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Fragrance release profile from sonochemically prepared protein microsphere containers

Oshrat Tzhayik [a], Artur Cavaco-Paulo [b], Aharon Gedanken [a,*]

[a] Department of Chemistry and Kanbar Laboratory for Nanomaterials, Bar-Ilan University Center for Advanced Materials and Nanotechnology, Bar-Ilan University, Ramat-Gan 52900, Israel
[b] University of Minho, Textile Engineering Department, P-4.800058 Guimaraes, Portugal

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ABSTRACT

Protein microspheres have been prepared by sonicating a mixture of pure fragrant oil (amyl acetate (AA)) with an aqueous protein (bovine serum albumin) solution. The prepared protein spheres are nano- to micrometer sized with an encapsulation efficiency of approx. 97% for the AA present on the surface and inside the BSA capsule. Containers were found stable for more than 6 months when stored sealed at 4 °C and 20 °C. For the release profile measurements, we used a simple, automated and direct method. We continuously weighed the encapsulated microspheres and measured the evaporation rates. The release profiles at 15 °C and 25 °C display two different evaporation rates. The higher rate is the sum of a few evaporation rates, including water molecules, while the slower rate is due to the evaporation of pure AA. The changes in the evaporation rates occur upon the collapse of the container. This event coincides with the full evaporation of water. For morphological characterization we dyed the AA with Nile red, and used SEM, ESEM, Cryo-SEM, light microscopy, and confocal laser scanning microscopy measurements.

1. Introduction

Durable fragrances are still one of the main aspirations in the cosmetics, food, textile, detergents and pharmaceutical industries. Fragrance compounds and essential oils are volatile substances; they react with other components and are very sensitive to the effects of light, oxygen, high temperature, humidity, and other factors.

Encapsulation is an effective technique for protecting active agents against the environment, rapid evaporation, contamination, and also for releasing the volatile substance from the enclosed capsules, as required [1]. The most common form of controlled release in many industries is encapsulation, where active molecules are surrounded by a layer of material (shell) that prevents their release and the penetration of environmental factors until desired. Thus, encapsulation improve the performance of a fragrance in regard to its tenacity or endurance [2]. Depending on the nature of the shell material, encapsulation can control the agent's release across the capsule to the bulk medium [3].

The release of the core from encapsulated material can be controlled through prolonged release, started release, or a combination of the two. In prolonged release, the core is released over an extended period of time, whereas started release is initiated by pH, heat, mechanical pressure, moisture, etc. [2]. The release measurements of the active agent in capsules are a key factor in encapsulation technology and govern the desired industrial applications of the capsules. Up to now, different methods for preparing capsules, based either on chemical or mechanical processes, have been considered and enhanced [3].

In this work we used amyl acetate, which has a characteristic apple–banana scent, as a fragrance model, and produced amyl acetate encapsulated protein (bovine serum albumin) containers using a one-step sonochemical method. Suslick and co-workers have carried out pioneering work on the ultrasonic synthesis of protein microspheres (PMs) [4,5]. The PMs are prepared by sonicating an aqueous solution of a protein with an over-layered organic liquid. The formation of microspheres under the influence of ultrasound is related to the oscillation of acoustic waves and the subsequent disruption of the solvent by ultrasound. The size of the microspheres is mainly determined by the energy input, which is a function of acoustic variables, such as the acoustic power employed in the sonication process, the sonication time, and the volume of the material being subjected to sonication [6]. Suslick proposed that for sulfur-containing proteins (e.g., bovine serum albumin (BSA) [7], human serum albumin (HAS) [4] and hemoglobin (Hb) [8]), the mechanism of the formation of stable oil- and gas-filled microspheres involves the cross-linking of cysteine residues through a disulphide bond formation. The

* Corresponding author.
E-mail address: gedanken@mail.biu.ac.il (A. Gedanken).

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inter-protein cross-linking is caused by the superoxide radical generated during the acoustic cavitation process leading to the formation of the PMs [7]. If the sonication is conducted using the same components, i.e., an aqueous solution of protein and an over-layered organic liquid, and a drug is dissolved in one of the phases, a PM is formed that encapsulates the drug. It has been claimed that the one-step sonochemical encapsulation process is extremely effective in producing a core–shell structure with high encapsulation efficiency [9].

