Title

An alternative encapsulation approach for production of active chitosan - propolis beads

Running title

Chitosan-propolis beads as active device

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Summary

Encapsulation is a promising technology to carry natural active substances, preventing their loss and maintaining their stability until use. Beads of chitosan containing propolis have been prepared using a mono-pore filter device, which permits the encapsulation of natural polyphenols avoiding heat treatments, high shear rates and the use of toxic solvents. Beads proved to be active against *Bacillis cereus*, *Escherichia coli*, *Listeria innocua*, *Pseudomonas fluorescens*, *Yarrovia lipolytica*, and three moulds strains; the

highest effect was found against *Staphylococcus aureus* (MIC 0.8 mg beads/mL). Results in liquid cultures of *S. aureus* evidenced that beads were able to release the flavonoids from propolis: the diffusion of the active compounds is a key factor in the exploitation of the microbial activity. The obtained chitosan-propolis beads represent an example of natural antimicrobial delivery system that could be used to prevent the growth of pathogenic/spoilage bacteria in food applications.

Keywords: propolis, chitosan, membrane encapsulation, active device, food packaging

Introduction

Propolis, a natural brownish resinous substance collected by honeybees (*Apis mellifera*) (Lu et al., 2005; Burdock, 1998; Salomao et al., 2004), is largely used in the pharmaceutical fields and recent studies have paved the way for potential applications of propolis also within the food and food packaging fields, to control primary factors of antimicrobial degradation and oxidation especially thanks to its great polyphenols content (Pastor et al., 2010, Guo et al., 2011; Tosi et al., 2007). Nowadays there is also a growing interest on employing natural additives with packaging technology designed to keep produce fresh, optimizing its shelf life (Weiss et al., 2009; Cutter et al., 2006). However, as propolis is a strongly adhesive, resinous mixture of insoluble or slightly soluble substances with bitter taste and no standard composition, the application of propolis in the food area has been limited (Sforcin and Bankova, 2011). In this perspective, encapsulation of propolis could be a promising technology to create standardized active delivery systems able to maintain propolis polyphenols active until use.

Not all the conventional encapsulating techniques are applicable due to propolis heat sensibility to temperatures below the room temperature and above 120° C for more than one minute (Gonzales et al., 2009). The membrane encapsulating technique could be a suitable technique, in fact one of the main advantages of this encapsulating technique respect to the conventional methods (Munin and Edwards-Lévy, 2011; Nedovic et al., 2011; Zhang et al., 2010), is the use of low energy density avoiding high shear rates and temperature; this helps to maintain quality and functionality of labile molecules, such as propolis polyphenols. Moreover, the encapsulation filter process employed is based on a membrane emulsification technique, which allows homogeneity of the matrix, the easy control of droplet sizes and size distribution of the obtained beads, by choosing suitable membranes and focusing on some operating process parameters (Piacentini et al., 2010). In particular, one of the main advantages of the membrane emulsification technique is the formation of active beads through the use of lower energy density and room temperature, in comparison with other techniques like extrusion blending, freeze drying or spray drying. In fact, in these conventional techniques, the high shear rates and the high variation of the process temperatures could have negative effects on sensitive active components.

One of the biopolymer that is largely used for encapsulation is chitosan. Chitosan is nowadays used for biomedical applications, drug delivery systems, coatings and tissue engineering, as well as applications in food, cosmetics and agricultural industries (Dutta et al., 2009; Senel and McClure, 2004). Even if it is not soluble in pure water, chitosan needs to be cross-linked in order to increase stability in contact with a lot of media (acidic solutions, oil/water emulsion, etc). One of the natural cross-linkers that is already used to cross-link biopolymers, to control swelling ratio and mechanical

properties, is genipin (Chen et al., 2004; Jin et al., 2004; Mi et al., 2001, 2005; Yuan et al., 2007; Liang et al, 2009).

