Visualization of the Process of a Nanocarrier-Mediated Gene Delivery: Stabilization, Endocytosis, Endosomal Escape and Exocytosis of Genes for Intercellular Spreading

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Short Report

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Abstract

Nanoparticles have been widely applied as gene carrier for improving RNA interference (RNAi) efficiency in medical and agricultural fields. However, the mechanism and delivery process of nanoparticle-mediated RNAi is not directly visualized. Here we synthesized a star polymer (SPc) consisted of a hydrophilic shell with positively-charged tertiary amine in the side chain as a gene nanocarrier, which was taken as an example to investigate the mechanism in gene delivery. The SPc could assemble with dsRNA spontaneously through electrostatic force, hydrogen bond and van der Waals force. Interestingly, the SPc could protect dsRNA from degradation by RNase A, insect hemolymph and immune cells, thus remarkably increasing the stability of dsRNA. Meanwhile, the SPc could efficiently promote the cellular uptake, endosomal escape and active exocytosis for systematic spreading of dsRNA. Transcriptome analysis revealed that the SPc could up-regulate some key genes such as *Chc*, *AP2S1* and *Arf1* for activating clathrin-mediated endocytosis. Furthermore, the suppression of endocytosis by a specific inhibitor hindered the cellular uptake of SPc-delivered dsRNA *in vitro*, and the subsequent RNAi effect was also disappeared *in vivo*. To our knowledge, our study is the first direct visualization of the detailed cellular delivery process and mechanism of nanocarrier-mediated gene delivery. Above mechanism supports the application of nanocarrier-based RNAi in gene therapy and pest management.

Introduction

RNA interference (RNAi) functions via post-transcriptional gene silencing and is highly conserved in eukaryotic organisms. RNAi technique is widely used to interfere gene function and shows a great potential for gene therapy and pest management in medical and agricultural fields.^{1–6} Gene therapy is a promising tool for the treatment of several diseases, including cancer, central nervous system disorders and metabolic disorders, but the major limitation of small interfering RNA (siRNA) or double-stranded RNA (dsRNA) is the inability of naked siRNA/dsRNA to passively diffuse through cellular membrane due to the electrostatic repulsion from the anionic cell membrane surface.^{7–10} Meanwhile, siRNA/dsRNA is highly susceptible to nuclease-induced degradation and thus their therapeutic actions are limited.^{8,11} Similar to the application in medical field, RNAi has also been recognized as a novel and safe strategy in pest green management.^{6,12–15} However, RNAi efficiency varies greatly among different insect species, and the major limitations for efficient RNAi include dsRNA instability, low efficiency of dsRNA cellular internalization, deficient core RNAi machinery and impaired systemic spreading of dsRNA, which constrains the application of RNAi-based gene therapy and pest management.^{16–21}

Nano-delivery systems for drugs and other bioactive molecules in medical and agricultural fields have been bloomed over the past 30 years.^{22,23} Several types of synthetic gene vectors such as cationic lipids, chitosan and quantum dots have been designed and constructed to overcome the delivery obstacle for efficient RNAi.^{24–27} Nanocarrier-mediated dsRNA delivery was firstly described in the silencing of *chitin synthase* genes in African malaria mosquito (*Anopheles gambiae*), and the *chitin synthase1* transcript level and chitin content were reduced by 62.8% and 33.8% in the larvae fed on chitosan-delivered

dsRNA.²⁸ Subsequently, the nanocarrier-mediated RNAi was tested in several insect species, and high RNAi efficiency has been observed in fall armyworm *Spodoptera frugiperda*,²⁹ Yellow fever mosquito *Aedes aegypti*,³⁰ beet armyworm *S. exigua*³¹ and German cockroach *Blattella germanica*³². Thus, the nanoparticle-mediated RNAi has a good potential in pest sustainable control, but the detailed delivery process and mechanism of nanoparticle-mediated RNAi have not been directly visualized.