PMs prepared by sonication usually have a broad size distribution due to the disruption of acoustic sound [6]. The prepared microspheres are nano- to micro-sized and biocompatible [10]. Generally, they have diameters between 100 nm and 50 μm with the 2.5 μm as the most abundant size [6]. The sono-prepared PMs are stable at room temperature for several months, which is ascribed to the protection of the stable cross-linking protein shell [10–12]. It has been found that the initial size and size distribution of fresh samples prepared at different protein concentrations are similar [13,14].

Bovine serum albumin (BSA) is the most commonly used solution for the preparation of protein microspheres due to its availability in a pure form, its biodegradability, non-toxicity, low cost, and non-immunogenicity [15]. Thus, it is not surprising that researchers have found BSA to be an excellent candidate for drug encapsulation [10,12,16–18].

Recent studies have investigated the fragrance-release profile using an indirect method. Feczkó and co-workers [1] prepared ethylcellulose (EC) capsules and chitosan (CS)-coated EC capsules by using an oil-in-water emulsion solvent evaporation method and compared the release of vanillin from both capsules. They air dried the capsules at elevated temperatures and sampled them at certain time intervals, after which the samples were dissolved in dichloromethane and the vanillin content determined by UV–VIS spectrophotometry. The release of vanillin from the capsules was investigated for 3 weeks at 50 °C. After 6 days, 80 mg of the CS-coated capsules released 20% of the total vanillin content, while the uncoated capsules lost the same vanillin percentage after 1 day. Their experiment showed that EC could efficiently sustain the delivery of vanillin, and an additional CS layer can elongate the release of the fragrance.

Gumi and co-workers [3] used the phase-inversion precipitation technique and prepared several polysulfone (PS) capsules containing different vanillin concentrations. In order to study the release properties, they added water to 1 g of capsules and stirred at 700 rpm during several days. They periodically sampled the bulk solution that was hermetically stored until analysis, and then checked the release of vanillin from the capsules by using high performance liquid chromatography (HPLC). Their release tendencies presented a rapid increase within the first 10 h, followed by an equilibrating period of a slow increase until reaching a plateau. The higher encapsulation of the initial vanillin amount, the sooner the plateau is attained. The maximum capacity of encapsulated vanillin was achieved when capsules were obtained from a 15% w/w of PS and 10% w/w of vanillin.

Sansukcharearnpon and co-workers [19] encapsulated six fragrances (camphor, citronellal, eucalyptol, limonene, menthol, and 4-tert-butylcyclohexyl acetate) with a polymer blend of ethylcellulose, hydroxypropyl methylcellulose and poly (vinyl alcohol) using the solvent-displacement method (water displacement of ethanol). The release profiles of the centrifuged, air-dried particles were acquired by quantifying the amount of fragrance remaining in the samples that had been left uncovered for specified times. Quantification was carried out using an electronic nose, GC–MS, and thermal gravimetric analysis (TGA). The release profile of the encapsulated fragrances indicated different characteristics amongst the six fragrances. Limonene showed the fastest release, while eucalyptol and menthol showed the slowest release. The release rate of the encapsulated fragrance was independent of the fragrance's vapor pressure. In general, these techniques require, among others, sampling the capsules at exact time intervals, transferring capsules from one container to another, stirring for days [3], heating, dissolving [1], etc., thus involving large release profile errors.

The aim of this study was to prepare fragrance-encapsulated protein spheres and investigate their release profile by using a very simple, in real-time, automated, low-cost and direct method. We prepared amyl acetate (AA) encapsulated in BSA durable spheres based on the ultrasonic synthesis method, and examined the release profile of AA by using continuous weight measurements in an open vial at ambient temperatures, and comparing them to the evaporation rates of the free components. As opposed to previous work, in this research we didn't dissolve the fragrance in either the aqueous or the organic phase. The pure fragrant liquid itself (AA) served as both the fragrance and the organic phase required for the formation of the protein spheres.