In the present work, beads of chitosan and propolis, with genipin as cross-linker, were produced using the membrane process concept to create an example of delivery system stable during storage time and active against pathogen and spoilage food microorganisms.

Material and methods

Materials

Propolis as hydroalcoholic extract (60 wt % ethanol) was used. The extract was obtained with a patented method of purification, starting from propolis of italian regions, that permits to eliminate waxes and resins to have a Dewaxed Hydrodispersible Propolis Extract (EPID). It is characterized by a standardized polyphenolic profile, determined by LC-DAD-MS analysis, reported in Table 2. The propolis extract was kindly supplied by Specchiasol (Bussolengo, Verona – Italy). Chitosan (Medium Molecular Weight) was purchased by Sigma Aldrich. Genipin, an aglycone derived from an iridoid glycoside named geniposide and extracted from the plant *Gardenia jasminoides Ellis,* was supplied by Wako Chemicals GmbH (Germany). All other reagents were of analytical grade.

Microrganisms and culture conditions

Several microbial strains were tested, in particular eight bacteria, five yeasts and three moulds, as follows: *Bacillus cereus* MIM 71 (MIM: Microbiologia Industriale Milano), *Enterobacter agglomerans* ATCC 29904 (ATCC: American Type Culture Collection),

Enterococcus faecalis MIM 109, *Escherichia coli* CECT 434 (CECT: Spanish Type Culture Collection), *Listeria innocua* DSMZ 20649 (DSMZ: Deutsche Sammlung von Mikroorganismen und Zellkulturen), *Pseudomonas fluorescens* MIM C20 and MIM S9, *Staphylococcus aureus* MIM 178, *Candida kefyr* CCY 29810 (CCY: Czeckoslovak Collection of Yeast), *Yarrowia lipolytica* CCY 29.26.5, *Kluyveromyces bulgaricus* IMI LT (IMI: International Mycological Institute), *Kluyveromyces marxianus* var. *lactis* IMI C1 69, *Rhodotorula mucilaginosa* IMAP 6484 (IMAP: Istituto Microbiologia Agraria Perugia), *Aspergillus niger* NRRL 565 (NRRL: Agricultural Research Service Culture Collection), *Penicillium notatum* MIM 29, *Cladosporium cladosporioides* MIM 259.

Yeasts and moulds were grown on MEA (Malt Extract Agar) medium, of the following composition (g/l): malt extract (Costantino, Favria, Turin) 20, soybean peptone 2 (Costantino), agar 15, glucose 20, pH 5.8, sterilisation at 118°C for 20 min. Bacterial strains were grown on TSA medium (Tryptic Soy Agar, Scharlau Chemie - Barcelona), incubated at 30 °C for 24-72 h. Cultures were maintained as frozen stocks at - 20 °C in the above mentioned media in liquid form added with glycerol (10 % w/v), and propagated twice before use in experiments.

Beads preparation

Beads were produced using the membrane process concept previously explored by some of the same authors (Figoli et al., 2007, Lakshmi et al, 2012). In this work, chitosan powder was added (2% wt.) to an aqueous solution containing 1% wt. of acetic acid. After complete solubilisation of chitosan, the solution was purged into a cylindrical Teflon module tank (50 ml volume) containing a polyethylene (PE) mono-pore film, produced by using a micro-driller of a local goldsmith shop, obtaining a pore-size dimension of 600 micron and thickness of about 1 mm. The chitosan solution, passing

through the mono-pore film by gravity, formed the droplets in the air-gap phase (distance between the film and the aqueous continuous solution of about 10 cm) and entered in contact with the continuous phase forming the chitosan beads. The continuous aqueous solution phase was made of an acetic acid solution (1% wt.) with propolis concentration of 0%, 2% and 10% wt., and genipin as cross linker (0.023% wt.). Wet beads were left in the mixture for reticulation and propolis encapsulation, under stirring (100 rpm, ARE, VELP Scientific, Italy) for 24 h, then recovered using a filter paper (wet beads) (Fig. 1). The natural cross-linker genipin was used with the aim of stabilizing the beads and to permit chitosan future swelling without its solubilisation in contact with different means. The beads were finally set in a climate chamber (Angelantoni E301, Italy) at 30°C for 24 h upon drying (dry beads) and stored at least for 30 days.

