Research Article

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Preparation and evaluation of alginate/chitosan microspheres containing pheromones for pest control of *Megaplatypus mutatus* Chapuis (Platypodinae: Platypodidae)

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Abstract

This work describes the optimization of an alginate/chitosan microsphere preparation for the encapsulation of a sexual pheromone, 6-methyl-5-hepten-2-ol (sulcatol), to realize a slow-release device for the biological control of the *Megaplatypus mutatus* pest. To evaluate and select the best encapsulation/release conditions three parameters were studied: alginate concentration, pH of gelling solution and Ca^{2+}/COO^{-} ratio. The preparation was optimized using biopolymers with improved mechanical properties and swelling behavior. The obtained microspheres were characterized using Fourier transform infrared spectroscopy, scanning electron and optical microscopies, swelling degree, mechanical properties and *in vitro* release of encapsulated pheromone. The microspheres performed best when they were synthesized using an alginate concentration of 4% w/v, at pH = 9 and with a Ca^{2+}/COO^{-} ratio of 3.5. The attractiveness of the alginate/chitosan microspheres towards *M. mutatus* was demonstrated by behavioral bioassay with the completed pheromonal blend of the species (sulcatol, sulcatone and 3-pentanol). The formulation can be considered as an efficient slow-release biological control system, with no negative environmental impact.

Keywords: microspheres; biopolymers; alginate/chitosan; *Megaplatypus mutatus*

INTRODUCTION

The excessive use of pesticides has led to an increase in the resistance of insects and mounting concerns about risks to human health and the environment. New strategies to control the damage caused by pests in forests and fields have been developed in recent decades, applying semiochemical slow-release devices to modify insect behavior.[1] This technology is based on the development of reservoirs using pheromones to mediate intraspecific interactions or allelochemicals to mediate interspecific interactions for their use in the control of a pest.[2] Since semiochemicals are highly volatile and extremely unstable, they need to be protected from degradation by UV light and oxygen. Their encapsulation within a polymeric matrix has proved to be successful, the polymer acting as the rate-controlling membrane to obtain the desired controlled release. The development of these devices must conform to certain specifications clearly defined by Heuskin *et al.*:[3] the aerial concentration after release must be sufficiently high for detection by insects; the release of semiochemicals must remain effective throughout the entire period of insect occurrence; and the production of the dispenser must be reproducible. Such devices should also be economically viable, devoid of harmful side effects and of course field-tested to prove their efficiency towards targeted insects before being legally authorized and commercialized.[4,5]

The current research focuses on *Megaplatypus mutatus* (= *Platypus mutatus*) Chapuis (Platypodinae, Platypodidae), an ambrosia beetle native to South America[6] that only attacks standing, live trees by burying into the xylem, forming tunnels that are later colonized by the fungus that the beetles transport. This tunnel system subsequently weakens the tree stem, causing breakage under conditions of severe stress and posing a serious threat to commercial plantations of poplar *Populus deltoides* Marshall (Salicaceae).[7,8] Chemical analysis, electro-antennogram and olfactometry studies show that male *M. mutatus* emits a sex pheromone composed mainly of 6-methyl-5-hepten-2-ol (sulcatol, STOL), its related ketone 6-methyl-5-hepten-1-one (sulcatone, STONE)[9] and 3-pentanol (3-PEN). Although there is considerable variability among insects, the mean relative amount of 3-PEN is estimated as $13.9 \pm 6.4\%$, of STONE as $34.9 \pm 9.3\%$ and of STOL as $51.2 \pm 10.7\%$.[10,11]

These compounds have also demonstrated their effectiveness for the purpose of disrupting both trapping and mating when delivered from reservoir systems made with polyethylene.

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However, an environmentally safe biodegradable matrix is preferable as a slow-release device for semiochemical delivery.[5,12] For this type of application, natural polysaccharides are the carrier material of choice.[13–15] The advantages of the use of biorenewable materials have also been taken into account.[16,17]

Alginate has attracted increasing attention due to its biodegradability and unique property of mild gel formation in the presence of multivalent cations in aqueous media,[18,19] although its high porosity and loose network constitute major obstacles to bead preparation. This latter impediment can be effectively diminished by the use of polycations such as chitosan.[20] Chitosan is a naturally occurring polysaccharide comprising D-glucosamine and *N*-acetylglucosamine with unique polycation characteristics. Upon mixing with alginate, the strong electrostatic interaction of the amino groups of chitosan with the carboxyl groups of alginate leads to the formation of a chitosan/alginate complex, which reduces the porosity of alginate beads and thus reduces leakage of encapsulated drugs.[21]

To date, a number of methods have been used for the preparation of alginate/chitosan microspheres, such as spray-drying, emulsification/solidification, coacervation and the membrane emulsification technique.[5,12,13,15,20,22] However, the ideal carrier is highly dependent on the type of application and on the chemical structure and concentration of the compounds to be incorporated into the microspheres. In a literature search we were unable to find any report on the use of alginate/chitosan microspheres as controlled-delivery carriers of pheromones for use in pest control.

