pH = 4.0. Addition of NaCas in the antisolvent aqueous phase caused a measurable increase in the final mean particle size (~190 nm), as well as a shift in the surface charge from positive to negative (ζ-potential = -30 mV), in agreement with previous observations (Davidov-Pardo et al., 2015; Patel, Bouwens, et al., 2010).

Figure 1 shows the appearance of the different colloidal suspensions prepared (either plain particles or loaded with EGCG), as well as the TEM images of the various particle morphologies. Zein, upon precipitation from aqueous ethanol in water, forms spherical, smooth particles (Patel, Hu, et al., 2010), according to mechanisms mainly influenced by nucleation and diffusion controlled Ostwald ripening (Patel, Heussen, Dorst, Hazekamp, & Velikov, 2013; Sitnikova, Sprik, Wegdam, & Eiser, 2005). TEM images (Figure 1) confirm that ZP particles are small, round and smooth, but also shows that they tend to aggregate easily during the drying process required for TEM sample preparation. This tendency to aggregate, which can be ascribed to the well-known zein inter-particle hydrophobic attraction (Davidov-Pardo et al., 2015), can also be noticed through the slow but relentless sedimentation in ZP dispersions, with the formation of a thin layer of particles at the bottom of the flask after several weeks of storage.

When NaCas is added to the antisolvent phase, the tendency of the particles to aggregate was significantly reduced. This observation is supported both by the TEM images, which shows mainly individual, detached particles and by macroscopic stability, with no measurable sedimentation occurring over several months of refrigerated storage. TEM images also suggest that NaCas mainly deposits around the zein particles after their formation (Fig. 1), contributing

to developing a core-shell type of structure. Despite the observed NaCas coating, the layer could still be an artifact created by the drying phase in sample preparation, as previously discussed for SEM images of similar particles (Davidov-Pardo et al., 2015). Other experimental evidence, such as the measured increase in mean particle size, as well as the ζ-potential reversal upon NaCas addition, supports the hypothesis of a core-shell particle architecture.

In general, the increase in viscosity induced by the addition of a biopolymeric additive such as NaCas to the antisolvent phase, is responsible for a reduction in molecular transport rate, collision frequency and hence coagulation and agglomeration of the precipitating particles (Joye & McClements, 2013), resulting in a final smaller particle size. However, DLS measurements show that NC-ZP particles are characterized by a twice larger mean particle size than ZP particles. TEM images show that the zein core of NC-ZP particles is not significantly smaller than in ZP particles. Finally, ζ-potential measurements as a function of pH of NaCas alone and of ZP and NC-ZP particles (Figure S1.1 in Supplementary Material), show that the isoelectric point of NC-ZP particles is close to that of NaCas alone (around a pH of 4.5), and quite different from ZP (in the pH range of 6.5-7.0). Therefore, in agreement with previous work (Patel, Bouwens, et al., 2010) and sustained by several experimental pieces of evidence, we can safely consider that in NC-ZP particles, a NaCas layer wraps a zein core, contributing to its electrostatic and steric stabilization of the composite particles.

Figure 1 also shows that EGCG loading does not affect the particle morphology of ZP and NC-ZP particles. Previous results have shown that the loading of zein particles of water-insoluble molecules, such as quercetin or curcumin, with which zein has been reported to have strong hydrophobic interactions, does not cause any significant effect on morphology (Patel et al., 2012; Patel, Hu, et al., 2010). However, the extent of molecular and physical interactions with both zein and NaCas are critical in determining the extent of encapsulation efficiency of water-soluble molecules, such as EGCG.

TEM images can not give any supporting information about such interactions; therefore, additional measurements, discussed in details in the Supplementary Material, have been carried out. EGCG scarcely interacts with zein (as from UV-Vis, DLS and nanoDSC measurements in Figure S2.1, Figure S2.2, and Figure S3.1), whereas it exhibits significantly stronger interactions with NaCas, as evident from nanoDSC measurements (Figure S1.1). Previous studies have shown that NaCas can form complexes with EGCG at interfaces and that such interfaces can load high ratios of EGCG to protein (Sabouri, Geng, & Corredig, 2015). In particular, the complex formed did not affect stability, size, and charge of emulsion particles stabilized by the protein, as no differences in the amount of NaCas adsorbed at the interface were observed, although the complex formed affected the viscoelastic properties of the protein layer formed at the interface (Sabouri et al., 2015).

3.2. Effect of EGCG loading

The data reported in Figure 2, where mean particle size, polydispersity index and ζ-potential are reported as a function of EGCG loading for EGCG/zein ratios in the range of 0-0.5 g/g, further confirm the limited effect of EGCG addition to zein-base particles, as discussed in Section 3.1. The fast anti-solvent precipitation method used resulted in fine particles with similar, monomodal distributions (d_H < 100 nm, PdI < 0.25), and ζ-potential (comprised between +25 and +30 mV). The anti-solvent precipitation in NaCas aqueous solution represents a facile and reproducible process for the preparation of zein particles with an oppositely charged biopolymeric layer coating their surface. These particles exhibited a mean particle size comprised between 170 and 250 nm, larger than what is observed for ZP, a PdI in general below 0.15 when loaded with EGCG, and ζ-potentials between -42 and -30 mV.

The limited effect of EGCG on size or ζ-potential of ZP suggests that EGCG interacts with zein only marginally. In particular, additional measurements, discussed in details in the

Supplementary Material, show that the addition of EGCG to zein in the binary solvent, before the precipitation step, does not have any significant impact on the mean size of zein self-associated molecules (Figure S2.2).

