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Keratin:Zein particles as vehicles for fragrance release on hair

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ABSTRACT

Hair perfumes are becoming a trend all over the world and new cosmetic formulations have been developed to address this recent need. In the present study, we developed a system for the controlled delivery of perfumes on hair, based on zein, a protein derived from maize, and on keratin. The Keratin:Zein particles, obtained using different strategies and proportions of zein and keratin, presented a high stability along storage, related with the presence of keratin on the particles' formulation. When applied on hair, the particles formed a film-like structure over the fibers. Fragrance diffusion from the hair-coated particles was dependent on the temperature, the method of particles' preparation and the fragrances' physicochemical properties (melting point and vapor pressure). The particles formulations showed also the capacity to improve hair's mechanical properties and hydration degree. The new system based on Keratin:Zein particles revealed high potential for the development of personalized hair cosmetic products allowing adjustments depending on the personal sensory preferences.

1. Introduction

Hair cosmetic market has been looking for new ingredients and technologies to create formulations for the protection, treatment and adornment of hair. Its role in the protection and thermal regulation of human body, together with is social relevance, makes hair a target for the development of innovative and appealing products (Jacob et al., 2010; Ueno et al., 2014). Fragrances have been extensively used in cosmetic products to an ample variety of consumer products including perfumes, soaps, and personal care products (Ciriminna and Pagliaro, 2013). Unfortunately, most of the fragrances are very sensitive presenting a low stability, which is associated with the molecules' reactive groups and low solubility. Additionally, the fragrances' degradation do not only cause changes in their sensory properties but may also induce some allergenic reactions (Sansukcharearnpon et al., 2010). Their encapsulation is thus foreseen as an strategy to protect the fragrances against chemical, thermal and/or physical instability and expand their applicability in several cosmetic products (Kaur et al., 2018; Salaün, 2016; Yang et al., 2014). Protein based-particles offer several advantages for the encapsulation and the delivery of fragrances. The encapsulation of fragrances in protein-based particles provides the stabilization and controlled release of the entrapped fragrance and improves the handling of the compounds and the fragrances' safety (Sansukcharearnpon et al., 2010).

Systems composed by keratin and zein were developed in this study for fragrance encapsulation. Prior to the application of the encapsulated fragrances on hair, this new delivery system has to satisfy several conditions including: (i) high encapsulation efficiency; (ii) long-term stability; (iii) small particle size with narrow size distribution and (iv) binding capacity to the hair (Günay et al., 2017; Kaur et al., 2018; Lima et al., 2016; van Soest, 2007).

Zein comprises approximately 45–50 % of the total proteins existent in maize, presenting high hydrophobicity (Lawton, 2002). Due to the special tertiary structure, composed 50–60 % by α-helix, zein can self-assemble to form micro- and nanoparticles through liquid–liquid dispersion or solvent evaporation approaches (Luo and Wang, 2014; Pascoli et al., 2018). The isolated zein is biodegradable, biocompatible and has high coating capacity which provides an advantage for its use as delivery system of drugs, enzymes, essential oils and other compounds (Lai and Guo, 2011; Pascoli et al., 2018; Rosa et al., 2015; Wang et al., 2019; Zhang et al., 2019). Keratin proteins refers to a broad category of insoluble proteins organized as intermediate filaments that form the

Abbreviations: DLS, Dynamic Light Scattering; SEM, scanning electron microscopy; TGA, thermogravimetric analysis; GC–MS, gas chromatography–mass spectrometry; RT, room temperature; PDI, polydispersity index.

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bulk of cytoplasmic epithelia and epidermal appendage structures like hair, horn, nails, wool and feathers (Rouse and Van Dyke, 2010). Taking in consideration the protein structure and the function, keratin proteins can be classified as soft or hard keratins. Hard keratins are the main responsible for the tough structure of epidermal appendages like hair, nails and horns. These proteins contain a high cysteine content and are capable to form inter and intramolecular disulphide bonds, giving keratin its high strength and stiffness (Barone et al., 2005; Martelli et al., 2006). Soft keratins are found in the stratum corneum and in the callus and present a lower cysteine content than the hard keratins (Li et al., 2011; Rouse and Van Dyke, 2010).

Due to its excellent mechanical and protective properties, keratin is largely explored for the development of cosmetic formulations. Keratin and mainly keratin hydrolysates have been widely used in personal and hair care products (Dias, 2015). Studies using functional keratin proteins demonstrated that these proteins were able to protect hair from the damaged caused by chemical treatments (relaxation and perming), while improving the mechanical strength of the hair fibers and decreasing the fading of color from dyed hair (Roddick-Lanzilotta et al., 2007). In the last years, our group has developed keratin-based particles for the improvement of the stiffness, tensile strength, thermal stability and smoothness of virgin and overbleached Asian hair (Tinoco et al., 2018a).

Here, we developed a system for the release of fragrances from hair, using zein and keratin proteins to encapsulate linalool or menthol. The Keratin:Zein particles were prepared by two different strategies: in strategy A, the zein nanoparticles were formed and then the keratin was associated by electrostatic deposition; in strategy B the keratin was co-precipitated with the zein during particle's formation (Fig. 1).

The stability of the prepared Keratin:Zein particles encapsulating the fragrances was evaluated using dynamic light scattering (DLS) and the particles' application on hair was characterized by scanning electron microscopy (SEM). The effect of the treatments on hair properties was evaluated in terms of hair mechanical properties and hydration degree. Gas chromatography-mass spectrometry (GC–MS) experiments and a human panel were selected to evaluate the fragrance release from the hair treated with the Keratin:Zein particles encapsulating the linalool or the menthol fragrances.

2. Materials and methods

2.1. Reagents and materials

Zein from maize (powder) was purchased from MerckSigma, Spain. Linalool (96 %) and (-)-menthol (99 %) were acquired from TCI chemicals, Belgium. Ethanol HPLC grade was obtained from Fluka, Portugal. All other reagents were acquired from MerckSigma, Spain, and used as received. Natural Asian hair was provided by International Hair Importers & Products Inc. (Glendale, New York U.S.A.). Gas chromatography vials (22.5 × 75.5 mm, septa of silicone blue transparent/PTFE white) were purchased from Enzymatic, Portugal.

2.2. Keratin extraction and purification

Hair donated from local hairdressing salons was used to extract the keratin, after washing the hair according to the IAEA/RL/50 1978 recommendations to remove contaminants and lipids (Tinoco et al., 2018b). A protocol adapted from Ayutthaya et al.(2015) was used for the keratin extraction from hair has previously reported (Tinoco et al., 2020, 2018b). Briefly, we used a solution comprising 0.2 M sodium dodecyl sulphate, 8 M urea and 0.5 M of sodium metabisulphite in a ratio of 10 mL of solution to 1 *g* of dry hair (Ayutthaya et al., 2015). The extraction solution was heated to 100 °C, the hair was added to this solution and the mixture was incubated for 30 min with agitation. After that time, the mixture was incubated over night at 37 °C, under constant agitation. The extraction solution was then centrifuged at 4000 rpm for 10 min, and the supernatant was filtered using paper filter to remove remaining hair fragments. The keratin solution was dialyzed for 5 days against distilled water using a dialysis membrane with a 14 kDa of cut-off (Tinoco et al., 2018a).

