Research Article

Received: 9 May 2023 Revised: 8 August 2023 Accepted article published: 28 September 2023 Published online in Wiley Online Library: 25 October 2023

(wileyonlinelibrary.com) DOI 10.1002/ps.7793

Effective delivery and selective insecticidal activity of double-stranded RNA via complexation with diblock copolymer varies with polymer block composition

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Abstract

BACKGROUND: Chemical insecticides are an important tool to control damaging pest infestations. However, lack of species specificity, the rise of resistance and the demand for biological alternatives with improved ecotoxicity profiles means that chemicals with new modes of action are required. RNA interference (RNAi)-based strategies using double-stranded RNA (dsRNA) as a species-specific bio-insecticide offer an exquisite solution that addresses these issues. Many species, such as the fruit pest *Drosophila suzukii*, do not exhibit RNAi when dsRNA is orally administered due to degradation by gut nucleases and slow cellular uptake pathways. Thus, delivery vehicles that protect and deliver dsRNA are highly desirable.

RESULTS: In this work, we demonstrate the complexation of *D. suzukii*-specific dsRNA for degradation of *vha26* mRNA with bespoke diblock copolymers. We study the *ex vivo* protection of dsRNA against enzymatic degradation by gut enzymes, which demonstrates the efficiency of this system. Flow cytometry then investigates the cellular uptake of Cy3-labelled dsRNA, showing a 10-fold increase in the mean fluorescence intensity of cells treated with polyplexes. The polymer/dsRNA polyplexes induced a significant 87% decrease in the odds of survival of *D. suzukii* larvae following oral feeding only when formed with a diblock copolymer containing a long neutral block length (1:2 cationic block/neutral block). However, there was no toxicity when fed to the closely related *Drosophila melanogaster*.

CONCLUSION: We provide evidence that dsRNA complexation with diblock copolymers is a promising strategy for RNAi-based species-specific pest control, but optimisation of polymer composition is essential for RNAi success.

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

Keywords: block copolymer; complexation; oral feeding; dsRNA delivery; *Drosophila suzukii*; dsRNA; RNAi; diblock copolymer; delivery

1 INTRODUCTION

Double-stranded RNA (dsRNA) is a versatile RNA interference (RNAi) biomolecule with applications in both therapeutic and agrochemical fields. In recent years, the use of dsRNA as a species-specific bio-insecticide has been the subject of intense investigation due to the need for pest-control agents with new modes of action and improved biosafety profiles to counter the rise in resistance to commonly used chemical pesticides and the widespread ecotoxicity concerns with the field-use of such chemicals.[1–9] The specificity of RNAi induced by the application of exogenous dsRNA offers a promising biological alternative to current chemical insecticides to address the aforementioned concerns.

RNAi, induced by dsRNA, acts within the cell cytoplasm, causing post-transcriptional degradation of mRNA. The judicious choice of the dsRNA sequence can provide a bio-chemical that is highly specific to the pest insect species, with a low risk of off-target toxicity.[10–12] For an in-depth explanation of the RNAi mechanism, readers can refer to previous literature reviews.[11,13–15]

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Prior work with *Drosophila suzukii* has shown a failed induction of RNAi when insects were fed naked dsRNA, whilst exhibiting effective RNAi when dsRNA was administered by injection.[16,17] A similar issue in RNAi efficacy is observed in many other insect pest species, such as those of the lepidopteran, coleopteran or hemipteran orders.[18–22] This phenomenon can be explained by the degradation of dsRNA by RNases in the haemolymph, saliva or digestive tract of the insect, or within the environment prior to ingestion.[13,15,23–30] Another cause of poor RNAi induction is the low cellular uptake of dsRNA. This is particularly relevant in *Drosophila* as they lack the SID-1-like transporter protein that ordinarily provides a faster cellular uptake pathway in comparison to endocytosis.[15,16,31–35] A method of delivery that provides effective protection of dsRNA against enzymatic degradation and facilitates efficient uptake through the cellular membrane is therefore highly desirable.

The delivery of genetic material is also of interest for therapeutic applications because of the developments in gene therapies,[36,37] CRISPR/Cas9 genome editing[38–40] and RNA therapeutics, such as RNAi or mRNA vaccines.[41] In this field, recent research has examined the use of branched polymers,[42] diblock copolymers[43,44] and triblock copolymers[39,45,46] to effectively deliver short interfering-RNA (siRNA) or plasmid DNA (pDNA).