Beads investigation

Diameter of wet beads and dry beads was determined using a digital micrometer (Carl Mahr D 7300 Esslingen A.N.) and an optical microscope (Olympus MIC-D). The morphology of chitosan beads was evaluated employing Scanning Electron Microscopy (SEM) at 20 kV (Cambridge Instruments Stereoscan 360). The efficiency of the cross-linking process was tested: chitosan beads, before and after cross-linking, were added to the distilled water and their solubility was evaluated as percentage of weight loss. Encapsulation efficiency was qualitatively evaluated by the identification of propolis polyphenols. A total extraction of polyphenols from beads was done putting in contact the beads with an ethanol solution (water:ethanol 50:50) for 24 hours under stirring and for 10 minutes of ultrasonic treatment. After filtration, the solutions were analysed by the LC-DAD-MS instrument as reported by Gardana et al. (2007). The chromatographic

system consisted of an Alliance 2695 (Waters, Milford, MA) equipped with a model 2996 (Waters) photodiode array detector and a triple quadrupole mass spectrometer mod. Quattromicro (Micromass, Beverly, MA). Identification of propolis polyphenols was achieved from a mother solution prepared by dissolving 10 mg of standard polyphenols (Sigma-Aldrich), in 10 mL methanol. The working solutions were prepared in the range of 0.5-50 µg/mL of Caffeic acid (CA), Ferulic acid (FA), 3,4-dimethyl-caffeic acid (DMCA), *p*-coumaric acid (pC), Chrysin (C), Galangin (G) from standard solutions while pinocembrin (P), pinobanksin (Pb) and pinobanksin-5-methyl-ether (Pb5ME) were assayed using pinocembrin.

Antimicrobial activity assay

Propolis antimicrobial activity was tested before and after encapsulation in the beads employing two liquid cultures, in particular TSB (Tryptic Soy Broth) for bacteria and MEB (Malt Extract Broth) for yeasts and moulds. The media were aliquoted (5 mL) in tubes and sterilized at 118°C for 20 min. Propolis, either in crude or as beads, was added after sterilization in order to obtain concentrations variable in the range 0-1 mg/mL. To avoid propolis sedimentation and favour the contact with microorganisms, cultures were subjected to magnetic stirring (150 rpm). Assays performed with moulds were set up in 100 mL Erlenmeyer flasks, each containing 10 mL MEB culture medium, maintaining the same propolis and beads concentrations range and stirring.

Microorganisms were inoculated (1% v/v) in form of a cell or spore suspensions, in the same culture medium, having an Optical Density (OD) at 600 nm of 0.300 ± 0.010. Cultures were incubated at 30 ± 1°C, up to 72 h for bacteria and yeasts and up to 14 days for moulds.

Control cultures (named Positive Control- PC) were always set up, without propolis. To determine propolis contribution to the increase of absorbance, series of tubes containing liquid medium and propolis at the tested concentrations (named Blank Propolis – BP) were also prepared.

Microbial growth for bacteria and yeasts was determined evaluating the increase in absorbance (A_c) (OD 600 nm) with the 6705 UV/Vis Spectrophotometer (Jenway). The autozero was done with the same base medium; cultures stirring was stopped 5 min before evaluation, to favor propolis but not microbial sedimentation. For each microorganism, growth in terms of absorbance (A_{mo}) was calculated as follows:

$$A_{mo} = A_c - A_{BP}$$

Where: A_c is the sample absorbance; A_{BP} is the absorbance of the liquid medium containing only propolis.