2. Materials and methods

2.1. Materials

Albumin, from bovine serum ⩾96% (BSA); amyl acetate ⩾99% (AA); albumin, fluorescein isothiocyanate conjugate bovine (f-BSA); Nile red ⩾98.0% (HPLC), were obtained from Sigma–Aldrich. All chemicals were used without any further purification. De-ionized H_2O was used in all the experiments.

2.2. Synthesis of fragrance-encapsulated protein containers

The AA-encapsulated BSA containers (BSA-AA) were synthesized by the sonochemical method, as described elsewhere [20]. Briefly, in a reaction cell we layered 20 ml of pure AA over 30 ml of 0.006%-0.2% (w/v) of an aqueous solution of BSA. We kept the volume ratio between the aqueous phase (BSA aqueous solution) and the organic solvent (AA) at 3:2, in all the experiments. The protein spheres were synthesized with a high- intensity ultrasonic probe (Sonics and Materials, VC-600, 20 kHz, 0.5in Ti horn). The bottom of the high-intensity ultrasonic horn was positioned at the aqueous-organic interface, employing an intensity of about 150 W cm^{-2}, with an initial temperature of 17 °C in the reaction beaker. The sonication lasted 3 min. We placed the reaction cell in an ice-water cooling bath during the sonication in order to maintain a low temperature, thus preventing the denaturizing of the BSA, which might occur due to the heat produced during the sonication process. At the end of the process the temperature in the reaction beaker reached 30 °C (measured by a thermocouple), which is much lower than the BSA denaturation temperature (65 °C). A separation flask was used to separate the product from the mother solution. The separation was accomplished within a few minutes due to the density differences between the spheres and the mother solution. To obtain a more complete separation of the spheres from the mother solution, the separation flask was placed in a refrigerator (4 °C) for 24 h. After separation the product was stored in a refrigerator (4 °C).

Using the same method, we also produced AA-encapsulated f-BSA containers (f-BSA-AA). For this purpose we layered 20 ml of pure AA stained with Nile red over 30 ml of a f-BSA 0.006% (w/v) aqueous solution.

2.3. Characterization

For imaging, scanning electron microscopy (SEM) measurements were conducted using *Inspect*, a FEI instrument. SEM

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samples were prepared by applying a drop of a BSA-AA sphere onto a glass wafer, and drying under room temperature. The glass wafer was then vacuum-coated for 3 min with a thin carbon film. The average size and size distribution of the BSA-AA spheres were evaluated by measuring over 100 spheres using light microscopy (ApoTome axioImager.z1) and an environmental-scanning electron microscope (ESEM) (*Ouanta* FEG, FEI) images at 20.00 kV. Confocal images were taken by a confocal laser-scanning system LSM 510 (Zeiss) equipped with a 63× oil immersion objective with a numerical aperture of 1.4. For the Cryo-SEM images, a suspension volume of 1.5 μl PM sample was sandwiched between two flat aluminum platelets with a 200 mesh TEM grid used as a spacer between them. The sample was then high-pressure frozen in a HPM010 high-pressure freezing machine (Bal-Tec). The frozen samples were mounted on a holder and transferred to a BAF 60 freeze fracture device (Bal-Tec) using a VCT 100 Vacuum Cryo Transfer device (Bal-Tec). After fracturing at a temperature of -120 °C, the samples were etched at −110 °C for 5 min and coated with 3 nm of Pt/C by double-axis rotary shadowing. Samples were transferred to an Ultra 55 SEM (Zeiss) using a VCT 100 and were observed using a secondary electron in-lens detector at 1.00 kV at a temperature of −120 °C.

2.4. Determination of the release profiles

The release profile of the AA from the BSA-AA containers was investigated by a direct method. 200 μl of the synthesized spheres were inserted into an open vial and weighed constantly during 60 h with time intervals of 5 s. The sample's weight loss profile *vs.* time served as the release profile of the fragrance (AA) from the spheres. For this purpose we used an AB135-S Mettler Toledo balance with a linearity of 0.2 mg. Each release study was recorded on a computer file using a special LabView environment program that was specifically programmed in our lab for this study. The program enabled us to choose the duration time of the experiment and the time intervals between each weight measurement. The temperature was monitored with a thermocouple.

