


Menthol-loaded PLGA Micro and Nanospheres: Synthesis, Characterization and Degradation in Artificial Saliva

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Received: May 12, 2017; Revised: October 10, 2017; Accepted: January 07, 2018

Menthol-loaded PLGA micro/nanospheres were synthesized using the multiple emulsion/solvent evaporation technique. Parameters such as stirring rate, external surfactant content, PLGA/menthol weight ratio, solvent evaporation and lyophilization time were evaluated as well as the degradation of micro/nanospheres in an artificial saliva medium. The menthol/PLGA micro/nanospheres thus obtained had a size distribution of between 217 nm and 13 μ m and particles with spherical and dense morphology were evidenced by SEM micrographs. FTIR and TGA data showed an increase up to 60% of menthol incorporated in the micro/nanoparticles. The degradation of menthol-loaded PLGA micro/nanoparticles in artificial saliva significantly affected the particles morphology and appears to be an effective medium for releasing menthol.

Keywords: *emulsion, menthol, micro/nanospheres, PLGA.*

1. Introduction

Producing micro/nanoparticles (micro/nanospheres and micro/nanocapsules) that enable controlled and/or sustained release of drugs and active principles is one of nanotechnology's most important research areas. To be effective, these micro/nanoparticles should have certain characteristics, such as high encapsulation efficiency and keep the drug or active principle preserved during storage. They should also be easily administered to the target site, present a controlled release rate aiming to achieve a therapeutic effect and minimize side effects, as well as increase the intracellular penetration and pharmacological activity¹⁻⁶.

Administering drugs or active principles *via* microspheres promotes both a protection under adverse conditions as well as a release profile to occur by the desired time^{5,7}.

Currently, there is particular interest in preparing micro- and nanospheres using biodegradable polymers, e.g. poly(lactic acid), PLA, and their copolymers, such as poly(lactic acid-co-glycolic acid), PLGA, considering its well-known biocompatibility and biodegradation characteristics^{5,8-10}.

Moreover, these polymers have interesting characteristics and possess a controllable degradation rate that depends on their molecular weight and lactic acid/glycolic acid ratio¹¹.

Menthol (cyclic monoterpene alcohol), a potential active principle in micro/nanospheres, is the major constituent of essential oils of plants such as peppermint and spearmint, in addition to having characteristics such as the cooling, minty taste and a characteristic odor. This compound has great commercial significance and is one of the main flavoring substances on the market, with an annual consumption of around 32,000 tonnes¹². Menthol also has applications in many pharmaceuticals products, cosmetics, sweets, chewing gum, drinks, toothpastes, shampoos, among others, in order to promote freshness and taste. Furthermore, menthol has various biological properties, such as being antimicrobial¹³, anticancer and anti-inflammatory¹⁴ and its plants can be used as insect repellent^{12,15}.

Microspheres or microcapsules can be used to protect the flavoring substances and the active agents from oxidation, which is caused by factors such as heat, light, humidity, or contact with other substances during storage, by preventing evaporation of volatile substances, such as menthol¹⁶. One method widely employed for preparing micro- and nanoparticles is emulsification followed by evaporation or multiple emulsion, consisting of several steps in which oil/water emulsion formation (o/w) occurs^{1,4,17-24}. A drying process can minimize the limited stability of these systems and lyophilization has been widely used for this purpose to dehydrate colloidal systems, especially liposomes and micro or nanospheres²².

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There is not study regarding micro- and nanospheres with menthol prepared by multiple emulsion and its behaviour in artificial saliva. Thus, in this context, the main goal of this study was to synthesize and characterize PLGA and menthol microspheres using the multiple emulsion (in this case, double emulsion) technique. Additionally, the degradation of these microspheres in a medium containing artificial saliva was evaluated, aiming to study them in a physiological environment.

2. Materials and Methods

2.1. Materials

The copolymer PLGA (PLG 8523 - 85:15 L-lactide:glycolide ratio, 99.4%, PURAC), menthol (99.95%, Vetec), poly (vinyl alcohol), PVA, (86.5-89.5%, Vetec) and dichloromethane (CH_2Cl_2 , 99.5%, Vetec) were used as receivers. Artificial saliva was prepared according to the standard composition used by patients with dry mouth symptoms (xerostomy) caused by radiotherapy for cancer. The artificial saliva was obtained from a 50/50 v/v mixture of solution A and solution B (Solution A: 0.1662% CaCl_2 , 0.0558% MgCl_2 , 0.625% KCl , 0.865% NaCl and water purified QSP. Solution B: 0.80% K_2SO_4 , 0.32% KHSO_4 , 1% sodium benzoate, 50% sorbitol, 0.04% sodium fluorate, 0.2% preservative-methyl/propyl paraben and water purified QSP).

