# Single Cell Encapsulation via Pickering Emulsion for Biopesticide Applications

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ABSTRACT: A new approach for single cell microencapsulation in an oil-in-water (o/w) Pickering emulsion is presented. The water/paraffin emulsions were stabilized by amine-functionalized silica nanoparticles. The droplet size of the emulsions was highly tunable, and ranged from 1 to 30 μm in diameter. The controllable droplet size along with the high colloidal stability of the Pickering emulsions was harnessed to obtain single cell microencapsulation. Successful encapsulation of the conidia entomopathogenic fungus *Metarhizium brunneum* by the studied Pickering emulsions was confirmed via confocal laser scanning microscopy. The resulting systems were implemented to develop a novel biopesticide formulation for arthropod pest control. The conidia incorporated in the emulsions were applied to *Ricinus communis* leaves by spray assay. After drying of the emulsion, a silica-based honeycomb-like structure with an ordered hierarchical porosity is formed. This structure preserves the individual cell encapsulation. The successful single cell encapsulation has led to a high distribution of conidia cells on the leaves. The Pickering emulsion-based formulation exhibited significantly higher pest control activity against *Spodoptera littoralis* larvae compared to the control systems, thus making it a promising, cost-effective, innovative approach for tackling the pest control challenge.
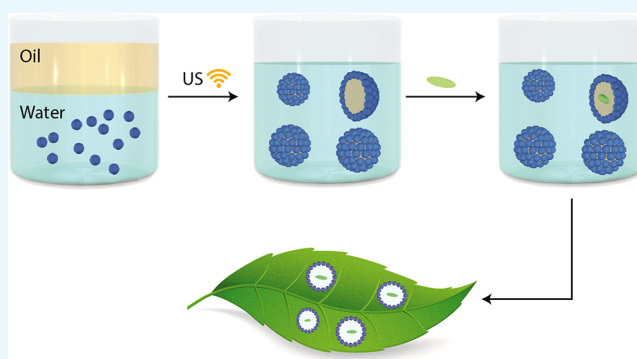
INTRODUCTION

Single cell encapsulation has many major applications in a wide variety of fields including medicine, food industry, and agriculture. Encapsulation protects against harsh external conditions, ensures lower exposure to contamination by foreign organisms, and provides improved isolation.[1,2] Cell encapsulation has become an important tool in gene therapy, controlling drug delivery,[3] fermentation industry,[4] image analysis tool, and genomic analysis in microorganisms.[5] The capsule provides synthetic membrane that will be removed in an appropriate environment. Several techniques for single cell microencapsulation have been developed, including solvent evaporation,[6] interfacial polymerization,[7] and layer-by-layer deposition.[8] Nevertheless, these methods may contain toxic chemicals and compounds such as organic solvents or corrosive surfactants that constitute risks for biological organisms.[9] This underscores the need for fine biocompatible single cell encapsulation techniques to maintain cell viability for example, hydrogel,[10] microfluidic-based methods[11] and emulsions.[12]

Pickering emulsions, which have been extensively studied and implemented in numerous applications during the last century, are excellent candidates for single cell encapsulation, given their high colloidal stability and controllable droplet size.[13−15] Pickering emulsions are stabilized by nanoparticles (NPs) that are self-assembled at the oil/water interface and act as a physical barrier.[16,17] Pickering emulsions come in the form of oil-in-water (o/w) or inverse (water-in-oil) emulsions. Inorganic NPs such as silica, clays, hydroxyapatites, and organic NPs can act as stabilizers for Pickering emulsions.[17] At the interface, the wettability of the particles determines their localization in the water and the oil phase.[18] The diameter of the droplets is dictated by the particle size and the composition of the system (o/w ratio and NP content).[19−21] Cell encapsulation by Pickering emulsions has become useful for various applications, such as encapsulation of microbial cells to protect from pH changes,[22] developing biopesticide formulations based on *Bacillus thuringiensis* (Bt),[23] platform for enzymatic reaction,[24,25] cellular capsules based on liquid marbles,[26] and encapsulation of adherent cells in water-in-water emulsions for tissue engineering.[27]

In recent years, in response to growing public demands, health concerns, and new regulations, in addition to the need for increased food production, synthetic pesticides have been

removed from the market. Biopesticides are low risk, environmental friendly pesticides based on living microbes. Their active compounds are designed to be a natural and environmentally safe alternative to the synthetic pesticides used in crop protection. However, the difficulties in running efficient field trials and the high cost of biopesticides have prevented them from making a significant global dent.[28] The development of new advanced formulation systems for biopesticides could thus lead to more efficient pest control, increased shelf life, and greater persistence in the field.

