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Chitosan, Carbon Quantum Dot, and Silica Nanoparticle Mediated dsRNA Delivery for Gene Silencing in *Aedes aegypti*: A Comparative Analysis

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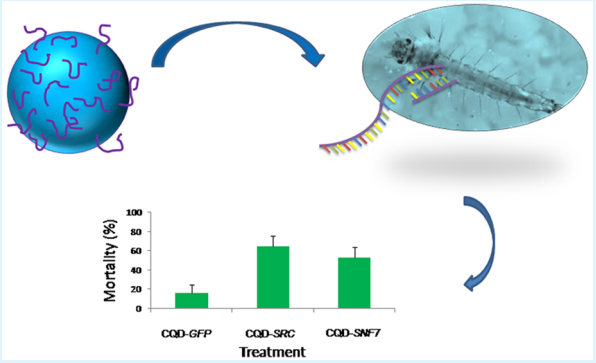
Supporting Information

ABSTRACT: In spite of devastating impact of mosquito borne pathogens on humans, widespread resistance to chemical insecticides and environmental concerns from residual toxicity limit mosquito control strategies. We tested three nanoparticles, chitosan, carbon quantum dot (CQD), and silica complexed with dsRNA, to target two mosquito genes (*SNF7* and *SRC*) for controlling *Aedes aegypti* larvae. Relative mRNA levels were quantified using qRT-PCR to evaluate knockdown efficiency in nanoparticle-dsRNA treated larvae. The knockdown efficiency of target genes correlated with dsRNA mediated larval mortality. Among the three nanoparticles tested, CQD was the most efficient carrier for dsRNA retention, delivery, and thereby causing gene silencing and mortality in *Ae. aegypti*.



KEYWORDS: *RNAi, dsRNA, mosquito, SRC, SNF7, CQD, Chitosan*

Mosquitoes transmit pathogens that cause deadly diseases including malaria, yellow fever, chickungunya, dengue, lymphatic filariasis, encephalitis etc. posing a major threat to human population worldwide.[1,2] The popular strategies of mosquito control include insecticides sprays, repellents, and insecticide-treated nets for adult control, and use of insecticides for larval control. However, these methods are gradually losing their effectiveness due to the development of widespread resistance to chemical insecticides[3,4] by mosquitoes and growing environmental concerns from residual toxicity. Use of bacterial proteins as larvicides are found to be effective but they are not highly used because of unpredictable efficacy under different environmental conditions.[5,6]

In this context, RNA interference (RNAi) technology is a promising environmental friendly method to control insects by double-stranded RNA (dsRNA) or small interfering RNA (siRNA) triggered post-transcriptional gene silencing. The ability of dsRNA to silence genes was discovered in the nematode, *Caenorhabditis elegans*.[7] RNAi technologies are being developed to apply this method in crop improvement, pest control and therapeutics.[8−16] Three general strategies have been elucidated for RNAi in insects: microinjection, soaking, and feeding of dsRNA.[17] dsRNA feeding is perhaps the most convenient and cost-effective RNAi approach for insect control. Delivery of dsRNA by expressing them in plants as well as direct feeding are being developed.[9,18] As the efficiency of RNAi in insects (especially those belonging to Lepidoptera, Hemiptera, and Diptera) is low because of dsRNA lower hydrophilicity and net negative charge, poor delivery and uptake efficiency, and sensitivity to nuclease degradation, there are only a few examples on insect control applications of RNAi.[19] The main challenge in widespread use of this technology is to develop inexpensive and reliable dsRNA production and delivery methods. Moreover, the success of RNAi technology largely depends on the stability of dsRNA or siRNA during and/or after delivery. The half-life of naked siRNA in serum ranges from several minutes to about 1 h.[20,21] Such a short half-life of the nucleic acids will not lead to an adequate RNAi response in an organism unless a high dose of dsRNA or siRNA is applied. Another determinant for successful RNAi in *Aedes aegypti* is lack of dsRNA transporter genes preventing robust systemic RNAi response.[22]

