Journal of Materials Chemistry B

Systemically interfering with immune response by a fluorescent cationic dendrimer delivered gene suppression

Journal:	*Journal of Materials Chemistry B*
Manuscript ID:	TB-ART-03-2014-000411.R2
Article Type:	Paper
Date Submitted by the Author:	07-May-2014

Complete List of Authors:	Shen, Dongxu; China Agricultural University,, Department of Entomology Zhou, Fan; China Agricultural University,, Department of Entomology Xu, Zejun; Beijing University of Chemical Technology, He, Bicheng; China Agricultural University, Department of Entomology Li, Miao; China Agricultural University, Department of Entomology Shen, Jie; China Agricultural University,, Department of Entomology Yin, Meizhen; Beijing University of Chemical Technology, ; Max Planck Institute for Polymer Research, An, Chunju; China Agricultural University, Department of Entomology

Journal of

Materials Chemistry B

RSCPublishing

ARTICLE

Systemically interfering with immune response by a fluorescent cationic dendrimer delivered gene suppression

Cite this: DOI: 10.1039/x0xx00000x

Received 00th January 2012,

Accepted 00th January 2012

DOI: 10.1039/x0xx00000x

www.rsc.org/

Dongxu Shen,[a] Fan Zhou,[a] Zejun Xu,[b] Bicheng He,[a] Miao Li,[a] Jie Shen,*[a] Meizhen Yin,*[b] and Chunju An*[a]

A water-soluble cationic dendrimer with a central fluorescent Perylenediimide (PDI) chromophore and many peripheral amines can rapidly penetrate into live hemocytes, gut and fat body. By double fluorescence tracing, the dendrimer is demonstrated with high gene transfection capacity. The synthesized dsRNA targeting at serpin-3, a key immune gene, is systemically delivered by the dendrimer to insect fat bodies and hemocytes out of midgut. Biological assays including PCR and immunoblotting show that the expressions of the target gene and its downstream immunity-related genes are largely suppressed. This study demonstrates for the first time that a PDI-cored cationic dendrimer-mediated dsRNA systemically interferes with the immune response in insects. This work provides an insect model for immunology research and a novel strategy for potential pest control.

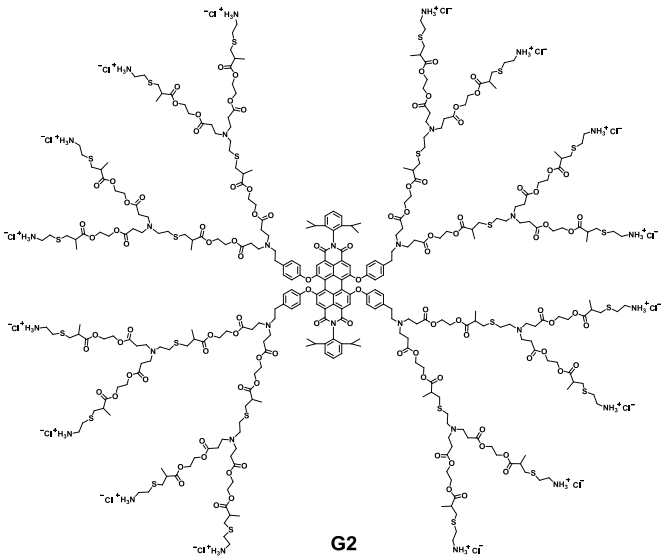
Introduction

Insects have been developed into popular models for scientific research in many biological disciplines, including genetics and development, immunology, biomedicine and chemical biology, due to their advantages of low cost, easy operation, and short experimental period.[1-6] Although the innate immune system of insect is different from that of human and mammals, findings in insects can be extrapolated to mammals due to the evolutionary conservation and homology of immune genes. RNA interference (RNAi) via direct injection or oral feeding of double-stranded RNA (dsRNA) is not only an increasingly important tool for functional analysis of genes, but also affords a new avenue for disease therapy and insect pest control.[7-11] Unfortunately, RNAi often results in failure due to the poor cellular uptake of dsRNA.[12] Therefore, many types of gene carriers have been designed and successfully applied with advantages of low cytotoxicity and efficient gene transfection.[13-16] Among them, functionalized fluorescent nanoparticles (FNPs) could be traced by fluorescence microscopy during the applications, thus, provide a great advantage for research and evaluation.[17-21]

Perylenediimides derivatives (PDIs) have been widely used in various fields because of their exceptional properties in chemical, thermal, and photochemical stability as well as high fluorescence quantum yields in organic solvents.[22-26] We further solved the problem about the easy aggregation of perylene chromophores by synthesizing water-soluble PDI-cored cationic dendrimers with different generations (G1-G3).[27] The central fluorescent PDI chromophore and the peripheral amines provide fluorescence tracing and DNA transfection efficacies for cultured cells, respectively.[27] However, it is still unknown whether this modified fluorescent cationic dendrimer could systemically deliver dsRNA to physiological tissues or organs. Although we had investigated the ability of a fluorescent nanoparticle (FNP) delivering dsRNA previously,[6] we only investigated the interfering effects of FNP-delivered dsRNA on the developmental genes expressed only in midgut but not in other organs out of the gut. Obviously, a general tool with high capacity of systemic gene-delivery would be desired. It would be of high interest and value to explore the dendirmers as a widely applicable tool for systemic gene delivery *in vivo*.