Entomopathogenic fungi are biopesticides with a unique mode of action in that they penetrate the body integument of the host directly. Most fungus-based biopesticides are ascomycetes belonging to the complex species *Metarhizium* and *Beauveria*.[29]

The development of advanced formulations for biopesticides that can meet the demands for high pest control efficiency is of paramount importance. These formulations must achieve high functionality under a range of environmental conditions. This can be done by (1) maximizing their distribution on the plant canopy, (2) protection against UV, (3) maintaining micro-environmental humidity control, and (4) increasing their shelf life.[30,31] The microencapsulation of biopesticides using Pickering emulsions and colloidosomes constitutes an excellent candidate for tailoring new advanced formulations with high protection performance. Pickering emulsions make it possible to tune the emulsion properties in terms of droplet diameter and proper surface functionalization of the NPs to achieve high activity and a prolonged shelf life of the incorporated microorganisms. Silica NPs is an eco-friendly[32] emulsion stabilizer, which enables the introduction of additional functionalities to the product, such as UV protection and humidity control. This can be done by chemical modification of the silica NPs with organosilanes.

Single microencapsulation of cells by Pickering emulsion presents a significant potential for the development of new biopesticide formulations. The most commonly used myco-biopesticides are conidia. These microorganisms must remain nongerminated before application in the field; that is, unexposed to water. This means that conidia must be encapsulated in the oil phase of the emulsion, which makes encapsulation in the w/o system impractical. Furthermore, the chemical nature of the outer surface of most conidia is hydrophobic, resulting in their arrangement in the oil phase. Hence, developing advanced o/w Pickering emulsions that can encapsulate conidia in the oil phase is a top priority.

In response to this need, this study presents a new approach for single encapsulation of conidia by an eco-friendly o/w Pickering emulsion. The overall process is illustrated in Figure 1. Silica NPs were functionalized by (3-aminopropyl)-triethoxysilane (APTES) to introduce amine-functionalized groups onto the NPs (silica-NH$_2$). Pickering emulsions were prepared in different paraffin/water ratios and at various concentrations of silica NPs (Figure 1a). *Metarhizium brunneum Ma7* was the model fungal strain chosen for this study, as it exhibits a broad spectrum of hosts, which makes it a suitable strain for commercialization.[33−36] The *Metarhizium*-based products are registered worldwide.[37]

The successful single encapsulation of *Metarhizium* conidia in the paraffin droplets (Figure 1b) was confirmed by confocal laser scanning microscopy. The hydrophobic outer layer of the conidia, which is critical for the attachment and germination process on the host surface,[38,39] is also responsible for their
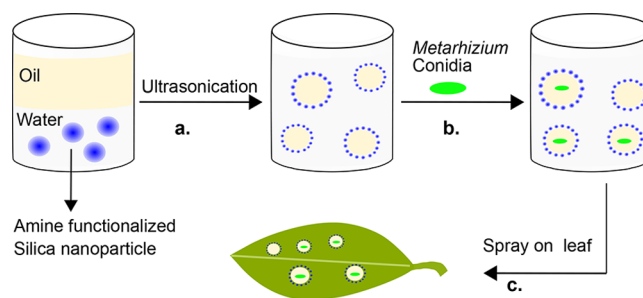
Figure 1. Schematic illustration of the silica-NH$_2$ oil/water Pickering emulsion for biopesticide formulation. (a) Silica-NH$_2$ particle dispersion in water sonicated with paraffin oil to form the silica-NH$_2$ Pickering emulsion. (b) Single cell encapsulation of *M. brunneum* conidia in the Pickering emulsion. (c) Spray assay on a leaf to assess cell distribution and biofunctionality.

individual arrangement in the oil phase. The biopesticide activity of the *Metarhizium* conidia was assessed on *Spodoptera littoralis* larvae. The formulation was sprayed on *Ricinus communis* leaves (Figure 1c) and exhibited significant pesticidal activity against *S. littoralis* larvae. These results confirm the high efficiency of the Pickering emulsion-based formulation developed in this study.

