Research Article

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Oral delivery of dsRNA lipoplexes to German cockroach protects dsRNA from degradation and induces RNAi response

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Abstract

BACKGROUND: In the past years, the concept of RNAi application for insect pest control has been proposed, considering the disruption of vital genes. However, the efficiency of RNAi is variable between different insect groups, especially by oral delivery of dsRNA. The purpose of this study is to assess the possibilities of RNAi as a tool for pest control using oral delivery of the dsRNAs encapsulated by liposome in the German cockroach *Blattella germanica*, which is highly sensitive to RNAi by injection of dsRNAs.

RESULTS: Injecting dsRNA into the abdomen of *B. germanica* caused dramatic depletion of essential *α-tubulin* gene and mortality. In contrast, oral delivery of the naked dsRNA resulted in lower RNAi efficiency, accounting for rapid degradation of the dsRNA in the midgut of *B. germanica*. Notably, we have further demonstrated that continuous ingestion of dsRNA lipoplexes in which dsRNA was encapsulated with a cationic liposome carrier was sufficient to slow down the degradation of dsRNA in the midgut and to increase the mortality of the German cockroach by significantly inhibiting *α-tubulin* expression in the midgut.

CONCLUSION: We provide empirical evidence that the formulation of dsRNA lipoplexes could be a plausible approach for insect pest control based on RNAi.

Supporting information may be found in the online version of this article.

Keywords: RNAi; ribonuclease; liposome; *α-tubulin*; pest control

1 INTRODUCTION

RNA interference (RNAi) is an endogenous mechanism that regulates gene expression at post-transcriptional level through the action of small non-coding RNAs, such as small interfering RNAs (siRNAs), microRNAs (miRNAs) or piwi-interacting RNAs (piRNAs), and it is conserved among eukaryotes.[1] Delivery of double-stranded RNA (dsRNA) in insects can induce the cascades of RNAi pathway by which the enzyme Dicer2 cleaves the dsRNA into fragments of about 21–22 nucleotides (the siRNAs) that bind to the RNA-induced silencing complex (RISC), which recognises the target mRNA, binds to it and blocks its translation.[2] Owing to its highly specific silencing effect on gene transcription, and because many insect taxa are very sensitive to the dsRNA-induced knock-down effect, RNAi has become an important tool for unveiling the functions and interactions of genes in insects.[3]

This immediately suggests that RNAi could also be envisaged as an insect control tool through targeting vital genes.[4] Many studies have suggested this possibility by different approaches of dsRNA administration, including feeding on transgenic plants expressing the dsRNAs or artificial diets, injection and even soaking.[5–11] However, oral delivery of dsRNAs, which might be a practical approach for the control of pest insects, has not worked in many insect species studied following this delivery system.[12–15] The negative results are considered to be instability of dsRNA, which is probably digested by nucleases, and their poor cellular uptake by different tissues.[1,16]

The purpose of this study is to assess the possibilities of RNAi as a tool for pest control, using oral delivery of the dsRNAs encapsulated by liposome and the German cockroach *Blattella germanica*, which is highly sensitive to RNAi by injection of dsRNAs, as a model. Whyard *et al.*[17] have shown that the RNAi response is facilitated when the insect is fed with dsRNAs encapsulated in liposome complexes. However, the mechanism by which liposome facilitates the RNAi effect in insects has been less well addressed. The use of liposomes as insecticide carriers has been applied previously in *B. germanica*, but not as dsRNA carrier.[18] Here, we report the use of liposomes to encapsulate the dsRNA and examine the RNAi response after oral administration. Owing to the vital function of the *α-tubulin* (*tub*) gene, silencing the expression of *tub* mRNA is expected to cause a lethal effect in the German cockroach. Our data have shown that encapsulating the dsRNA of the vital *tub* gene with a liposome carrier, which slows down enzyme degradation of dsRNA in the midgut juice, results in a significant

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reduction in target gene expression in the midgut. In addition, the significant depletion of *tub* expression in the midgut, but not apparently in other tissues, most likely accounts for the mortality of the German cockroach after feeding on dsRNA lipoplexes for 16 days continuously.