In this context, a carrier system is pertinent for delivering dsRNA to target site. Liposomes are being used as a delivery agents to knockdown target genes (e.g., EphA2, FAK, neuropilin-2, or IL-8) in mouse models of cancer diseases.[23] Another class of nanoparticles (NPs), broadly termed as polymeric NPs are a group of solid, biodegradable, colloidal systems that are widely used as drug or gene carrier.[24] During the past few years, NP mediated RNAi is being developed as an

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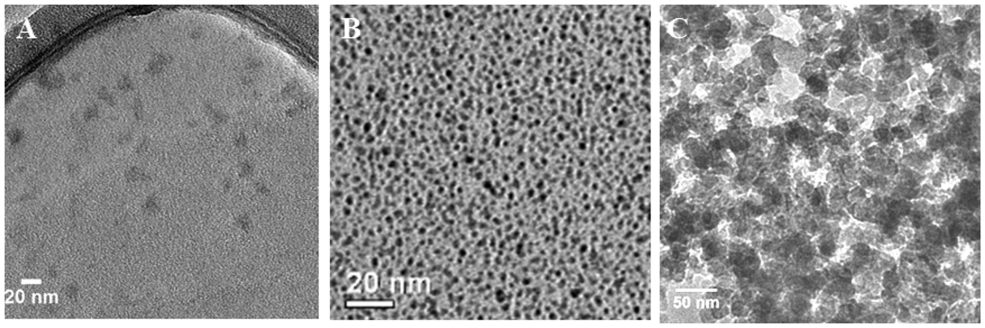

Figure 1. Transmission electron micrograph of (A) chitosan-dsRNA complex, (B) carbon quantum dots, and (C) silica nanoparticles.

alternative pest management strategy.[18,25,26] Zhang et al. found that chitosan NP mediated silencing of chitin synthase genes (*AgCHS1* and *AgCHS2*) in *Anopheles gambiae* through feeding increased pesticide susceptibility of larvae.[26] A simple and cost-effective method to conduct successful RNAi in mosquitoes has been developed.[17] Here, we have evaluated efficiency of NP mediated dsRNA delivery method to knockdown *Ae. aegypti* genes. NP mediated delivery methods could increase the stability of dsRNA and facilitate its uptake and endosomal release for efficient silencing of target genes. We have compared the efficiency of three NP mediated dsRNA delivery methods to control mosquitoes using the RNAi technology.

DsRNA of two target genes (*SNF7* and *SRC*) and one control gene (green fluorescence protein, *GFP*) were synthesized using T7MegaScript transcription kit. These genes were chosen based on the previous published reports on their effectiveness in RNAi-mediated growth arrest in western corn rootworm and on the basis of recent data from our laboratory using RNAi in *Tribolium castaneum*.[27,28] SNF7 is a protein involved in transport and is required for survival of insects.[27] SRC is a steroid receptor coactivator and is required for survival of *Tribolium castaneum*.[28] Three NPs were used as the delivery aids of dsRNA: carbon quantum dots (CQD), chitosan and amine functionalized silica NP (ASNP). CQDs were synthesized by a microwave-assisted method. First, 9 mL of polyethylene glycol (M. W. 200; PEG-200) was mixed with 3 mL of water. Then 100 mg of polyethylenimine (PEI) in 2 mL H_2O was added to it and the mixture was heated in a microwave for nearly 3 min at 800 W. Chitosan NP-dsRNA complex was synthesized following the method described previously by Zhang et al.[17] 0.16 wt %/vol solution of deacetylated chitosan from crab shells was prepared by dissolving in sodium acetate buffer. Thirty-two micrograms of dsRNA was diluted in 100 μL of sodium sulfate solution (50 mM) and was then added to 100 μL of chitosan solution. This mixture was further heated at 55 °C for 1 min, followed by immediate high speed vortexing for 30 s to allow the formation of the NPs.[29] For amine functionalization of nanosilica (purchased from M K Implex, Canada) 0.5 g nanosilica powder was dispersed in dimethyl sulfoxide (DMSO) under ultrasonic treatment for 15 min. The dispersion was then transferred to a round-bottom flask. Four-hundred microliters of (3-Aminopropyl)triethoxysilane (APTEOS) was added into the dispersion. The mixture was then refluxed at 120 °C for 3 h. NP was recovered by centrifugation at 12000 rpm for 15 min followed by repeated washing with DMSO and ethanol to remove unreacted APTEOS. The particles were then dried at 60 °C for overnight to obtain amine functionalized silica nano powder (ASNP). As CQDs were functionalized with PEI, overnight incubation of dsRNA suspended in sodium sulfate solution at 4 °C was enough for making dsRNA-CQD complexes. In the case of dsRNA-ASNP conjugation, dsRNA suspension was mixed with 1000 ppm ASNP solution at the ratio of 1:30, followed by overnight incubation. In both the cases the complex was ethanol washed and the pellet was used in further experiments.