In this paper we report the optimization of the synthesis of microspheres prepared from alginate/chitosan polyelectrolyte complexes and the encapsulation of the pheromone STOL into these microspheres. Our goal was to develop a new device with desired release kinetics for potential use in the control of the *M. mutatus* pest. After optimization of the microsphere formulation with STOL, STONE and 3-PEN were encapsulated.

It is known[5,12,24,25] that the diffusion speed of volatile compounds through beads depends on characteristics such as the size, shape and thickness of the matrix and the distribution of a semiochemical in the matrix. In order to optimize the release performance we therefore evaluated these parameters by carrying out a physicochemical characterization of the beads. Behavioral bioassays showed a good percentage of attraction for released semiochemical volatiles for *M. mutatus* with the pheromones STOL, STONE and 3-PEN as attractants.

EXPERIMENTAL

Materials

Chitosan with an average viscosity molecular weight of 50–190 kDa and 85% deacetylation and analytical grade STOL (98%), STONE (99%) and 3-PEN (98%) were purchased from Sigma-Aldrich. Sodium alginate (LF 120 M, fG 35–45%, viscosity 70–150 mPa s) was obtained from Protanal FMC Biopolymers. Glacial acetic acid (analytical grade) was purchased from Anedra. Sodium hydroxide beads and calcium chloride were purchased from Cicarelli.

The solvents used were of analytical grade and no purifications were performed on them. Ethyl alcohol, dichloromethane, chloroform and tetrahydrofuran were purchased from Anedra. The pH changes were carried out with standard buffer solutions.

Table 1. Experimental design for the synthesis of alginate/chitosan microspheres

Run order	$(Ca^{2+})/(COO^-)$	Alginate concentration (% w/v)	pH of gelling solution
1	1.5	4	3
2	1.5	4	6
3	1.5	4	9
4	1.5	6	3
5	1.5	6	6
6	1.5	6	9
7	3.5	4	3
8	3.5	4	6
9	3.5	4	9
10	3.5	6	3
11	3.5	6	6
12	3.5	6	9
13	5.4	4	3
14	5.4	4	6
15	5.4	4	9
16	5.4	6	3
17	5.4	6	6
18	5.4	6	9

Preparation of alginate/chitosan microspheres

Solutions of sodium alginate (3 mL) were prepared at concentrations of 4 and 6% (w/v). Each solution was dropped via a 2 mm syringe into a solution of $CaCl_2$ (2% w/v). The spheres that formed in the $CaCl_2$ gelling solution were left there for 10 min, then collected and placed in 20 mL of a solution of chitosan (1% w/v, dissolved in 0.1 mol L^{-1} HCl) for 10 min and finally collected and placed in a solution of $CaCl_2$ (1% w/v) for a further 10 min.

To encapsulate the semiochemical, it was previously incorporated into the alginate solution in a ratio of 0.78 mol of STOL, STONE or 3-PEN to 100 g of alginate (120 mg semiochemical per 100 g of alginate). Drops were added into an agitated (magnetic stir bar at 600 rpm) $CaCl_2$ solution to form alginate gel beads containing the semiochemical compounds.

Three values of pH of the gelling solution were used to evaluate the effect of pH on the preparation of microspheres: 3, 6 and 9. The effect of the Ca^{2+}/COO^- ratio on the release of the semiochemicals was also evaluated, using values of 1.5, 3.5 and 5.4 (Table 1).

Microsphere characterization

Fourier transform infrared (FTIR) spectra of alginate, chitosan and alginate/chitosan complex were recorded with a Nicolet 5-SXC spectrometer. Samples were mixed with KBr, after which discs were prepared.

The morphology of the microspheres was investigated using SEM and watershed transformation (optical microscopy). SEM images (JEOL JSM 6060, resolution 3.0 nm) were obtained by applying the following protocol. The spheres were placed in turn for 20 min in 10 mL of the following proportions of water–acetone solutions: 70:30%, 50:50%, 10:90% and 0:100%. The microspheres were cut in half immediately after the last immersion, dehydrated by 'critical point' and metalized with gold, taking care to place the cut-side areas of interest upwards.

Optical microscopy analysis was performed with a digital CMOS (20×) microscope to test the samples' ability to reflect the light

emitted from the microscope using the watershed transformation program.

The water sorption capacity of the microspheres was determined by swelling them in distilled water at room temperature. Dried microspheres (W_d) were immersed in distilled water for 24 h. The swollen samples (W_s) were removed from the solution, quickly wiped with filter paper and weighed. The swelling degree (Sd) of microspheres was determined according to the following expression:

$$S_d = \frac{W_s - W_d}{W_d} \times 100 \quad (1)$$

The test of the compression capacity of the microspheres was performed with an Instron Texturometer universal testing machine (model 3342, Norwood, MA, USA) equipped with a cell capacity of 500 N. The compression capacity (gf) of each microsphere was measured in terms of a 50% deformation of the original volume. Each microsphere was measured with a gauge in order to set the compression distance that the cylinder had to move to produce a 50% deformation.