2.2. Synthesis of micro/nanospheres of PLGA/Menthol

Initially the biodegradable copolymer PLGA and the menthol were solubilized in sufficient amounts of CH_2Cl_2 (approximately 1g polymer/100 mL CH_2Cl_2), yielding the oil phase (O). Next, 200 mL of distilled water as first aqueous phase (A_1) was added to the oil phase (O) by stirring (3,600 rpm) with Ultra-turrax® (IKA, model T18 basic), thus producing the primary emulsion (A_1/O). This emulsion was added to the 400 mL of PVA suspension (second aqueous phase, A_2) by stirring (24,000 or 7,000 rpm) for 5 minutes, thus yielding a multiple emulsion ($A_1/O/A_2$), forming nanoparticles in suspension. Next, the organic solvent was evaporated at room temperature for 12h or under reduced pressure with a rotary evaporator (40°C for 1h). Then the micro/nanospheres were isolated by centrifugation (5000 rpm equivalent to 1,400g) in 20 min and washed with distilled water (3 times) to remove the excess PVA. Lastly, the micro/nanoparticles suspensions were frozen with liquid nitrogen and lyophilized (condenser temperature of -110°C to -100°C, pump pressure of 0.66 mbar and sample temperature of -40°C) for the desired time and stored in a desiccator. Nano/microparticles samples with menthol incorporated were submitted the smell essay and it was considered positive (presence of menthol) when the smell was discernible for at least three different individuals.

Table 1 shows the parameters studied (weight ratio for menthol/PLGA, PVA content in the water phase (A_2), stirring rate, time of solvent evaporation and lyophilization) in the preparation of the PLGA/menthol micro/nanospheres and PLGA particles (without menthol).

2.3. Preparing the samples for degradation in artificial saliva

Degradation tests in artificial saliva were carried out with the NP-3 and NP-5 samples (Table 1). These samples (five samples of each) were weighed on an analytical balance (accuracy of $\pm 0.0001\text{g}$, Bel Engineering Ltda). Next, the samples were placed in Falcon tubes containing 10mL of artificial saliva (pH=7) at 37°C and removed at determined times (7, 14, 30, 60 and 90 days). The samples removed from the artificial saliva were centrifuged (500 rpm and 20 minutes) and washed with distilled water (3 times) to remove the excess of saliva solution. Next the samples were dried in an oven (Marconi Ltda) at 100°C until a constant weight was achieved and then stored in a desiccator.

2.4. Characterization of micro/nanoparticles

2.4.1. Fourier transform infrared spectroscopy (FTIR)

The chemical structures of the PLGA/menthol micro/nanoparticles were characterized by FTIR (PerkinElmer Instruments, Spectrum One FTIR Spectrometer or ABB Bomem-MB Series, model Arid-zone). Samples were analyzed in the ATR mode in the 4000-650 cm^{-1} range, using SeZn cell or the transmission mode, in the 4000-450 cm^{-1} . The samples were prepared as a film (in THF) or in a KBr pellet.

2.4.2. Scanning electron microscopy (SEM)

The PLGA/menthol micro/nanoparticle morphology was evaluated using the SEM technique (Philips microscope, model XL30 with a resolution of 3.5 nm in the secondary electron mode), with an accelerating voltage of 20 kV and gold plating samples. After degradation in the artificial saliva, the PLGA/menthol micro/nanoparticles were analyzed using SEM (JEOL microscope, model JSM-6510LV with a resolution of 3.5 nm in the secondary electron mode), with an accelerating voltage of 5kV and gold plating samples. The particle size data were obtained using the digital program "Image Tool" (version 2, GPL) that was applied the SEM images. Measurements were taken on average (different images of same sample) from a set of 18 particles to NP-1 and at least 100 particles for others particles.

2.4.3. Differential scanning calorimetry (DSC)

Glass transition (T_g), melting (T_m) and crystallization (T_c) temperatures were performed with DSC equipment (TA Instruments, model Q20) in a temperature range of -90°C to 200°C, with a heating rate of 10°C/min, in a nitrogen

Table 1. Evaluated parameters in preparing micro/nanoparticles using the multiple emulsion technique.

Sample	Weight ratio Menthol/PLGA	PVA content (%m/v)	Stirring rate (rpm)	Solvent evaporation time (h)	Lyophilization time (h)
NP-1	1:10	1	7,000	12	24
NP-2	1:5	1	24,000	12	24
NP-3	1:5	1	24,000	13*	24
NP-4	1:5	0.5	24,000	1	72
NP-5	1:5	0.5	24,000	1	24
NP-6	1:5	1	24,000	12	24
NP-7	1:5	0.75	24,000	12	24
NP-8	1:5	0.5	24,000	12	24
NP-9	1:2.5	1	24,000	12	24

* Organic solvent was evaporated at room temperature and atmospheric pressure for 12h and then under reduced pressure at 40°C for 1h.

atmosphere and using an aluminum sample holder. Analyses were performed in duplicate.

2.4.4. Thermogravimetric analysis (TGA)

Thermogravimetric analyses were performed with SDT equipment (TA Instruments, Q600 model), from room temperature up to 1200°C, at a heating rate of 20°C/min, in a nitrogen atmosphere and using a platinum sample holder. Analyses were performed in duplicate.

