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Encapsulation of R-(+)-Limonene in Edible Electrospun Nanofibers

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Bio-polymeric (pullulan and β-cyclodextrin) emulsions in water were electrospun to fabricate nanofibrous membranes and to encapsulate a bioactive volatile compound (R-(+)-limonene) simultaneously. The morphology of the polysaccharide membranes obtained can be described as a pullulan nanofibrous matrix with small crystals homogeneously dispersed. Encapsulation occurs because the conical cavity of the β-cyclodextrin is hydrophobic and able to bind non-polar molecules in water solutions, combined to the high evaporation rate of the solvent during the electrospinning process. The methodology developed allows encapsulation of the volatile compound in a rapid and efficient way. Moreover, the release of the limonene from the membranes was modulated by relative humidity changes, which enables controlled release applications as an active device for food or active packaging. Thermodynamic and kinetic models were proposed to describe the system and the volatile release..

1. Introduction

Promotion of health and prevention of disease through improved nutrition demands the development of food-grade delivery systems, including active packaging, to encapsulate, protect and deliver bioactive components (Lesmes and McClements, 2009, Sozer and Kokini, 2008). Volatile substances with antimicrobial features, such as limonene, are of great interest for the active packaging industry and their efficient encapsulation and controlled release represent a major challenge, considering their high liability towards environmental factors.
Conventionally, studies done on active packaging have involved the dispersion of the active agent in carriers with limited surface areas, e.g. polymer films and layers, with no negligible losses of volatile compounds during production and storage (Appendini and Hotchkiss, 2002; Guillard et al., 2009). The controlled release of active substances from these structures is mainly based on concentration-dependent passive diffusion (Vega Lugo and Lim, 2009).
Recently, electrospinning, has received great attention in functional food and active food packaging systems (Sanchez-Garcia et al., 2010; Lagaron et al., 2011). This simple technique enables the production of nanofibrous polymeric membranes, using electric fields to spin polymer fibers with diameters ranging from hundreds to tens of nanometers (Frenot and Chronakis, 2003). Because of their submicron-scale diameter and very large surface area, electrospun fibers are ideal for sensors (Arecchi et al., 2010), biomolecules and cells immobilization and controlled release (Jiang et al., 2005), filtration (Ramakrishna et al., 2010) and tissue engineering applications (Fiorica et al., 2012). Nanofibers may offer additional advantages compared to film and sheet carriers, for instance, being more responsive to changes in the surrounding atmosphere (e.g., relative humidity and temperature gradients), that allows for a more specific triggered release (Vega Lugo and Lim, 2009). Moreover, since electrospinning takes place at ambient

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conditions, electrospun fibers are more suitable for encapsulating thermally-labile molecules as compared to the fibers made by melt spinning or extruded films (Qi et al., 2006). Electrospinning technique seems to be suitable to hold molecular Inclusion Complexes structure, as for example cyclodextrins that form inclusion complexes with hydrophobic substances, i.e., the Aroma Compound Inclusion Complexes (AC-IC). In this type of encapsulates, a shell is present around the active agents (core-shell structure) (Zuidam and Shimoni, 2007), thus being more effective for the stabilization/protection of hydrophobic volatile additives including aroma compounds (Koontz et al., 2009). In literature, few works are focalized on electrospinning of edible biopolymers (Kayaci and Uyar, 2012; Li et al, 2009), and even less on edible polysaccharides biopolymers for a controlled release of bioactives. In general, edible polysaccharides do not need toxic solvents to be electrospun (Stijnman et al, 2011), are commonly used in food applications as coating and thickening agents, or additives for technological aims. The drawback of these polymers is that, because of their hydrophylicity, they are not suitable to encapsulate hydrophobic volatiles.
In this work, we overcome the aforementioned problem by developing an alternative encapsulation process. This method consists of a single-step electrospinning process in which β-cyclodextrin (β-CD) encapsulates limonene forming AC-IC that are simultaneously attached to pullulan nanofibers. The resulting system is fully edible and stable for a long time span. The retentive capacity of the edible nanofibrous system for D-limonene was evaluated, and the release behaviour was investigated, both in storage conditions and as a function of relative humidity.

2. Materials and methods

2.1 Chemicals

A food grade preparation of pullulan (PF-20 Grade, 200kD) of Hayashibara Inc. (Okayama, Japan) was kindly supplied by Giusto Faravelli (Milan, Italy). β-CD and R-(+)-limonene, were purchased from Sigma Aldrich (Milan, Italy). Doubly distilled water was used as solvent to prepare the emulsions. Sodium chloride, potassium chloride and potassium nitrate were purchased from Sigma Aldrich (USA). Methanol and ethanol were supplied by Fluka analytical (Spain).

