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# Guanidinium-Functionalized Interpolyelectrolyte Complexes Enabling RNAi in Resistant Insect Pests

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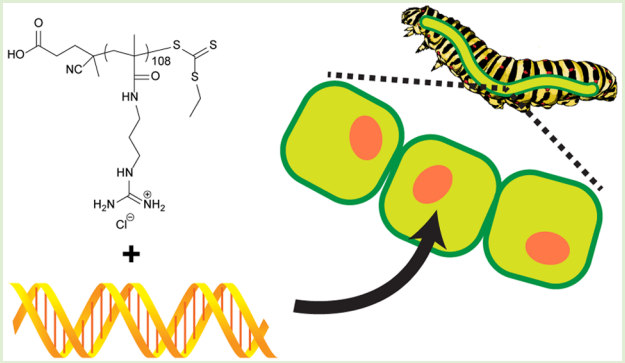
Supporting Information

**ABSTRACT:** RNAi-based technologies are ideal for pest control as they can provide species specificity and spare nontarget organisms. However, in some pests biological barriers prevent use of RNAi, and therefore broad application. In this study we tested the ability of a synthetic cationic polymer, poly-[*N*-(3-guanidinopropyl)methacrylamide] (pGPMA), that mimics arginine-rich cell penetrating peptides to trigger RNAi in an insensitive animal—*Spodoptera frugiperda*. Polymer−dsRNA interpolyelectrolyte complexes (IPECs) were found to be efficiently taken up by cells, and to drive highly efficient gene knockdown. These IPECs could also trigger target gene knockdown and moderate larval mortality when fed to *S. frugiperda* larvae. This effect was sequence specific, which is consistent with the low toxicity we found to be associated with this polymer. A method for oral delivery of dsRNA is critical to development of RNAi-based insecticides. Thus, this technology has the potential to make RNAi-based pest control useful for targeting numerous species and facilitate use of RNAi in pest management practices.




## INTRODUCTION

Insect crop pests are a major global concern that exacerbate increasing pressures on food supplies from overpopulation and global warming. Unfortunately, use of chemical pesticides cause collateral environmental damage and kill nontarget insects.[1] Transgenic strategies such as Bt toxin can alleviate these concerns;[2] however, resistance can emerge, which limits their effectiveness.[3−5] An increasingly exciting option for control of plant pests is the use of RNA interference- (RNAi-) based technologies.[6,7] RNAi is a process in which small, 19−30 nucleotide RNA molecules trigger the destruction or decay of complementary transcripts.[8] RNAi-based pest control improves upon traditional small molecule pesticides by providing high specificity to the target species.[9]

RNAi in insects can be induced through introduction of double stranded RNA (dsRNA), which is processed into small interfering RNA (siRNA) effectors.[10−12] Feeding of dsRNA to crop pests is effective at inhibiting gene expression in some species.[13] Indeed, transgenic corn expressing dsRNA is currently being used to control western corn rootworm (WCR) by targeting vacuolar ATPase (V-ATPase).[14] dsRNAs can also be applied as crop sprays,[15] which enables use of synthetics to increase efficiency. Use of dsRNA in sprays is a very attractive mode of delivery as it eliminates the need for transgenics, which are not feasible to generate for some crops.

Unfortunately, while attempts at RNAi-based pest control have been successful in some species, many insect orders seem refractory to ingested RNAi.[16−19] Although feeding is ineffective in these insects, dsRNA injection is often capable of eliciting RNAi,[16−21] indicating that barriers to dsRNA uptake primarily exist in the digestive tract. Indeed, high nuclease activity in the migratory locust gut renders dsRNA feeding ineffective.[16] Furthermore, additional barriers may exist, such as the endosomal entrapment of dsRNA found in lepidopterans (i.e., moths and butterflies).[22] To address this problem we sought to develop a polymeric dsRNA vector that can circumvent barriers to uptake via ingestion and facilitate the use of RNAi in crop sprays.

Polycations have gained interest for their ability to electrostatically complex the negatively charged RNA phosphodiester backbone to form interpolyelectrolyte complexes (IPECs).[23,24] Recently, we demonstrated that polymers synthesized from *N*-(3-guanidinopropyl)methacrylamide (GPMA) are able to enter cells readily via both endocytotic and nonendocytotic routes,[25] and Tabujew et al. subsequently demonstrated these polymers can bind and protect siRNAs.[26] pGPMA guanidinium groups provide moieties similar to

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arginine-rich cell penetrating peptides (CPPs), which are observed to accumulate in endomembrane vesicles, where they can cross membranes.[27−29] CPPs have also been found to enter cells through nonendocytotic routes.[30] In Sf9 cells, an RNAi-insensitive cell line derived from fall armyworms (*Spodoptera frugiperda*), naked dsRNAs are eliminated in endosomal compartments.[22] In this study, we test the ability of pGPMA to enable RNAi in fall armyworms, through cytoplasmic delivery of dsRNAs.