3. Results and Discussion

The PLGA/menthol micro/nanospheres were prepared using different parameters, such as stirring rate, external surfactant content, PLGA/menthol weight ratio, evaporation time for the solvent and lyophilization time. Different conditions were used in preparing the micro/nanoparticles in order to achieve narrow size distribution and regular spherical morphology. The degradation of these micro/nanospheres in artificial saliva was also analyzed and the results are presented below.

3.1. Effect of stirring rate

NP-1 and NP-2 microspheres (Table 1) were prepared with a stirring rate of 7,000 rpm and 24,000 rpm, respectively. As shown in Figure 1b, the NP-2 microspheres have a smooth surface and an average particle size of between 1.12µm and 2.75µm (average particle size of 1.6µm ± 0.7µm). The NP-1 microspheres (Figure 1a) presented an average particle size of between 5.6µm and 13.1µm (average particle size of 9µm ± 3µm), with crowded and irregular structures and some ruptured particles, probably due to the fact of not being fully formed. The higher stirring rate (24,000 rpm) allowed the formation of dense particles with smaller sizes as found by Oliveira²¹. The formation of particles with characteristics of dense matrix is evidenced in Figure 1a, in which it shows some broken particles (poorly formed) where it can observe

the inside of the particles indicating that are not hollow particles. Figure 1c shows PLGA microparticles (without menthol, Table 1) used as control particles for comparison with the PLGA/menthol particles obtained. It was obtained aggregated PLGA microspheres with heterogeneity in the particle size (broad particle size distribution) of between 0.7µm and 11.8µm (average particle size of 1.8µm ± 1.4µm).

3.2. Effect of the evaporation time of the solvent

In the systems where the organic solvent (CH₂Cl₂) was removed after only 1 hour, nanospheres without a characteristic smell (NP-4 and NP-5, Table 1) were produced. In the others systems (NP-1 to NP-3 and NP-6 to NP-9), where the CH₂Cl₂ was slowly evaporated, microspheres with the odor were produced. It was identified by sensorial analysis of qualitative form.

Figures 2a and 2b show the typical spectra of PLGA/menthol microspheres (NP-3) in the attenuated total reflectance (ATR) and the transmission techniques, respectively. The presence of characteristic bands of the PLGA polymer at 2955 cm⁻¹ and 2876 cm⁻¹ assigned to C-H stretching of the CH₃ is highlighted; 1757 cm⁻¹ characteristic of the carbonyl (C=O) sym ester stretch present in the polymer backbone; 1183 cm⁻¹ assigned to asym stretching of the C=O group and at 1090 cm⁻¹ assigned to sym. C-O-C stretch. Typical bands of menthol at 3276 cm⁻¹ assigned to O-H group stretching were also observed, as confirmed by the presence of a broad band in figure 2b; 1044 cm⁻¹ assigned to alicyclic secondary alcohol (6-membered ring); 919 cm⁻¹, 876 cm⁻¹ and 769 cm⁻¹ assigned to asym angular deformation in the isopropyl group's plane, as described in the literature^{8,25,26}.

The NP-5 nanospheres, obtained by fast solvent evaporation, exhibited a spherical shape and a homogeneous particle size distribution of 217nm to 783nm (average particle size of 391nm ± 160nm) (Figure 3a). However, the NP-3 microspheres obtained by slow solvent evaporation had an oval shape and an aggregate particle structure with a particle

