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Using magnetic nanoparticles to explore symbiotic interactions

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Abstract

All plants and animals form symbiotic associations with microbes, yet many of the underlying mechanisms associated with these interactions remain uncharacterized. There are inherent challenges to studying the cellular and metabolic interactions between eukaryotes and their microbial symbionts, thus new methodologies that enable the discovery of symbiotic processes are continually needed. Here, we explored the use of magnetic nanoparticles (MNPs) as a tool to track aspects of the host innate immune response to symbionts under both *ex vivo* and *in vivo* conditions. The symbiotic association between the Hawaiian bobtail squid *Euprymna scolopes* and its bioluminescent partner *Vibrio fischeri* was used as a model to explore the potential of MNPs as non-toxic, manipulable agents to investigate aquatic symbiotic associations. Results suggest that host cells can be effectively labeled with MNPs under *ex vivo* conditions and that the particles can be visualized and tracked within the host animal *in vivo* using magnetic particle imaging. Proteomic and metabolomic analyses also revealed minimal changes to the host innate immune cells after uptake of MNPs in the presence and absence of *V. fischeri*. Together, these results suggest that MNPs have minimal biochemical impact on the host cells and can serve as an effective tool to explore aquatic symbiotic interactions.

Introduction

Symbiosis is a ubiquitous life strategy on Earth and plays a key role in

shaping how ecosystems are structured and function [1,2]. Symbiotic interactions affect biodiversity on both ecological and evolutionary scales [3,4] and help maintain host health by influencing their developmental, physiological and immune responses [5,6]. Despite increased awareness of the importance of symbioses in all aspects of life, numerous persistent questions remain about the underlying establishment, maintenance and persistence of host-microbe interactions.

To address these critical questions, important advancements in tools (e.g., CRISPR-Cas9, NanoSIMs imaging, and single cell genomics) have deepened our understanding of fundamental processes associated with symbiosis [7,8]. However, the wide array of host-microbe associations requires versatile tools to explore various aspects of these interactions. One technology that has emerged as a valuable tool for biomedical and environmental studies has been magnetic nanoparticles (MNPs) [9,10]. The unique properties of MNPs have made them a versatile technology for advancements in diagnostics, drug delivery therapies, imaging activities and *in situ* manipulation with external fields [11-15]. MNPs have a unique architecture that consist of a magnetic core (e.g. Fe, Ni, Co) with a coating shell that improves biocompatibility and reduces particle aggregation [16]. In addition, the surfaces of MNPs can be engineered for targeting certain cells or delivering molecules to discreet locations [17].

As the use of MNPs in environmental remediation and biomedicine is growing rapidly, in this study we explored the uptake of commercially

available MNPs by host innate immune cells *ex vivo* in the presence and absence of beneficial microbes to assess whether there were changes to the protein and small molecule profiles after uptake. We used the well-established Hawaiian bobtail squid *Euprymna scolopes* as a model organism as it forms extracellular symbiotic associations with environmental microbes [18,19].

Symbiotic associations between bobtail squid and marine bacteria have provided critical insights into the factors required for colonization and establishment of beneficial symbioses [20,21]. In the bobtail squid *E. scolopes*, the host harbors two distinct symbiotic organs, the accessory nidamental gland and the light organ (Fig. 1A). Both organs recruit symbiotic bacteria from the environment using fields of ciliated epithelial cells (Fig. 1B, C), the recognition of which is mediated by the innate immune cells of the host squid, known as hemocytes [22]. Within both organs, the symbiosis influences development of the hemocytes, resulting in recognition of the beneficial microbes and trafficking of the immune cells into the symbiotic tissues (Fig. 1D, E). The trafficking of these hemocytes in response to the presence of the symbionts represents critical processes in both the onset and maturation of symbiosis in the host organs [23-25]. Here, we explore the impact of MNP uptake on the proteome and metabolome of these critical immune cells to evaluate the potential for using MNP technologies to explore the beneficial symbiotic associations with aquatic organisms.

Results and discussion

Hemocytes can be effectively labeled and isolated with MNPs

To assess whether MNPs can be effectively taken up by the host immune cells, hemocytes were isolated from adult *E. scolopes* and exposed to fluorescent MNPs that contained an iron oxide core and polyethylene glycol (PEG) coating. Transmission electron microscopy revealed the MNPs exhibited a uniform shape and disbursement (Supplemental Fig. S1) with a mean effective hydrodynamic diameter of 63.1 nm ± 5.2, as determined with dynamic light scattering. Total iron content of the MNPs was 1.07 mg/mL as determined with a 1,10-o-phenathroline assay. An overview of the general work plan in which hemocytes were labeled, isolated and examined with a multi-omics approach is visualized in Fig. 1F-H. Hemocytes purified from the hemolymph of the host animal were incubated for up to 24 h with the MNPs and examined with confocal microscopy (Fig. 2). Results demonstrated that the hemocytes effectively internalized MNPs at concentrations as low as 20 µg per mL, although not all hemocytes were labeled at this lower concentration (Fig. 2C).