2 MATERIALS AND METHODS

2.1 Insects

B. germanica colonies were maintained with dog chow and water in an environmental chamber under a 16:8 light:dark cycle at 28 °C. Further information on cockroach maintenance has been previously described in detail.[19] In this study, 10–13-day-old adult virgin males were used in the following experiments.

2.2 Preparation of dsRNA

The dsRNA was synthesised using MEGAscript T7 kit (Ambion, Grand Island, NY). The sequence of fragment targeting 151 nucleotides of the *tub* gene (GeneBank accession number KX228233) (supporting information Fig. S1) was amplified by PCR with the forward primer 5′-GGG ACA AGC CGG AGT GCA GA-3′ and reverse primer 5′-TCC TGC TCC TGT CTC GCT GA-3′. The dsRNA synthesis template was constructed using the PCR product with primers containing the T7 promoter sequence 5′-TAA TAC GAC TCA CTA TAG GG-3′. In addition, the dsRNA of *enhanced green fluorescence protein* (*EGFP*) sequence was used as a control, and the PCR product targeting 520 nucleotides was amplified by the forward primer 5′-TAA TAC GAC TCA CTA TAG GGT ATG GTG AGC AAG GGC GAG GAG-3′ and reverse primer 5′-TAA TAC GAC TCA CTA TAG GGT GGC GGA TCT TGA AGT TCA CC-3′, which contained the T7 promoter sequence from the plasmid containing *EGFP* fragment constructed in our lab previously.[20] Synthesis of the dsRNA reaction followed the manufacturer's protocol, and the dsRNAs were stored at −20 °C until use.

2.3 Preparation of dsRNA lipoplexes

To prepare a dsRNA lipoplex solution, the given amount of dsRNA was first diluted in the same volume of 5% glucose solution and then mixed with an equal volume of liposome solution, which was prepared by mixing GenJet™ Plus DNA *in vitro* Transfection reagent (SignaGen Laboratories, Rockville, MD) and 5% glucose solution (1:1 ratio in volume), following the manufacturer's protocol. Different amounts of dsRNA (0.25, 0.5, 1.0 and 2.0 μg) were used to examine the efficiency of the encapsulation with 1 μL of GenJet Transfection reagent. For the dsRNA feeding assay, the dsRNA lipoplex solution (0.25 μg of dsRNA and 1 μL of GenJet Transfection reagent) was freshly prepared as previously mentioned before use.

2.4 RNAi by injection and ingestion

For the dsRNA injection experiments, 1 μL of dsRNA solution (2 or 4 μg μL^{-1}) was injected into the abdomen of each male cockroach using a Microliter syringe (Chrom Tech, Apple Valley, MN).

For the dsRNA ingestion experiments, each cockroach was fed twice a day with 4 μL of dsRNA lipoplex solution (0.0625 μg μL^{-1} of dsRNA with liposome) or naked dsRNA solution (0.0625 μg μL^{-1} without liposome), and other control groups without dsRNA treatment were fed with the same amount of liposome solution, 5% glucose solution or saline buffer respectively. All the cockroaches were deprived of water in the period of the feeding assay and acquired water only through manual feeding using a pipetman (supporting information Fig. S2). Note that all the cockroaches in the period of the feeding experiments acquired the exact amount of dsRNA.

For the survival assay, each cohort of 12–15 individuals with injection or feeding of dsRNA as mentioned previously was monitored every day, and three independent experiments were conducted.