Controlled release studies

Optimization of microsphere synthesis and volatile extraction

The effect of the pH (3, 6 and 9) of the gelling solution was studied in order to evaluate its impact on the final release of the compounds of interest from the microspheres, initially using STOL only. Once it was determined which pH gave the best results, the release of STONE and 3-PEN was also studied at this optimal pH.

The alginate/chitosan spheres (500 mg) containing each of the pheromones were placed in vials for solid-phase microextraction (SPME) and left for 24 h at 25 °C. A polydimethylsiloxane fiber (Supelco, Bellfonte, PA) was then placed for 2 min in the vial head space and subsequently injected into a Shimadzu 14B gas chromatography (GC) instrument equipped with flame ionization detector. The column was a β DEX 120 of 30 m length, 0.25 mm inner diameter and 0.25 μm film thickness. Injector and detector temperatures were 230 and 250 °C, respectively. The carrier gas was nitrogen at a constant flow rate of 0.5 mL min^{-1}. Flame composition was hydrogen flow at 30 mL min^{-1}, air flow at 300 mL min^{-1} and make up gas flow at 30 mL min^{-1}. The temperature program was as follows: initial temperature 60 °C/3 min, ramp at 10 °C min^{-1} up to 120 °C/20 min hold.

After collecting the head space, the vial was cleaned with a stream of nitrogen, closed again, and the procedure was repeated every 24 h until no further signal was observed.

We did not attempt to quantify the volatiles since SPME is not suitable for this purpose; however, by plotting the total area of the pheromone *versus* time we established the temporal pattern of the release, i.e. the maximum number of days that the microspheres are active.

Quantification of remaining pheromone

After no more pheromone was detected in the head space of the vial, the spheres were placed in vials with 3 mL of dichloromethane in order to remove any residual compound not released to the gaseous state. The remaining pheromone was calculated using GC, with the following GC program using a β DEX 120 column: initial temperature 90 °C, held for 3 min, ramp at 15 °C min^{-1} to 200 °C, and held for 40 min. The carrier gas was nitrogen. The volume injected was 1 μL. The total amount of pheromone liberated in the gaseous state was calculated from the difference between initial load and residual amount.

Behavioral activity of microspheres

A glass Y-tube olfactometer was used to investigate the attraction of female *M. mutatus* to semiochemical odors released by the alginate/chitosan microspheres. The two arms of the tube were connected to small glass chambers (5 cm in diameter) each containing alginate/chitosan microspheres loaded with one of the compounds of the blend, or alginate/chitosan microspheres with no compounds inside them used as a control. The internal diameter of the olfactometer arms was 4 mm, similar to that of the natural gallery, so that the insect could walk easily upwind in the airstream. The length of each arm was 9 cm.

Air was pushed at a rate of 15 mL min^{-1} into each glass chamber. The experiments were conducted in a room at 24 ± 2 °C with homogeneous light, between 12 and 4 p.m. Three alginate/chitosan microspheres with and without semiochemicals, respectively, were deposited in the chambers of the olfactometer 20 min before experiments. After each essay, the odor source and the control chambers were changed (interchange of olfactometer arms) in order to avoid any bias towards one of the arms. Between each experiment, the arms of the olfactometer were cleaned with *n*-hexane to eliminate residual odors and dried at room temperature for 30 min.

For each experiment, one female *M. mutatus* was individually introduced into the third arm at the opposite side of the two test arms. In the center of the olfactometer, where the three arms were connected, females had a choice between control or semiochemical odor arms. The test ended when the female entered one of the olfactometer arms and walked through it towards the source. Ten females were used for STOL, nine for STONE and eight for 3-PEN.

Statistical analysis

For statistical analysis, the results were subjected to Fisher's least significant difference test and analysis of variance using the Infostat program, with a significance level of 0.05.

RESULTS AND DISCUSSION

The optimization of alginate/chitosan beads was achieved by taking into account the concentration of pheromone encapsulated and its controlled release as a function of time. Parameters playing a role in the microsphere formation process were modified according to three experimental parameters – alginate concentration, Ca^{2+}/COO^- ratio and pH of the gelling solution – in order to determine their effect on the encapsulation/liberation of pheromone and the mechanical stability of the beads.

Finally, an olfactometer bioassay was performed with the optimized microsphere formulations in order to determine the attractiveness efficiency of the alginate/chitosan beads towards *M. mutatus* insects. STOL, STONE and 3-PEN were individually tested.