For agrochemical applications, recent work by Parsons *et al.* and Christiaens *et al.* targeting *Spodoptera* used guanidinium-functionalised homopolymers and (co)polymers, respectively, to orally deliver dsRNA.[33,47] The strongly basic guanidine moieties are important for lepidopteran species due to the insects' alkaline gut contents.[24,25,33,48]

Vha26 codes for the 26 kDa E subunit of the vacuolar H^+-ATPase and has been shown to be a valid insecticidal target for RNAi in several insect species, including *D. suzukii*.[17,51] Taning *et al.* used Lipofectamine 2000 for encapsulation of dsRNA to show that feeding *D. suzukii* larvae with species specific *vha26* dsRNA mixed in an artificial diet resulted in 42% silencing efficiency and 42% larval mortality.[17] Here, we report the effectiveness of a polymethacrylate-based hydrophilic homopolymer and a series of diblock copolymers, synthesised via reversible addition-fragmentation chain-transfer (RAFT) polymerisation, to protect *vha26* dsRNA from nuclease attack in the gut of *Drosophila suzukii*, an important insect pest of fruit.[49,50] One of the polymer blocks contains quaternised ammonium groups, possessing a permanent positive charge to electrostatically interact with the anionic phosphodiester backbone of dsRNA.[52] The interaction at specific N/P ratios results in the formation of polyplexes. N/P ratio is the ratio between the number of positively-charged ammonium groups of the polymer with respect to the number of negatively-charged phosphate groups of the dsRNA.[53]

Following investigation of the effect of N/P ratios on complexation characteristics in our previous work, polyplexes were formed at N/P ratio ≥2 and analysed for their capacity to protect dsRNA against *ex vivo* enzymatic degradation by *D. suzukii* adult and third-instar (L3) larvae gut enzymes.[54] When complexed with either homopolymer or diblock copolymer, successful *in vitro* delivery of dsRNA to HEK-293T cells was observed via flow cytometry and confirmed by confocal microscopy. *In vivo* oral feeding of *D. suzukii* larvae demonstrated successful uptake of complexed dsRNA through a decreased survival rate only when using polyplexes formed with the diblock copolymer possessing the longest length of neutral block. These results suggest that hydrophilic diblock copolymers are a promising potential candidate to protect and deliver dsRNA for bio-insecticidal applications in *D. suzukii*, but tailored polymer design and complexation optimisation are required for successful RNAi induction. As far as the authors are aware, our study represents the first to use block copolymers to orally deliver dsRNA for bio-insecticidal applications.

2 MATERIALS AND METHODS

2.1 Materials

[2-(Methacryloyloxy)ethyl] trimethylammonium chloride solution (QDMAEMA, 80 wt% in H_2O), *N,N*-dimethylacrylamide (99%), Dulbecco's Modified Eagle's Medium (DMEM), sodium chloride (NaCl, 99.5%), D_2O (99.9%), ethanol (100%), hydrochloric acid (HCl, 12 M) and 10X phosphate-buffered saline were purchased from Sigma Aldrich. 4-((((2-carboxyethyl)thio)carbonothioyl)thio)-4-cyano-pentanoic acid (95%) was purchased from (Boron Molecular, Victoria, Australia). 4,4′-Azobis(4-cyanovaleric acid) (97%) was purchased from Acros Organics. *Vha26* (V-ATPase) 222 bp dsRNA was synthesised by (Genolution, Seoul, Republic of Korea) AgroRNA (4.68 μg μL^{-1}), with the nucleotide sequence specific to the pest insect, *Drosophila suzukii*, sequence provided in Fig. S1.[17] Regenerated cellulose dialysis membrane (3500 g mol^{-1} molecular weight cut-off [MWCO]) was purchased from Fisher Scientific. 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium (CellTiter 96® AQueous One Solution Cell Proliferation Assay) was purchased from (Promega, Southampton, UK). ProLong™ Gold Antifade Mountant with 4′,6-diamidino-2-phenylindole (DAPI) was purchased from Thermo Fisher Scientific. Label IT® Tracker™ Intracellular Nucleic Acid Localization Kit Cy3 was purchased from (MirusBio, Madison, USA). Ultrapure Milli-Q water (resistivity of minimum 18.2 MΩ.cm) was used for synthesis, solution preparation and dialysis, and nuclease-free water was used for biological assays.

2.2 Polymer synthesis

Synthesis of homopolymer (Q, macro-chain transfer agent [CTA]) and diblock copolymers was conducted as described in a previous publication,[54] but is mentioned in brief below.

2.2.1 *Hydrophilic homopolymer*

Poly[2-(methacryloyloxy)ethyl] trimethylammonium chloride (Q) was synthesised by aqueous RAFT polymerisation as a macro-CTA. Quaternised monomer (QDMAEMA) was stirred at 70 °C for 1.5 h before quenching by exposure to air. The Q macro-CTA, with a degree of polymerisation of 110, was purified by dialysis in Milli-Q water (<3500 g mol^{-1}) and lyophilised.

2.2.2 *Hydrophilic diblock copolymers*

Q macro-CTA was chain extended with poly(*N,N*-dimethyl acrylamide) (D) (to measured degrees of polymerisation of 57, 89 and 219) in aqueous solution at 70 °C for 4 h prior to dialysis in Milli-Q water (<3500 g mol^{-1}) and lyophilisation.