We find that pGPMA-dsRNA IPECs can elicit RNAi in fall armyworm cells and larvae that are otherwise insensitive to ingested RNAi.[22] Confocal microscopy revealed successful dsRNA delivery to Sf9 cells, and RT-qPCR analysis showed potent gene suppression. These IPECs, when fed to fall armyworm larvae, resulted in a similar degree of knockdown. Through targeting a gene known to have a role in digestive physiology, IPECs induced larval mortality and significant gut hypertrophy. To our knowledge, this is the first study demonstrating successful gene suppression via orally ingested RNAi in an otherwise insensitive lepidopteran species.

## EXPERIMENTAL SECTION

**Materials.** All reagents were purchased from Sigma-Aldrich at the highest available purity and used as received unless otherwise noted. 4-Cyano-4-[(ethylsulfanylthiocarbonyl)sulfanyl]pentanoic acid (CEP)[31] and *N*-(3-guanidinopropyl)methacrylamide (GPMA)[32] were synthesized as previously reported. Gibco Sf-900 II serum free media was purchased from Fisher. Sf9 (*S. frugipera*, ovarian) cells were purchased from Millipore. Fall armyworm (*S. frugiperda*) larvae were obtained from Benzon Research through USDA permit P526P-17−00512. For reactions requiring nitrogen, ultrahigh purity nitrogen (purity ≥99.998%) was used. Spectra/Por regenerated cellulose dialysis membranes (Spectrum Laboratories, Inc.) with a molecular weight cutoff of 12−14 kDa were used for dialysis.

**Synthesis of pGPMA.** Poly[*N*-(3-guanidinopropyl)-methacrylamide] (pGPMA) was prepared employing 4,4′-azobiscyanovaleric acid as the primary radical source and CEP as the chain transfer agent at 70 °C. GPMA (1.46 g, 6.6 mmol), CEP (15.6 mg, $59.2 \times 10^{-6}$ mol), and 4,4′-azobiscyanovaleric acid (3.3 mg, $11.8 \times 10^{-6}$ mol) were added to a 25 mL round-bottomed flask and dissolved in 1 M acetate buffer (pH = 4.5) with 1 mL MeOH (to improve CTA and initiator solubility) with a final volume of 10 mL ($[M]_0 = 0.65$ M). 50 μL dioxane was added as an internal standard for $^1$H NMR analysis. The round-bottomed flask was septum-sealed and purged with nitrogen for 1 h. prior to polymerization. The polymer was prepared with $[M]_0/[CTA] = 110$ while $[CTA]/[I]$ was kept at 5, and the reaction was allowed to proceed for 19 h. Aliquots were taken via degassed syringe to monitor monomer conversion. The polymerization was quenched by rapid cooling in liquid nitrogen followed by exposure to air. The product was isolated by dialysis (pH = 3−4) at 4 °C and recovered by lyophilization.

***In Vitro* Transcription of dsRNA.** Using *Taq* DNA polymerase, ∼500 nucleotide (nt) of exonic sequence was amplified by polymerase chain reaction (PCR) for GFP, and the *S. frugiperda* genes: sfV-ATPase, sfKIF, and sfCDC27 genes (See Supporting Information). Fragments were ligated into pGEM-T Easy plasmid (Promega), and sequence verified. PCR products were generated from these constructs to add T7 promoter sequences to create templates for bidirectional transcription. Using MEGAscript T7 Transcription Kit (Thermo Scientific), *in vitro* transcription reactions were carried out, followed by LiCl precipitation of products. RNAs were resuspended in nuclease free water and denatured at 95 °C. After 2 min, the heat block was turned off to allow gradual reduction of temperature to anneal RNAs. Annealing was carried out for 1 h, after which purity, concentration, and quality was determined via UV spectroscopy with a nanoDrop-1000 and gel electrophoresis. dsRNAs were stored at −80 °C.