Preparation of the alginate/chitosan beads

The alginate/chitosan microspheres were successfully prepared using the coacervation technique following the procedure shown in Fig. 1. Microspheres with a monodisperse size distribution and a uniform diameter of approximately 3 mm were obtained.

This novel two-step methodology puts the microspheres in contact with a $CaCl_2$ solution twice, providing microspheres in very good yields and with a perfectly spherical shape that is maintained over time, with a marked improvement in stability. A possible explanation for this is that the free carboxylate groups that do

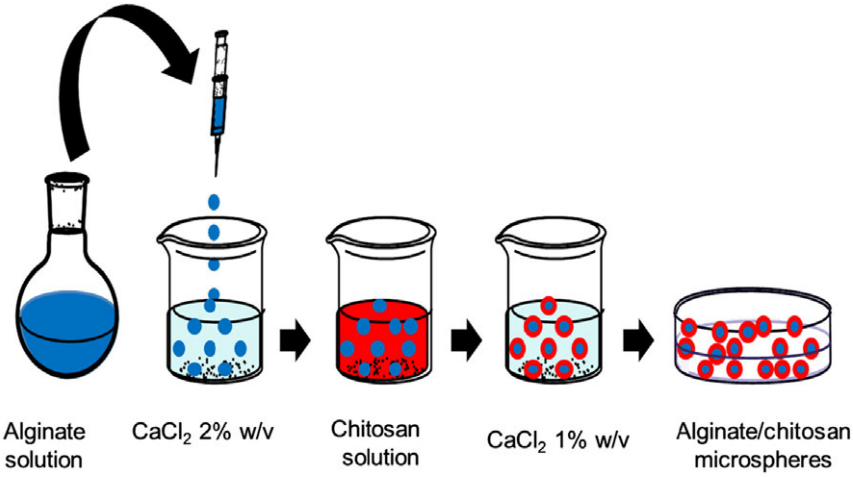

Figure 1. Methodology used for obtaining alginate/chitosan microspheres.

not react with Ca^{2+} ions in the first stage or that do not interact with the amino of chitosan have a second opportunity to crosslink with calcium ions after the relaxation of the system. Experimental conditions, namely alginate concentration (4 and 6% w/v), pH of the gelling solution (3, 6 and 9) and Ca^{2+}/COO^- ratio (1.5, 3.5 and 5.4), were studied in order to evaluate their effect on the final properties of the beads (Table 1).

The electrostatic attraction between the cationic amino groups of chitosan (pK_a value about 6.3) and the anionic carboxyl groups of alginate (mannuronic acid monomers $pK_M = 3.38$ and guluronic acid monomers $pK_G = 3.65$) is the main interaction leading to the formation of polyelectrolyte complexes but is strongly dependent on the pH of the reaction solution.[25] The crosslinking reaction of Ca^{2+} ions with carboxylate groups of alginate is markedly favored at higher pH. The pH of the reaction, the alginate concentration and the Ca^{2+}/COO^- ratio are the parameters exerting the greatest effect on the physicochemical properties of the beads. These parameters will be discussed further in relation to the swelling behavior and mechanical properties of the microspheres.

Characterization of microspheres

FTIR spectral analysis

The FTIR spectra were analyzed to characterize the chemical formation of the alginate/chitosan microspheres. For the isolated polymers, the spectra are in agreement with data reported in the literature (results not shown). The main chitosan bands are at 3451 cm^{-1} (OH), 2875 cm^{-1} (C—H), 1652 cm^{-1} (amine I), 1590 cm^{-1} (NH_2) and 1150 cm^{-1} (C—O—C); and for alginate at 3442 cm^{-1} (OH), 2923 cm^{-1} (C—H), 1616 and 1415 cm^{-1} (COO—), 1030 cm^{-1} (C—O—C) and 820 cm^{-1} (C—O).

Broadening of the band at approximately 3433 cm^{-1} is observed in the spectrum of the alginate/chitosan microspheres and can be attributed to overlapping of the stretching frequencies of —OH and —NH_2 groups and to hydrogen bonds between chitosan and alginate. The bands at 2923 and 1418 cm^{-1} represent stretching and bending frequencies of —CH_2 groups. The bands at 1622 (broadening) and 1088 cm^{-1} are due to the overlapping of —NH_2 (chitosan) and —COO^- (alginate) and stretching of —COO^- groups, respectively. The presence of —C—O and —C—N linkages is confirmed by the peaks at 1316 and 1033 cm^{-1}.[1,3,20]

The N—H bending vibration of non-acylated 2-aminoglucose primary amines (band at 1590 cm^{-1}) and asymmetric and symmetric —C—O stretching at 1616 and 1415 cm^{-1} show clearly reduced relative absorbance, indicating that the —NH_3^+ of the chitosan had reacted with the —COO^- of the alginate.[23]

Optical microscopy and SEM studies

Optical microscopy and SEM techniques were used to determine the morphology of the microspheres obtained with various alginate concentrations and pH.