3. Results and discussion

3.1. Characterization of protein containers

The morphology and approximate sizes of the BSA-AA containers were characterized using an environmental scanning electron microscope (ESEM), a cryo scanning electron microscope (cryo-SEM), and light microscopy. All three devices revealed the BSA-AA containers as smooth surface-spherical particles, and no surface defects were observed (Fig. 1a, b, and c respectively). As Fig. 1 shows, the prepared BSA-AA spheres are nano- to micrometer-sized with a broad size distribution.

Fig. 2 displays the size distribution of BSA-AA containers based on light microscopy and ESEM measurements. During the sonication process, we produced two major types of BSA-AA container size, which are reflected as a clear peak around 1300 nm and a rise in the particle's size towards smaller sized spheres. However, the exact location of the second peak could not be located because of the detection limit of light microscopy (∼300 nm). It is likely that the difference in the size of the containers is caused by the uneven distribution of acoustic energy in the ultrasonic vessel. The region with intense energy is restricted close to the sound-emitting surface of the ultrasonic probe [6]. Furthermore, it has been concluded in previous work [21] that smaller BSA microcapsules are obtained when the encapsulated organic solvent has a higher viscosity. Hence, it is expected that AA as the organic solvent will produce a narrower size distribution and smaller BSA-AA containers

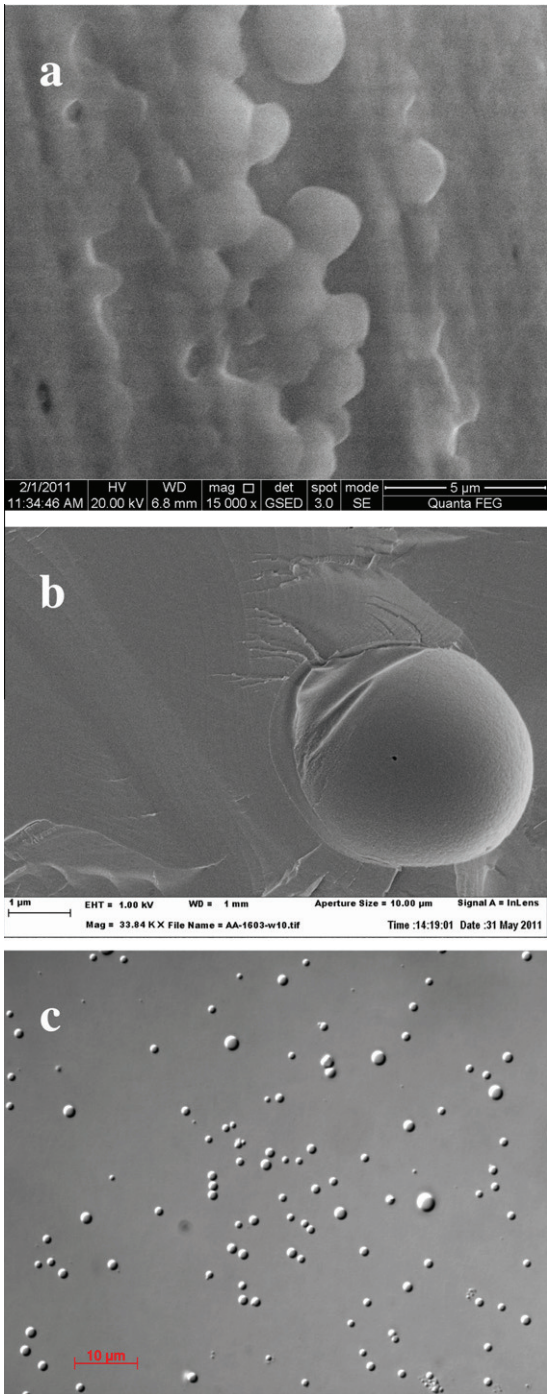

Fig. 1. Images of BSA-AA containers. (a) ESEM; (b) Cryo-SEM; (c) light microscopy. The initial concentration of BSA is 0.006% (w/v).

relative to other organic solvents, with a lower viscosity, like toluene and chloroform, if used in identical conditions during the sonication process.