**Polymer Characterization.** pGPMA was characterized by aqueous size exclusion chromatography (ASEC) with an eluent of 1 wt % acetic acid and 0.1 M LiBr (aq) at a flow rate of 0.25 mL/min at 25 °C, Eprogen Inc. CATSEC columns (100, 300, and 1000 Å), a Wyatt Optilab DSP interferometric refractometer ($\lambda$ = 690 nm), and a Wyatt DAWN-DSP multiangle laser light scattering (MALLS) detector ($\lambda$ = 633 nm). Absolute molecular weight and molecular weight distribution were calculated using Wyatt Astra (version 4) software; $dn/dc$ measurement for polymer was performed utilizing a Wyatt Optilab DSP interferometric refractometer ($\lambda$ = 690 nm) at 25 °C and Wyatt DNDC (version 5.90.03) software. $^1$H NMR spectroscopy was performed using a Varian MercuryPLUS 300 MHz NMR spectrometer in $D_2O$ utilizing a delay time of 5 s. Monomer conversion was calculated from the $^1$H NMR spectra by monitoring the disappearance of the GPMA vinyl peaks (5.27 and 5.51 ppm) relative to the dioxane internal standard (3.58 ppm) (Figure S1).

**Light Scattering.** Variable-angle dynamic light scattering (DLS) measurements of copolymer−dsRNA complexes under aqueous conditions were performed using an incident light of 633 nm from a Research Electro-Optics Model 31425 He−Ne laser operating at 35 mW. The angular dependence (60−120° in 10° increments) of the autocorrelation function was determined with a Brookhaven Instruments BI-200SM goniometer with an Avalanche photodiode detector and TurboCorr autocorrelator. DLS measurements were carried out at a complex concentration (dsRNA + polymer) of 0.1 mg/mL in phosphate buffer (10 mM $P_i$, pH = 7.4 or 10) at 25 °C. To remove dust, polymer and dsRNA solutions were individually passed through a 0.45 μm Millipore filter (PVDF) directly into the scattering cell. The solution was gently mixed and allowed to equilibrate for 30 min prior to analysis. The mutual diffusion coefficient ($D_m$) was determined from the relation

$$\Gamma = D_m q^2$$

in which $\Gamma$ and $q^2$ represent the decay rate of the autocorrelation function and the square of the scalar magnitude of the scattering vector, respectively. The hydrodynamic radius ($R_h$) was then calculated from the Stokes−Einstein equation:

$$D_m \approx D_0 = (k_B T)/(6\pi\eta R_h)$$

in which $\eta$ is the solution viscosity, $k_B$ is Boltzmann's constant, and $T$ is the temperature in K.

Static light scattering (SLS) measurements were performed using the same instrumentation and samples as described above. The angular dependence of the inverse excess scattering intensity ($I_{ex}$) was analyzed via Berry analysis by plotting $I_{ex}^{-1/2}$ vs $q^2$, yielding the radius of gyration ($R_g$) from the slope.

Zeta-potential measurements were carried out a complex concentration (dsRNA + polymer) of 0.1 mg/mL in phosphate buffer (10 mM $P_i$, pH = 7.4 or 10) at 25 °C using a Malvern Zetasizer Nano ZEN3600. To remove dust, polymer and dsRNA solutions were individually passed through a 0.45 μm Millipore filter (PVDF) directly into the folded capillary cell. The solution was gently mixed and allowed to equilibrate for 30 min prior to analysis. Measurements were performed in triplicate.

**Polymer−dsRNA Binding Assay.** pGPMA-dsRNA solutions were prepared to complex 1 μg dsRNA at varying polymer−dsRNA weight ratios (0.25−100 μg of polymer/μg of dsRNA, ± = 0.5−180). Briefly, an appropriate volume of a 1 μg/μL or 10 μg/μL pGPMA stock solution in 10 mM PBS was added to 2 μL of a 0.5 μg/μL dsRNA solution in nuclease-free di$H_2O$. The solutions were gently mixed and allowed to equilibrate for 30 min before being diluted with 15 μL of 2× RNA loading buffer (Ambion). Gel electrophoresis was then performed on a 1% agarose gel in 1× TAE buffer stained with ethidium bromide. The gel was soaked in di$H_2O$ for 30 min to remove excess ethidium bromide before being imaged.

**Gene Suppression in Sf9 Cell Culture.** Sf9 cells were grown in Sf-900 II SFM at 28 °C. Sf9 cells (1 million cells/mL, 2 mL) were seeded in a 6 well plate (Corning Inc.). pGPMA-dsRNA complexes were formed to deliver a total of 5 μg of dsRNA complexed with 20,


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30, or 40 $\mu$g of pGPMA per well. Briefly, 20, 30, or 40 $\mu$L of a 1 $\mu$g/$\mu$L pGPMA stock solution in 10 mM PBS was added to 10 $\mu$L of a 0.5 $\mu$g/$\mu$L stock solution of dsRNA targeting CDC27 in nuclease-free di$H_2O$. The solution was gently mixed and allowed to equilibrate for 30 min before being added to the cell media, resulting in [dsRNA] = 7.4 nM. Identical complex solutions using dsRNA targeting KIF were used as controls. After 24 h, total RNA was extracted with TRI Reagent following manufacturer protocol. CDC27 transcript abundance was determined via RT-qPCR. First strand cDNA was synthesized with the Reverse Transcription Kit (Fermentas). Amplification and quantification was carried out with qPCR mix containing SYBR green (Fisher Scientific) and a BioRad CFX 96. All amplifications were performed in quadruplicate (primers listed in Supporting Information).