2.3 Polymer characterisation

The polymers were characterised by ^1H NMR spectroscopy (400 MHz) to confirm degree of polymerisation (DP) and molecular weight (M_n), and by aqueous gel permeation chromatography (aq-GPC) to confirm dispersity ($Đ$) (Agilent 1260 Infinity 2, with two PL aquagel-OH Mixed-H columns, eluent 0.8 M $NaNO_3$, 0.01 M NaH_2PO_4, 0.05 wt% NaN_3 in Milli-Q water, adjusted to pH 3 using 37% HCl). These data can be found in our previously published work.[54]

2.4 *Ex vivo* degradation

Intestines were dissected from third-instar *D. suzukii* larvae (L3), after 2 h starvation to clear the gut of food, and homogenised in Milli-Q water using a plastic homogeniser (30 × intestines/100 μL). For adults, male and female flies were starved for 2 h, and the intestines were then removed from flies of mixed sex and homogenised in Milli-Q water (7 × intestines/100 μL). Subsequently, all homogenates were centrifuged at 13 000× g for 20 min and the supernatant collected and stored at −20 °C until required.

Pre-determined aliquots of Q_{110}, Q_{110}-*b*-D_{57}, Q_{110}-*b*-D_{89} or Q_{110}-*b*-D_{219} polymers were added to 1 μg of dsRNA to provide polyplexes with a N/P ratio = 2.5. These solutions were incubated at room temperature for 1.5 h. Adult or L3 larval gut solution (equivalent of half a gut was used per 1 μg of dsRNA (i.e., per gel lane), which was sufficient to fully degrade naked dsRNA[55,56])) were added to samples of naked dsRNA and polyplex solutions. Following this, the samples were incubated for 30 min or 24 h, respectively, at 26 °C, 60% relative humidity (RH). Each solution was loaded (with 2 μL of 6X blue/orange loading dye) onto a 2% (wt/vol) agarose gel stained with ethidium bromide (EB, 20 ng mL^{-1}), prepared with 1X Tris base, acetic acid and EDTA buffer. Assays were run for 25 min at 90 V. A 100 bp DNA ladder (New England Biolabs, UK) was run for comparison. Gels were imaged under a UV transilluminator at 365 nm.

2.5 Cell culture

HEK-293T cells were cultured in 75-cm^2 flasks in an incubator at 37 °C, with 5% CO_2 and 95% RH. The medium was DMEM supplemented with 10% fetal bovine serum (FBS) (vol/vol) and 1% (wt/vol) penicillin–streptomycin. Cell confluence/density was monitored and cells were passaged as required.

2.6 Cy3-labelling of dsRNA

DsRNA (*vha26*, 222 bp) was labelled with Cy3 labelling kit fluorophore (MirusBio) according to kit protocols, purified by precipitation in ethanol and stored at −20 °C until required.

2.7 Flow cytometry

HEK-293T cells in a 12-well plate were seeded at 0.1 × 10^6 cells/well density, 24 h before treatment. Polyplexes were prepared 24 h prior to treatment through addition of 20 μL of Cy3-labelled dsRNA (50 ng μL^{-1}) to 20 μL of polymer (concentrations varied to provide a N/P ratio = 5). Polyplexes, Cy3-labelled dsRNA and polymer solutions were individually applied to HEK-293T cell wells. Nuclease-free water was applied in the absence of either polymer or dsRNA, and a nuclease-free water control was conducted. After 4 h, cells were placed on ice and then lifted from wells by physical disturbance. Cells were pelleted by centrifuge, and media/treatment was removed before washing and resuspension of cells in fresh, ice-cold DMEM media. Cy3-positive cells were analysed by flow cytometry using a Cytoflex S (Beckman Coulter) with filters set at 561 nm excitation and 585 nm emission wavelegnths. Data was analysed using FlowJo software. Examples of gating and dead cell determination by 7-amino actinomycin D (7-AAD) are presented in Figs S2–S5.

2.8 Confocal microscopy

Solution preparation and cell seeding were performed as described above. Cells were cultured on microscope slides, introduced to the 12-well plate and after incubation the cells were fixed with 4% (wt/vol) paraformaldehyde. A DAPI-stain liquid mountant was used to prepare slides for cell imaging on a confocal microscope (Zeiss LSM700).

2.9 Cell viability

HEK-293T cells were seeded onto 96-well plates 2 days prior to assay, at a 20 000 cells/well density. A range of polymer concentrations was prepared in Milli-Q water (0.01–10 mg mL^{-1}). Aliquots of polymer (100 μL/well) at each concentration were added to cells with three replicates, and incubated for 24 h at 37 °C, 5% CO_2 and 95% RH. 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium (MTS, 20 μL) was then added to each well. Cells were incubated with the MTS for 4 h prior to analysis using a plate reader set at a wavelength of 495 nm. Cells treated with media only were normalised to 100% cell viability and other treatments were normalised with respect to the media cells.