Changing the BSA concentration from 0.006% to 02% (w/v) in the aqueous phase, maintaining the 3:2 volume ratio of an aqueous:organic layer, doesn't influence the particle size and the size distribution, and only affects the concentration of the particles, i.e., increasing the BSA concentration in the aqueous phase increases the amount of BSA-AA microcapsules. This observation is consistent with the work of Makino and co-workers, [22] who found that as the concentration of BSA increases from 0.005% to 0.02% (w/v), the microencapsulation yield increases. They

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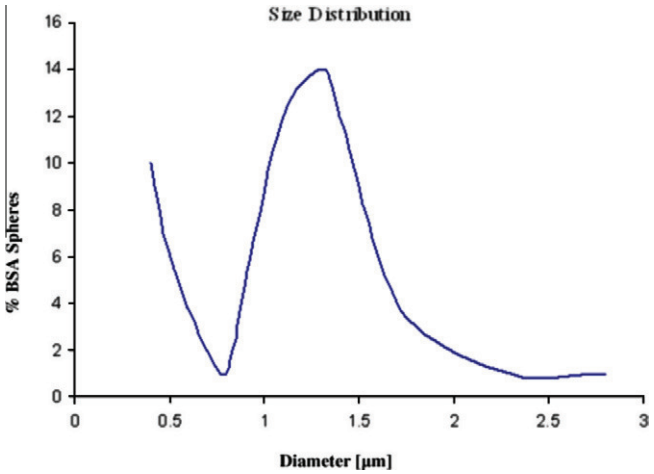

Fig. 2. Size distribution of BSA-AA containers. The initial concentration of BSA is 0.006% (w/v).

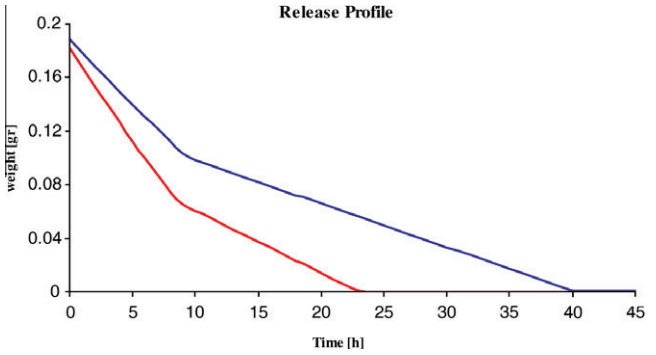

Fig. 4. Release profile of BSA-AA containers at 15 ± 2 °C (blue line) and 25 ± 2 °C (red line) during the first 45 h. The initial concentration of BSA is 0.05% (w/v). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

interpreted the results as originating from the diluted solution in which the frequency of the collision of BSA molecules is low and the density of the microcapsule membrane also seems to be low, which in turn diminishes the yield of the sphere formation.

Encapsulation, or loading efficiency, is defined as the weight percent of encapsulated AA related to the initial AA weight. Typically, at the end of the sonication-encapsulation process (initial concentration of BSA is 0.05% (w/v)) we usually obtained a residue of not more than ~500 μL of AA. Hence, if we take into account the solubility of AA in water (1.24×10^{-3} mol/L at 303 K) [23], this means that in the worst case scenario we have a total of ~506 μL AA that was not encapsulated, leaving us with ~97% encapsulation efficiency. This number includes AA molecules present on and inside the spheres.

Further experiments were carried out in order to confirm the encapsulation of the AA inside the BSA spheres. The fluorescent dye, Nile red, was dissolved in the AA phase before sonication. Nile red is an uncharged heterocyclic molecule and thus is soluble in organic solvents. However, its solubility in water is negligible, less than 1 μg/ml. This property makes Nile red suitable for probing the encapsulation process [24,25]. Confocal laser scanning microscopy (CLSM) images of f-BSA-AA spheres show that the fluorescence of the red color is distributed homogeneously across the sphere's cross-section, indicating that the sphere is loaded with AA containing Nile red [24]. Furthermore, at the same time, the presence of the protein shell is proven by using the labeled BSA protein coupled with a green florophor (FITC). The green proteins are seen to assemble at the surface of the droplets, forming a dense spherical protein layer, as shown in Fig. 3. The high stability of the protein layer is attributed to the formation of disufide bonds among the protein molecules, which is induced by sonication [12]. This observation confirms that the inner side of our spherical BSA containers has a hydrophobic (non-polar) character.