Previous studies have implied that the application of nano-delivery system might solve the systemic and intracellular barriers such as rapid degradation and poor cellular uptake.^{33–36} Nanoparticles, such as polymers and cationic lipids, may protect dsRNA molecules from enzymatic degradation and improve their stability in various insect tissues.^{31,37} On the other hand, nanoparticles may promote the translocation of dsRNA across cell membranes, thus improving dsRNA delivery. Based on current knowledge, receptor-mediated endocytosis may be the major route for nanocarriers to enter into the cells.^{38–40} Nanocarriers are usually coated by membrane-bound vesicles called endosomes following cellular uptake, and the late endosomes ultimately fuse with degradative lysosomes.^{41,42} Therefore, endosomal escape has become another key step for nano-delivery systems.⁴³ Although there are some studies focusing on nanoparticle-mediated dsRNA delivery in therapeutic applications, the mechanism for nanoparticle-mediated RNAi should be further directly visualized.

Our group has constructed a nanocarrier-based platform for pest management to deliver various pesticidal substances, such as dsRNA^{44,45}, insect virus DNA⁴⁶, narrow-spectrum pesticides⁴⁷ and Bt toxins⁴⁸. To decrease the production cost of nanoparticles for field application, a facile-synthesized star polycation (SPc) is designed to construct a transdermal dsRNA delivery system for controlling soybean aphids, which has been selected for Research Highlights from China collection by Springer Nature^{49,50}. Subsequently, the SPc-delivered dsRNA is applied to disrupt the wing development of fruit flies and green peach aphids and inhibit the feeding behavior of oriental fruit moths^{51–53}. So far, the SPc has been widely shared with researchers in China, and the transdermal dsRNA delivery system has been successfully applied in more than 30 insect species. The current study focused on the mechanism and delivery process of nanoparticle-mediated RNAi. We took SPc as an example, determined the interaction of SPc with dsRNA, tested the stability of SPc-complexed dsRNA, investigated the cellular uptake and intracellular fate of dsRNA/SPc complex, and finally confirmed the major gene pathway for the cellular uptake of dsRNA/SPc complex. Our study directly visualized the detailed cellular process and mechanism of polymer-mediated RNAi, which supports the development and practice of RNAi-based gene therapy and pest management.

Results And Discussion

Loading Capacity of SPc and Its Interaction with dsRNA

The SPc was designed and constructed as a gene vector for efficient dsRNA delivery, achieving good gene silencing effects on insects.^{49–53} However, the interactions between SPc and dsRNA were not very

clear. To investigate the loading capacity of SPc toward dsRNA, *dseGFP* was mixed with SPc at various mass ratios and analyzed using gel retardation test. As shown in Fig. 1A, the band's intensity of the migrated *dseGFP* gradually decreased with the increasing mass ratios, indicating that the SPc had excellent performance in combining with dsRNA. The SPc is consisted of a hydrophilic shell with positively-charged tertiary amine in the side chain. We deduced that the tertiary amine could combine with negatively-charged nucleic acids through electrostatic interaction, which resulted in the electronegativity loss of dsRNA.

ITC was then performed to further illustrate the interaction between dsRNA and SPc (Fig. 1B). A high affinity constant (K_a) of $5.526 \times 10^7 \text{ M}^{-1}$ and a low dissociation constant (K_d) of $1.810 \times 10^{-8} \text{ M}$ indicate that there is an effective and strong interaction between SPc and *dseGFP*, and this interaction is automatic due to the negative ΔG of -43.9 KJ/mol . The negative ΔH and ΔS suggest that the hydrogen bond and van der Waals force also play an important role in the self-assembly of *dseGFP*/SPc complex.⁵⁴ Based on the chemical structure, multifunctional SPc can be complexed with exogenous substances through various interaction forces such as hydrogen bond and van der Waals force with chitosan, electrostatic interaction with eugenol, thiamethoxam and osthole, and hydrophobic interaction with matrine.⁵⁵⁻⁵⁸ Different self-assembly mechanisms of SPc with exogenous substances are beneficial for expanding the application area of SPc.