Additionally, the spectrum of the zein-EGCG mixture in the binary solvent merely results from the superimposition of the spectrum of zein and EGCG (UV-Vis and DLS measurements in Figure S2.1 and Figure S2.2). NanoDSC thermograms on the aqueous suspensions containing ZP colloidal particles (Figure S3.1) also show no significant changes upon addition of EGCG. In contrast, the addition of EGCG to NC-ZP particles caused some changes in the nanoDSC thermogram, which can be related to the well-known interactions between EGCG and NaCas. In particular, previous studies have shown that the complexes formed by caseinate and EGCG at interfaces, despite the high EGCG loading capability (Sabouri et al., 2015), did not affect stability, size, and charge of emulsion particles stabilized by the protein, but affected only the viscoelastic properties of the protein layer formed at the interface (Sabouri et al., 2015). This aspect is important to consider when analyzing the interfacial antioxidant activity of NC-ZP particles.

3.3. Encapsulation efficiency of EGCG and antioxidant activity

On the basis of the results reported in Section 3.2, it is questionable if zein particles can efficiently encapsulate a hydrophilic compound such as EGCG, with which they have limited chemical interactions, and which does not phase-separate upon water dilution to foster its physical entrapment.

Therefore, specific experiments were designed to understand the contribution of the precipitation stage on EGCG encapsulation, and on the resulting antioxidant activity. In particular, EGCG was added to ZP or NC-ZP particles either before or after precipitation. In the first case, EGCG was mixed with zein in the binary solvent (EGCG added *before*

328 *precipitation*), whereas in the second case, EGCG was added at the same final concentration of
329 the aqueous suspension containing the formed colloidal particles (EGCG added *after*
330 *precipitation*). In both cases, the EGCG/zein ratio was 0.12 g/g.
331 The different systems were characterized in terms of encapsulation efficiency and antioxidant
332 activity, with the results being shown in Table 1.
333 Encapsulation efficiency is calculated by measuring the concentration of EGCG in the filtrate
334 compartment of centrifuge filters, and correcting this concentration by the recovery yield of
335 pure EGCG upon filtration (88.2 %). The concept of encapsulation efficiency has been
336 consciously used in a broader sense also when EGCG is added *after precipitation*, despite
337 EGCG can only adsorb onto the surface of ZP or NC-ZP particles, and is not physically
338 encapsulated inside the colloidal particles.
339 When EGCG is added *before precipitation*, its encapsulation efficiency in ZP is 37 %, which
340 is significantly lower ($p \leq 0.05$) than in NC-ZP (46 %). However, both these values are lower
341 than the values reported for hydrophobic, water-insoluble compounds, such as curcumin
342 (between 70 and 95 %) (Patel, Hu, et al., 2010).
343 In particular, the addition of a biopolymer in the antisolvent has already been reported to have
344 a positive effect on encapsulation of compounds such as resveratrol, with an increase from 65
345 and 85 % following the addition of NaCas (Davidov-Pardo et al., 2015), not only through the
346 promotion of the physical entrapment of the payload molecules in the zein matrix, but also
347 through the formation of complexes with them, either at the interfaces, or dispersed within the
348 continuous phase.
349 Interestingly, when EGCG is added *after precipitation*, the measured encapsulation efficiency
350 in ZP and NC-ZP particles (17 %) is significantly lower ($p \leq 0.05$), than the encapsulation
351 efficiency for EGCG added *before precipitation*. In the latter case, the "encapsulation effect"

is a result of some adsorption of EGCG on the surface of the colloidal particles, giving rise to a slight decrease in the bulk concentration.

Table 1. Encapsulation efficiency of EGCG and its antioxidant activity. *Encapsulation efficiency and antioxidant activity (FRAP assay) are reported for ZP and NC-ZP particles in the presence of EGCG, added either before or after precipitation. For comparison, the FRAP value of free EGCG is reported at the same concentration as in particles. EGCG/zein ratio is 0.12 g/g.*

	Sample	Encapsulation efficiency (%)	Ascorbic acid equivalent (μM)	
			FRAP of filtrate	FRAP of colloidal particles
	EGCG	-	2189.1±28.2[a]	-
EGCG before precipitation	ZP	36.6±5.7[b]	1708.2±52.1[d]	1784.4±147.4[d]
	NC-ZP	46.3±4.6[a]	1459.5±14.3[e]	1727.2±35.6[d]
EGCG after precipitation	ZP	17.4±5.8[c]	2205.5±38.0[a]	2129.7±31.1[a,b]
	NC-ZP	17.0±2.5[c]	1942.1±26.6[c]	2070.2±42.9[b]

Values are means ± standard deviations (n = 3). Means with different uppercase letters within the same type of measurement (Encapsulation efficiency or FRAP) are significantly different (P ≤ 0.05).

The EGCG antioxidant activity (FRAP assay) has been measured both on the filtrate of centrifuge filters and directly on the colloidal suspension (followed by centrifugation to remove the interference of suspended particles on UV-Vis reading). In the first case, the FRAP value derives only from the activity of free, unbound or molecularly bound EGCG. In the second case, it corresponds to the combined activity of unbound EGCG and of that fraction of EGCG

adsorbed onto the particle surfaces or encapsulated in the particle matrix, which in both cases is still bioaccessible and able to induce the reduction of the Fe(III)-TPTZ complex. In particular, the methodology proposed for this last case has been previously tested and validated to characterize the surface antioxidant activity of bioactive compounds physically or chemically bound to particles (Wang et al., 2014).