Figure 2 shows SEM images of microspheres, with diameters ranging from 2 to 3 mm. The SEM analysis of the external and internal surface of the beads reveals that most spheres present rough surfaces, the roughness of the external surface increasing with the pH of the gelling solutions and showing a highly heterogeneous shell which could imply a closer interaction between alginate and chitosan. The typical drying process of the samples for SEM analysis gives rise to folds which are most evident in those samples with a lower degree of crosslinking (pH = 3 and 6).

Figure 3 shows images of the microspheres after applying the watershed transformation. Knowing that the reflection of light is high in crystalline zones and low in amorphous zones, the difference in topography between the core formed by alginate, an amorphous polymer, and the shell formed by an interpenetrating network of alginate and crystalline polymer chitosan becomes clear.

Furthermore, when the pH of the gelling solution is increased from 3 to 9, a broadening of the shell is observed. This could be explained by the increase in the electrostatic interaction between alginate and chitosan and also by an improvement in the crosslinking reactions of alginate with Ca^{2+} ions, giving rise to a more compact core and higher interpenetration of alginate/chitosan chains in the shell.

An increase in the alginate concentration from 4 to 6% (w/v) gives rise to microspheres with a more heterogeneous outer layer. A higher alginate concentration without Ca^{2+} ions and no increase in the chitosan concentration weakens the crosslinked network and the electrostatic interaction between alginate and chitosan.

Effect of pH and Ca^{2+}/COO^- ratio of gelling solution on swelling behavior of microspheres

Since the degree of swelling of the alginate/chitosan microspheres depends on the process used for the preparation of the beads,

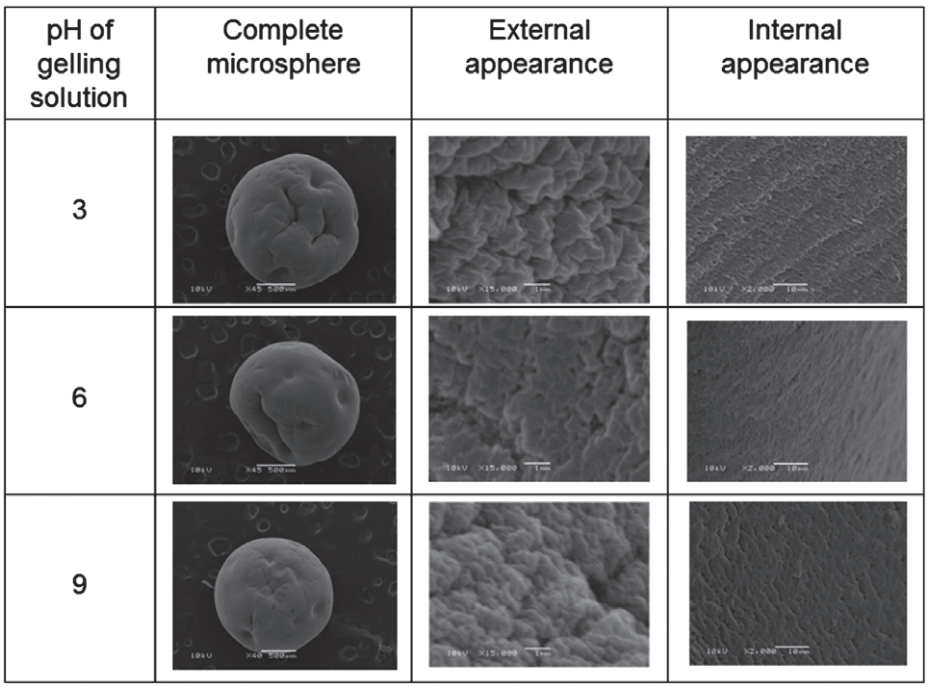

Figure 2. SEM images of alginate/chitosan microspheres synthesized with gelling solution of three pH values (3, 6 and 9).