The Minimal Inhibitory Concentration (MIC) was defined as the lowest amount of crude propolis or beads that inhibited microbial growth, with A_{mo} values obtained lower than the initial absorbance or without significant difference ($p > 0.05$) from each other by the Least Significant Different (LSD) test (Williams and Abdi, 2010).

Subsequent trials performed with *Staphylococcus aureus* were set up as mentioned before; culture samples were taken at appropriate intervals and microbial growth (log cfu/ml) determined employing the plate count technique. The polyphenols components were identified inside culture samples. After incubation, cultures were centrifuged to separate cell mass from culture filtrates which were subjected to HPLC analysis as reported in section 2.4.

Results and Discussion

Beads characterization

198 Wet chitosan cross-linked beads produced with and without propolis are shown in Fig.
199 2. The successful loading of different quantity of propolis in chitosan beads resulted in a
200 visible change in colour from yellow (without propolis), to dark brown: the darker the
201 colour, the higher the concentration of propolis (from 2% to 10% wt) in chitosan beads.
202 The efficacy of the cross-linking process was determined by the beads solubility in
203 water that was found less than 10% after 24 h of contact, whereas more than 50%
204 solubilisation was observed in the case of the chitosan beads without the cross-linker.
205 A series of Scanning Electron Microscopy (SEM) pictures of the surface of dry chitosan
206 beads, with and without the active compound, is shown in Fig. 3(a). These
207 characterizations confirm the spherical shape of the dry active devices and highlight that
208 the presence of propolis determine the formation of a rough surface, visible at high
209 magnification (X 60K). The result of the encapsulation process is biopolymeric beads in
210 which propolis is dispersed in the chitosan according to the model presented in Fig.
211 3(b).
212 Size distribution was found to be different in wet and dry beads with and without
213 propolis, as shown in Fig. 4. In particular, wet beads either with or without propolis,
214 evidenced similar average capsule size diameter (1650±20 μm), Fig 4A, due to the
215 chitosan swelling phenomena presents in both types of beads. Desiccation obviously
216 produced a reduction in size dimension and such modification resulted in different
217 extent for beads with and without propolis. In fact, 40% of dry chitosan beads without
218 propolis had a mean diameter of about 500 μm, that increases to an average diameter of
219 600 and 700μm for 2% wt. and 10% wt. of propolis concentration in solution during
220 encapsulation (Fig.4B). The absence of water and the residual presence of propolis
221 molecules respectively in the two types of dry beads (with and without propolis),

resulted in a different steric hindrance and so in a different size of the beads in dry conditions.

To better understand which active propolis molecules are present in the beads, and so to evaluate the encapsulation quality, the identification of propolis polyphenols in the chitosan beads was done, by HPLC analysis. The chromatogram reported in Fig. 5 evidenced no significant presence of phenolic acids, whereas a significant presence of the main propolis phenolic esters and flavonoids was detected; this suggests that the phenolic acids are not encapsulated in the beads probably due their polar nature that leads to a greater affinity of the acids to the aqueous solution compared to the cross-linked chitosan. Contrariwise, the flavonoids and the esters, due to their amphiphilic nature, are able to interact with chitosan occupying areas of the beads structure.

Crude propolis and chitosan-propolis beads antimicrobial activities

Preliminary experiments were carried out in order to evaluate antimicrobial spectrum of activity of crude propolis. Trials were performed in liquid cultures, employing bacteria, yeasts and fungi selected among the most commons spoilage and/or pathogen microorganisms that might be present in fresh food products. Obtained results are reported in Table 1. Propolis was found to be active against *Staphycococcus aureus* (MIC 0.2 mg/mL), *Listeria innocua* (MIC 0.6 mg/mL) and *Enterococcus faecalis* (MIC 1 mg/mL), all Gram-positive bacteria, slightly against the yeast *Yarrovia lipolytica* (MIC 1 mg/mL after 72 h) and the moulds *Penicillium* and *Cladosporium* (MIC < 1 mg/mL only in the first 5 days), but not against *Bacillus,* Gram-negatives and yeasts in general. Similar results were reported in the literature, employing propolis of different geographical origin with differences in chemical compositions (Stepanovic et al., 2003; Koru et al., 2007; Kujumgiev et al. (1999). To be noted that the propolis used in this