■ RESULTS AND DISCUSSION

Preparation of Silica-NH$_2$ Pickering Emulsion. Silica NPs were functionalized by APTES through silanization to introduce amine groups on the surface of the NPs.[40] Oil-in-water Pickering emulsions based on amine-functionalized silica NPs were prepared. Different contents of amine-functionalized silica (wt %) and oil/water (vol %) ratios were implemented to determine the proper conditions for a stable Pickering emulsion system that would meet the demands of single cell encapsulation. The silica contents were varied (0.1, 1, 2, 3, and 5 wt %) at oil/water ratios of 5:95, 10:90, 20:80, and 30:70, respectively. A major objective of the current study is to develop an efficient formulation for biopesticides that will maintain the conidia nongerminated. Therefore, the encapsulation of the conidia cells in the oil phase is mandatory.

The stability of the Pickering emulsions was characterized by visual observation over time. Most of the prepared emulsions were stable for 6−7 months (see Table 1). The highest

Table 1. Stability of the Silica-NH$_2$ Pickering Emulsions

silica-NH$_2$ (wt %)	oil/water ratio (vol %)			
	5:95	10:90	20:80	30:70
0.1	no emulsion	no emulsion	no emulsion	no emulsion
1	6 months	6 months	6 months	6 months
2	6 months	6 months	7 months	7 months
3	7 months	7 months	7 months	7 months
5	6 months	6 months	>7 months	>7 months

stability was observed at a silica content of 5 wt % at oil/water ratios of 20:80 and 30:70, which were stable for more than a year. However, no emulsification was obtained at a silica content of 0.1 wt %. For highly stable systems, we used confocal microscopy to observe changes in the droplet diameter as a result of droplet coalescence and creaming (data not shown).

Confocal microscopy images of Pickering emulsions with 1 wt % silica NPs at different oil/water ratios is depicted in Figure 2. The diameter of the paraffin droplets are highly
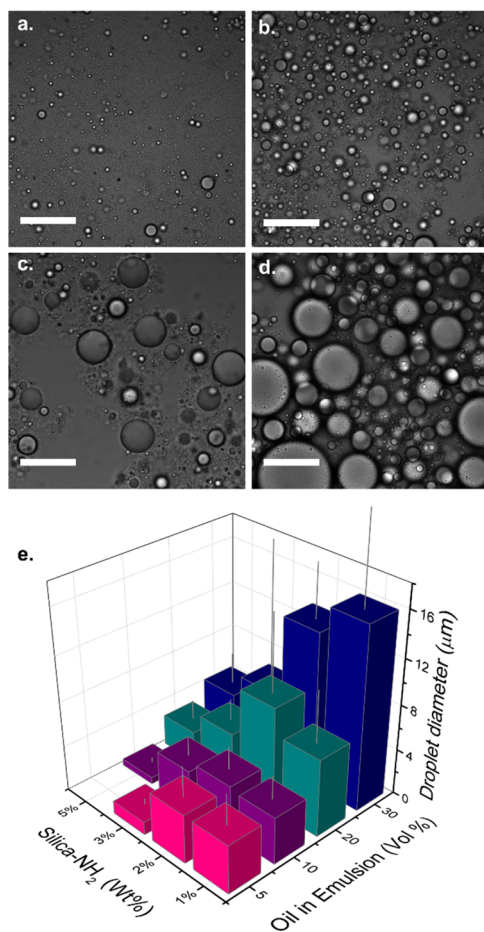
Figure 2. Pickering emulsions with 1% silica-NH$_2$ at different oil/water ratios. (a) 5:95. (b) 10:90. (c) 20:80. (d) 30:70. Scale bar is 50 μm. (e) Droplet diameter as a function of silica-NH$_2$ contents (wt %) and oil percentages in the emulsion (vol %).

tunable and can be varied in a relatively wide range of 1−30 μm. Figure 2e depicts the paraffin droplet diameter of the emulsions versus the content of the silica-NH$_2$ NPs at four different paraffin/water ratios. It is observed that the higher the silica content, the smaller is the droplet diameter at any studied volume fractions of paraffin oil. This phenomenon is explained by the increase of the total surface area of the o/w interface.[20] In addition, under a given content of silica-NH$_2$, the increase of the volume fraction of the paraffin resulted in larger droplet sizes.[20] The tuning of the emulsion composition enabled us to fine-tune the resulting droplet size, which is essential for single cell encapsulation. The relatively high stability of the Pickering emulsion is derived from the low coalescence rate of the droplets due to the presence of the NPs at the interface.

Single Cell Encapsulation. *M. brunneum Ma7*-green fluorescent protein (GFP) conidia[41] were added to the different silica-NH$_2$ Pickering emulsions. Single cell encapsulation of the conidia in the paraffin phase of the emulsions was confirmed by confocal microscopy (Figure 3). The confocal microscopy images are representing one focal plane, thus the conidia cells that appear inside the boundaries of the oil

droplets are actually located in the internal part of the paraffin droplets.