2.10 *In vivo* oral feeding

First/second-instar (L1/2) *D. suzukii* and *Drosophila melanogaster* larvae were starved on moist filter paper for 3 h prior to addition to artificial diet. *Vha26* dsRNA (4.68 g L^{-1}, 8 μL) was complexed with Q_{110}, Q_{110}-*b*-D_{57}, Q_{110}-*b*-D_{99}, or Q_{110}-*b*-D_{219} (8 μL) at concentrations to provide N/P ratio = 5 (see Fig. S6) and incubated for 1.5 h. Polyplex (16 μL), dsRNA (8 μL) + nuclease-free water (8 μL), polymer (8 μL) + nuclease-free water (8 μL) or nuclease-free water (16 μL) solutions were added to 50 mg of diet and mixed thoroughly for an even distribution. Five starved L1/2 larvae were moved to each 50 mg of treated diet, $n = 25$ total, and fed for 24 h. Larvae were then removed to fresh diet and monitored for the next 15 days for mortality, assessed by the failure to reach the pupal stage and the absence of any live larvae in or on the surface of the food. Survival results were averaged across assay repeats and normalised against nuclease-free water-fed larvae. Standard deviations were calculated and represented as error bars. Statistical significance was determined using binary logistic regression, performed in SPSS Statistics 26.

3 RESULTS

3.1 Polymer synthesis and characterisation

A polymethacrylate-based, linear homopolymer was synthesised via RAFT polymerisation as a macro-CTA. A neutral, hydrophilic polymer block was then chain extended onto the macro-CTA to create a series of hydrophilic diblock copolymers, with three different lengths of neutral block (three different DPs). The polymethacrylate-based polymer, poly[2-(methacryloyloxy)ethyl] trimethylammonium chloride (Q), with a degree of polymerisation (DP) of 110, contains a quaternised ammonium moiety and thus a positive charge on each unit regardless of pH conditions. Three samples of the Q macro-CTA were chain extended by a second poly(*N,N*-dimethyl acrylamide) (D) block with DPs of 57, 89 and 219, respectively. Chemical structures of the Q macro-CTA (homopolymer (a) and diblock copolymer (b)) are shown in Fig. 1. Further details of the synthesised polymers (including conversion, molecular weight, dispersity etc.) can be found in our previous article.[54]

3.2 Homopolymer and diblock copolymer complexation prevents dsRNA degradation by *D. suzukii* gut enzymes

The relative proportions of polymer/dsRNA were varied to ascertain an optimal N/P ratio (≥2) for full complexation as determined in previously published work.[54] It was found that an N/P ratio of 2.5 was efficient in ensuring complexation of the dsRNA, thus this ratio was used in the experiments testing for dsRNA protection through complexation with the diblock copolymer. Whilst free

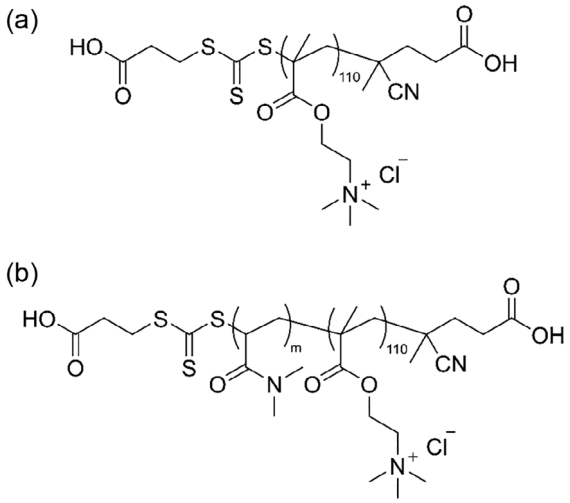

Figure 1. Chemical structures of (a) Q macro-CTA and (b) Q-*b*-D diblock copolymers used for dsRNA complexation. Figure created in ChemDraw Prime 17.0.

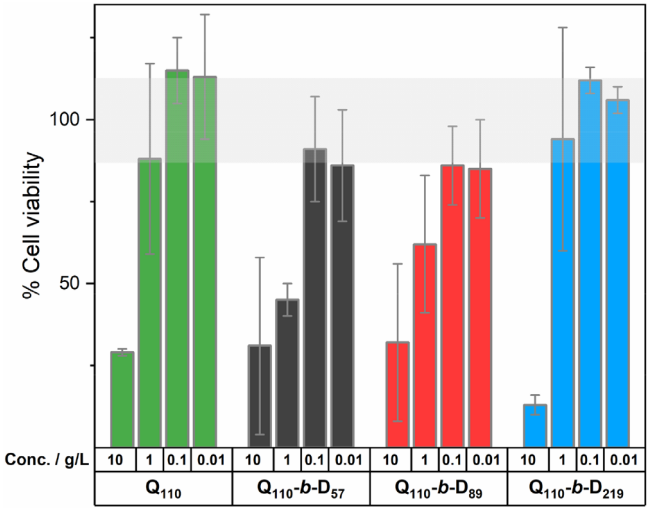

Figure 3. Cell viability of HEK-293T cells, normalised with respect to untreated cells (standard deviation shown by grey band), via MTS assay with polymers. The concentration of stock polymer solution added to cell culture was varied from 0.01 to 10 g L^{-1}. Cell viability was assessed in triplicate after 24 h of incubation with each polymer. Figure created in Origin(Pro), (2020). OriginLab Corporation, Northampton, MA, USA.

dsRNA can freely migrate through an agarose gel, polyplexes remain retarded in the well due to their large size when fully complexed. Enzymes from the midgut of *D. suzukii* L3 larvae and adults were prepared by tissue homogenisation and centrifugation.