2.5 Quantitative real-time PCR (qRT-PCR)

Total RNA was extracted from the head, antennae, midgut, Malpighian tubules or fat body of male cockroaches. The samples were homogenised with 200 μL of TRIzol (Invitrogen, Carlsbad, CA), and total RNA was separated from protein and DNA by adding 40 μL of chloroform. About 100 μL of the upper layer (aqueous phase) of each sample was transferred into a new tube, and total RNA was purified using the HiYield™ Total RNA Extraction kit protocol (ARROWTEC, Taipei, Taiwan). All the RNA samples were treated with DNase to remove genomic DNA contamination. Synthesis of complementary DNA with 1 μg of total RNA was performed using High Capacity cDNA Reverse Transcription kit (Applied Biosystems, Foster City, CA), following the manufacturer's protocol. To design the qPCR primers, the sequence of the *tub* gene was cloned and obtained in our transcriptome data. The forward primer was 5′-GGA CCG CAT CAG GAA ACT GGC-3′ and the reverse primer was 5′-CCA CAG ACA GCC TCT CCA TGA GC-3′. In addition, the *elongation factor 1A* (*EF1A*) gene (GeneBank accession number KX228232) of *B. germanica* was selected as the housekeeping gene, and its forward primer was 5′-ACC AAT CTC TGG ATG GCA TGG-3′ and its reverse primer was 5′-GAG GCT TCT CAG TGG GTC TG-3′. All primers used in the present study had an efficiency of >96%. Quantitative real-time PCR was performed in triplicate with Fast SYBR Green Master Mix (ABI) on the StepOnePlus real-time system. Cycling conditions were 95 °C for 3 min, followed by 40 cycles of 95 °C for 30 s and 60 °C for 30 s. The reaction was concluded with the melt curve beginning from 60 to 95°C in 0.5 °C increments at 5 s $step^{-1}$. Moreover, each assay was run with a non-template control reaction to avoid nucleic acid contamination.

2.6 Collection of haemolymph and midgut juice

A haemolymph sample was collected from an incision on the coxa of a hind leg of *B. germanica* from six individuals by using a pipetman; then the haemocytes were removed by centrifuging at 3000 rpm for 10 min at 4 °C. For collecting midgut juice, midguts from six individuals of *B. germanica* were explanted under a microscope and directly transferred into 100 μL of saline. For complete retention of the gut fluid from the midgut and removal of the haemocytes, the sample was vortexed slightly for 10 s and then centrifuged at 3000 rpm for 10 min at 4 °C. The resulting supernatants from the centrifuged haemolymph or midgut juice samples were regarded as the original concentration (1×) for later use. All samples were measured at the A280 spectrum by Nanodrop and normalised to the same protein concentration.

2.7 dsRNA degradation assay

The naked dsTub or dsTub lipoplexes containing 1 μg of dsRNA were added to 10 μL of midgut juice or haemolymph respectively. In the separated samples, 2 μL of EGTA (20 mM) was added to inhibit the enzyme activity. After treatment, all the dsRNA samples were processed using TRIzol extraction to isolate dsRNA from liposome conjugation. Thereafter, the purified dsRNA was analysed by electrophoresis in 1.5% agarose gel to examine the results of degradation.