Successful single cell encapsulation in the paraffin oil droplets was obtained in emulsions with an o/w ratio of 20:80 at three different silica content values of 2, 3, and 5 wt % (Figure 3). The droplet concentration in these emulsions is approximately an order of magnitude higher than the conidia cell concentration, thus very few droplets that host conidia cells could be detected in a given confocal microscopy image even at very low magnification. Figure 3 depicts characteristic confocal microscopy images that conclusively confirm the successful single cell encapsulation of the conidia cells in the paraffin droplets of the emulsions.

The Pickering emulsions that have shown successful single cell encapsulation (at silica contents values of 2, 3, and 5 wt %) had an average droplet diameter of 9.1 ± 6.8, 4.7 ± 1.7, and 2.7 ± 1.5 μm, respectively (Figure 2e), close in their values to the size of the conidia cells, which are ~4 μm in their length. These results indicate that individual encapsulation can be achieved when the sizes of the droplets and the cells are of the same order of magnitude.

The conidia cells assemble at the oil phase in the water/paraffin biphasic system because of their hydrophobic surface.[42,43] Nevertheless, the conidia cells have to overcome the barrier made by the NPs that are located at the interface. The preparation of the silica-NH$_2$ Pickering emulsions is performed by an ultrasonic probe. The exposure of a liquid sample to ultrasonic waves results in vigorous agitation characterized with high shear forces that are responsible for emulsification. However, ultrasonication cannot take place in the presence of the conidia cells because it will lead to cell lysis. Therefore, the conidia cells were incorporated only after the emulsion formation, through gentle agitation by vortex, which enables the penetration of the conidia cells into the paraffin droplets.

The ability of cells or particles to enter the droplets of Pickering emulsions by introduction of shear forces to the system is explained by the work of French et al.[44,45] They have shown that the particles in the o/w interface undergo transformations between the droplets upon introducing shear forces, leaving temporary voids at the interface. This phenomenon explains the successful encapsulation of the conidia cells in the paraffin droplets through gentle agitation that is apparently generating proper shear forces. Furthermore, the implementation of NPs with a relatively low diameter (~40 nm) for the preparation of the Pickering emulsions might also decrease their ability to form a barrier (at the interface) against the penetration of the conidia cells that are 2 orders of magnitude larger (~4 μm in length) than the NPs.

The obtained fluorescent signal of the GFP conidia is a clear indication of the viability of the cells when encapsulated in the oil phase of the silica-NH$_2$ Pickering emulsions. The conidia in the emulsions remained viable for three weeks. The viability of the *Metarhizium* conidia cells while encapsulated in the silica-NH$_2$ Pickering emulsions was further characterized by culturing the conidia on a Sabouraud Dextrose Agar (SDA) growth medium (see Materials and Methods). The germination percentages of the encapsulated conidia were 85 ± 8.3%, demonstrating their viability and their ability to germinate. The control system (without emulsion) had germination percentages of 95 ± 5%.

