Sustained Release of Fipronil Insecticide *in vitro* and
in vivo from Biocompatible Silica Nanocapsules

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ABSTRACT

A pesticide delivery system made of biocompatible components and having sustained release properties is highly desirable for agricultural applications. In this study, we report a new biocompatible oil-core silica-shell nanocapsule for sustained release of fipronil insecticide. Silica nanocapsules were prepared by a recently-reported emulsion and biomimetic dual-templating approach under benign conditions and without using any toxic chemicals. The loading of fipronil was achieved by direct dissolution in the oil core prior to biomimetic growth of a layer of silica shell surrounding the core, with encapsulation efficiency as high as 73%. Sustained release of fipronil *in vitro* was tunable through control of the silica shell thickness (i.e., 8–44 nm). *In vivo* laboratory tests showed that the insecticidal effect of the fipronil-encapsulated silica nanocapsules against economically important subterranean termites could be controlled by tuning the shell thickness. These studies demonstrated the effectiveness and tunability of an environmentally-friendly sustained release system for insecticide, which has great potential for broader agricultural applications with minimal environmental risks.

KEYWORDS

silica; nanocapsules; sustained release; insecticides; fipronil; subterranean termites

29 INTRODUCTION

30 Over recent decades, there has been an increasing interest in the development of pesticide
31 delivery systems to prevent and control pest populations, especially in countries with agriculture-
32 driven economies.¹⁻⁵ Encapsulation and delivery of active ingredients such as pesticides using
33 carriers offer substantial advantages including protection of the actives against premature
34 decomposition (e.g., photolysis, hydrolysis, microbial, or oxidation/reduction) and loss (e.g.,
35 evaporation or leaching), protection of the environment against toxicity of the actives, enhanced
36 bioavailability especially for hydrophobic actives, and the possibility of delivering high amounts
37 of actives in a sustained manner. Sustained release of pesticides is highly desirable in agriculture
38 as it likely reduces periodic applications by maintaining an effective local concentration in a
39 prolonged duration, thereby lowering potential for development of pest resistance and
40 undesirable environmental side effects. Polymeric matrices,⁶⁻⁸ solid lipid particles⁹ and
41 composites^{10,11} are among commonly investigated carriers in pesticide delivery systems.

42 Silica-based nanomaterials mainly evaluated for biomedical applications¹² have emerged as
43 relatively new nanocarriers for agricultural applications.¹³⁻²⁵ Silica materials can offer distinct
44 advantages over other materials, as they provide more mechanically stable structures than
45 polymeric materials¹⁵ and have structural flexibility in forming nanomaterials with high-capacity
46 loading of actives.²⁶ Furthermore, silica in an amorphous structure is biocompatible as it is
47 categorized as a “Generally Recognized As Safe (GRAS)” material by the U.S. Food and Drug
48 Administration.²⁷ Previous studies have shown the loading and release of various agrochemicals
49 from silica-based nanomaterials, including non-porous silica nanoparticles,¹⁶ mesoporous silica
50 nanoparticles¹⁷⁻²⁰ and hollow-core silica-shell nanoparticles.²¹⁻²⁵ The latter are more
51 advantageous as the core provides higher loading capacity of actives than non-porous and

mesoporous silica nanoparticles.²⁸ Generally, there are two techniques of active-loading in core-shell silica nanoparticles i.e., post-loading and pre-loading. The post-loading is based on diffusion of actives into the interior space via immersion^{21,22} or high-pressure supercritical fluid.²²⁻²⁴ The drawback with this method is that it promotes initial burst release in aqueous-based environments due to the presence of surface-associated active molecules,²¹⁻²⁴ especially when water-soluble actives were used.²⁴ On the other hand, the pre-loading technique is based on the solubilization of actives in template materials prior to formation of silica shells,²⁵ which is more advantageous as to reduce the possibility of attachment of actives at the external surfaces of the shells, thus increasing the flexibility to protect and release the actives. Despite these benefits, synthesis of the core-shell silica nanoparticles most commonly involves chemical surfactants, templates and toxic organic solvents which must then be completely removed from the product.²⁵ It has been shown that residual chemical surfactants embedded in the silica-based nanomaterials can cause severe cytotoxicity¹² which would pose significant environmental risks. In this respect, a superior strategy for synthesis of nanocarriers should enable the pre-loading of actives with high loading capacity and involve only biocompatible components, thus avoiding time-consuming and tedious monitoring-procedures for ensuring complete removal of residual toxic components down to the levels that might be considered as environmentally safe.