The primary particle size of the chitosan and CQD-dsRNA complexes and ASNPs were measured using a high-resolution field emission gun transmission electron microscope (HR-TEM, Jeol 2010F, Tokyo, Japan). The primary particle diameter of the chitosan dsRNA complexes as determined by TEM was 15.6 ± 3.5 nm, n = 166. The primary particle diameter of the CQDs was 3.7 nm ± 0.7, n = 77, whereas the same for SNP was 15.8 nm ± 0.7, n = 265 (Figure 1). The hydrodynamic diameter and zeta (ζ) potential of the particles was determined using dynamic light scattering and phase analysis light scattering, respectively (Malvern Zetasizer nano ZS, Malvern, United Kingdom). The electrophoretic mobility of the CQD's in deionized water (pH 5.8) was 0.79 ± 0.42 μm cm/(V s) with a corresponding ζ potential of 15 ± 8 mV. The chitosan-dsRNA complexes had an electrophoretic mobility of 2.25 ± 0.28 μm cm/(V s) with a corresponding ζ potential of 29 ± 4 mV. The positive values of surface charge (zeta potential) for chitosan-dsRNA, CQD-PEI, and ASNP complexes showed the confirmation of overall positively charged surfaces of the NPs. This positive charge on the surface of NPs is required for the binding of negatively charged dsRNA. Apart from this, dsRNA binding capacity was determined for functionalized and unfunctionalized NPs by gel retardation assay. The results showed that naked (without surface functionalization) CQDs and SNPs were unable to conjugate with dsRNA. But functionalization of CQDs with positively charged PEI and amine functionalization of SNP with APTEOS enabled these NPs to effectively conjugate with dsRNA molecules (Figure 2). This gel retardation assay also showed that the capping materials were successfully incorporated in CQD and SNP surfaces resulting in their surface positive charge which helped in binding to negetively charged dsRNAs. Complete binding of all dsRNA (32 μg dsRNA in 100 μL of 50 mM sodium sulfate solution) with the chitosan NP was confirmed by the absence of any fast migrating dsRNA band in gel retardation assay. The optimum ratio for chitosan NP to dsRNA was found to be 10:1. For ASNPs, 100% dsRNA binding efficiency was obtained at the ASNP to dsRNA ratio of 30:1. The most efficient conjugation of CQDs with dsRNA was obtained when CQD was mixed with dsRNA at a 20:1 ratio. Due to the unique chemical properties of chitosan and dsRNA molecules, the chitosan/dsRNA conjugate was formed by self-

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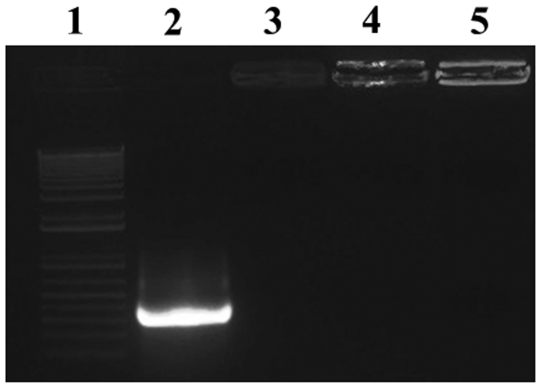

Figure 2. Gel retardation assay of nanoparticle-dsRNA complexes. Lane 1, 1 kb ladder; Lane 2, dsRNA; Lane 3, carbon quantum dot-dsRNA complexes; Lane 4, chitosan-dsRNA complexes; and Lane 5, amine-functionalized silica nanoparticle-dsRNA complexes.

assembly of polycations with dsRNA through the electrostatic forces between positive charges of the amino group in the chitosan and negative charges carried by the phosphate group on the backbone of the dsRNA. As already discussed, the positive charge on ASNP contributed by amine group from APTEOS, and the positive charge on CQDs contributed by PEI helped the binding of negatively charged dsRNA to these surface-functionalized NPs.