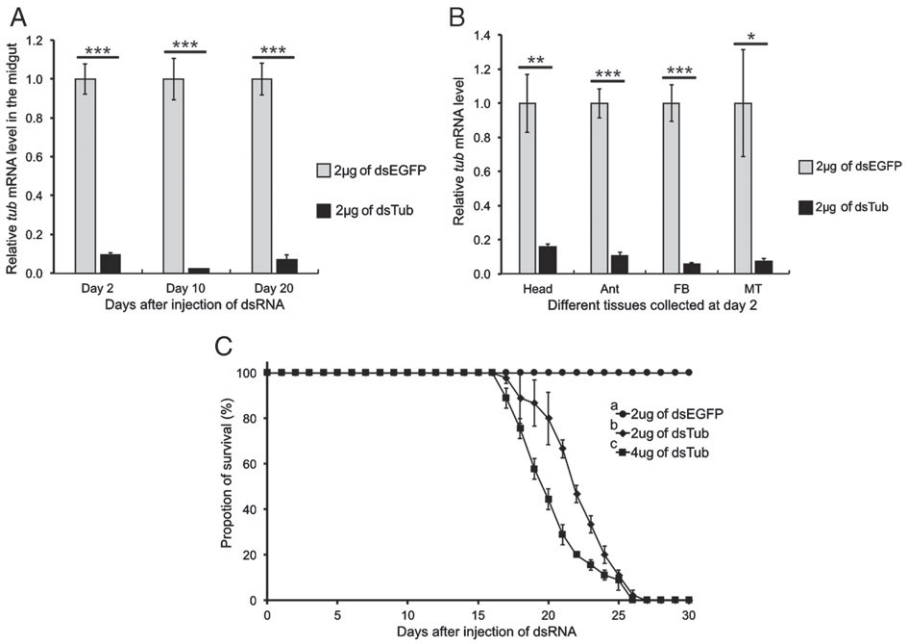

Figure 1. Effects of dsTub injection on *B. germanica*. (A) Persisting depletion of α-*tubulin* expression in the midgut. (B) RNAi-mediated effect on α-*tubulin* expression in heads (Head), antennae (Ant), fat body (FB) and Malpighian tubules (MT) 1 day after injecting dsTub. Data presented in (A) and (B) are the mean ± SE from three independent experiments ($n = 3$), each with 3–5 biological replicates. Asterisks indicate significant differences: *at $P < 0.05$, **at $P < 0.01$ and ***at $P < 0.001$ (*t*-test). (C) Survivorship after injecting 2 μg of dsEGFP and 2 or 4 μg of dsTub ($n = 3$). Different letters on the treatment group indicate significant differences at $P < 0.05$ (Kruskal–Wallis test).

2.8 Statistical analysis

The data were given as mean ± SE and analysed by *t*-test between two groups or ANOVA following Tukey's HSD *post hoc* test for multiple comparisons. The survival analyses were carried out by Kaplan–Meier (KM) test following the Kruskal–Wallis rank sum test for post-test calculations. The calculation of statistical differences was analysed using R software, v.3.0.2 (R Core Team, Vienna, Austria).

3 RESULTS

3.1 Systemic depletion of α-*tubulin* by RNAi in *B. germanica*

Results of dsTub injection are shown in Fig. 1. The mRNA levels of *tub* in the midgut were significantly depleted 1 day after injecting 2 μg of dsTub, and the depletion effect was maintained for at least 20 days (Fig. 1A). Systemic RNAi after injecting dsTub successfully depleted *tub* expression not only in the midgut but also in different tissues, including the head, antennae, fat body and Malpighian tubules (Fig. 1B). Lethal effects were observed 17 days after the single injection of either 2 or 4 μg of dsTub, and all the cockroaches died 26 days after treatment (Fig. 1C). In addition, the survival curves revealed that the lethal effect of *tub* RNAi required at least 17 days, and that the effect was dose dependent.

3.2 Liposomes protect dsRNA from degradation by midgut juice

In our pilot experiments, no depletion of *tub* expression in the gut was observed by single feeding of naked dsTub (2 and 4 μg), in contrast to the robust inhibition caused by injecting dsTub (Fig. 1) into the German cockroach. The immediate hypothesis was that the dsRNA was degraded in the gut after ingestion. To examine the hypothesis of dsRNA degradation in the gut, and whether liposome was able to protect dsRNA from degradation, we performed *ex vivo* experiments to test the integrity of dsRNA, using gel electrophoresis after incubation in haemolymph or midgut juice. The results (Fig. 2) showed that strong nuclease activity was present in the midgut juice, leading to the complete disappearance of the naked dsTub band on the gel after exposure to midgut juice for just 1 h, whereas the effect was much reduced when the midgut juice was diluted, or even when the chelating agent EGTA, which mostly inhibits enzyme activity, was used (Fig. 2A). However, in the case of liposome-conjugated dsTub in the *ex vivo* assay, a discrete dsTub band was still present after 1 h incubation in the midgut juice (Fig. 2A). Furthermore, the resistance of liposome-conjugated dsRNA to degradation was time dependent and declined following incubation for 6 and 12 h in the midgut juice according to observation of the smearing and weakening dsRNA bands (Fig. 2B). However, the dsTub encapsulated by liposomes was significantly degraded after 24 h exposure in the midgut juice. By contrast, the haemolymph of the German cockroach appears to contain low concentrations of active ribonucleases, so the naked dsRNA and liposome-conjugated dsTub remained practically intact after 24 h incubation (Fig. 2B).