Efficient Protective Effects of Nanocarriers on dsRNA

The degradation of dsRNA is very fast,⁵⁹ which was also confirmed by our results that the *dseGFP* could be degraded quickly by RNase A (Fig. 2A). Interestingly, there was no significant change in band density of SPc-complexed *dseGFP* treated with RNase A (Fig. 2B-B'). The SPc-complexed *dseGFP* was purified and quantified, and the results demonstrated that RNase A could not degrade SPc-loaded dsRNA (Fig. 2C). Similarly, the SPc could also prevent *dseGFP* from degradation by hemolymph, providing a strong protection (Fig. S1). Meanwhile, a perylenediimide-cored cationic dendrimer (G2) previously constructed by our group was also tested in the current study.⁶⁰ The stability of G2-complexed dsRNA was also improved remarkably (Fig. S2 and S3). Similar to previous studies, a guanidine-containing polymer is able to protect dsRNA against nucleolytic degradation at pH 11 in gut juice of beet armyworms for up to 30 h.³¹ Cationic lipids can protect dsRNA from degradation by endonuclease present in Sf9 cells conditioned medium, hemolymph and midgut lumen contents collected from the larvae of *S. frugiperda*.³⁷

In addition to the fast digestion of free dsRNA by dsRNases, immune cells in hemolymph also attack exotic dsRNA.^{61,62} Fluorescent *dseGFP* was synthesized for the stability test of nanocarrier-complexed dsRNA in confrontation with immune cells. The combination of G2 with *dseGFP* changed the fluorescence intensity of both *dseGFP* and G2, and the addition of SPc did not lead to such effects, suggesting that the SPc is fit for investigating the fate of nanocarrier-delivered fluorescent dsRNA in immune cells (Fig. S4). Fluorescent signal was strongly detectable in immune cells treated with fluorescent *dseGFP*/SPc complex (Fig. 2E-E'), whereas the signal of naked *dseGFP* was undetectable in

immune cells (Fig. 2D-D'). Our results indicate that the dsRNA/SPc complex could remain relatively stable in immune cells of *S. frugiperda*.

SPc-Mediated Efficient Delivery of dsRNA

Previous study has confirmed that the dsRNA could be taken up by Sf9 cells within 30 min of incubation.⁶³ In the current study, both dseGFP/SPc complex and naked dseGFP could be taken up by Sf9 cells quickly. However, much higher fluorescent intensity was observed with dseGFP/SPc complex, and this phenomenon was more obvious after 12 h incubation, indicating that the SPc could promote the cellular uptake of dsRNA (Fig. 3A-B and Fig. S5). Compared to dsRNA, delivery of naked siRNA remains a considerable hurdle owing to inefficient cellular uptake.⁶⁴ Saleh et al.⁶⁵ demonstrated that long dsRNA bound to cells and was localized in large puncta in the cell interior, and low-level binding and no obvious internalization of siRNA were observed. As shown in Fig. S6, siRNA alone exhibited no uptake by Sf9 cells, but the cellular uptake of siRNA was remarkably improved with the help of SPc, suggesting that the SPc could efficiently deliver both long and short dsRNA across the cell membrane. Our previous study has confirmed that the SPc could combine with nucleic acids and the zeta potential of formed complex was still positive.⁴⁹ The slight positive electricity of dsRNA/SPc or siRNA/SPc complex was beneficial for its adhesion to cell membrane.

Exogenous substances are commonly taken up through endocytosis, and the endocytic vesicles generated by dsRNA/nanoparticle complex travel along microtubules and subsequently fuse with early endosomes.^{27,66} As shown in Fig. 3C-E' and Fig. S7, the fluorescence signal was firstly located in the early vesicles close to the inner membrane of cells treated with dseGFP/SPc complex following the cellular uptake, and the complex was then dispersed into the cytoplasm quickly, suggesting that the dsRNA could be released from the vesicles. The dsRNA/SPc complex should exit endosome to avoid the degradation in late endosome (lysosome).^{67,68} The "proton sponge" hypothesis is the most generally accepted mechanism of endosomal escape, although it is heavily debated.⁶⁹⁻⁷¹ Many cationic polymers have a strong buffering capacity over a range of pH between 5 and 7, and the acidic environment in late endosome can lead to the protonation of their amine groups, thereby causing a water influx that leads to endosome lysis.^{27,72-73} Compared to naked dsRNA, there was no accumulation of SPc-delivered dsRNA in the late endosomes labelled by a specific antibody (Fig. 3F-G'), indicating that the SPc could promote the endosomal escape of dsRNA. Similar to a previous study, lipid formulated dsRNA exhibits reduced accumulation in the endosomes of Sf9 cells.³⁷