We found that BSA-AA containers are stable for more than 6 months; they can be stored in a sealed bottle either in the refrigerator (4 °C) or at room temperature (~20 °C). The spheres were found to be unstable when they were self dried by placing them in an open glass vial at ambient temperature. The spheres broke at room temperature under the vacuum formed in the SEM chamber and the broken shells are observed (Fig. 5).

3.2. Release profile measurements

We used a very simple and direct method for measuring the release profile of the BSA-AA containers. We placed a measured volume of the BSA-AA containers inside an open vial and placed them on a balance. We predicted that as the AA is released from the BSA-AA containers and evaporates, the sample's weight will be reduced accordingly. Using computer software, we constantly followed the sample's weight during 60 h. These weight measurements are the mirror image of the release profile of the BSA-AA containers. We measured the release profile of AA from BSA-AA containers at two different temperatures, 15 ± 2 °C and 25 ± 2 °C. As described in the experimental section, at the end of the sonication process,

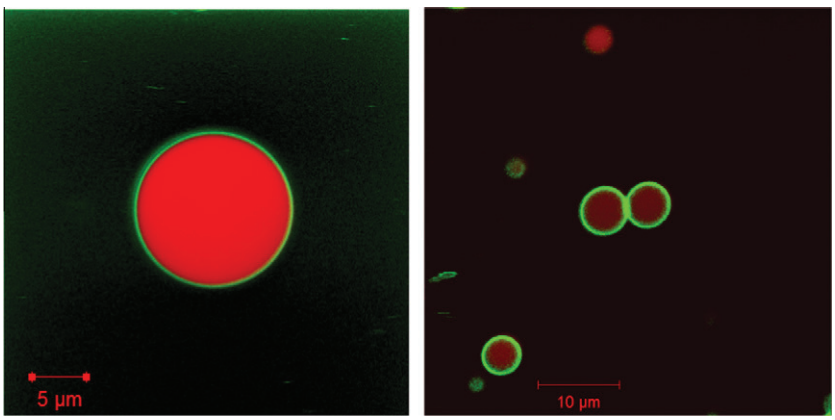

Fig. 3. Confocal images of f-BSA-AA spheres loaded with red dye (Nile red). The BSA protein shell is labeled with a green dye (FITC. The initial concentration of f-BSA is 0.006% (w/v). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

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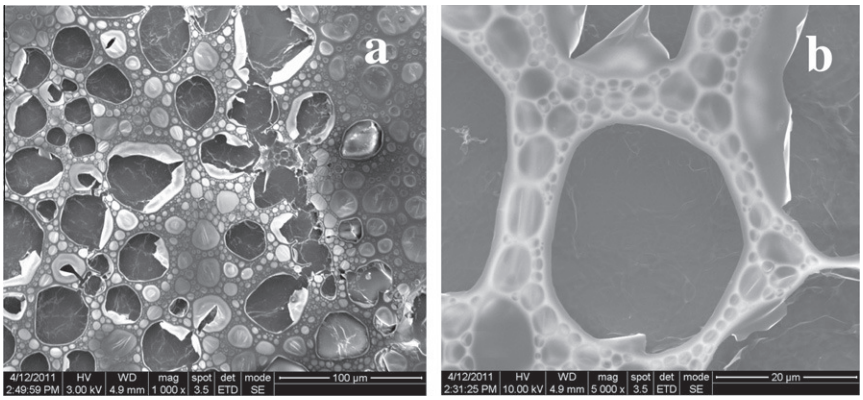

Fig. 5. SEM images of the broken shells of dried BSA-AA containers, magnified by 1000 (a) and by 5000 (b). The initial concentration of BSA is 0.05% (w/v).

the product consists of three layers; water, AA and BSA containers. Thus, identical measurements were carried out for each of these layers and the results compared to those of the BSA-AA containers.

