Pollen-mimicking, enzyme-loaded microparticles to reduce organophosphate toxicity in managed pollinators

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Abstract

Pollinators support the production of 87 of the leading food crops worldwide and contribute over $15 billion to U.S. farm income. Organophosphates are a heavily used group of insecticides that pollinators can be exposed to, especially during crop pollination. Exposure to lethal or sub-lethal doses can impair fitness of wild and managed bees, risking pollination quality and food security. Here, we report a low-cost, scalable in vivo detoxification strategy for organophosphate insecticides involving encapsulation of phosphotriesterase (OPT) in pollen-mimicking microparticles (PMMs). We developed uniform and consumable PMMs capable of loading OPT at 90% efficiency and protecting OPT from degradation in the pH of a bee gut. Microcolonies of bumble bees (*Bombus impatiens*) fed malathion-contaminated pollen patties demonstrated 100% survival when fed OPT-PMMs but 0% survival with OPT alone and 0% survival with plain sucrose within 5 and 4 days, respectively. Thus, the detrimental effects of malathion were eliminated when bees consumed OPT-PMMs. This design presents a versatile and scalable treatment for managed pollinators to reduce risk from organophosphate insecticides, which can be integrated into supplemental feeds such as pollen patties or dietary syrup.

Introduction

Pollinators provide vital pollination services to crops by facilitating fertilisation and subsequent production of seeds and fruit. A frequently cited figure is that one third of the food we consume is dependent on pollinators[1–3]. Both wild and managed pollinators are critical for ensuring pollination services; each contributes approximately equally to crop pollination[4,5]. However, wild and managed pollinators are currently experiencing declines in the form of range contractions, population declines, and unsustainable hive losses. For example, beekeepers in the U.S. are losing a third of their hives each year on average[6]. These losses pose a risk to global food security[7] since beekeepers now spend a third of their income on replacing hives[8]. These costs have forced beekeepers to increase fees for pollination, which adds cost to farmers' operations. For example, almond growers have experienced a 300% increase in pollination costs since 2003[9].

Chemical insecticides are a key tool used in modern agriculture; without their use, crops could suffer a 20% loss of yield, in an industry which provides 5.5% of U.S. GDP [10–12] . However, insecticide usage can cause unintended consequences by harming non-target organisms such as pollinators [13] . In particular, exposure to insecticides is one of the key global drivers of declines in pollinator health [14] . For example, organophosphates (OPs), a group of insecticides which have a market of over $7 billion and account for more than a third of insecticide sales worldwide, often lead to pollinator exposures and exhibit high toxicity towards honey bees and bumble bees [15–20] . OP insecticides influence insect cholinergic neural signaling through inhibition of carboxyl ester hydrolases, particularly acetylcholinesterase (AChE) which breaks down acetylcholine. OPs inactivate AChE through irreversible covalent inhibition, causing a build-up of acetylcholine and overstimulation of nicotinic and muscarinic receptors [21,22] . Malathion and parathion are the two of the most widely applied OPs in commercially pollinated crops [23] . Malaoxon,

malathion's metabolite, is 1000-fold stronger at inhibiting AChE than malathion [24]. Malathion and parathion exhibit oral LD_{50}'s of 0.38 and 0.175 µg/bee respectively [25].

Phosphotriesterases are metalloenzymes that hydrolyze the triester linkage found in OP insecticides[26]. There are several variants of phosphotriesterase; the most frequently used, amidohydrolase phosphotriesterase (OPT), is isolated from bacteria *P. diminuta* or *Flavobacterium* ATCC 27551 and exhibits a TIM-barrel fold structure[27,28]. OPT can be easily produced from transfected *E. coli* culture with the appropriate OPT plasmid sequence[29,30]. OPT has a wide substrate specificity; it exhibits optimal hydrolysis upon encountering paraoxon (parathion's metabolite), at a rate approaching the limit of diffusion[31]. OPT performs best hydrolyzing substrates which possess phenol leaving groups, yet it will also successfully degrade thiol linkages as in the case of malathion[32,33]. Previously, OPT has been considered in a bioremediation capacity to detoxify heavily contaminated soils, as well as a treatment for OP insecticide or nerve agent poisoning[34–36]. However, OPT application has demonstrated poor efficacy in industry due to its poor stability at a low pH and high temperatures[37]. Bioactivity rapidly declines at pH < 8.0. At pH 7.0, activity is less than half of its maximum potential. At the optimum pH range of 8.0-9.5 the Co^{2+} OPT complex maintains thermostability < 45°C, above which, the stability rapidly declines until deactivation at 60°C[38]. Efforts to improve stability and function have focused on engineering OPT via rational design[39–42], directed evolution[29] and the incorporation of non-canonical amino acids[37,43].