2.2. Electrospinning solutions preparation

A polymer solution was prepared by dissolving pullulan dry powder in water (20 wt %) at room temperature under 4 hour stirring. After this, the pullulan solution was mixed with an amount of dry free β-CD (25 wt % with respect to dry pullulan) and with 10% wt % (AC/ β-CD), containing more than 90 wt % of the active compound D-limonene. The solution was emulsified using an Ultra Turrax T25 IKA blender (IKA Works, Guangzhou, China) running at 10.000 rpm for 5 min. It must be noticed that on adding cyclodextrins the system turns to a water-in-water emulsion because of the thermodynamic uncompatibility of the two polymers (Grinberg and Tolstoguzov, 1997): cyclodextrin rich aqueous droplets are dispersed within an aqueous pullulan rich phase.

2.3. Electrospinning

Plastic syringes (10 mL) with a metallic needle were filled with the polymeric emulsions and placed in a syringe pump (KD-Scientific, New Hope, PA). Pump was set as 0.5 mL/h. The needle was linked to a Spellman SL150 high voltage power supply and a grounded foil-covered copper tray, positioned in front of the needle. The production of a single membrane was stopped at 15 min, the membrane were removed from the collector and dried.

2.4. Field-Emission Scanning Electron Microscopy (FE-SEM).

SEM images were obtained from a Sigma Field Emission microscope (Carl Zeiss Microscopy, LLC) at accelerating 5KV voltage and 6 mm working distance, with a 30 μm width slit. The samples were first gold sputtered (Sputtering Polaron E 5100) for 30 s (rate 1 nm s-1) with argon and 18 mA current intensity.

2.5. Thermogravimetric analysis (TGA).

TGA were performed under nitrogen atmosphere with a Perkin Elmer TGA 4000 instrument. Scans at a constant 20 °C/min heating rate (30 °C- 450 °C). Raw data were converted into time derivative trace, DTG, and expressed in mg/K.

2.6. Extraction and quantification of limonene in the nanofibrous membranes.

Limonene was extracted by immersing the membrane in 5 mL of methanol and stirring by 24 h / 500 rpm followed by an ultrasonic for 10 min. Alcoholic phase containing the aroma was analyzed via total vaporization by head space gas cromatography (HSGC) (Mod HS 40, Perkin Elmer) equipped with a TRB-

WAX column (30 m x 0.53 mm, film thickness of 1μm) and a flame ionization detector (FID). Helium was used as carrier gas (2 mL/min). Injector and detector were set at 230 and 260 °C. The residual quantity was quantified with an external standard. The extraction efficiency was >90%. For quantifying losses during storage, membranes were stored at 55% RH and 23 °C for 45 days, and then analyzed with HSGC.

2.7. Release from membranes as a function of relative humidity.

Single membranes were weighed and put in different chambers at 23 ± 2 °C and constant relative humidity (RH) of 55 %, 75 %, 85 % and 92 %. Membranes were removed from the chambers at given time intervals; the amount of volatile was immediately determined with the extraction method described above.

3. Results and Discussion

3.1 Morphology of the encapsulation membranes.

In a series of previous experiments, 20 wt% pullulan solution, 0.5 mL/h flow rate, 15kV applied voltage and 12 cm tip-to-collector distance, were determined as optimal parameters for obtaining pure pullulan nanofibers. These same conditions were found suitable for electrospinning of the emulsions of pullulan, β-CD and limonene. The macroscopical appearance of the membranes was homogeneous and smooth, (Figure 1, top left). SEM micrographs (Figure 1, top right and bottom) showed randomly oriented fibers with diameters in the submicron scale (370 ± 15 nm) with crystals distributed along the fibers.

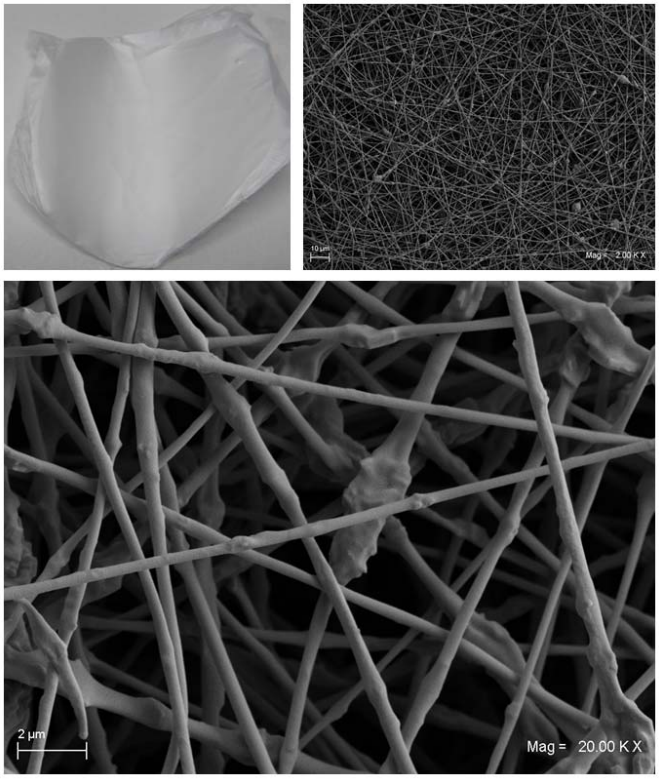

Figure 1: Macroscopical appearance of the membranes (left top); scanning electron micrographs of nanofibrous membranes under magnification 2 KX (right top) and 20 KX (bottom).