Therefore, the difference between the FRAP value for EGCG added *after precipitation* and the encapsulation efficiency for EGCG addition *before precipitation* can be considered as the fraction of EGCG, which has been physically entrapped in the zein particles during the antisolvent precipitation process. The results (Table 1) show that for EGCG added *after precipitation* to ZP particles, the antioxidant activity is not significantly different ($p > 0.05$) from free reference EGCG at the same concentration, both in the filtrate and in the colloidal suspension. This result can be linked to the pH changes the sample undergoes during the measurement. The pH of the ZP suspension is 4.5 whereas the NC-ZP suspension has a pH = 6.5.

When EGCG is added after precipitation to NC-ZP particles, the antioxidant activity of the filtrate is significantly reduced ($p \leq 0.05$) with respect to free reference EGCG, likely due to EGCG complexation with the NaCas layer. However, the formation of complexes with NaCas does not prevent EGCG from expressing surface antioxidant activity, with FRAP overall values significantly higher ($p \leq 0.05$) than when measured in the filtrate.

When EGCG is added *before precipitation*, the FRAP values measured on the filtrate and the colloidal suspension do not differ significantly ($p > 0.05$), suggesting that the fraction of EGCG encapsulated inside the zein core is not able to express any antioxidant activity.

In contrast, in the case of EGCG encapsulated in NC-ZP particles, the FRAP value of the filtrate is significantly lower than in the colloidal suspension ($p \leq 0.05$), because of the significant contribution of surface EGCG. Remarkably, the FRAP values of the colloidal suspensions of

ZP and NC-ZP particles are not significantly different ($p > 0.05$), supporting the hypothesis not only that the observed difference in encapsulation efficiency can be ascribed to EGCG adsorbing onto the NaCas layer, but also that this EGCG fraction remains bioaccessible and active.

3.4. EGCG release during simulated digestion

The pH of the system often plays a significant role in the release and bioaccessibility of encapsulated compounds. Therefore, before investigating their behavior during simulated digestions, the surface antioxidant activity of ZP and NC-ZP particles loaded with EGCG was characterized at different pH values over time. The experiments were carried out by diluting (ratio 1:4 v/v) the zein-based colloidal particles in 0.1 M HCl (pH = 1.0) or in a phosphate buffer solution (pH = 7.4). The results, reported in Figure 3, show that at pH = 1.0, where free EGCG is chemically stable (Zhu, Zhang, Tsang, Huang, & Chen, 1997), the FRAP values of both particles do not change significantly over time, and only for ZP particles a slight increase can be observed after 5 hours, which suggest a moderate release of EGCG.

In contrast, at pH = 7.4, which is just past the zein isoelectric point (See Figure S1.1), ZP particles aggregate and precipitate, exhibiting high physical instability, whereas NC-ZP particles remain well dispersed and stable. The measured FRAP values of both free and encapsulated EGCG rapidly decrease over time, as a consequence of the well known EGCG chemical instability at neutral or basic pH. Previous data have shown that after 6 and 12 hours at pH = 7.5, respectively 65 % and 95 % of EGCG is degraded (Hirun & Roach, 2011), which is in agreement with our data. However, other authors indicate even faster degradation rates in sodium phosphate buffer at pH = 7.4, with complete degradation after 4 h (Zhu et al., 1997).

The rapid EGCG degradation hinders any relevant information in EGCG release at pH = 7.4. However, the reduced difference in antioxidant activity between encapsulated EGCG and free

EGCG (initially it is of more than 100 μM ascorbic acid equivalent, and after 60 min at pH = 7.4 it is less than 50 μM ascorbic acid equivalent) suggests the occurrence of some release from both ZP and NC-ZP particles.

To assess more rigorously the EGCG release, the particles were preliminary dried, and EGCG concentration was measured in simulated intestinal fluids. In this case, because of the presence of proteolytic enzymes, some differences might be expected by particles formulation. To carry out spray drying, some additional carriers were added to the particles, such as maltodextrins (MD) alone and in combination with gum arabic (GA), as described in Section 2.2. The resulting dried particles were the reference system GA/MD, based on the combination of maltodextrins and gum arabic, according to a previously tested formulation (Peres et al., 2011), ZP particles dried using as carrier maltodextrins alone (ZP+MD), or a combination of maltodextrins and gum arabic (ZP+GA/MD), and NC-ZP particles using as carrier maltodextrins alone (NC-ZP+MD). The addition of MD does not significantly affect the size or ζ-potential of ZP or NC-ZP particles. In contrast, upon addition of GA, the ZP particles become oppositely charged, with a ζ-potential of -24 mV, because of the electrostatic interactions with zein (data reported in Supplementary Material, Table S4.1).

The reference system (GA/MD) is completely soluble in the aqueous phase, and only biopolymeric micelles are measured by dynamic light scattering. The drying process induces some changes in the used biopolymers, which are revealed by an increase in particle size upon rehydration. In contrast, ζ-potential is not significantly affected after rehydration for all the particles. The GA/MD system, despite still forming a transparent dispersion, exhibits slightly larger bodies, which can be ascribed to partial dissolution. ZP+MD and ZP+GA/MD also exhibit significantly larger mean particle sizes than before drying, which are likely due to zein aggregation during water removal, as well as to a temperature effect. Apparently, the particle size increase becomes more critical in the presence of GA. In the case of NC-ZP+MD, the size

increase is only marginal, thanks to the presence of a NaCas layer, which likely prevents zein aggregation phenomena.