In this work, we report a biomaterial approach to control organophosphate toxicity aimed at managed bees (i.e., bumble bees such as the common eastern bumble bee, *Bombus impatiens*, or the western honey bee, *Apis mellifera*) using phosphotriesterase (OPT) loaded microparticles (Fig. 1). We used the common eastern bumble bee (*Bombus impatiens*) for our *in vivo* assays, though similar gut pH exist for *A. mellifera*[44,45], thus our results may be relevant to *A. mellifera* as well. We chose calcium carbonate microparticles to deliver OPT based on several design considerations. First, the microparticles mimic pollen grains in size and are therefore easily consumed by bees. Both bumble bees and honey bees have a GI tract composed of a crop and ventriculus, separated by a proventricular valve which mechanistically extracts micro-sized particles for digestion[46]. Second, by harnessing the acid scavenging capability of $CaCO_3$, the microparticles can protect OPT from unfavorable acidic GI conditions to maintain enzyme bioactivity, once consumed by bees. The pH of the crop and ventriculus are 4.8 and 6.5 respectively[45–47], well below the optimal pH conditions of OPT[38]. Third, $CaCO_3$ microparticles (2–50 µm) are relatively easy and inexpensive to produce in large quantities and are capable of loading biomacromolecules during production[48]. With optimized fabrication parameters and importantly the inclusion of gelatin as an additive, we produced homogenously sized microparticles that encapsulated OPT at 90% efficiency and displayed a superior suspension stability in sucrose. *In vitro* studies confirmed the protective effect of the microparticles on OPT bioactivity. The OPT encapsulating pollen-mimicking microparticles (OPT-PMMs) allowed 100% survival of microcolonies of bees fed malathion-contaminated pollen patties, while 0% survival was observed for those fed with OPT alone or plain sucrose after 5 and 4 days, respectively. To understand the protective properties of PMMs, we fed bees PMMs loaded with a FITC-labelled protein

human serum albumin (HSA-PMMs) and free FITC-HSA. Fluorescent imaging and spectrophotometric assay confirmed stability and bioactivity of HSA-PMMs throughout digestion for 4 hrs, whilst HSA alone remained bioactive for only up to 1 hour and was undetectable in the posterior section of the GI tract. This versatile, scalable, low-cost detoxification strategy can act as a precautionary or remedial measure for managed pollinators when pollinating in areas of organophosphate application, to address the issue of pollinator exposures.

Results And Discussion

Calcium carbonate microparticles can be easily fabricated by rapidly mixing equimolar 0.33 M volumes of $CaCl_2$ and $NaCO_3$. Size and shape can be acutely controlled by altering synthesis parameters such as stirring speed, time and additive inclusion[49,50]. Initially, we fabricated $CaCO_3$ microparticles with no inclusion of an additive, nor control of stirring time. The product displayed high incidences of aggregation, calcite crystal growth and poor size homogeneity (Fig. 2A), the average size is around 8.2 µm with large size distribution under pH 7.4, which caused poor suspension stability. To circumvent these challenges we restricted stirring time to 10 seconds and included gelatin (24 mg/mL) as an additive, which resulted in smaller and consistently homogenously sized (3.9 µm) microparticles. Given these microparticles mimic certain pollen grains, we refer to them as pollen-mimicking microparticles or PMMs. (Fig. 2B) PMMs displayed a superior suspension stability in sucrose. The significantly improved suspension stability was confirmed using a biophotometer which measured the uppermost layer of the microparticle suspension. After 2 days, ~ 90% of unmodified microparticles had settled whilst > 75% of PMMs maintained good suspension stability. PMMs took 6 days to fully settle whereas unmodified microparticles only took 3 (Fig. 2C). The sucrose media used to suspend the microparticles was at a typical concentration used to feed wintering honey bees (2 g/mL). Although the molecular mechanism behind crystal growth and aggregation is unclear[51,52], scanning electron microscope (SEM) imaging confirmed that the gelatin modified microparticles maintained a highly porous nanoparticle aggregation structure (Fig. 2F). Nanometer size pores are known to provide accessible channels for biomacromolecule diffusion and a high internal surface area to allow physical adsorption, with high substrate loading[53].

Since OPT-PMMs need to maintain function when passing through acidity presented by the crop stomach, we tested the PMMs stability in pH 4.8. PMMs at pH 4.8 displayed a fractional shift to a smaller size distribution (3.4 µm) (Fig. 2B). Despite the partial dissolution of microparticles in acid, PMMs largely retained their size. We then repeated our PMM fabrication process using high reagent volumes to demonstrate the capacity for large scale manufacture. PMMs were successfully produced at a 1L total volume (Fig. 2D) and displayed a size distribution comparable to that of PMMs fabricated at small scale, with an average size of 4.3 µm (Fig. 2E). Microparticle pore size was analyzed in accordance with density functional theory using N_2 adsorption isotherms. PMM nanochannel volumes dropped from 0.0067 to 0.0043 cm^3/g following HSA encapsulation which further confirmed protein loading (Fig. 2G). Nanochannel diameters only dropped from 14 to 12 nm which indicated that protein loading did not block channels and would still permit OPs to enter for enzymatic degradation.

Protein loading and gelatin modification of PMMs was further confirmed through confocal laser scanning microscopy (CLSM). HSA was used in this instance as a model protein. Microparticles exhibited an overlay of Cy5.5-conjugated gelatin and FITC-conjugated HSA (FITC-HSA) in the full morphology of each microparticle (Fig. 3A). The CLSM imaging indicated gelatin conjugation and protein loading throughout the microparticle volume. The protein loading efficiency (PLE) of our microparticle design when feeding different enzyme concentrations was first characterized spectrophotometrically using FITC-HSA. HSA-PMMs presented high loading efficiencies of 85.5% for gelatin-modified and 83.6% for unmodified microparticles when feeding 5% protein loading content (PLC) (Fig. 3B), consistent with previous studies[48]. The loading efficiency decreased as the protein loading content increased (67.1% PLE for 10% PLC; 52.1% PLE for 15% PLC), with a slightly smaller efficiency decrease (64.1% & 47.1%) for unmodified relative to gelatin-modified microparticles (Fig. 3B). Considering the loading efficiency decrease of higher PLCs and the intrinsic value of OPT, we selected 5% as a baseline to test OPT loading. OPT presented 88.1% PLE at 5% PLC and 90.1% PLE at 2% PLC (Fig. 3C). We found that a concentration of 0.5 mg/mL OPT was sufficient to initiate rapid hydrolysis of methyl-paraoxon to visibly form nitrophenol (Supplementary Fig. 2). A 2% protein loading content yields an OPT concentration of 1.21 mg/mL in OPT-PMMs, which can be diluted 1.4-fold to 0.5 mg/mL, we found this dilution offered adequate sucrose to render the solution sufficiently attractive to bumblebees for consumption. Furthermore, it was evaluated that no protein was released from the PMMs, up to 7 days following fabrication whilst suspended in 2 g/mL sucrose (Supplementary Fig. 3).