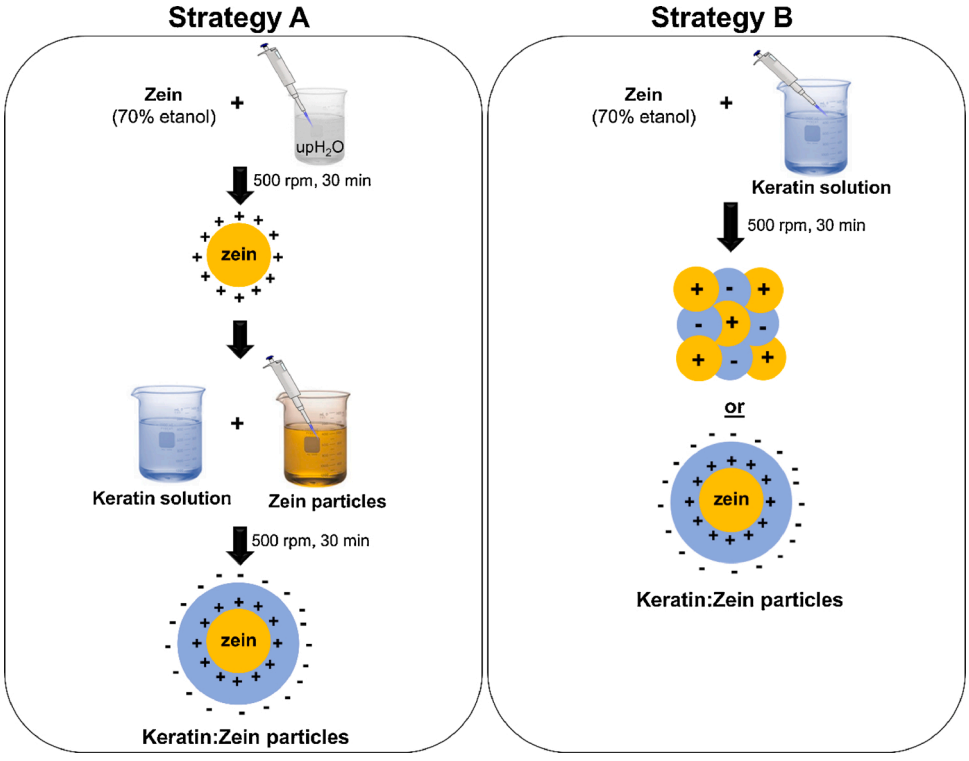


Fig. 1. Schematic representation of the procedures (strategy A and B) used to prepare the Keratin:Zein particles.

2.3. Preparation of the Keratin:Zein particles

To prepare the Keratin:Zein particles, two strategies represented on Fig. 1 were used (strategy A and B). In strategy A, 2 mL of a zein solution (50 mg/mL, pH 5, in ethanol 70 %) was added to a beaker containing 8 mL of ultrapure water (upH_2O), to obtain the zein particles. The particles were then subjected to agitation at 500 rpm for 30 min, at room temperature (RT). The zein particles were then coated with keratin, considering different Keratin:Zein ratios of 2:1, 1:1, 1:2; with the final zein concentration being maintained constant at 5 mg/mL (Table 1). The particles were then stirred at 500 rpm for 30 min, at RT. In strategy B, 1 mL of the zein solution (50 mg/mL, pH 5, in ethanol 70 %) was added to a beaker containing 9 mL of a keratin solution and the particles solution was stirred at 500 rpm for 30 min, at RT. The final keratin concentration was determined considering the zein concentration of 5 mg/mL and the Keratin:Zein ratios of 2:1, 1:1, 1:2. The concentration of zein and keratin, here used, were optimized to improve the particles' stability over time and particles' physical properties like size, surface charge and polydispersity index.

To quantify the particles formation efficiency, the free protein was separated using a centrifugal filter tubes with a 100 kDa of cut-off (Vivaspin 20, GE Healthcare, Spain). The free protein was quantified using DC™ Protein Assay (Bio-Rad, Portugal) according to manufacturer, and bovine serum albumin (BSA, MerckSigma) was used to perform the calibration curve. The efficiency of particles formation was determined by Eq. 1:

$$Eff.\ particles\ (\%) = \frac{[IProtein]i - [Protein]f}{[Protein]i} \times 100 \quad (1)$$

Being Eff.particles the efficiency of particles formation; $[Protein]_i$ is the protein concentration used to prepare the particles; $[Protein]_f$ is the free protein concentration quantified by DC™ Protein Assay.

2.4. Characterization of the Keratin:Zein particles

The mean size diameter, polydispersity index (PDI) and surface charge of the Keratin:Zein particles were measured using a Zetasizer Nano ZS (Malvern Instruments) at 25 °C. The samples were measured without any dilution, and read in triplicate, being the results described as mean ± standard deviation.

2.5. Preparation of the Keratin:Zein particles encapsulating the linalool or the menthol fragrances

Considering the Keratin:Zein particles physical characteristics and stability, the ratio 2:1 was selected for the fragrances encapsulation. Briefly, in strategy A, 2 mL of the zein solution (50 mg/mL, pH 5, in ethanol 70 %) were added to 8 mL of the fragrance solution (previously prepared in ethanol 70 % HPLC-grade) dissolved in upH_2O. The zein particles encapsulating the fragrances were then incubated for 30 min, with constant agitation at 500 rpm. The beaker was sealed to avoid the fragrance evaporation. The zein particles encapsulating the fragrances were then coated with the keratin as described in 2.3. The Keratin:Zein particles encapsulating the fragrances were then incubated with constant agitation (500 rpm) for 30 min, at RT, and were stored completely sealed at 4 °C until further use. In strategy B, 2.5 mL of a zein solution (50 mg/mL, pH 5, in ethanol 70 %) were added to 22.5 mL of keratin solution where the fragrances (prepared in ethanol 70 % HPLC-grade) were previously dissolved. The particles formulation with the fragrances were then stirred at 500 rpm for 30 min, at RT, and were sealed and stored at 4 °C to avoid the fragrance evaporation until further use. The particles, prepared by the two strategies, presented the final concentrations of the components: $[keratin]_{final}$ =10 mg/mL, $[zein]_{final}$ =5 mg/mL, and $[fragrance]_{final}$ =0.5 mg/mL.

Table 1
Final concentration of keratin and zein proteins in the Keratin:Zein particles formulation, according to the different ratios used for particles preparation.

Keratin:Zein Ratio	[Keratin] mg/mL	[Zein] mg/mL
2:1	10	5
1:1	5	5
1:2	2.5	5

2.6. Fragrance encapsulation efficiency

To determine the fragrance encapsulation efficiency into the Keratin:Zein particles, the free fragrance was separated from the particles formulations, by size-exclusion chromatography (SEC) using a 5 kDa of cut-off PD-10 Desalting Column (GE-Healthcare). The amount of fragrance encapsulated into the particles was indirectly quantified by GC–MS by the difference between the initial concentration of fragrance added to the particles (0.5 mg/mL) and the non-encapsulated fragrance separated through the PD-10 Desalting Column. Calibration curves using known concentrations of linalool and menthol were previously performed by GC–MS for encapsulation efficiency determination. Relatively to the fragrance encapsulation stability during storage at 4 °C, the fragrance release was measured over time. Briefly, after each storage time points (1, 3 and 7 days), the SPME fiber was inserted in the middle of the vial and the samples were incubated for 1 h at 4 °C. The fragrance release was then evaluated by GC–MS and compared with calibration curves prepared in the same conditions.

2.7. Characterization of the Keratin:Zein particles encapsulating linalool or menthol

The physical characteristics of the Keratin:Zein particles encapsulating the linalool or the menthol fragrances were measured using a Zetasizer Nano ZS (Malvern Instruments), as described in 2.3.1. Changes in the chemical structure of the Keratin:Zein particles were studied by Fourier Transform Infrared Spectroscopy (FTIR). The spectra were obtained using a Bruker Alpha II (Massachusetts, USA) and acquired by the software Opus 8.22.28. Samples were mounted directly over the crystal and the spectra were acquired between 400−4000 cm^{-1} wavenumbers with a 2 cm^{-1} resolution.

3. Hair treatment and characterization

3.1. Hair treatment with the Keratin:Zein particles

Virgin Asian hair samples were washed using a commercial shampoo (Pantene® Basic, Portugal) and dried at room temperature, prior treatment. Hair tresses with about 90 mg of weight were treated with 1.5 mL of Keratin:Zein particles (~15 mg/mL for the 2:1 Keratin:Zein ratio), a volume sufficient to cover the hair fibers, at room temperature, for 1 h. After this period, the hair samples were removed from the particles solution and let dry at room temperature.