These results are in agreement with previous literature data. Colloidal particles based on hydrophobic polymers, such as zein, often form aggregated lumps on drying, which are difficult to rehydrate into a stable colloidal dispersion. The presence of NaCas has already been shown to improve drying stability, especially in the case of plain zein colloidal particles, which are very difficult to redisperse due to strong aggregation (Patel, Bouwens, et al., 2010). The stabilizing activity of NaCas, in particular if used as a cryoprotectant, has been related to its capacity to bind water (Alvarez, Canet, & Fernández, 2007).

The cumulative release of EGCG from dried particles during simulated gastric (0 – 120 min) and intestinal (120 – 240 min) digestion is reported in Figure 4.

During the gastric digestion phase, the EGCG release is quite fast and is initially due to the rehydration process, which releases unbound EGCG (see Table 1). However, in the case of NC-ZP+MD particles, also the gastric digestion of NaCas by pepsin significantly contributes to the release of that significant EGCG fraction, which is directly complexed with NaCas (see Table 1). After 30 min, already 84 % of EGCG has been released by NC-ZP+MD particles, whereas only 75 and 78 % from GA/MD and ZP+GA/MD, respectively, and 59 % for ZP+MD. After 60 min, the cumulative release from NC-ZP+MD particles reaches 91 %, it remains around 75 and 78 % for both GA/MD and ZP+GA/MD and increases to 69 % for ZP+MD. In fact, zein is known for its slow digestibility (Patel & Velikov, 2014), which results in a more controlled and sustained release of EGCG. During intestinal digestion, where the action of pancreatin contributes to further protein digestion and carbohydrate hydrolysis, any additional release of EGCG is hindered by EGCG degradation at pH = 7.4 (see Figure 4). However, at the end of the digestion phase, a higher concentration of EGCG can be observed for NC-ZP+MD particles.

3.5. Effect of EGCG on lipolysis

Green tea catechins, and, in particular, EGCG, have been reported to decrease absorption of nutrients, probably by inhibiting gastrointestinal enzymes involved in their digestion (Rains, Agarwal, & Maki, 2011). Zein colloidal particles have also been previously reported to interfere with fat digestion preventing the action of both bile salts and digestive enzymes (Filippidi, Patel, Bouwens, Voudouris, & Velikov, 2014), because of their low digestibility coupled with their tendency to adsorb at the interface of fat droplets (de Folter, van Ruijven, & Velikov, 2012).

Figure 5 reports the results of a pH stat study, regarding the extent of hydrolysis (reported as mL of base consumption) of a model lipid emulsion (Intralipid®) in the presence of free EGCG and of EGCG encapsulated in ZP and NC-ZP particles.

In particular, the hydrolysis rate of triacylglycerides (TAG) of the surfactant-stabilized soybean oil emulsion at 2.0 % total fat concentration has been investigated. In the control sample (no additive), the lipase action immediately starts to hydrolyze the TAG at a fast initial rate as compared to the addition of EGCG or even of the plain zein-based colloidal particles (Figure 5), in agreement with previous results (Filippidi et al., 2014). The addition of free EGCG causes a significantly slower initial rate of hydrolysis, and correspondingly significantly lower amounts of base consumption in the first 35 min of simulated digestion.

ZP and NC-ZP particles without EGCG also cause a slower initial rate of hydrolysis, likely owing to the effect of the adsorption of the colloidal particles on fat droplets, in agreement with what was previously observed and described for zein-coated emulsion droplets (Filippidi et al., 2014). However, after about 10 min, the action of the pancreatin proteolytic enzymes on zein and NaCas causes a significant acceleration of base consumption, with the hydrolysis process involving at the same time protein and TAG. Interestingly, the base consumption is slightly higher for NC-ZP particles, whose NaCas external layer is more rapidly digested, than for ZP particles.

When EGCG is added to the colloidal particles, the curve of base consumption is very close to that of free EGCG with very similar initial rates of hydrolysis. Only after 20 min, the base consumption for EGCG@ZP particles exhibits higher values than for free EGCG. However, for EGCG@NC-ZP particles, an increased base consumption with respect to free EGCG can be observed already after 15 min. In both cases, the protein digestion can be considered the main responsible for increased NaOH volumes consumed.

Overall, in the presence of zein-based particles, the rate-limiting step appears to be the full or partial zein digestion by the proteases trypsin. However, with the progression of the digestion process, after 7-8 min, also the contribution of EGCG becomes evident, which limits lipase activity. As digestion proceeds, the digestion of zein and NaCas causes an increase in the extent of hydrolysis, which cannot be distinguished from the TAG digestion. Nonetheless, loading EGCG in both ZP and NC-ZP particles beneficially contributes to the slowing down of the fat digestion process with respect to plain zein-based particles.