In this study, to explore a widely applicable tool for systemic gene delivery *in vivo*, by carefully comparing the synthesis procedure and the transfection efficacy of the three generation dendrimers, we here choose the second generation cationic dendrimer (G2, **Scheme 1**) for the research on the level of live organs and insects to evaluate gene transfection efficacy. G2 not only efficiently entered into all tested physiological tissues and cells, but also delivered dsRNA and DNA into

insect midgut and fat bodies. We demonstrate that the immune response of insect is successfully interfered through systemic G2-delivered dsRNA targeting at a key immune gene. This is the first report that a PDI-cored cationic dendrimer-mediated dsRNA systemically interferes with the immune response in insects. According to the evolutional conservation of immune response between invertebrates and vertebrates, this work provides a potential tool to study the functions of immune genes in humans and other vertebrates as well as agricultural pest control.



Scheme 1. Chemical structure of G2.

Experimental section

Materials

The second generation PDI-cored cationic dendrimer (G2) was produced as described previously.[27] Schneider's *Drosophila* Medium was purchased from Sigma-Aldrich (St. Louis, MO, USA), and was manually supplemented with 10% fetal bovine serum (FBS) (Gibco, USA), 100 U/mL penicillin and 100 μg/mL streptomycin. For the synthesis of DNA and dsRNA, specific primers (Table S1) were designed based on the cDNA sequence of serpin-3 gene (GenBank™ accession number: KF5014490) (Figure S1). An 821 bp-length serpin-3 DNA fragment was obtained by PCR amplification with the reaction conditions as 95 °C for 5 min and 35 cycles of 95 °C for 30 s, 55 °C for 30 s, and 72 °C for 1 min followed by incubating at 72 °C for 5 min. dsRNA of serpin-3 and green fluorescent protein (GFP) were prepared using T7 RiboMAX expression RNAi system (Promega, USA) according to the manufacturer's protocol. The resulted DNA or dsRNA (6.4 μM) was labeled with 0.5 μM CXR Reference Dye (Promega, USA) according to the manufacturer's instructions.

Insect rearing, tissue dissection, and hemocyte isolation

All insect experiments were performed in compliance with the relevant laws and institutional guidelines and had been approved by the institutional committee. Asian corn borer, *Ostrinia furnacalis* (Guenée), were reared on an artificial diet at 26℃ and 80% RH with a photoperiod of 16:8 L:D. Day 1 third-instar larvae were rinsed with 70% alcohol, and dissected to obtain intact midgut and fat bodies under microscope. For hemocyte collection, hemolymph from 5 fifth-star larvae was centrifuged at 1,800 rpm for 2 min. The hemocyte pellet was washed three times with sterile phosphate-buffered saline (PBS), and suspended in 50 μL of Schneider's insect medium for use.

In vitro and *in vivo* tissue uptake of G2 alone

The newly-synthesized fluorescent cationic dendrimer G2 was incubated with dissected midguts, fat bodies, and hemocytes at the final concentration of 6.33 μM for 8 hours at room temperature. The tissue and cell uptake of G2 was recorded by fluorescence microscopy. In the assay for the tissue uptake G2 *in vivo*, 15 μg of G2 was mixed with 55 mg of fresh artificial diet, and then fed to the newly-hatched larva. 72 hours later, the midgut was dissected and imaged under fluorescence microscopy.

In vitro delivery of dsRNA and DNA by G2

The above CXR-labeled *serpin-3* dsRNA or DNA (3.2 μmol) was pre-treated with G2 (332.5 μmol) at N/P ratio (charge ratio of G2/DNA) of 2:1 at room temperature, and then incubated with dissected midguts, fat bodies, or hemocytes in a total volume of 50 μL. After 8 h, the fluorescent images were obtained by fluorescence microscopy.

In vivo delivery of dsRNA by G2

Totally, 14 μg *GFP* or *serpin-3* dsRNA was mixed with 18 μg G2 (N/P ratio of 2:1), then injected into 55 mg of fresh artificial diet. This diet was separated into 4 portions, and then fed to the newly-hatched larvae individually to ensure that each portion was completely swallowed by the larvae. On day 5, all larvae were injected with a bacteria *Micrococcus luteus* (10 μg/μL, 3 μL/larva) for the stimulation of immune response,[28] and then moved to the normal artificial diet without dsRNA and G2. 24 hours later, total RNA samples were collected from these larvae, and subjected to the analysis of transcriptional changes of serpin-3 and other immune-related genes by quantitative reverse transcript-PCR (qRT-PCR).[29] One μL of hemolymph was subjected to 12 % SDS-PAGE and immunoblot analysis[30] to check the serpin-3 protein level change.

Effects of feeding with G2 alone on the larval growth and development

Immediately after hatching from eggs, twenty Asian corn borer larvae were fed with the artificial diet containing 55 mg of G2 or water (as a control), respectively, until they developed into pupae. During the whole experimental period, the survival and developmental status of each larva in two groups was observed and recorded. Prior to the pupal stage, the body size and body weight of each larva were measured and analyzed statistically.