Time-dependent gene suppression followed a similar procedure. Cells were seeded as described above, and pGPMA-dsRNA complexes targeting CDC27 were formed to deliver a total of 5 $\mu$g of dsRNA complexed with 40 $\mu$g of pGPMA. Lipofectamine 3000 (Invitrogen) was used as a positive control, and the Lipofectamine-dsRNA complexes were prepared according to manufacturer protocol. Untreated cells were used as a negative control. After 24, 48, or 72 h, total RNA was extracted, and RT-qPCR was performed as described above.

**Cell Viability Assay.** Cells (1 M cells/mL, 100 $\mu$L) were seeded in a 96 well plate (Corning Inc.). Cells were treated with 1, 1.5, or 2 $\mu$L of a 1 mg/mL pGPMA stock solution to yield polymer concentrations equivalent to those used in the gene suppression studies. Cell proliferation was determined via a standard MTT assay (Vybrant MTT Cell Proliferation Assay Kit; Invitrogen). Cells were incubated for 48 h before adding 10 $\mu$L of a 12 mM MTT reagent to each well. The cells were further incubated for an additional 4 h, followed by adding 100 $\mu$L of a SDS (10%)/HCl (0.01 M) solution to each well. The absorbance was then determined utilizing a Biotek Synergy2-MultiMode Microplate Reader. All studies were performed in triplicate.

**Confocal Microscopy.** Sf9 cells (200 000 cells/mL, 500 $\mu$L) were seeded in a 48 well plate (Corning Inc.). pGPMA-dsRNA complexes were formed to deliver a total of 25 ng Cy5-labeled dsRNA complexed with 150 ng pGPMA per well. Briefly, 1.5 $\mu$L of a 0.1 $\mu$g/$\mu$L pGPMA stock solution in 10 mM PBS was added to 1.02 $\mu$L of a 24.5 $\mu$g/$\mu$L dsRNA solution in nuclease-free di$H_2O$. The solution was diluted to 25 $\mu$L with 10 mM PBS, gently mixed, and allowed to equilibrate for 30 min before being added to cell media. A 25 $\mu$L solution containing 25 ng Cy5-labeled dsRNA was also prepared and added to cells as a control. After 24 h, the cells were collected and spun down at 4.5k RPM. The supernatant was removed, and the cells were washed with 500 $\mu$L PBS. After spinning down again, the cells were resuspended in 40 $\mu$L PBS and placed on precleaned microscope slides. The cells were then fixed with 4% formaldehyde, washed with PBS, and stained with 12 $\mu$L 4′,6-diamidino-2-phenylindole (DAPI) mounting medium before adding coverslips. Fluorescence cell images were taken using a Zeiss LSM 510 scanning confocal microscope and processed with manufacturer software. Multiple fields were imaged for each sample to document uniform cytoplasmic distribution of complexes.

**Larvae Feeding Experiments.** pGPMA-dsRNA complexes targeting V-ATPase or GFP (control) were formed in 8:1 weight ratio as previously described. Fall armyworm larvae were immobilized, and either pGPMA alone or pGPMA-dsRNA complex solution (~100 ng/$\mu$L dsRNA) was put directly on larval mouth parts, and ingestion verified by observation under a stereomicroscope. Animals were then kept in a 26 °C incubator on larval food. Insect midguts were dissected and homogenized in TRI reagent for total RNA extraction following manufacturer protocol. V-ATPase transcript abundance was determined via RT-qPCR as described above. For survival assay, the number of larvae/pupae was counted in regular intervals (days) for mortality.

## RESULTS AND DISCUSSION

**pGPMA Synthesis and IPEC Characterization.** Employing aqueous reversible addition−fragmentation chain transfer (*a*RAFT) polymerization, poly[*N*-(3-guanidinopropyl)-methacrylamide] (pGPMA, Figure 1a,b) was synthesized to serve as a dsRNA delivery vehicle. This cationic polymer shares features with other synthetic carriers of nucleic acids that can electrostatically bind to negatively charged RNA phosphodiester groups to form interpolyelectrolyte complexes (IPECs).[23,24] Formation of IPECs confers enhanced RNA stability, providing protection from RNase-mediated degradation.[33−35] pGPMA is chemically similar to CPPs, which can traverse biological membranes and enter cells through both endocytotic and nonendocytotic pathways,[30] and we have previously shown similar behavior for pGPMA.[25] It has been demonstrated that pGPMA has a modest capacity to deliver plasmid DNAs to nuclei, though toxicity was observe in one cell line.[36] However, studies in other cell culture systems have demonstrated negligible toxicity for similar polymers,[37−40] suggesting that any toxicity may be confined to certain cell types or configuration of IPECs. Use of pGPMA-dsRNA IPECs also addresses the alkalinity and high RNase activity in insect guts: lumen pH ranges from 10 to 11,[41] where commonly used