Literature has shown OPT catalytic efficiency and conformational stability can vary upon structural mutagenesis and variation in the central metal cation[39,54,55]. In our experimentation, we used wild-type Co^{2+}-bound phosphotriesterase (molecular weight 39 kDa) (Supplementary Fig. 1), which is the optimum metalloenzyme complex capable of a k_{cat}/k_m of 7.6×10^7 M^{-1} s^{-1} in hydrolyzing paraoxon. For the successful function of our design, it is critical OPT-PMMs are able to maintain bioactivity in the conditions of a bee digestive tract (pH 4.8 in the crop stomach). Therefore, bioactivity and enzyme stability of OPT-PMMs and free-OPT were assessed *in vitro* over a pH range using OPT 0.5 mg/mL and either 0.5 mM paraoxon or 0.44 mM malathion. Paraoxon assays were carried out by measuring the absorbance of nitrophenol as it is produced from the OPT-catalyzed degradation of paraoxon. The relative enzymatic activity was obtained by normalizing the absorbances of both free OPT and OPT-PMMs to that of OPT-PMMs incubated at pH 7.4. As shown in Fig. 3D, free OPT yielded an activity of 49.7%, approximately 1-fold lower than that of OPT-PMMs at pH 7.4. We suspect the higher performance of OPT-PMMs is because microparticle encapsulation facilitates enhanced enzyme kinetics[56–58]. We anticipated the $CaCO_3$ element of the microparticle structure would bear an ‘acid scavenging’ ability which could neutralize acid in the microparticle’s immediate vicinity, allowing encapsulated OPT to outperform free enzyme in acidic conditions[59]. As expected, OPT-PMMs at pH 4.8 displayed lower activity (73.4%) than that at pH 7.4. However, free enzyme assays almost did not function at all (1.7%) at pH 4.8.

An absorbance from malathion hydrolysis was characterized using Ellman's reagent (5,5'-dithiobis-(2-nitrobenzoic acid) or DTNB) which can react with the thiol group of malathion’s breakdown product to

form 2-nitro-5-thiobenzoate or TNB^{2-} which has an absorbance at 412 nm (Supplementary Fig. 4). In malathion degradation assays, a similar trend could be detected at both pH 7.4 and pH 4.8. OPT is less adept at cleaving thiol groups and therefore enzymatic degradation of malathion is relatively slow. Free OPT therefore displayed much lower activity of 17.1% at pH 7.4 and 0.6% at pH 4.8, respectively (Fig. 3E). However, OPT-PMMs could still maintain high activity of 82.2% at pH 4.8. This indicates the benefits of the microparticle design are more pronounced when degrading OPs that OPT can degrade relatively slowly. The superior catalytic performance of PMMs in pH 4.8 demonstrates the importance of utilizing a biomaterial element to protect enzyme catalysts in oral consumption.

We wished to understand the stability of our system under significant thermal stress, as any treatment could experience high summer temperatures when administered to bees. OPT has been found to denature at temperatures exceeding 45^{o}C. We aimed to determine whether microparticle encapsulation offers any protection from thermal denaturation. We tested the capacity for the microparticle design to withstand elevated temperatures by measuring paraoxon breakdown following enzyme incubation at temperatures ranging from 30-60^{o}C. The relative enzymatic activity was obtained by normalizing the absorbances of both free OPT and OPT-PMMs to that of OPT-PMMs incubated under 30℃. As shown in Fig. 3F, free OPT displayed half bioactivity of OPT-PMMs under 30℃. The enzymatic activity of free OPT dramatically dropped to 36.5% after incubation at 50 °C, whereas the activity of OPT-PMMs remained at 66.3% at the same temperature. Further, increases in temperature ≥ 60 °C resulted in little catalytic activity (5.0%) of the enzyme in OPT systems, which is much lower than that of OPT-PMMs (17.7%). We found OPT maintained greater bioactivity when encapsulated in PMMs, relative to the case of free OPT as temperatures increased. This is important for the potential application of our design, as it may be administered at elevated temperatures.

In order to gauge the time taken for treatment to lose functionality, bioactivity of each group treatment relative to OPT-PMMs was measured over time when kept at room temperature (25^{o}C). Microparticle activity maintained around 60% of original activity after 7 days and 49.5% after 14 days, whereas free enzyme activity reduced to ~ 30% and < 10%, respectively. OPT-PMMs stored at 4^{o}C maintained almost 100% activity after 14 days (Fig. 3G). The microparticle's capacity for long term bioactivity and protein sequestration indicates a practical shelf life of the design.