247 work has been standardised by applying a patented method of purification: this assures a
248 precise polyphenols profiles and permits to correlate microbiological activity to the pool
249 type of polyphenols and to their relative percentages. It is in fact well known that
250 different substance combinations are essential for propolis biological activity
251 (Stepanovic et al., 2003) (Table 2).
252 Considering that propolis concentration inside the dry beads, and consequently its
253 polyphenols content, is lower than in the crude propolis samples, it is possible to say
254 that beads generally present a similar spectrum of action respect to crude propolis
255 (Table 1). This means that propolis antimicrobial activity is maintained also after
256 encapsulation, drying and storage. Results also evidenced that propolis-chitosan
257 combination increased propolis spectrum of activity, with the contribution of chitosan
258 against Gram-negative bacteria (*Pseudomonas* and *E. coli*). This behaviour has also
259 been evidenced in literature with chitosan and other natural antimicrobials (Dutta et al.,
260 2009; Scazzocchio et al., 2006, Rodriguez-Nunez et al., 2012).
261
262 **Antimicrobial activity against S. aureus liquid cultures**
263 In view of the importance of *S. aureus* in food poisoning, this microorganism was
264 chosen for the prosecution of the research. Beads were added (different concentration
265 comparatively) in liquid TSB cultures immediately after the inoculum, and microbial
266 growth monitored at appropriate intervals in terms of total viable count (log CFU/mL).
267 Table 3 reports the obtained results. In control samples without beads, *S. aureus*
268 population was found to increase from 6.6 to 10.2 log cfu/mL in about 24 h incubation.
269 The presence of chitosan beads without propolis did not produce a statistically
270 significant growth reduction. Instead, propolis beads added even at low concentration
271 (0.7 mg/mL culture) were found to inhibit *S. aureus* growth, and after 30 h incubation

272 microbial population remained at the same level or even lower than the initial inoculum
273 (5.9 ± 0.3 - 6.9 ± 0.4 log cfu/mL).
274 Filtrates obtained by centrifugation of culture samples at 30 h incubation were analysed
275 through HPLC. The presence of peaks with the characteristic Retention Times (Rt) of
276 the most representative flavonoids (Chrysin, Pinobanksin and Galangin) of the crude
277 propolis, and their absence in control cultures with beads without propolis, indicates
278 that flavonoids are effectively released in the surrounding media, leading to the
279 hypothesis that only in this way they can play their antimicrobial activity. The
280 antimicrobial activity can thus be attributed to the presence of flavonoids in the beads
281 that are the polyphenolic components most represented also in samples of crude
282 propolis.
283

284 ## Conclusions

285 Obtained results evidenced that the membrane emulsification technique is a promising
286 encapsulation technology to create dry active devices. These devices are stable during
287 their storage time and maintain their activity until use when they release in a controlled
288 way. Moreover, the laboratory membrane technology applied in this work, that can be
289 considered as precursor of the membrane emulsification process, can be considered a
290 promising encapsulation technique for propolis, thanks to the possibility of using mild
291 (room temperature and no shear stress) and "green" (no toxic solvent) process
292 conditions on a resinous complex matrix that is not water soluble.
293 Propolis-chitosan beads were found to inhibit the growth of several microbial strains
294 selected among the most commons spoilage and/or pathogen microorganisms that might
295 be present in fresh food products. Beads were found to inhibit microbial growth of *S.*

