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Poly(ε-caprolactone)/propolis extract: microencapsulation and antibacterial activity evaluation

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Received April 5, 2006, accepted August 8, 2006

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Pharmazie 62: 287–290 (2007) *doi: 10.1691/ph.2007.4.6058*

Spherical and homogenous microparticles of poly(ε-caprolactone) (PCL), containing propolis were prepared by the emulsification-solvent evaporation technique. Using this method of preparation, a solid formulation of propolis, free of ethanol and suitable for manipulation and storage, was obtained from an ethanolic extract of propolis. The incorporation efficiency of propolis in the microparticles was almost 30% and around 60% of the substance was released in 48 h. *In vitro* propolis microparticles exhibited similar halo zones in the Petri plate test against *Streptococcus mutans* (GS5) with a 10-fold lower concentration than the free propolis extract showing that the encapsulated propolis in microparticles is more efficient as antibiotic.

1. Introduction

Propolis is the generic name for the resinous material collected by honey bees from various plant sources. The literature search indicates the renewed interest in its chemical and pharmacological properties, because biological activities have been ascribed either to propolis or its extracts (Moreno et al. 2000; Sforcin et al. 2000; Maciejewicz et al. 2001; De Castro 2001; Paraventi and Esposito, 2002; Gebara et al. 2002; Murad et al. 2004; Sawaya et al. 2004).

Propolis can prevent dental caries since it demonstrated significant antimicrobial activity against microorganisms like *Streptococcus mutans*, *Streptococcus sobrinus* and *C. albicans*, which are involved in oral diseases (Uzel et al. 2005). *Streptococcus mutans* triggers dental caries establishment by two major factors: synthesis of organic acids, which mineralize dental enamel, and synthesis of glucans, which mediate the attachment of bacteria to the tooth surface. Propolis is a natural product that may prevent dental caries. Brazilian propolis from different regions showed different antibacterial activities against *S. mutans*. The effect of a new variety of propolis from several regions in Brazil on growth of mutans Streptococci, cell adherence and water insoluble glucans synthesis were evaluated. The results show that the new variety of propolis was exceptionally effective in all *in vitro* parameters tested against S*treptococci* mutants, biological effects of propolis are likely not to be due solely to flavonoids and hydroxy cinnamic acid derivatives (Koo et al. 2000). More recently, it was found that green propolis inhibited the synthesis of insoluble glucans ($IC_{50} = 12.9$ μg/mL), soluble glucans ($IC_{50} = 50.4$ μg/mL), and organic acids ($IC_{50} = 0.34$ μg/mL, Leitão et al. 2004). The minimal bactericidal concentration (MBC) for *S. mutans* was from 2.5 mg/mL to 10 mg/mL (Sawaya et al. 2004).

Biodegradable and biocompatible materials such as gelatin, dextran, collagen, alginate and polyesters like poly(ε-caprolactone) (PCL) and poly(DL-lactic-co-glycolic) and others have been investigated on microencapsulation processes, but very few publications are related to encapsulation of propolis at the microparticle levels in the literature. Pepeljnjak et al. (1981) prepared gelatin-acacia microcapsules with propolis extract and inhibition microbial assay against *Bacillus subtilis* IP 5832 were measured for microencapsulated propolis extract and the propolis solution. The microcapsules produced narrower zones of inhibition than the solutions, but released the active ingredient in a sustained manner. The properties of the encapsulated propolis extract in alginate microparticles were evaluated by Hai et al. (2002). They verified that the microencapsulated propolis extract showed good solubility and dispersion in water. More recently, the propolis extract (from Paraná State, Brazil) was encapsulated in gelatin microparticles by spray-drying technique. The microencapsulation by this technique maintained the activity against *Staphylococcus aureus*. These gelatin microparticles containing propolis would be useful for developing propolis dosage forms without strong and unpleasant taste, aromatic odor, and presence of ethanol (Bruschi et al. 2003). However, no publications with propolis extract encapsulated in biodegradables polyesters are available. Thus, the aim of this paper was to encapsulate the propolis extract in biodegradable PCL, evaluate the sustainable release of the microencapsulated compounds and to analyze the antibacterial activity of the free and encapsulated propolis.

2. Investigations, results and discussion

2.1. Scanning electronic microscopy analysis of the propolis microparticles

2.1.1. Particle sizes and distribution

Propolis microparticles were prepared by an emulsion/evaporation technique using the experiments 1–9. Typical microparticles (Fig. 1) had a mean diameter of 5–10 μm and the efficiency of encapsulation was around 25% (experiment 6, Table 1).