Bombus impatiens (the common eastern bumble bee) were used for in vivo experimentation because colonies can be maintained indoors during the winter in a practical and accessible box. Bumble bees have displayed a susceptibility to OPs comparable to *A. mellifera*[60]. In order to understand the retention performance of PMMs once consumed, we fed bumblebees microparticles loaded with FITC-labelled HSA (FITC-HSA-PMMs) and free FITC-HSA for 30 minutes, before extracting digestive tracts over a 12-hour period for fluorescent microscopy analysis (Fig. 4). FITC displayed PMMs successfully in the crop stomach and ventriculus sections of the GI tract for samples collected at 0/1/4/12 hrs. During the first hour of digestion, microparticles were distributed across both the crop stomach and ventriculus. By 4 hours, the majority of microparticles had travelled out of the crop stomach, before full clearance after 12

hours into the ventriculus, suggesting proventricular filtering of PMMs (Fig. 4A). Free enzyme was imaged throughout the digestive tract at all time points. As expected, the crop stomach maintained fluorescence unlike PMM, indicating free enzyme was not extracted into the posterior section of the GI tract. The data suggests PMMs are digested akin to pollen grains, increasing the number of microparticles drawn into the ventriculus alongside pollen. This maximizes PMM function in detoxifying pollen as it is digested. This is significant because OPs are often found in high quantities in pollen, which may be held in the posterior section of the ventriculus for digestion for up to 12 hours or more. Fluorescence of PMMs was maintained throughout digestion[46,61], whereas free enzyme fluorescence considerably diminished over the 12-hour period, with clear fluorescence loss after 4 hours (Fig. 4B). We were not able to quantify the fluorescence because FITC fluorescence is pH dependent[62], the presence of microparticles would have altered stomach pH to the point where fluorescence readings would have been inaccurate. However, these images qualitatively suggest the PMM design improved retention and provided protection from denaturation of loaded proteins.

We were able to further validate our characterization of OPT-PMM efficacy via quantification of AChE activity when mixed with our treatment and paraoxon. As AChE is inhibited by OPs such as paraoxon, high AChE activity would indicate effective detoxification through our treatment. Acetylthiocholine cleavage via AChE can be used to quantify AChE activity, as the thiocholine product reacts with DTNB to form TNB^{2-} which has an absorbance at 412 nm (Fig. 5A). Homogenized honey bee cells were able to maintain 91.5% of AChE activity when treated with 0.5 mM paraoxon and OPT-PMMs, relative to the positive control. This was a stark improvement in AChE functionality relative to no treatment, which resulted in a relative activity reduction of ~ 72%. Samples treated with free OPT retained 18.8% less activity than that of samples treated with OPT-PMMs (Fig. 5B).

Groups of 50 bumble bees were treated with paraoxon or malathion contaminated pollen balls and OPT-sucrose treatments, to determine the efficacy of treatments in reducing mortality under OP exposure (Fig. 5C). Paraoxon and malathion, present oral LD_{50}'s for honey bees at 0.0175 and 0.38 µg/bee respectively[63]. These data set a benchmark for OP doses we would attempt to administer and subsequently detoxify, to demonstrate OPT-PMM efficacy. Bumblebees approximately consume 40.5 µg pollen/day depending on body mass[64]. Based on this figure, we initially formed pollen balls containing 0.432 µg/g paraoxon and 9.383 µg/g malathion to feed without enzyme treatment as a negative control. We found these pollen balls caused no health deterioration after one week. Subsequently, through trial and error, we significantly increased contamination to concentrations which caused significant mortality. We tested pollen balls containing 50 µg/g paraoxon over 12hrs to measure the OPT-PMM impact against acute exposure. In this trial OPT-PMMs at 500 µg/mL of OPT was able to maintain a 70% survival rate, whereas free enzyme and sucrose treated groups sustained 62.5 and 72.5% fatalities respectively (Fig. 5D). Although OPT-PMMs largely detoxified acute exposure, the catalytic efficiency was not able to fully mitigate mortality.

A moderate level of toxicity was tested using 15 µg/g paraoxon and 750 µg/g malathion contaminated pollen balls against 50 µg/ml OPT treatments. Free-OPT and no treatment resulted in 100% mortality after 5 and 4 days respectively following paraoxon toxicity. OPT-PMMs were able to maintain a slower incidence of mortality relative to other treatments. After 10 days 38% of the group sample had survived. Groups containing non-contaminated pollen balls and either pure sucrose or PMM-sucrose maintained 100% and 96% survival respectively. It is expected to see some minor mortality in any sample after 10 days (Fig. 5E). In malathion contaminated samples, OPT-PMM at 800 µg/mL of OPT was able to maintain 100% survival for the duration of observation, a lower concentration of 500 µg/mL maintained above 80% survival over 10 days. Free-OPT and sucrose treated groups presented 100% mortality after 5 and 4 days respectively, analogous to the paraoxon trial (Fig. 5F). We suspect the poor performance of free, unprotected OPT is in part driven by its higher denaturation in the acidic conditions of the digestive tract. Pollen grains release their internal contents as they progress along the midgut[65,66]. We assume that any OPs absorbed into pollen grains during incubation, are also made available at this stage of digestion. This means for the effective detoxification of contaminated pollen, OPT must retain bioactivity until it makes passage into the ventriculus. In addition, it is critical a high concentration of OPT is drawn into the midgut to intercept paraoxon or malathion as pollen is digested. Both of which we have facilitated via microparticle encapsulation in PMMs.

Experimentation has shown that PMMs are able to enhance the bioactivity of OPT. OPT-PMMs outperforms free OPT when tested under unfavorable conditions of temperature, storage and pH. The microparticle design has rendered OPT suitable for use in addressing pollinator OP intoxication, as it bestows functionality in gastric acidity and can maintain performance for longer durations, under elevated thermal stress. The microparticle design has also improved functionality of OPT under digestion in consideration of a bee's digestive system. Microparticles are extracted into the midgut and retained for a greater duration than free OPT. The aforementioned advantages ultimately result in a lower rate of mortality when treated with OPT-PMM relative to free OPT. The benefits are most appreciable when degrading OPs which are typically hydrolyzed at a lower relative rate (as in the case of malathion). This work has produced a viable product to mitigate insecticide damage to pollinator colonies and has revealed new ways in which research can work to address the impacts of insecticide application, through improving this current design, or by exploring new microparticle treatments.