Results

G2 efficiently enters into insect physiological tissues and cells

We previously synthesized water-soluble PDI-cored cationic dendrimers with different generations which showed good water solubility (>10 mg/mL) and high photostability.[27] By comparing carefully the synthesis procedure and the transfection efficacy of the three generation dendrimers, in this study, the second generation cationic dendrimer G2 was selected for the current research. The fluorescent dendrimer G2 shows good water solubility (>10 mg/mL) and high photostability. The maximum absorption and emission of G2 in water are around 591 and 617 nm, respectively (Figure S2). Previous study only demonstrated that G2 could efficiently enter into cultured *Drosophila* cells with high gene transfection efficacy.[27] G2 can be rapidly internalized into cells within 1 h incubation[27] while traditional gene carrier PEI can not enter into cells within 6 h incubation[31]. G2 also has an advantage of low cytotoxicity. Cell viability of 2μM G2 treatment (>93%)[27] is much higher than the 1μM PEI treatment (<10%)[31]. Here we further investigate the ability of G2 to internalize into more complicated physiological tissues or cells. We dissected midguts and fat bodies (major tissue for the expression of immunity-related genes, and analogous to mammal lives) from an important agriculture insect pest, Asian corn borer, *O. furnacalis*, larvae. The obtained tissues were incubated with G2 medium. After 8 h, the presence of G2 in insect tissues was examined under fluorescence microscopy. As shown in Figure 1, strong fluorescent signals were detected in both midguts and fat bodies. This suggests that G2 is able to internalize into insect tissues efficiently and rapidly. Additionally, we collected hemocytes directly from *O. furncalis* hemolymph to investigate the ability of G2 to enter insect autologous cells other than commercial cells cultured *in vitro*. Six types of hemocytes, including granulocyte (Gr), plasmatocyte (Pl), prohemocyte (Pr), oenocytoid (Oe), spherulocyte (Sp) and cystocyte (Cy), are present in *O. furnacalis* hemolymph.[32] Among them, granulocytes and plasmatocytes are the two hemocyte classes involved in *O. furnacalis* immune responses, and granulocyte is most abundant among all six hemocyte classes.[32] We have isolated all types of hemocytes and detected the G2 fluorescence in them. Due to the variance of cell contents in insect hemolymph, Figure 1C-1C'' only shows the images of granulocytes, plasmatocytes, and spherulocytes as examples. Altogether, G2 has a strong ability to enter into insect physiological tissues and cells.

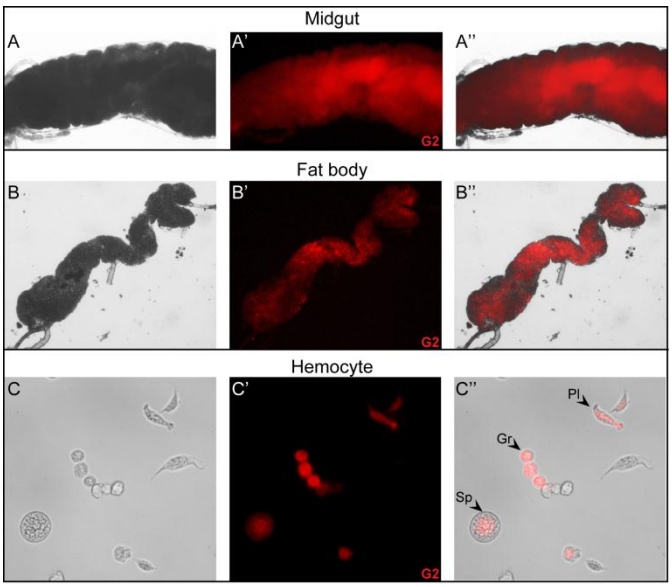


Figure 1. Fluorescence images of G2 internalized into *O. furnacalis* tissues or cells, including midgut (A-A''), fat body (B-B''), and hemocyte (C-C''). A, B, and C: separated channels for tissues or cells only. A', B', and C': separated channels for G2 (red). A'', B'', and C'': merged images. Three types of hemocyte classes are indicated by arrowheads. Gr: granulocyte; Pl: plasmatocyte; Sp: spherulocyte.

G2/DNA complexes

The peripheral positive charges in G2 contribute to its high tendency to bind to negatively charged macromolecules such as DNA and dsRNA.[27] For efficient gene delivery, G2 and nucleic acid have to form stable complexes in solution. To test the complex stability of G2/nucleic acid, gel electrophoresis was performed to test the gel retardation of the complexes at various N/P ratios. As shown in Figure S4, the movement of DNA band was prevented at the N/P ratio of 2:1. This suggests that G2 has high potential to form stable complexes with nucleic acid at low N/P ratio. While commercial PEI requires high N/P ratio for delivering DNA.[33] The measured size of the G2/DNA complexes is 136.5±3.4 nm (Table S2), suggesting an efficient DNA condensation. The zeta-potentials of G2 before and after it forms complexes with DNA are shown in Table S3. The zeta-potential value of the complexes is an indicator of surface charges of the complexes, indicating the efficient DNA binding to G2.

G2 efficiently delivers both dsRNA and DNA into various tissues and hemocytes

Comparing with the efficient tissue uptake of G2 alone, the potential of G2 delivering DNA or dsRNA into insect tissues is more attractive. Therefore, we synthesized a 473-bp dsRNA fragment with T7 RiboMAX expression RNAi system, and labeled it with a blue-fluorescence CXR Reference Dye. After mixed with G2 at an N/P ratio of 2:1, the dissected *O. furnacalis* tissues or isolated hemocytes were incubated with G2/dsRNA complexes solution. The delivery of dsRNA by G2 *in vitro* was determined by observing the fluorescence distributions of G2 and the CXR Reference Dye labelled dsRNA in tissues or cells. Under fluorescent microscope, the

red signals from G2 and the blue signals from dsRNA completely merged (Figure 2). Meanwhile, we carried out the similar experiments to investigate the ability of G2 delivering DNA into insect tissues. As shown in Figure S3, the fluorescent signals for G2 and DNA also merged well in the observed midguts, fat bodies, and hemocytes. These results suggest that the G2 is indeed able to deliver both dsRNA and DNA into various tissues and hemocytes. This endues G2 with high potential as a non-viral vector to deliver genes into the complicated tissues or cells.