3.2. Scanning electron microscopy analysis

The analyses of the hair samples treated and untreated with the Keratin:Zein particles were performed using a desktop Scanning Electron Microscope (Phenom ProX, Netherlands), with the ProSuite software. The hair samples were placed on aluminium pin stubs covered with electrically conductive carbon adhesive tape (PELCO Tabs™) and observed at 5 kV, with a spot size of 3.3. Before analysis, the samples were covered with 20 Å of gold to improve samples electrical conductivity.

3.3. Hair fiber tensile test

Hair fibers' mechanical properties (Young's modulus and Tensile

Strength) were assessed using a dynamometer Hounsfield machine, in order to study the effect of the Keratin:Zein particles on hair. The differences in hairs' mechanical properties were determined following the guidelines outlined in ASTM D1145−95 for fiber tensile testing. For these tests, 20 single hair fibers with low variability in diameter were selected and were individually attached to a tensile jig (paper template with a fixed gauge length of 20 mm). Initially, the hair fibers in the tensile jigs were placed in an excicator for 48 h to avoid excessive humidity. Before the tensile test, the paper template was cut across so the measurements could be performed in the middle section of the hair fiber. The stretching measurements were performed at a rate of 1.5 mm/min, with a 0.01 N preloaded force, until the fiber broke (Tinoco et al., 2018a). The data are presented as a mean ± SD of twenty samples.

3.4. Thermal gravimetric analysis

The water content loss from the hair fibers was determined using a TGA 4000 (Perkin Elmer, Waltham, MA, US) and acquired using the Pyris software (version 13). Hair samples (8−10 mg) were analyzed between 25–800 °C at 20 °C/min under a nitrogen atmosphere (flow rate: 20 mL/min) and the weight loss, in percentage, and its derivative were represented as function of temperature (Sessa et al., 2011). The temperature calibration was done by Curie temperatures of reference materials: alumel, nickel and perkalloy at the same conditions of the tested samples.

Fragrance release from the hair treated with the Keratin:Zein particles encapsulating the menthol or the linalool fragrances

The release of the fragrances from the hair treated with the Keratin:Zein particles encapsulating the menthol or the linalool fragrances, was evaluated by headspace GC–MS using manual injection of the SPME fiber (100 μm polydimethylsiloxane), as performed in Gonçalves et al. (2019) (Gonçalves et al., 2019). Briefly, 500 μL of the Keratin:Zein particles (~15 mg/mL for the 2:1 Keratin:Zein ratio) encapsulating the fragrances were added to 30 mg of virgin Asian hair with 1.5 cm of length and were incubated at 25 °C for 1 h to promote the adsorption of the particles onto the hair. The hair samples were then transferred to a GC vial, and a SPME fiber was inserted in the middle (~40 mm from top) of the vial. The samples were incubated for 16 h at 25 or 37 °C and the release of the fragrances was evaluated by GC–MS. The GC analyses were carried out using a GC Varian 4000 system with a split/splitless injector coupled to a mass spectrometer (MS). The GC–MS conditions for this analysis were based on Gonçalves et al. (2019) (Gonçalves et al., 2019). Injections were operated at 250 °C in the split mode, with ratio 1:10 and using a Rxi-5Sil MS (Restek) column (30 m Å~ 0.25 mm, and 0.25 μm film thickness), with a column-head pressure of 7.3 psi using helium as carrier gas. The oven temperature started at 45 °C for 5 min, and the temperature increased until 250 °C at a rate of 7 °C/min. A full scan mode (50−750 m/z) was applied for the identification of the target compound. The mass spectrometer (MS) was operated in the electron ionization (EI) mode at 70 eV with total ion chromatogram (TIC) detection mode for quantitative determination and S/N ratio of 5. Calibration curves for linalool and menthol fragrances were performed using the same conditions of the samples (volume, temperature and time) and were measured in duplicate. The amount of fragrance released from the hair treated with the particles was determined by integration of the peaks from chromatograms and the area value was substituted on the calibration curves.

3.5. Sensorial assessment using a human panel

A sensorial olfactory test was performed with 31 volunteers to evaluate if the hair samples treated with the Keratin:Zein particles with linalool or menthol presented a pleasant smell. The cohort included 31 volunteers, 18 females and 13 males, with ages between 20 and 42 years. A blind control (untreated hair) was included in each set of hair samples to validate the results. The olfactory test was performed in the following order: the volunteer smelt first the untreated Asian hair and then the hair samples treated with the fragrance-encapsulated Keratin:Zein particles and the blind control in a random order. Afterwards, it was asked to the volunteer to select the sample (corresponding to a strategy and a fragrance) which conferred the best smell to the hair.

3.6. Statistical analysis

Statistical comparisons were performed by one-way ANOVA with GraphPad Prism 5.0 software (La Jolla, CA, USA). Tukey's post hoc test was used to compare all the results between them, and a Dunnet's test was used to compare the results with a control. A p-value$\leq$0.05 was considered to be statistically significant.

4. Results and discussion

4.1. Keratin:Zein particles characterization

Zein colloidal particles can be prepared by controlled precipitation in ethanol/water solutions of zein by changing the characteristics of the solvent using water as an antisolvent (Patel et al., 2010; Zhong and Jin, 2009). The stability of the zein particles is often limited because of its sensitivity to temperature, pH and ionic strength (Dai et al., 2016; Davidov-Pardo et al., 2015). To circumvent these problems, copolymers like proteins are normally incorporated into the particles' formulation to improve the dispersion stability (Davidov-Pardo et al., 2015; Joye et al., 2015). In this work, we selected keratin extracted from hair to stabilize the zein particles and to promote the interaction of the particles with hair, based on the natural affinity of this protein towards the hair fibers (C. F. Cruz et al., 2017a). Two strategies were explored to include the keratin into the particles' formulations. In strategy A, the keratin was associated to the zein nanoparticles by electrostatic deposition after zein particle formation (Joye et al., 2015) while in strategy B, the keratin was co-precipitated with the zein during particles formation (Patel et al., 2010).

Particles formulation was optimized regarding the methodology used (strategy A and strategy B), zein concentration (1, 2.5 and 5 mg/mL) and keratin concentration (2.5, 5 and 10 mg/mL). When the zein particles (5 mg/mL) were prepared at pH 5, they presented positive charge (≈29−34 mV) and an average size of 148 ± 14.11 nm, however at the end of 1 week of storage at 4 °C they started to precipitate and formed a clump at the bottom of the vial – Fig. 2.

The same behavior was observed in the work of Cheng and Jones (2017) where zein nanoparticles prepared at pH 5.25–6.75 presented significant precipitation (Cheng and Jones, 2017). However, when the keratin was included into the zein particles' formulation, the particles revealed a high stability for at least 90 days – Figs. 2 and 3. The interaction between the two proteins was promoted by electrostatic forces since zein and keratin present, respectively, a positive and a negative charge at pH 5. It was also observed that, the addition of the keratin into the zein formulation caused the particles' surface charge to shift from positive (29.7 ± 1.27 mV) to negative (≈ −24 mV), corroborating the hypothesis of the presence of keratin coating the zein particles. The mechanism involved in the stabilization of the zein particles by the keratin could be either by electrostatic repulsion, steric stabilization, or a combination of both effects that is known as electrosteric stabilization (Patel et al., 2010). Keratin could have the tendency to localize and adsorb on the surface of zein particles due to their amphiphilic and charged nature, providing an electrostatic and steric repulsion against aggregation. This behavior was also observed for sodium caseinate that stabilized zein colloidal particles (Patel et al., 2010).

The formation efficiency of the Keratin:Zein particles was determined by quantifying the free protein separated using centrifugal filter tubes (100 kDa cut-off) and the results are present in Table 2.

Despite the particles' formation efficiencies higher than 80 %, there was an influence of the strategy used for the particles' preparation.

