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Creating functional nanostructures: Encapsulation of caffeine into α-lactalbumin nanotubes

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ABSTRACT

This work evaluated the stability and functionality of nanotubes obtained from α-lactalbumin (α-LA). α-LA nanotubes' structure was highly stable during a freeze-drying process but not after grinding. The ability of α-LA nanotubes to encapsulate caffeine, used as a model molecule, was evaluated. α-La nanotubes were highly effective for this purpose as encapsulation efficiency (*%EE*) was near 100% and loading capacity (*%LC*) near 10% at 1.5/20 and 2/20 ratios (caffeine/α-LA, *w/w*). α-LA nanotubes' structure was not affected by the presence of caffeine. Also, in general, refrigeration temperatures and neutral or alkaline conditions, under which the adverse effect of chelating agents was prevented, helped to stabilise α-LA nanotubes' structure and maintain caffeine encapsulated. At 8 °C and pH 7.5, in the presence of 75 μg mL^{-1} of EDTA, >50% of the caffeine remained encapsulated into α-LA nanotubes.

Industrial relevance: Linear and hollow structures could be highly effective to increase the viscosity while encapsulating bioactive compounds to protect them from degradation, provide on-demand release, or mask unpleasant organoleptic properties. However, until now, carbon nanotubes are the most commonly used carriers for the controlled release of bioactive molecules and these are not food-grade materials. Enzymatic partial hydrolysis of α-lactalbumin (α-LA) in the presence of a divalent ion results in the formation of food-grade nanotubes with high potential in the food and cosmetic industry. In this work the functionality of α-LA nanotubes was evaluated considering their applicability in real food.

1. Introduction

The addition of ingredients with functional properties into food products allows developing new functional foods. However, some factors can limit their application. The bioavailability of these compounds depends on their stability under conditions that can occur in foods during processing or in the gastro-intestinal (GI) tract (Chen, Remondetto, & Subirade, 2006; Chen & Subirade, 2007). Besides, many ingredients used in the food industry have an unpleasant flavour that should be masked (Nedovic, Kalusevic, Manojlovic, Petrovic, & Bugarski, 2013), as is the case of caffeine (MW: 194.19 g mol^{-1}). Caffeine is commonly used as an active component of energy drinks due to its many effects on the central nervous system, reducing drowsiness, and restoring concentration (Belscak-Cvitanovic et al., 2015). Although many people desire these benefits of caffeine, not everyone likes the characteristic bitterness associated with caffeine. This fact limits the use of caffeine in certain food applications or involves adding sugars and flavours to mask the bitterness (Sobel, Gundlach, & Su, 2014). Encapsulation can provide healthier technological solutions to these problems by preventing caffeine release into the food and allowing it into the gastrointestinal tract, avoiding sugar addition usually added to masking the bitter taste of caffeine (Belscak-Cvitanovic et al., 2015; Bohannon, 2008; Gunasekaran, Ko, & Xiao, 2007; Madadlou, Jaberipour, & Eskandari, 2014; Nedovic et al., 2013). Also, the controlled release of caffeine once ingested will allow those people with caffeine sensibility to reduce gastrointestinal problems associated with the ingestion of high amounts of caffeine (Gudas et al., 2000). Besides caffeine absorption from the gastrointestinal tract is too rapid (Belscak-Cvitanovic et al., 2015), so controlling its release after ingestion may be beneficial for food and nutraceutical applications maintaining its positive effects for a longer time, in the same way as the controlled release of other bioactive compounds.

A large number of delivery systems for medical and pharmaceutical applications have been developed using different biopolymers (Dumitriu & Popa, 2013; Langer & Peppas, 2003). However, the application here addressed needs materials considered as GRAS (Generally Recognised as Safe). In this regard, food biopolymers, specifically food proteins, are widely used due to their high nutritional value and functional properties. These features include their ability to form gels and

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emulsions, their availability from abundant renewable sources, and their acceptability as naturally occurring food components (Chen & Subirade, 2007; Chen et al., 2006; Mohammadi, Jafari, Esfanjani, & Akhavan, 2016). α-Lactalbumin (α-LA) from cheese whey, a by-product of the cheese industry, has a high nutritional and functional value. Besides, it can self-assemble into nanotube structures that could be used to modify food texture and to encapsulate micronutrients.

Whey protein nanotubes can be formed by partial hydrolysis of α-lactalbumin (α-LA). The reaction is catalysed by a serine endoprotease from *Bacillus licheniformis* (BLP) in the presence of a divalent cation, which promotes the formation of salt-bridges between two deionized carboxylic groups (Graveland-Bikker, Ipsen, Otte, & De Kruif, 2004; Ipsen & Otte, 2007). The type and concentration of cation and the reaction temperature will determine the correct nanotube formation with around 20 nm in outer diameter and 7 nm in inner diameter (Graveland-Bikker et al., 2004).

Decreasing the particle size of food ingredients increases their surface area improving their solubility, dispersion and therefore their delivery properties as improves their availability and absorption (Abbasi, Emam-Djomeh, Mousavi, & Davoodi, 2014; Augustin, 2003; Chen et al., 2006; López-Rubio & Lagaron, 2012). Particle size reduction also improves the sensory properties (extends flavour perception, provides better mouth feel, transparent appearance), and enhances the processability (Bagheri, Madadlou, Yarmand, & Mousavi, 2013; Moraru et al., 2003). Smaller sizes also increase the adhesiveness and extend the GI transit time, leading to a higher drug bioavailability (Chen et al., 2006).