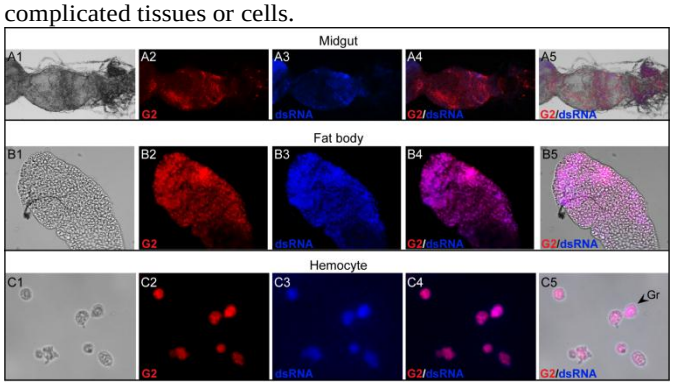


Figure 2. Fluorescence images of G2/dsRNA complexes (N/P= 2:1) after 8 h incubation with *O. furnacalis* midgut (A1-A5), fat body (B1-B5), and hemocyte (C1-C5). A1, B1 and C1: separated channels for tissues or cells only. A2, B2, and C2: separated channels for G2 (red). A3, B3, and C3: separated channels for dsRNA labeled by CXR Reference Dye (blue). A4, B4, and C4: merged images from the second and third one on the left. A5, B5, and C5: merged images from the three ones on the left. Gr: granulocyte.

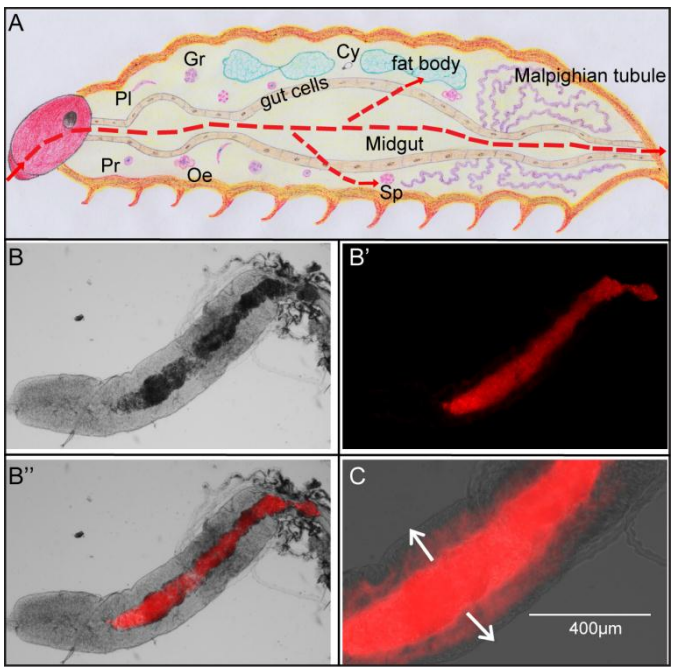


Figure 3. Fluorescence images of dissected *O. furnacalis* gut containing G2. (A) Schematic diagram for longitudinal section of *O. furnacalis* larva. The red dashed arrow indicates the spreading line of ingested G2 in insect. The abbreviations of hemocytes mean as follows: granulocyte (Gr), plasmatocyte (Pl), prohemocyte (Pr), oenocytoid (Oe), spherulocyte (Sp), and cystocyte (Cy). (B-B'') *O. furnacalis* larvae are fed with artificial diet containing G2. B: separated channels for midgut only. B': separated channels for the G2 (red). B'': merged image from B and B'. C: enlarged one for B'', red: the G2. The diffusion of G2 to gut cells is indicated by white arrows.

G2 can enter into the insect gut cells through the simple oral feeding

To develop G2 into a promising tool for systemic gene delivery *in vivo*, we need to consider the way G2 delivers genes. Given that oral feeding is the simplest way to deliver dsRNA into insects compared to the microinjection and transgenetic expression, we applied efforts in the investigation about the efficacy of G2 to deliver genes via oral feeding. For this aim, we firstly checked whether strategy of oral feeding can make G2 itself enter into insect gut cells, and even into other tissues out of the gut (Figure 3A). We mixed 15 μg of G2 with 55 mg of fresh artificial diet, fed this diet to the newly-hatched larvae, and observed the distribution of G2 in midgut three days later. As displayed in Figure 3B-3B'', red fluorescence was still stable in the midgut after three-day feeding. When the fluorescence images were recorded at higher magnification for details, red fluorescence denoting G2 penetrated into the gut cells (Figure 3C). The result indicates that G2 has efficiently entered into the gut cells through oral feeding method.

