Uptake and bioactivity of chitosan/double-stranded RNA polyplex nanoparticles in *Caenorhabditis elegans*

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Abstract

In this study, we have investigated chitosan/dsRNA polyplex nanoparticles as RNAi agents in the nematode *Caenorhabditis elegans.* By measurement of an easily observed phenotype and uptake of fluorescently labeled dsRNA, we demonstrate that chitosan/dsRNA polyplex nanoparticles are considerably more effective at gene knockdown on a whole body concentration basis than naked dsRNA. Further, we show that chitosan/dsRNA polyplex nanoparticles introduce dsRNA into cells via a different mechanism than the canonical *sid-1* and *sid-2* pathway. Clathrin-mediated endocytosis is likely the main uptake mechanism. Finally, although largely reported as non-toxic, we have found that chitosan, as either polyplex nanoparticles or alone, is capable of downregulating expression of myosin. Myosin is a critical component of growth and development in eukaryotes, and we have observed reductions in both growth rate and reproduction in chitosan exposed *C. elegans*. Given the increased potency, non-canonical uptake, and off-target effects we have identified, these findings highlight the need for rigorous safety assessment of nano-RNAi products prior to deployment. Specifically, potential adverse effects of the nanocarrier and components thereof need to be considered.

Introduction

RNA interference (RNAi) is a system by which double-stranded RNA (dsRNA) is used by cellular machinery as a template for the degradation of a corresponding messenger RNA (mRNA) [1]. Although

the potential of RNAi as a pest control agent was recognized in its early days, it has taken nearly twenty years for RNAi-enabled products to reach the market[2]. As a pesticide, an mRNA encoding an essential gene is targeted, leading to mortality in the pest species. Due to the high level of sequence homology required for efficacy, RNAi is generally understood to be considerably less toxic and more specific compared to traditional small molecule pesticides. In fact, the specificity of RNAi pest control is such that a well-designed targeting sequence is likely only to affect very close relatives of the pest species[3], leaving non-target species unharmed. Several delivery methods for RNAi sequences exist, the most common of which is stable integration of a dsRNA coding sequence into the genome of a crop species[4]. In this system, dsRNA is transcribed by the host plant, then ingested by the pest species during feeding. Though effective, this method is not without limitations, as many relevant crop species are not amenable to genetic transformation, and the extreme specificity of RNAi limits control to a single pest for each incorporated sequence. As such, products utilizing *in-vitro* synthesized dsRNA applied as a surface treatment are in development.

Naked dsRNA, though more stable than single-stranded RNA, is still vulnerable to rapid degradation by nucleases in the environment[5]. dsRNA is also poorly assimilated in many highly destructive pest species[6]. It is well established that complexation of dsRNA with nanoscale carriers such as polycations[7], liposomes[8], or solid particles[9] greatly improves RNAi response and environmental stability. dsRNA nanocomposites are far less susceptible to nuclease degradation compared to naked dsRNAs[10], with chitosan derivatives receiving particular attention in this regard[11]. Chitosan (poly β-1,4-D-glucosamine) possesses numerous characteristics which make it an excellent substrate for dsRNA delivery. The chemical and physical properties of chitosan are fairly simple to manipulate, with numerous different degrees of deacetylation and molecular weights being commercially available[12]. Further, being comparatively non-toxic[13] and produced from abundant seafood waste[14] differentiate chitosan from synthetic polycations. When combined at low (<6) pH, the protonated amine groups of

chitosan are electrostatically attracted to the anionic phosphate backbone of nucleic acids to form polyplex nanoparticles (PNs) [15, 16]. Previous studies have demonstrated the efficacy of chitosan/dsRNA PNs for induction of an RNAi response in insects, including species in which RNAi is normally ineffective[17, 18], but little work has focused on the specific mechanism by which chitosan/dsRNA PNs improve RNAi response.

In many species, an ingested dsRNA can trigger an RNAi response systemically to tissues and cells far removed from the initial site of dsRNA entry, via a series of RNA polymerases and transporters[19]. Termed environmental RNAi (eRNAi), this process was first observed in the nematode *Caenorhabditis elegans*[20], where ingested dsRNA is internalized into cells by a series of proteins collectively known as the *sid* (systemic RNA interference defective) genes[21]. *Sid-1*[22] is a multipass transmembrane protein, with an extracellular domain capable of specifically binding dsRNA[23]. It is essential for the efficient import of dsRNA into cells, but is not required for the export of RNAi signals[24]. *Sid-2* localizes to the nematode midgut, and participates in the internalization of environmental dsRNA[25], possibly in concert with *sid-1*[21]. Notably, *C. elegans* with mutations to *sid-2* are still capable of systemic RNAi, provided that dsRNA is directly injected into a founder cell outside the midgut, whereas *sid-1* mutants are completely incapable of systemic RNAi, no matter the site of introduction. Homologs of *sid-1* have been reported in a diverse range of organisms[26, 27], but many of these are non-functional or absent[28] in relevant pest species. Surprisingly, systemic RNAi can still be observed in many *sid-1* analog deficient species. In these cases, receptor mediated endocytosis plays a key role in the uptake of dsRNA from the environment[29], and systemic spread is facilitated by extracellular vesicles[30].

Though the environmental concerns associated with nanomaterials have received much attention over the past decade, little work has been conducted on biologically active nanomaterials that are intended to be deliberately introduced at field scales. In addition to this knowledge gap, studies investigating the potential for off-target and non-target effects associated with pesticidal RNAi tend to

focus on the nucleic acid component, with little attention paid to the potential of any co-delivered agents to alter these effects. In this work, we investigate the potential for off-target effects of chitosan/dsRNA PNs in *C. elegans.* We assessed whether gene knockdown in *C. elegans* using chitosan/dsRNA PNs is as effective as naked dsRNA when using a soaking assay as an analogue for environmental exposure. We also tracked the uptake of naked dsRNA and chitosan/dsRNA PNs using fluorescence microscopy. We investigated the mechanism of uptake of chitosan/dsRNA PNs using mutant strains for the sid-1 and sid-2 genes, both of which are required for naked dsRNA activity, and used chlorpromazine as a pharmacological probe for the role of clathrin-mediated endocytosis, Lastly, we investigated the effects of chitosan and of chitosan/dsRNA PNs on *myo-3,* a myosin isoform that contributes to development and musculature in *C. elegans*, growth, and reproduction.