Additionally, proteins nanotubes have advantages over other protein structures that make them more suitable to transport and release different bioactive compounds (Graveland-Bikker, 2005; Ipsen & Otte, 2007; Sadeghi et al., 2013). Their open ends, on both sides of the tubes, may provide a more efficient delivery and controlled release of bioactive compounds. Besides, as both outer and inner layers of nanotubes have different chemical characteristics, they could be functionalized differently, for example immobilising various bioactive compounds on both sides. Once encapsulated, bioactive compounds could be released from nanotubes in a controlled manner taking advantage of the ability of α-LA nanotubes to disassemble by lowering the pH below 3, which may be exploited for the transport and controlled release of bioactive compounds to specified sites of the GI tract. Besides all these advantages, tubular nanostructures could be very useful as thickener agents as structures with linear alignment are highly efficient to improve viscosity.

Despite the interesting properties of protein nanotubes and their potential as delivery structures, to our knowledge, only Sadeghi et al. (2013) described the application of BSA nanotubes to incorporate curcumin as a bioactive compound.

On the other hand, commercialization of α-LA nanotubes in solution is not possible because they could be contaminated or degraded. For this reason, α-LA nanotubes should be dried to improve their marketability and stability during storage. We propose two steps to obtain a dry powder of α-LA nanotubes: freeze-drying followed by a grinding process. α-LA nanotubes resistance to a freeze-drying process has already been tested in a previous work (Graveland-Bikker, 2005). However, to our knowledge, the effect of grinding on α-LA nanotubes has never been studied.

In this work were evaluated α-LA nanotubes' physical stability and their functional ability by determining their capacity and effectiveness to encapsulate caffeine and retain it under environmental conditions commonly found in food products, which may compromise α-LA nanotubes' chemical stability.

2. Materials and methods

2.1. Standards and chemicals

α-LA (α-lactalbumin) was gently supplied by Davisco Foods International, Inc. (Eden Prairie, MN, USA). Trifluoroacetic acid CHROMASOLV®, for HPLC (TFA, ≥99.0%), acetonitrile CHROMASOLV® Plus, for HPLC (ACN, ≥99.9%), ethylenediaminetetraacetic acid (EDTA, 99.4–100.6%), piperazine (anhydrous, ≥99.0%), 3-(*N*-Morpholino) propanesulfonic acid (MOPS, ≥99.5%) and sodium chloride (NaCl) were purchased from Sigma-Aldrich (Munich, Germany). TRIS buffer (tris-(hydroxymethyl)-aminomethane, extra pure) was from Scharlau. Hydrochloric acid (HCl, 37%), sodium hydroxide (NaOH) and Glycine were from Panreac (Barcelona, Spain). Serine protease from *Bacillus licheniformis* (BLP) was gently supplied by Novozymes A/S (Bagsværd, Denmark). Manganese (II) chloride tetrahydrate ($MnCl_2 \cdot 4H_2O$) was from Merck (Darmstadt, Germany). Sodium cacodylate EM and glutaraldehyde EM 25%, were purchased from TAAB (Reading, UK). Caffeine anhydrous was purchased from VWR (Pennsylvania, US).

2.2. Nanotube synthesis

Nanotubes were prepared following the method described in Graveland-Bikker et al. (2004) with some modifications. Briefly, for all the assays, the α-LA powder was dissolved (30 g L^{-1}) in buffer 75 mM Tris-HCl (pH 7.5). $MnCl_2 \cdot 4H_2O$ was added to the α-LA solution to obtain the final desired concentration of Mn^{2+} ($R = 2$ mol of ion/mol of α-LA). To begin the hydrolysis, 4% BLP (*w*/*w*), respect to the amount of protein sample, was added. After stirring, the solution was immediately filtered using a 0.1 μm low protein binding filter from Millipore Ibérica, S.A. (Madrid, Spain) and incubated at 55 °C in a thermostatic bath for 3 h.

According to previous works (Gunasekaran et al., 2007; Madadlou et al., 2014) caffeine does not bind with whey proteins, so apparently encapsulation was only due to physical entrapment. Thus, caffeine-loaded nanotubes were prepared by adding caffeine to the reaction before the nanotube formation to be trapped during self-assembly. The binding constant of caffeine with manganese is very low (Kolayli, Ocak, Küçük, & Abbasoglu, 2004), so chelating capacity at the concentrations of caffeine used is negligible.

The desired amount of caffeine was added to the α-LA solution in Tris-HCl 75 mM (pH 7.5) with $MnCl_2 \cdot 4H_2O$ before adding the BLP so that caffeine was trapped inside during nanotubes' self-assembly. Six caffeine/α-LA (*w*/*w*) ratios have been tested: 0/20, 0.2/20, 1/20, 1.5/20, 2/20, and 4/20.

2.3. Determination of nanotubes' physical stability

To determine the stability of α-LA nanotubes, under two common industrial processes such as freeze-drying and grinding, α-LA nanotubes samples were immediately frozen at −80 °C after their synthesis and then freeze dried. Once the samples were freeze-dried, they were resuspended in the same volume of buffer 75 mM Tris-HCl (pH 7.5) used during their synthesis. From each sample, a 25 μL aliquot was withdrawn and analysed by TEM. Three replicates were analysed for each condition.

Grinding resistance was examined using freeze-dried nanotubes prepared as explain in the above paragraph. Glass beads (425–600 μm acid washed glass beads, Sigma-Aldrich) were added to the freeze-dried nanotube samples in a relation 1:1 (*w*/*w*). Samples were then shaken in a Tissuelyser® LT (Qiagen, Venlo, The Netherlands) for 3 min at 32.5 Hz. Finally, samples were resuspended in the same volume of buffer 75 mM Tris-HCl (pH 7.5) used during their synthesis. From each sample, a 25 μL aliquot was withdrawn and analysed by TEM. Three replicates were analysed for each condition.