4. Conclusions

Encapsulation of water-soluble compounds, such as EGCG, in zein particles, differently from water-insoluble compounds, is limited by the weak physical interactions occurring during the precipitation phase, as a consequence of the EGCG affinity for the antisolvent. Chemical interactions with additional biopolymers dissolved in the antisolvent, such as NaCas, has been exploited to improve the encapsulation efficiency of EGCG significantly by forming structured core-shell particles (zein colloidal particles coated with a layer of NaCas, NC-ZP) with respect to uncoated zein particles (ZP). Remarkably, the higher amount of EGCG encapsulated in NC-ZP particles appears to be mainly localized to the outer particle layer, where it is bioaccessible and can express its antioxidant activity. Such structured particles exhibit interesting properties, especially during digestion. Simulated digestion processes proved that the release rate of EGCG

519 can be regulated by the use of NaCas, which is more easily digestible than zein alone. The
520 encapsulation of EGCG into zein-based particles can also be exploited to modulate the rate of
521 fat digestion, combining the Pickering stabilization effect of the colloidal particles with EGCG
522 release and interaction with lipase enzyme. Therefore, despite the low affinity of EGCG for
523 zein, by tuning particle composition, it is possible to enable a certain extent of control in EGCG
524 release and bioactivity.
525

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630

631

Figure captions

Figure 1. TEM images of zein-based colloidal particles without and with EGCG. A set of three TEM images at different magnification has been reported for ZP, for zein co-precipitated with EGCG (EGCG@ZP), for NC-ZP, and for zein co-precipitated with EGCG in NaCas (EGCG@NC-ZP). EGCG/zein was always 0.12 g/g. Each set of images is grouped in a single line and is identified by a legend accompanied by an image of the corresponding colloidal dispersion.

Figure 2. Effect of EGCG loading on size distribution and ζ-potential of zein-based particles. Mean particle size (a), polydispersity index (b) and ζ-potential (c) of ZP and NC-ZP particles are reported as a function of EGCG/zein ratio.

Figure 3. Antioxidant activity of free and encapsulated EGCG. Antioxidant activity of EGCG (added before precipitation) encapsulated in ZP and in NC-ZP particles is reported in comparison with free reference EGCG, upon dilution (1:4 v/v) in 0.1 M HCl (pH = 1.0) and in a phosphate buffer solution (pH = 7.4).

Figure 4. Cumulative release of EGCG during simulated digestion. Cumulative release of EGCG is reported during 120 min of simulated gastric digestion and 120 min of intestinal digestion of zein-based spray-dried particles containing EGCG (ZP+MD, ZP+GA/MD, NC-ZP+MD), in comparison with a reference system (GA/MD).

Figure 5. Effect of free and encapsulated EGCG on fat digestion. Lipolysis of Intralipid emulsion (2% total fat), upon the addition of free EGCG (EGCG), and of EGCG encapsulated in ZP particles (EGCG@ZP) and NC-ZP particles (EGCG@NC-ZP) is reported in comparison to plain ZP and NC-ZP particles and to lipolysis in the absence of additives (no additives). The

655 extent of lipolysis is expressed in terms of the amount of base consumption (mL of 0.1M NaOH)
656 against time of incubation during intestinal digestion.