Scanning electron microscopy (SEM) characterization of the applied emulsions (see Materials and Methods) revealed a
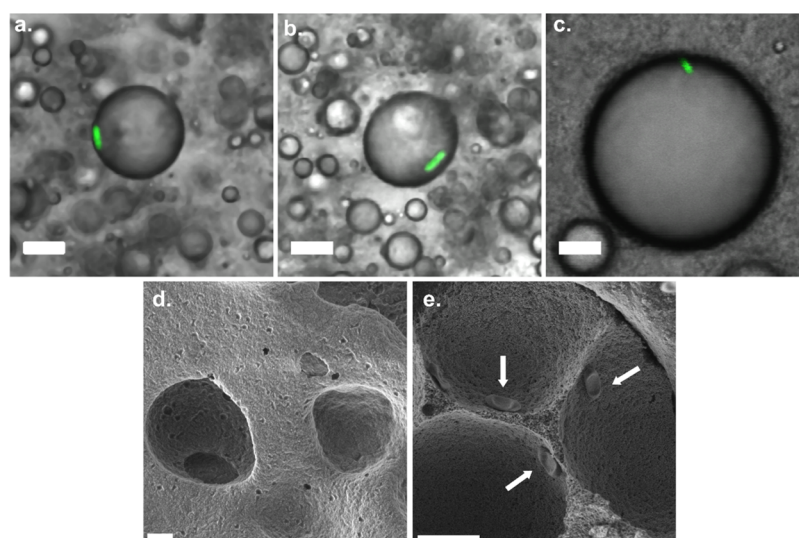
Figure 3. Confocal microscopy images of single cell encapsulation of *M. brunneum Ma7*-GFP conidia in a silica-NH$_2$ Pickering emulsion (o/w ratio, 20:80) with different NPs contents of (a) 2, (b) 3 and (c) 5 wt %. Scale bar is 10 μm. SEM micrographs of dried silica-NH$_2$ Pickering emulsion, (d) without conidia and (e) with conidia (arrows).
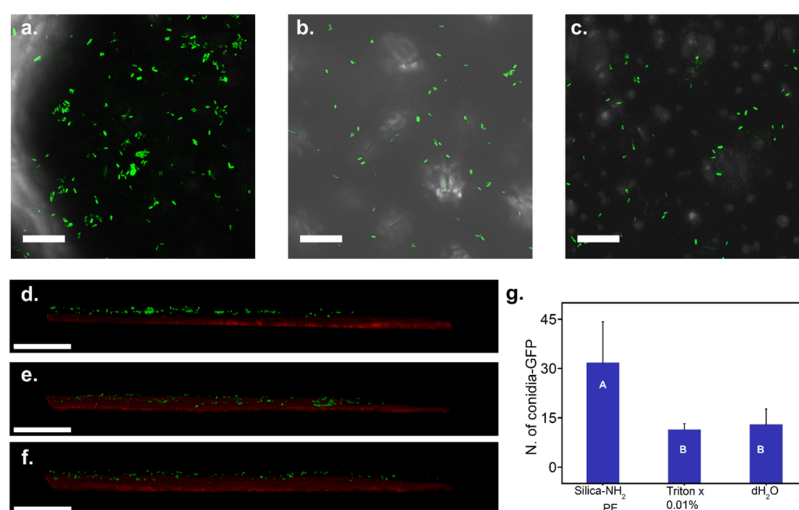
Figure 4. Distribution of *M. brunneum* conidia-GFP on *R. communis* leaves. (a−c) Z projection and (d−f) cross section of confocal microscopy images of *M. brunneum*-GFP conidia after spray application on the surface of the leaves. (a,d) Conidia in silica-NH$_2$ Pickering emulsion; (b,e) conidia in 0.01% Triton X-100 in water; (c,f) conidia in distilled water. (g) Number of conidia on leaves. Bars with the same letter do not differ significantly in the number of spores; Students *t*-test (*P* = 0.05). Scale bar: (a−c), 100 μm. (d−f), 200 μm.

silica-based honeycomb structure with ordered hierarchical porosity (Figure 3d,e). This structure is formed during the drying process of the emulsion through emulsion templating.[46,47] It maintains the basic morphology of the Pickering emulsion and preserves the single cell encapsulation, as shown in Figure 3e. This finding is important as it demonstrates the individual arrangement of the conidia on the leaves, which will lead to high efficiency of the biopesticide activity against the target insect.

Application of *M. brunneum* Conidia on *R. communis* Leaves. To test the functionality of the Pickering emulsions as a biopesticide formulation, *M. brunneum* conidia-GFP encapsulated in silica-NH$_2$ Pickering emulsions were sprayed on *R. communis* leaves until full coverage (see Materials and Methods).

The distribution of the conidia cells on the leaves was characterized by confocal microscopy. The Z projection

(Figure 4a−c) and the three-dimensional (3D) images (Figure 4e,f) are presented in Figure 4. The silica-NH$_2$ Pickering emulsion (Figure 4a,d) exhibited a significantly higher distribution of conidia cells on the leaves compared to the controls (0.01% Triton X-100, Figure 4b,e; and water, Figure 4c,f). The higher cell distribution of the silica-NH$_2$ Pickering emulsion is thus derived from the single cell encapsulation in the emulsions. These findings were supported by SEM analysis showing individual encapsulation of the conidia in the compartments of the silica/paraffin honeycomb structure (Figure 3e). Single cell encapsulation is required for efficient distribution of conidia cells on the leaf surface. The conidia cells, which are hydrophobic, will localize in the oil phase. Obtaining the suitable droplet size and composition has led to single cell encapsulation. After the spray assay on the leaves with the Pickering emulsion formulation, higher cell

distribution was observed on the leaf surface compared to water and Triton X-100 samples.