For delivering dsRNA to knockdown *Ae. aegypti* genes, we evenly mixed NP-dsRNA conjugates with mosquito larval food. The larvae were fed on NP-dsRNA containing food once a day for a total of 3 days. The mRNA levels of the target genes were determined on the fifth day after initiation of dsRNA feeding, while the mortality data were recorded until the control larvae became adults. Mosquito larvae (*Ae. aegypti*, Waco strain) were reared at 27 ± 1 °C under a photoperiodic regime of 16:8 h (L:D). The effectiveness of NP based RNAi method for silencing both *AaSNF7*and *AaSRC* through mosquito larval feeding was assessed by quantitative real-time PCR (qRT-PCR) and mortality assay. The qRT-PCR results clearly showed that feeding mosquito larvae with either ds*AaSNF7* or ds*AaSRC* conjugated to CQD or chitosan NPs effectively triggered RNAi in the larvae. Specifically, feeding of ds*AaSNF7* and ds*AaSRC* CQD complex reduced their mRNA levels to 60 and 29%, respectively (Figure 3A). Feeding of ds*AaSNF7* and ds*AaSRC* chitosan NP conjugate reduced their mRNA levels to 38 and 90%, respectively (Figure 3B). In contrast, feeding of ASNP-ds*AaSRC* did not cause a reduction in the mRNA levels of target gene (Figure 3C). These data showed that PEI-coated CQD is the most potent NP for delivering dsRNA to *Ae. aegypti* larvae. Mortality was assessed until the larvae in the controls became adults. It may be mentioned that toxicity profiling of the NPs used for dsRNA mediated larvicidal assay showed that the NPs alone were not toxic at the doses used for dsRNA delivery (Table S1). As expected, larval mortality was induced by RNAi. Compared to control chitosan-ds*GFP* treatment, chitosan-ds*AaSRC* and chitosan-ds*AaSNF7* treated larvae showed moderate mortality. On the third day after initiation of chitosan−dsRNA feeding, mortality was observed to be 8 and 20% for ds*AaSRC* and ds*AaSNF7*, respectively (Table 1). By the fifth day, the mortality increased to 13 and 38% respectively in ds*AaSRC* and ds*AaSNF7* fed larvae. By the seventh day, the mortality of larvae reached 27 and 47% for ds*AaSRC* and ds*AaSNF7* treatment, respectively. On the third