Further research is required to determine the impact of the design on a colony as a whole, in colony-scale testing. A characterization of how microparticles are distributed around colony castes and brood chambers when administered, would give an indication of the potential for the design at a colony level. Emphasis should also be placed upon testing the effect upon pollination efficiency of crops, to comprehend the economic potential of the design if applied in agricultural purposes. Field studies should also look into the possibility of reaching native pollinators. By studying differences in diets and feeding mechanisms between crop pests and beneficial insects, OPT-PMMs could potentially be administered in suitable feeding carriers in areas of intensive agriculture to exclusively reach pollinators and other non-target organisms.

Declarations

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Conflict of Interest: The technology described in this paper is being licensed to Beemmunity, a start-up company co-founded by J.W.

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Figures

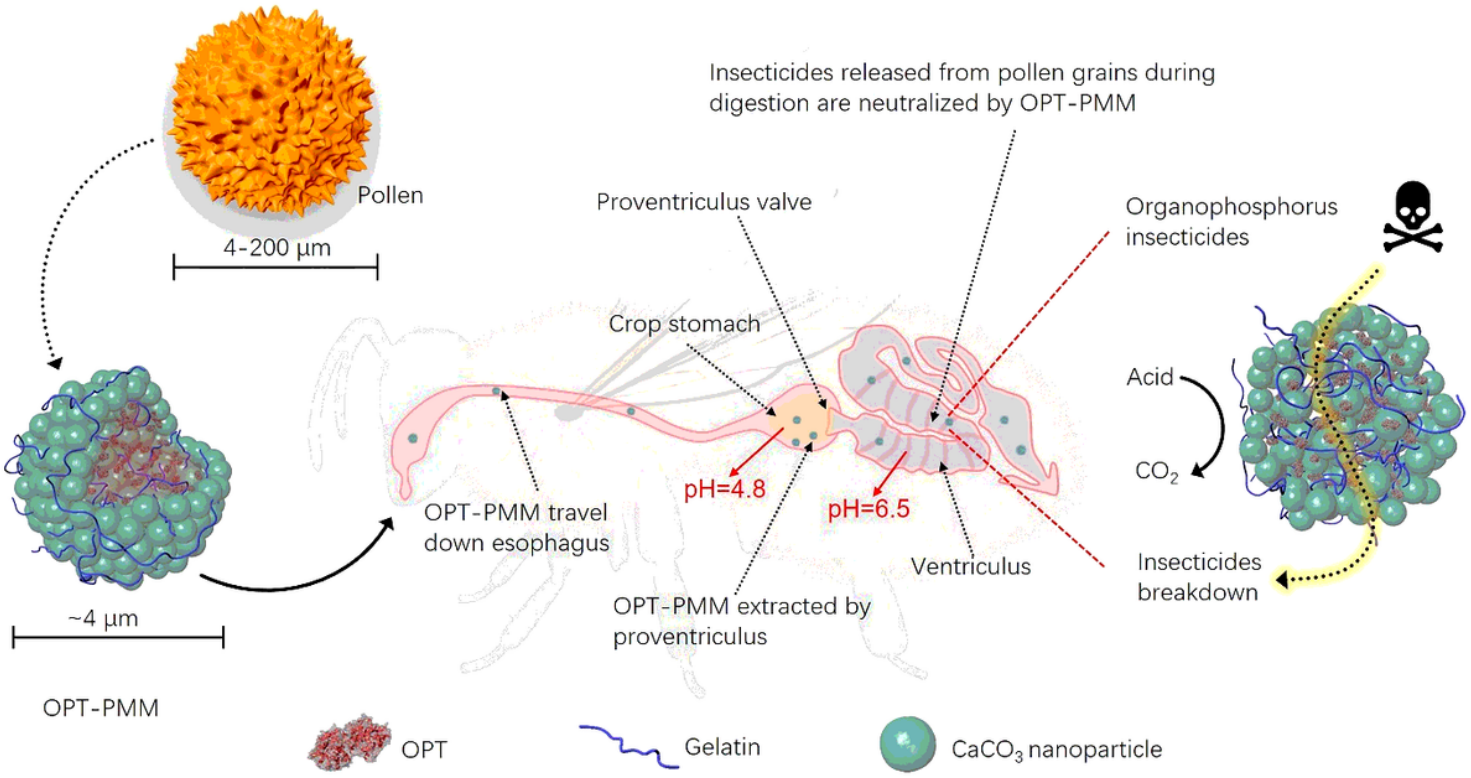

Figure 1

A schematic of the passage of microparticles through a bee digestive tract. Microparticles move into the midgut analogous to pollen grains as they are extracted by the proventriculus which draws particulates out of the crop stomach. The PMM structure protects the encapsulated protein from gastric acidity. PMMs are retained in the midgut to detoxify pesticides as they are released during pollen digestion.