Recently, we developed a novel emulsion and biomimetic dual-templating approach to synthesize oil-core silica-shell nanocapsules with tunable shell thickness.²⁹ Bifunctional SurSi peptide (Ac-MKQLAHSVSRLEHARKKRKKRKKRKKGGGY-CONH₂) was designed by modularizing a surface active peptide sequence (Sur module, MKQLAHSVSRLEHA) with a sequence for biosilicification (Si module, RKKRKKRKKRKKGGGY). Typically, oil-in-water (O/W) nanoemulsions are formed with a pharmaceutically safe oil, Miglyol[®] 812, dispersed in

the SurSi aqueous solution. Then a silica precursor tetraethoxysilane (TEOS) is added to facilitate the biosilicification at the O/W interfaces forming the oil-core silica-shell nanocapsules under room temperature, and at nearly neutral pH without use of any toxic chemicals. In contrast to the chemical methods mentioned above, our method is more biocompatible and environmentally friendly. An additional advantage of using peptide biosurfactant is the ability to be produced renewably at large scale and relatively low cost using biological expression.^{30,31} Furthermore, our dual-templating approach offers easy pre-loading of active components by simply dissolving in the oil phase with a high loading capacity. It has been demonstrated that fipronil saturated in Miglyol[®] 812 (at 10 mg·mL⁻¹) was easily encapsulated in silica nanocapsules by a pre-loading technique and without the need for template removal.²⁹

Fipronil (5-amino-1-[2,6-dichloro-4-(trifluoromethyl)phenyl]-4-[(trifluoromethyl)sulfinyl]pyrazole-3-carbonitrile) is a systemic insecticide commonly used as soil treatment, seed coating, or baits³² due to its non-repellent property, broad-spectrum activity against agricultural, forest, pastoral, domestic and veterinary pests,³³ and high specificity for insect pests over mammals.³⁴ The biochemical mode of action of fipronil is by blocking γ -aminobutyric acid (GABA) receptor of the GABA-gated chloride channel, thereby disrupting central nervous system activity and, at a sufficient dose, causing death.³⁴ It has low water solubility ($\sim 2 \mu\text{g}\cdot\text{mL}^{-1}$ at 25 °C) and low Henry's constant ($6.6 \times 10^{-6} \text{ m}^3\cdot\text{atm}\cdot\text{mol}^{-1}$ at 25 °C) suggesting low volatility, and its soil organic carbon partition coefficient (K_{oc}) of 727 indicates low mobility in soil, hence, low potential for ground water contamination.^{32,35}

In this study, sustained release of fipronil *in vitro* and *in vivo* from biocompatible oil-core silica-shell nanocapsules were investigated and compared. Fipronil-encapsulated silica nanocapsules with different shell thicknesses were synthesized using the dual-templating

approach aforementioned. The *in vitro* release of fipronil was studied in water. The efficacy of the fipronil-encapsulated silica nanocapsules was evaluated by conducting *in vivo* laboratory tests, i.e. direct- and feeding-treatments, against economically important subterranean termites (i.e., *Coptotermes acinaciformis*). Subterranean termites cause substantial damages to cultivated plants, wooden structures, pastures, forests and other agricultural crops.³⁶ Based on the sales estimation of insecticides in 2010, the worldwide annual control and repair costs can reach US\$40 billion with subterranean termites accounted for 80% of the costs.³⁷ This study would thus be expected to bring significant impact in agricultural fields.

MATERIALS AND METHODS

Chemicals. SurSi peptide (Ac-MKQLAHSVSRLEHARKKRKRKRKRKGGGY-CONH₂, M_w 3632 g·mol⁻¹) was custom synthesized by Peptide 2.0 Inc. (Chantilly, VA, USA) with purity >95% and was lyophilized prior to use. Fipronil in a powder form (C₁₂H₄Cl₂F₆N₄OS, M_w 437.15 g·mol⁻¹, mp 196–201 °C, and d 1.55 g·cm⁻³) was of analytical standard obtained from Sigma-Aldrich. Miglyol[®] 812 was purchased from AXO Industry S.A. (Wavre, Belgium) and was passed through heat-activated silica gel (Sigma-Aldrich) prior to use. Termidor[®] (9.1% fipronil in a form of suspension concentrate) was obtained from BASF Australia Ltd. Water with >18.2 MΩ·cm resistivity was obtained from a Milli-Q (Millipore, Australia) system with a 0.22 μm filter. Other chemicals were of analytical grade purchased from either Sigma-Aldrich or Merck and used as received.