2.4. Determination of encapsulation efficiency and loading capacity

Encapsulation efficiency (*%EE*) and loading capacity (*%LC*) were determined by filtration separating the caffeine-loaded nanotubes from the supernatant containing free caffeine by filtration. The procedure was as follows. Once caffeine-loaded nanotube samples were obtained

as explained above, 8 mL of 75 mM Tris-HCl buffer (pH 7.5 at 25 °C) were added to 2 mL of nanotube samples. Then samples were subjected to a gentle shaking (favouring sample washing without damaging the nanotube structure) for periods of 15 min in a rocking shaker. At different time intervals, shaking was stopped, and 1 mL of the supernatant was removed and filtered through a 0.2 μm filter. Supernatant aliquots were stored at −20 °C until caffeine quantification by RP-HPLC. Three replicates were analysed for each condition.

%EE and *%LC* were calculated as follows:

$$\%EE = \frac{Caf_{total} - Caf_{free}}{Caf_{total}} \times 100 \quad (1)$$

$$\%LC = \frac{Caf_{total} - Caf_{free}}{\alpha\text{-}LA_{total}} \times 100 \quad (2)$$

where Caf_{total} is the total amount of caffeine initially added to the reaction to be incorporated into nanotubes during their synthesis, Caf_{free} is the amount of free caffeine determined in the filtrated supernatant. $\alpha\text{-}LA_{total}$ is the amount of α-LA employed for nanotube synthesis.

2.5. Determination of nanotubes' disassembly and caffeine release

Ideally, caffeine would remain protected into the nanotubes when it is added to food products. Therefore, nanotube stability and caffeine release was studied under different environmental conditions that may occur in food products.

For that purpose, we studied temperatures from refrigeration till pasteurization (8–80 °C), pH values from 2 to 8, the presence of chelating agent (EDTA, usually added as antioxidant agent at maximum concentrations of 75 μg mL^{-1}), and the addition of salt (NaCl, usually added as preservative and flavouring at concentrations around 1000 μg mL^{-1}).

A 2^4 full-factorial design (Box, Hunter, and Hunter $2^{K\text{-}p}$ type) was used to analysed the effect of the four variables above exposed (T, pH, EDTA, NaCl) on the nanotube disassembly and caffeine release. For each experiment of the design, caffeine release kinetics were performed by adding 1.5 mL of the corresponding buffer, indicated in Table 1, over 0.380 mL of caffeine-loaded nanotubes prepared with a caffeine/α-LA ratio of 1.5/20 (*w*/*w*). α-LA nanotubes samples without caffeine were also evaluated in the same way to be used as controls. Samples were then incubated in a thermostatic bath at the temperatures indicated in Table 1. One sample was prepared for each sampling time. The supernatants were filtered through 0.2 μm filters and aliquots were stored at −20 °C until caffeine quantification by RP-HPLC. TEM analyses were performed with 25 μL aliquots removed from the samples at the end of the assay.

The percentage of free caffeine released from nanotubes ($\%Caf_{free}$) was calculated as follows:

$$\%Caf_{free} = \frac{Caf_{free}}{Caf_{total}} \times 100 \quad (3)$$

where Caf_{total} is the total amount of caffeine initially added to the reaction for incorporation into the nanotubes during their synthesis, and Caf_{free} is the amount of free caffeine determined in the filtered supernatant.

The maximum percentage of free caffeine released from nanotubes ($\%Caf_{free\ max}$) was evaluated as the dependent variable in the factorial design and calculated by fitting the experimental data with the following equation (Fuciños et al., 2012):

$$\%Caf_{free\ t} = \%Caf_{free\ max} + \left(\%Caf_{free\ 0} - \%Caf_{free\ max}\right) e^{-m \cdot t} \quad (4)$$

where $\%Caf_{free\ 0}$ and $\%Caf_{free\ t}$ are the percentages of free caffeine released from nanotubes at the initial time and at time *t*, calculated with Eq. (3) for each sampling time. The parameter *m* (h^{-1}) is the maximum release rate, and *t* (h) is the sampling time.

The full-factorial design consisted in 20 experiments with 16 factorial points (2^4) and four central points for replication. The range and codification of the independent variables are shown in Table 1.

Statistical analysis was performed using Statistica 8.0 (StatSoft, Inc.). Response values from the experiments of the factorial design were fitted to the following polynomial linear model:

$$y = \alpha_0 + \alpha_1 \cdot X_1 + \alpha_2 \cdot X_2 + \alpha_3 \cdot X_3 + \alpha_4 \cdot X_4 + \alpha_{12} \cdot X_1 \cdot X_2 + \alpha_{13} \cdot X_1 \cdot X_3 + \alpha_{14} \cdot X_1 \cdot X_4 + \alpha_{23} \cdot X_2 \cdot X_3 + \alpha_{24} \cdot X_2 \cdot X_4 + \alpha_{34} \cdot X_3 \cdot X_4 + \alpha_{123} \cdot X_1 \cdot X_2 \cdot X_3 + \alpha_{124} \cdot X_1 \cdot X_2 \cdot X_4 + \alpha_{134} \cdot X_1 \cdot X_3 \cdot X_4 + \alpha_{234} \cdot X_2 \cdot X_3 \cdot X_4 \quad (5)$$

where *y* is the response ($\%Caf_{free\ max}$). X_1, X_2, X_3, and X_4 are the coded levels of the independent factors, i.e. temperature (T), pH, the concentration of EDTA (EDTA) and the concentration of NaCl (NaCl), respectively. $X_1 \cdot X_2$ to $X_2 \cdot X_3 \cdot X_4$ represent the interaction terms. α_0 is the

Table 1
Range and codification criterion for independent variables in the fractional factorial design at 2 levels (Box, Hunter, and Hunter 2^4 type, *R* = FULL).