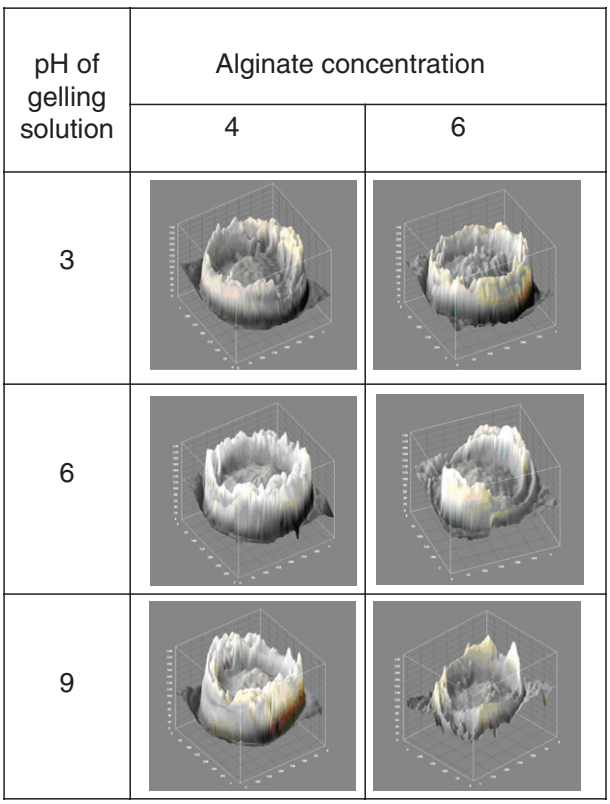

Figure 3. Optical microscopy and watershed transformation images of alginate/chitosan microspheres synthesized with gelling solution of three pH values (3, 6 and 9) and with two alginate concentrations (4 and 6% w/v).

swelling studies were carried out in water in order to determine the impact of three reaction condition parameters on the water uptake ability of the beads: pH, alginate concentration and Ca^{2+}/COO^{-} ratio.

Figure 4(a) shows the variation in the degree of swelling in relation to pH and alginate concentration. At both alginate concentrations (4 and 6%), the lower the pH (pH = 3) the higher is the degree of swelling, and the higher the pH (pH = 9) the lower is the degree of swelling of the microspheres. The highest degree of swelling at the lowest pH is attributed to the protonation of chitosan. The deprotonation of alginate begins to take on importance at pH = 6, but the improvement in the crosslinking reactions of alginate and the electrostatic interaction between alginate and chitosan when the pH increases lead to a decrease in the degree of swelling.

The concentration of alginate in the preparation of the beads also affects the degree of swelling of the microspheres. In all cases, the degree of swelling of the beads increases with increasing concentration of alginate at the same pH. However, at a 4% alginate concentration, the differences are significant at pH = 9 and non-significant at pH = 3 and 6, whereas at a 6% alginate concentration the differences are significant at pH = 3. The former result could be explained by the increasing formation of a crosslinked network and the latter may be ascribed to the lower level of crosslinking, since when the alginate concentration increases the Ca^{2+} ion concentration remains the same. This same reasoning explains the crosslinking reactions based on the electrostatic interaction between the two polyelectrolytes. This phenomenon is clearly visible at pH = 3, when the microspheres present the highest degree of swelling. A further point to mention is that alginate is a more hydrophilic polymer than chitosan. The pH also has a significant effect on the swelling properties: at 4 and 6% alginate concentrations the differences in the degree of swelling are significant at the two extremes of pH = 3 and 9.

The Ca^{2+}/COO^{-} ratio has a direct impact on the degree of crosslinking of the microspheres and significant differences are found for all the studied conditions. Figure 4(b) shows that the degree of swelling varies with pH (6 and 9) for three different Ca^{2+}/COO^{-} ratios (1.5, 3.5 and 5.4). The higher level of crosslinking found at pH = 9 produces a lower degree of swelling compared

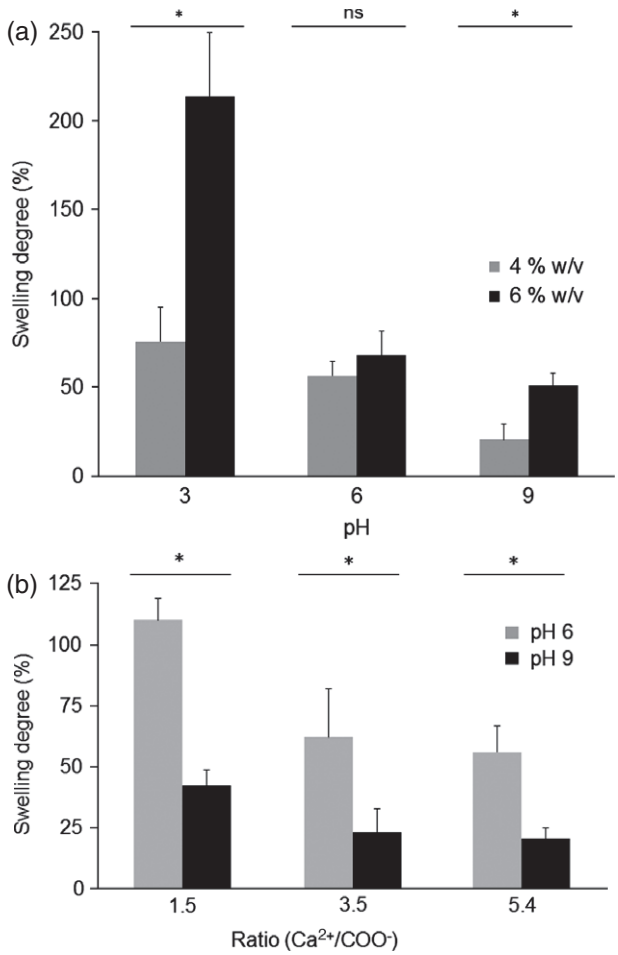

Figure 4. Degree of swelling of alginate/chitosan microspheres (a) synthesized with gelling solution of three pH values (3, 6 and 9) and with two alginate concentrations (4 and 6% w/v); and (b) synthesized with variation of pH (6 and 9) and with three Ca^{2+}/COO^{-} ratios (1.5, 3.5 and 5.4). Asterisk indicates significant difference between treatments ($p \leq 0.05$).

with that at pH = 6. This effect is seen for the three Ca^{2+}/COO^{-} ratios analyzed.