Synthesis. Oil-core silica-shell nanocapsules were synthesized using an emulsion and biomimetic dual-templating approach as we previously reported.²⁹ Briefly, fipronil was dissolved in Miglyol[®] 812 oil at a concentration of 0.05 mg·mL⁻¹ or 1 mg·mL⁻¹, and then the fipronil-

loaded oil was added into a solution of SurSi peptide (400 μM) containing ZnCl_2 (800 μM) in sodium 4-(2-hydroxyethyl)-1-piperazine ethanesulfonate (HEPES) buffer (25 mM, pH 7), and the mixture was sonicated at 10 W (Branson Sonifier 450 ultrasonicator) to obtain O/W nanoemulsions which were dialyzed against HEPES buffer (25 mM, pH 7.5) at 4 °C for 20 h to remove residual peptide in the bulk solution. Then the nanoemulsions (pH 7.5) were mixed with tetraethoxysilane (TEOS) at a concentration of 16 μmol , 32 μmol or 96 μmol under stirring at room temperature for 30 h to form fipronil-encapsulated silica nanocapsules having three different shell thicknesses. Subsequently, the nanocapsules were dialyzed against water at 4 °C for 20 h to remove the residual TEOS and prevent further growth of the silica shell.

Characterization. *Encapsulation efficiency.* The suspensions of fipronil-encapsulated silica nanocapsules were freeze-dried for 20 h, subsequently mixed with water and sonicated at 20 W (Branson Sonifier 450 ultrasonicator) for 60 s to finely disperse the nanocapsules. Subsequently, acetonitrile was added into the suspensions with final acetonitrile concentration of 60 v/v%, and vigorously stirred at room temperature for up to 72 h. At different time intervals, the supernatants were collected by centrifugation, then fresh acetonitrile (60 v/v%) was added to the pellets to repeat the release of fipronil from the nanocapsules until no residual fipronil in the supernatants was detected via reversed-phase high-performance liquid chromatography (RP-HPLC).

Reversed-Phase High-Performance Liquid Chromatography (RP-HPLC). Concentrations of fipronil were assayed by RP-HPLC using a Shimadzu system equipped with a Jupiter C18 column (5 μm , 300 Å, 150 mm $\times$ 4.6 mm). Mobile phase A was water containing 0.1 v/v% phosphoric acid, while phase B contained 90 v/v% acetonitrile and 0.1 v/v% phosphoric acid. The elution gradient was increased from 50% B to 70% B over 20 min at a monitoring

wavelength of 220 nm. The concentration of fipronil was determined based on the peak area at retention time of approximately 14.5 min according to calibration curve obtained from standard solution of fipronil in acetonitrile solution (60 v/v%).

Fourier Transform-Infrared (FT-IR) spectroscopy. Silica nanocapsules without and with fipronil in the oil cores were prepared as described previously. The nanocapsules were washed twice with water and subsequently freeze-dried. FT-IR spectra of lyophilized SurSi peptide and the nanocapsules were determined by using a Perkin-Elmer Spectrum 100 FT-IR Spectrometer equipped with a universal attenuated total reflection (ATR) attachment. Spectra data were collected in the transmission mode by averaging 135 scans over wavenumber range 4000–600 cm^{-1} at a spectral resolution of 4 cm^{-1} .

Dynamic Light Scattering (DLS). Size distributions of nanoemulsions and nanocapsules were determined by DLS using Malvern Zetasizer Nano ZS (Malvern Instrument Ltd, UK) at a scattering angle of 173° and a temperature of 25 °C. Samples were diluted by a factor of 100 prior to the measurements to avoid multiple scattering effects.

Transmission Electron Microscopy (TEM). Morphology of the fipronil-encapsulated silica nanocapsules was characterized by depositing the suspension (2 μL) onto Formvar-coated copper grids (ProSciTech Pty Ltd, Australia) and was then visualized via TEM (JEOL 1010, accelerating voltage of 100 kV). Size of the nanocapsules was analyzed using iTEM software (version 3.2, Soft Imaging System GmbH) equipped with the TEM. Structure of the silica shell was determined using selected area electron diffraction (SAED) technique via high-resolution (HR) TEM (JEOL 2100, accelerating voltage of 200 kV).

164 ***In vitro* release study of fipronil into water.** Silica nanocapsules with three different shell
165 thicknesses encapsulating 1 mg·mL⁻¹ fipronil in Miglyol[®] 812 were used to study the release
166 profile of fipronil *in vitro*. The releases of fipronil from the silica nanocapsules to water were
167 carried out by stirring the suspensions at room temperature for up to 142 h. At different time
168 intervals, aliquots were taken, replaced with fresh water and centrifuged to obtain clear
169 supernatants which were then analyzed using RP-HPLC.