Materials and Methods

Strains

Caenorhabditis elegans strains (**N2**; **CGC4** [umnTi1 III.]; **HC196** [sid-1(qt9) V]; **HC271** [ccIs4251 I; qtIs3 sid-2(qt42) III; mIs11 IV]; **PD4251** [ccIs4251 I; dpy-20(e1282) IV]) were maintained on K-medium agar plates seeded with OP50 *Escherichia coli* at 20°C, according to established methods[31].

dsRNA and Polyplex Nanoparticle Synthesis

Genomic DNA was isolated from *C. elegans* using phenol-chloroform extraction and ethanol precipitation, according to established methods[32]. Templates for in-vitro transcription were generated by PCR using T7 promoter appended primers[32], and purified using the Qiagen PCR Cleanup Kit (28104, Germantown, MD). A complete list of primers used in this study is included in supplemental materials. dsRNA was synthesized using the Thermo Scientific TranscriptAid T7 High Yield Transcription Kit (K0441, Waltham, MA) according to manufacturer's instructions, and purified using phenol-chloroform and ethanol precipitation[32]. Typical yield from each reaction was ~150 µg of RNA, as confirmed by

measurement of absorbance at 260 nm, using a Varian Cary 50 Bio UV-Vis Spectrophotometer equipped with a Hëllma TrayCell. Chitosan/dsRNA PNs were synthesized using a modification of Zhang's method[17]. A 0.58% w/v solution of low molecular weight chitosan (Polysciences 21161, Warrington, PA) was prepared in 0.2 M acetate buffer at pH 4.5. dsRNA was diluted to 1 µg/µL in 50 mM Na_2SO_4. For each preparation, equal volumes chitosan solution and dsRNA solution were combined in a 1.5 mL microcentrifuge tube. The mixture was placed in a water bath at 55°C for 1 minute, followed by vigorous vortexing for 30 seconds to generate PNs. PN solution was then used for exposures without further purification. We have previously reported detailed characterization of these PNs[33]; average diameter was found to be 15.6 ± 3.5 nm, with a zeta potential of 29 ± 4 mV.

Fluorescent Labeling of dsRNA and Chitosan

GFP dsRNA was labeled with cy3 using the Ambion Silencer siRNA Labeling Kit (AM1632, Foster City, CA) according to the manufacturer's instructions. Fluorescein-5-Isothiocyanate (Thermo Fisher Scientific F143, Waltham, MA) was dissolved in methanol at 1 mg/mL. A 1% solution of low molecular weight chitosan in 0.2 M acetate buffer was prepared as above. Equal volumes of FITC/Methanol and chitosan solution were combined and vigorously stirred for 3 hours in the dark at room temperature. Following completion of the reaction, chitosan was precipitated by the dropwise addition of 0.2 M NaOH to a final pH of 7. The resulting solution was centrifuged at 12,000 x g, and the supernatant was discarded. Labeled chitosan was subsequently washed five times with 18 MΩ sterile distilled H_2O and centrifugation, to remove residual FTIC. The resulting chitosan was air dried overnight in a desiccator, and gently pulverized with a mortar and pestle. The labeled chitosan powder was then dissolved in 0.2 M acetate buffer to a final concentration of 0.58% w/v and used as above for the synthesis of chitosan/dsRNA PNs.

***Caenorhabditis elegans* dsRNA and polyplex nanoparticle exposures**

In a soaking RNAi assay, *C. elegans* are suspended in liquid medium with a given concentration of dsRNA, and then scored based upon observed phenotype[34]. Although developed for ease of use in the laboratory, this assay is also roughly analogous to a feeding assay, as dsRNA internalized by this method exclusively enters organisms via ingestion. Using a soaking assay, we were able to compare the efficacy of naked dsRNA and chitosan/dsRNA PNs at inducing an RNAi response. We targeted a stable transgene, green fluorescent protein (GFP). In this case, the measured phenotype is a reduction in the fluorescence intensity of exposed nematodes. In order to determine if *C. elegans* assimilates naked dsRNA and chitosan/dsRNA PNs by the same mechanism, we used *sid-1* and *sid-2* null mutant strains in soaking assays targeting a stable and non-essential gene, *pmp-3*. We note that the *sid-2* null mutant strain contains a GFP construct; however, this was the only null mutant available from the CGC.

C. elegans were age synchronized according established protocols[31] and incubated for 24 hours at 20°C on 10 cm K-agar plates seeded with *E. coli* strain OP50. To maximize the observable phenotype, L1/L2 stage nematodes were used in this assay[34]. At 24 hours post age synchronization, nematodes will be at late L1/early L2 stage[35].L1 stage nematodes were then gently washed from plates with K-medium (51.3 mM NaCl, 31.6 mM KCl) into 15 mL polypropylene centrifuge tubes and centrifuged at 130 x g. The supernatant was drawn off and replaced with fresh K-medium, followed by centrifugation and removal of supernatant, leaving a gently compacted nematode pellet. Exposure solutions were prepared by diluting dsRNA or chitosan/dsRNA PNs in K-medium. Exposures were conducted in PCR tubes, with 2 µL compact nematode pellet (~50 worms) in 18 µL exposure solution at the final concentration indicated, with incubation at 20°C for 24 hours. This methodology remained consistent among experiments, with changes in the concentration of dsRNA and chitosan/dsRNA PNs detailed following. Exposures for imaging were conducted at 100 ng/µL dsRNA as naked dsRNA or chitosan/dsRNA PNs. Exposures for GFP knockdown were conducted at 0, 5, 40, 100, and 400 ng/µL dsRNA as naked dsRNA or chitosan/dsRNA PNs. All exposures for qRT-PCR were conducted at 0 or 100 ng/µL dsRNA, as either

naked dsRNA or chitosan/dsRNA PNs. Chlorpromazine (Sigma-Aldrich C8138, St. Louis, MO) was prepared at 350 µg/mL in K-medium as a stock solution, and used at a final concentration of 35 µg/mL in exposures[36].