Vha26 dsRNA was degraded by gut enzymes from adults and L3 larvae with a total absence of EB staining of the agarose gel after 30 min and 24 h incubation, respectively (Fig. 2). The homopolymer and all three diblock copolymers are seen to efficiently protect dsRNA against degradation by *D. suzukii* gut enzymes at a N/P ratio = 2.5, as shown by the strong fluorescence maintained in the agarose gel wells for both the presence and absence of gut enzymes.

3.3 Cell viability of homopolymer and diblock copolymers assessed in HEK-293T cells

Cell viability in the presence of Q_{110}, Q_{110}-*b*-D_{57}, Q_{110}-*b*-D_{89} and Q_{110}-*b*-D_{219} was assessed via MTS assay in HEK-293T cells (0.01–10 mg mL^{-1}). HEK-293T cells are widely used in the study of RNAi delivery to cells in culture and are used here for comparison with results from similar studies designed for therapeutic applications.[57–59] Across the polymer concentration range tested, as shown in Fig. 3, a significant decrease in cell viability was

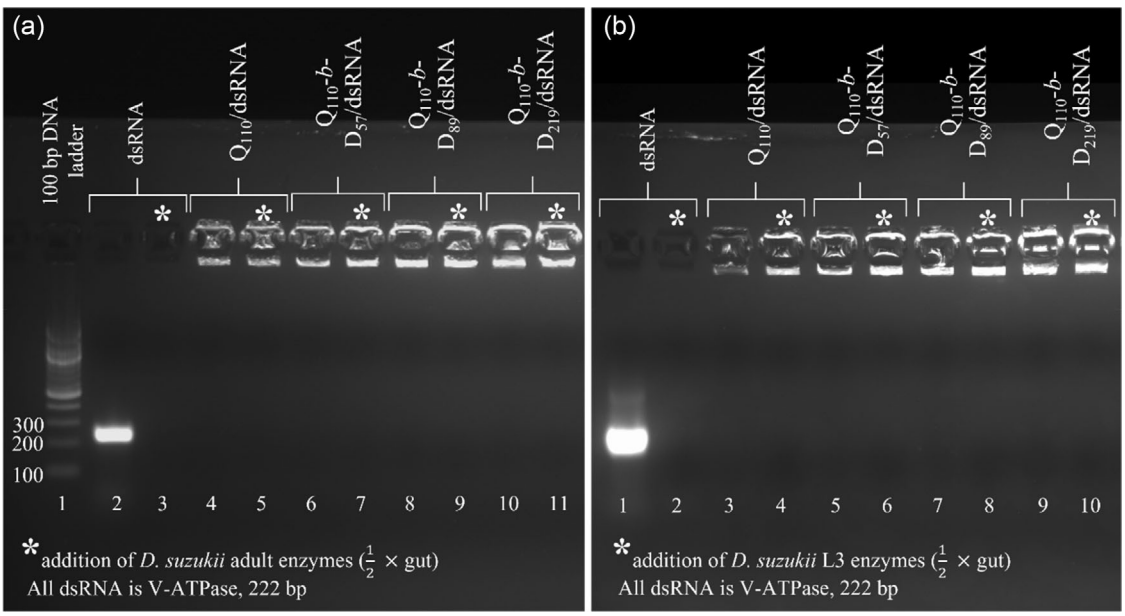

Figure 2. dsRNA complexation with homopolymer or diblock copolymers protects against *D. suzukii* adult (a) and L3 larvae (b) gut enzyme degradation, as shown in agarose gel electrophoresis of free and complexed dsRNA at N/P ratio = 2.5 in the absence and presence (*) of gut enzymes (equivalent to half a gut per 1 μg of dsRNA). The fluorophore, EB, intercalates between the base pairs of dsRNA to give a strong fluorescent band on the gel with free and complexed intact dsRNA, but not with highly fragmented dsRNA. A 100 bp DNA ladder was run for comparison. There appears to be fluorescence above lanes 3 in (b), which is likely due to contamination or a small amount of positively charged complexes migrating towards the negative electrode.

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observed at 10 mg mL^{-1} for all polymers, and cell viability was variable at 1 mg mL^{-1}, with Q_{110}-*b*-D_{57} and Q_{110}-*b*-D_{89} appearing to induce lower cell viability at this concentration. Below 1 mg mL^{-1} there was no significant decrease in cell viability caused by any of the polymers. Cell viability was also determined through flow cytometry by introducing a dead cell stain (7-AAD), the results of which can be found in Fig. S7.

3.4 Complexation with homopolymer and diblock copolymers enhances *in vitro* uptake of dsRNA in HEK-293T cells

Cellular uptake was assessed in HEK-293T cells using flow cytometry and confocal microscopy.