170 ***In vivo* study against termites.** Termites (*C. acinaciformis*) were collected from field site in
171 Esk (Queensland, Australia). Plastic containers filled with moist wood were inverted (lid off)
172 onto a timber-filled trench known to contain termite infestation, and each container was then
173 covered with insulating material secured with soil. After harvesting, actively moving termites
174 were carefully selected from the containers for *in vivo* tests.

175 ***Direct treatment.*** Six samples, including water, nanoemulsions, silica nanocapsules
176 (encapsulating 0.05 mg·mL⁻¹ fipronil in Miglyol[®] 812) with three different shell thicknesses (8
177 nm, 25 nm, and 44 nm), and Termidor[®] were used in the direct treatment, and they all contained
178 the same final fipronil concentration except for the water sample. Water was used as control, and
179 nanoemulsion was used as nanocapsules with zero silica shell thickness. All six samples were
180 used in five replicates for the direct treatment. Prior to the treatment, termites were placed in a
181 group of ten termites in a Petri dish (92 mm × 16 mm), and incubated at 2 °C for 2 min to slow
182 their movement. Each of the fipronil formulations or water (5.5 μL) was topically applied on the
183 dorsal thorax of the worker termite. The termites were then transferred to clean Petri dishes
184 containing a filter paper (42.5 mm, Whatman[®]). The Petri dishes were placed into a controlled-
185 environment chamber (65 cm × 47 cm × 37.5 cm) containing a thermometer, a lid and a 11.5-L
186 metal container with water and heater to maintain the temperature at 28 ± 1 °C and high

humidity, and, once covered with insulating material, total darkness (except during observation). At different time intervals, the Petri dishes were removed from the chamber, and the lids were opened with the least possible disturbance in order to examine termite response (mortality). A termite was considered dead when it was on its back or side (not supported by legs) and not able to move even after prodding with a soft brush.

Feeding treatment. Silica nanocapsules (encapsulating 1 mg·mL⁻¹ fipronil in Miglyol[®] 812) with the thickest shell (44 nm) were used in the feeding treatment. Termidor[®] with the same final fipronil concentration and water were used as controls. Each fipronil formulation and water samples were used in four and two replicates for the feeding treatment, respectively. To mimic natural environment of termite colonies, a 750-mL container was filled with *Nasutitermes magnus* mound material (150 g) which was roasted (200 °C, 60 min), ground and mixed with water (50 g) prior to use. A clear vinyl tubing (150 mm × 6 mm) was connected from the container to a 70-mL feeding vial containing α -cellulose (20 g) bait with a moisture content of 70 w/w%. Then termites (7.5 g $\approx$ 1500 worker and soldier termites) were placed into the container. Each of the assembled feeding devices was placed into a controlled-environment chamber having dimensions and conditions similar to that in the direct treatment (described above). The termites in the ten assemblies were allowed to acclimatize to these conditions for 4 days and began feeding on the baits as indicated by the presence of enclosed tunnels and chambers built in the baits. Then, 0.5 mL of the sample (water, Termidor[®] or the nanocapsule) was transferred into each feeding vial. The assemblies were removed from the chamber and observed daily with the least possible disturbance in order to detect movement of living termites using a Termatrac[®] T3i device (Termatrac[®] Pty Ltd, Australia). Lack of movement indicated 100% mortality of termites and inspections in the containers were conducted to confirm. During the treatment, termite

groups in one container connected to Termidor[®]-treated bait and in two containers connected to nanocapsule-treated baits remained alive for up to 8 days as the tube entrances to the vials were blocked with mud built by the termites. These containers were not considered for further study. Therefore, three treatment results were included in the analysis for the Termidor[®]-treated baits and two treatment results for the nanocapsule-treated baits. The termites in two containers connected to water-treated baits survived throughout the study.

RESULTS AND DISCUSSION

Encapsulation of fipronil in silica nanocapsules. Fipronil was easily dissolved in Miglyol[®] 812 due to its high solubility in the oil (i.e., 10 mg·mL⁻¹).²⁹ The fipronil-loaded oil was emulsified in the presence of SurSi peptide via sonication, and nanoemulsions with narrow droplet size distribution were obtained due to the presence of the surface active Sur module²⁹ (Figure 1) in the emulsifying peptide. Following addition of the silica precursor tetraethoxysilane (TEOS) to the nanoemulsions, silica shell encasing the nanoemulsion core was formed at near-neutral pH and room temperature without using any toxic chemicals. The biosilicification process at the oil–water interface was due to the catalytic role of densely packed cationic arginine (R) and lysine (K) residues of Si module (RKKRKKRKKRKKGGGY) as well as polar residues of Sur module exposed toward the bulk aqueous solution that could induce hydrolysis of TEOS and interfacial poly-condensation of the hydrolysis products i.e., silanol ($\equiv\text{Si}-\text{OH}$) and negatively-charged silanolate ($\equiv\text{Si}-\text{O}^-$)²⁹ (Figure 1).