Within the *ex vivo* hemocytes, actin staining revealed the MNPs were localized to the cell cytoplasm primarily in the cell body (Fig. 2C-F). At higher MNP concentrations (100 µg per mL), all observed hemocytes exhibited MNP labeling (Fig. 2E) and were similarly localized to the cell body of the hemocytes (Fig. 2D-F). No statistical changes in hemocyte viability were observed up to three days when labeled and unlabeled

hemocytes were stored in Squid Ringer's buffer at 4□C as determined with live-dead staining (Supplemental Fig. S2). Additional imaging of the MNP-labeled cells using live-dead staining showed viable cells for up to one week post labeling. The localization results are consistent with intracellular uptake of MNPs in mammalian immune cells [26] and the results suggest that MNP labeling approaches could enable longitudinal imaging studies of host immune cells over time.

Minimal impact of MNP-labeling on proteome of host innate immune cells

To determine whether the labeling of host immune cells with MNPs affected cell protein physiology, peptides from hemocytes labeled with 100 µg per mL of MNPs and unlabeled controls were identified and quantified using liquid chromatography-mass spectrometry (LC- MS/MS) (Fig. 3). The proteome of hemocytes with and without MNPs were compared with Scaffold [27] and analysis revealed 3,934 recovered proteins in both labeled and unlabeled populations of hemocytes (Fig. 3A). Of these recovered proteins, 3748 proteins were initially found to be shared between hemocytes labeled with and without the MNPs (Supplemental Fig. S3; Supplemental Datasets S1-S2). When the results were compared after Benjamini-Hochberg correction for false discovery rate no significant difference was observed between the two treatments (Fig. 3A).

Analysis of the proteome showed that the most common proteins

found in the hemocytes (Fig. 3C) were histone-related, which can be key components of the innate immune system and can act as damage-associated molecular patterns (DAMPs) [28]. In some cases, histones can act as DAMPs and bind to pattern recognition receptors (PRR) thereby triggering the innate immune response [29]. In addition to the numerous histone related proteins, the PRR *E. scolopes* peptidoglycan recognition protein 5 (EsPGRP5) was also slightly enriched in the host hemocytes labeled with MNPs, although this increase was not significant. Previous work also identified histones and EsPGRP5 in host hemocytes as being regulated by light organ colonization state in *E. scolopes* [30]. These analyses showed that MNP uptake did not have a significant impact on the hemocyte proteome.

Effects of MNP-labeling on the metabolomic profile and distribution of *E. scolopes* hemocytes

In addition to proteomic analysis, metabolites of MNP-labeled and unlabeled hemocytes controls were extracted and compared using an untargeted metabolomics approach (Fig. 3B; Fig. 4; Supplemental Fig. S3; Supplemental Datasets S1, S3). A total of 7,474 mass spectrometric features were found from the hemocyte extracts and there were no statistical differences observed between the two treatments as determined using a one-tailed t-test ($t_{(5)} = 2.7$ $p > 0.05$) and an analysis of similarity (ANOSIM) test (R: 0.1812, $p = 0.1191$). The lack of extensive statistical variation between the hemocyte metabolic profiles could reflect several

technical limitations of the study. First by using the immune cells of wild-caught male and females, the variation of hemocyte metabolomic profiles between individual animals may be greater than any statistical variation caused by the presence of MPNs. Additionally, the use of only five animals may have limited the statistical power of the analyses, thus we acknowledge that there could be complicating factors to explain why there is no pronounced statistical differences in the biochemical profiling. Previous research, however, has shown that using hemocytes from multiple animals is sufficient to detect differences in the metabolome and proteome of hemocytes collected from wild-caught or antibiotic cured animals [30], suggesting that the MNP treatment is not having a pronounced impact on the host immune cells biochemical profiles.