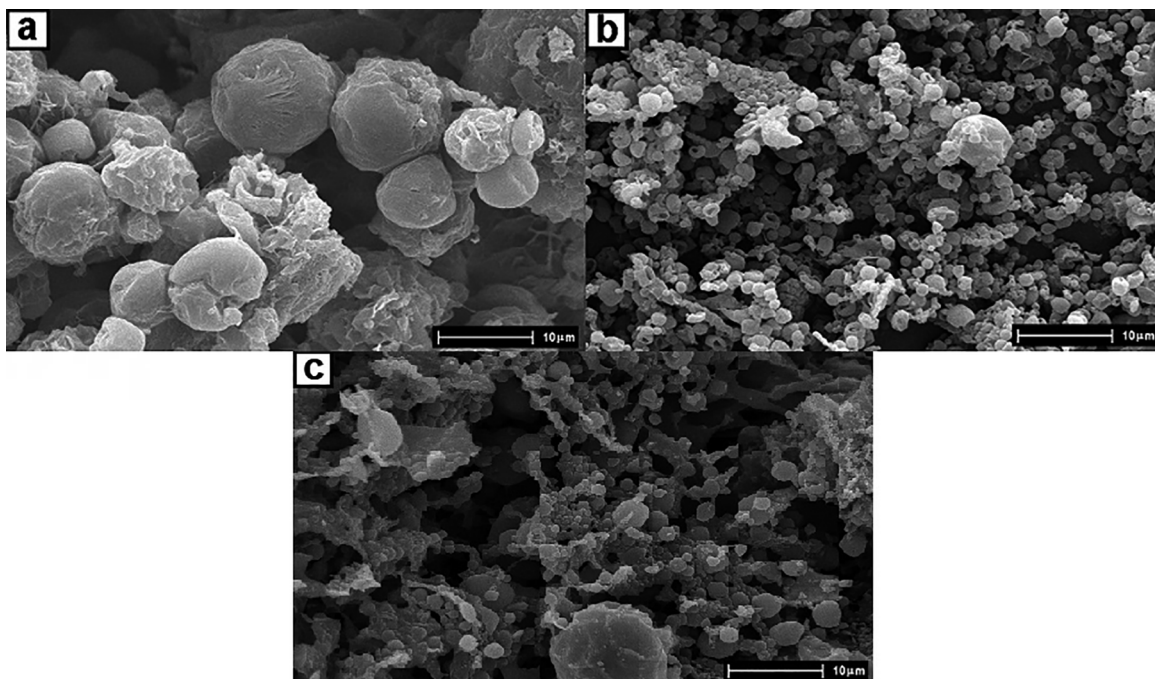


Figure 1. Micrographs of the microparticles: (a) NP-1, (b) NP-2 and (c) PLGA particles (4000x magnification)

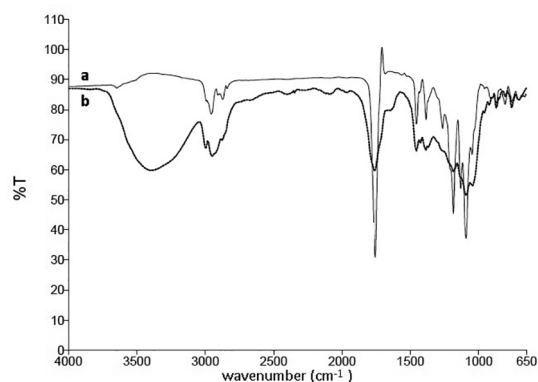


Figure 2. FTIR spectra of the NP-3 microspheres obtained by (a) ATR and (b) transmission techniques

size distribution of 1.9 μm to 5.8 μm (average particle size of 3 $\mu\text{m} \pm 1 \mu\text{m}$) (Figure 3b). Comparing the NP-3 with the NP-2 microspheres (same the amount of PVA), this last have a smaller average particle size of between 1.12 μm and 2.75 μm (average particle size of 1.6 $\mu\text{m} \pm 0.4 \mu\text{m}$) indicating the real effect solvent evaporation process.

Polymer nanoparticles are often defined as particles with dimensions of between 10 nm and 1000 nm, including nanospheres and nanocapsules (Feczko et al., 2011). In this work, micro- and nanospheres with dense matrix structures were prepared, wherein the menthol molecules can be adsorbed on the surface or incorporated into the polymer matrix. The literature shows that nanoparticles with a non-spherical shape can also be produced^{27,28}. Abdelwahed et al. prepared poly(caprolactone) nanospheres with an oval

shape and they attributed this peculiar morphology to the surfactant (PVA) used²⁹.

3.3. Effect of lyophilization time

NP-4 Nanoparticles were obtained under the same conditions as the NP-5 except for the lyophilization time (72h for the NP-4 and 24h for the NP-5, Table 1). It could be seen that a longer lyophilization time led to the formation of nanospheres with heterogeneous sizes and in clusters like beads on a chain (Figure 4a) or similar to loose spheres (Figure 4b). Figure 5 shows the NP-5 nanospheres obtained after 24h of lyophilization (Table 1). These spheres had a more homogenous distribution and smaller particle size (average particle size of 391 nm \pm 160 nm) when compared to NP-4 (average particle size of 1.0 $\mu\text{m} \pm 0.6 \mu\text{m}$). This behavior can be explained by effect of lyophilization time, where longer times can lead the morphology modification and increase of solute nucleation^{22,30}.

Process parameters influence of different form the particle size distribution. A larger stirring rate in the emulsion step led to smaller particle sizes in micrometer scale. For the other side a lesser solvent evaporation time and of lyophilization resulted in nanometric particles. This behavior was also described in the literature^{17,22}.

3.4. Effect of PVA content and menthol/PLGA ratio

The increase in the menthol/PLGA ratio from 1:2.5 to 1:5 (NP-9 and NP-6, respectively) resulted in the increase of intensity of the menthol bands as highlighted