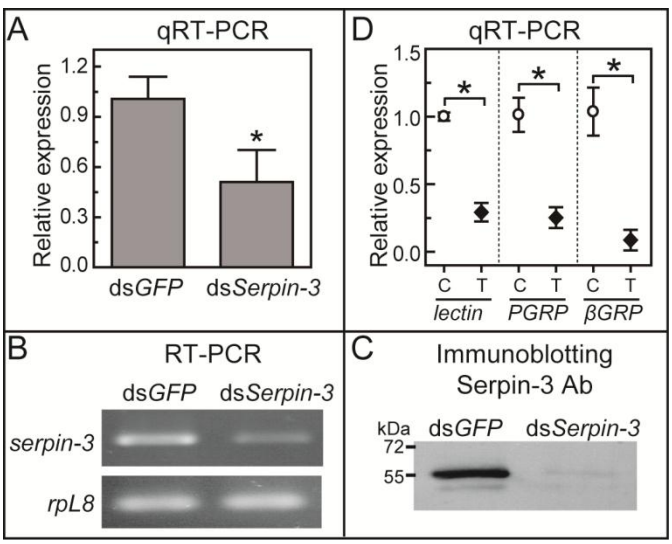


Figure 4. Gene silencing by the complex of dsRNA/G2 via oral feeding. The newly-hatched larvae are fed on 55 mg of fresh artificial diet containing 14 μg of G2-bound *serpin-3* or *GFP* (used as control) dsRNA within 4 days. (A) qRT-PCR analysis of serpin-3 transcript level. The *serpin-3* mRNA level is reduced roughly by 51%. Asterisk indicates a significant difference (unpaired *t* test, $p < 0.05$). (B) RT-PCR analysis. The transcription levels of unaffected ribosomal gene *rpL8* are used as an internal standard. Note that serpin-3 transcription is apparently suppressed as revealed by the weaker band in gel electrophoresis. (C) Immunoblot analysis with serpin-3 antiserum as primary antibodies. The serpin-3 protein is severely abolished from the hemolymph, presented as undetectable in SDS-PAGE. (D) Relative transcript levels of indicated genes in the larvae fed on G2/*serpin-3* dsRNA (*T*) or G2/*GFP* dsRNA (*C*). Symbols represent mean ± S.D. (n =3). Asterisks indicate means that are significantly different from the control (unpaired *t* test, $p < 0.05$). Note the expressions of all tested immunity-related genes are significantly reduced in serpin-3-suppressed larvae.

G2-delivered dsRNA systemically interfere the immune response of insect pest

In order to study the efficacy of G2-delivered dsRNA systemically interfering with the immune response, we chose a serine protease inhibitor gene, *O. furncalis serpin-3*, as the tested gene. Serpin-3 is a vital gene for immune response, and expressed mainly in fat bodies and hemocytes.[34] We firstly synthesized *serpin-3* dsRNA, and *GFP* dsRNA as control. Immediately after hatching from eggs, the *O. furnacalis* larvae were individually fed on an artificial diet containing G2-bound *serpin-3* or *GFP* dsRNA. Then all larvae were injected with a bacteria *M. luteus* to stimulate the immune response. After 24 hours, *serpin-3* knock-down efficiency was assayed by three different methods including qRT-PCR, RT-PCR, and immunoblotting. The *serpin-3* transcript level was reduced by 51% as compared with larvae fed on the diet containing *GFP* dsRNA (Figure 4A). In RT-PCR analysis, the band corresponding to *serpin-3* was consistently present at lower intensity in *serpin-3* dsRNA-fed larvae (Figure 4B). RNA analysis even underestimated the extent of gene silencing. Indeed, immunoblot analysis using specific antibodies against *O. furnacalis* serpin-3 showed a complete absence of serpin-3 protein after feeding of *serpin-3* dsRNA (Figure 4C). The results obtained from three different methods all verified the expression of *serpin-3* was effectively knocked down, with the help of G2.

We also performed qRT-PCR analysis to check the effects of the repression of *serpin-3* on the expression of immunity-related genes. Three serpin-3-downstream genes such as *lectin*, *PGRP* and *βGRP* were chosen as indicators. As shown in Figure 4D, compared with the control with G2-bound *GFP* dsRNA, G2-mediated *serpin-3* depletion significantly reduced the transcription levels of *lectin*, *PGRP* and *βGRP* in response to bacterial stimuli. Therefore, the immune response of insect pest is successfully and systemically interfered by G2-delivered dsRNA targeting.

On the other hand, we also investigated the effects of G2-mediated *serpin-3* silencing on the expression of *serpin-3*-unrelated immune genes such as *cecropin*, *gloverin*, *moricin*, *Imd* and *Toll*. As shown in Figure 5, there was no significant difference for the expression of these *serpin-3*-unrelated immune genes in the larvae fed on G2/serpin-3 dsRNA or G2/GFP dsRNA. It suggests that the interfered immune response observed in Figure 4D is determined by G2-delivered dsRNA, but not by G2. G2 itself has no effect on the immune response. Taken together, these results not only suggest that G2-mediated dsRNA indeed successfully interferes with insect immune response, but also suggest that this interfering is targeted and determined by the specific dsRNA. Therefore, this work provides a valuable tool to study the *in vivo* function of special immune genes in insects.

Figure 5. Relative transcript levels of indicated genes in the larvae fed on G2/serpin-3 dsRNA (T) or G2/GFP dsRNA (C). Symbols represent mean ± S.D. (n =3). Note the expression of these 5 serpin-3-unrelated immune genes has no significant difference in serpin-3-suppressed larvae although the transcript levels of the serpin-3-downstream genes including lectin, PGRP and βGRP are indeed significantly reduced (Figure 4D).

G2 alone has no long term toxicity on the larval growth and development

Although G2 does not affect insect immune response, we still need to check whether G2 alone has long term toxicity on the larval growth and development. Newly-hatched *O. furnacalis* larvae were continually fed on an artificial diet containing G2 or normal artificial diet, respectively. All tested larvae in both control and treated groups survived to adulthood (Figure 6A). The body size and body weight of fifth instar larvae in each group had no significant difference (Figure 6B and 6C). Moreover, all larvae developed normally into pupae (Figure 6D). Altogether, it suggests that the feeding of G2 alone has no toxicity on the larval growth and development, and any possible abnormal performance after feeding with G2/dsRNA should come from the gene interference of special dsRNA. This ensures the necessary safety of G2 as a potential delivery tool for the *in vivo* application.