Although analysis of the overall metabolome suggested no significant difference between MNP-labeled and unlabeled hemocytes, pairwise comparisons of several individual metabolites did show a few metabolites with differential abundance (Fig. 3D). There were decreases in hexadecyl acetyl glycerol, an inhibitor of protein kinase C, and heptanoyl carnitine, an acylcarnitine that is involved in transporting fatty acids into the mitochondria (Fig. 3D) [31]. The only metabolite increased in MNP-labeled hemocytes was the glycerophospholipid octadecenyl-sn-glycero-3-phosphoethanolamine (oleoyl-PE). Glycerophospholipid metabolism plays an important role in the production of the biological membrane and previous research has shown that in oral administration of MNPs in rats also resulted

in increased metabolites associated with this pathway [32], which may suggest a potential impact on the cell membrane architecture. However, oleoyl-PE has a wide range of functions including anti-inflammation and the synthesis of other glycerophospholipids [33]. A more detailed analysis of the role of this metabolite in hemocytes is required to better assess whether MNP-induced increases of this metabolite would negatively impact symbiotic interactions between bacteria and the host immune cells.

Assessing the effect of symbiont *V. fischeri* exposure on the metabolomic profiles of *E. scolopes* hemocytes labeled with MNPs.

Previous research has shown that colonization of the *E. scolopes* light organ by *V. fischeri* affects hemocyte-bacteria binding behavior, migration, host gene expression, and the proteome of hemocytes [24,30,34,35]. Therefore, to explore whether hemocytes exposed to *V. fischeri* would alter their response to MNPs, the immune cells were pre-exposed to symbiosis-competent *V. fischeri* for 2 h and then co-incubated with MNPs for 24 h. After incubation, the hemocytes were washed to remove non-phagocytosed MNPs and then assessed using LC-MS/MS to evaluate changes in the metabolome (Fig. 4).

No significant difference was found between the metabolome of *V. fischeri*-exposed hemocytes, regardless of whether they were MNP-labeled ($t_{(5)} = 3.5$ $p > 0.05$) or unlabeled ($t_{(6)} = -5.8$, $p > 0.05$). An NMDS plot of the four treatments showed that *V. fischeri* exposure was the strongest driver of

variation between the hemocytes (Fig. 4A) reinforcing previous research on the impact of symbiont exposure on host innate immune cells, although the impact of MNPs on immune cells with and without symbiont exposure was not significant (ANOSIM R: 0.15, $p = 0.17$; PERMANOVA $F_3 = 1.3$, $p > 0.05$). Comparing unexposed and *V. fischeri*-exposed hemocytes, both labeled with MNPs (Fig. 4B), resulted in distinctive groupings of these samples but no significant differences in their metabolome profiles ($t_{(5)} = 3.25$ $p > 0.05$; ANOSIM R = -0.111, $p = 0.8$).

Despite the lack of larger trends, there were some separation patterns observed in the Bray-Curtis dissimilarity heatmap (Fig. 4C). As in the NMDS plot, results suggested that *V. fischeri*-exposed hemocytes clustered closer to each other regardless of whether the cells were labeled with MNPs, suggesting minimal impact of the nanoparticles on the hemocytes or on the interaction. Further exploration of the metabolomic profiles indicated that 34% of the features were shared by all treatment groups (Fig. 4D inset) and that more of the features differed in response to treatment with *V. fischeri* compared to MNP treatments (Fig. 4D).

Together, these results suggest that the hemocytes were not negatively impacted by the presence of the symbiont nor were the MNPs expelled due to the presence of *V. fischeri*. In other symbiotic associations, such as in cnidarians and sponges, often the phagocytosis of non-symbiotic microbes or inert particles, such as microplastics or latex beads is indiscriminate and can impact host morphology and function [36-38]. In many

invertebrates, non-symbiotic phagocytosed particles are quickly expelled through vomocytosis as symbiosis-competent strains can trigger the suppression of host immune responses [37]. The results of this study, however, suggest that MNP uptake was stable even in the presence of symbionts and did not have a significant impact on host metabolome.

MNPs can be visualized within host adult symbiotic organs using magnetic particle imaging.

To explore whether the MNPs could be visualized *in vivo*, three adult female squid were injected with either MNP-labeled hemocytes or free MNPs into the cephalic artery under anesthesia. The MNPs were allowed to circulate through the blood stream of the host animal for 2 h to evaluate the trafficking pattern. Upon dissection, the results indicated that the injected MNPs dispersed extensively through the squid body with visible accumulation and clustering of the brown MNPs in the heart, gills, and brain (Fig. 5A), but that the patterns of accumulation were not evenly distributed throughout the body.

Although MNP distribution could be visibly observed in dissected animals, we also explored the visualization of these particles *in vivo* in live, anesthetized adults using magnetic particle imaging (MPI). MPI is a non-invasive, imaging technique in which animals can be screened to assess the spatial patterns of MNPs within the host [39]. Results showed that concentrated MNP-labeled hemocytes gave strong signals under the MP

imager, which was replicated in three animals (Fig. 5B). After injection, the anesthetized animals were then loaded into a custom 3D-printed tray to minimize movement and imaged using the CT option of an IVIS Lumina system to provide a companion orientation image (Fig. 5C, D).