Fluorescence Imaging

Strain CGC4 has a stable transgenic array incorporated at a known location in the *C. elegans* genome, ensuring that no protein coding genes are disrupted, and the transgene will not suffer from any effects associated with multiple copy insertion[37]. This array is composed of GFP driven by a translation elongation promoter, giving recipient organisms ubiquitous, constitutive expression of GFP. After 24 hours of exposure, CGC4 nematodes treated with GFP dsRNA and chitosan/dsRNA PNs were imaged for fluorescence. Exposure solution and nematodes (8 µL) were gently placed on a glass microscope slide. Two microliters of 1M sodium azide (Sigma-Aldrich S2002, St. Louis, MO) was added to each drop of solution to anesthetize the nematodes, which were then secured with a coverslip. Imaging was performed using a Nikon Eclipse 90i microscope equipped with Nikon Intensilight C-HGFI Epifluorescence Illuminator, Nikon cy3 and GFP filter cubes, and a Nikon DS-Qi1Mc camera (Tokyo, Japan). Multichannel images of individual nematodes were taken at 10x magnification, consisting of DIC images (autoexposure) and GFP images (5s exposure). Using the image analysis software Fiji[38], Regions of interest were drawn around individual nematodes using the DIC image, background was subtracted using the 'rolling ball' method with a radius of 50 pixels, and mean fluorescence from the GFP channel was determined as mean pixel intensity. Length measurements were obtained by drawing and measuring a segmented line on individual nematodes, from the tip of the tail, along the midline, to the opening of the pharynx.

To determine the uptake and localization of dsRNA and chitosan/dsRNA PNs within exposed *C. elegans*, we labeled chitosan and dsRNA with separate fluorophores, FITC and Cy3 respectively.

Nematodes were then exposed to naked labeled dsRNA, chitosan/dsRNA PNs made with labeled chitosan, dual labeled (dsRNA and chitosan) PNs, and labeled chitosan without dsRNA. For dsRNA uptake measurements, the above method was utilized, but at 20x magnification and with the cy3 channel serving as the source of the mean pixel intensity (1s exposure). Exposures were conducted as above, with treatments of FITC-chitosan alone, FITC-Chitosan/dsRNA PNs, cy3-dsRNA alone, and dual labeled FITC-chitosan/cy3-dsRNA dual labeled particles. Fluorescently labeled chitosan/dsRNA PN exposed nematodes were imaged at 20x magnification for cy3 (2s exposure), GFP (2s exposure), and DIC (autoexposure) images. dsRNA uptake was measured using the cy3 channel alone from cy3-dsRNA and cy3-dsRNA/chitosan PN exposed nematodes. As above, the background was subtracted from each image, and a region of interest was drawn around each nematode. Mean pixel intensity was then measured in each region of interest.

Images of dual labeled chitosan/dsRNA PN solution was performed as above, at 20X magnification with autoexposure for each channel. Background from each channel was subtracted, and colocalization analysis was performed on the whole image using the coloc2 Fiji plugin.

Reproduction Assay

Wild type N2 *C. elegans* were age synchronized as previously described[31]. Eggs were hatched on 10cm OP50 seeded K-agar plates, and allowed to mature for 24 hours. L1/L2 stage nematodes were subsequently rinsed from plates with K-medium, followed by centrifugation at 130xg. The supernatant was drawn off and replaced with fresh K-medium. This process was repeated 2X to ensure removal of OP50. Worms were then placed in K-medium supplemented with the indicated concentration of chitosan, as described previously. Exposures were conducted for 24 hours in the absence of OP50, due to the known bactericidal and bacteriostatic effects of chitosan[39], as this would be a confounding variable in the assay. Following exposures, 4-6 worms per treatment group were placed on 10 cm OP50

seeded K-agar plates, with three replicates per treatment group. Upon the onset of egg-laying (8-12 hours after exposure), adults from each replicate were transferred to fresh OP50 seeded K-agar plates daily, for three days. Following removal of adults, plates with young worms were stained with 1.5 mL 0.5 g/L Rose Bengal (Acros Organics 189450250, Morris Plains, NJ) and heated to 55°C for 30 minutes. Plates were then scored for total number of hatched young worms, using a LeicaS6D dissection microscope.

Quantitative Real Time PCR (qRT-PCR)

Following exposure to dsRNA and chitosan/dsRNA PNs as above, treated nematodes were washed in 5 mL K-medium, and centrifuged at 130 xg. Supernatant was discarded, and remaining nematode pellet was suspended in 850 µL TRIzol (Thermo Fisher Scientific 15596026, Waltham, MA). Solutions were flash frozen in LN_2 and thawed in a 37°C water bath five times to fully lyse all cells. After final thaw, 50 µL chloroform was added to each lysate. The aqueous layer was removed, and 2 µL glycogen (Thermo Fischer Scientific R0551, Waltham, MA) was added to each sample. Ethanol precipitation was then carried out according to established protocol[32]. The resulting whole RNA was resuspended in sdH_2O and quantitated using a Cary 50 Bio UV-Vis Spectrophotometer. cDNA was synthesized using a RevertAid First Strand cDNA synthesis kit (Thermo Fischer Scientific K1622, Waltham, MA), according to the manufacturer's instructions. qRT-PCR was performed using an Applied Biosystems StepOnePlus thermocycler, TaqMan Fast Advanced Master Mix (Applied Biosystems 4444557, Foster City, CA) and TaqMan probes, as listed in supplemental material (**Fig. S2**). This assay targets regions of exons which do not overlap with the region homologous to our dsRNA, ensuring that we would not amplify our dsRNA and were only measuring mRNA levels. We subjected our dsRNA to a reverse transcription reaction and assessed for amplification using this assay and did not detect an amplification. Three biological replicates per treatment group were analyzed, with three technical replicates used per biological replicate. *Y45F10D.4* was used as the endogenous control for all

experiments, owing to the stability of this gene under most environmental conditions, including nanomaterial exposure.[40] The C_T values reported were compiled, and relative expression was calculated after normalization to the reference gene according to Pfaffl using Relative Expression Software Tools (REST) software[41], and reported here as the $\log_2$ transform of the expression and standard error.