2.1.2. Effect of the surfactant and solvent in the encapsulation

Table 1 shows the best results of many attempts to encapsulate propolis. This Table also shows that the presence of a larger quantity of polyvinylalcohol (PVA) (MM 127 kDa) (5 g) is better than the presence of two surfactants like PVA/Tween (Experiments 1–2). Moreover, the molar mass of the PVA (30–70 kDa or 89–98 kDa) did not interfere in the particles morphology when the PVA was mixed with Pluronic (Experiment 3 and 4). Compared to PVA (MM 30–70 kDa) (Experiment 6 and 9) the utilization of chloroform was better than the mixture of chloroform and acetone. Moreover, an important aspect is that the use of chloroform instead of acetone improved particle morphology (Table 1, Experiments 8 and 9, respectively) and an alteration in terms of encapsulation efficiency was not verified when the chloroform quantity was reduced (Experiment 6 and 7).
Other conditions and experiments with PCL and different polymers (PLGA and poly (lactide co-caprolactone) were tested. However, they were not satisfactory in the propolis encapsulation (data not shown).
In conclusion, 1% of PVA (MM 30–70 kDa) gave the best formulation of propolis extract in low ethanol and chloroform concentrations (Experiment 7, Table 1) showing a spherical form and low distribution size profile (Fig. 1).

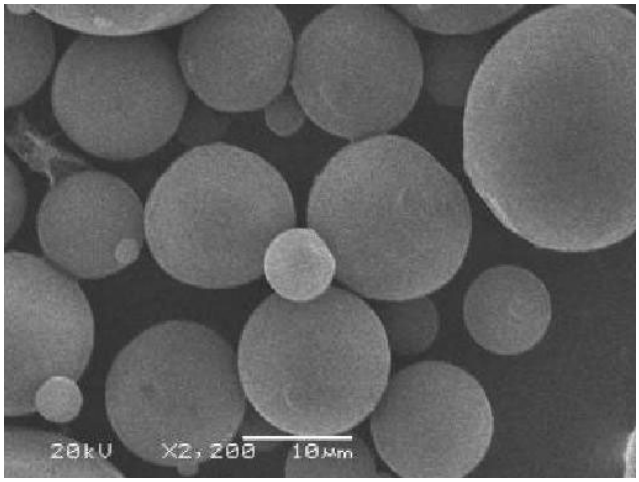

Fig. 1: SEM micrographs of emulsion/evaporation propolis microparticles (PCL MM 65 kDA; PVA MM 30–70 kDA, using ethanol and chloroform) showing the morphological profile of experiment 7 (Table 1) conditions x 2,200.

2.2. In vitro release study

The PCL microparticles in Experiment 7 (Table 1) were easily dispersed in water after lyophilization and the *in vitro* release of the microencapsulated propolis was evaluated. The release profile showed a gradual release of the encapsulated propolis within the first 40 h. The release percentage of theses microparticles was around 60%. The free propolis was almost immediately dissolved in the release media (Fig. 2).

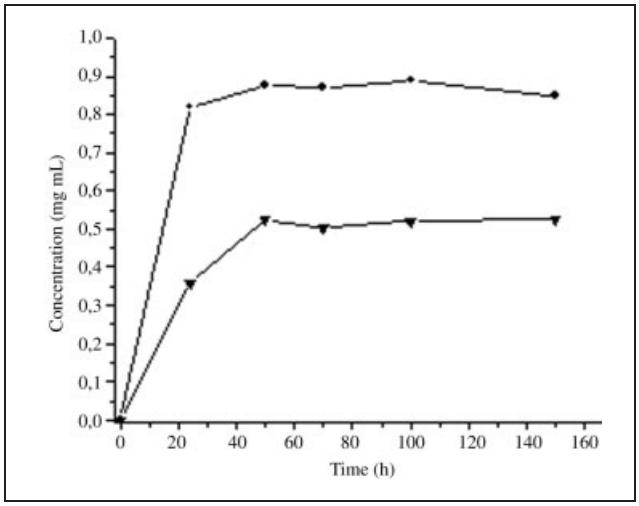

Fig. 2: *In vitro* release profile of the entrapped propolis from PCL microparticles (Experiment 7 – Table 1).