Direct injection of MNP-labeled hemocytes, however, did not enable a strong enough signal in the symbiotic organs despite the sensitivity of the MP imager. However, when concentrated MNP particles were directly injected into the cephalic artery, after 2 h there was a pronounced signal of MNPs within both accessory nidamental gland and light organ (Fig. 5E). To ensure that the MNPs were concentrating to the light organ and not the hind gut, the animals were euthanized, and the light organs were dissected out and re-imaged with MPI. A pronounced signal was observed using the MPI suggesting successful trafficking of the MNPs to the light organs (Fig. 5F), where the window/leveling of these images was set to where the maximum is the maximum voxel value in the image and the minimum was set to half of this maximum (i.e., 0.5*Max).

Although the MPI efforts were effective in tracking the MNPs, there were limitations with the spatial resolution and the mode of delivery of the nanoparticles to the host. For example, the gill on the right side of the body and the left brachial heart had higher levels of MNPs within the tissues compared to the other side (Fig. 5A) suggesting that injection in the cephalic artery might not be enough to effectively administer the labeled cells in aquatic animals. An alternative solution to more precisely distribute

MNP-labeled immune cells or molecules to targeted locations in the animals is to use an external magnetic field to assist in the delivery of the MNP-labeled products. Previous work has shown that MNPs can be captured and guided by strong magnetic field gradients [40,41], which has been shown to increase the concentration and magnetically guide MNPs to key locations within the host. Future efforts to manipulate the MNP-labeled hemocytes or other functionalized MNPs to better understand variation within the circulatory system and how hydrodynamic conditions may change within different organs can provide an avenue to improve delivery and retention of MNP-labeled targets.

Taken together, MNPs have become a versatile tool for a range of applications in medicine, environmental remediation, and industry [42]. In this study, we evaluated whether MNPs could effectively probe the symbiosis between the bobtail squid, *E. scolopes*, and its symbiotic bacterium, *V. fischeri*. Our results show that host innate immune cells can be reliably labeled with iron oxide MNPs without significantly altering their biochemical profiles or without negatively impacting their interactions with beneficial symbionts. The labeling of host cells, such as immune cells, has wide applications in other symbiotic systems. For example, the method could be applied to label target cells, and then using a magnetic field manipulate the physical movement or spatial arrangement of the MNP-labeled host cells in controlled studies [43,44]. Additionally, the MNPs could be pre-labeled to deliver key molecules to targeted cells or locations. The

approach is now becoming widely used in cancer research to deliver therapeutics to specific cell types [45]. Additionally, the approach could be used to collect extracellular vesicles form both host and bacterial cells. For example, MNPs could be labeled with antibodies or membrane insertion peptides to capture shed extracellular vesicles to understand how production rates and cargo changes under different environmental and symbiotic conditions [46,47]. Although more research is needed to develop specialized MNPs that target molecular features of different symbiotic associations and delivery of these functionalized particles to a wider range of cell types, these findings support the potential of MNPs as a promising tool for investigating aquatic symbiotic associations.

Materials and Methods

MNP characterization and property measurements

Briefly, commercially available polyethylene glycol MNPs that emit fluorescence at 610 nm were used for all experiments (Core Quantum Technologies, Columbus, OH). The particles were imaged using transmission electron microscopy and the hydrodynamic core diameter was measured using dynamic light scattering (DLS). DLS was performed at ambient temperature with backscatter detection using MNPs diluted in molecular-grade water. The hydrodynamic diameter was calculated from the translational diffusion coefficient D obtained from the intensity autocorrelation function via the Stokes–Einstein relation $D_h = kT/(3\pi\eta D)$,

assuming spherical particles and using η and T for the medium viscosity and temperature. The total Fe within the MNPs was quantified by the 1,10-0-phenanthroline colorimetric assay as previously described [48]. The Fe concentration was determined from a standard curve of Fe^{2+} standards at 510 nm.

Animal husbandry, hemocyte isolation, and symbiont exposures

The cephalopod procedures described in the paper were approved by the University of Florida (Protocol 201910899) and University of Connecticut (Protocol A25-004) Institutional Animal Care and Use Committees. Adult squid were collected from O'ahu, Hawai'i, and transferred to either the University of Florida or the University of Connecticut, where they were housed in aquaria within an environmentally controlled room that was maintained at 23°C under a diel light cycle. Prior to all experimental procedures, adult animals were anesthetized with 2% ethanol in filtered seawater as previously described [49]. Animals that were euthanized were overanesthetized in 2% ethanol, which is the current practice for euthanasia in cephalopods [50].

Hemocytes were isolated from the adult squid using previously optimized protocols [51,52].