Statistical Analysis

Statistical analysis was conducted using PROC GLM in SAS 9.4 for the fluorescence and reproduction assays. In each case, Dunnett's test was used to test for differences between treatment and control[42]. For the length assay, comparisons were made between treatments at the same concentration using a Student's T-test.

Results and Discussion

Comparison of naked dsRNA and chitosan/dsRNA PN RNAi efficacy in whole organisms - As expected, a reduction in fluorescence occurred as treatment concentration increased (**Fig. 1**). From 40 and 100 ng/µL onward, the difference in fluorescence intensity was always significantly different from control for PNs and naked dsRNA, respectively. At concentrations of 40 and 400 ng/µL, there was a significant decrease in fluorescence intensity for PNs relative to naked dsRNA at the same concentrations. Curiously, at 5 ng/µL, the PN treatment showed a significant increase in fluorescence. Overall, these results clearly demonstrate that on an exposure concentration basis, chitosan/dsRNA PNs are at least as effective at gene knockdown as naked dsRNA, and likely slightly more effective.

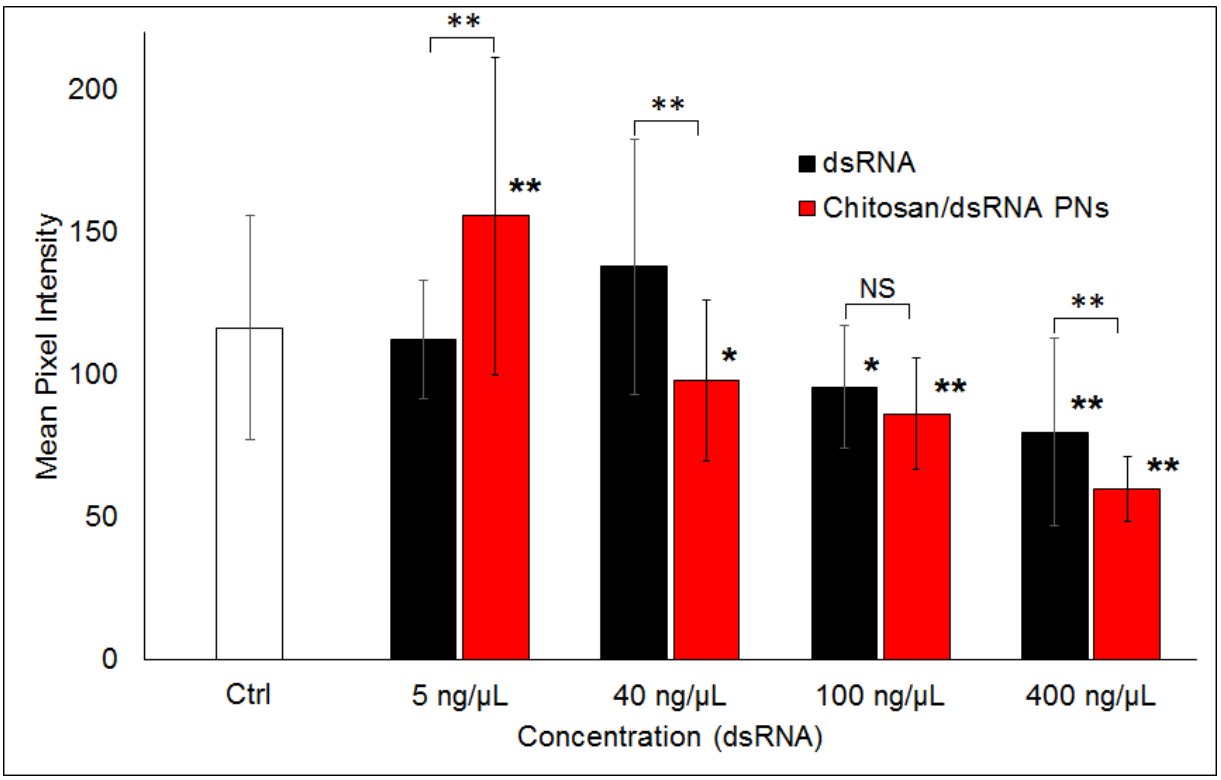


Figure 1 – Mean pixel intensity of CGC4 *Caenorhabditis elegans* exposed to varying concentrations of naked dsRNA and chitosan/dsRNA polyplex nanoparticles (PNs). Data presented is mean ± standard deviation. Asterisks over brackets are comparisons between treatments within concentrations; asterisks over bars are comparisons to control. (NS = $p>0.1$; * = $p<0.1$; ** = $p<0.05$; *** = $p<0.01$; n=20)