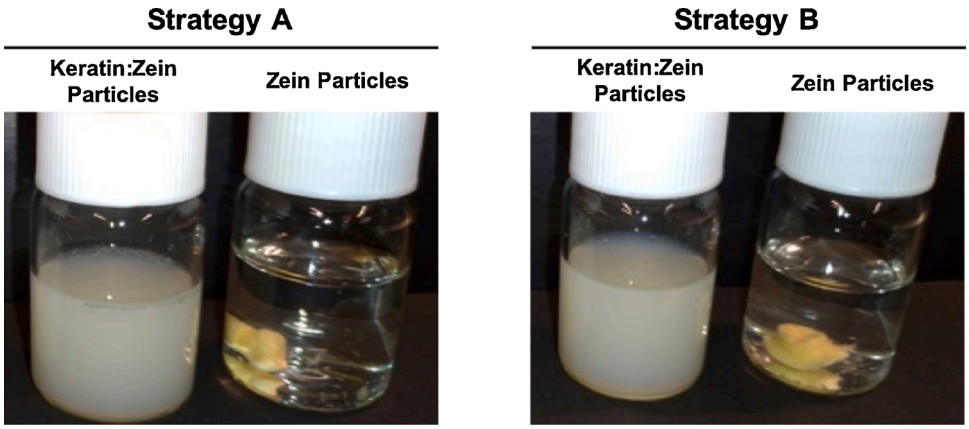


Fig. 2. Effect of the keratin on the stability of the zein particles, prepared at pH 5 by the strategy A or B. The photographs were taken after 1 week of storage at 4 °C.

While for the particles prepared using strategy A, the formation efficiencies were higher than 96 %, for the particles prepared by strategy B, the formation efficiencies were around 83.0 %. The differences in the formation efficiency between both strategies could be related with the particles' structure: core-shell particles for strategy A or mixed particles for strategy B (Davidov-Pardo et al., 2015). The formation of the core-shell particles is likely to be a more efficient process for the synthesis of the particles, which is corroborated by the higher particle formation efficiency obtained for strategy A. This phenomenon of particle formation could also affect the particles capacity to encapsulate different compounds/fragrances, as demonstrated later in this study.

The size, PDI, and surface charge of the Keratin:Zein particles were measured by dynamic light scattering (DLS), and the stability of the particles was evaluated until 90 days of storage at 4 °C (Table 2 and Figure S1). Similar sizes, PDI and zeta-potential values were obtained for the Keratin:Zein particles prepared using different ratios of keratin and zein and the two strategies. After 90 days of storage at 4 °C, the size of the Keratin:Zein particles (ratio 2:1) obtained using strategy A varied between 231.8–243.7 nm with a PDI of 0.108–0.166, and the particles produced by strategy B varied between 239.9–259.7 nm with a PDI of 0.096–0.127. The small variations measured along time (not statistically significant) indicate a great stability of the Keratin:Zein particles and of their components. Due to the presence of the keratin on the particles' surface, all formulations presented a negative surface charge (~ from −19 to −25 mV). The formulation Keratin:Zein (ratio 2:1) was selected for further assays due to the higher content of keratin, which might increase the particles' affinity towards the hair. This greater affinity is associated with the high cysteine content of keratin and with specific hair-binding amino acidic sequences present on the keratin sequence (C. F. Cruz et al., 2017a).

Relatively to the particles structure, different organizations between the zein and the keratin could be obtained depending on the strategy used for the particles production. In strategy A, the particles' structure is most likely characterized by a positively charged core-shell of zein with the negatively charged keratin protein adsorbed on the surface. For strategy B, the final structure depends on the kinetics of two competing processes that occur during particle formation: precipitation of the hydrophobic protein (zein) and association of the keratin with the zein. Depending which process is predominant in the beginning of the particles' formation process, the Keratin:Zein particles could have a zein core-shell surrounded by the keratin or be mixed particles composed by keratin and zein. If the zein particle formation occurs more rapidly than the association between the keratin and the zein, it would be expected a zein core-shell surrounded by keratin. In another way, if the association between zein and keratin occurs more rapidly than the zein particle formation, it would be expected a mixed internal structure to be formed, with both proteins distributed through the particle matrix (Davidov-Pardo et al., 2015; Patel et al., 2010).

Although a detailed understanding of the particles' formation mechanism is outside of the scope of this paper, the absorption of keratin on the surface of zein particle during zein precipitation seems to be the most likely mechanism to happen on strategy B since zein is a highly hydrophobic protein that will rapidly organize and form particles when in contact with water (Davidov-Pardo et al., 2015; Patel et al., 2010).

4.2. Keratin:Zein particles encapsulating linalool and menthol fragrances

After particles optimization, relatively to the concentration of keratin and zein, the ratio 2:1 was selected to produce the Keratin:Zein particles with the linalool or the menthol fragrances. The strategies A and B were used to prepare the particles encapsulating the fragrances in order to evaluate the potential effect of the particles structure on the fragrance release. To determine the fragrance encapsulation efficiency into the Keratin:Zein particles, the free fragrance was separated from the particle formulations by size-exclusion chromatography using a PD-10 Desalting Column (GE-Healthcare) with a 5 kDa of cut-off. The free fragrance was quantified by GC–MS and this value was subtracted to the initial concentration of fragrance (0.5 mg/mL) used to prepare the particles. Encapsulation efficiencies upper than 77.6 % were obtained for all the tested particles, with a higher encapsulating efficiency being verified for the particles prepared by strategy A when compared with the particles obtained using strategy B (Table 3).

These differences could be explained by the higher efficiency of particle formation obtained for strategy A (around 97 %) and the core-shell structure of the particles prepared by the same strategy, that could promote a higher fragrance encapsulation when compared to the mixed structure obtained for the particles prepared by strategy B. The same behavior was observed by Li et al. (2017), where higher encapsulation efficiencies were obtained for the core-shell zein particles encapsulating coumarin 6, when compared with the mixed structure zein particles (Li et al., 2017).

The efficiency of the encapsulation was evaluated by measuring the releasing rates of linalool and menthol over time by GC–MS, when the particles were stored at 4 °C (**Figure S2**). It was observed an increase in the fragrances release until day 3, however after this day the release of the two fragrances was constant, reaching a plateau around 5% of the initial encapsulated fragrance. These results indicate that the Keratin:Zein particles are excellent systems for the encapsulation of these fragrances with a high encapsulation efficiency.

4.3. Particles size, size distribution and surface charge characterization

The particles produced through the two strategies remained stable through the 90 days of storage at 4 °C (last measurement), exhibiting along of these 90 days sizes ranging between 280−310 nm (Fig. 3). Both strategies presented a monodisperse character and surface charge with