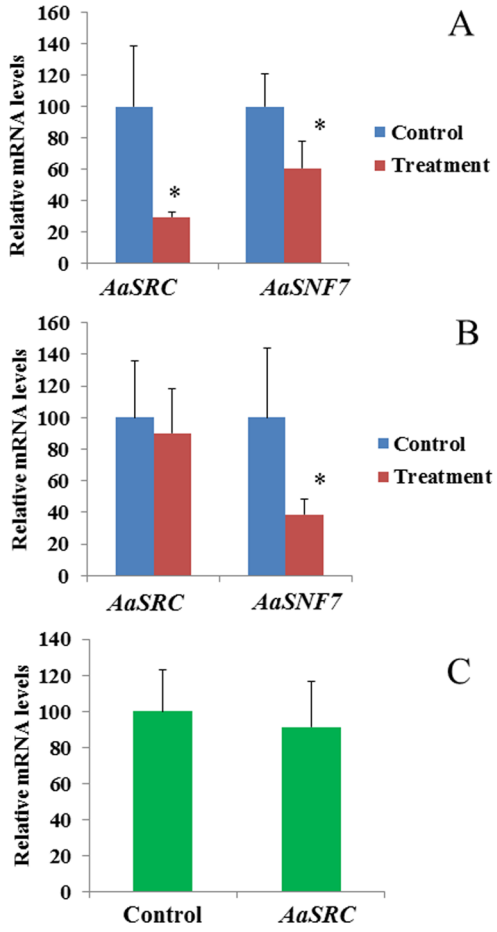

Figure 3. Nanoparticle-dsRNA mediated knockdown assay for two *Ae. Aegypti* genes, *AaSRC* and *AaSNF7*. Gene coding for green fluorescence protein (*GFP*) was used as a control. Relative mRNA levels of these genes were determined on fifth day after initiation of feeding the larvae with nanoparticle-dsRNA complexes. Relative mRNA levels of *AaSRC* and *AaSNF7* in larvae fed on (A) carbon quantum dot-dsRNA complexes, (B) chitosan-dsRNA complexes, and (C) ASNP-AaSRC were determined. Relative mRNA levels of only *AaSRC* was determined in case of amine functionalized silica nanoparticle and dsRNA complexes as these had no effect on larval mortality. Total RNA isolated from pooled larvae was used in qRT-PCR to determine relative mRNA levels of target genes using S7RP expression for normalization. The data shown are means ± SE (n = 3, Student's t test. * $P < 0.05$).

day after initiation of CQD−dsRNA feeding, larval mortality was observed to be 38 and 32% for ds*AaSRC* and ds*AaSNF7*, respectively (Table 1). The mortality increased to 52 and 47%, respectively, by the fifth day. By the seventh day after initiation of treatment, the mortality of larvae reached 75% for CQD-ds*AaSNF7* and 53% for CQD-ds*AaSRC* treatment, respectively. ASNP-ds*AaSRC* was found to be ineffective and caused no mortality in *Ae. aegypti* larvae (Table 1).

Knockdown efficiency of three NP-dsRNA complexes was studied by oral administration of NP-dsRNA complexes to second instar mosquito larvae. Although, similar amount of dsRNA was complexed with three types of NPs and each of the NP showed 100% loading efficiency, we observed differential knockdown efficiency based on NPs and dsRNA used. CQD conjugated dsRNA showed the best gene suppression, chitosan is moderately efficient carrier system for dsRNAs in suppression of gene expression. But most surprisingly ASNP

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Table 1. Mean Mortality of *Aedes aegypti* Larvae Induced by RNAi[a]

treatment	day 3	day 5	day 7
Chitosan-*GFP*	0 ± 0 Aa	1.67 ± 4.08 Aa	5 ± 5.48 Aa
Chitosan-*AaSRC*	8.33 ± 4.08 Aa	13.33 ± 5.16 Ba	26.67 ± 10.33 Bb
Chitosan-*AaSNF7*	20 ± 6.32 Ab	38.33 ± 11.69 Bb	46.67 ± 10.33 Bc
CQD-*GFP*	10 ± 6.3 Aa	10 ± 8.94 Aa	18.33 ± 9.83 Aab
CQD-*AaSRC*	38.33 ± 9.83 Ac	51.67 ± 11.69 Abc	75 ± 10.49 Bd
CQD-*AaSNF7*	31.67 ± 9.83 Ac	46.67 ± 8.16 ABbc	53.33 ± 10.06 Bc
ASNP-*GFP*	0 ± 0 Aa	8 ± 4.94 Ba	13.33 ± 5.16 Ba
ASNP-*AaSRC*	8.33 ± 4.08 Aa	8.33 ± 4.08 Aa	15 ± 8.36 Aab

[a]Mortality caused by dsRNA targeting *AaSNF7* and *AaSRC* was compared with mortality in control larvae fed on *GFP* dsRNA. Within each column, means followed by the same lower case letter are not significantly different; within each row, means followed by the same upper case letter are not significantly different. Tukey–Kramer HSD test; $P = 0.05$. CQD, carbon quantum dot; ASNP, amine-functionalized silica nanoparticle.