Uptake and localization of naked dsRNA and chitosan/dsRNA PNs –

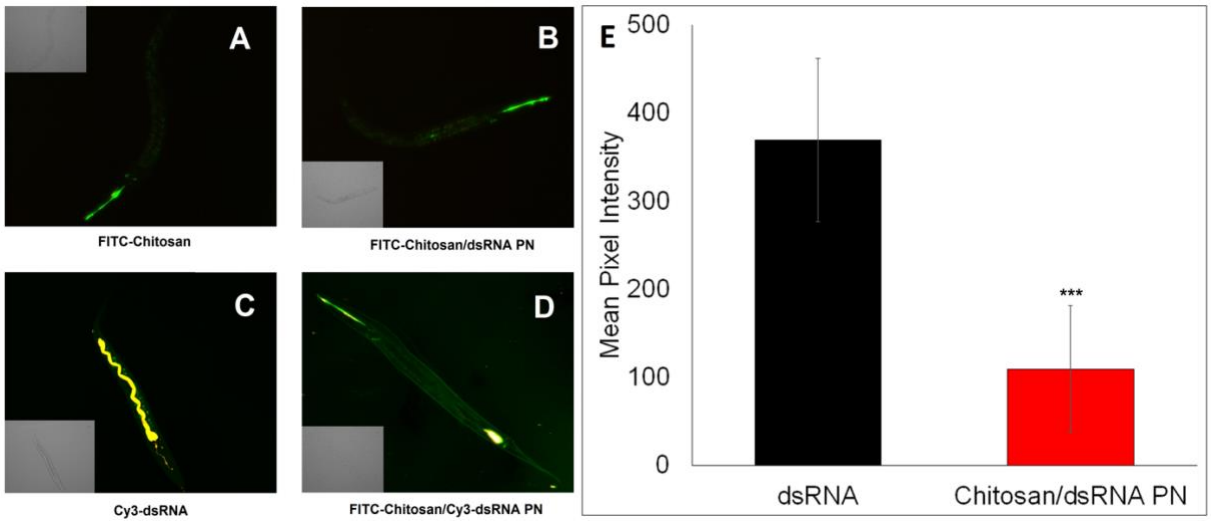


Figure 2 – Localization of fluorescently labeled PNs and components within *Caenorhabditis elegans.* All exposures were conducted with equivalent concentrations of constituent components. Brightfield image shown in panel insets. A – FITC-Chitosan alone; B – FITC-Chitosan/dsRNA PNs; C – Cy3/dsRNA; D – FITC-Chitosan/Cy3-dsRNA PNs; E - Mean pixel intensity of *Caenorhabditis elegans* exposed to cy3 tagged dsRNA as either naked dsRNA or chitosan/cy3-dsRNA polyplex nanoparticles for 24 hours. Data presented is mean ± standard deviation (*** = $p<0.01$; n=15).

Uptake of chitosan alone had similar localization to that of chitosan as PNs (**Fig. 2A and 2B**). The majority of the ingested materials collects in the pharynx and posterior intestine, just above the hindgut. Given that the pH of the *C. elegans* gut is entirely acidic[43], chitosan/dsRNA PNs should remain stable prior to cellular entry. Chitosan and chitosan/dsRNA PNs show a strong affinity for the cuticle, with a slight accumulation of materials seen on the surface of individual nematodes (**Fig. 2A, 2B, 2D**). However, *C. elegans* internalized far less dsRNA as chitosan/dsRNA PNs than as naked dsRNA (**Fig. 2C,**

2D, 2E). When exposed to naked labeled dsRNA, intense fluorescence was seen throughout the intestine, with near equal distribution throughout (**Fig. 2C**). Dual labeled chitosan/dsRNA PNs distributed in a similar fashion to that of labeled chitosan alone, with significant co-localization of chitosan and labeled dsRNA (**Fig. 2D, Table S5**). Small amounts of dsRNA coated the intestine as in naked dsRNA exposures, but with markedly less intensity.

Concurrently, images of dual labeled PN solution were captured for colocalization analysis. Images of labeled dsRNA solution were uniform and homogenous, with no particular accumulations of dsRNA outside nematodes. Images of labeled chitosan/dsRNA PNs had a distinctly different appearance, with mixed size particulates being dominant. Dual labeled particle solutions were significantly colocalized (**Table S5**).

Efficacy of chitosan/dsRNA PNs in RNAi deficient mutants - When soaked in *pmp-3* dsRNA (100 ng/µL), the *sid-2* mutant showed no significant difference in *pmp-3* transcripts compared to control (**Fig. 3**). However, when soaked in an equivalent concentration of dsRNA as chitosan/dsRNA PNs (100 ng/µL), a drastic reduction in *pmp-3* was detected. Since *sid-2* is specific for dsRNA uptake in the intestine, the sid-2 null mutant is not responsive to eRNAi, but still possesses systemic RNAi when dsRNA is introduced via injection. Although this result indicates that chitosan/dsRNA PNs are capable of entry into gut cells, it is quite possible that further uptake and spreading of the RNAi signal was facilitated by other endogenous systems, including *sid-1*. Thus, we conducted a *pmp-3* soaking assay with a *sid-1* null mutant. As expected, exposure to naked dsRNA (100 ng/µL) caused no significant change in *pmp-3* transcript levels compared to control (**Fig. 3**). However, significant reductions in *pmp-3* transcripts were seen when chitosan/dsRNA PNs (100 ng/µL) were used (**Fig. 3**). Evidence of a robust RNAi response when using chitosan/dsRNA PNs in both *sid* mutants strongly indicates that PNs are internalized into cells with intact dsRNA payloads, using a mechanism independent of the traditionally understood means of naked dsRNA uptake.

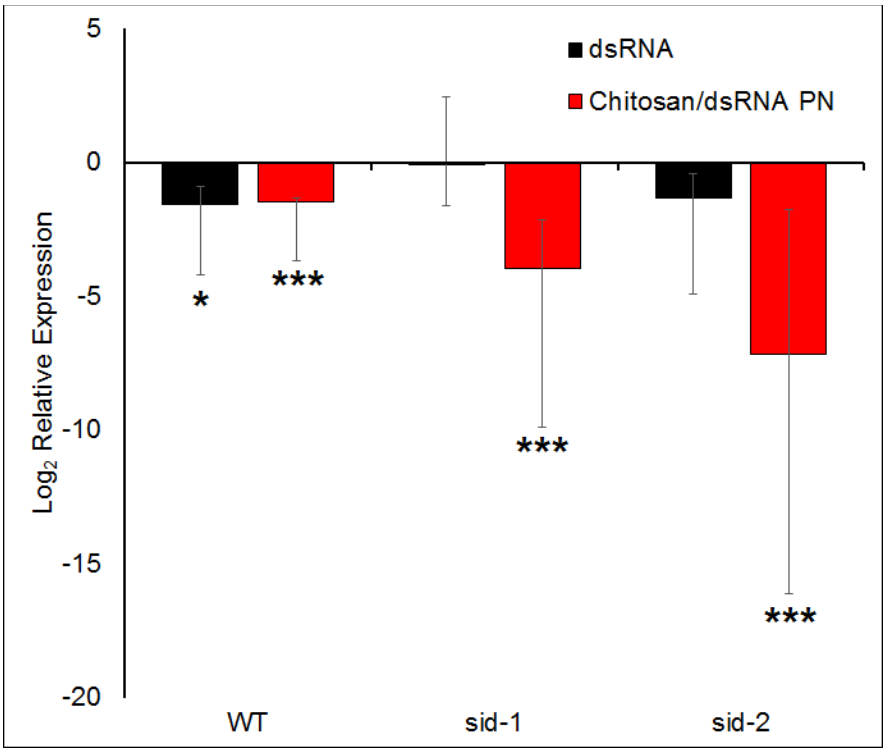


Figure 3 - Fold-change in *pmp-3* expression compared to control for wild-type and *sid* null mutant *Caenorhabditis elegans* under dsRNA and chitosan/dsRNA polyplex nanoparticle treatment. Error bars represent standard error as reported by REST. (NS = p>0.1; * = p<0.1; ** = p<0.05; *** = p<0.01; n=3 for WT and *sid-2* exposures, n=5 for *sid-1* exposures)

Role of clathrin-mediated endocytosis in chitosan/dsRNA PN uptake – Clathrin-mediated endocytosis is a major means by which eukaryotes selectively import extracellular materials. Since we were able to rule out *sid* mediated import of dsRNA delivered by chitosan/dsRNA PNs, clathrin-mediated endocytosis emerged as a possible mechanism of uptake. To test this hypothesis, we conducted soaking assays as previous, but with chlorpromazine added to the exposure medium.