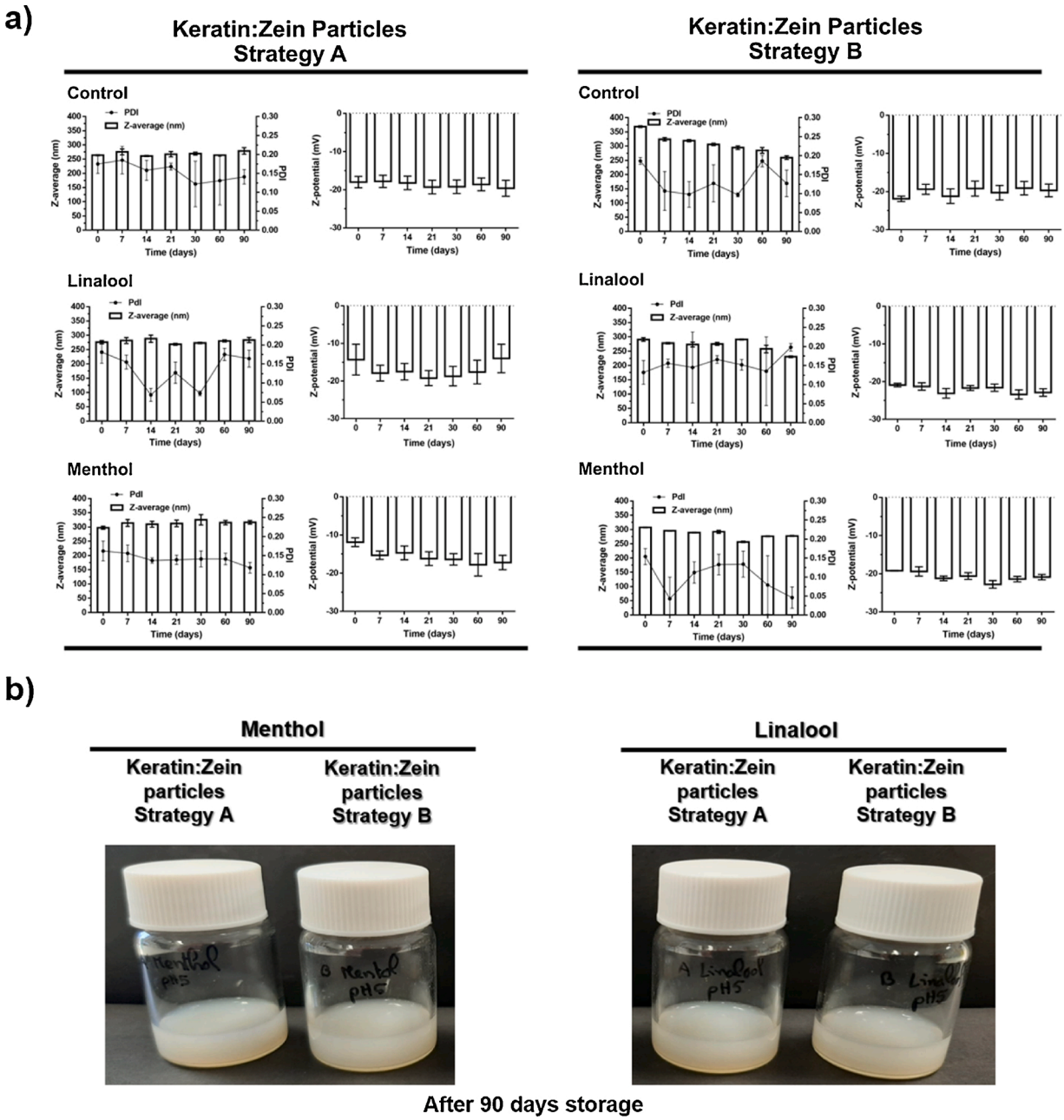


Fig. 3. a) Effect of storage at 4 °C on the physicochemical characteristics of the Keratin:Zein particles ('Control') and the Keratin:Zein particles encapsulating linalool ('Linalool') or menthol ('Menthol'), when prepared by strategy A or strategy B; b) Images of Keratin:Zein particles' formulations after 90 days storage at 4 °C. Values represent the mean ± SD of 3 independent experiments.

approximately −20 mV, indicating that both strategies produced uniform and stable particles.

4.4. Particles analysis by FTIR

FTIR analysis was performed to assess the intermolecular interactions between zein and keratin proteins during the formation of the Keratin:Zein particles, and to evaluate the encapsulation of the linalool and menthol into the particles (Fig. 4). The spectra of keratin and zein, showed the characteristic peaks of proteins, corresponding to the amide I and amide II (C═O bonds) (Davidov-Pardo et al., 2015). For the keratin, these peaks, amide I and amide II, appeared at 1653 and 1538 cm^{-1}, respectively; while for the zein a small shift in the peaks (1645 and 1585 cm^{-1}) was observed. Some other characteristic peaks were observed for these proteins. The peaks at 632, 1024 and 3282 cm^{-1}, which correspond to C–S bonds present in cystine, C—O stretching vibration and to N—H bonds, respectively, were detected for the keratin (Estévez-Martínez et al., 2013). For the zein, it was detected a peak at 3304 cm^{-1}, which result from the stretching vibration of N—H and —OH from the amides in the amino acid residues (Dai et al., 2016). Comparing the spectra of the keratin and zein proteins with the spectra of the Keratin:Zein particles, prepared by strategy A or strategy B, it was observed the appearance of two new peaks at 878 and 2030−2043 cm^{-1} for the particles (* in Fig. 4). These peaks could be due to the interaction between the proteins during the particle's preparation. The peak at 2030−2043 cm^{-1} could be attributed to an overtone, indicating the presence of aromatic residues from the amino acids of keratin and zein proteins present on the particles' surface, while the peak at 878 cm^{-1} could be due to the C═C bending from these aromatic compounds (Khademi-Azandehi and Moghaddam, 2015; Lassenberger et al., 2016).

Table 2
Formation efficiency of Keratin:Zein particles prepared by strategy A and B and using different Keratin:Zein ratios. Physicochemical properties (size, PDI and surface charge) of the particles after 90 days of storage at 4 °C.

Strategy	Particles	Keratin: Zein ratio	Formation efficiency (%)	Size (nm)	PDI	Surface Charge (mV)
	Zein (control)	–	97.41 ± 0.003	–	–	–
A	Keratin: Zein	1:2	96.88 ± 0.002	243.7 ± 4.8	0.166 ± 0.066	−20.8 ± 2.2
		1:1	97.51 ± 0.005	231.8 ± 2.8	0.132 ± 0.066	−20.9 ± 2.5
		2:1	96.89 ± 0.012	233.6 ± 8.1	0.108 ± 0.018	−23.5 ± 4.0
B	Zein (control)	–	97.66 ± 0.001	–	–	–
	Keratin: Zein	1:2	83.58 ± 0.002	251.6 ± 4.6	0.096 ± 0.005	−20.3 ± 1.8
		1:1	82.34 ± 0.007	239.9 ± 3.2[a]	0.150 ± 0.029	−20.6 ± 1.1
		2:1	84.64 ± 0.005	259.7 ± 6.6[a]	0.127 ± 0.035	−19.7 ± 1.7

Size, PDI and surface charge of zein particles were not represented in this table since the particles precipitated during storage at 4 °C.
The size results with the superscript 'a' are significantly different between them ($p-value \leq 0.01$).

Table 3
Fragrance encapsulation efficiency (%) of linalool and menthol in the Keratin: Zein particles (ratio 2:1) prepared using strategy A and B. The fragrance encapsulation efficiency was determined by quantification of free fragrance using GC–MS and linalool and menthol standard curves.

Sample	Strategy	Fragrance	Fragrance encapsulation efficiency (%)
Keratin:Zein particles	A	Linalool	80.2 ± 2.3
	A	Menthol	88.8 ± 3.5
	B	Linalool	77.6 ± 0.8
	B	Menthol	87.3 ± 2.4

Moreover, the presence of the keratin and zein characteristic peaks, 632 cm^{-1} of the C–S bonds in the keratin and 3304 cm^{-1} of the stretching vibration of N—H from amides for the zein, in the Keratin:Zein particles spectra corroborate the presence of the two proteins in the final particles.

Regarding the fragrances, the menthol spectra presents peaks at 3226 cm^{-1} which corresponds to the OH group; at 2867 and 2929 cm^{-1} which are attributed to the C–H stretching vibrations; at 1344 cm^{-1} that corresponds to the isopropyl group and at 1024 and 1045 cm^{-1} which are attributed to C—O bonds (Al-Bayati, 2009). For the linalool fragrance, the spectra show significant peaks at 3383 cm^{-1} that are attributed to the stretching of O—H bond of an alcohol; at 2924−2970 cm^{-1} corresponding to the C–H stretching vibrations and at 1109 cm^{-1} that is assigned to the C—O stretching vibration (Al-Moghrabi et al., 2018). Most of the characteristic peaks of linalool or menthol are not detected or are masked when the fragrances were encapsulated into the Keratin:Zein particles. The absence of the fragrances' characteristic peaks, in conjugation with the encapsulation results determined by GC—MS (Table 3), shows that the fragrances were successfully encapsulated using both strategies. Also, the absence of new peaks in the particles encapsulating the fragrances suggested that the fragrances and the proteins might be physically attached without the formation of any new chemical bond (Davidov-Pardo et al., 2015).