Briefly, hemocytes were extracted from the cephalic artery of anesthetized adult bobtail squid and placed in a microfuge tube. Cells were pelleted by centrifuging for 15 min at 2000 RCF at 4□C and then were ready for

downstream applications. For those hemocytes exposed to symbiotic *V. fischeri*, purified hemocytes were incubated with 1×10^5 cells of *V. fischeri* ES114 per ml of filtered seawater for 2 h before the addition of MNPs. Hemocytes were co-incubated with *V. fischeri* and MNPs for 24 h, and then the mixture was resuspended using a pipette. Labeled hemocytes were then purified away from unlabeled cells using a magnetic separator. To remove unincorporated MNPs and bacteria, the suspension was centrifuged at 1000 RCF for 15 min to pellet the hemocytes. The labeled cells were then rinsed three times in Squid Ringer's solution. *V. fischeri* concentrations were determined spectrophotometrically (A_{600nm})[53].

Labeling and imaging of hemocytes with magnetic nanoparticles

A detailed protocol of the magnetic particle labeling technique is available on protocols.io [54]. MNPs were resuspended using a pipette to separate the particles and diluted in Squid Ringer's solution for hemocyte labeling [54].

Hemocytes from five adult *E. scolopes* were collected and individually examined. From each animal, collected hemocytes were split into four equal volumes that were then used for the downstream imaging, proteomic and/or metabolomic analyses including: untreated hemocyte controls, hemocytes exposed to MNPs, hemocytes exposed to only *V. fischeri* cells, and hemocytes exposed to both MNPs and *V. fischeri*. After exposures, the hemocytes were then concentrated by centrifugation at 1000 x g for 15 min, supernatants were removed, and the cells were frozen at -80□C until

processing. Isolated hemocytes were then incubated with the resuspended MNPs so that the final concentrations were either 20 or 100 µg per mL of Squid Ringer's solution. Hemocytes were incubated with the MNPs for 24 h in the dark at room temperature and then the mixture was resuspended using a pipette. A magnetic separator was used for 15 min to remove unlabeled hemocytes from the suspension as described in protocol.io [54]. The remaining suspension of labeled hemocytes was centrifuged at 1000 RCF for 15 min to pellet the hemocytes, leaving any residual unincorporated MNPs in suspension and rinsed in Squid Ringer's solution. The rinsing and centrifugation process was repeated three times prior to downstream analysis. Viability of the hemocytes with and without MNP labelling was assessed with a LIVE/DEAD Cell Imaging kit according to manufacturer's instructions (Thermo Fisher, Waltham, MA). Imaging of the labeled hemocytes was performed on a Nikon A1R confocal microscopy located in the Microgravity Simulation Support Facility at the Kennedy Space Center.

Untargeted proteomic analysis of hemocytes labeled with and without magnetic nanoparticles

For untargeted proteomic analysis, subsets of pelleted cells from two hemocyte treatment groups (n = 5 biological replicates each) (i.e., untreated hemocyte controls and hemocytes exposed to MNPs) were extracted by adding 1X lysis (5% SDS, 50 mM triethylammonium using a BCA Assay. Proteomic analysis was conducted at the Proteomics and

Metabolomics Facility at the University of Connecticut. The ten samples were suspended in 2 mM $MgCl_2$ and then treated with benzonase for 15 min on ice. Next, samples were dried, resuspended in 10% SDS, and processed with S-Trap columns (ProtiFi, LLC, Fairport, NY) before incubating for 1 h at 25°C with dithiothreitol, alkylated in the dark with iodoacetamide for 30 min at 25°C, acidified with phosphoric acid, and finally diluted at 1 1:6 ratio with binding/wash buffer containing 90% methanol and 100 mM TEAB. Samples were loaded onto S-trap micro columns, washed 4x with binding/wash buffer, and digested at 25°C overnight with trypsin/LysC at 1:10 enzyme:protein (w/w, Promega #V5073). Elution of peptides was carried out using a series of washes: first with 50 mM ammonium bicarbonate, followed by 0.2% formic acid in water, and finally with 0.2% formic acid in 50% acetonitrile. The resulting peptides were thoroughly dried and desalted by reverse-phase chromatography with Pierce peptide desalting spin columns (Thermo Fisher, Waltham, MA). After another drying step, peptides were reconstituted in Solvent A containing 0.1% formic acid in Optima LC/MS grade water (Fisher Chemical, Pittsburgh, PA). The peptide concentrations in each sample were determined spectrophotometrically (A_{280} nm), and sample injection volumes were adjusted to ensure equal amounts were analyzed.