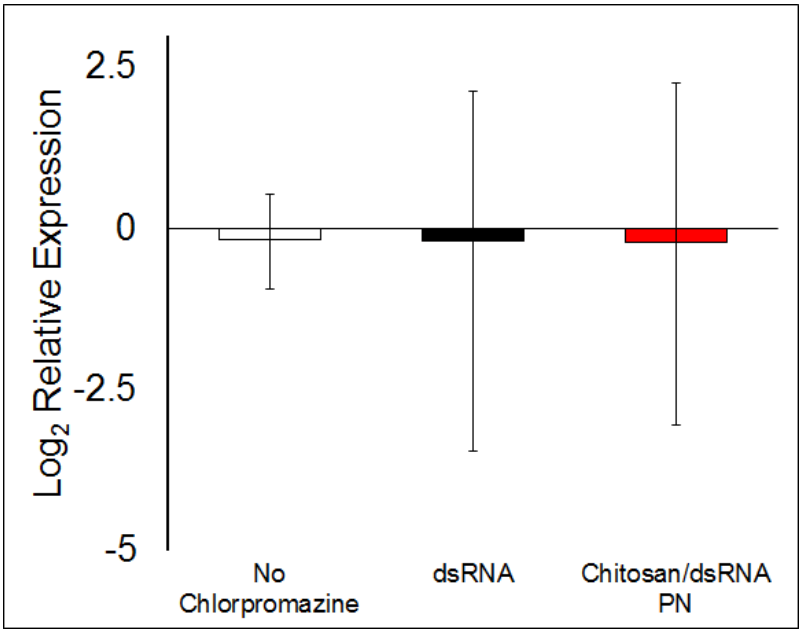
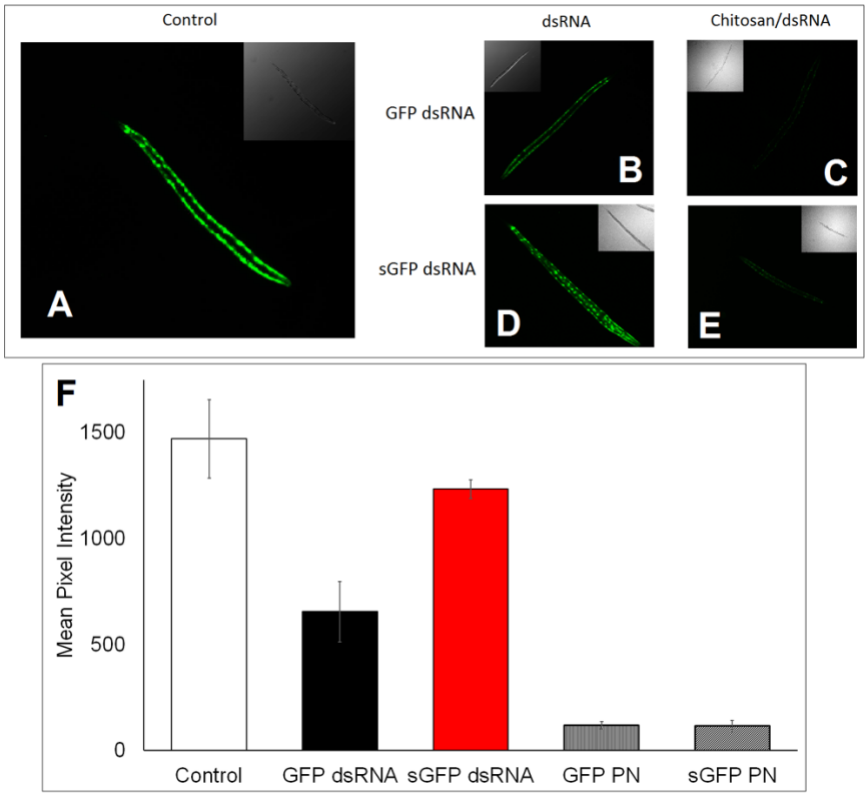


Figure 4 – Relative expression of *pmp-3* during concurrent *Caenorhabditis elegans* exposure to chlorpromazine and dsRNA or chitosan/dsRNA polyplex nanoparticles. Neither treatment was significantly different than control ($p>0.1$, $n=5$). Error bars represent standard error as reported by REST.

As before, *pmp-3* was targeted (100 ng/µL dsRNA) in wild-type strain N2, and quantitated by qRT-PCR. In both treatments, there was no significant change in *pmp-3* expression when compared to chlorpromazine only control (**Fig. 4**). There was also, no significant difference between chlorpromazine control and the no-chlorpromazine control, indicating that chlorpromazine had no effect. The implications of this are twofold. First, as has been reported previously[29, 44], because there is no significant knockdown in naked dsRNA treated groups, *sid* mediated uptake must be tied to clathrin-mediated endocytosis in some fashion. Additionally, since knockdown is suppressed in chitosan/dsRNA PN treated groups, clathrin-mediated endocytosis likely plays a key role in the internalization of chitosan/dsRNA PNs. These findings are similar to those of other studies investigating the uptake of nanoparticles in *C. elegans*[45], though this work represents the first to specifically investigate the uptake

mechanisms of chitosan/dsRNA PNs. The enhanced response observed in chitosan/dsRNA PNs in *sid-1* and *sid-2* mutants relative to wildtype is surprising and hints at a role of these genes in regulating the RNAi response that has not been fully characterized. Elucidation of that role remains a topic for further study.