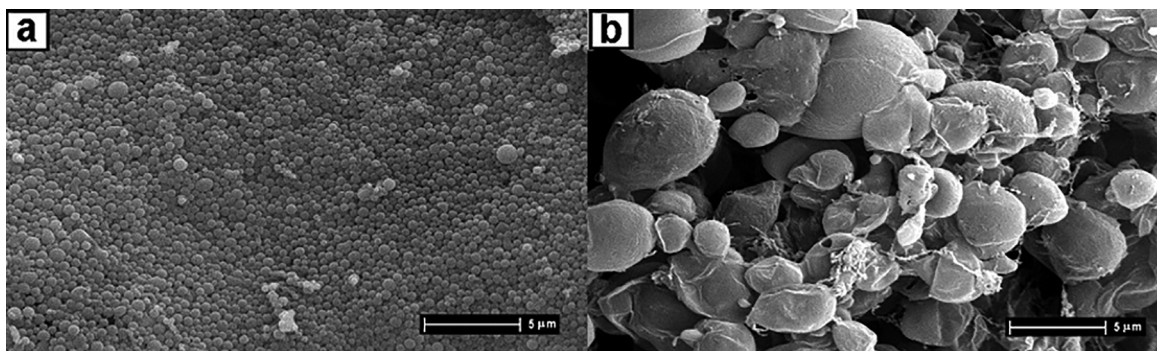


Figure 3. Micrographs of nano/microspheres: (a) NP-5 and (b) NP-3 (8000x magnification)

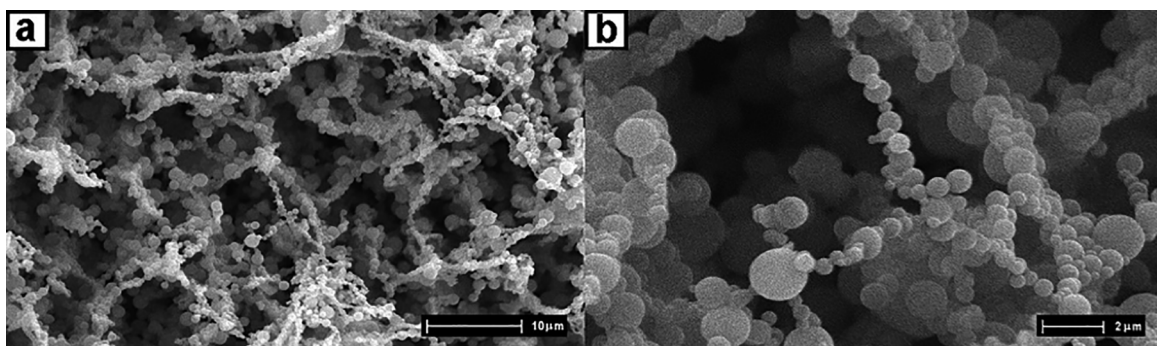


Figure 4. Micrographs of NP-4 microspheres: (a) 4000x and (b) 16000x

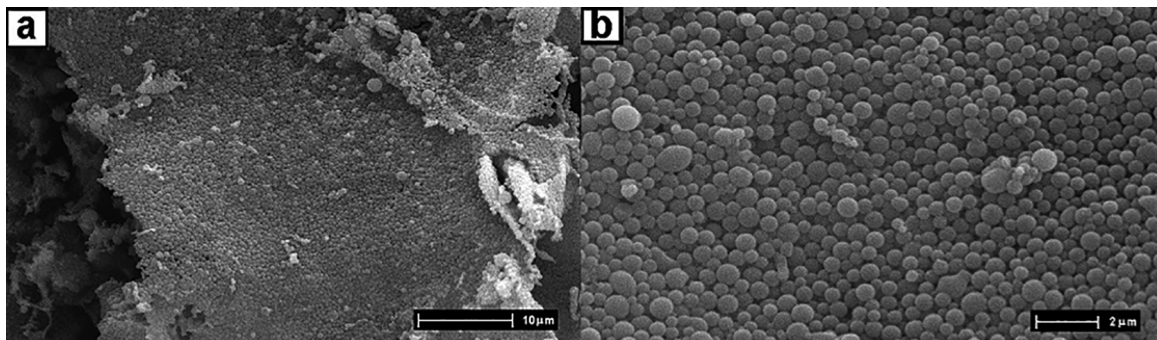


Figure 5. Micrographs of NP-5 nanospheres: (a) 4000x and (b) 16000x

in Figure 6. Comparing the relative heights of the bands at 1044 cm^{-1} (menthol) and 1754 cm^{-1} (PLGA), one can estimate approximately a 60% increase in menthol in the NP-9 nanospheres compared to the NP-6 obtained under the same conditions. This result indicates that the double in the menthol/PLGA ratio (1:2.5 in NP-9 compared with 1:5 in NP-6, Table 1) leads an increase in menthol incorporation of approximately 60%. In the same way, a decrease in the PVA content (Table 1) led to an increase in the amount of incorporated menthol in the NP-7 and NP-8, approximately 25% and 30% respectively, compared to NP-6.

Figure 6a shows NP-6 microparticles spectrum, what is very similar to PLGA microparticles (without menthol) spectrum (Figure 6c) indicating the presence of a small menthol quantity observed by intensity of band at 1044

cm^{-1} (Figure 6a). However in NP-9 spectrum (Figure 6b) observes a higher intensity of the bands at 1044cm^{-1} and 919 cm^{-1} characteristics of menthol highlighting a higher this compound quantity in NP-9 particles.