657

FIGURES

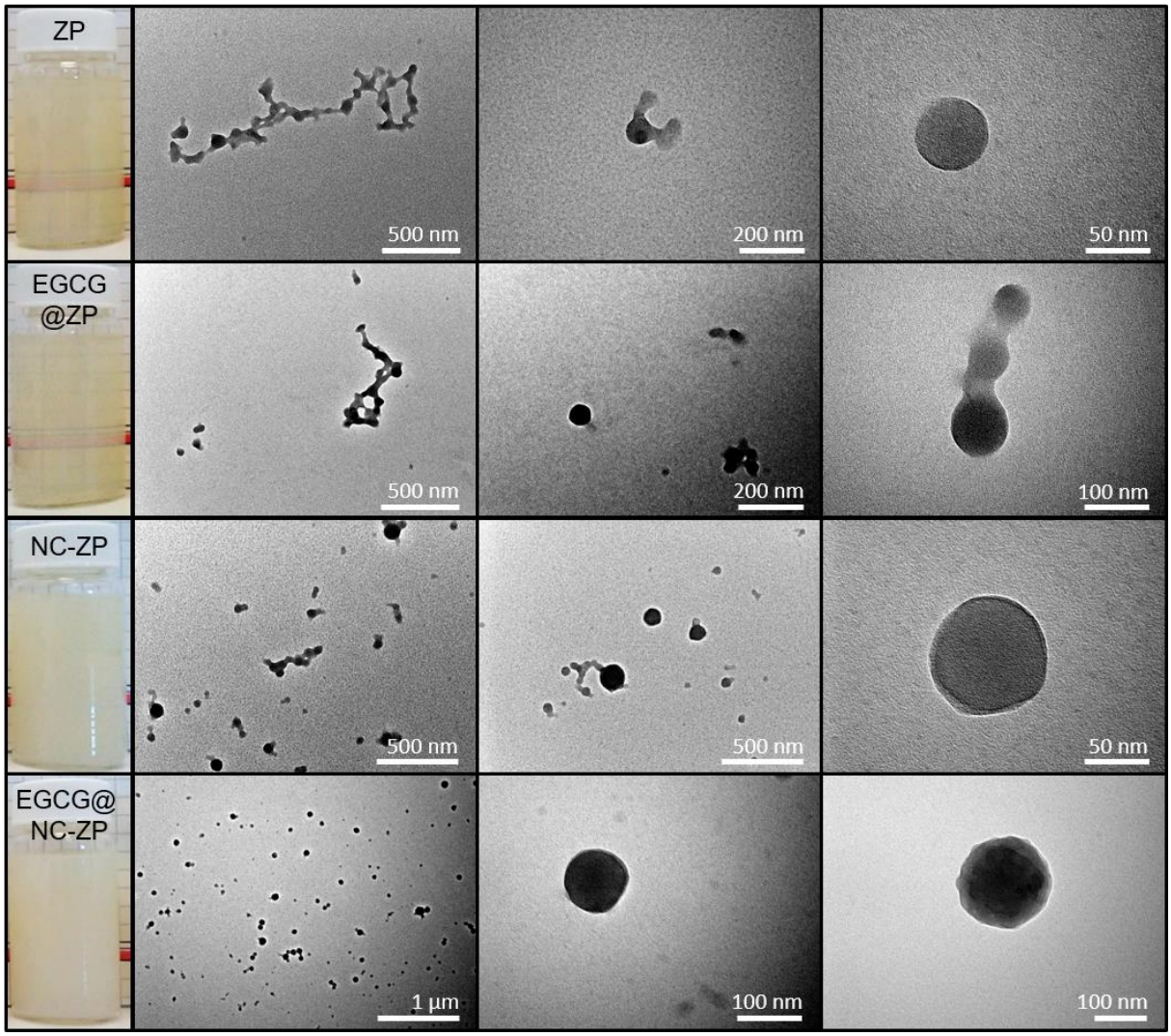
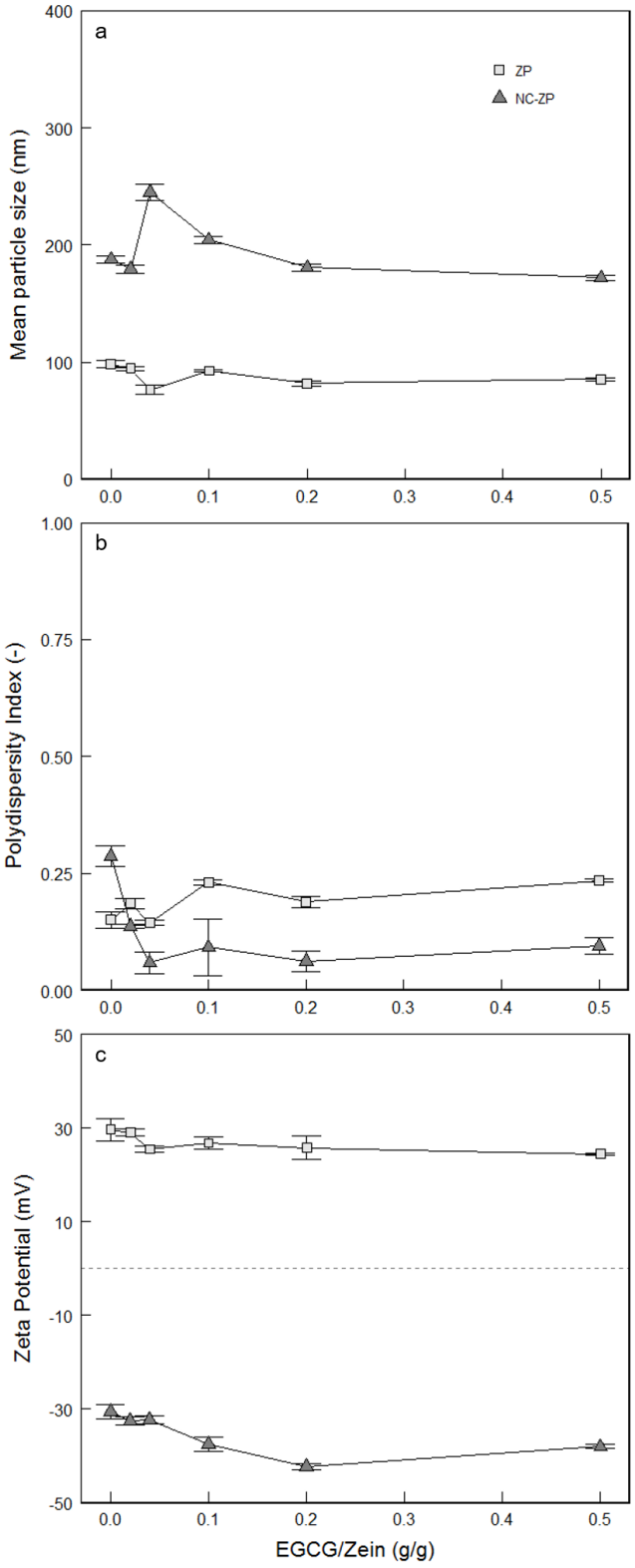