Myosin dysregulation associated with chitosan exposure – The body of work assessing the potential for off target effects of RNAi has largely focused upon sequence-specific phenomenon. However, when delivered via a nanocarrier, effects associated with the nanocarrier and components thereof must also be considered. Although chitosan is regarded as largely non-toxic[13], the potential for sub-lethal or other innocuous but relevant effects is present.



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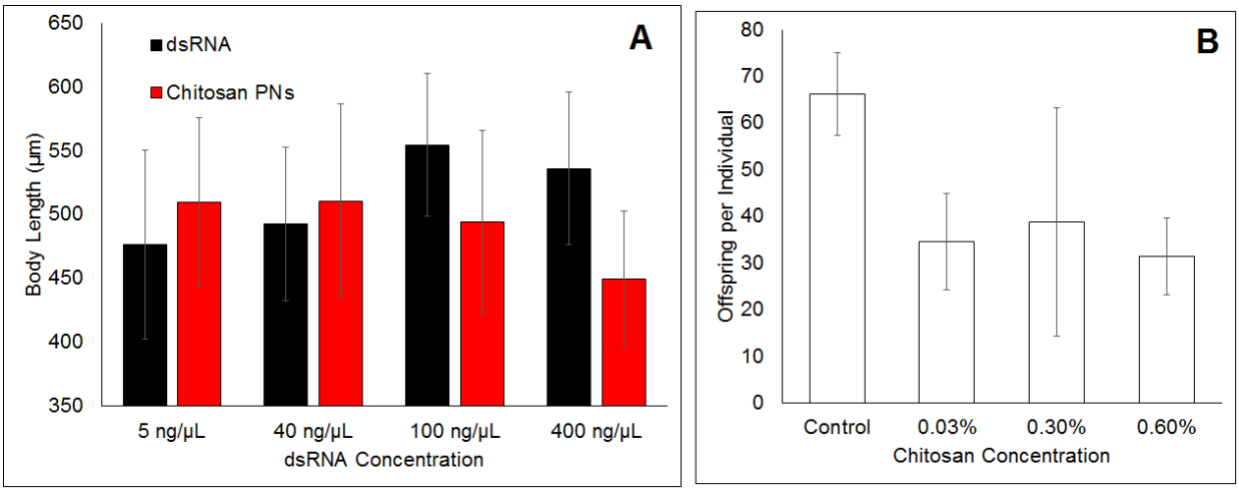
325 **Figure 5** – A – *Caenorhabditis elegans* strain PD4251 possesses bright, constitutive fluorescence
326 in body wall muscles. B – When exposed to GFP dsRNA, a slight reduction in fluorescence is visible. C –
327 A more profound reduction in fluorescence is demonstrated when exposed to chitosan/dsRNA polyplex
328 nanoparticles (PNs). D – Exposure to a scrambled GFP sequence (sGFP) dsRNA causes no detectable
329 change in fluorescence. E – Exposure to chitosan/dsRNA PNs constructed with sGFP dsRNA causes a
330 reduction in fluorescence comparable to PNs with a valid GFP sequence. F – Mean pixel intensity of
331 exposure groups indicated in figures 5A-E (n=5).

332 *C. elegans* strain PD4251 possesses several stable GFP transgenes driven by the *myo-3* promoter
333 (**Fig. 5A**). When exposed to a sequence of GFP dsRNA (400 ng/µL), a reduction in fluorescence was
334 observed (**Fig. 5B**). Exposure to dsRNA corresponding to a random scrambling of the same sequence
335 failed to produce any knockdown (**Fig. 5D**). Likewise, exposure to an equivalent mass of dsRNA as

chitosan/dsRNA PNs resulted in a far greater reduction in fluorescence (**Fig. 5C**). Unexpectedly, exposure to scrambled dsRNA as chitosan/dsRNA PNs also resulted in reductions in fluorescence similar to those seen using PNs bearing the valid sequence (**Fig. 5E**).

After confirming that this effect was not a result of dsRNA contaminating solutions or apparatus, we concluded that chitosan must be affecting myosin expression. To confirm this hypothesis, we investigated expression of *myo-3* under treatment by chitosan and chitosan PNs, and measured body length of treated CGC4 *C. elegans* from the previous imaging study. Since *myo-3* is strongly associated with body wall muscle, we theorized that downregulation would result in a reduction in overall body length. Indeed, PN exposed nematodes showed a significant decrease in length, starting at the 100 ng/µL exposure (**Fig. 6A**). Next, we conducted a reproduction assay using chitosan exposed *C. elegans.* As myosin is heavily involved in basic cellular processes, we hypothesized that severe downregulation of a major myosin isoform would result in a reduction in the fecundity of exposed nematodes. As expected, exposure to chitosan caused a measurable reduction in the number of offspring produced over the course of a three day laying period (**Fig. 6B**). Even relatively low concentrations induced this response, but increasing concentration did not appear to increase severity. Notably, our control groups produced a brood size noticeably smaller (~65 offspring/individual) than the typically encountered brood size (~150 offspring/individual). This can likely be attributed to the starvation period the nematodes are subject to during the exposure assay. The lack of concentration-response for reproduction data may indicate that we have reached an asymptote in the concentration-response curve and cannot reduce reproduction below a minimum value. In a previous study with another cationic NP (diethylaminoethyldextran coated CeO_2 NPs), we observed a similar phenomenon.[46]



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358 **Figure 6** – A - Mean body length of *Caenorhabditis elegans* exposed to the concentration of GFP
359 dsRNA , or chitosan/dsRNA polyplex nanoparticles indicated on the x-axis (n=20). B - Total offspring
360 produced per individual *Caenorhabditis elegans* over the course of a three day laying period, following
361 24 hours of exposure to the concentration of chitosan indicated on the x-axis (n=3). Data presented are
362 mean ± standard deviation. (NS = $p>0.1$; * = $p<0.1$; ** = $p<0.05$; *** = $p<0.01$)