Fig. 4 displays the release profile of the BSA-AA containers. The blue and the red lines display the release profile of the BSA-AA containers at 15 ± 2 °C and 25 ± 2 °C, respectively. Each of the release profiles appears as a straight line with one deflection point, which divides the profile into two lines with different slopes, A and B. In order to evaluate the BSA-AA release profile, we compared these slopes to the evaporation rates that we measured for pure AA, pure de-ionized H_2O, and the aqueous phase that was left after the sonication. Table 1 summarizes all the slope values related to the measurements at 15 ± 2 °C.

In general, our proposed explanation for the behavior described in Fig. 4 is as follows: Line A displays the evaporation of the molecules (AA and water) that are located inside the sphere and on the surface of the BSA-AA containers. Line B displays the evaporation of the molecules that are only located inside the BSA-AA containers. The intersection point, C, represents the time at which the microspheres collapse, and the evaporation rate is equal to that of free AA as seen in Table 1. Indeed, we can observe with the naked eye the appearance of liquid drops inside the glass vial after about 10 h of leaving the PMs at 15 ± 2 °C.

In more quantitative terms, our proposed explanation is substantiated if we take into account the rate values in Table 1. Adding the pure AA evaporation rate (slope = 3.1 mg/h) and the evaporation rate of the 'aqueous phase after sonication' (slope = 7.0 mg/h) gives 10.1 mg/h. This value can be assigned to line A at 15 ± 2 °C, depicting the release profile that has almost the same slope (slope = 9.7 mg/h). This calculation strongly implies that Line A actually describes the molecule evaporation of both pure AA and the 'aqueous phase after sonication' that are located inside and on the surface of the BSA-AA containers. Although most of the evaporation is from the outer surface of the capsule, we cannot exclude the diffusion of the AA molecules from inside the sphere, since the small AA molecules can easily penetrate the BSA protein shell. The interactions between the fragrant AA molecules and the BSA matrix, together with the vapor pressure of the volatile substances on each side of the shell, are the major driving forces influencing diffusion [19]. Fig. 4 and Table 1 enable us to calculate roughly the distribution of the 97% of the AA encapsulated in the BSA spheres between the surface and inside the spheres. The calculation is based on the assumption that in the first 10 h the release of the AA originates from the surface of the sphere, while in the next 30.5 h the AA is released only from inside the spheres. Thus, the ratio of 10/(10 + 30.5) ~25%. This calculation also assumes that the rate of evaporation of the AA molecules from the surface is equal to the rate of evaporation from inside the sphere.

Table 1
Evaporation rates at 15 ± 2 °C* [mg/h].

BSA-AA containers		Pure de-ionized H_2O	Pure AA	Aqueous phase after sonication
A	**B**			
9.7 ± 0.2	3.2 ± 0.2	6.2 ± 0.2	3.1 ± 0.2	7.0 ± 0.2

* The experimental error of these values is ± 5%. The initial concentration of BSA is 0.05% (w/v).

After ~10 h the slope decreases to 3.2 mg/h (Fig. 4. Line B 15 ± 2 °C), which is a value that is almost identical to the slope of the pure AA evaporation rate (3.1 mg/h). After 10 h of drying at room temperature on the balance, the BSA-AA containers had no surrounding moisture, and therefore they started to collapse and spilt their AA content, which evaporated. The evaporation slope of Line B is almost identical to the evaporation rate of pure AA. As suggested by Zhou and co-workers [24], it can be speculated that BSA adsorption at the AA droplet/water interface (when an emulsion is produced by sonication) is weaker and not effective enough due to the relatively high surface tension of AA, which leads to the formation of a relatively weak, or thin, BSA cross-linked shell wall that collapses upon AA evacuation [24].

The difference between the red and the blue lines in Fig. 4 originates at the different temperatures that these two curves represent. Therefore, it is not surprising that the measurements at a higher temperature reveal a faster evaporation rate.

4. Conclusions

In this paper we efficiently encapsulated a fragrance (amyl acetate) in a protein (BSA) container by a one-step sonication method and investigated the fragrance release profile of the encapsulated AAr. We detected two disappearance rates of the AA from the spheres. The higher rate is the sum of a few evaporation rates, also including the water molecules, while the slower rate is due to the pure evaporation of the AA resulting from the collapse of the spheres. Our manuscript manifests the importance of moisture on the surface of the PMs.

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