In order to quantify the number of conidia cells on the leaves, a Z series was taken and a Z projection was performed. The results of the Z projection showed a higher count of conidia on the studied system than the controls (Figure 4g, one-way ANOVA: $F = 4.5042$, df = 2, $P = 0.0372$). The single cell encapsulation in the Pickering emulsions maintains the dispersibility of the cells during the spray assay resulting in a higher number of conidia cells on leaves. On the contrary, the lower amount of conidia cells in the control samples resulted from cell agglomeration during the spraying process because of poor dispersibility of the cells in the aqueous medium. Triton X-100-based suspension was used as a standard in the screening of entomopathogenic fungi in many studies.[41,48,49] In addition, surfactants including Triton X-100, are being extensively used in commercial formulations of microbial control agents.[50,51]

Biological Functionality of Conidia Incorporated in Silica-NH$_2$ Pickering Emulsions. The *S. littoralis* larvae were indirectly exposed to the conidia by inoculation of the *R. communis* leaves with conidia (see Materials and Methods and Table 2) incorporated in the silica-NH$_2$ Pickering emulsions.

Table 2. List of Samples Used in the Biological Functionality Bioassay

#	sample	conidia presence
1	dH$_2$O	−
2	0.01% Triton X-100	+
3	silica-NH$_2$ Pickering emulsion	−
4	silica-NH$_2$ Pickering emulsion	+

The larvae were kept for seven days and the mortality of the larvae was monitored at days 3, 4, and 7 (Figure 5a). Larvae treated with conidia incorporated in the Pickering emulsion showed 75% mortality, whereas the formulation alone resulted in a mortality of only 25%. Treatment with conidia in 0.01% Triton X-100 led to 40% mortality. Corrected mortality and

probability tests were performed on the data. The analysis of corrected mortality is based on Abbott's formula[52] and is the adjustment of insect mortality rates used worldwide in insecticide trials. The results are presented in Figure 5b and Table 3, respectively. Table 3 clearly shows the higher

Table 3. Median Lethal Time in Days (LT50) of the Treatments in the *S. littoralis* Larvae Assay

type	conidia	LT50	lower 95%	upper 95%	prob > ChiSq
dH$_2$O	−	11.48	7.94		
0.01% Triton X-100	+	8.25	6.99	13.77	0.5594
silica-NH$_2$ Pickering emulsion	−	16.97	8.8		<0.001
silica-NH$_2$ Pickering emulsion	+	4.89	4.23	5.69	<0.013

pesticidal activity of conidia incorporated in the Pickering emulsions compared to the controls. The sporulation processes started seven days postinoculation. The dead larvae were kept separately under moist conditions to promote sporulation and confirm mycosis. Cadavers were monitored daily for sporulation. Of the 14 dead larvae, 13 were mycosed. An example of a sporulated larva cadaver is presented in Figure 5c; nontreated larva from the control group is shown in Figure 5d.

These results confirm the successful bioassay of encapsulated *M. brunneum* conidia on *S. littoralis* third instar larvae after spray application. The results also demonstrate the low toxicity of the Pickering emulsions (without conidia) against the larvae and leaves. The activity of the conidia was enhanced by their incorporation in the o/w silica-NH$_2$ Pickering emulsions. The increased efficiency of the Pickering emulsion-based formulation can thus be attributed to the successful single encapsulation of the conidia cells, which resulted in a significantly higher cell distribution on the leaves.

In addition, the silica-based honeycomb structure should decrease the exposure rate of the conidia to humidity, resulting in a controlled germination rate that will ensure the higher
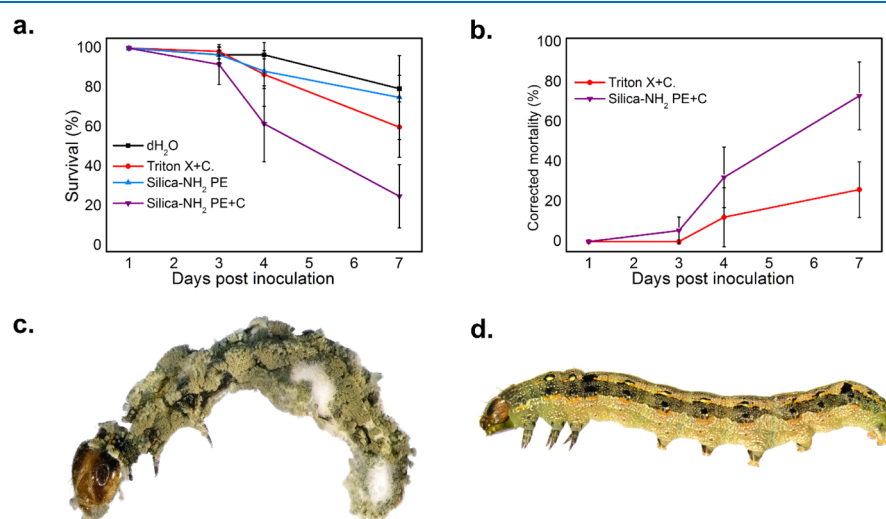
Figure 5. (a) Survival curve of *S. littoralis* third-instar larvae after spray application of dH$_2$O, 0.01% Triton X-100 with conidia, silica-NH$_2$ Pickering emulsion with and without *M. brunneum* formulation. Larvae were incubated for seven days at 25 °C, 85% RH. (b) Corrected mortality % of *S. littoralis* larvae seven days postinoculation with *M. brunneum* conidia in a water based formulation (Triton X-100) and silica-NH$_2$ Pickering emulsion. Mortality correction was calculated for each system with the respective blank. Data are the average of three independent experiments. *S. littoralis* third instar larva assay. (c) Larva treated with *M. brunneum* Pickering emulsion formulation 10 days postinoculation. (d) Nontreated larva.