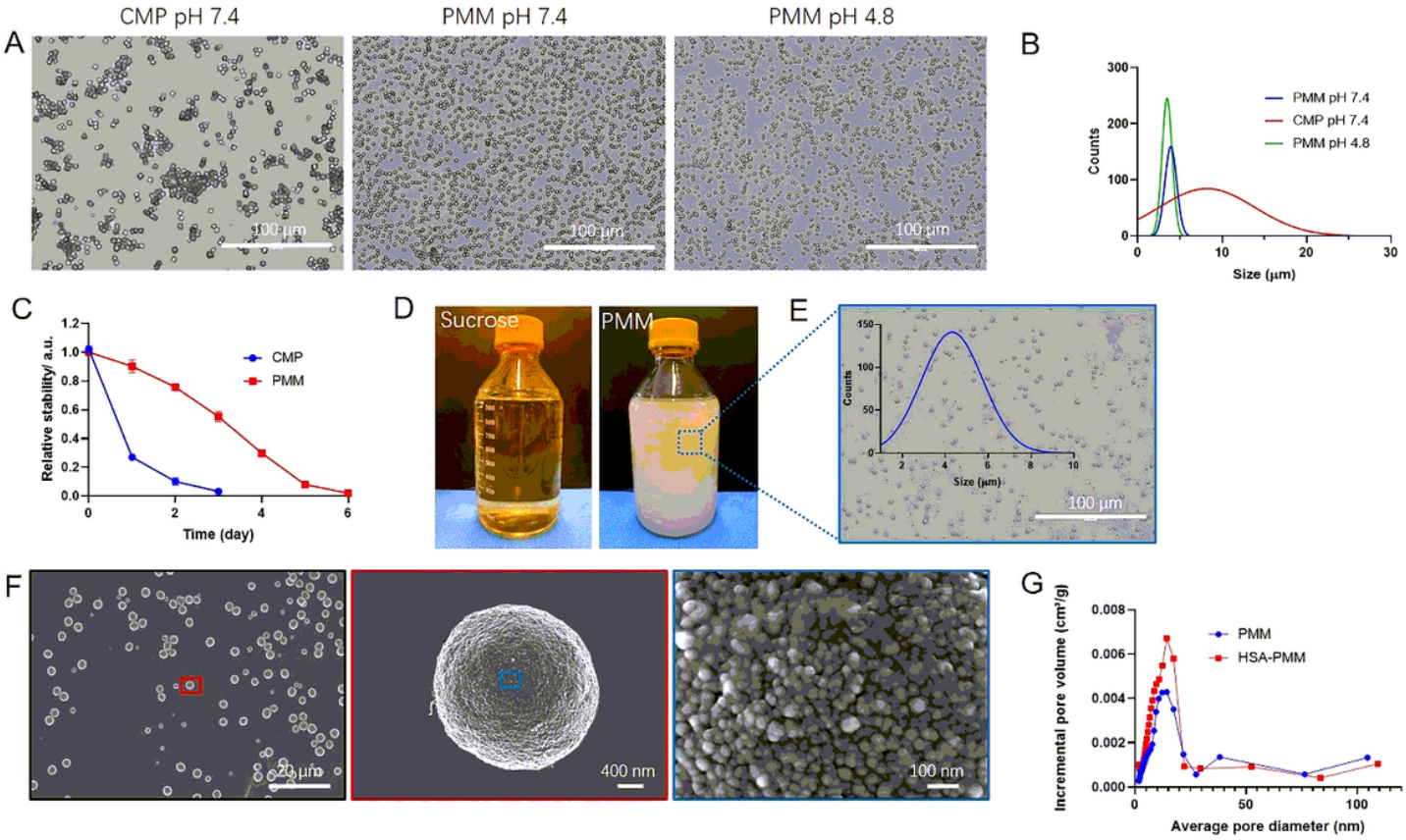

Figure 2

Physical characterization of PMMs. (A) Morphological analysis of unmodified CaCO3 microparticles (control) in pH 7.4 and PMMs in pH 7.4 and 4.8. (CMP denotes unmodified CaCO3 microparticles, include as control.) (B) Size distribution analysis of PMMs and unmodified microparticles in pH 7.4 and PMMs in pH 7.4 and 4.8. (C) Relative suspension stability of unmodified microparticles and PMMs in 2 g/mL sucrose. (D) Large scale fabrication of PMMs. (E) Size distribution analysis of PMMs fabricated at a large scale. (F) SEM imaging of microparticles to determine PMM surface morphology. (G) Pore size distribution analysis of PMMs and PMMs loaded with HSA.