Microsphere texture analyses

The compression was studied on microspheres prepared with 4% (w/v) of alginate concentration and under different pH conditions (6 and 9) and Ca^{2+}/COO^{-} ratios (1.5, 3.5 and 5.4). Figure 5 shows the results.

The highest compression of the alginate/chitosan microspheres is found at pH = 9 concomitantly with the highest Ca^{2+}/COO^{-} ratio (5.4). However, at this pH significant differences are found between microspheres at all the molar ratios tested.

Networks formed at higher pH and Ca^{2+}/COO^{-} ratios tend to be more rigid and resistant. These results corroborate the finding under these same conditions of the highest level of alginate crosslinking and electrostatic interaction between alginate and chitosan, giving rise to a more rigid network with improved mechanical properties and stability. These results are also in agreement with the findings of the swelling studies.

Semiochemical release measurements

The effect of the pH of the gelling solution was analyzed in order to determine the optimal value for achieving the highest

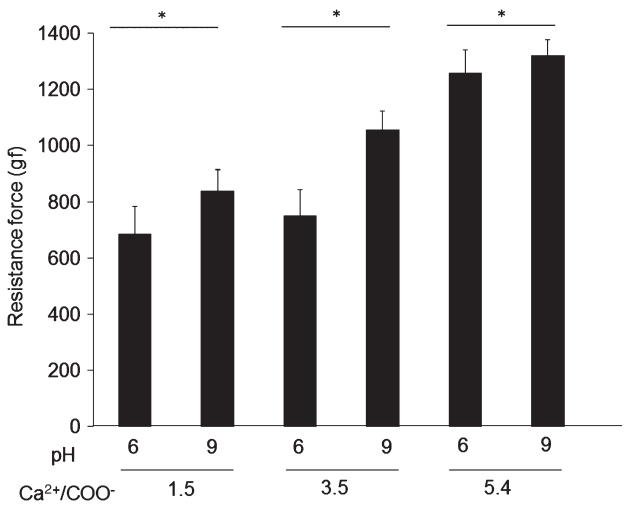

Figure 5. Resistance force of alginate/chitosan microspheres synthesized with an alginate concentration of 4% (w/v) and with different conditions of pH of the gelling solution (6 and 9) and Ca^{2+}/COO^{-} ratio (1.5, 3.5 and 5.4). Asterisk indicates significant difference between treatments ($p \leq 0.05$).

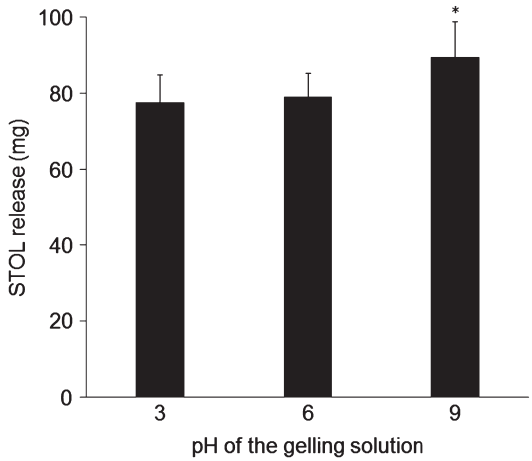

Figure 6. STOL release as a volatile from alginate/chitosan microspheres in closed glass vials (25 °C and without airflow). The devices were synthesized with an alginate concentration of 4% (w/v) and Ca^{2+}/COO^{-} ratio of 3.5 and with various values of pH of the gelling solution (3, 6 and 9). Asterisk indicates significant difference between treatments ($p \leq 0.05$).

amount of released pheromone. To this end we quantified the total release of STOL in the gaseous state as the difference between the total amount of compound introduced at the synthesis and the residual amount found after the volatile emission experiment.

The Fisher test shows significant differences between the releases achieved at pH = 9 (89.51 mg), whereas no differences are found between those at pH = 3 (77.56 mg) and 6 (79.03 mg) (Fig. 6). Likewise, the microspheres synthesized at pH = 9 released STOL for seven days, whereas those synthesized at pH = 3 and 6 released the compound for five days.