3.3 Capacity of liposome for encapsulating dsRNA

According to our results (Fig. 2B), the maximum time for the protection of liposome-conjugated dsRNA against degradation by midgut juice was about 12 h. We then investigated whether liposome and repeated administration (twice a day) would increase RNAi response efficiency. In order to avoid confusion of naked dsRNA for RNAi response during oral administration, we first ran a gel electrophoresis to examine the capacity of liposomes to encapsulate the dsRNA. In principle, dsRNA encapsulated in a cationic liposome to develop a neutral lipoplex charge loses its mobility on gel electrophoresis and remains in the loading well. Results (Fig. 3) showed that quantitites of 0.25 and 0.5 μg of dsEGFP were well

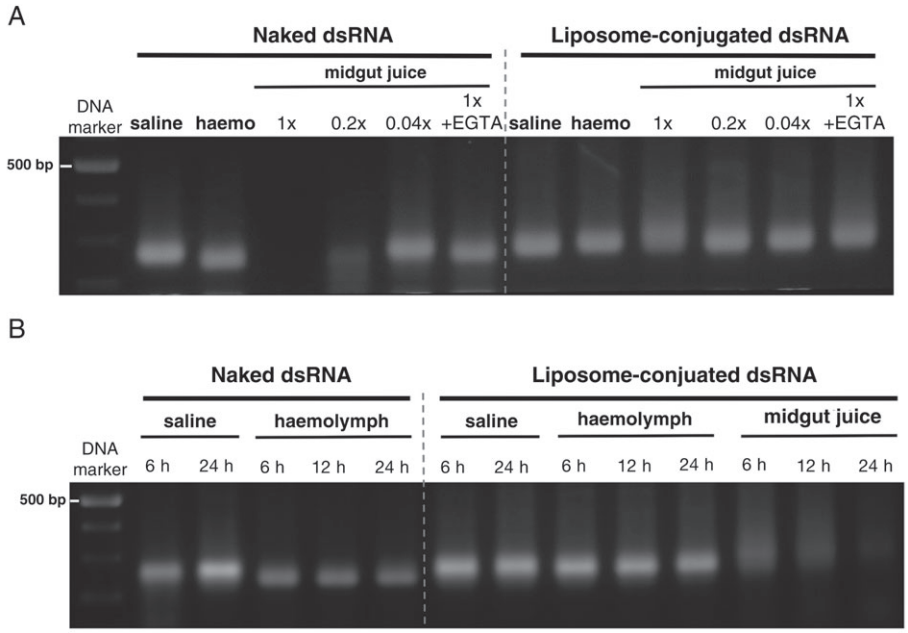

Figure 2. Degradation of dsRNA *ex vivo* by midgut juice or haemolymph (Haemo) of *B. germanica*. (A) Resistance of degradation against midgut juice of naked and liposome-encapsulated dsTub for 1 h incubation. Incubation of 1 μg of naked dsTub and dsTub lipoplexes in a serial dilution of midgut juice for 1 h. Degradation was inhibited by ion chelation due to EGTA treatment as control. (B) Time-dependent protection of liposome-encapsulated dsTub against degradation by incubation of the haemolymph or midgut juice. The haemolymph and midgut juice were used in the original concentration for different time periods.