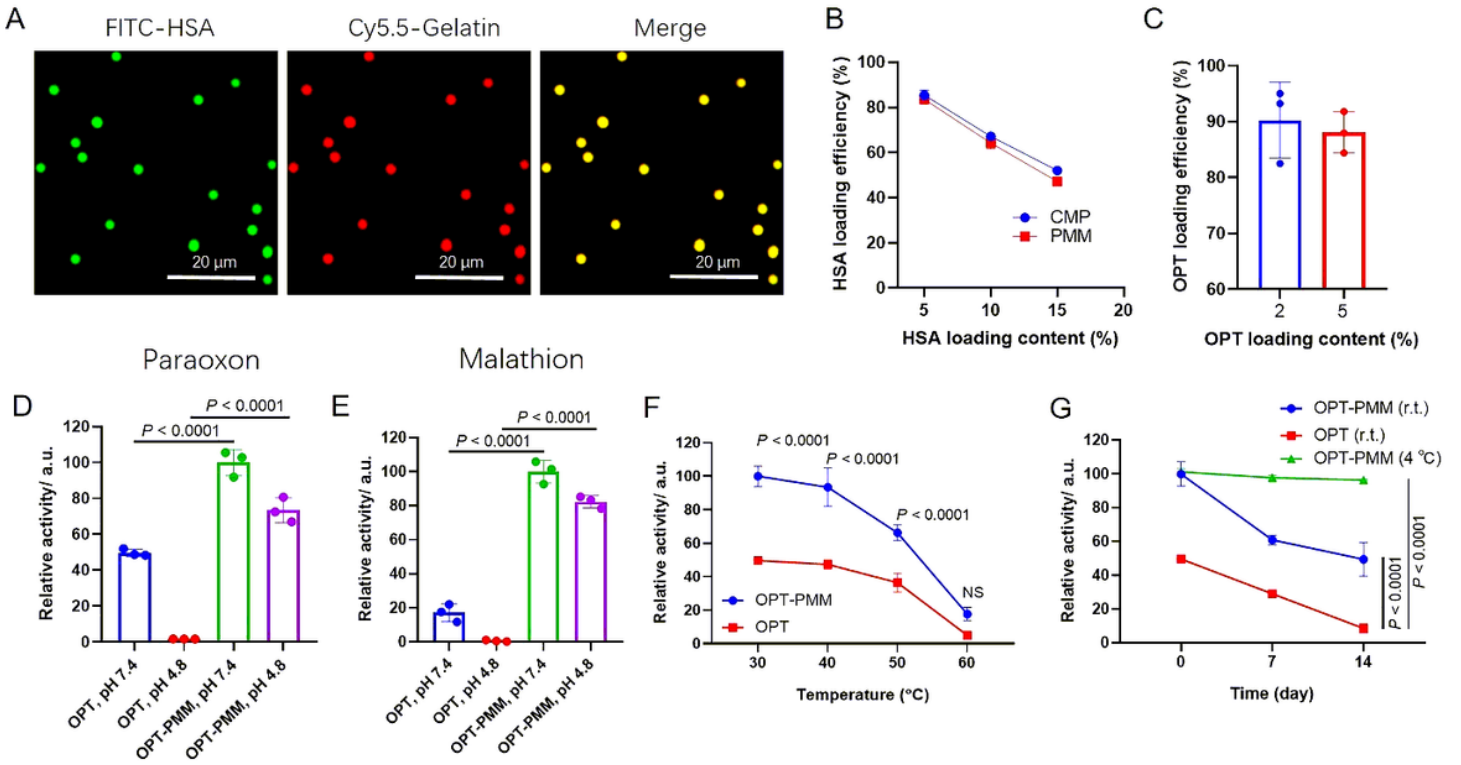

Figure 3

Characterizations of OPT-PMMs. (A) Fluorescent imaging of PMMs containing Cy5.5 modified gelatin and FITC-conjugated HSA. (B) Protein loading efficiency (PLE) of unmodified CaCO3 microparticles (CMP) and PMM loaded with HSA at 5, 10 and 15% protein loading content (PLC). (C) PLE of PMM loaded with OPT at 2 and 5% PLC. (D) Relative activity of OPT-PMM and free OPT in paraoxon hydrolysis under pH 7.4 and 4.8 (n=3). (E) Relative activity of OPT-PMM and free OPT in malathion hydrolysis under pH 7.4 and 4.8 (n=3). (F) Temperature-dependent relative activity of OPT-PMM and free OPT in paraoxon hydrolysis when incubated at temperatures 30, 40, 50, 60oC (n=3). (G) Long-term relative activity of OPT-PMM and free OPT in paraoxon hydrolysis when stored at room temperature and 4oC (n=3). Statistical analysis was performed by analysis of one-way ANOVA tests (D & E) and two-way ANOVA tests (F & G).

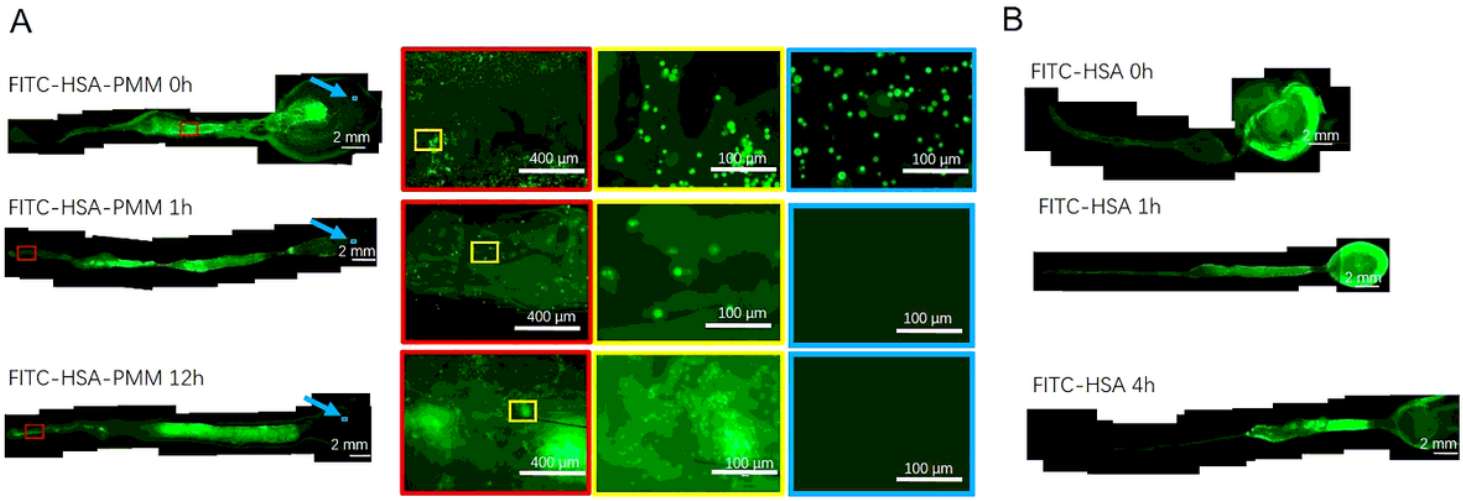

Figure 4

Tracking of digested PMMs by fluorescent imaging of bumblebee GI tracts. (A) GI tracts following HSA-PMM treatment, fluorescence was maintained up to 12 hrs post-consumption. Microparticle morphology is clearly visible and microparticles are successfully drawn into the midgut (n=3). (B) GI tracts following free-HSA treatment, fluorescence diminished over the 12 hrs after consumption. Free enzyme is not actively drawn into the midgut (n=3).