The exocytosis and intercellular spreading of dsRNA are important for systemic RNAi, which seems to be less efficient in lepidopteran species.^{63,74} To directly visualize the potential exocytic vesicles of dseGFP/SPc complex, when the cellular uptake reached saturation, the culture medium containing fluorescent dseGFP/SPc complex was immediately replaced with fresh medium. The fluorescent signal was accumulated and located close to the inner membrane of cells (Fig. 3H-H' and Fig. S8), which might represent the exocytosis of the complex. Therefore, we deduced that the SPc could promote the excretion

and intercellular spreading of dsRNA to improve systemic RNAi although we failed to detect the fluorescence signal outside the cells, which might be due to the rapid diffusion of dsRNA in culture medium. A model was further proposed to illustrate the cellular uptake and intracellular trafficking of SPc-delivered dsRNA (Fig. 3I).

SPc Activates the Clathrin-Mediated Endocytosis for Enhanced dsRNA Delivery

To identify the crucial regulators during cellular uptake of SPc-delivered dsRNA, transcriptome sequencing was performed *in vitro*. About 77% (116/150) of up-regulated genes and 74% (73/99) of down-regulated genes were enriched in the section of $\log_2$ (fold change) = [0.58, 1] (Fig. 4A). The unigenes were divided into three main categories such as biological process, cellular component and molecular function (Fig. 4B). As expected, differentially expressed genes (DEGs) were enriched in various signaling pathways, and the endocytic pathway was activated by the application of dseGFP/SPc complex significantly. As crucial genes regulating endocytosis and exocytosis, *AP2S1*, *Arf1*, *Rab11*, *CHMP5* and *GRK* were significantly up-regulated in cells treated with dsRNA/SPc complex (Fig. 4C). *AP2S1* encodes the sigma subunit of the Adaptor Protein 2 complex, which drives endocytic vesicle formation at the plasma membrane.^{75,76} ARF protein regulates vesicular traffic and organelle structure by recruiting coat proteins, which plays a crucial role in fundamental biological processes, such as endocytosis, secretion, phagocytosis etc.^{77,78} Surprisingly, the expression of *Chc* gene, which encodes a major structural polypeptide of the surface lattice of clathrin-coated pits and vesicles,^{79,80} was not significant changed. Thus, we further performed a qRT-PCR analysis of *Chc*, *AP2S1* and *Arf1* at various incubation time points (Fig. 4D). Three genes were all up-regulated in cells treated with dsRNA/SPc complex, suggesting that the SPc might promote the cellular uptake of dsRNA by activating clathrin-mediated endocytosis.

Previous studies revealed that the clathrin-mediated endocytosis might be involved in RNAi responses of *Drosophila* S2 cells and red flour beetles using pharmacological block.^{65,81} The role of clathrin-mediated endocytosis in the cellular uptake of dsRNA/SPc complex was further confirmed *in vitro* and *in vivo* using the similar method. Our results indicated that the inhibitor application hindered the cellular uptake of SPc-delivered dseGFP, and the SPc-delivered dsATP-d lost biological function toward larvae fed with inhibitor, confirming that the clathrin-mediated endocytosis was the major pathway for the cellular uptake of SPc-delivered dsRNA (Fig. 5).

Conclusions

In summary, a star polymer (SPc) consisted of a hydrophilic shell with positively-charged tertiary amine in the side chain was synthesized as a gene nanocarrier, which was taken as an example to illustrate the mechanism and detailed process of nanoparticle-mediated RNAi. The SPc could assemble with dsRNA spontaneously through electrostatic force, hydrogen bond and van der Waals force. Interestingly, the SPc and another nanocarrier G2 could protect dsRNA from degradation by RNase A, insect hemolymph and immune cells remarkably. With the help of SPc, dsRNA could be efficiently taken up by cells, successfully

released from early endosome, diffused into the cytoplasm, and transmitted among cells for systemic RNAi. Transcriptome analysis revealed that the SPc could up-regulate some key genes such as *Chc*, *AP2S1* and *Arf1* for activating clathrin-mediated endocytosis pathway. Furthermore, the application of a specific inhibitor hindered the cellular uptake of dsRNA/SPc complex *in vitro*, and the RNAi effect was also disappeared *in vivo*. Our study thoroughly revealed the process how nanocarrier deliver dsRNA and this mechanism supports wide application of nanocarrier-delivered gene for therapy and pest control.