effectivity of the conidia against target insects, and prolonged field persistence.

CONCLUSIONS

In summary, we presented a new approach for single cell encapsulation by eco-friendly o/w Pickering emulsions stabilized by amine-functionalized silica NPs. The emulsions were stable for months. *M. brunneum* conidia cells, a well-studied biopesticide, were incorporated into the Pickering emulsions. Single cell encapsulation of the conidia was confirmed by confocal microscopy. The *M. brunneum* is localized in the oil droplets due to the hydrophobic nature of their outer layer. The high tunability of the droplet size along with high colloidal stability of the emulsions enabled single cell microencapsulation. The silica-NH$_2$ Pickering emulsions were implemented to develop formulations for biopesticides. The conidia incorporated in the emulsions were applied on leaves via the spraying process. The studied formulation resulted in a higher cell distribution on the leaves, leading to a high mortality rate of *S. littoralis* larvae. The silica-based honeycomb-like structure resulting from emulsion drying preserves the single cell encapsulation on the leaf surface. The formulation exhibited significantly higher pest control activity in comparison to the controls. This successful single cell encapsulation along with its high biopesticide activity is thus a promising, cost-effective, innovative approach for tackling the pest control challenge.

MATERIALS AND METHODS

Salinization of Silicon Dioxide Surfaces with APTES. Silica (1 g) (AEROSIL OX 50, with an estimated primary particle size of 40 nm, obtained from Evonik, Germany) was added to 40 mL of methanol and stirred for complete dispersion. Then, APTES (99% Sigma-Aldrich) was added slowly to the solution for a final concentration of 0.5 M. The reaction was carried out at ambient temperature for 45 min. After silanization, the particles were collected by centrifugation (9000 rpm, 10 min) and rinsed four times with methanol. Afterward, the silica-NH$_2$ NPs were dried at 35 °C under vacuum for ca. 3 h.

Silica-NH$_2$ Pickering Emulsion Preparation. Pickering emulsions were prepared from amine-functionalized silica in water and paraffin oil (Sigma-Aldrich, analytical grade). First, silica-NH$_2$ NPs were dispersed in distilled water by sonication for 5 min (Sonics Vibra-Cell 750 W, 25% amplitude) with increasing silica content: 0.1, 1, 2, 3, and 5 wt %. Then, paraffin oil was added at the o/w ratios of 5:95, 10:90, 20:80, and 30:70 vol %, respectively. The mixture was sonicated for 5 min for emulsification.

Fungal Strains and Culture Conditions. *M. brunneum* Ma7[53] and *M. brunneum* Ma7-GFP mutant[41] were cultured on SDA (Difco, Becton–Dickinson, MD) for 2 weeks at 28 °C until sporulation. Conidial suspensions were prepared by harvesting conidia by scraping the fungal colony, suspending the collected material in sterile distilled water containing 0.01% Triton X-100, followed by vortexing. The suspension was filtered through three layers of gauze, and conidial concentrations were determined using a hemocytometer.

Viability Assay. Conidia viability in different suspensions was determined by germination assay. Aliquots of conidial suspension were applied over SDA plates and incubated for 18 h at 28 °C. Conidia viability in the emulsion was measured at different time points using confocal microscopy. Conidial fluorescence was the indication for viability as described previously.[41] Viability was satisfactory if rates were above 95% for germination and fluorescent conidia.