In this study, fipronil dissolved in Miglyol[®] 812 oil at a concentration of 0.05 mg·mL⁻¹ or 1 mg·mL⁻¹ was encapsulated in silica nanocapsules and used for the sustained release study *in vitro* and *in vivo*. The amount of fipronil encapsulated in silica nanocapsules was determined based on

the lethal dose of fipronil sufficient to eliminate a termite for the *in vivo* tests. It has been reported that the lethal dose of fipronil for *Coptotermes* spp. is in the range of 2–10 ng per termite.^{38,39} Based on this consideration, the encapsulated fipronil which was dissolved in Miglyol[®] 812 at concentrations of 0.05 mg·mL⁻¹ and 1 mg·mL⁻¹ was equivalent to 1 ppm and 20 ppm in the silica nanocapsule suspensions and, at an applied volume, would give fipronil dose of around 6 ng/termite in the direct- and feeding treatments, respectively.

The encapsulation efficiencies (%*EE*) of fipronil in silica nanocapsules were determined by monitoring the concentration of fipronil released in acetonitrile solution (60 v/v%) over a period of time compared to initial concentration of fipronil dissolved in Miglyol[®] 812 oil. Higher solubility of fipronil in acetonitrile (60 v/v%) i.e., 71.5 mg·mL⁻¹ than in Miglyol[®] 812 i.e., 10 mg·mL⁻¹ promotes easy partition of the fipronil and its diffusion out of the silica nanocapsules. The results of practical encapsulation efficiencies of fipronil were calculated using Equation 1:²⁹

$$\%EE = \frac{\sum_{j=1}^n (C_f V_f)_j}{C_i V_i} \times 100\% \quad (1)$$

where C_f (mg·mL⁻¹) and V_f (mL) are the fipronil concentration released in acetonitrile solution and volume of the acetonitrile solution, respectively, C_i (mg·mL⁻¹) and V_i (mL) are the initial fipronil concentration in Miglyol[®] 812 and volume of the Miglyol[®] 812, respectively, and n is the number of repeated-release of fipronil. At an initial fipronil concentration of 0.05 mg·mL⁻¹, %*EE* of fipronil in silica nanocapsules obtained at reactions with 16 μmol, 32 μmol and 96 μmol of TEOS were 72.8%, 73.2%, and 73.6%, respectively, while similar %*EE* of 71.6%, 72.2%, and 72.9%, respectively, were obtained when initial fipronil concentration of 1 mg·mL⁻¹ was used.

The encapsulation of fipronil in the silica nanocapsules demonstrated high encapsulation efficiency which is suitable for agricultural applications.

Characterization of Silica Nanocapsules Containing Fipronil. To determine the molecular components of fipronil-encapsulated silica nanocapsules, SurSi peptide and silica nanocapsules without and with fipronil in the oil cores were analyzed using Fourier transform-infrared (FT-IR) spectroscopy, and the results were compared in Figure 2. For SurSi peptide, four characteristic peaks of the peptide backbone were observed (Figure 2a) which are assigned to amide A (N–H stretch), amide B (N–H stretch), amide I (C=O stretch), and amide II (C–N stretch and N–H in-plane bend) at 3284, 3071, 1648, and 1542 cm^{-1} , respectively.⁴⁰ The functional groups of peptide side chains can also be identified (Figure 2a) i.e., peak at 1298 cm^{-1} corresponds to O–H deformation of hydroxyl group of serine (S), shoulder at 1248 cm^{-1} and doublet at 837 and 800 cm^{-1} are associated with C–O stretch and phenyl ring of tyrosine (Y), respectively, and doublet at 1180 and 1130 cm^{-1} can be attributed to NH_3^+ rocking of lysine (K).^{40,41} FT-IR spectrum of the silica nanocapsules without fipronil (Figure 2b) shows a broad signal centered at 3330 cm^{-1} which reflects the vibration of O–H stretching of silanol group Si–OH. A strong absorption band at about 1060 cm^{-1} , with a shoulder at 1160 cm^{-1} , and a band at 780 cm^{-1} correspond to asymmetric and symmetric Si–O–Si stretching vibrations of siloxane group, respectively. An absorption shoulder at 960 cm^{-1} arises from the Si–O[−] stretching of Si–OH. The major peaks of N–H stretch of peptide and other peptide side chains are overlapped by the characteristic bands of silica and thus are unobserved (Figure 2b). However, the presence of peptide in the silica nanocapsules can still be characterized by the absorption bands of amide I and amide II although the intensities are significantly reduced. The FT-IR spectrum of fipronil-encapsulated silica nanocapsules (Figure 2c) retained most of the major peaks of the silica nanocapsules without