3.4.1 *Flow cytometry*

After incubation with Cy3-labelled dsRNA alone or complexed with each polymer, cells were fixed and analysed for fluorescence intensity at 585 nm. There was a large shift (10-fold increase) in the mean fluorescence intensity of cells treated with polyplexes (formulated at N/P ratio = 5). In comparison, cells treated with naked Cy3-labelled dsRNA alone did not cause a significant increase in fluorescence intensity. Gating strategies to exclude dead cells and cell doublets are shown in Figs S2–S5.

The percentage of Cy3-positive, live, single cells after incubation is shown in Fig. 4. Greater than 80% of HEK-293T cells exhibited a shift in fluorescence when incubated with homopolymer or diblock copolymer/Cy3-labelled dsRNA polyplexes. In comparison, less than 5% of cells showed a shift in fluorescence when incubated with naked Cy3-labelled dsRNA, homopolymer or diblock copolymer alone, or when untreated.

3.4.2 *Confocal microscopy*

Z-stack images of representative HEK-293T cells were captured after incubation with Q_{110}-*b*-D_{219}/Cy3-labelled dsRNA polyplexes (used as a representative polyplex, as flow cytometry data was similar between polyplexes), naked Cy3-labelled dsRNA, Q_{110}-*b*-D_{219} alone or nuclease-free water (Fig. 5). Orthogonal mid-section images of representative cells and histogram profiles of the Cy3 fluorescence intensity at each Z-slice confirm the *in vitro* delivery of Cy3-labelled dsRNA and its perinuclear intracellular localisation, indicating successful cytoplasmic delivery.

3.5 Complexation of *vha26* dsRNA with diblock copolymer with the longest neutral block is important for larval toxicity in *D. suzukii*

Drosophila diet was mixed with homopolymer or diblock copolymer/dsRNA polyplexes, dsRNA, polymer alone or nuclease-free water. L1/2 larvae were starved for 3 h before transfer to the various treated diets for 24 h and then monitored for survival on fresh diet for 15 days. The normalised (with respect to untreated diet-fed *D. suzukii* or *D. melanogaster*) survival rate of *D. suzukii* (or *D. melanogaster*) is shown in Fig. 6. Mortality, when observed, was found between the larval and pupal stages of *Drosophila* development.

A significant lower survival rate was observed in *D. suzukii* larvae fed diet treated with Q_{110}-*b*-D_{219}/dsRNA polyplexes, decreasing to ~30%. In contrast, larvae fed diet treated with polyplexes formed with homopolymer or diblock copolymers with shorter neutral block lengths, naked *vha26* dsRNA, polymers only or nuclease-free water (untreated) did not show any significant effect on survival. It is important to note that the presence of polymer alone in the diet did not induce larval toxicity or visually prevent feeding. It is also important to note the larvae fed Q_{110}-*b*-D_{219} polymer had the highest decrease in survival when compared to the larvae fed the other polymers at this