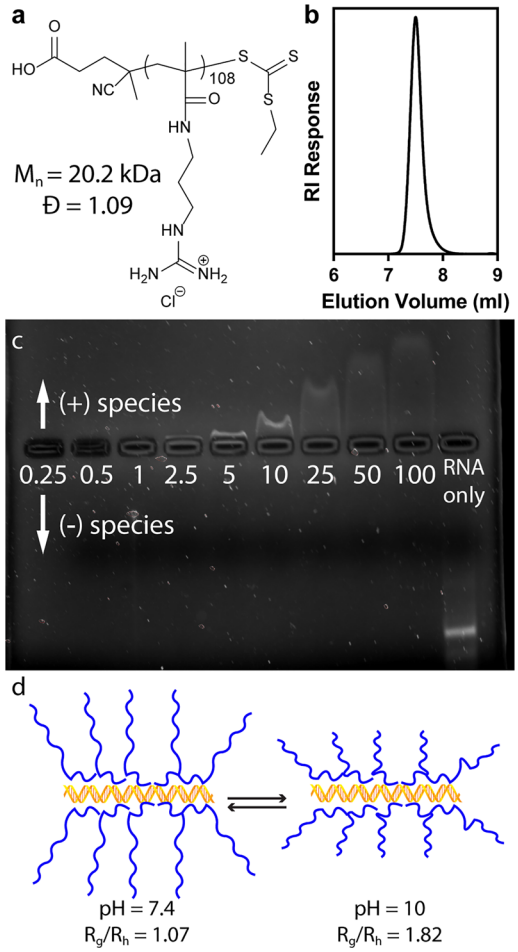


**Figure 1.** (a) Structure, number-average molecular weight ($M_n$), and dispersity (Đ) of pGPMA. (b) SEC trace of pGPMA. (c) Gel electrophoresis of pGPMA-dsRNA IPECs. Numbers indicate polymer/dsRNA weight ratio. (d) Proposed morphological changes in IPEC structure between pH = 7.4 and pH = 10.



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tertiary amine-containing nucleic acid carriers become deprotonated, leading to IPEC dissociation. However, the guanidinium functionalities of pGPMA, with p$K_a$ = 12.5, should retain cationic charges in the same pH range. Additionally, GPMA-based polymers can form multiple hydrogen bonds with the dsRNA phosphodiester moieties to provide greatly enhanced binding to siRNAs,[26] which subsequently should increase protection from enzymatic degradation of dsRNA within the insect gut.

In order to form IPECs with a several hundred nucleotide dsRNA, a pGPMA homopolymer with a degree of polymerization (DP) = ∼ 100 repeat units was synthesized using a protocol previously developed in our laboratories.[25] Because the GPMA guanidinium moieties serve as sites for both dsRNA complexation and cell penetration, polymer−dsRNA IPECS must be formed at IPEC charge ratio (±) > 1 (i.e., net cationic charge) to ensure solubility as well as uncomplexed GPMA units to interact with cell membranes. Gel electrophoresis was performed to determine the polymer/dsRNA weight ratio(s) at which ± > 1 (Figure 1c). On the basis of moderate IPEC gel migration toward the anode, subsequent experiments used weight ratios of 4×, 6×, or 8× (± = 7, 11, 15, respectively). At these ratios, the IPECs possess the desired net cationic charges while not being so cationic that they encourage excessive protein opsonization and IPEC exchange *in vivo*.

$\zeta$-potential measurements were also performed using these ratios to confirm net cationic charges, both at neutral pH and under the alkaline conditions found in the lepidopteran gut. As demonstrated in Table 1, $\zeta$-potential values increase with increasing polymer−dsRNA weight ratio at both pH conditions. As one might expect for a system approaching the GPMA repeat unit p$K_a$, some deprotonation likely occurs at pH = 10, leading to overall lower $\zeta$-potential values relative to pH = 7.4 However, the positive $\zeta$-potential values for each IPEC indicate that a net cationic charge is maintained, even under alkaline conditions.