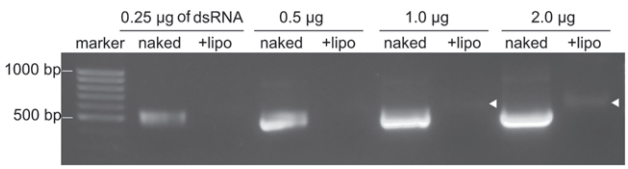

Figure 3. Capacity of liposomes to encapsulate dsRNA. Different amounts of dsEGFP were encapsulated in 1 μL of GenJet Transfection reagent. Naked dsEGFP (naked) and dsEGFP lipoplexes (+lipo) were assayed on 1.5% agarose gel.

encapsulated in 1 μL of GenJet Transfection reagent, but when the amount of dsEGFP was increased to 1 μg or more, the efficiency of conjugation was reduced, as indicated by the detection of weak dsRNA bands on the agarose gel. Therefore, a ratio of 0.25 μg of dsRNA and 1 μL of GenJet Transfection reagent, following the manufacturer's suggested protocols, was used in the following experiments.

3.4 Effect of ingestion of dsRNA lipoplexes on RNAi response

The depletion effect of *tub* mRNA expression in the midgut was investigated at different time points by qRT-PCR after continuous ingestion of dsRNA (Fig. 4A). There was no significant difference in *tub* expression among treatments 1 day after ingestion assay (one-way ANOVA, $P = 0.17$), although the dsTub lipoplexes caused approximately 25% reduction on average. However, *tub* expression was significantly depleted in the midgut (by approximately 35%) after continuous ingestion of dsTub lipoplexes for 8 days, in contrast to dsEGFP lipoplex control (Fig. 4A, day 9). When continuous ingestion of dsRNA lasted up to 16 days, *tub* expression in the midgut was significantly depleted with oral administration of naked dsTub and dsTub lipoplexes (by approximately 24 and 60% respectively), in contrast to the dsEGFP lipoplex control treatment (Fig. 4A, day 17). Consequently, these results indicate that encapsulation of dsRNA by liposome and continuous ingestion increase the RNAi efficiency and suggest that dsTub is efficiently released in the midgut epithelium where it depletes *tub* mRNA levels. Moreover, a significant, though slight, reduction in *tub* expression was observed in the head, whereas no significant effect was observed in other tissues after the continuous feeding of dsRNA lipoplexes for 16 days (Fig. 4B).

To investigate further the effect of oral delivery of dsRNA, the survivorship of experimental animals was monitored. There was no significant difference in cockroach survival with continuous feeding of dsTub lipoplexes for 8 days, naked dsTub and dsEGFP lipoplexes. Conversely, significant and consistent mortality was observed after continuous ingestion of dsTub lipoplexes for 16 days (Fig. 4C).

4 DISCUSSION

RNAi has been demonstrated to be an effective way to study the function of a particular gene of *B. germanica* by knocking down its expression through injecting dsRNA into the haemocoel.[20–23] The open circulatory system of insects allows dsRNA to reach the target sites in a short time, aided by the practical absence of operative extracellular RNA nuclease in the haemolymph (Fig. 2), as previously reported.[24] Moreover, the depletion effect of RNAi can be maintained for at least 20 days by a single injection in this species (Fig. 1). However, dsRNA administered by ingestion results in much milder effects, if any, apparently because the ingested dsRNA is rapidly degraded by the RNA nucleases present in the midgut juice (Fig. 4). This finding is in agreement with the strong dsRNA degradation due to the activity of alkaline nuclease in the midgut of other insects, including *Locusta migratoria*, *Schistocerca gregaria* and *Bombyx mori*.[13–15] The present study, however, has further demonstrated that liposomes can be a protective vehicle