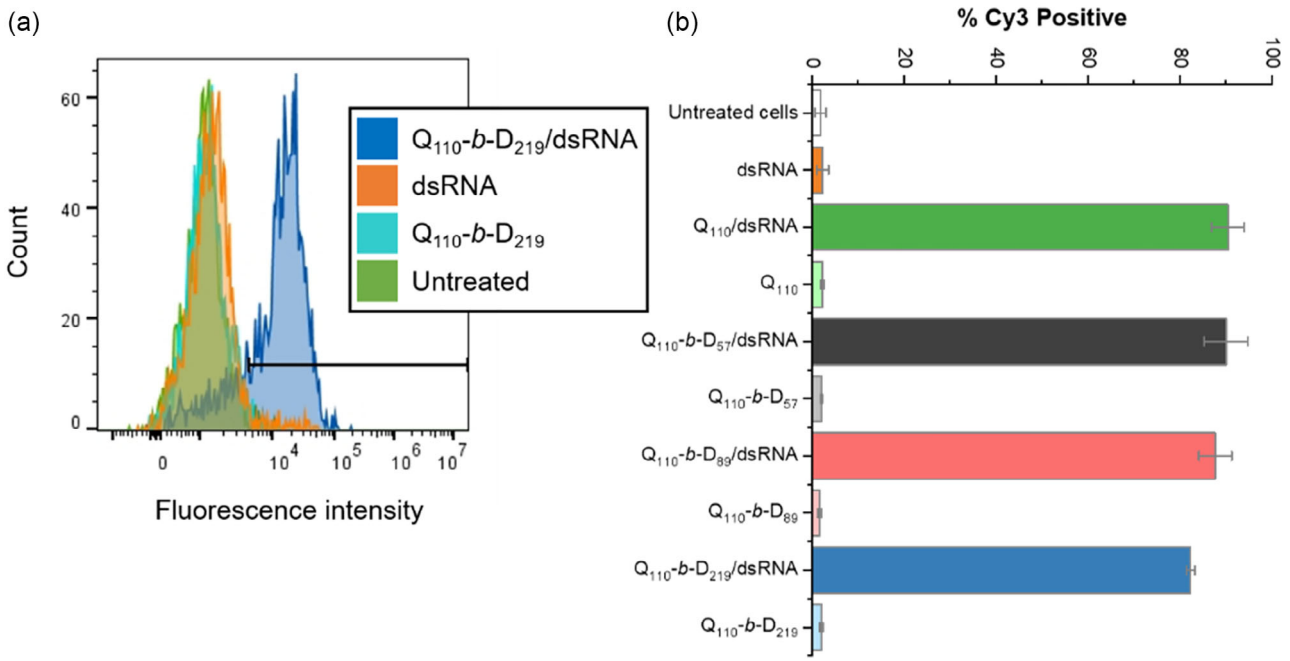

Figure 4. Complexation of dsRNA with either homopolymer or diblock copolymer greatly enhances interaction with eukaryotic cells. Flow cytometry data was gated with respect to live and single cells. (a) Cy3 fluorescence intensity (arbitrary units) of differently treated cells (separated intensity plots in Fig. S5). The Q_{110}-*b*-D_{219}/dsRNA polyplex is provided here as an example to highlight the gating used in analysis (>95th percentile of the negative control). (b) Percentage of Cy3 positive events, determined via flow cytometry of untreated or treated HEK-293T cells. All N/P ratios = 5. Figures created in Origin(Pro), (2020). OriginLab Corporation, Northampton, MA, USA.

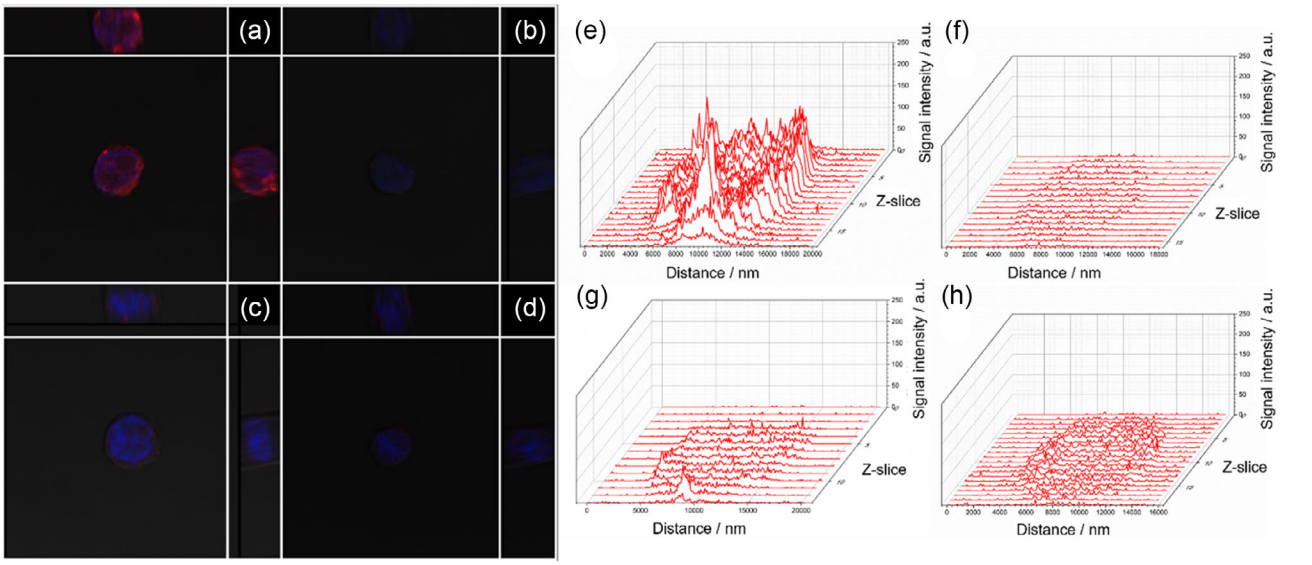

Figure 5. Representative confocal and light microscopy merged images of mid-section Z-slices of HEK-293T cells treated with (a) Q_{110}-*b*-D_{219}/dsRNA (N/P ratio = 5), (b) Q_{110}-*b*-D_{219}, (c) dsRNA and (d) water. The images show the red fluorescence of Cy3-labelled dsRNA. DAPI was used as the mountant to stain nuclei. Orthogonal projections are shown above and right of the primary image. Images were captured using identical microscope settings. Histogram profiles of Cy3 fluorescence intensity at each Z-slice across a horizontal middle line of cell images are shown of HEK-293T cells treated with (e) Q_{110}-*b*-D_{219}/dsRNA, (f) Q_{110}-*b*-D_{219}, (g) dsRNA and (h) water. Figures created in Origin(Pro), (2020). OriginLab Corporation, Northampton, MA, USA.

concentration. There could therefore be some level of toxicity induced by the diblock copolymers, and thus polymer concentration must be carefully considered. Binary logistic regression indicated a significant association between larvae fed Q_{110}-*b*-D_{219}/dsRNA polyplex-treated diet and survival to the pupal stage ($P \leq 0.0001$), with an 87% decrease in the odds of survival. No significant effect of feeding other polyplex-treated diet, naked dsRNA-treated diet or polymer only-treated diet, compared to untreated diet, was observed when analysing the data with binary logistic regression despite an apparent slight decrease in survival rate for the polymers alone when the polymer chain length increases.