3.5. Thermal properties of menthol/PLGA nano/microparticles

DSC parameters (T_g , T_m and T_c) of PLGA, menthol, PVA and some microparticles are shown in the Table 2.

Glass transition temperature (T_g) of pure PLGA appears around 53°C (Table 2) and there was no melting peak or crystallization peak observed in the DSC analysis due to the amorphous nature of this copolymer^{25,31}. However the menthol has a melting peak at around 32°C and crystallization peak at 18°C , whereas the poly (vinyl alcohol) has a T_g around

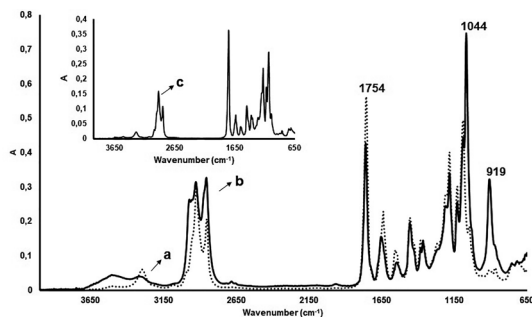


Figure 6. FTIR spectra of the microspheres: (a) NP-6, (b) NP-9 and (c) PLGA particles

Table 2. Some parameters obtained from the DSC analyses of the initial components and nano/microparticles.

Sample	Tg (°C)	Tm (°C)	Tc (°C)	ΔH_m (J/g)
PLGA	52.9	-	-	-
PLGA nanoparticles	48.9	177.5	126.1	2.0
Menthol	-	32.5	18.3	45
PVA	35.0	175.3	116.2	17.8
NP-3	41.5	189.2	154.1	3.2
NP - 5	48.0	-	-	-
NP - 6	37.2	178.5	124.6	2.2
NP - 9	39.8	181.4	128.6	1.9

35°C and Tm and Tc around 175°C and 116°C respectively (Table 2). According to Table 2, the microparticles with a characteristic aroma, such as NP-3, NP-6 and NP-9, showed a Tg between 37°C and 41°C, lower than the PLGA (pure polymer) Tg and higher than that of PVA. They also showed melting and crystallization points (Table 2) close to those of the PVA. This behavior indicates menthol incorporation in the PLGA matrix, as observed by Owen *et al.* in the study of ketoprofen-loaded PLGA nanoparticles³¹. Conversely, microparticles without a characteristic aroma, such as NP-5, only presented Tg at around 48°C, indicating non-incorporation of menthol. This Tg value is close to the Tg of PLGA (Table 2). PLGA nanoparticles without menthol showed a lower Tg (48.9°C, Table 2) than pure PLGA (52.9°C) due to the preparation process. These results are in line with those found in literature³¹. The values of 177.5°C and 126.1°C obtained for PLGA nanoparticles (Table 2) refer to the Tm and Tc, respectively, of the PVA, the external surfactant used in the preparation process of PLGA nanoparticles.

Typical thermogravimetric curves for PLGA/menthol micro/nanospheres are shown in Figure 7. The thermal degradation behavior of the NP-5 (particles in which no incorporation of menthol was detected) exhibited two degradation steps, from 267°C to 389.7°C with higher weight loss (91.5%) and from 389.7°C to 492.7°C with lower weight loss (3.5%), essentially due to the PLGA polymer decomposition³².

However, the NP-3 (particles in which incorporation of menthol was detected) showed four thermal degradation steps: the first two steps between 65°C to 155°C for menthol and between 155°C and 262°C for the thermal degradation of PVA and the last two between 262°C and 507°C due to the thermal decomposition of PLGA nanoparticles (weight loss of 88.2%). Similar thermal degradation behavior was observed for the NP-6 and NP-9 (Figure 7), which presented a higher weight loss of 4.0% and 3.8% respectively, compared to NP-3 (2.4%) in the 56°C to 158°C range (corresponding to the thermal degradation of menthol), thus indicating that there was a higher incorporation of menthol in NP-6 and NP-9 particles. Weight loss in the step of menthol degradation in the NP-9 was of 3.9%, i.e. approximately 60% high than NP-3 that was of 2.4%, this result is corroborated by FTIR data.

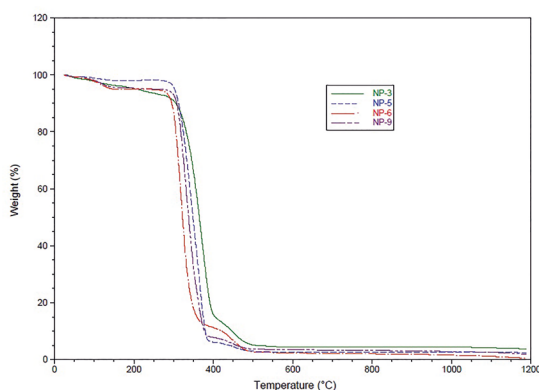


Figure 7. TGA curves of the NP-3, NP-5, NP-6 e NP-9 particles

3.6. Degradation in artificial saliva

The menthol/PLGA micro/nanospheres behavior in artificial saliva was evaluated with regard to the weight change of the samples and the pH of the medium (artificial saliva) by the ninety-day mark. The particles' morphology was also assessed before and after the degradation assay in artificial saliva (Figures 8 and 9).