Analysis was performed using a Thermo Scientific Ultimate 3000 RSLCnano UPLC system in tandem with a high-resolution Thermo Scientific Eclipse Tribrid Orbitrap mass spectrometer. Individual samples were

introduced to a nanoEase M/Z Peptide BEH C18 column (Waters Corporation, Milford MA) and separated over 150 min under reversed-phase conditions with a 4 - 30% gradient of Solvent B containing 0.1% formic acid in Optima LC/MS grade acetonitrile followed by an increase to 30 - 90% Solvent B during a subsequent 30-min ramp, totaling 180 min of gradient separation using a flow rate of 300 nL per min.

Peptide elution proceeded by nanoflow electrospray ionization in the positive mode at a capillary voltage of 2200 V, delivering samples to the Eclipse. MS1 spectra were recorded at 120,000 resolution, using an AGC target of 4e5, RF lens at 30%, maximum ion time set to Auto, and a scan range of 300-1800 m/z. MS2 data-dependent acquisition operated at a 15,000 resolution in the Orbitrap for precursor ions exceeding a 5.0e4 intensity threshold; AGC target was Standard, mass range and ion time were set to Auto, and a 1.6 m/z isolation window was applied. Each cycle lasted 3 sec. Peptides were fragmented by HCD with 30% energy, and dynamic exclusion was enabled for 30 seconds following a single detection, targeting ions with charge states 2-8.

Peptide identification was conducted using MaxQuant software (v 2.0.2.0) using the integrated Andromeda search engine, and quantification utilized a label-free approach [55]. The raw mass spectrometry data were compared against both the *E. scolopes* genome [56] and the MaxQuant contaminants database. During analysis, peptides were required to have at least five amino acids and a maximum mass of 4600 Da. The search allowed

up to five possible variable modifications per peptide, including N-terminal protein acetylation, asparagine or glutamine deamidation, methionine oxidation; the carbamidomethylation of cysteine was identified as a fixed modification. Tryptic digestion was defined with trypsin/P specificity, permitting up to two missed cleavages. A 1% false discovery rate for both peptides and proteins was used as a filter for the results using the target-decoy method, whereas other settings remained as defaults. The MaxQuant results were then imported into the program Scaffold (Proteome Software, Inc.) for visualization and further analysis.

Mass spectrometry data acquisition and analysis for metabolomics

For metabolomic analyses, hemocytes from five individual animals were examined for the data shown in Fig. 3, with four additional animals used for the experiments shown in Fig. 4. Collected hemocytes from each animal were divided into either two treatments (i.e., □ MNPs for Fig. 3) or four treatments (i.e., □ MNPs, □ *V. fischeri* for Fig. 4) and metabolites were extracted with 50% methanol in LC-MS grade water (Fisher Scientific, Waltham, MA). Specifically, 50% methanol (50 µl) was added to each sample and sonicated for 30 sec. Samples were then centrifuged at 13,000 x g for 5 min at room temperature to remove cellular debris. Supernatant was removed and transferred to an LC-MS vial. Extraction was repeated two more times, without sonication and then immediately injected for mass spectrometry data acquisition.

To acquire the data, a Waters Synapt G2-Si Q-ToF mass spectrometer (Waters Corporation, Milford, MA) coupled with an Acquity UPLC system was used. For chromatographic separation, a HSS T3 column (i.e., 2.1 × 150 mm, 1.8 µm) with an Acquity VanGuard precolumn (i.e., 2.1 x 5 mm, 1.8 µm). The mobile phase consisted of water with 0.1% formic acid (Solvent A), and acetonitrile with 0.1% formic acid (Solvent B). Flow rate was set at 0.45 mL per min. The gradient separation was as follows: 0.5 min hold at 95% Solvent A and 5% Solvent B, a 3.5 min ramp to 40% Solvent B, with a 4 min ramp to 100% Solvent B, 1 min hold at 100% Solvent B, 0.2 min ramp back to 5% Solvent B and a re-equilibration hold at 5% of Solvent B for 1.8 min.

Electrospray ionization was performed in positive mode and data were acquired using MS^E with 0.1 sec MS1 survey scans from 50-2000 m/z in resolution mode, followed by 0.1 sec high energy MS2 scan. Ionization and acquisition parameters included an 800 L per h desolvation gas flow, 100°C temperature, 2 kV capillary voltage, 20 V sampling cone, 80 V source offset, and 30 V MS2 collision energy. Mass correction was conducted in real-time and used a lockspray of 400 pg per µL leucine enkephalin solution containing an equal ratio of methanol to water solution containing 0.1% formic acid. The injection volume (10 µl) was used in a closed-loop configuration.