The crystals correspond to AC-IC of β-CD, probably formed during electrospinning due to the rapid evaporation of the solvent (water), leading to the nanofibrous matrix that embed crystalline structures. Apparently, pullulan fibers and cyclodextrin crystals form a core-shell structure. This is because, in spite of the chemical similarity (saccharide nature), β-CD and pullulan are thermodynamically incompatible, forming separate aqueous phases in the presence of excess solvent, because of different exclusion volumes (Grinberg and Tolstoguzov, 1997). These phases create a dispersed system, (droplets of aqueous β-CD dispersed in the aqueous pullulan rich solution). The thermodynamic incompatibility that takes the β-CD droplets apart from the surrounding pullulan-rich dispersion medium is equivalent to a surface tension effect between the β-CD aqueous droplets and solvated bunches of pullulan molecules (Schiraldi et al., 2012). While solvent is spun during electrospinning, β-CD rich droplets generate the

characteristic small crystals (with a 50-100 nm size) around the pullulan fibers that come from the starting solvated polymer. The fact that the formation of the AC-IC and their attachment to the nanofibrous membrane take place in a single step, is clearly advantageous and the membranes can be directly used as active devices, for instance in active packaging without further use of adhesives.

3.2 Encapsulation capacity of the system and volatile loss during storage.

The amount of limonene in the membranes, as quantified immediately after their preparation was 3.1 wt% limonene/dry membrane, corresponding to 16.1 wt% limonene/β-CD. In order to evaluate the volatile losses during storage, the amount of limonene in the membranes was assessed after 3, 7, 15 and 45 days at ambient conditions. Results are shown in Figure 2. The loss of limonene after 3 days was 13.7 % with respect to the freshly prepared membranes. There were no further losses up to 45 days, proving the stability of the encapsulation system under normal storage conditions.

The encapsulation occurs because the hydrophobic conical cavity of the cyclodextrin binds non-polar molecules, in this case R-(+)-limonene, in aqueous environment. Each molecule of β-CD (MW 1134.98 g/mol) is able to bind one molecule of the volatile (MW 136.24 g/mol). Therefore, the maximum amount of limonene that can be encapsulated by the β-CD should be around 12 % of its weight. Since retention capacity of pure pullulan nanofibers is negligible (<0.1 wt% over total dry matter in the membranes), the overall encapsulation capacity of the membranes will be determined by the β-CD. In effect, after 3 days, the encapsulated limonene was 13.4 wt% limonene/β-CD, and after 7 to 45 days this remains stable around 12.7 wt% limonene/β-CD. Thus, the evidences show that release of aroma during the early stage of storage is due to the excess of volatile that cannot be effectively encapsulated inside the β-CD. Once this excess is quickly lost, the system remains stable without losses during months if kept at a relatively low humidity. The nanofibrous device is suitable to preserve the volatile compound and masking its odour until use at high relative humidity.

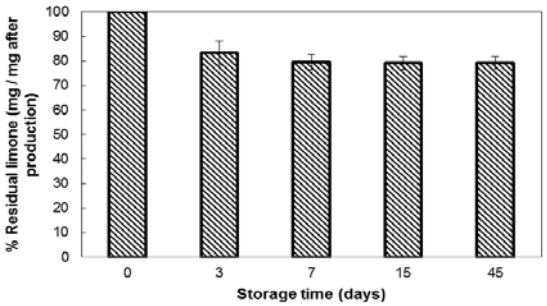

Figure 2: limonene encapsulated in the nanofibrous membranes after different storage times (% referred to the to the amount of limonene as quantified right after membrane production)

3.3 Thermal characteristics.

TGA was used to assess the thermal stability of pure limonene and limonene – β-CD complexes fixed within the pullulan membranes. The record of a TGA run encompasses a wide temperature range where mass loss occurs because of various events such as release of water, release of the aroma compound, degradation of limonene and pullulan, which take place in different temperature spans with partial overlaps. It is expedient to use the corresponding time derivative (DTG) trace where the different contributions to the overall mass loss appear as peaks or shouldered peaks. In the present case, DTG data were referred to the mass released as function of Temperature (dW/dT). Figure 4 indicates that the release of limonene started at ambient temperature and reached a maximum rate at 170°C (dashed line). The same Figure (continuous line) shows the DTG trace collected from limonene β-CD complexes fixed within the pullulan membrane. The mass lost in the 30-to-150°C was related to the release of moisture and the mass lost in 260-300 °C temperature range was related to limonene encapsulated in β-CD pullulan matrix. It is clear the great increase of stability of limonene when encapsulated in the nanofibrous matrix and this is explained also by the morphological characteristics described above.