The highest weight change was approximately 26% for NP-3 in 7 days and approximately 36% for NP-5 in 30 days. The greatest weight change was observed up to the 30-day mark for two particles (NP-3 and NP-5). This behavior can be associated with hydrolytic degradation of the PLGA polymer, where the ions present in artificial saliva may contribute to accelerating this degradation process³³. Additionally, the presence of acidic species, coming from the glycolic and lactic acid segments of the polymer chain, promotes a small decrease in the pH values up to 30 days (from 6.6 to 6.45).

The micrographs of NP-3 and NP-5 (Figures 8 and 9) show the artificial saliva's effect on micro/nanoparticles morphology, where there was a significant change of the spherical or oval particles (Figures 8a and 9a) for wires or ribbons within 30 days (Figures 8b, 8c and 9b,9c).

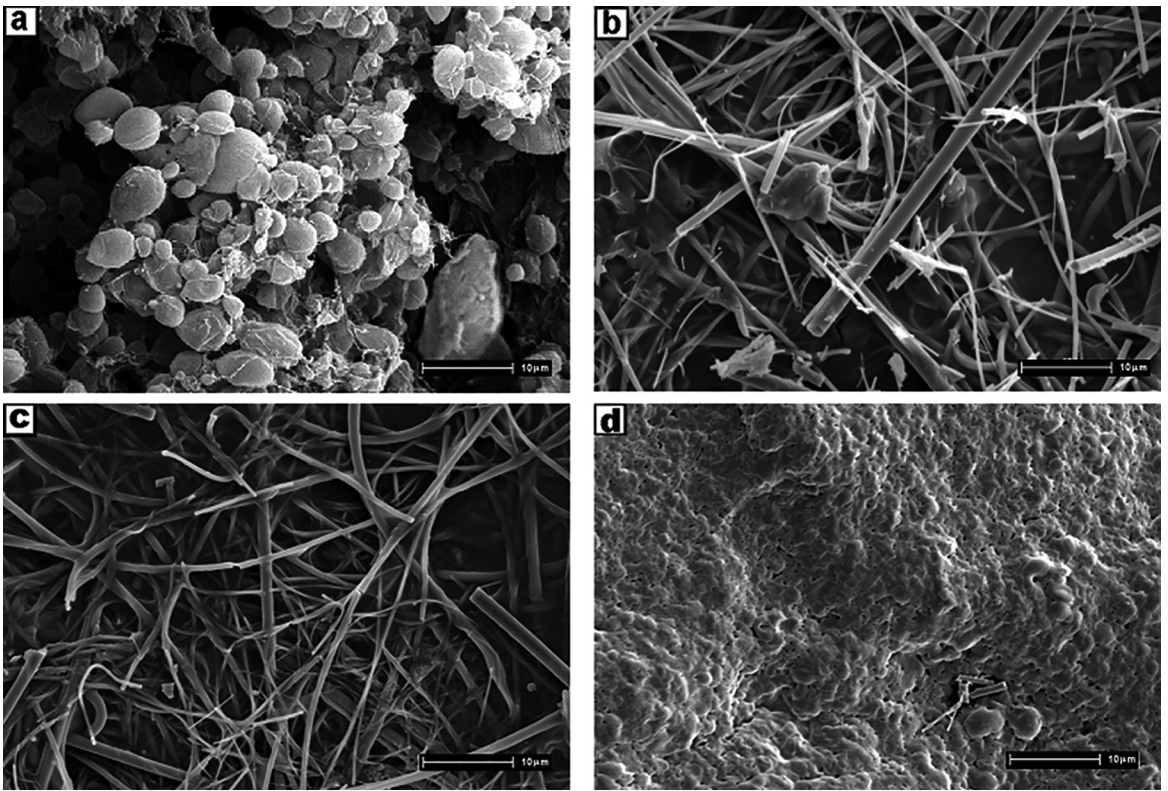


Figure 8. Micrographs of the NP-3 surface after artificial saliva assay: (a) sample at time= 0 days; (b) at 7 days; (c) at 30 days and (d) 90 days