Following the acquisition, data were processed using Progenesis Qi™ for peak picking and alignment. The parameters for peak picking included fragment sensitivity of 0.2% of the base peak and adducts (i.e., M+H,

M+2H, 2M+2H, 3M+3H, and M+Na). Any feature whose highest abundance occurred in the methanol blanks or that had an ANOVA p-value higher than 0.005 (i.e., no difference in variance among groups) was removed from analysis. Data were transformed using root square transformation and Pareto scaling. Feature annotation was performed in Progenesis using MS-DIAL and NIST mass spectral databases [57,58].

Metabolomics data visualization and statistical analysis

Multivariate analysis was performed on the transformed feature table using the R packages vegan (v2.7-1), and stats (v4.6.0) [59,60]. Volcano plots were built using multiple two tailed t-tests, and q-values were calculated using a false discovery rate (FDR) correction with the Benjamini-Hochberg procedure [61]. Similarly, two tailed t-tests were performed on the most abundant metabolites, as measured by area under the curve (AUC), found within hemocytes from the control group and those exposed to MNPs. Bray-Curtis dissimilarity distance matrices were used to conduct non-metric multidimensional scaling (NMDS) discriminant analyses, with statistical comparison via analysis of similarity (ANOSIM) and two-way t-tests.

For metabolomics comparisons of hemocytes exposed to MNPs with or without *V. fischeri,* a Bray-Curtis dissimilarity distance matrix was calculated and used to build a heatmap using the Ward clustering algorithm [62]. The same Bray-Curtis dissimilarity distance matrix was subsequently used for NMDS analysis to compare all four experimental groups.

Comparisons of metabolite presence or absence amongst the four experimental groups used the eulerR package to generate Euler diagrams [63] and the UpSetR package to generate upset plots [64].

Magnetic particle imaging of whole animals

To visualize the magnetic nanoparticle distribution within the host animals *in vivo*, three anesthetized adult squid were injected with 100 µg per mL of MNPs into the cephalic artery and incubated for 2 h. The anesthetized animals were then individually loaded into a 50 mL tube containing filtered sea water, placed in a customized holder and then imaged using an IVIS Lumina system with auto exposure and medium binning to acquire overall images of the adult animal. After which, the animal holder containing the squid was then imaged with a MOMENTUM field free line (FFL) imager (Magnetic Insight, Inc, Alameda, CA) at the University of Florida, as previously described using v2022.01.1 of the software [65]. Briefly, two-dimensional projections with High Sensitivity/High resolution settings (5.7T/m, 16mT for X and Z excitation strengths) in isotropic mode were generated taking ~2 min to acquire images within a 12 x 6 cm field of view. Optical images were registered to the MPI images by using three fiducial markers to construct a registration matrix that was applied to all images.

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Author Contributions

H.G. completed the magnetic particle characterization. E.K. completed the hemocyte labeling optimization and imaging. R.S. completed the initial hemocyte extraction and acquisition of mass spectrometry data. N.V. and S.N. completed the proteomics analyses. D.G.M. and M.B. completed the metabolomics analyses. H.G., C.R., S.N. and J.F. completed the magnetic particle imaging. D.A., C.R., S.N., M.B. and J.F. conceived of the experimental plan and design. All authors contributed to the writing and editing of the manuscript.

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Competing Interest Statement

The authors declare no competing interests.

Data Availability Statement

All data generated as part of this study is available within this manuscript, including figures, and supplemental materials. The mass spectrometry proteomics data have been deposited in the EMBL-EBI PRoteomics IDEntifications Database (PRIDE) under the dataset identifiers PXD074352 and 10.6019/PXD074352. Spectra are available in the MassIVE data repository MSV000098647.

Additional Information

Supplementary Information: The online version contains supplementary materials.

ARRIVE Statement

All animal experiments were performed in accordance with ARRIVE guidelines (Animal Research: Reporting of *In Vivo* Experiments). For example, all cephalopod procedures were approved by both the University of Florida (Protocol 201910899) and the University of Connecticut (Protocol A25-004) Institutional Animal Care and Use Committees and were performed in accordance with the approved protocols and guidelines.

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Figure Legends

Figure 1. Overview of magnetic nanoparticle methodology for labeling host innate immune cells typically associated with the symbiotic organs within the bobtail squid *Euprymna scolopes*. (**A**) Ventral dissection of adult squid revealing the two symbiotic organs found within *E. scolopes* including the light organ (LO) and the accessory nidamental gland (ANG), which is found only in females. (**B - C**) Micrographs of ciliated fields of epithelial cells in juvenile animals used to recruit symbiotic microbes from the environment including the light organ (**B**) and accessory nidamental gland [66] (**C**). (**D – E**) Transmission electron micrographs of innate immune cells trafficking to the host symbiotic organs in response to colonization including the light organ (**D**) and accessory nidamental gland (**E**). (**F**) Magnetic nanoparticles (MNP) with a fluorescent nanocrystal core conjugated to a polyethylene glycol (PEG) were used to label hemocytes. (**G**) Magnets were used to purify away unlabeled cells. (**H**) Multi-omics techniques were used to assess the proteomic and metabolomic profiles of the labeled and unlabeled cells.