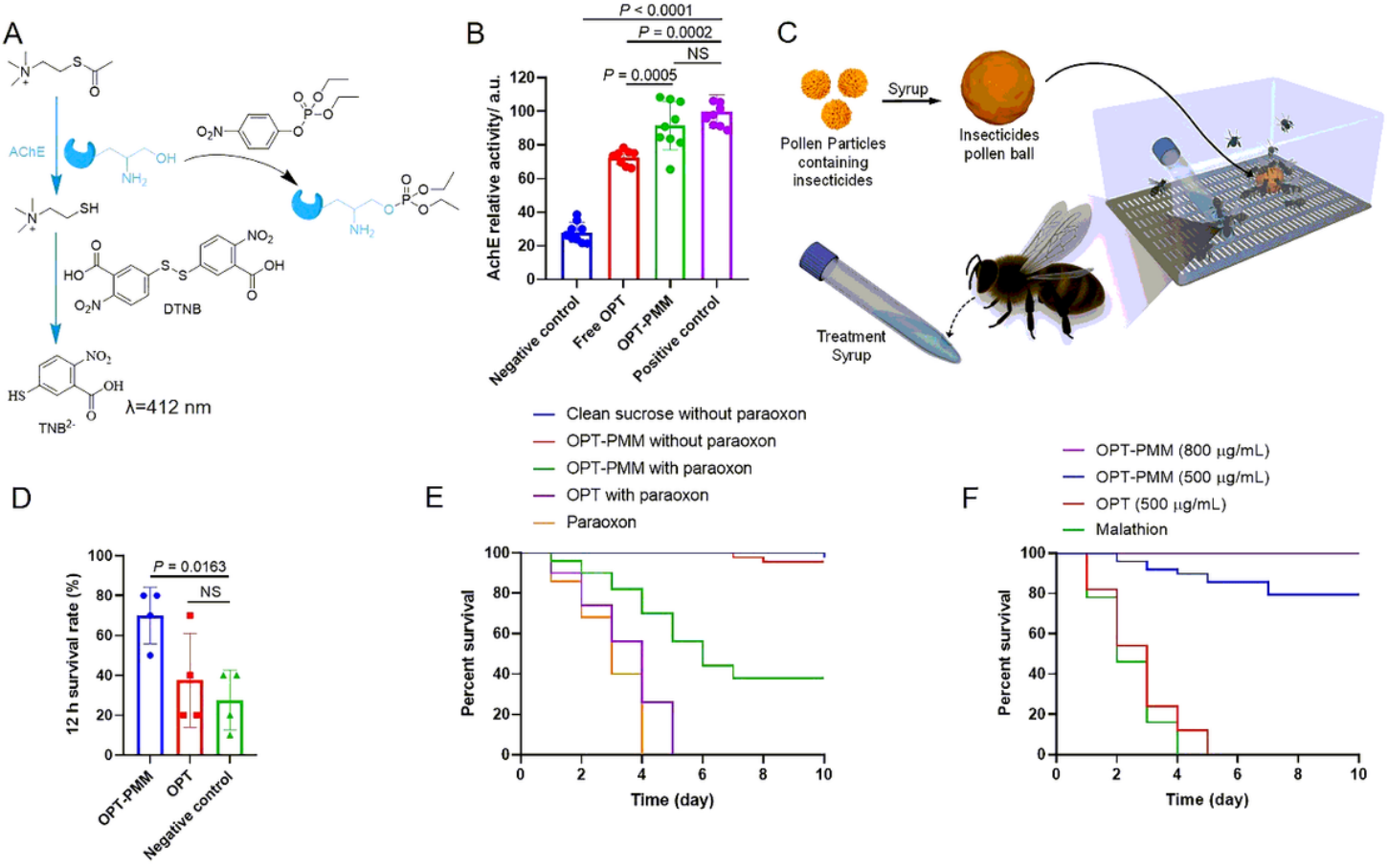

Figure 5

Function of PMMs. (A) The formation of thiocholine from acetylthiocholine through AChE cleavage can be characterized using DTNB. DTNB and thiocholine react to form TNB2-, the absorbance of which can be measured at 412 nm. (B) Relative activity of AChE from homogenized honey bees when incubated in 0.5 mM paraoxon or DI water (positive control) and treated with samples of free OPT, OPT-PMM and DI water (positive and negative control, n=9). (C) The apparatus for determining mortality following contaminated pollen ball consumption against PMM treatment in syrup. (D) Survival rate of bumblebees following acute exposure to paraoxon (50 μg/g pollen) over 12 hrs when treated with 500 μg/mL OPT treatments (n=40) (E) exposure to paraoxon (15 μg/g pollen) over 10 days (n=50). (F) exposure to malathion (750 μg/g pollen) over 10 days (n=50). Statistical analysis was performed by analysis of one-way ANOVA tests (B & D).

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