4.5. Application of the Keratin:Zein particles in virgin Asian hair

After optimizing the conditions for the synthesis of the Keratin:Zein particles, the ability of the particles to adsorb to the hair fibers was tested. The hair was incubated with the particles' formulations and, after hair drying, the hair remained soft and loose (**Figure S3**). The ability of the Keratin:Zein particles to bind to the hair fibers was evaluated by SEM (Fig. 5). Comparing the fibers of the untreated virgin Asian hair (control) with the fibers treated with the Keratin:Zein particles prepared by both strategies, it is possible to verify the deposition of the particles onto the hair surface on the treated samples, while the control samples present a smooth surface with cuticles without any type of material deposited. No differences were observed in the particles' deposition pattern between the two strategies of particles preparation and no alteration in the hair thickness was detected after particles application. In both samples the particles are tightly bound to the hair and present a random distribution along the hair fibers, with a higher accumulation in the interlayer zone of the cuticles. It is noteworthy that no differences can be found

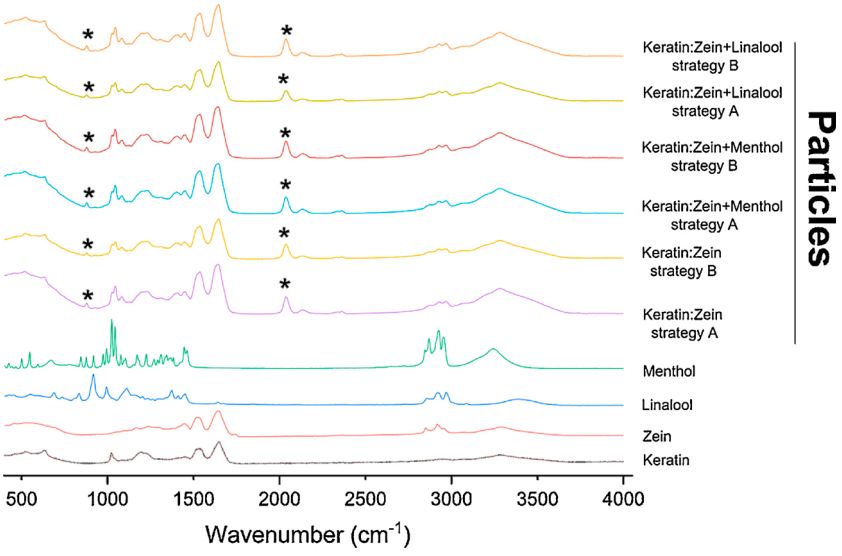


Fig. 4. FTIR spectra of Keratin:Zein particles and Keratin:Zein particles encapsulating linalool or menthol, when prepared by strategy A or strategy B. Keratin, zein, linalool and menthol were used as references. Bands marked with * represent changes observed during particles formation.

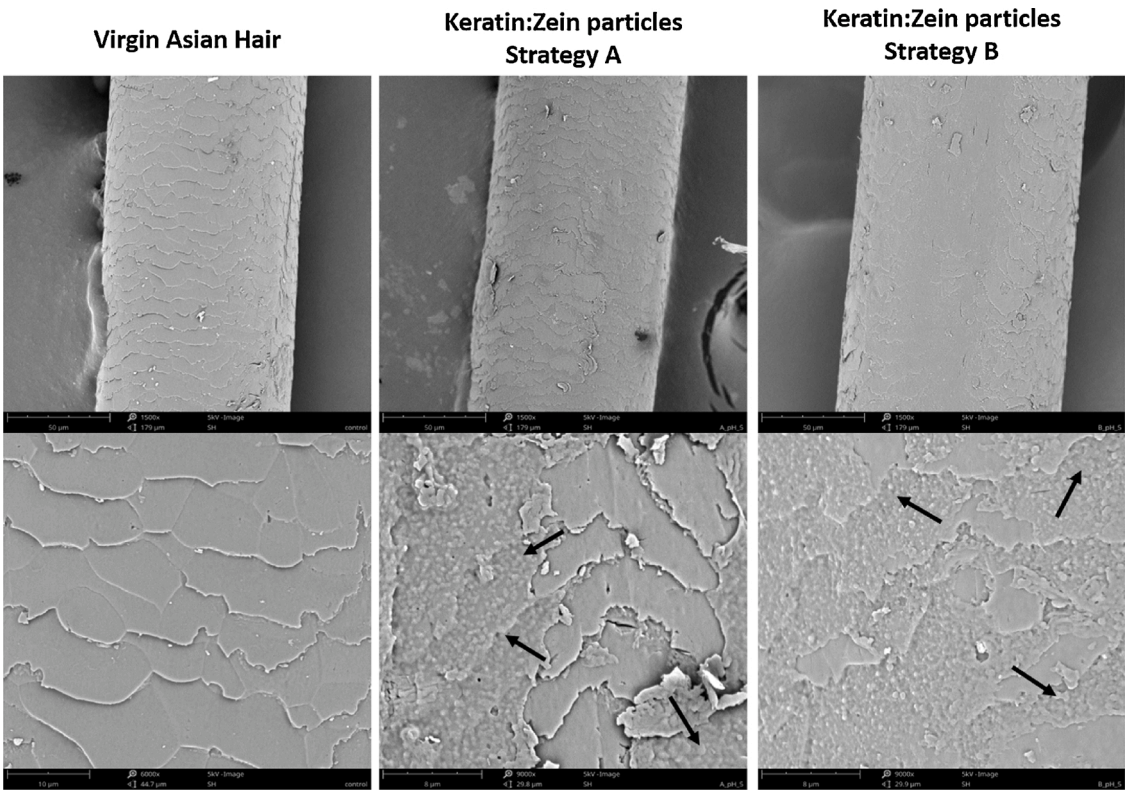
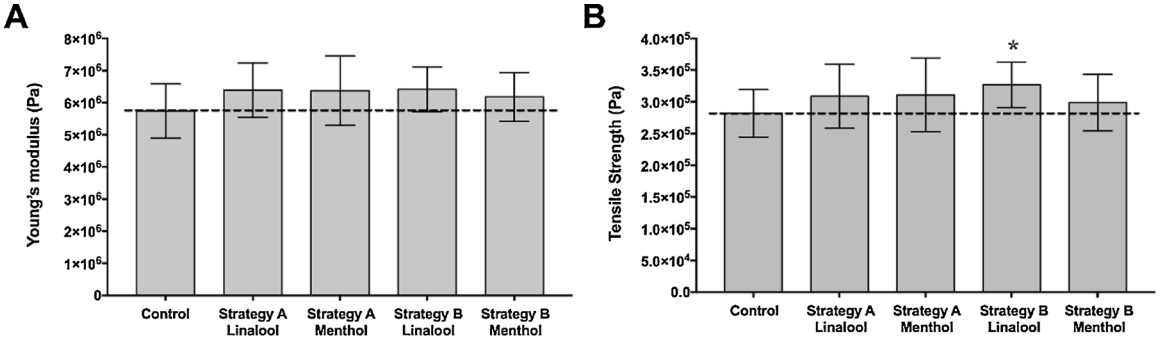


Fig. 5. SEM micrographs (1500x and 9000x) of untreated virgin Asian hair (left), Asian hair treated with the Keratin:Zein particles prepared by the strategy A (middle) and Asian hair treated with the Keratin:Zein particles produced by strategy B (right). The presence of the Keratin:Zein particles on hair surface is highlighted by arrows.

regarding the morphology of the Keratin:Zein particles prepared by strategy A (zein core – keratin shell morphology) and strategy B (zein core – keratin shell morphology or mixed morphology of particles composed by keratin and zein). Since zein is a highly hydrophobic protein, which rapidly organizes into particles when in contact with water, the Keratin:Zein particles prepared by strategy B most likely display a predominant zein core – keratin shell morphology.