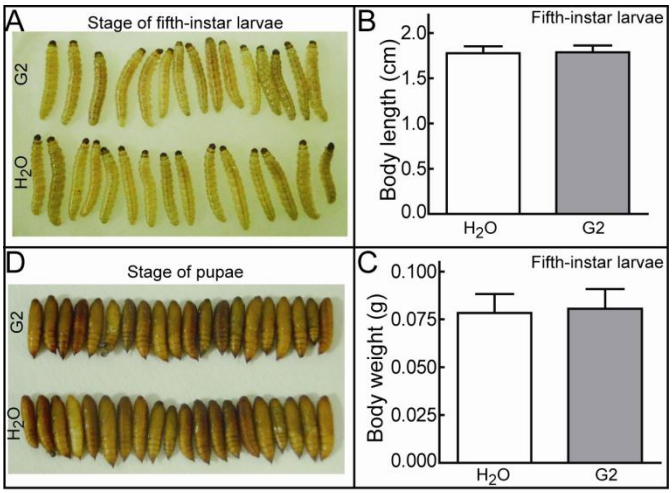


Figure 6. Feeding with G2 alone has no toxicity on the larval growth. The newly-hatched larvae (20 larvae/group) are continually fed with the artificial diet containing G2 or water until they developed into pupae. (A) Compare with the control, no difference about the growth of the larvae is observed. Here only shows the image of fifth-instar larvae as an example. (B) The body sizes of fifth-instar larvae in two groups have no significant difference ($P > 0.1$ in pairwise comparison, $n = 20$). (C) The body weights of fifth-instar larvae in two groups have no

significant difference (P > 0.1 in pairwise comparison, n = 20). (D) All larvae in two groups develop into pupae normally.

Discussion

In this study, we demonstrated that a water-soluble PDI-cored cationic dendrimer G2 not only efficiently penetrated into all tested physiological cells, tissues and organs, but also delivered dsRNA and DNA into insect midgut and fat bodies. Via the simple oral feeding, G2-delivered dsRNA successfully diminished the expression of a key immune gene, *serpin-3*, in the fat bodies. The immune response of insect was thus systemically interfered. This is the first time to report that a PDI-cored cationic dendrimer-mediated dsRNA systemically interferes with the immune response in insects. As regards the evolutional conservation of immune response between invertebrates and vertebrates, this work provides a potential tool to study the functions of immune genes in humans and other vertebrates.

Our previous work investigated the ability of G2 to internalize into cultured exogenous S2 cells.[27] It is significantly different for a delivery system to internalize into cultured cells *in vitro* or into live animals *in vivo*. Here we described that G2 also efficiently entered into various complicated tissues or organs including dissected midgut and fat bodies, and isolated hemocytes (Figure 1). Moreover, G2 could excellently bind to negatively charged dsRNA and DNA in virtue of the positive charges provided by its peripheral primary amines, and delivered them into the physiological tissues and cells (Figure 2). One main challenge that needs to be overcome before RNAi can really reach wide application in human disease treatment and pest control is that dsRNA cannot enter a cell without the aid of a delivery vehicle.[35] Our results made G2 highly potential to work as a non-viral gene delivery system.

It is noteworthy that the simple oral feeding was enough for G2 to penetrate into gut cells (Figure 3). So far, direct injection of dsRNA is the most commonly used delivery method for RNAi. However, injection method is highly technically demanding and time consuming, and sometimes is restricted in certain insect species such as small and/or aquatic species.[36, 37] Oral feeding methods undoubtedly avoids these drawbacks. Oral feeding of G2 alone had no long-term toxicity on insect growth and development (Figure 6). It further demonstrates that G2 fulfill the requirements of gene carrier for the *in vivo* application.

It is known that gene silencing through dsRNA is difficult to achieve in Lepidoptera (moth and butterflies).[7-11] We also failed to acquire the detectable *serpin-3* silencing by feeding the larvae with serpin-3 dsRNA alone (data not shown). In a few successful cases, gene silencing by feeding dsRNA alone requires high concentrations (higher than 1 μg dsRNA/mg diet).[8, 9] Based on the efficient tissue uptake and high gene delivery efficacy of G2, *serpin-3* was successfully knocked down at the concentration of as low as 0.25 μg dsRNA/mg diet in our study. Furthermore, it is noteworthy that *serpin-3* is not a midgut-specific gene. We previously reported the silencing effects of FNP-delivered dsRNA on the developmental genes expressed only in midgut.[6] As shown in Figure 3A, it is much more difficult for fed dsRNA to systemically spread to other tissues out of the midgut such as fat bodies and hemocytes, compared with entering into the gut cells. The results from the current study firstly demonstrated that an ingested PDI-cored cationic dendrimer G2 carries dsRNA penetrates gut cells, spreads from gut cells to the circulating hemolymph, and then enters into fat bodies and hemocytes, where the expression level of targeted gene, *serpin-3*, is suppressed. Moreover, serpin-3 is a vital gene for immune response. The *serpin-3* depletion significantly reduced the transcription levels of *serpin-3*-downstream genes, such as *lectin*, *PGRP* and *βGRP* in response to bacterial stimuli (Figure 4). Therefore, our results indicated that oral feeding with G2/dsRNA could achieve systemic RNA interference and act on the complicated immune response. It gives insights to the future studies about developing new methods for pest control through dsRNA targeting at immune or other genes expressed in midguts or not.

Except for G2, the third generation PDI-cored cationic dendrimer G3 also possesses the high transfection efficacy, even slightly higher than G2.[27] However, the synthesis procedure of G2 is much easier than that of G3.[27] Since we aimed to afford a widely usable tool for gene delivery *in vivo*, here we still chose G2 as the tested dendrimer. In view of the similarities of G2 and G3 on the structure and efficacy, we believe that the results about G2 could be reasonably extrapolated to G3. Further experiments are required to test this hypothesis.