Coded values				Natural values				Responses	Buffer (75 mM)
T	pH	EDTA	NaCl	T (°C)	pH	EDTA (μg mL^{-1})	NaCl (μg mL^{-1})	$\%Caf_{free\ max}$	
−1	−1	−1	−1	8	2.0	0.0	0.0	67.41 ± 9.30	Glycine-HCl
1	−1	−1	−1	80	2.0	0.0	0.0	104.30 ± 9.85	Glycine-HCl
−1	1	−1	−1	8	7.5	0.0	0.0	56.78 ± 5.00	MOPS-NaOH
1	1	−1	−1	80	7.5	0.0	0.0	79.68 ± 3.84	Piperazine-HCl
−1	−1	1	−1	8	2.0	75.0	0.0	88.44 ± 17.97	Glycine-HCl
1	−1	1	−1	80	2.0	75.0	0.0	85.28 ± 1.33	Glycine-HCl
−1	1	1	−1	8	7.5	75.0	0.0	45.42 ± 3.73	MOPS-NaOH
1	1	1	−1	80	7.5	75.0	0.0	79.12 ± 9.09	Piperazine-HCl
−1	−1	−1	1	8	2.0	0.0	1000	61.61 ± 3.62	Glycine-HCl
1	−1	−1	1	80	2,0	0,0	1000	101.80 ± 3.90	Glycine-HCl
−1	1	−1	1	8	7.5	0.0	1000	42.85 ± 3.40	MOPS-NaOH
1	1	−1	1	80	7.5	0.0	1000	94.41 ± 4.75	Piperazine-HCl
−1	−1	1	1	8	2.0	75.0	1000	128.8 ± 41.61	Glycine-HCl
1	−1	1	1	80	2.0	75.0	1000	95.91 ± 4.55	Glycine-HCl
−1	1	1	1	8	7.5	75.0	1000	47.83 ± 3.57	MOPS-NaOH
1	1	1	1	80	7.5	75.0	1000	87.17 ± 0.77	Piperazine-HCl
0	0	0	0	44	4.8	37.5	500	70.28 ± 9.77	Piperazine-HCl
0	0	0	0	44	4.8	37.5	500	87.26 ± 9.98	Piperazine-HCl
0	0	0	0	44	4.8	37.5	500	76.89 ± 6.44	Piperazine-HCl
0	0	0	0	44	4.8	37.5	500	85.78 ± 10.18	Piperazine-HCl

independent term. α_1 to α_{234} are the regression coefficients of factors and interaction terms. The objective function in this study was that which minimises the $\%Caf_{free\ max}$ by investigating its relationship with the factors mentioned above. The signification of coefficients for the model, and the model adequacy was analysed by an Analysis of Variance (ANOVA).

2.6. Protein profile analysis and caffeine quantification by reverse-phase high-performance liquid chromatography

The determination of the profile of α-LA fragments that appear during nanotube disassembly and the caffeine quantification were performed by RP-HPLC following the method described by Estévez (2015). Briefly, the separation was performed employing an ACE® C18 column (4.6 mm × 250 mm, 5 μm, 300 Å) at 30 °C. The flow rate was 0.7 mL min^{-1}, the injection volume was 20 μL and the detection wavelength 220 nm. Mobile phase A was 0.1% (*v*/*v*) TFA in Milli-Q water, and mobile phase B was 0.1% (*v*/*v*) TFA in 100% (*v*/*v*) ACN. Mobile phase A was maintained at 100% for 5 min, then it decreased linearly to 50% over 50 min. Mobile phase B was increased linearly to 100% over the next 2.5 min and then was maintained at 100% of mobile phase B for another 2.5 min. Finally, mobile phase A was increased linearly to 100% over 5 min and the column was re-equilibrated at 100% of mobile phase A for a further 5 min.

A commercial standard of α-LA was used to determine its retention time. Calibration curves of caffeine were constructed the commercial standard, which resulted highly linear within the concentration range 0.01–1 mg mL^{-1}. All the analysis were performed in triplicate.

2.7. Transmission electron microscopy (TEM)

α-La nanotube samples were 1:1 diluted with fixative (1.5% glutaraldehyde in 0.1 M sodium cacodylate buffer) and stored at 4 °C until their analysis. Samples were prepared by the conventional negative staining method. A Formvar layer on a copper grid was coated with carbon, immersed in 5 μL of the sample 2× (samples from physical stability and encapsulation efficiency and loading capacity) and 1× (samples from nanotubes' disassembly and caffeine release kinetics) diluted, and blotted after 1 min. Then, the copper grid was washed with water for 1 min and subsequently blotted, to remove the excess of protein material. After that, the copper grid was immersed in 3% (*w*/*w*) uranyl acetate, to enhance the contrast, during 1 min and blotted again. Samples were then dried for 1 h in a desiccator. Micrographs were obtained with a JEOL JEM-1010 100 kV high contrast transmission electron microscope (CACTI, University of Vigo, Spain).

3. Results and discussion

3.1. Nanotubes' physical stability

To commercialise α-LA nanotubes as a dry powder, the stability of α-LA nanotubes was evaluated under industrial processing conditions, such as freeze-drying and grinding. α-LA nanotubes were frozen and freeze-dried after their synthesis. Fig. 1 shows TEM micrographs of redispersed freeze-dried nanotubes in the synthesis buffer. Nanotubes' structures resisted the freeze-drying process without significant morphological changes. These results are in agreement with those obtained by Graveland-Bikker (2005) with α-LA nanotubes synthesised in the presence of calcium, suggesting that the type of cation does not affect the mechanical resistance of structures under mild stress conditions. However, intense mechanical stress, at which the nanotube samples were subjected during grinding, destroyed their original tubular structure (Fig. 1).