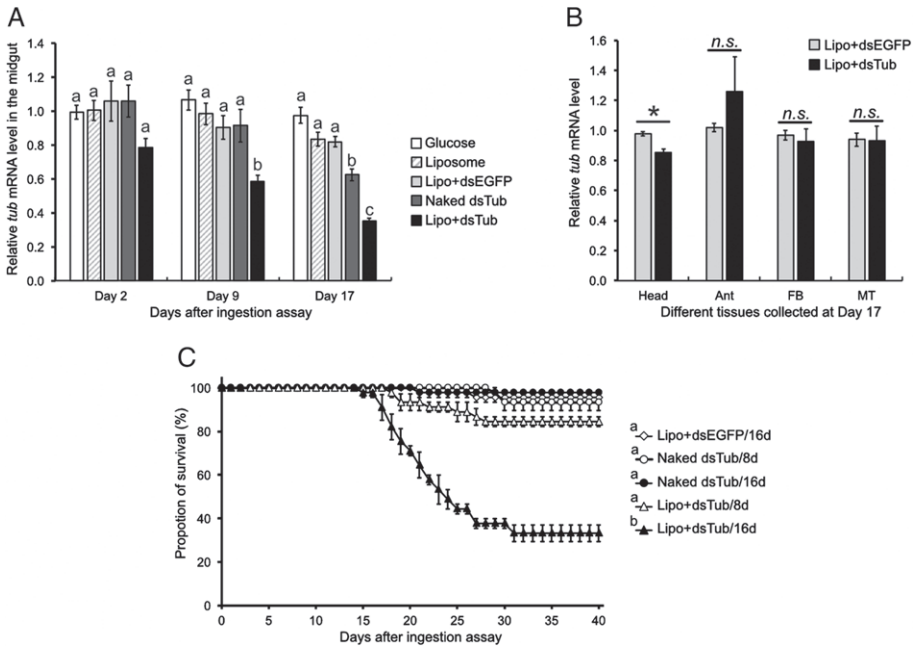

Figure 4. RNAi response by feeding with dsRNA in *B. germanica*. (A) The *tub* expression in the midgut of the German cockroach after different feeding treatments. Different letters on the treatments indicate significant differences at $P < 0.05$ (ANOVA following Tukey's HSD *post hoc* test). (B) Expression of *tub* mRNA in different tissues, including heads (Head), antennae (Ant), fat body (FB) and Malpighian tubules (MT), after feeding on dsRNA lipoplexes for 16 days. The asterisk indicates significant difference at $P < 0.05$; n.s. indicates no significance (*t*-test). Data presented in (A) and (B) are the mean ± SE from three independent experiments ($n = 3$), each with 3–5 biological replicates. (C) Survivorship after ingesting naked dsTub or dsTub lipoplexes ($n = 3$). Different letters on the treatment group indicate significant differences at $P < 0.05$ (Kruskal–Wallis test).

of dsRNA against the degradation that takes place in midgut juice when it is administered orally (Fig. 2). In addition, our results with successful RNAi responses (Fig. 4) suggest a potential use of RNAi for pest control through liposome encapsulation delivery.

In a previous study, Wynant *et al.*[25] injected 5 μg of dsRNA (0.0025 μg mg^{-1} fresh weight) and depleted *tub* expression by ca 80% in the brain of adult locusts *S. gregaria* 1 day after injection. Accordingly, the single injection of 2 μg of dsTub, which corresponds to a concentration of 0.02 μg mg^{-1} fresh weight of an adult male *B. germanica*, was sufficient to result in a similar depletion of *tub* expression (Fig. 1). Although the transcriptional level can be suppressed within 1 day in both insect species, the initial lethality occurred 7 and 17 days after the injection of dsRNA for locusts and cockroaches respectively. This implies that the lethal effect caused by RNAi is species specific. In addition, initial lethality consistently occurred 17 days after the injection of the highest dose (4 μg of dsTub) and continuous ingestion of dsTub lipoplexes (0.5 μg day^{-1}) in *B. germanica*. Therefore, the longer time elapsing before observation of a lethal effect after depletion of *tub* expression was not due to the insufficient dosage of dsRNA applied either by injection or ingestion. The plausible explanation is that the turnover rate of Tub protein in the male adult *B. germanica* is probably slower, or the low quantity of Tub protein might be enough to maintain the cellular function for a period of 17 days.