296 *aureus* in liquid culture, and flavonoids were found to be more responsible of this
297 activity respect to phenolic acids that are not encapsulated.
298 The obtained chitosan-propolis beads represent an example for the creations of an
299 innovative antimicrobial delivery system to prevent the growth of pathogenic and
300 sometimes also spoilage bacteria in food applications. Beads should be posed in direct
301 contact with the surrounding mean to be active. This study, that describes the efficacy of
302 a device based on propolis, could be completed undergoing the device to a risk
303 assessment procedure. This could permit to consider the active solution by potential
304 users for a real food application, for example on the internal surface of trays or bottles.
305

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310

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404

Table 1: Antimicrobial activity of crude propolis and chitosan-propolis beads obtained with a chitosan solution containing 10% of propolis, and MIC (Minimum Inhibitory Concentration, mg/mL) determination. Assays carried out in liquid culture.

Microrganism	MIC crude propolis (mg/mL)	MIC chitosan-propolis beads (mg/mL)
Bacillus cereus	> 1.2	1
Enterobacter agglomerans	> 1.2	> 2
Enterococcus faecalis	1	> 2
Escherichia coli	> 1.2	1
Listeria innocua	0.6	1
Ps. fluorescens MIM 151	> 1.2	1
Ps. fluorescens MIM 153	> 1.2	1
Staphylococcus aureus	0.2	0.8
Candida kefyr	> 1.2	> 2
Yarrowia lipolytica	0.2 (1 after 72 h)	1
Kluyveromyces bulgaricus	> 1.2	> 2
Kluyver. marxianus var. *lactis*	> 1.2	> 2
Rhodotorula mucilaginosa	> 1.2	> 2
Aspergillus niger	>1, different morphology	1, different morphology
Penicillium notatum	0.6 (> 1 after 5 days)	1.2
Cladosporium cladosporioides	0.6 (> 1 after 5 days)	1.2

417 Table 2: Propolis composition in crude propolis dry extract as phenolic acid end esters

418 (no bold), and flavonoids (Bold) (polyphenols correspond to 23% of the crude propolis).

419

Component	**%**
Caffeic acid	0.53
P-coumaric acid	0.44
Ferulic acid	0.44
Isoferulic acid	0.85
Caffeic acid dimethyl ether (DMCA)	1.02
Cinnamic Acid	0.26
Caffeic acid phenethyl ester (CAPE)	0.89
Chrysin	**3.54**
Pinocembrin	**4.72**
Pinobanksin-acetate	**2.53**
Pinobanksin-5-Methyl ether	**1.68**
Pinobanksin	**3.62**
Galangin	**2.83**

420

421

Table 3. Time course of *S. aureus* growth (expressed as log cfu/mL) in liquid cultures in absence and presence of different concentration of beads.

Beads added (mg/ml)	**Log cfu/ml**					
Time (hour)	**0**	**6**	**7**	**24**	**26**	**30**
0 (control)	6.6 ± 0.2	7.0 ± 0.5	7.3 ± 0.4	10.2 ± 0.7	10.3 ± 0.6	10.2 ± 0.8
+ 0.4	6.4 ± 0.3	6.9 ± 0.3	7.1 ± 0.2	8.3 ± 0.4	8.7 ± 0.4	9.4 ± 0.3
+ 0.7	6.5 ± 0.2	6.0 ± 0.4	6.5 ± 0.2	6.4 ± 0.3	6.7 ± 0.2	6.9 ± 0.4
+ 1.4	6.6 ± 0.4	6.5 ± 0.2	6.5 ± 0.5	6.2 ± 0.5	6.4 ± 0.5	6.3 ± 0.2
+ 2.8	6.4 ± 0.3	6.4 ± 0.2	6.1 ± 0.4	5.9 ± 0.3	5.9 ± 0.3	5.9 ± 0.3
+ 2.8 w/o*	6.4 ± 0.3	7.1 ± 0.3	7.7 ± 0.4	9.5 ± 0.5	9.3 ± 0.4	9.3 ± 0.5

* w/o: beads without propolis