**Table 1. $\zeta$-Potential Measurements of pGPMA-dsRNA Complexes at Varying Weight Ratios and pH**

| | $\zeta$-potential (mV) | |
|---|---|---|
| weight ratio | pH = 7.4 | pH = 10 |
| 4× | 16.0 | 12.0 |
| 6× | 18.4 | 12.8 |
| 8× | 19.9 | 13.8 |
| dsRNA | −11.8 | [a] |

[a] $\zeta$-potential was not measured due to dsRNA hydrolytic instability at pH = 10.

A similar trend was revealed in dynamic light scattering (DLS) analysis of IPECs formed at a weight ratio of 8 (Table 2). IPECs formed at pH = 7.4 resulted in a uniform population (see Figure S2 for histograms) with hydrodynamic radius $R_h$ = 318.1 nm, but at pH = 10, some pGPMA deprotonation leads to a partial collapse of the IPEC corona, resulting in $R_h$ = 239.2 nm. Additionally, the large increases in $R_h$ for the IPEC vs naked dsRNA may suggest multiple dsRNAs per complex. Static light scattering (SLS) analysis was performed to determine IPEC and dsRNA radii of gyration ($R_g$) and thus $R_g/R_h$, which serves as an indicator of morphology. dsRNA alone exhibits $R_g/R_h$ = 2.01, indicating the rigid rod-like morphology expected of long dsRNA. The $R_g/R_h$ values for the IPECs at both high and low pH also indicate high aspect ratio morphologies as one would expect from pGPMA chains binding to and forming a corona around a rigid rod. The increase in $R_g/R_h$ at pH = 10 further supports this notion: as the pGPMA corona slightly collapses upon partial deprotonation, the IPEC increasingly adopts the morphology of the dsRNA (Figure 1d).

**Table 2. Dynamic and Static Light Scattering Measurements of pGPMA-dsRNA Complexes at Varying pH (Weight Ratio = 8)**

| sample | $R_h$ (nm) | $R_g$ (nm) | $R_g/R_h$ |
|---|---|---|---|
| IPEC, pH = 7.4 | 318.1 | 341.7 | 1.07 |
| IPEC, pH = 10 | 239.2 | 436.5 | 1.82 |
| dsRNA | 35.7 | 71.8 | 2.01 |

**pGPMA-dsRNA IPEC Transfection and Gene Suppression in Lepidopteran Cell Culture.** The IPECs were tested for their ability to enter Sf9 cells and affect gene expression. This cell line is derived from embryonic fall armyworms and, unlike some insect lines (e.g., *Drosophila* S2), is insensitive to dsRNA added to growth media.[22] To verify the ability of pGPMA to facilitate uptake of dsRNA, Cy5-labeled dsRNA was complexed with pGPMA (8×) and added to Sf9 cell culture media. Cells were imaged following incubation with the complex for 24 and 48 h. Significant accumulation of the Cy5 signal could be observed in the pGPMA-dsRNA complex-treated cells after both 24 (Figure 2a) and 48 h. (Figure 2b). Conversely, cells treated with Cy5-dsRNA alone (Figure S3) exhibited no Cy5 signal. Accumulation appears constant, likely due to continued uptake from media. Primarily the dsRNA localized to cellular bodies that are likely endosomal, consistent with observations that guanidinium-functionalized oligomers facilitate uptake of nucleic acids through an endocytosis-dependent mechanism.[37] Significantly, treatment with the polymers resulted in negligible cytotoxicity (Figure 2c).

The CDC27 gene, which was targeted by RNAi in Sf9 cells in a previous study[42] that relied on *Caenorhabditis elegans* SID-1 to transport dsRNA into the cytoplasm, was used to test the ability of pGPMA to enable gene knockdown. pGPMA was complexed either with CDC27-dsRNA or control dsRNA and added to Sf9 media. After a 48-h incubation, expression levels were quantitated by RT-qPCR (Figure 3a). We observed extensive knockdown of CDC27 (>90%) that was sequence dependent. Time-dependent gene suppression at an 8× weight ratio was then evaluated relative to untreated cells and those transfected using Lipofectamine 3000 (Figure 3b). pGPMA-dsRNA IPECs induced knockdown comparable to Lipofectamine and showed better performance after 72 h. To ensure that changes in gene expression were not induced by the polymer itself, CDC27 expression was evaluated after treatment with uncomplexed pGPMA equivalent to that of 8× weight ratio. No gene suppression from the polymer alone was observed (Figure S4).