3.2. Encapsulation efficiency and loading capacity

The ability of α-LA nanotubes to encapsulate caffeine, used as a model molecule, was evaluated through the determination of encapsulation efficiency (*%EE*) and loading capacity (*%LC*). At low caffeine/α-LA ratios (*w*/*w*) we could not detect caffeine encapsulation (Fig. 2). However, nanotubes seem to be highly effective in caffeine encapsulation at 1.5/20 and 2/20 ratios, as *%EE* was near 100% (96.96 ± 2.12% and 97.16 ± 1.91%, respectively) and *%LC* was around 10% (9.21 ± 0.20% and 12.30 ± 0.24%, respectively) (Fig. 2). High concentrations of polymer and bioactive compound increase the encapsulation efficiency as low levels may decrease the contact between the polymer and the bioactive compound. So, increasing the bioactive compound/polymer ratio could result in an encapsulation increase although the encapsulation efficiency may decrease at higher ratios (Pridgen, Alexis, Langer, & Farohkzad, 2009). In fact, the growth in caffeine/α-LA ratio led to higher *%LC*, but *%EE* rose until reaching a plateau of which the growth in caffeine/α-LA ratio resulted in a *%EE* decrease. This behaviour could be explained by considering that caffeine-coupling sites into nanotubes are limited. At high caffeine/α-LA ratios, coupling sites were less available, so encapsulation was less efficient. Indeed, prolonged washing times (60 min) promoted caffeine decapsulation at the highest caffeine/α-LA ratio assayed, at which nanotubes coupling sites could be saturated and not bound caffeine could be more easily released. For this reason, a compromise caffeine/α-LA ratio must be achieved such that *%EE* was higher enough without caffeine decapsulation when

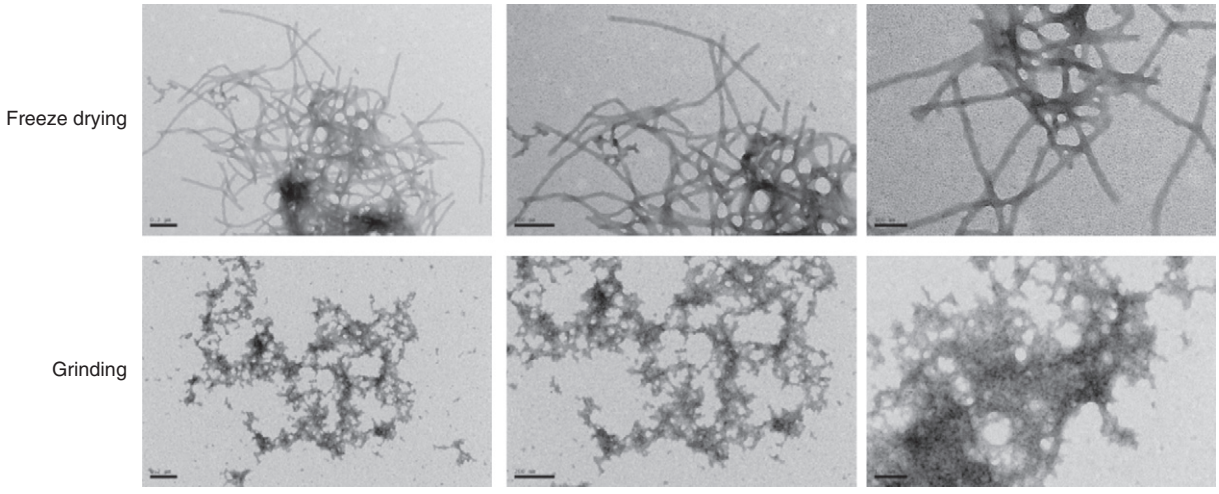

Fig. 1. Transmission electron micrographs (TEM) of α-LA nanotubes redispersed after freeze-drying and after freeze-drying and subsequent grinding. Scale bar of the images is 200 nm.

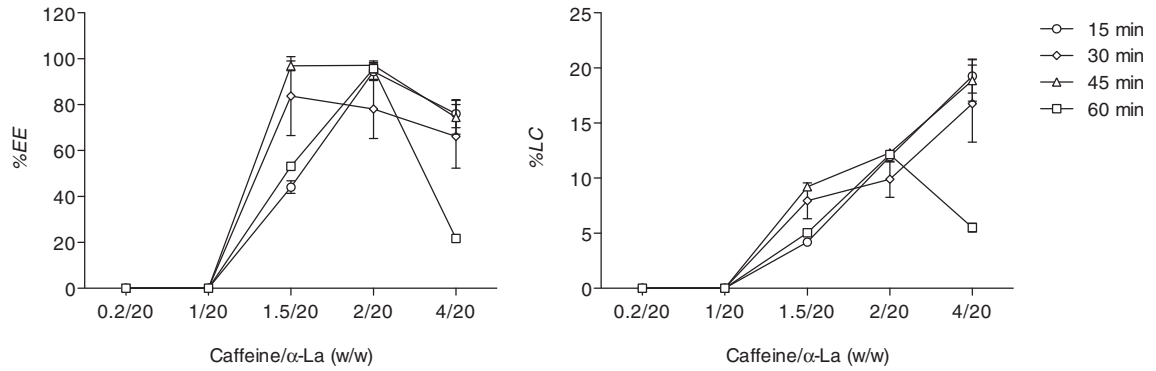

Fig. 2. Encapsulation efficiency (*%EE*) and loading capacity (*%LC*) of α-LA nanotubes synthesised in the presence of different ratios of caffeine/α-LA and after different washing times.

nanotubes remain in suspension for a long time, for example in liquid food products.

TEM micrographs (Fig. 3), taken just after caffeine-loaded nanotube synthesis, agree with the results described above. Up to the ratio 1.5/20, the structure of nanotubes appeared intact. However, at higher ratios nanotubes' surface seem to be modified by the presence of free caffeine. At the ratio 2/20, α-LA nanotubes showed a sort of coating, which blurs nanotubes' surface. At the ratio 4/20, nanotubes probably already exist but can no longer be identified due to the presence of a large amount of free caffeine on their surface, which could be easily released to the liquid medium in which the nanotubes were incorporated.