3.4 Effect of relative humidity on limonene release.

Figure 4 shows the fraction of limonene released at different values of water activitiy (a_w = *RH*/100). As it can be observed, the relative humidity plays a critical role on the limonene release. The figure shows that the release occurs at a_w > 0.9 whereas at dryer conditions it is much smaller. Hydrophobicity of the limonene molecule is important in the releasing phenomenon: the water molecules can weaken the host-guest interaction in the AC-IC due to conformational changes, stimulating the expulsion of hydrophobic

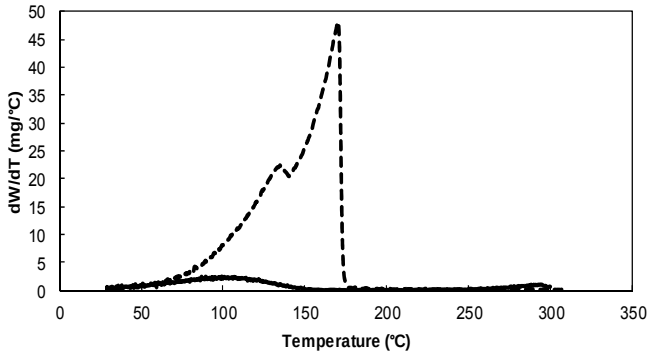

Figure 4: DTG traces of pure limonene (dashed line) and of nanofibrous pullulan matrix with R-(+)-limonene – β- CD complexes.

limonene towards the external environment.
The state of the system can be described as two co-existing thermodynamic equilibriums:

$$CD + n_1(H_2O) \leftrightarrow CD(aq) \qquad K_h = \frac{c(CD,aq)}{c(CD) \times a_w^{n_1}} \tag{1}$$

$$c(CD,aq) + AC \leftrightarrow CD - AC(aq) + n_2 H_2O \qquad K = \frac{c(CD-AC,aq) \times a_w^{n_2}}{c(CD,aq) \times c(AC)} \tag{2}$$

where *CD*, *AC* and *CD-AC* stand for β-CD, free aroma compound (limonene) and inclusion complex, respectively, *c* is the symbol for any suitable kind of concentration. Both equations indicate the major role played by the relative humidity (*RH*), on the release. Considering that the overall mass of limonene is split in the free and bound species, i.e., *M*(*AC*) = [*m*(*AC*) + *m*(*AC* in *CD-AC*)], the relevant concentration ratio, *c(AC)/c(CD-AC)*, in Eq (2) can be replaced by the corresponding mass fractions of AC, namely *φ/(1-φ)* Combining Eq (1) and Eq (2), the following expression for *φ* can be obtained:

$$\varphi = \frac{a_W^{\ n}}{a_W^{\ n} + K_{app}} \tag{3}$$

where $n = (n_2 - n_1)$ and $K_{app} = K \times K_h \times c(CD)$. This equation was used to fit the trend of the experimental data that are reported in Figure 4.

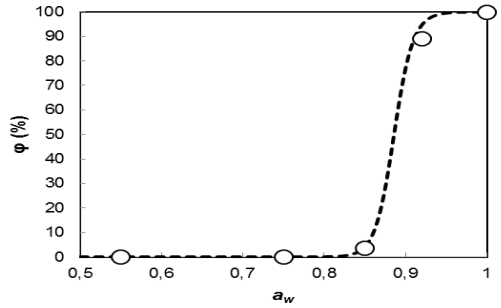

Figure 4: Limonene release at equilibrium (φ) at different water activities (23 °C): circles represent the experimental data and dotted line the corresponding fit (Eq (3)).

4. Conclusions

A novel single-step methodology for encapsulation of bioactive/antimicrobial hydrophobic volatile compounds (e.g., limonene) was developed. The process consists in electrospinning of a dispersed aqueous solution containing pullulan, β-CD and the volatile, resulting in nanofibrous non-woven membranes that are ready-to-use. The system is stable during months without significant loss when kept

in relatively dry conditions, even at high temperatures (up to 260 °C). The release of the volatile from the membranes is triggered by relative humidity changes, taking place at $a_w \geq 0.9$. This system can be potentially used in active packaging, in particular of fresh foods, for which the risk of microbial degradation increases at high a_w conditions, and therefore demand special protection.

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