2.3. Antibacterial activity evaluation

As the use of microparticles could enhance the therapeutic effect of antibiotics (Bruschi et al. 2003), vaccines (Baras et al. 2000), antivirals (Santoyo et al. 2002) and cytotoxics

Table 1: Methods, conditions, morphology, efficiency and release of propolis encapsulated in microparticles

Water (g)	PVA (g)	Tween′(g)	Pluron (g)	PCL (g)	Propolis (g)	Ethanol (mL)	Chlorf. (mL)	Acet. (mL)	Stirring (rpm)	Redisp.	Effic. (%)	Release (%)	Morphol.
1) 100	2[a]	1	0	0.179	0.1	2.5	0	2.5	magnet.	regular	nd	nd	regular
2) 97	5[a]	0	0	0.200	0.2	3.0	0	3.0	magnet.	good	20	30	good
3) 99	0.25[d]	0	0.15	0.150	0.3	7.5	0	7.5	16,000	regular	nd	nd	regular
4) 99	0.25[b]	0	0.15	0.150	0.3	7.5	0	7.5	16,000	regular	nd	nd	regular
5) 99	1[b]	0	0	0.180	0.3	7.0	4.5	7.0	1,060	regular	12	nd	regular
6) 99	1[b]	0	0	0.200	0.1	1.8	5.0	0	1,060	good	25	60	good
7) 99	1[b]	0	0	0.200	0.1	1.5	2.5	0	1,060	good	22	nd	good
8) 99	1[b]	0	0	0.200	0.1	1.8	4.5	0	1,060	good	nd	nd	good
9) 100	1[b]	0	0	0.200	0.3	3.0	0.0	3.0	1,060	regular	nd	nd	regular

MM PVA: a) 127 KDa; b) 30–70 KDa; c) 9–10 KDa; d) 89–98 KDa.
MM PCL: 65 KDa.
MM Tween 40: 1.248 KDa.
MM Pluronic F-68: 8.400 KDa

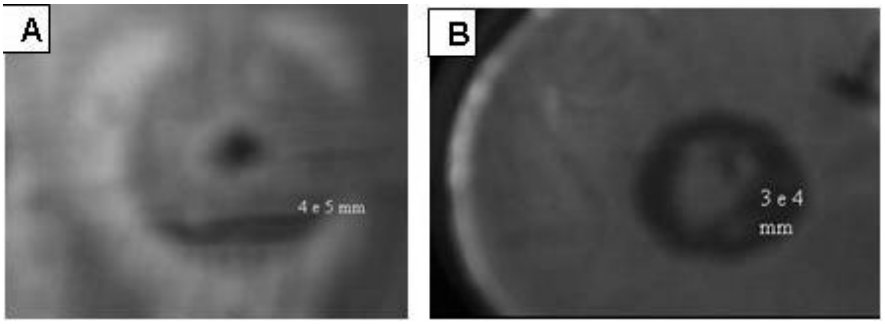

Fig. 3:
Agar diffusion method TSA medium at 37 °C incubated 105 cells of *Streptococcus mutans* (GS5) in Petri plates for 24 h: a) 243.5 microgrames of propolis encapsulated in PCL microparticles/50 microliters of ethanol and b) 2,470 microgrames of ethanolic extract of propolis.

Table 2: Evaluation of antibacterial activity on *Streptococcus mutans*

	Weight (mg)	Ethanol (μL)	Propolis extract (μg)	Halo (mm)
Microparticles Control	38	50	–	–
Microparticles with propolis	38	50	243.5	3–4
Control/ethanol	–	–	–	–
Control/free	–	–	2470.0	4–5

Data: 1 g of microparticles = 25.41 μL of free propolis solution; 1 μL of propolis extract = 252 μg of propolis

(Melo et al. 2005), a qualitative antibacterial experiment was carried out with the microparticles containing propolis. The results are presented in Table 2 showing the antibacterial activity on *Streptococcus mutans.* The *S. mutans* strain was sensible to free propolis in a dose of 806.4 μg propolis in 1M acetate buffer pH 7.4, and similar results were found with the addition of ethanol in the buffer (results not shown).

Under that condition it was not possible to observe any antibacterial effect of propolis encapsulated in PCL microparticles, probably due to the low ethanol concentration that gave a low release of the propolis. Table 2 shows that an amount of 243.5 μg of propolis encapsulated in PCL microparticles using 50 μL of pure ethanol in the Petri plates clearly inhibits the *S. mutans* growth (halo 3–4 mm) (Fig. 3a), similar values were obtained under the same conditions with 2,470 μg of ethanolic extract of propolis (Fig. 3b). Then, the *in vitro* evaluation of antibacterial activity against *S. mutans* showed that propolis encapsulated in PCL microparticles was 10-fold more efficient free propolis. These results show that the antibacterial action was probably achieved via membrane disruption changing the membrane penetrability due to the PCL microparticles surface properties.