Compared with commercial DNA carrier such as PEI, G2 has many advantages including fluorescence detection, low cytotoxicity, rapid cellular internalization, and low N/P ratio for delivering nucleic acid. Cell viability of 2μM G2 treatment (>93%)[27] is much higher than the 1μM PEI treatment (<10%)[31]. Cellular G2 fluorescence can be observed within 1 h incubation,[27] while PEI is not fluorescent and needs longer incubation time for gene delivery[31]. As shown in the gel retardation test (Figure S4), the movement of DNA band was prevented at the N/P ratio of 2:1, suggesting a high capacity of G2 for gene delivery at low N/P ratio. While PEI delivers DNA only at high N/P ratio.[33] These advantages can be attributed to G2's nice structure with precise surface charges which contribute to the high efficiency of gene delivery and low cytotoxicity.

Conclusions

In this study, we chose an insect pest, Asian corn borer larvae, to explore a way of systemically suppressing immune response. A water-soluble fluorescent PDI-cored cationic dendrimer G2 could rapidly penetrate into all tested physiological cells, tissues and organs. This dendrimer was also able to deliver negatively charged dsRNA and DNA into insect cells and tissues. More importantly, only via oral feeding, G2

successfully delivered the dsRNA of serpin-3, a key immune gene, into fat bodies and hemocytes out of midgut. This systemic delivery induced the large suppression on the expression of target gene, and further interfered with insect immune response after the bacteria stimuli. This is the first example of a PDI-cored cationic dendrimer-mediated dsRNA that systemically interferes with the immune response on insect model. This work provides a novel strategy for pest control and a potential tool to study the functions of immune genes in other vertebrates because of the evolutional conservation of immune response between invertebrates and vertebrates.

Acknowledgements

This work was supported by the 973 Program (2013CB114102 and 2013CB127603), the National Natural Science Foundation of China (31172090, 21174012, 51103008 and 51221002), the Beijing Natural Science Foundation (2142026), the Program for New Century Excellent Talents in University (NCET-10-0215 and NCET-11-0476), and the Special Fund for Agro-scientific Research in the Public Interest (201003025).

Notes and references

[a] Department of Entomology, China Agricultural University, 100193 Beijing, China, E-mail: shenjie@cau.edu.cn; anchunju@cau.edu.cn

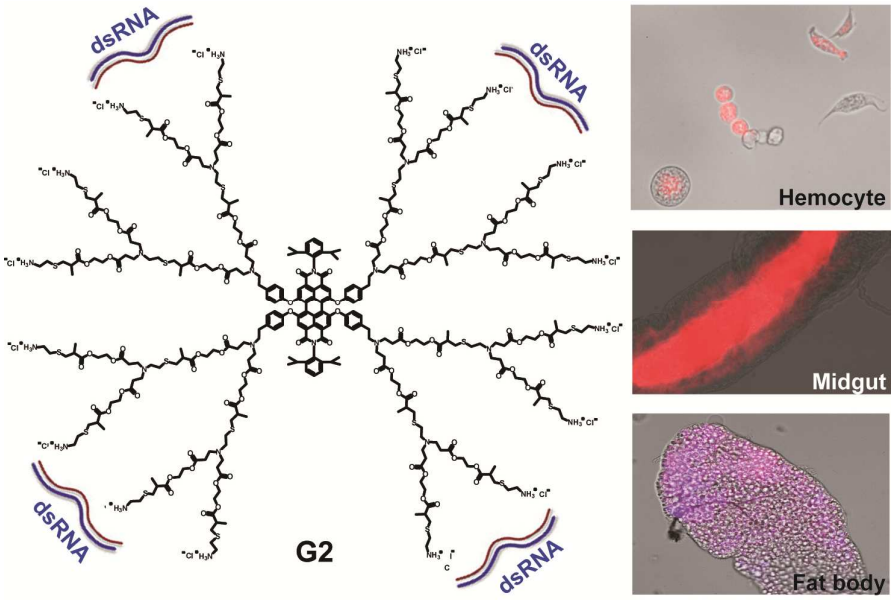
[b] State Key Laboratory of Chemical Resource Engineering, Key Laboratory of Carbon Fiber and Functional Polymers, Ministry of Education, Beijing University of Chemical Technology, 100029 Beijing, China, E-mail: yinmz@mail.buct.edu.cn