The release for the different systems increases with pH; the swelling studies and mechanical properties corroborate that at high pH the microspheres are less amenable to swelling, giving rise to microspheres with more rigid and stable pores (lower gel structure), enabling them to release higher concentrations of pheromones for a longer period of time.

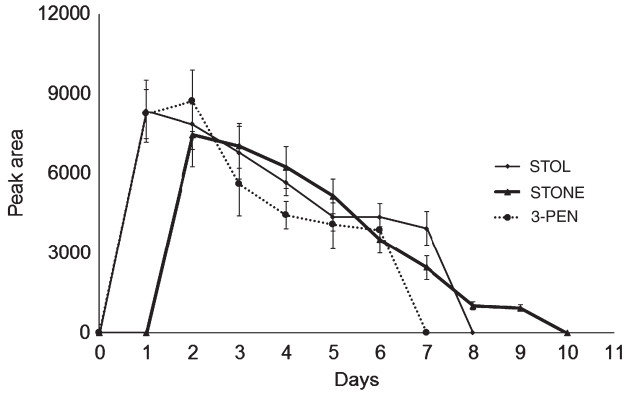

Figure 7. Volatile release patterns for STOL, STONE and 3-PEN from alginate/chitosan microspheres analyzed by SPME–GC. GC program: initial temperature 60 °C, held for 3 min, ramp at 10 °C min^{-1} to 120 °C, and held for 20 min. Carrier gas: nitrogen.

Once the optimal pH of the gelling solution was established at 9, the release of STONE and 3-PEN was analyzed with the following synthesis conditions: alginate concentration of 4% (w/v), Ca^{2+}/COO^{-} ratio of 3.5 and pH of the gelling solution of 9. The microspheres with 3-PEN release 95.7 mg in six days and those with STONE release 107.65 mg in nine days.

The total amounts of pheromones released corroborate our previous results that the aerial concentrations obtained are biologically relevant. Furthermore, Fig. 7 shows the peak areas of the three compounds of the blend as they are present in the head space. Although we did not attempt a quantification, the temporal pattern of release follows the kinetic behavior expected for monolithic systems of pheromone delivery, with a high initial release of the compound followed by an exponential decrease in the speed of release. The biological relevance of this result was analyzed with olfactometry studies.

Response of *M. mutatus* females to alginate/chitosan microspheres

Olfactometry bioassays were performed with optimized formulations of the microspheres in order to determine the attractiveness efficiency of the alginate/chitosan microspheres towards *M. mutatus* females.

Attractiveness studies were performed with alginate/chitosan microspheres synthesized with a 4% (w/v) concentration of alginate, a Ca^{2+}/COO^{-} ratio of 3.5 and a pH of 9. The results obtained indicate that the concentration of STOL and STONE emitted by the alginate/chitosan microspheres is adequate to elicit attraction on the part of the females tested (Table 2). The third pheromonal blend compound assayed, 3-PEN, does not show a significantly positive effect on the attraction response of the insects. However, this result is not a limitation when defining the attractiveness of the full blend: it is known that although the three pheromonal compounds are emitted by the insect in nature, there are no significant differences between insect attraction to a blend formed by STOL and STONE and one formed by STOL, STONE and 3-PEN.[10] In our previous field work we found that there was no significant difference in insect catches for a blend formed by STOL and STONE and that formed by STOL, STONE and 3-PEN.[25]

The results suggest that the alginate/chitosan microspheres are suitable for use in pheromone-baited traps in fields for catching female *M. mutatus*. The novelty of the study is that the devices used as polymeric carriers can release these compounds in the necessary amounts to cause an attraction response.

Table 2. Number of female *M. mutatus* responding to STOL, STONE and 3-PEN in a Y-tube olfactometer

Test chemical	No. of females walking to control	No. of females walking to test chemical
STOL[a]	2	8
STONE[a]	1	8
3-PEN	2	5

[a] χ^2 test on the number of insects responding to either STOL or STONE indicated that both chemicals elicited an equally significant attraction response on female *M. mutatus* compared with control (χ^2 STOL = 3.26; $\alpha = 0.1$; d.f. = 1; χ^2 STONE = 6.26; $\alpha = 0.025$; d.f. = 1).

The study of other factors such as the speed of evaporation, which depends mainly on environmental parameters like air temperature, wind speed and relative humidity, and the physical properties of the compound itself are in progress as a second phase of this research.

CONCLUSIONS

Microspheres with an alginate core and alginate/chitosan shell were successfully prepared and in laboratory tests demonstrated their potential application as carriers for the controlled release of insect sex pheromones. The best performance was observed when the microspheres were synthesized with a 4% (w/v) alginate concentration, at a pH of 9 and with a Ca^{+2}/COO^{-} ratio of 3.5. Under these conditions they showed improved mechanical properties and optimal swelling behavior for controlled release. The great advantage of this delivery system is the ability to fraction the set of microspheres in order to achieve the desired concentration in the environment.

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