was found to be totally ineffective in carrying dsRNA to kill mosquito larvae. According to the previous literature survey on mosquito gut physiology it is well-known that a wide range of pH conditions exist within the mosquito alimentary canal. Anterior midgut is nearly neutral, whereas a moderately alkaline pH conditions (between 8 and 8.5) are detected between esophagus and the junction of the ceca.[30] Just behind the cecal junction (the third or fourth abdominal segment), both within and outside the peritrophic membrane, the pH is above 9.6. Posterior to this strongly alkaline region, in the midgut the pH declines rather abruptly over a region comprising about a tenth of the total midgut length. The pH is slightly alkaline or nearly neutral at the terminal region of the midgut (abdominal segments 5 to 6). Orally administered NP-dsRNA complex needs to go through these variable physiological ionic modalities. For successful functioning of RNAi the NP-dsRNA complex needs to stably cross these physiological barrier to reach the cells in the alimentary canal. To determine the effect of pH on the stabiliy of NP-dsRNA complex, the NP-dsRNA complexes were incubated in a buffer of pH 4, 7, and 10 for 72 h and the dsRNA retention capacity of different NPs was studied by gel retardation assay. After 48 h incubation, ASNP-dsRNA complex was unstable both at pH 4 and pH 10 (Figure S1). With an increase in pH, an increase in the rate of degradation of ASNP-dsRNA complex was observed. At 72 h after incubation, almost all the dsRNA was found to be disintegrated from ASNP at pH 10, as seen by the presence of a distinct unbound dsRNA band. These data suggest that although ASNP was able to load 100% dsRNA initially, the complex may not be able to survive PH conditions encountered in the alimentary canal of mosquito larvae. This may be one of the reasons why ASNP-ds*AaSRC* was unable to trigger RNAi and did not cause mortality in the mosquito larvae. dsRNA disintegration in chitaosan-dsRNA complexes incubated in pH 10 buffer started at or beyond 48 h after incubation (Figure S2). After 72 h incubation in pH 10 buffer, no retardation of dsRNA was observed at pH 7 or pH10 suggesting disintegration of the dsRNA from the chitosan NP. In contrast, CQDs were found to retain 100% dsRNA even after 72 h incubation in all the pH conditions tested (Figure S3). Their stabilty under a range of pH conditions may be one of the factors contributing to their efficiency to bind and carry the dsRNAs to trigger successful RNAi and mortality in mosquito larvae. In addition to eficient dsRNA delivery and hence causing mortality of mosquito larvae, use of CQD as dsRNA carrier has an additional advantage. CQD has excellent fluorescent property due to its quantum size effect. Hence, distribution of dsRNA within the larval body could easily be monitored. A few CQD-dsRNA complex were detected in the tissues after 24 h of exposure through food. Presence of this complex within larval tissues gradually increased with increasing time (Figure S4). Similar trend was obtained when we compared the effect of these nanocarrier dsRNA complexes on the survival of mosquito larvae. Knockdown of *SNF7* and *SRC* genes in mosquito larvae by feeding CQD-*AaSNF7* and CQD-*AaSRC* dsRNA caused high mortality. Larval death due to Chitosan-*AaSNF7* and Chitosan-*AaSRC* dsRNA was also corroborated with their moderate RNAi efficiency. Although the same amount of the dsRNA complexed with ASNP was fed, it did not cause significant mortality in mosquito larvae. Similar to CQD, cationic core–shell fluorescent NP of perylene-3,4,9,10-tetracarboxydiimide chromophore can also deliver dsRNA and trigger RNAi in insect pests. In addition, the NP-dsRNA complexes can be monitored in vivo by means of fluorescence detection.[18]

Here, three NPs (chitosan, CQD, and SNP) were used as a delivery system of dsRNA molecules for successful RNAi through oral route. Chitosan, a naturally occurring polymer, has received a lot of interest in encapsulation of active compounds and is being widely used in medical science because of its biocompatibility, low toxicity, and biodegradability.[31] Similarly, CQDs, synthesized from a biocompatible carbon source (PEG), are nontoxic and now being applied widely in bioimaging, medical diagnosis, and treatment.[32] SNP is another nontoxic and eco-friendly delivery system, which is able to adhere and carry nucleic acid.[33] This technology has the potential to become a sustainable, targeted, and eco-friendly insect management method.

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acsami.5b05232.

Detailed description of experimental methods, Table S1 on mortality caused by NP alone, and Figures S1–S4 (PDF)

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Notes

The authors declare no competing financial interest.

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