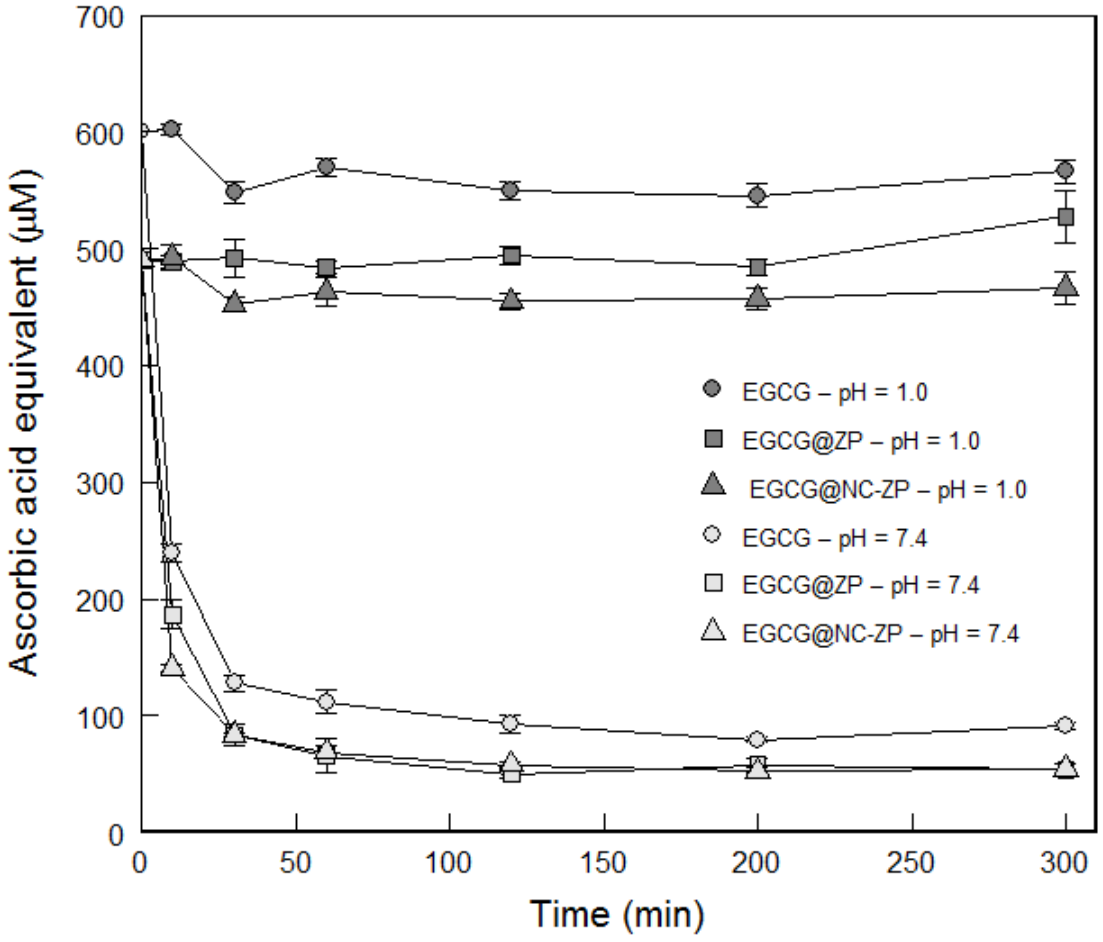
Figure 1. TEM images of ZP and NC-ZP colloidal particles without and with EGCG (EGCG/zein = 0.12 g/g). A set of three TEM images at different magnification has been reported for ZP, for zein co-precipitated with EGCG (EGCG@ZP), for NC-ZP, and for zein co-precipitated with EGCG in NaCas (EGCG@NC-ZP). Each set of images is grouped in a single line and is identified by a legend accompanied by an image of the corresponding colloidal dispersion.



667

668 **Figure 2.** Effect of EGCG/zein ratio on (a) mean particle size, (b) polydispersity index and (c)

669 ζ-potential of ZP and NC-ZP particles.



670

671 **Figure 3.** Antioxidant activity of EGCG (added before precipitation) encapsulated in ZP and in
672 NC-ZP particles in comparison with free reference EGCG, upon dilution (1:4 v/v) in 0.1 M HCl
673 (pH = 1.0) and in a phosphate buffer solution (pH = 7.4).

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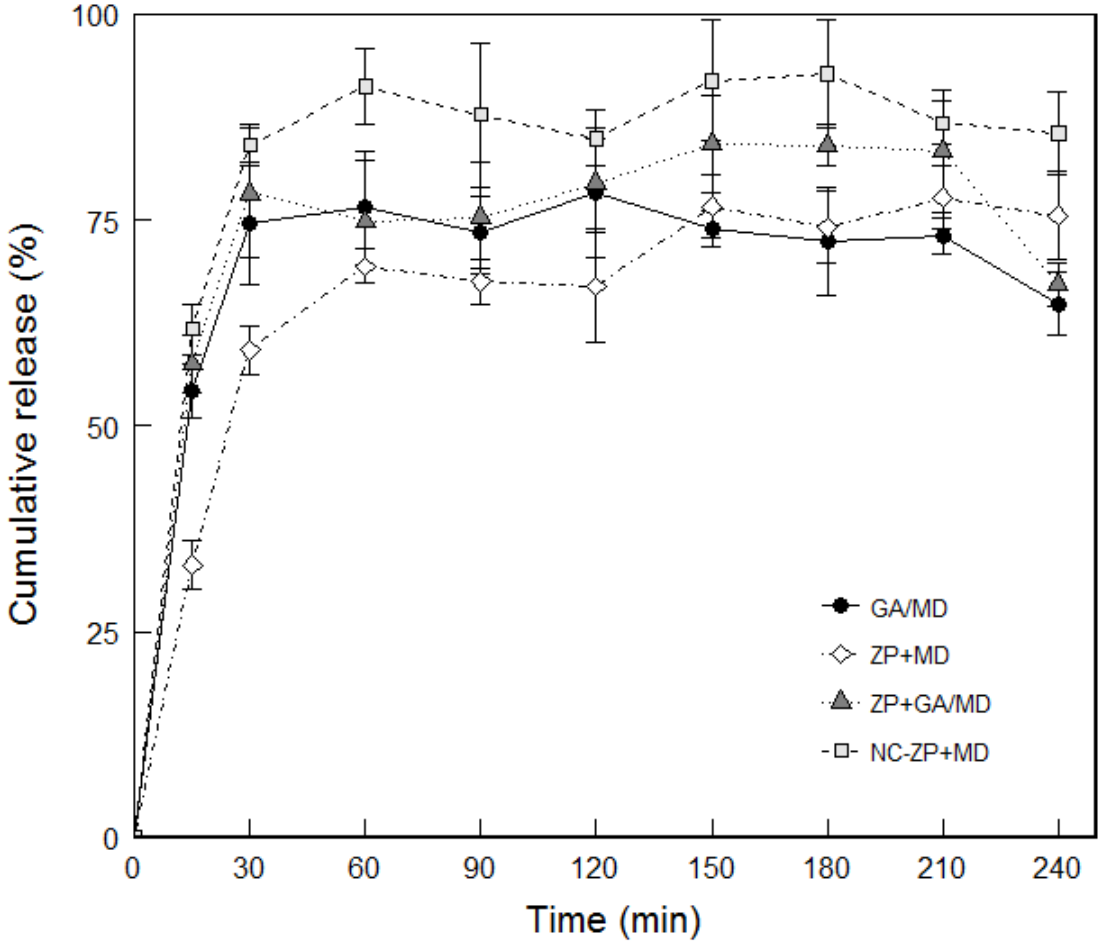