Methods

Chemicals

The SPc and PDI-cored cationic dendrimer G2 were synthesized according to the methods described by Li et al.⁴⁹ and Xu et al.⁶⁰, respectively. For SPc synthesis, the star initiator Pt-Br was synthesized using pentaerythritol, and the polymerization of star initiator with DMAEMA was then carried out. For G2 synthesis, the synthesis strategy was started from amine-functionalized PDI. The four primary amines selectively reacted with the acrylate group in 2-methacryloyloxyethyl acrylate to prepare the intermediate product, and the methacrylates at the periphery reacted with the thiol group in cysteamine to further prepare the G2.

Cell Culture and Insect Rearing

The Sf9 cells from ovaries of fall armyworms (*S. frugiperda*) were cultured in Sf-900 II SFM Medium (Gibco, USA) at 27°C supplemented with 10% fetal bovine serum, penicillin (100 unit/mL) and streptomycin (100 µg/mL). Larvae of *S. frugiperda* were fed on an artificial diet bought from Tuidongzhe Biotechnology Co. (China), and reared under a photoperiod of 16 h light: 8 h dark at 25°C.

Gel Retardation Test and Isothermal Titration Calorimetry (ITC) Assays

The *enhanced green fluorescent protein* gene (*eGFP*) was selected for dsRNA synthesis using the T7 RiboMAX expression (Promega, USA). All primers (Table S1) were synthesized by Tsingke (China). A gel retardation test was firstly performed to determine the best mass ratio for the combination of dsRNA with nanoparticle. One µg *dseGFP* was mixed with SPc or G2 at various mass ratios, and the mixture (10 µL) was incubated at room temperature for 15 min and then analyzed by agarose gel electrophoresis. To determine the interaction of dsRNA with SPc, the 5.1 µM SPc was titrated with 0.333 µM *dseGFP* in Nano ITC (TA Instruments Waters, USA). The heats of interaction during each injection were calculated by the integration of each titration peak via the Origin7 software (OriginLab Co., USA). The test temperature was 25°C, and the ΔG was calculated using the formula of $\Delta G = \Delta H - T\Delta S$.

Stability Test of Nanocarrier-Complexed dsRNA

To determine the stability of dsRNA, one µg *dseGFP* was added with RNase A (Sigma-Aldrich, USA) to prepare the reaction solution (*dseGFP*: 100 ng/µL), and the mixture was incubated for 20 min at 37°C.

The hemolymph collected from 5th instar larvae of *S. frugiperda*⁸² was diluted with 1×PBS and mixed with 1 µg *dseGFP*, and the mixture was incubated for 1.5 h at room temperature. The agarose gel electrophoresis was used to detect the integrity of dsRNA treated with RNase A or hemolymph.

To investigate the stability of nanocarrier-complexed dsRNA, dsRNA was mixed with SPc and G2 at the best mass ratio, respectively. The RNase A was used to treat *dseGFP*/SPc or *dseGFP*/G2 complex (*dseGFP*: 1 µg). For the test of SPc-complexed dsRNA, the *dseGFP*/SPc complex was decomplexed in 0.3% SDS solution and analyzed using agarose gel electrophoresis. The relative band density was determined using ImageJ 1.48v (National Institutes of Health, USA). Meanwhile, decomplexed *dseGFP* was purified using MEGAclear Kit (Thermo Fisher Scientific, USA), and quantified using NanoDrop 2000 spectrophotometer (Thermo Fisher Scientific). Naked *dseGFP* was applied as control, and each treatment was repeated 3 times. The stability test of G2-complexed dsRNA was performed using the method described by Liu et al.⁴⁷ Changes in absorption spectra of *dseGFP*/G2 complex treated with RNase A were detected using NanoDrop 2000 spectrophotometer. The stability of *dseGFP*/SPc or *dseGFP*/G2 complex treated with hemolymph was analyzed according to the method of *dseGFP*/SPc complex treated with RNase A.