This deposition pattern is similar to the one observed for the hair conditioners which accumulate near the cuticle edges, masking the damages caused by environmental factors, daily care routines or



C

Sample	Strategy	Encapsulated fragrance	Young's modulus improvement (%)	Tensile strength improvement (%)
Keratin:Zein particles	A	Linalool	11.46	9.63
	A	Menthol	11.00	10.47
	B	Linalool	11.96	15.97
	B	Menthol	7.82	6.07

Fig. 6. Effect of the treatment with the Keratin:Zein particles encapsulating the linalool and menthol fragrances on the mechanical properties of Asian virgin hair: (A) Young's modulus, (B) tensile strength. Statistically significant differences from the control and the treated hair meshes are indicated as *p-value$\leq$0.05. (C) Improvement of Asian hair mechanical properties measured in terms of Young's modulus and tensile strength after treatment with the Keratin:Zein particles, compared with the untreated Asian virgin hair.

cosmetic procedures like bleaching, straightening, relaxing, coloration, among others (Bhushan, 2008; Latorre and Bhushan, 2005). A film-like structure surrounding the hair fibers was also observed. This behavior was already expected due to the excellent film forming properties characteristic of the zein protein (Cabin-Flaman et al., 2018; Lai and Padua, 1997; Torres-Giner et al., 2008). The keratin protein contain peptide sequences that have been designed by nature to interact with the keratin from hair through several hydrophobic, hydrogen and disulphide interactions (C. F. Cruz et al., 2017a, 2017b). This formulation was developed to be applied daily like a normal perfume, and it can be easily washed off after hair shampooing (**Figure S4**).

4.6. Effect of Keratin:Zein particles treatment on the mechanical properties of hair

Cosmetic formulations are described to affect the mechanical properties of hair. Depending on the composition, hair cosmetic formulations can protect hair from damage and recover its properties (Tinoco et al., 2019, 2018b) or decrease the overall performance of the hair fibers (Jeong et al., 2010; Nogueira et al., 2004). To study the effect of the treatment with the Keratin:Zein particles on the hair mechanical properties, the hair mechanical resistance was determined taking in consideration the fibers' Young's modulus and tensile strength (Fig. 6 **A-B**). Analyzing Fig. 6 all of the tested formulations increased the Young's modulus and the tensile strength values of the hair fibers when compared with the untreated Asian virgin hair (control).

The improvement of the mechanical resistance caused by the Keratin:Zein particles application was determined comparatively to the values obtained for the control samples, and are presented in Fig. 6 **C**. Although an increase in both parameters was verified, no significant differences were obtained for the tested formulations, with exception for the hair treated with the particles encapsulating linalool and prepared using the strategy B (increase of 16 % for the tensile strength parameter). From previous works, we determine that the effect of keratin-based particles on the mechanical properties of hair could be related with the particles interaction with the hair and with the number of particles and their distribution along the hair (Tinoco et al., 2018b). Relatively to the particles interaction with the hair, they may re-establish some of the hydrophobic, hydrogen and disulphide bonds lost during daily routine, resulting in an improvement of hair's mechanical properties (Célia F. Cruz et al., 2017).

4.7. Effect of the Keratin:Zein particles on hair hydration

Water content is an important factor in the maintenance of a healthy hair. However, daily routines and cosmetic procedures damage the keratin structure, inducing a decrease in fibers' water content (Barba et al., 2009). The effect of the treatment with the Keratin:Zein particles encapsulating the linalool or the menthol, on the thermal stability and water content of hair was followed by thermal gravimetric analysis (TGA) (Table 4). Analyzing the TGA curves (**Figure S5**), different decomposition profiles and temperatures were observed depending on the treatment. The first weight loss stage was observed between 25−165 °C due to the loss of loosely (25−65 °C) and tightly bound water (65−165 °C) (Aluigi et al., 2007; Barba et al., 2010). The second loss weight loss is related to the organic degradation of the hair fiber and destruction of lateral chains of keratin protein, happening in the range of 165−550 °C (Li and Wang, 2013). In the last step, from 550−800 °C, it was observed a complete degradation of the hair keratin structure (Aluigi et al., 2007; Barba et al., 2009). The remaining weight percentage verified at 800 °C is associated with the carbon molecules existent in the protein and others structures that did not decompose at this temperature, and remain as ashes (Thonpho and Srihanam, 2016). In the thermograms of the hair treated with the Keratin:Zein particles formulations, various loss steps were observed between 165−500 °C that were not observed in the control sample. The additional peaks could be related with the presence of the zein and the keratin proteins in the hair fibers, which can be localized in the hair surface or deeper in the hair cortex (Bragulla and Homberger, 2009).

Table 4
Effect of Keratin:Zein particles application on the moisture content of hair characterized by TGA.

Sample	Strategy	Fragrances	Moisture content* (%)	Residue weight at 800 °C (%)
Virgin Asian hair	–	–	7.18 ± 0.04	11.85
Virgin Asian hair treated with Keratin:Zein particles	A	Linalool	8.31 ± 0.06****	19.14
	A	Menthol	7.72 ± 0.04**	17.22
	B	Linalool	7.36 ± 0.05	18.50
	B	Menthol	8.46 ± 0.07****	18.84

*the moisture content is the fibers' weight loss calculated between 25−165 °C. Statistically significant differences from the control and the treated hair meshes are indicated as **p-value≤0.01 or ****p-value≤0.0001.

Considering only the temperature range between 25 and 165 °C from the thermograms, whose percentage corresponds to the loss of water molecules from the fibers, it is possible to evaluate the moisture content of the different hair samples (Aluigi et al., 2007; Barba et al., 2010). Through the analysis of Table 4, we observe an increase of the total moisture content of the treated hair samples when compared with the untreated hair. The increase in the fibers' water content after application of the Keratin:Zein particles results from: (i) the ability of the proteins to bind water molecules, forming an appropriate environment for healthy hair (Ribeiro et al., 2013); and (ii) the film-forming properties of zein that may confer a moisture barrier surrounding the hair, decreasing the water loss from the fibers (Lai and Padua, 1997; Tihminlioglu et al., 2010).

Considering the remaining weight percentage after hair samples heating at 800 °C, it was observed that the treated hair samples show a higher weight percentage comparatively to the control samples (increase between 5.4–7.3 %). This increase could result from a stabilization of the keratin structure given by the mixture of zein and keratin proteins, making the hair fiber more resistant to higher temperatures.

Fragrances release from hair treated with Keratin:Zein particles encapsulating linalool or menthol

The main goal of the present study was the development of an efficient system for the release of fragrances from hair. Two temperatures, 25 and 37 °C, were selected to perform the fragrance release experiments. These temperatures correspond to the room temperature and the body temperature or the temperature in a hot day in summer. The release of the fragrances was evaluated along 16 h, which corresponds to the normal period where an individual is awake. The calibration curves for linalool and menthol were prepared determining the area of GC–MS peaks of increasing concentrations of these fragrances at the same conditions of the samples (**Figure S6**). From the GC–MS data, a unique peak was obtained for linalool and for the menthol corresponding to a mass spectrum with retention time of 13.7 min and 15.8 min characteristic of linalool and menthol, respectively (**Figure S7**). The chromatograms of the hair treated with the Keratin:Zein particles without the encapsulated fragrances did not reveal any peak (data not shown). This indicates that the matrix did not have any effects on the release of the fragrances, validating the GC–MS (SPME-HS-GC–MS) experiments.

The GC–MS data showed an increased release of both fragrances when they were encapsulated into the Keratin:Zein particles following strategy B and at 37 °C (Fig. 7). Regarding the fragrance release at 25 °C, a release of 33 % and 44 % was measured for linalool encapsulated using strategy A and strategy B, respectively. While for linalool, at 37 °C, the method of particle preparation/fragrance encapsulation significantly influenced the release profiles (p-value≤0.5), no significant differences