It is anticipated that propolis containing microparticles could be applied broadly as antimicrobial agents in medicine for their high antibacterial activity and acceptable biocompatibility. Moreover it is easy to obtain and probably scaling up to an industrial level could be economically feasible.

3. Experimental

3.1. Propolis extracts

The propolis was obtained from MN Própolis Indústria Comercio e Exportação Ltda., from Mogi das Cruzes, S.P, Brazil and used without any other purification or extraction (Propolis Ouro, Lot 273). Containing cereal alcohol, propolis extract >25% (w/v), 6 g carbohydrates, 1 g protein, 21 g total lipids and 1 g sodium, 45–50% resins, 25–35% wax, 10% essential lubricating elements, 5% pollen, 5% of organic elements and 20% of flavonoids and pH 5.23 (South-east region, Brazil).

3.2. Preparation and microparticles characterization

The microparticles were prepared by the emulsion/solvent evaporation technique, using poly(ε-caprolactone) (PCL) (MM 65 kDa) poly(vinyl alcohol) (PVA) (MM 30–70 or 127 or 89–98 kDa) as stabilizants, the surfactants Tween 40 (MM 1,248 kDa) or Pluronic F-68 (MM 8.4 kDa) and acetone or chloroform as the solvents. The propolis was solubilized in absolute ethanol (Table 1). Briefly: Into 0.25–5 g PVA and 1 g Tween 40 or 0.15 g of Pluronic F-68 were dissolved in distilled water under magnetic or mechanic stirring (400 mL beaker). In another 50 mL beaker 0.179–0.200 g PCL were dissolved in acetone and ethanol (in some cases chloroform was used). An equivalent amount of 0.1–0.3 mg of ethanolic extract of propolis was added slowly (micro syringe) to the organic phase and then to aqueous phase containing PVA/Tween 40 or PVA/Pluronic F-68 or without any surfactants. After 24 h stirring all the organic solvent was removed and the microparticles were centrifuged at 10,000 rpm for 30 min and washed five times with distilled water. The microparticles were dispersed in water and lyophilized.

3.3. Morphology of microparticles

The PCL microparticles with and without propolis extract were characterized by scanning electron microscopy – energy dispersive spectroscopy (SEM-EDS) at a voltage of 20 kV (Jeol – JSM-6360LV) and previously coated with gold/paladium under vacuum by sputtering using a BAL-TEC's apparatus. Secondary electron images were obtained.

3.4. Determination of encapsulated propolis

Microparticles (0.4 mg) were dissolved in 2 mL of chloroform and 2 mL of ethanol. The sample was analyzed spectrophotometrically using a UV-Vis Hitachi U-2000 spectrophotometer at 380 nm. The amount of propolis encapsulated was determined from a standard curve.

3.5. In vitro release of encapsulated propolis

Microparticles (20 mg) were dispersed in 3.5 mL of 0.1 M phosphate buffer (pH 7.4) and 1.5 mL of ethanol in a closed container in an incubator at 37 ± 0.01 °C and stirred at 120 rpm. Samples of dissolved propolis (supernatant) were removed at specific intervals for analysis and replaced with fresh buffer. All the *in vitro* release was carried out at sink conditions. Absorbance was determined with an UV-Vis spectrophotometer Hitachi U-2000 at 380 nm. Concentration of dissolved propolis was calculated from a standard curve.

3.6. In vitro test of antibacterial activity

The agar diffusion method was used in detecting the biological response to ethanolic solution of propolis well as to microparticles with propolis. Sterile agar-trypticase (TSA) kept at 37 °C incubated with 10^5 cells of *Streptococcus mutans* (GS5) was poured into sterile Petri plates forming a layer of 4 mm thickness. After the gelification of Agar, a cavity of 6 mm in diameter in the central region of the plate was formed. The controls (50 μL of ethanol and 38 mg of PCL microparticles without propolis in 50 μL of ethanol), 2,470 μg of ethanolic extract of propolis and 38 mg of PCL microparticles with propolis in 50 μL of ethanol were added in the cavities of each plate. The microparticles used for *in vitro* test of antibacterial activity were prepared under the conditions of the experiment 7 (Table 1). The plates were incubated at 37 °C for 24 h and the inhibition zones were measured using calipers. Subsequently the Petri plates were kept at room temperature for one month and the inhibition zones were checked at the end of this period.

Acknowledgement: Support from Brazilian Nanobiotechnology Network, CNPq/MCT and FAPESP are acknowledged.

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