For the stability test of nanocarrier-complexed dsRNA in confrontation with immune cells, 100 µL of above hemolymph was added with 400 µL Sf-900 II SFM Medium. Fluorescent *dseGFP* was synthesized,⁶³ and then mixed with SPc and G2 at the best mass ratio, respectively. Changes in fluorescence intensity of *dseGFP* complexed with SPc or G2 were then tested using NanoDrop 2000 spectrophotometer. The complexation of *dseGFP* with G2 influenced the fluorescence intensity of *dseGFP*, and therefore SPc was used in the following experiments. The *dseGFP* and *dseGFP*/SPc complex were incubated with hemolymph for 3 h (*dseGFP*: 500 ng). The cells were washed, fixed with 4% paraformaldehyde, deposited on the slides using an Antifade Mounting Medium with DAPI (Vector Laboratories, USA), and then examined using a confocal microscope (Leica SP8, Germany).

Cellular Uptake and Intracellular Trafficking of SPc-Delivered dsRNA/siRNA

Fluorescent *dseGFP* and *sieGFP* (GenePharma Co., China) were used to determine the cellular uptake of SPc-delivered dsRNA/siRNA. The cells were treated with *dseGFP*, *dseGFP*/SPc complex, *sieGFP* and *sieGFP*/SPc complex, respectively (*dseGFP*: 500 ng; *sieGFP*: 280 ng). Cells were imaged using a confocal microscope at various time points after incubation. The time-lapse imaging for tracing *dseGFP*/SPc complex was conducted using an inverted fluorescence microscope (Olympus, Japan) after 1 h of incubation, followed by a qualification of the intracellular fluorescence intensity by Surface Plot Program using ImageJ 1.48v at various time points.

To investigate the intracellular trafficking of SPc-delivered dsRNA, cells incubated for 6 h were re-suspended in fresh medium for 30 min, and then imaged using an inverted fluorescence microscope. The intracellular fluorescence intensity was determined using Surface Plot Program similarly. Meanwhile, above cells were deposited on the slides, fixed with 4% paraformaldehyde, treated with 0.1% Triton X-100,

and then blocked with 0.2% bovine serum albumin. Blocked samples were incubated with primary antibody mouse anti-Rab 7 (1:200, DSHB, USA) overnight, incubated with secondary antibody goat anti-mouse Cy5 (1:200, Jackson, USA) for 1.5 h, and then examined using a confocal microscope.

Transcriptome Analysis

Cells were collected at 6 h after incubation with *dseGFP* and *dseGFP*/SPc complex (*dseGFP*: 500 ng), respectively. Total RNA was extracted from three biological replicates using the TRNzol formulation (TIANGEN, China). The transcript libraries were constructed via Illumina HiSeq sequencing platform. Raw reads containing connectors and with low-quality ($Q \leq 10$) were removed. The resulting clean reads were assembled using Trinity software.⁸³ TopHat2 was used to achieve the sequence alignment of clean reads with the reference genome (<http://www.insect-genome.com/Sfru/>).⁸⁴ BLASTX was used to compare unigene sequences with the Non-Redundant protein sequence database (NR) and Swiss-Prot database for annotation of unigenes. The expression level of each transcript was presented by FPKM value. Deseq was applied for differential expression analysis between transcripts, and fold change ≥ 1.5 and FDR < 0.05 were screen conditions.⁸⁵

The expression levels of endocytosis-related gene *AP2S1*, *Arf* and *Chc* were determined using quantitative real-time PCR (qRT-PCR) at various time points after the treatment of *dseGFP* or *dseGFP*/SPc complex (*dseGFP*: 500 ng). All primers for qRT-PCR were shown in Table S2. The qRT-PCR was performed with Step One Plus Real-Time PCR system (Applied Biosystems, USA) using Power SYBR® Green Master Mix (Applied Biosystems). The *actin* and *ribosomal protein S15 (RPS-15)* were selected as reference genes, and the relative mRNA levels of target genes were normalized to the abundance of two genes using the $2^{-\Delta\Delta CT}$ methods.⁸⁶

Pharmacological Inhibitors of SPc-Delivered dsRNA

Bafilomycin-A (Baf A) (MCE, USA) was used to inhibit clathrin-mediated endocytosis to determine the major pathway for SPc-mediated cellular uptake.⁶⁵ For *in vitro* test, the cells were exposed to 0.2 μ M Baf A (MCE, USA) for 30 min, treated with fluorescent *dseGFP*/SPc complex, deposited on the slides using an Antifade Mounting Medium with DAPI, and then examined using a confocal microscope. For *in vivo* test, the *V-type proton ATPase subunit d (ATP-d)* was selected as a target gene, and the ds*ATP-d* was mixed with SPc and 1% volume of surfactant Alkyl Polyglucoside (Wanhua, China) to prepare the ds*ATP-d*/SPc complex formulation (dsRNA and SPc: both 1 μ g/ μ L). The 2-day-old larvae of *S. frugiperda* were fed with Baf A for 2 d, and the 0.5 μ L formulation was applied directly on the notum of 4-day-old larvae. The relative mRNA level of *ATP-d* was evaluated using qRT-PCR at 24 h after the topical application. Each treatment consisted of 3 replications.