Figure 2. Host hemocytes labeled with magnetic nanoparticles and imaged with confocal microscopy. **(A)** Isolated hemocytes incubated for 24 h in Squid Ringer's solution and counter stained with nucleic (blue) and actin (green) labeling depict the wide range of morphologies exhibited by the host innate immune cells. **(B)** Higher magnification image of negative

control hemocyte with no magnetic nanoparticle (MNP) exposure. **(C)** Hemocytes exposed to 20 µg of MNPs per mL of Squid Ringer's exhibited pronounced uptake of the MNPs (purple). **(D)** Higher magnification of hemocyte exposed to MNPs (20 µg per mL of Squid Ringer's solution) showing the localization pattern of the MNP within the hemocyte cytoplasm. **(E)** Hemocytes exposed to higher concentrations of MNPs (100 µg per mL Squid Ringer's solution) showing increased uptake within the host hemocyte. **(F)** Same image as (**E**) but with the actin staining removed showing the localization of the MNPs to cell cytoplasm.

Figure 3. Uptake of magnetic nanoparticles has no significant effect on the proteomes or metabolomes of *E. scolopes* hemocytes. **(A)** Volcano plot and Venn diagram of shared proteins between hemocytes with and without magnetic nanoparticles (MNPs) after Benjamini-Hochberg correction ($q < 0.0001$). **(B)** Volcano and Venn diagram of shared metabolites between hemocytes with and without MNPs after Benjamini-Hochberg correction ($q < 0.05$). **(C)** Top 15 most abundant proteins identified from unlabeled (light blue) and MNP-labeled (dark blue) hemocytes. Shown are mean normalized average precursor intensities (APIs) of peptide spectra with error bars representing the standard error of the mean. **(D)** Top 15 most abundant compounds putatively annotated by mass spectrometry in unlabeled hemocytes (light blue) compared to MNP-labeled (dark blue) hemocytes. Shown are mean normalized areas under the curve (AUCs) of

peaks along with pairwise t-tests of significance (* $p < 0.05$, ** $p < 0.01$) with error bars representing the standard error of the mean.

Figure 4. Effect of the symbiont *Vibrio fischeri* on host hemocyte metabolomes labeled with MNPs. (**A**) NMDS plot showing no separation of hemocyte samples with or without MNPs, although some separation was observed due to presence of *V. fischeri* (ANOSIM R = 0.1534, p = 0.17; PERMANOVA F3 = 1.3, $p > 0.05$). (**B**) NMDS plot highlighting separation observed between hemocytes labeled with MNPs, with or without *V. fischeri* exposure ($t_{(5)} = 3.25$ $p > 0.05$; ANOSIM R = -0.111, 0.8). (**C**) Bray-Curtis dissimilarity heatmap of the four treatments: unexposed hemocytes, MNP-labeled hemocytes, hemocytes exposed to *V. fischeri* and MNP-labeled hemocytes exposed to *V. fischeri*. D) UpSet and Euler plots of metabolite profiles show similarity across all four treatment groups, although there were a substantial number of metabolites found only in MNP-labeled hemocytes in the presence of *V. fischeri*.

Figure 5. Trafficking of magnetic nanoparticles through circulatory system of host squid. (**A**) Ventral dissection of male adult squid showing prominent symbiotic light organ (lo) injected directly with magnetic nanoparticles (MNPs) in the cephalic artery and incubated for 1 h. Extensive movement of MNPs (brown) through the circulatory system into the heart (h) and gills (g). (**B**) Isolated hemocytes from adult squid labeled with MNPs and imaged

with a magnetic particle imager (MPI). (**C**) Anesthetized adult squid injected with labeled cells and within holder to limit movement for MPI visualization. (**D**) IVIS Lumina scan of adult squid within the MPI holder to show overall morphology of adult squid. (**E**) High resolution MPI scan revealing localization of MNPs within both symbiotic light organ (lo) and accessory nidamental gland (ang). (**F**) Representative light organ dissected from body of animal and imaged with MPI conforming localization of MNPs specifically to the light organ. The color scale is defined relative to the global maximum voxel value of each image, with the maximum color corresponding to this peak value and the minimum corresponds to half that global maximum value, '0.5*max'. Voxels with values below this half-maximum threshold are rendered transparent to enhance visualization. Bar = 1 cm.