fipronil (Figure 2b). Additionally, new absorption bands appear and are clearly discernable at 1320 and 888 cm^{-1} which can be attributed to $-\text{CF}_3$ stretching of trifluoromethyl group and C–H out-of-plane bending of benzene ring⁴² of the fipronil molecule, respectively (Figure 2c). These vibrational frequencies further verify the incorporation of fipronil into silica nanocapsules.

Morphology of silica nanocapsules can be visualized by using TEM without the need for staining or cryo-preservation. The TEM images showed morphology of a core–shell structure of fipronil-encapsulated silica nanocapsules having three different shell thicknesses (Figure 3a–c). In contrast, fipronil-loaded nanoemulsion having no silica shell was hardly observed in TEM as its structure was prone to disintegrate under high vacuum and electron beam irradiation (data not shown). The measured shell thicknesses, as determined by using TEM, of silica nanocapsules obtained at TEOS concentrations of 16 μmol , 32 μmol and 96 μmol were 8 nm, 25 nm and 44 nm, respectively, while the outer diameters were 140 nm, 198 nm and 288 nm, respectively. These values are consistent with our previously-reported silica nanocapsules that encapsulated fipronil at concentration of 10 $\text{mg}\cdot\text{mL}^{-1}$ in Miglyol[®] 812.²⁹ Additionally, the representative selected area electron diffraction (SAED) of silica nanocapsules shows diffuse ring pattern (Figure 3d), indicating the amorphous structure of the silica shell. The formation of amorphous silica via peptides has also been reported for natural biogenic silica^{43,44} and biomimetic silica.⁴⁵

Dynamic light scattering (DLS) confirmed a size increase for silica nanocapsules in comparison with the nanoemulsion template (Figure 4). Furthermore, increasing TEOS concentrations corresponded to an increase in number-averaged diameters of the nanocapsules (Figure 4a–c). The number-weighted size distribution results showed that silica nanocapsules having 8-nm, 25-nm, and 44-nm shells have narrow size distributions (polydispersity index, PDI < 0.350).

298 **Fipronil Release Study *in vitro*.** The release profiles of fipronil *in vitro* from silica
299 nanocapsules with three different shell thicknesses encapsulating 1 mg·mL⁻¹ fipronil in Miglyol®
300 812 i.e., 1F-NC8, 1F-NC25 and 1F-NC44 are shown in Figure 5. The concentration of fipronil
301 released in the water phase was normalized against the saturated concentration of fipronil in
302 water considering the low solubility of fipronil in water (2 µg·mL⁻¹). The release of fipronil from
303 1F-NC8 was detected after 8 h of release study at 2.4%, while a longer lag time of 16 h was
304 observed for fipronil released at 2.5% and 1.6% from silica nanocapsules having thicker shells
305 i.e., 1F-NC25 and 1F-NC44, respectively (Figure 5). After 142 h, fipronil was released at
306 percentages of 40.7%, 24.3% and 15.2% from 1F-NC8, 1F-NC25 and 1F-NC44, respectively.
307 The rates of fipronil released in water show the order of 1F-NC8 > 1F-NC25 > 1F-NC44,
308 demonstrating that increasing shell thickness resulted in an increased diffusion pathway and
309 hence enhanced barrier to diffusion of fipronil across the silica shell, thus the slower release of
310 fipronil.

311 **Fipronil Release Study *in vivo*.** Sustained release of fipronil from silica nanocapsules was
312 also investigated *in vivo* against termites. Two experiments were carried out, including direct
313 treatment and feeding treatment.