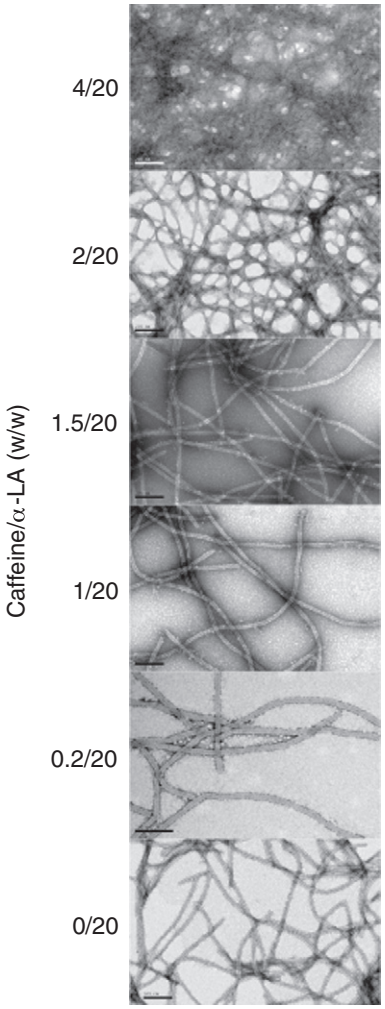

Fig. 3. Transmission electron micrographs (TEM) of caffeine-loaded α-LA nanotubes at different relations of caffeine/α-LA. Scale bar of the images is 200 nm.

The values of *%EE* obtained with α-LA nanotubes were higher than those achieved by caffeine encapsulation into other biopolymer nanoparticles, like the alginate nanoparticles with around 85% *EE* (Bagheri et al., 2013), or lactoferrin-glycomacropeptide nanohydrogels with around 90% *EE* (Bourbon, Cerqueira, & Vicente, 2016). This fact is even more evident when the results obtained here are compared with those obtained when other bioactive compounds, different from caffeine, were encapsulated in whey protein nanostructures. For example, the 70–80% *EE* reached by metoprolol succinate encapsulation into α-LA microparticles (Vijayaragavan et al., 2014), the 55% *EE* obtained by anthocyanin extract (Arroyo-Maya et al., 2012) or the encapsulation of date palm pit aqueous extract into WPI nanoparticles (Bagheri et al., 2013).

3.3. Nanotubes' disassembly and caffeine release

To evaluate how different environmental factors, that may occur in food products, could affect nanotube stability, a full 2^4 full-factorial design was performed using as independent variables temperature (8–80 °C), pH (2–8), EDTA concentration (0–75 μg mL^{-1}), and NaCl (0–1000 μg mL^{-1}).

The maximum percentage of caffeine released from α-LA nanotubes ($\%Caf_{free\ max}$), obtained by fitting experimental release kinetics for each experiment of the factorial design (data not shown) to the Eq. (4), ranged from ~40% to 100% (Table 1).

Fig. 4 showed TEM micrographs and RP-HPLC chromatograms of samples under those conditions which hindered the lowest and the highest caffeine release (Table 1). Both micrographs and chromatograms showed a strong correlation with the $\%Caf_{free\ max}$ values. Conditions in which the release was low were those corresponding to intact or less degraded nanotubes in TEM micrographs (A and B). The absence of the characteristic peak of α-LA at around 55 min indicates that there were no free α-LA fragments in solution, evidencing that it was still part of the nanotube's structure. Conversely, conditions in which caffeine release was high were those corresponding to nanotubes that appeared more degraded in TEM micrographs (C and D). Accordingly, peaks associated with large fragments of α-LA appeared in the RP-HPLC chromatograms.

Mathematical modelling of the 2^4 full-factorial design data was carried out using Eq. (5) to obtain a polynomial empirical model. Coded values of the independent variables (T, °C; pH; EDTA, μg mL^{-1}; and NaCl, μg mL^{-1}) and their products, as shown in Eq. (5), along with the dependent variable ($\%Caf_{free\ max}$) were subjected to multiple regression analysis to determine the coefficients (α_1 to α_{234}) and *p*-values of each term of the equation. An analysis of variance was performed to determine which factors significantly affected the $\%Caf_{free\ max}$ (Table 2).