The amount of dsRNA delivered at an 8× weight ratio was quantified via RT-qPCR employing primers specific to the dsRNA, rather than the targeted mRNA (Figure 3c). After 24 h, pGPMA transfected similar amounts of dsRNA to Lipofectamine. However, at 48 and 72 h, cells treated with IPECs maintained significantly higher levels of transfected dsRNA than did those treated with Lipofectamine. The relatively high levels of dsRNA transfected by pGPMA resulted in consistent levels of gene suppression over 3 days. Lipofectamine, on the



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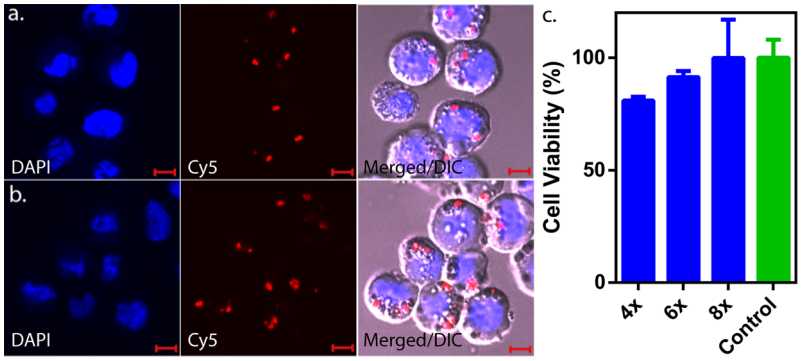


**Figure 2.** Sf9 cells treated with Cy5-labeled dsRNA (red) complexed with pGPMA after (a, top row) 24 h or (b, bottom row) 48 h. Nuclei were stained with DAPI (blue). Scale bars = 5 $\mu$m. (c) Cell viability assay of pGPMA after 48 h employing polymer concentrations identical to the indicated weight ratios used in IPECs. Cell viability was determined relative to the untreated control. Error bars represent the standard deviation from triplicate experiments.

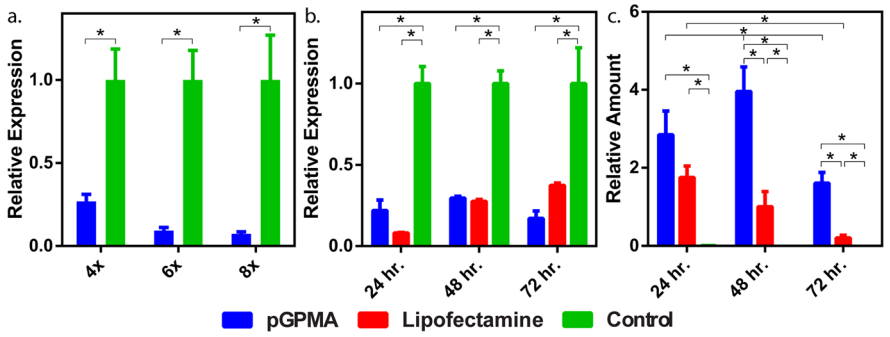


**Figure 3.** (a) Expression of CDC27 determined by RT-qPCR in Sf9 cells following incubation with pGPMA complexed with either CDC27- or control-dsRNA. Numbers indicate polymer/dsRNA weight ratios. Values are normalized to CDC27 expression in respective control (KIF-dsRNA-treated) samples. Errors bars represent SEM. (b) Expression of CDC27 determined by RT-qPCR in Sf9 cells following incubation with CDC27 dsRNA complexed with either pGPMA (8×) or Lipofectamine 3000. Values are normalized relative to respective untreated controls. Error bars represent SEM. (c) RT-qPCR quantification of CDC27-dsRNA transfected by pGPMA, Lipofectamine 3000, or untreated control. Values are relative to zero. Error bars represent SEM. For plots a–c, groupings indicated with asterisks (*) were found to be significantly different after Tukey analysis.

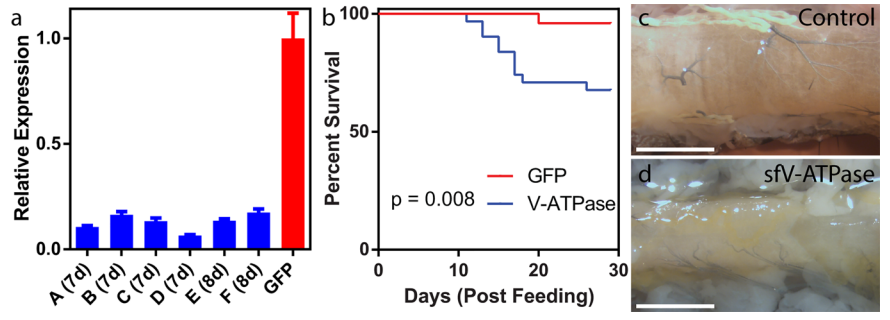


**Figure 4.** (a) Expression of V-ATPase mRNA in midgut tissue from second instar fall armyworm larvae fed with pGPMA complexed with either V-ATPase dsRNA or GFP dsRNA determined by RT-qPCR. Letters indicate individual animals. Days between feeding and harvesting are indicated in parentheses. Values are normalized to V-ATPase expression in control sample. Error bars represent SEM. (b) Percent survival of second and third fall armyworm larvae fed pGPMA complexed with dsRNA targeting V-ATPase ($N$ = 25) or control dsRNA ($N$ = 31). (c) Image of fall armyworm larval gut after feeding with pGPMA complexed with dsRNA targeting GFP or (d) sfV-ATPase. Scale bars =2 mm.

other hand, yielded decreasing levels of transfected dsRNA over the observed time period that correspond to a trend of decreasing knockdown. These results suggest that the IPEC provides greater dsRNA protection and retention within the cells, traits that would be advantageous when delivering dsRNA through feeding.