363 For gene expression studies, we conducted exposures of N2 strain *C. elegans* to GFP dsRNA (400
364 ng/µL), chitosan/dsRNA PNs (400 ng/µL dsRNA), and a chitosan sample prepared in the same fashion as
365 PNs, but with DI water rather than dsRNA solution. For this last exposure, an equivalent amount of
366 chitosan solution was used as in the PN exposure, to ensure an equivalent concentration of chitosan in
367 each assay. Following 24 hours of exposure, *myo-3* transcripts were quantitated by qRT-PCR. *myo-3*
368 levels were slightly downregulated in the presence of dsRNA alone (**Fig. 7**). However, both the
369 chitosan/dsRNA PNs and chitosan groups showed marked downregulation of *myo-3.*

370

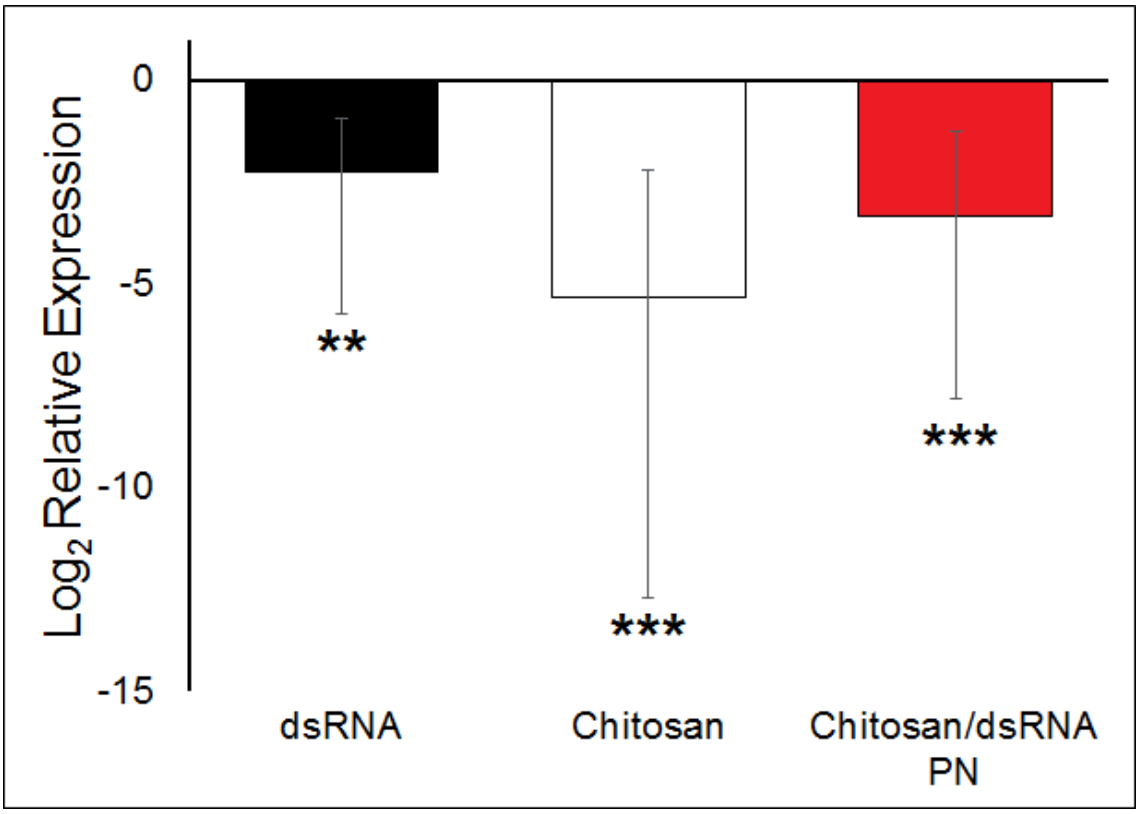

Figure 7 – Relative expression of *myo-3* under treatment with GFP dsRNA, chitosan alone, and chitosan/GFP dsRNA polyplex nanoparticles. All treatments were significantly different than control. Error bars represent standard error as reported by REST. (NS = $p>0.1$; * = $p<0.1$; ** = $p<0.05$; *** = $p<0.01$; n=3)

The ability to selectively knock down genes has been a boon to the research community, but the applications of RNAi extend far beyond reverse genetics and functional genomics. Products utilizing RNAi for pest control are dependent on dsRNA ingestion being sufficient to trigger an RNAi response in the target organism. Oral delivery being key, many products currently in development utilize transgenic RNAi constructs incorporated into the genome of crop species. This method is effective, but limited in that specific products must be developed for each pest and each crop. GM crops are also not able to be used in some countries.

The identification of alternative means of dsRNA uptake has significant toxicological implication for target and non-target species alike. Clathrin-mediated endocytosis is a highly conserved mechanism in eukaryotes, indicating that internalization of dsRNA in a wide variety of species is possible when a nano-carrier is utilized. This is of concern when exposure to vertebrates is a possibility, as long dsRNA is known to activate innate immune responses[47]. Most toxicological assessments of RNAi have focused upon sequence homology as the key driver of biological effects, be they target or off-target effects. However, differences in uptake of dsRNA, internalization into cells, and endosomal escape are important as well. If nano-carriers increase uptake of dsRNA they may increase effects in both target and off-target species, as RNAi is known to be dose-dependent[48]. Further studies are warranted to confirm the role of clathrin mediated endocytosis in dsRNA PNs. It is also important to consider the potential toxicity of the nanomaterial itself and the components of the nanomaterial, in this case chitosan. While chitosan is typically viewed as being benign, it had significant biological activity in this study. *myo-3* is crucial for muscle development, locomotion[49] and reproduction in *C. elegans*[50], and is required for numerous functions in other eukaryotes as well[51]. Further studies into the effects of chitosan on myosin are warranted, especially considering the considerable amount of work that has been put into the development of chitosan as a possible human therapeutic and for delivery of dsRNA as an insecticide. Such work could refine the concentration-response relationship for effects discovered in this study. With these concerns in mind, chitosan/dsRNA based nanomaterials do possess great potential for a number of applications. Further improvements to this delivery system could yield materials which reduce the total mass of dsRNA required for crop pest control, a highly desired outcome given the extreme expense associated with *in-vitro* synthesis of dsRNA.

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