Conidia Encapsulation in Silica-NH$_2$ Pickering Emulsion. For single cell encapsulation in the Pickering emulsion, for each of the silica-NH$_2$ NPs content, two different ratios of oil/water were chosen: 20:80 and 30:70. 10 mg of *M. brunneum* Ma7-GFP conidia were added to 10 mL of emulsion. The mixture was vortexed at high speed in vortex mixers for 5 min. Cell samples (10 μL) were placed onto a glass slide and analyzed by confocal microscopy.

Conidia Distribution on *R. communis* Leaves. For the leaf spray assays, three conidia samples of *M. brunneum* Ma7-GFP were prepared: silica-NH$_2$ Pickering emulsion, 0.01% Triton X-100, and distilled water. Conidia (10 mg) were added to 10 mL of each sample, mixed well, and then sprayed on *R. communis* leaves using a 50 mL hand sprayer (~100 μL liquid per spray) for full coverage. The plant tissues were left to dry at room temperature. To characterize the distribution of conidia on plant tissues, leaf discs from each treatment were analyzed by confocal microscopy.

Biological Functionality of Microencapsulated Conidia as a Biopesticide Formulation. To test the functionality of the silica-NH$_2$ Pickering emulsion as a biopesticide formulation, a bioassay was conducted to assess the LT$_{50}$ of the different samples. *R. communis* leaves were sprayed with Pickering emulsions and controls with and without conidia (see Table 2). Both sides of the *R. communis* leaves were treated using a 50 mL hand sprayer (~100 μL liquid per spray). The suspension contained 10 mL of 10^8 conidia/mL. After spraying, the leaves were dried for 30 min in the hood. The dried treated leaves were hand cut and inserted into a 55 mm Petri dish lined with filter paper impregnated with 500 μL distilled water to maintain high humidity and a single *S. littoralis* third-instar larva. For each sample, 20 larvae were used. The plates were sealed and incubated at 25 °C under a 12:12 L/D photoperiod. The larvae were examined at days 3, 4, and 7 for mortality. Food was supplied during examination. Dead larvae were removed from the Petri dishes and incubated in a moist chamber until sporulation occurred. The experiment was repeated three times.

Confocal Laser Scanning Microscopy and Image Analysis. The samples were analyzed by laser scanning confocal microscopy (Olympus, FluoView 500) using argon laser 488 nm excitation. Fluorescence emission of GFP was recorded at 500−520 nm. For 3D images, acquisition used a Leica SP8 laser scanning microscope (Leica, Wetzlar, Germany) equipped with a solid state laser with 488 nm light, HC PL APO CS 20×/0.75 objective (Leica, Wetzlar, Germany) and Leica Application Suite X software (LAS X, Leica, Wetzlar, Germany). Imaging of the GFP signal was done using the argon laser, and the emission was detected in a range of 500−525 nm. Autofluorescence of the chloroplasts was detected in a range of 650−700 nm. For *Metarhizium* conidia counting, image stacks were first projected using a Z projection (as maximum intensity) to find all the fluorescent conidia, then counted by a cell counter using Fiji software.[54] The droplet average diameter was measured for every sample by the particles analysis tool of Fiji software based on confocal microscopy images. 12 droplets were sampled from each image and plotted as a 3D graph with Origin (OriginLab, Northampton, MA).

Scanning Electron Microscopy. SEM measurements were performed using a MIRA3 field-emission SEM microscope (TESCAN, Brno/Czech Republic) with an acceleration voltage of 1.0 kV and a secondary electron detector. Pickering emulsion samples were drop-cast on a conductive double stick carbon tape and dried under ambient conditions. Prior to imaging, a thin layer of carbon was evaporated onto them to render them electrically conductive and to avoid surface charging by the electron beam.

Statistical Analysis. The JMP package (SAS Institute, 2002) was used for all statistical analyses. Mortality data were corrected by Abbott's formula.[52] Differences in the number of conidia on the leaves and larvae survival were analyzed by a one-way ANOVA followed by a Tukey–Kramer honestly significant difference (HSD) for comparisons for all pairs. Difference in the number of attached conidia to the leaf surface was analyzed to the Student-t test. Probit analysis was used to obtain the LT50 data.

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Notes
The authors declare no competing financial interest.

■ ACKNOWLEDGMENTS

■ ABBREVIATIONS

NPs, nanoparticles; o/w, oil-in-water; w/o, water-in-oil; SEM, scanning electron microscope; APTES, (3-aminopropyl)-triethoxysilane

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