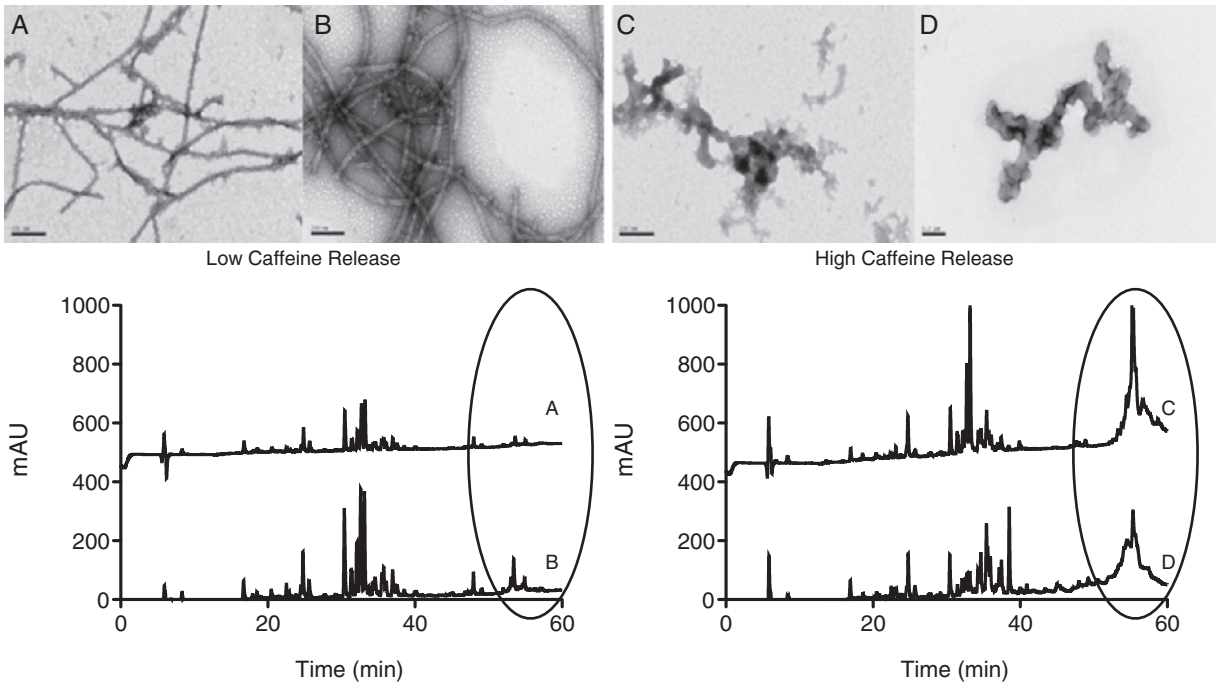

Fig. 4. Transmission electron micrographs (TEM) and chromatograms of samples with α-LA nanotubes subjected to different environmental conditions that may occur in food products: A) 8 °C, pH 7.5, 0 μg mL^{-1} EDTA, 1000 μg mL^{-1} NaCl; B) 8 °C, pH 7.5, 75 μg mL^{-1} EDTA, 0 μg mL^{-1} NaCl; C) 8 °C, pH 2, 75 μg mL^{-1} EDTA, 1000 μg mL^{-1} NaCl; D) 80 °C, pH 2, 0 μg mL^{-1} EDTA, 1000 μg mL^{-1} NaCl. Scale bar of the images is 200 nm.

After neglecting the insignificant terms ($p > 0.10$), the fitted equation which describes the $\%Caf_{free\ max}$ released from nanotubes is the following ($r^2 = 0.8327$ and $r^2\ adjusted = 0.7555$):

$$\begin{aligned}\%Caf_{free\,max} = {} & (79.40 \pm 1.78) + (11.85 \pm 1.99)\cdot T \\ & + (-12.46 \pm 1.99)\cdot pH + (6.72 \pm 1.99)\cdot T\cdot pH \\ & + (-7.10 \pm 1.99)\cdot T\cdot EDTA + (-4.78 \pm 1.99)\cdot pH\cdot EDTA \\ & + (7.04 \pm 1.99)\cdot T\cdot pH\cdot EDTA \end{aligned} \quad (6)$$

The model was statistically significant ($p < 0.10$), and the lack of fit was not significant ($p > 0.10$) (Table 2). The relatively good r^2 value ($r^2 = 0.8327$ and $r^2\ adjusted = 0.7555$) indicated a good correlation between the adjusted and predicted values, which supports the statistical validity and significance of the equation obtained.

The large values of coefficients for the terms T and pH (Eq. (6)) evidence their effect on $\%Caf_{free\ max}$ ($p < 0.05$). NaCl did not have any significant ($p > 0.10$) effect on $\%Caf_{free\ max}$. The interaction of EDTA with T and pH had a significant effect on $\%Caf_{free\ max}$ ($p < 0.05$). Fig. 5 demonstrated the effect of interactions between T, pH, and EDTA on $\%Caf_{free\ max}$. In the absence of EDTA (Fig. 5A), the effect of T on $\%Caf_{free\ max}$ was intense, with the amount of free caffeine increasing with increasing temperatures, for all the pH-values assayed. Free caffeine also increased by reducing pH, for all the T assayed, but the effect was less intense than that observed with T. Graveland-Bikker (2005) just explained that α-LA nanotubes synthesised in the presence of Ca^{2+} are resistant to mild heat treatments like a pasteurization process. However, they could not resist longer heating processes and high temperatures (>70 °C). The pH effect could be explained by an increase in the net charge of the building blocks at low pH, which could cause an increase in electrostatic repulsion leading to the nanotubes' disassembly (Graveland-Bikker, 2005).

Introducing EDTA in the release solution (Fig. 5B and C) increased the effect of pH at low T, being $\%Caf_{free\ max}$ clearly higher at low pH. At high T, the effect of the other parameters could not be seen probably because the nanotube stability was so low that other effects could be neglected.

It is interesting to note that the interaction of EDTA with T and pH had a substantial impact on the nanotubes' stability and caffeine release, resulting in a significant ($p < 0.10$) effect on $\%Caf_{free\ max}$ (Table 2). At pH 7.5 the effect of EDTA was small, for all the T assayed (Fig. 5F). However, free caffeine increased by increasing the concentration of EDTA when pH was lowered to 4.8 (Fig. 5E) and, more intensely, to pH 2 (Fig. 5D), at low T. The effect of EDTA at high T was probably masked by the significant effect of these temperatures on nanotube's disassembly.

At high pH values, EDTA predominates in its fully deprotonated form ($EDTA^{4-}$). $EDTA^{4-}$ can chelate or complex metal ions in 1:1 metal-to-EDTA complexes (Singh, 2007). In our experimental units, the ratio of Mn^{2+}:EDTA was at least 5.33 (mol:mol). So the excess of Mn^{2+} could prevent nanotube disassembly caused by the chelating activity of EDTA. At low pH values, the metal-EDTA complexes become unstable because H^+ competes with the metal ions for the EDTA, predominating the fully protonated form H_6EDTA^{2+} (Harris, 2013). Nevertheless, the presence of chelating agents like EDTA can induce structural changes in Ca^{2+}-binding proteins like α-LA, due to electrostatic repulsion between molecules of similar charge (Stemmer & Klee, 1990). So, at low pH values, protonated H_6EDTA^{2+} may contribute to enhance the effect of pH on the nanotube disassembly due to repulsion between positively charged groups.

Table 2
Analysis of variance (ANOVA) for the results of the fractional factorial design at 2 levels (Box, Hunter, and Hunter 2^4 type, R = FULL) ($r^2 = 0.8327$, $r^2\ adjusted = 0.7555$).