Statistical Analysis

The ANOVA with Tukey HSD test was conducted using SPSS 20.0 (SPSS Inc., USA) at the $P=0.05$ level of significance. A two-tailed Student's *t*-test was performed using Prism 7.0 (GraphPad Software Inc., USA)

at the $P=0.05$ level of significance. The descriptive statistics were shown as the mean value and standard errors of the mean.

Declarations

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Authors' contributions

Conceptualization, SY and JS; Methodology, SY, JS, ZM and MY; Formal Analysis, SY, JS, ZM and MY; Investigation, ZM, YZ, ZC, HC and YZ; Writing-Original Draft, SY; Writing-Review & Editing, SY, JS, ZM and MY; Funding Acquisition, SY, JS and YZ; Supervision, SY and JS.

ZM and YZ contributed equally to this work.

Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Conflicts of Interest

The authors declare no competing financial interest.

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Figures

Figure 1

Self-assembly mechanism of dsRNA/SPc complex. (A) Gel electrophoresis assay of *dseGFP* retardation by SPc at various mass ratios. M: DNA marker. (B) ITC titration of *dseGFP* (0.333 μM) into SPc solution (5.1 μM).

Figure 2

Enhanced stability of SPc-complexed dsRNA. (A) The *dseGFP* degradation by RNase A. M: DNA marker. (B-B') Gel electrophoresis assay (B) and relative band density (B') of SPc-complexed *dseGFP* treated with RNase A. (C) Relative dsRNA amount of SPc-complexed *dseGFP* treated with RNase A. Statistical analysis was conducted using independent *t*-test at the $P=0.05$ level of significance. (D-E') Stability test of naked *dseGFP* (D-D') or SPc-complexed *dseGFP* (E-E') treated with immune cells. Blue: DAPI. Green: *dseGFP*.

Figure 3

Improved endocytosis and exocytosis of dsRNA/SPc complex. (A-B) Cellular uptake of naked dseGFP (A) and dseGFP/SPc complex (B). Blue: DAPI. Green: dseGFP. (C-E') Vesicle release of SPc-delivered dseGFP into cytoplasm. A surface plot test was conducted using ImageJ. Three typical regions were marked. (F-G') Endosomal escape of SPc-delivered dsRNA. Red: Rab7 marking the late endosome. (H-H') Potential exocytosis of dsRNA/SPc complex. The dseGFP/SPc complex was accumulated and located close to the inner membrane of cells. (I) Schematic illustration of cellular uptake and intracellular trafficking of SPc-delivered dsRNA.

Figure 4

Comparative transcriptome analysis of Sf9 cells treated with naked dseGFP and dseGFP/SPc complex. (A-B) Analysis of differentially expressed genes (DEGs) with volcano plot (A) and GO annotation (B). (C) Heatmap of the endocytic pathway. (D) QRT-PCR analysis of the genes related to clathrin-mediated endocytosis. Asterisk indicates significant difference in gene expression between dseGFP/SPc complex and naked dseGFP treatment according to independent *t*-test (ns.: no significant difference, **P*<0.05, ***P*<0.01, ****P*<0.001 and *****P*<0.0001).

Figure 5

Pharmacological inhibitor blocks the cellular uptake of dsRNA/SPc complex. (A-B') Inhibitor application hindered the cellular uptake of dseGFP/SPc complex. Blue: DAPI. Green: dseGFP. (C) Schematic diagram of pharmacological assay toward *S. frugiperda*. The 2-day-old larvae were fed with Baf A for 2 days. Then, RNAi was performed using 4-d-old larvae. (D) QRT-PCR analysis of *ATP-d* gene expression in larvae fed with inhibitor. Different letters on columns indicate significant differences (Tukey HSD test, $P < 0.05$).

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