**pGPMA-dsRNA IPEC Gene Suppression in Lepidopteran Larvae after Oral Ingestion.** Having demonstrated that pGPMA-dsRNA IPECs successfully elicit gene knockdown in an otherwise refractory cell line, we evaluated their ability to trigger RNAi in live caterpillars through feeding. RNAi has been used to target WCR V-ATPase through feeding.[13] Thus, we sought to similarly target a fall armyworm V-ATPase ortholog (sfV-ATPase) using pGPMA. Larvae were fed pGPMA-dsRNA IPECs targeting either sfV-ATPase or Green Fluorescent Protein (GFP, control dsRNA). 100 ng of dsRNAs were fed to second or third instar larvae in complex with 8× pGPMA (w/w). Seven days after feeding, total RNA was extracted from


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midguts, and RT-qPCR was performed to determine changes in sfV-ATPase expression (Figure 4a). As in the cell culture experiments, dsRNA delivered by pGPMA resulted in >80% knockdown of the target gene, indicating that pGPMA-dsRNA IPECs can successfully navigate the hostile environment of lepidopteran guts, resulting in gene suppression after feeding.

Because suppression of sfV-ATPase leads to decreased nutrient uptake,[13] such extensive knockdown was expected to result in large increases in larval mortality. However, only moderate larval death (Figure 4b), was observed after 29 days. Such low mortality suggests that the inhibition of gene expression by RNAi is transient, or that knockdown of a different gene may prove more effective. This could be addressed with multiple doses of the IPEC, similar to what would be ingested through continuous feeding on sprayed foliage. In any case, larval mortality was associated with the significant gut hypertrophy expected from decreased nutrient uptake (Figure 4d), as would be expected from sfV-ATPase knockdown. Additionally, when larvae were fed pGPMA alone, no death was observed, even when fed 100× the amount used in the IPEC feeding experiments (Figure S5). These results, along with those of the Sf9 viability assay, suggest low pGPMA toxicity, a necessary requirement for full implementation into crop sprays.

## CONCLUSIONS

*a*RAFT polymerization of pGPMA and complexation with dsRNA has been described. pGPMA successfully delivered dsRNA, targeting genes in a sequence specific manner in otherwise refractory in Sf9 cells. Feeding pGPMA-dsRNA IPECs to fall armyworm larvae likewise caused suppression of target mRNA accumulation, resulting in moderate animal mortality. Furthermore, pGPMA alone seems to be relatively nontoxic to the larvae and exhibited no significant toxicity in Sf9 culture. As previously mentioned, pGPMA has exhibited cytotoxicity toward one cell line,[36] but similar guanidinium-functionalized polymers have exhibited negligible cytotoxicity in a myriad other cell lines.[37−40] To account for this variance, extensive toxicology studies across multiple cell lines will be necessary before implementation into a commercial product.

This is the first time to our knowledge that pGPMA-based polymers have been shown to elicit RNAi in lepidopterans after oral ingestion, a strategy that has heretofore been unsuccessful. The species specificity of RNAi makes this approach attractive from an environmental perspective, and insect inability to develop resistance points to long-term efficiency of this strategy. Furthermore, *a*RAFT polymerization provides access to higher order polymer architectures with tailorable functionalities while maintaining precise control. Thus, RNAi-based pesticides built on this IPEC platform could be candidates for commercial development into crop sprays. Dosing optimization, toxicity studies in animal models, and alterations to the polymer architecture for spray formulation will be necessary to progress this technology and are the subjects of ongoing investigation in our laboratories.

## ASSOCIATED CONTENT

### Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acs.biomac.7b01717.

Primers used in this study and Figures S1−S5, showing $^1$H NMR spectra, hydrodynamic size histograms, Sf9 cells treated with free Cy5-labeled dsRNA, expression of CDC27, and survival of fall armyworm larvae after ingestion of pGPMA (PDF)

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The manuscript was written through contributions of all authors. All authors have given approval to the final version of the manuscript and have contributed equally.

### Notes

The authors declare no competing financial interest.

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