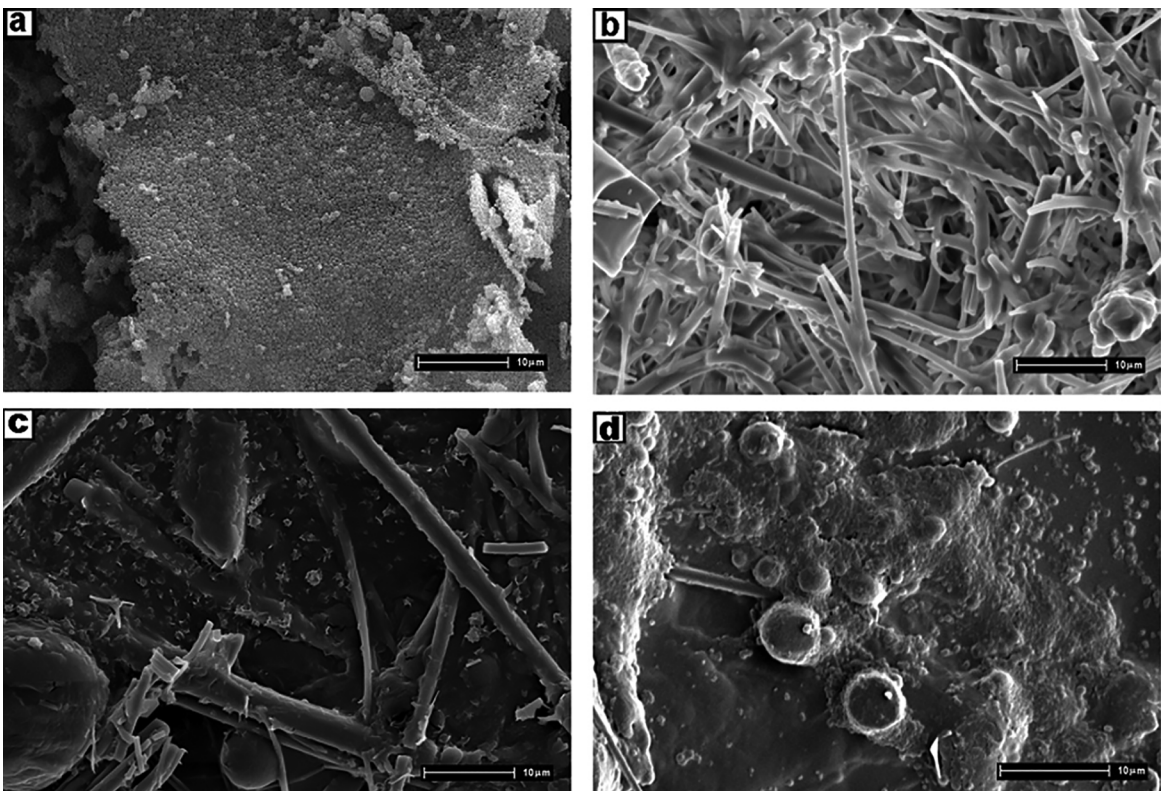


Figure 9. Micrographs of the NP-5 surface after artificial saliva assay: (a) sample at time= 0 days; (b) at 7 days; (c) at 30 days and (d) 90 days

However, in longer periods of time (60 and 90 days), the wires or ribbons were observed to disappear and irregular and dense surfaces formed on both particles NP-3 (Figure 8d) and NP-5 (Figure 9d). The literature indicates that this behavior can be associated to the mineralization process since the degradation in artificial saliva was carried out in media with high ions content³⁴.

Additionally, menthol appears to have an additive effect on the morphology, since in the NP-3 (particles with menthol) wires or ribbons are formed in greater quantities while the NP-5 present some spheres (Figure 9c) after being in the saliva medium for a long time. In a study where menthol was encapsulated in starch, it could be seen that full aroma release was achieved after 4 hours of hydrolysis in artificial saliva, the time period when the particles are partially degraded and starch hydrolysis is significantly initiated³⁵. In another degradation study of a natural thermoplastic polymer in simulated saliva fluid, the surface and bulk degradation were seen to take place at the same time, completely changing the polymer morphology³⁶.

TGA analyses were performed for NP-3 and NP-5 samples after degradation in artificial saliva (TGA curves not shown). After 7 days there was no degradation event observed between 65°C to 155°C, relating to the menthol degradation in the NP-3 particles, while with the NP-5 particles, there were no signals present under the same conditions.

4. Conclusion

Using the multiple emulsion/solvent evaporation technique made it possible to prepare the micro-nanoscale for a controlled release delivery system of aroma. The menthol/PLGA spheres in micro/nanometric size (13 µm to 217 nm) were obtained with high mixing speed (24,000 rpm) for the emulsion and a long solvent evaporation time (12h) and lyophilization time (24h). The presence or absence of aroma in the micro/nanospheres was evaluated using the FTIR, DSC and TGA techniques. Moreover, a preliminary assessment made by FTIR and TGA showed that the increase in the menthol/PLGA ratio (weight ratio= 1:2.5, NP-9) resulted in approximately 60% increase in incorporated menthol when compared with NP-6 (weight ratio=1:5). It should be highlighted that menthol was released from the PLGA under simulated saliva conditions. This result indicates that the PLGA polymer can serve as an efficient matrix for the control release of aroma in the oral cavity.

5. Acknowledgements

The authors would like to thank Hewlett-Packard Brasil Ltda for the scholarship (JPH), FINEP and PRONEX/FAPERGS for financial support and LabCEMM/PUCRS, SE and RL acknowledge CNPq for the DT grant.

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