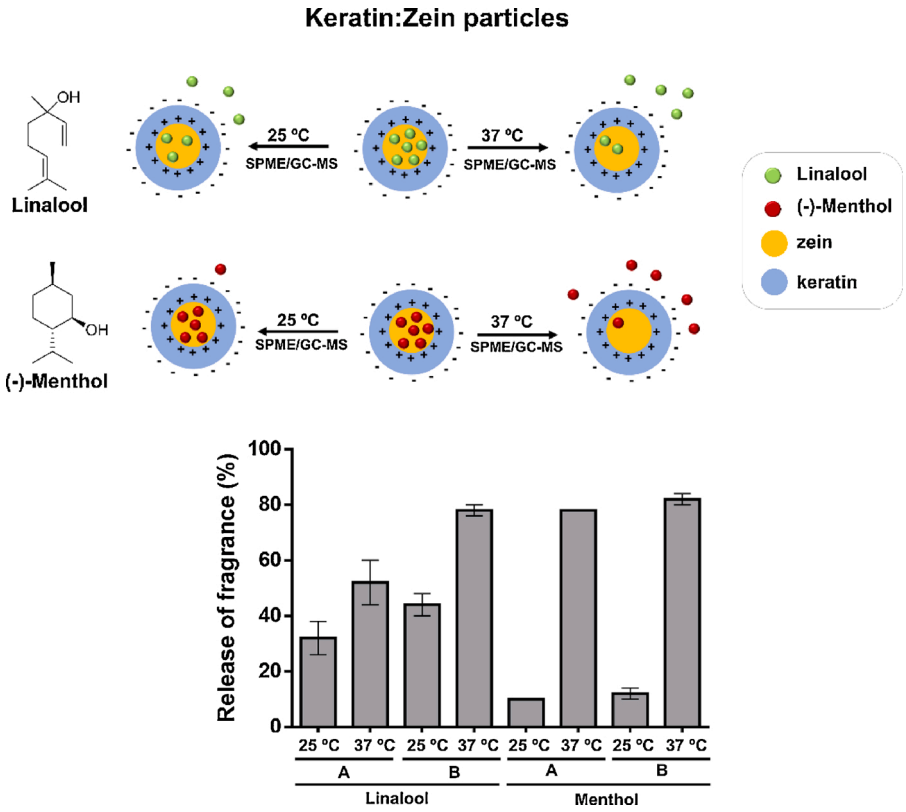


Fig. 7. Effect of temperature and method of encapsulation on the release of linalool and menthol from treated virgin Asian hair. The SPME fiber was exposed for 16 h at 25 or 37 °C and the fragrances were quantified by GC–MS.

were detected for the menthol when comparing the two strategies of preparation. However, the increase of the temperature from 25 to 37 °C promoted a higher release of the fragrances from the hair. This higher release promoted by the temperature was particularly relevant for the hair samples treated with the Keratin:Zein particles with menthol, with an increase up to 68–70 % when compared with the release measured at 25 °C.

The differences in the release profiles of linalool and menthol can also be associated with the structure and physicochemical properties of these fragrances. Despite the two fragrances belong to the class of monoterpenes, there are some structural differences between the linalool and the menthol. While the first is linear, the menthol has a ring in its structure which confers the molecule with some molecular rigidity. This might influence the molecule mobility with the temperature – less mobility at room temperature and higher mobility near the menthol melting point (39 °C). During encapsulation, the menthol most likely formed crystalline structures in the Keratin:Zein particles core. However, when the temperature increased to values near the menthol melting point, the molecule mobility and volatility increased, boosting the release of this fragrance from the particles. Moreover, the vapor pressure at 25 °C for the linalool and menthol is 0.159 mm Hg and 0.00767 mm Hg, respectively (PubChem database). Therefore, the linalool can have a greater ability to release from the particles at 25 °C comparatively to the menthol.

4.8. Sensorial assessment of the fragrance release from the hair using a human panel

The effect of cosmetic formulations incorporating fragrances on hair is an essential parameter during the development of new hair cosmetic products. Most of end-users prefer products that leave the hair with a pleasant smell instead of a neutral scent. A subjective assessment with a cohort of 31 volunteers (18 females and 13 males) was performed to study the effect of the Keratin:Zein particles encapsulating the linalool or the menthol on the hair odor (Fig. 8).

Analyzing Fig. 8 **'Total'**, 77 % of the volunteers classified as 'Favorite' the formulations encapsulating the linalool (including both strategy A and B), while only 16 % of the volunteers gave the same classification for the formulations encapsulating the menthol fragrance. Comparing the results regarding sex, 83 % of the females and 69 % of the males classified as favorite the hair treated with the formulations containing the linalool. Although the majority of the females and males' participants classified as 'Favorite' the formulations containing linalool, only a few percentage of the participants gave the same classification for the formulations containing menthol. The formulation containing menthol were classified as 'Favorite' by only 23 % of males and by 11 % of females. The differences between the results from the formulations containing linalool and menthol could be justified by the variances in the fragrance's perception according to the volunteer's gender. Donna (2009) described that fresh scents appear more in masculine fragrances and they are present in most personal care products for men (Donna, 2009). This corroborate the results obtained in our study, where the hair treated with the Keratin:Zein particles with menthol presented a higher preference by the male gender. In the other hand, floral scents tend to be a feminine category and are present in 42 % of woman's products and perfumes (Donna, 2009). In this olfactory test, it was also observed that a small number of volunteers, 6.45 %, classified the neutral scent (hair without fragrance application) as the 'Favorite' sample.

According to the volunteers, the low preference for the formulations encapsulating menthol was mainly due to the intensity of this fragrance. Therefore, a reduction of the menthol concentration could increase the pleasantness of the tested formulations with menthol.

5. Conclusions

This work showed a promising and low-cost delivery system for the

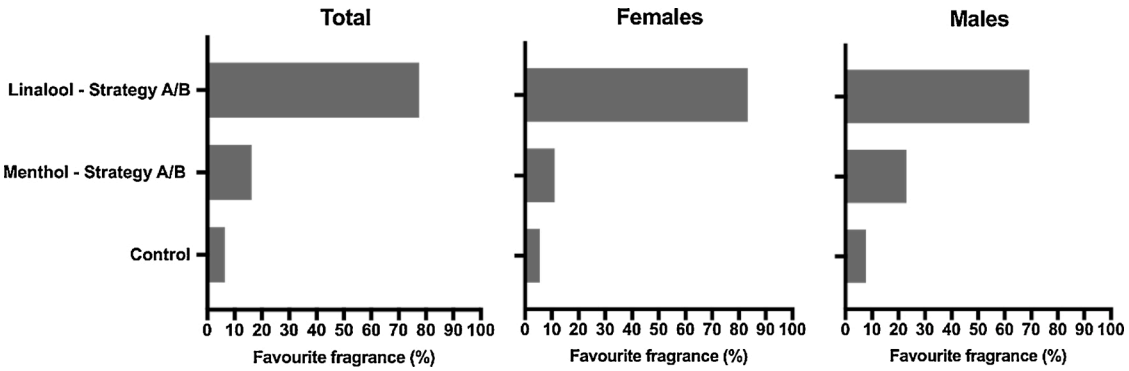


Fig. 8. Percentage of volunteers that classified as 'Favorite' the fragrance obtained from the hair treated with Keratin:Zein particles encapsulating linalool or menthol, when compared with the control sample (untreated virgin Asian hair). The olfactory test was performed by 31 volunteers (18 females and 13 males).

release of fragrances from hair. The Keratin:Zein particles, prepared using two different approaches (strategies A and B), were stable when stored at 4 °C and were capable to encapsulate the linalool or the menthol fragrances. The inclusion of keratin on the particles' formulation increased the particles stability and the adhesion of the particles to the hair. Additionally, the formulations revealed high efficiency of fragrance release, and were able to protect the fragrances during storage. The treatment of Asian hair with the developed formulations demonstrated to be effective in the improvement of hair's properties, traduced on the increase of Young's modulus, tensile strength, and hydration degree. An olfactory test revealed, that the majority of the 31 volunteers preferred the formulations encapsulating linalool independently of the strategy used for the particles' preparation. The fragrances used in this study were selected as a model, proving the capacity of these particles to be personalized according to the personal perception of smell.

CRediT authorship contribution statement

Ana Tinoco: Methodology, Validation, Formal analysis, Investigation, Writing - original draft. **Filipa Gonçalves:** Methodology, Validation, Formal analysis, Investigation, Writing - original draft. **André F. Costa:** Methodology, Formal analysis. **David S. Freitas:** Methodology, Formal analysis. **Artur Cavaco-Paulo:** Writing - review & editing, Supervision. **Artur Ribeiro:** Conceptualization, Methodology, Writing - review & editing, Supervision.

Declaration of Competing Interest

The authors report no declarations of interest.

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Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:https://doi.org/10.1016/j.indcrop.2020.113067.

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