675

676 **Figure 4.** Cumulative release during 120 min of simulated gastric digestion and 120 min of
677 intestinal digestion of zein based spray-dried particles containing EGCG (ZP+MD,
678 ZP+GA/MD, NC-ZP+MD), in comparison with a reference system (GA/MD).

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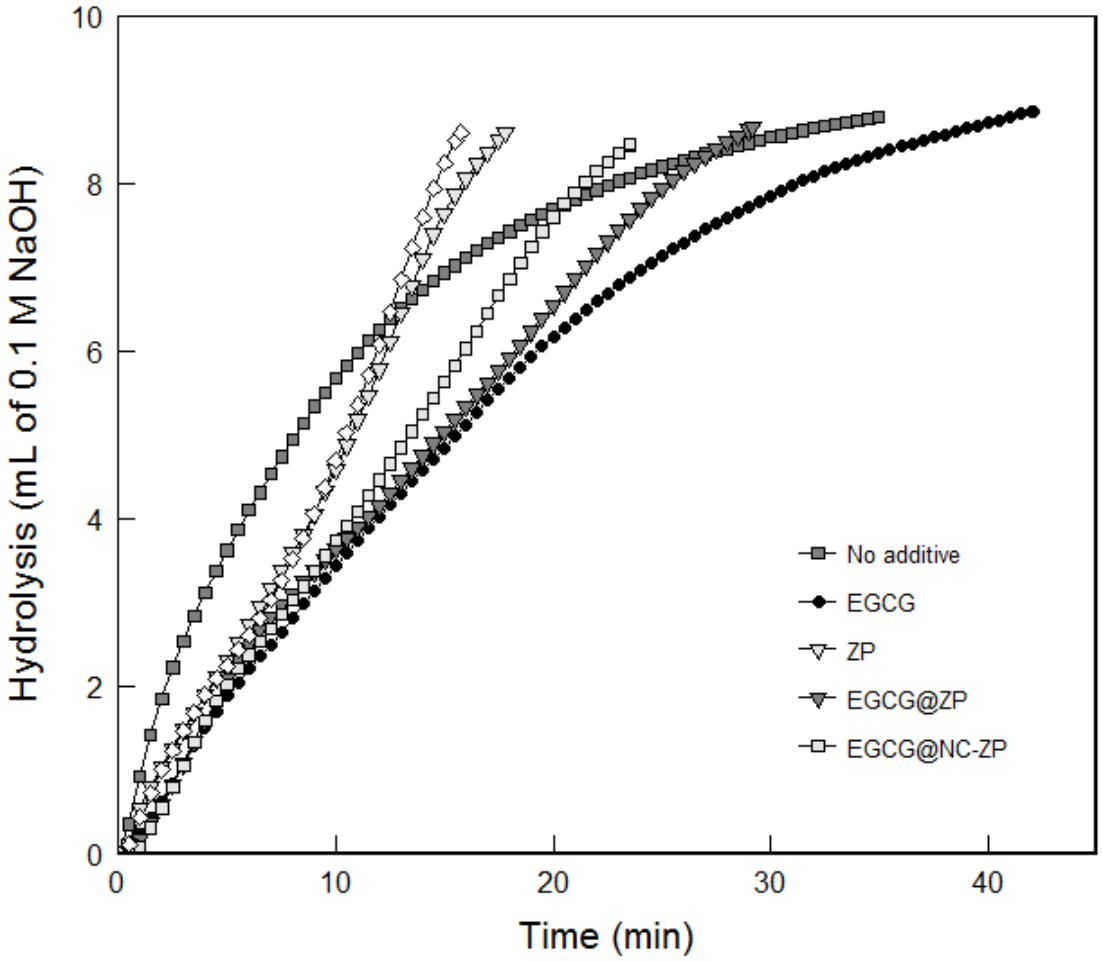

680

681 **Figure 5.** Lipolysis of Intralipid emulsion (2% total fat), upon the addition of free EGCG
682 (EGCG), and of EGCG encapsulated in ZP particles (EGCG@ZP) and in NC-ZP particles
683 (EGCG@NC-ZP) in comparison to plain ZP and NC-ZP particles and to lipolysis in absence
684 of additives (no additives). The extent of lipolysis is expressed in terms of amount of base
685 consumption (mL of 0.1M NaOH) against time of incubation during intestinal digestion.