Factor	SS[a]	DF[b]	MS[c]	F	p
T	2245.101	1	2245.101	35.40137	0.009494
pH	2482.282	1	2482.282	39.14129	0.008240
T·pH	721.863	1	721.863	11.38252	0.043289
T·EDTA	805.850	1	805.850	12.70686	0.037695
pH·EDTA	365.670	1	365.670	5.76598	0.095767
T·pH·EDTA	793.972	1	793.972	12.51956	0.038410
Lack of fit	1299.458	10	129.946	2.04902	0.301762
Pure error	190.255	3	63.418		
Total SS	8904.451	19			

[a]SS, sum of squares, [b]DF, degrees of freedom, [c]MS, mean squares.
$p < 0.10$ was considered significant.

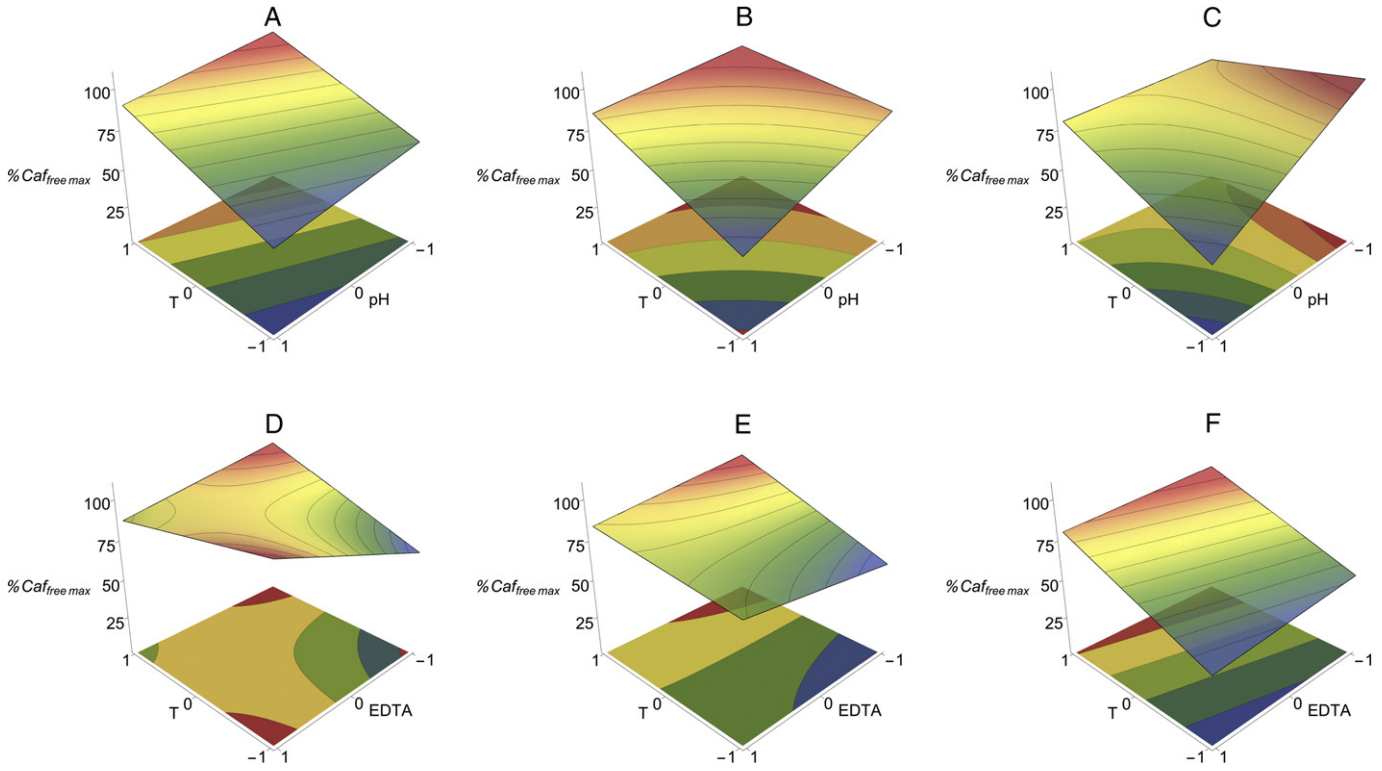

Fig. 5. Response surfaces corresponding to the combined effect of temperature (T) and pH and EDTA on the maximum percentage of free caffeine released from nanotubes ($\%Caf_{free\ max}$) at A) 0 μg mL^{-1}, B) 37.5 μg mL^{-1}, C) 75 μg mL^{-1} of EDTA, D) pH 2, E) pH 4.8, and F) pH 7.5, according to the Eq. (6).

Within the domain evaluated, the predicted minimum $\%Caf_{free\ max}$ was 43.65 (i.e. ~56.35% remained retained) and occurred at 8 °C, pH 7.5 and 75 μg mL^{-1} of EDTA. Our experimental results confirmed the prediction. Thus, the $\%Caf_{free\ max}$ values obtained experimentally under these conditions were 45.42 ± 3.73 and 47.83 ± 3.57 at 0 and 1000 μg mL^{-1} of NaCl, respectively. Therefore, the conditions that will help to maintain caffeine encapsulated into α-LA nanotubes, during food processing and storing until their consumption, are refrigeration temperatures at neutral or alkaline pH. In acidic conditions the absence of chelating agents would be preferable.

4. Conclusions

α-LA nanotubes resulted highly stable during a freeze-drying process, which could be very useful considering their preservation and future commercialization. Also, α-LA nanotubes seem to be highly effective in caffeine encapsulation, achieving values near of 100% of encapsulation efficiency and close to 10% of loading capacity. Nanotubes' structure did not appear to be damaged by the presence of caffeine so that they could maintain their functionality as a thickener agent. There was a clear correlation between those conditions where nanotubes appeared more damaged and higher caffeine release ratios. An equation was developed using a 2^4 full-factorial design to determine the percentage of caffeine that remains trapped into α-LA nanotubes as a function of environmental conditions that may occur in food products.

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