314 Direct treatment was demonstrated by topical application of six different formulations
315 including water, Termidor®, nanoemulsions (0.05F-NE), silica nanocapsules with three different
316 shell thicknesses of 8 nm (0.05F-NC8), 25 nm (0.05F-NC25) and 44 nm (0.05F-NC44), on the
317 dorsal thorax of worker termite *C. acinaciformis* (Figure 6). Rapid mortality was observed when
318 termites were treated with commercial Termidor® or fipronil-loaded nanoemulsions having no
319 silica shells (0.05F-NE). The fipronil penetrated through the cuticle layer on the body surface of
320 worker termites and eventually caused death depending the fipronil doses.³⁹ Termidor® gave

burst release of fipronil resulting in 50% mortality within the first hour and 100% mortality at 4 h (Figure 6). Fipronil-loaded nanoemulsions having no silica shells (0.05F-NE) exhibited a two-step release profile; a significant initial burst effect, with mortality of almost 50% within the first 4 h, and then more sustained release over 22 h (Figure 6). In contrast, silica nanocapsules showed sustained release as a result of the silica shell. First mortality was at 4 h (6%), 6 h (4%), and 10 h (8%) for silica nanocapsules having 8-nm (0.05F-NC8), 25-nm (0.05F-NC25) and 44-nm (0.05F-NC44) shells, respectively (Figure 6). Further, the termite mortality gradually increased in a way dependent on shell thickness suggesting that the silica shell provided an effective barrier for the controlled diffusion of the encapsulated fipronil, which corresponds well with the *in vitro* release study. The lag time, which was also observed in *in vitro* experiments, prior to termite mortality in the topical applications of silica nanocapsules strongly evidenced the sustained release of fipronil from the nanocapsules with no indication of burst release. Fipronil-encapsulated silica nanocapsules having 44-nm shell demonstrated the most significant delay in termite mortality and thus was chosen for the subsequent *in vivo* test.

We further evaluated the sustained release performance of fipronil-encapsulated silica nanocapsule having 44-nm shell (1F-NC44) *in vivo* by remote feeding treatment against *C. acinaciformis*. Prior to the treatment, groups of orphaned worker and soldier termites (≈ 1500) were allowed to adapt to the living conditions maintaining their biological activities in a container filled with nutritious materials at set temperature.⁴⁶ Food in the form of α -cellulose was supplied in peripheral feeding vials which were accessible through clear vinyl tubes. After 4 days, we observed that termites built mud and cellulose surrounding the interior space of the connector tubes as well as galleries within the baits, indicating that termites were responding to the α -cellulose. The α -cellulose in the feeding vials was then treated with water containing no

344 fipronil, Termidor[®] or 1F-NC44. As termites foraged in the feeding vials, they acquired and
345 carried the α -cellulose back to the container, and inadvertently transferred it to other termites
346 within the groups. These interactions could be through trophallaxis in which foraging worker
347 termites feed other termites that do not or cannot forage on their own including soldier termites
348 due to their anatomical and behavioral specializations.⁴⁷ Termidor[®]-treated α -cellulose caused
349 100% mortality to termite groups after 3 days, whereas more delayed 100% mortality (6 days)
350 was observed after treatment using nanocapsule-treated α -cellulose (Figure 7). The α -cellulose
351 treated with water containing no fipronil in the feeding vials caused negligible termite mortality
352 during the 8 days of the test. The feeding experiment results further confirmed the sustained
353 release property of silica nanocapsules as a result of the presence of silica shell which plays a
354 role as barrier to diffusion of fipronil.

355 Dead and decaying bodies of termites can elicit avoidance behaviour of other termites,^{48,49}
356 and consequently any transmission through those termites is ineffective.³⁹ Therefore, the
357 sustained release property of the fipronil-encapsulated silica nanocapsules is important to ensure
358 constant exposure of fipronil to the termites in the colony until 100% mortality of the colony can
359 be achieved. Furthermore, the more delaying insecticidal effect demonstrated by the silica
360 nanocapsules as compared to the commercial fipronil formulation would be highly desirable as it
361 increases the likelihood of horizontal transfer of fipronil and hence allowing improved area-wide
362 control of larger populations of termite colonies.

363 **Conclusion.** Biocompatible oil-core silica-shell nanocapsules synthesized using an emulsion
364 and biomimetic dual-templating approach under environmentally-friendly conditions were
365 investigated as nanocarriers for the sustained release of fipronil insecticide. *In vitro* release
366 studies of fipronil showed decreased release rate with increasing the shell thickness. *In vivo*

direct treatment experiments using silica nanocapsules with three different shell thicknesses (8-nm, 25-nm and 44-nm) against worker termites demonstrated that the time interval between the application of the nanocapsules and the occurrence of the termite mortality could be prolonged in a way dependent on silica shell thickness as compared to the commercial formulation Termidor[®] and fipronil-loaded nanoemulsions having no silica shells. *In vivo* efficacy of 44 nm-shell nanocapsules against orphaned groups of ≈ 1500 worker and soldier termites by dispersing the nanocapsules in α -cellulose feeding bait proved that the silica nanocapsules could be taken up from the bait vials by foraging workers, transmitted to recipient termites in the groups probably through horizontal transfer, and eventually caused 100% mortality of the termite groups in 6 days after the application which was 3 days longer than the commercial Termidor[®]. Both the *in vitro* and *in vivo* fipronil-released studies presented in this paper demonstrated the effectiveness of a pesticide delivery system having biocompatibility, high encapsulation efficiency and sustained release property, which open potential utilities of the silica nanocapsules for other agricultural applications.