Oral delivery of dsRNA opens up many possibilities for insect pest control, but there are still many drawbacks to be overcome. For example, continuous feeding is a major requirement to cause a conspicuous lethal effect, in particular for insects with strong RNA nuclease activity in the gut. The failure of RNAi response by oral delivery of dsRNA has been reported, even the administration of a high amount of dsRNA, e.g. 50 μg in *B. mori*, and continuous feeding, e.g. 1 μg day^{-1} in *S. gregaria* and 6 μg day^{-1} in *L. migratoria* for 8 days.[13–15] However, our experiments have demonstrated that continuous feeding of a relatively low amount of dsRNA (0.5 μg day^{-1}) for 16 days depletes *tub* gene expression in the gut and causes dramatic mortality in *B. germanica* (Fig. 4). Moreover, our methodology of feeding dsRNA was carefully conducted in order to reduce instances of any naked dsRNA in the dsRNA lipoplex solution causing RNAi response by chance (Fig. 3), and to ensure that the exact amount of dsRNA in the droplet was ingested completely by the cockroaches (supporting information Fig. S2). Note that the RNA nuclease enzymes in the gut of *B. germanica* are highly efficient, as shown by the degradation of the dsRNA encapsulated in the liposome after 6 h of incubation (Fig. 2). Our method of feeding dsRNA lipoplexes twice a day is most likely to protect and maintain the dsRNA taken up by gut cells to achieve the knockdown effect.

The restrictive depleting effect in the midgut by dsRNA lipoplex ingestion indicates that dsRNA encapsulated by liposome might not pass through the gut cells into the haemocoel to cause a strong silencing effect in other tissues of the German cockroach (Figs 4A and B). The midgut cells of insects are closely packed on the gut epithelium which serves as a line of defence to prevent unwanted materials or organisms from getting into the haemocoel.[26] Although systemic RNAi response through oral delivery of dsRNA has been reported in several insect species,[11] the larger size of the dsRNA lipoplexes used in the present study compared with the single dsRNA molecule applied in other studies would impair penetration through the gut epithelium. More recently, a proposed mechanism of dsRNA uptake by the gut epithelium demonstrated the involvement of the endocytotic pathway in the midgut of Colorado potato beetle *Leptinotarsa decemlineata*.[27] Nevertheless, an important property of the liposome delivery system is that it enhances the uptake of encapsulated dsRNA into the target cell owing to the biocompatibility of its phospholipid structure.[28] Therefore, most dsRNA encapsulated

by liposome would be taken up by gut epithelial cells because of the biocompatibility of liposome. However, the spread of dsRNA through the gut epithelium might occur to a lesser extent in the German cockroach, based on our results showing a mild or even no silencing effect in other tissues (Fig. 4B). As a result, the best strategy for applying oral RNAi using liposome encapsulation systems for pest control should be based on targeting the genes expressed in the digestive system, many of which are vital, as shown by a number of studies.[29–32] Another strategy that has been explored is to increase insecticide susceptibility by depleting detoxification genes, which might be other suitable targets for implementing RNAi pest control.[33–35] In any case, prolonging the stability of dsRNA in field conditions is another crucial challenge. Apparently, liposome-encapsulated dsRNA can meet the challenges of stabilising dsRNA and allowing more exposure time in the field, and it appears to be a promising approach for pest control, at least for insect pests in the semi-protected (e.g. net houses) farms with limited space.

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SUPPORTING INFORMATION

Supporting information may be found in the online version of this article.

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