Electronic Supplementary Information (ESI) available: See DOI: 10.1039/b000000x/

References

1 X. Zhang, D. Luo, G. O. Pflugfelder and J. Shen, *Development*, 2013, **140**, 2917.
2 C. An, A. Budd, M. R. Kanost and K. Michel, *Cel. Mol. Life. Sci.*, 2011, **68**, 1929.
3 M. Yin, J. Shen, G. O. Pflugfelder and K. Müllen, *J. Am. Chem. Soc*., 2008, **130**, 7806.
4 M. Yin, J. Shen, R. Gropeanu, G. O. Pflugfelder, T. Weil and K. Müllen, *Small*, 2008, **4**, 894.
5 J. Li, K. Guo, J. Shen, W. Yang and M. Yin, *Small*, 2014, **10**, 1351.
6 B. He, Y. Chu, M. Yin, K. Müllen, C. An and J. Shen, *Adv. Mater.*, 2013, **25**, 4580.
7 O. Terenius, A. Papanicolaou, J. S. Garbutt, I. Eleftherianos, H. Huvenne, S. Kanginakudru, M. Albrechtsen, C. An, J. L. Aymeric, A. Barthel, P. Bebas, K. Bitra, A. Bravo, F. Chevalier, D. P. Collinge, C. M. Crava, R. A. de Maagd, B. Duvic, M. Erlandson, I. Faye, G. Felföldi, H. Fujiwara, R. Futahashi, A. S. Gandhe, H. S. Gatehouse, L. N. Gatehouse, J. M. Giebultowicz, I. Gómez, C. J. Grimmelikhuijzen, A. T. Groot, F. Hauser, D. G. Heckel, D. D. Hegedus, S. Hrycaj, L. Huang, J. J. Hull, K. Iatrou, M. Iga, M. R. Kanost, J. Kotwica, C. Li, J. Li, J. Liu, M. Lundmark, S. Matsumoto, M. Meyering-Vos, P. J. Millichap, A. Monteiro, N. Mrinal, T. Niimi, D. Nowara, A. Ohnishi, V. Oostra, K. Ozaki, M. Papakonstantinou, A. Popadic, M. V. Rajam, S. Saenko, R. M. Simpson, M. Soberón, M. R. Strand, S. Tomita, U. Toprak, P. Wang, C. W. Wee, S. Whyard, W. Zhang, J. Nagaraju, R. H. Ffrench-Constant, S. Herrero, K. Gordon, L. Swevers and G. Smagghe, *J. Insect Physiol.*, 2011, **57**, 231.
8 M. A. Bautista, T. Miyata, K. Miura and T. Tanaka, *Insect Biochem. Mol. Biol.*, 2009, **39**, 38.
9 S. Whyard, A. D. Singh and S. Wong, *Insect Biochem. Mol. Biol.*, 2009, **39**, 824.
10 S. Tomita and A. Kikuchi, *Dev. Biol.*, 2009, **328**, 403.
11 D. R. G. Price and J. A. Gatehouse, *Trends. Biotechnol.*, 2008, **26**, 393.
12 S. C. Miller, S. J. Brown and Y. Tomoyasu, *Dev. Genes. Evol.*, 2008, **218**, 505.
13 F. Dai, W. Liu, *Biomaterials*, 2011, **32**, 628.
14 J. Xia, L. Chen, H. Tian, X. Chen, A. Maruyama and T. Park, *J. Control. Release*, 2011, **152**, 176.
15 R. Dong, L. Zhou, J. Wu, C. Tu, Y. Su, B. Zhu, H. Gu, D. Yan and X. Zhu, *Chem. Commun.*, 2011, **47**, 5473.
16 W. Qu, S. Chen, S. Ren, X.-j. Jiang, R.-x. Zhuo and X.-z. Zhang, *Chin. J. Polym. Sci.*, 2013, **31**, 713.
17 L. Albertazzi, B. Storti, L. Marchetti and F. Beltram, *J. Am. Chem. Soc.*, 2010, **132**, 18158.
18 Q. Zhu, F. Qiu, B. Zhu and X. Zhu, *RSC. Adv.*, 2013, **3**, 2071.
19 C. Zhu, L. Liu, Q. Yang, F. Lv and S. Wang, *Chem. Rev.*, 2012, **112**, 4687.
20 M. Chen and M. Yin, *Prog. Polym. Sci.*, 2014, **39**, 365.
21 M. Yin, C. Feng, J. Shen, Y. Yu, Z. Xu, W. Yang, W. Knoll and K. Müllen, *Small*, 2011, **7**, 1629.
22 T. Weil, T. Vosch, J. Hofkens, K. Peneva and K. Müllen, *Angew. Chem. Int. Ed.*, 2010, **49**, 9068.
23 O. Bozdemir, M. Yilmaz, O. Buyukcakir, A. Siemiarczuk, M. Tutas and E. Akkaya, *New. J. Chem.*, 2010, **34**, 151.
24 L. Zhu, W. Wu, M. Zhu, J. Han, J. Hurst and D. Li, *J. Am. Chem. Soc.*, 2007, **129**, 3524.
25 J. Pan, W. Zhu, S. Li, J. Xu and H. Tian, *Eur. J. Org. Chem.*, 2006, **4**, 986.
26 T. Nguyen, D. Türp, D. Wang, B. Nölscher, F. Laquai and K. Müllen, *J. Am. Chem. Soc.*, 2011, **133**, 11194.
27 Z. Xu, B. He, J. Shen, W. Yang and M. Yin, *Chem. Commun.*, 2013, **49**, 3646.
28 C. An, J. Ishibashi, E. J. Ragan, H. Jiang and M. R. Kanost, *J. Biol. Chem.*, 2009, **284**, 19716.
29 K. J. Livak and T. D. Schmittgen, *Methods*, 2001, **25**, 402.
30 C. An and M. R. Kanost, *Insect Biochem. Mol. Biol.*, 2010, **40**, 683.
31 Z. Xu, B. He, W. Wei, K. Liu, M. Yin, W. Yang, and J. Shen. *J. Mater. Chem. B*, 2014, DOI: 10.1039/C4TB00195H
32 M. D. Lavine and M. R. Strand, *Insect Biochem. Mol. Biol.*, 2001, **32**, 1295.
33 Y. Chen, L. Zhou, Y. Pang, W. Huang, F. Qiu, X. Jiang, X. Zhu, D. Yan and Q. Chen, *Bioconjugate Chem.*, 2011, **22**, 1162
34 Y. Zhu, Y. Wang, M. J. Gorman, H. Jiang and M. R. Kanost, *J. Biol. Chem.*, 2003, **278**, 46556.
35 D. Peer and J. Lieberman, *Gene. Ther.*, 2011, **18**, 1127.
36 F. M. Nunes and Z. L. Simões, *Insect Biochem. Mol. Biol.*, 2009, **3**9, 157.
37 D. P. Walshe, S. M. Lehane, M. J. Lehane and L. R. Haines, *Insect Mol. Biol.*, 2009, **18**, 11.

A table of contents entry.



A water-soluble fluorescent cationic dendrimer systemically delivers dsRNA into insect cells and tissues resulting in the suppression of immune gene.