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399 **ABBREVIATIONS USED**

400 Ac, acetyl; M, methionine; K, lysine; Q, glutamine; L, leucine; A, alanine; H, histidine; S, serine;
401 V, valine; R, arginine; E, glutamic acid; G, glycine; Y, tyrosine; O/W, oil-in-water; M_w ,
402 molecular weight; mp, melting point; d , density; TEOS, tetraethoxysilane; HEPES, sodium 4-(2-
403 hydroxyethyl)-1-piperazine ethanesulfonate; 0.05F-NE, nanoemulsions loaded with 0.05 mg·mL⁻¹
404 ¹ fipronil in Miglyol[®] 812 oil; xF-NCy, silica nanocapsules (encapsulating x mg·mL⁻¹ fipronil in
405 Miglyol[®] 812 oil) with shell thickness of y nm; TEM, transmission electron microscopy; HR-
406 TEM, high-resolution transmission electron microscopy; SAED, selected area electron
407 diffraction; DLS, dynamic light scattering; FT-IR, Fourier transform-infrared; RP-HPLC,
408 reversed-phase high-performance liquid chromatography

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FIGURE CAPTIONS

Figure 1. Illustration of the synthesis of fipronil-encapsulated silica nanocapsules formed using an emulsion and biomimetic dual-templating approach for sustained-release agroformulations against termites. Step (I), solubilization of fipronil in Miglyol[®] 812 oil; step (II), sonication of the oil in SurSi peptide solution to form fipronil-loaded nanoemulsions; step (III), addition of tetraethoxysilane (TEOS) in the nanoemulsions to form silica shell encapsulating the nanoemulsion core; step (IV), *in vivo* efficacy tests of the fipronil-encapsulated silica nanocapsules against economically important subterranean termites.

Figure 2. FT-IR spectra of (a) SurSi peptide, (b) silica nanocapsules without fipronil in the oil cores and (c) fipronil-encapsulated silica nanocapsules.

Figure 3. Fipronil-encapsulated silica nanocapsules with tunable shell thickness. a–c) Representative transmission electron microscopy (TEM) images of fipronil-encapsulated silica nanocapsules having (a) 8-nm, (b) 25-nm, and (c) 44-nm shells. Scale bars are 200 nm. Arrows indicate the boundary of the silica shell. d) Representative selected area electron diffraction (SAED) pattern of silica nanocapsules confirmed the amorphous state of the silica shell.

Figure 4. Number-weighted size distributions of fipronil-encapsulated silica nanocapsules and the nanoemulsion template. The measured number-averaged diameters (d_n) include silica nanocapsules having (a) 8-nm, (b) 25-nm, and (c) 44-nm shells, (*) fipronil-loaded nanoemulsions before reaction with TEOS, and (**) control fipronil-loaded nanoemulsions incubated over the reaction period.

558 **Figure 5.** *In vitro* release profile of fipronil from silica nanocapsules having 8-nm (1F-NC8, ▲),
559 25-nm (1F-NC25, ■) and 44-nm (1F-NC44, ●) shells into water.

560 **Figure 6.** *In vivo* efficacy test via topical application on the dorsal thorax of worker termites.
561 Fipronil-encapsulated silica nanocapsules having 8-nm (0.05F-NC8, ▲), 25-nm (0.05F-NC25,
562 ■) and 44-nm (0.05F-NC44, ●) shells are tested in comparison with fipronil-loaded
563 nanoemulsions without silica shells (0.05F-NE, □), Termidor[®] (○), and water containing no
564 fipronil (control, △). The lines are drawn to assist in reading the trends.

565 **Figure 7.** *In vivo* efficacy test via feeding treatment against groups of ≈1500 worker and soldier
566 termites. The delayed 100% mortality of the termite groups caused by fipronil-encapsulated
567 silica nanocapsules having 44-nm shell (1F-NC44) is presented as compared to Termidor[®].

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569 **Figure 1.**

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572 **Figure 2.**

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574 **Figure 3.**

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Figure 4.

581 **Figure 5**

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Figure 6.

587 **Figure 7.**

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