The *vha26* dsRNA used in this study was designed to specifically target *D. suzukii* V-ATPase subunit transcripts.[17] As expected, feeding the Q_{110}-*b*-D_{219}/dsRNA polyplex to the closely related species *D. melanogaster* did not result in any significant lethality.

4 DISCUSSION

The cationic ammonium moieties of the Q polymer block are the basis of homopolymer and 15eblock copolymer complexation with dsRNA through electrostatic interaction with the anionic phosphate groups of the phosphodiester-bonded backbone.

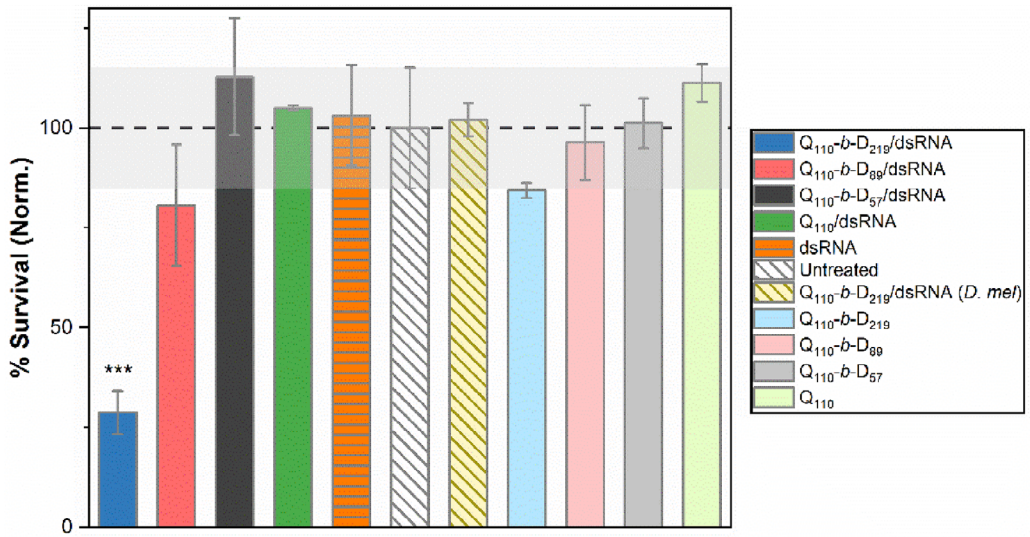

Figure 6. Q_{110}-*b*-D_{219}/dsRNA polyplexes induce species-specific targeting of RNAi. Survival of *Drosophila suzukii* after feeding larvae (L1/2) for 24 h on a diet treated with polyplexes, naked dsRNA or polymers alone, as well as survival of *Drosophila melanogaster* larvae (L1/2) when diet was treated with Q_{110}-*b*-D_{219}/dsRNA polyplex. Survival was assessed after 15 days by the failure to reach the pupal stage and the absence of any live larvae in or on the surface of the food. Results were normalised against larvae fed on diet treated with nuclease-free water (untreated). Error bars represent standard deviation, and the grey shading represents the standard deviation of the survival of larvae fed on diet treated with nuclease-free water (untreated). ***P value ≤0.0001 according to binary logistic regression analysis. Figure created in Origin(Pro), (2020). OriginLab Corporation, Northampton, MA, USA.

The entropically favourable complexation, due to the associated release of counterions, has previously been demonstrated through the use of agarose gel electrophoresis.[54,60,61] In the present work, gel electrophoresis following *ex vivo* *vha26* dsRNA degradation was used to highlight the protective properties of both the homopolymer and 15eblock copolymers against *D. suzukii* gut enzymes of both adult flies and L3 larvae. As described by Yoon *et al.*, late-stage *D. suzukii* L3 larvae (and pupae) express lower levels of dsRNases in comparison to early-stage L1 or L2 larvae and the adult flies.[30] Hence, samples were incubated with L3 larvae gut enzymes for 24 h, rather than the shorter 30 min for adult gut enzymes. The extent of degradation of *vha26* dsRNA by L3 gut enzymes after incubation for 1, 2 and 24 h is shown in Fig. S8. The maintained strong fluorescence of the dsRNA complexed with polymer (and therefore stability) in the presence of dsRNA-degrading enzymes illustrates the potential for delivery of intact dsRNA to *D. suzukii* by protection from enzymatic degradation.

Following confirmation that homopolymer and 15eblock copolymers efficiently complex with dsRNA and provide protection against *ex vivo* enzymatic degradation, it was important to determine the cellular delivery potential of the polyplexes. Homopolymer and 15eblock copolymers were not toxic to HEK-293T cells, a model cell line for assessing cellular uptake of dsRNA, within the range 0.01–0.1 mg mL^{-1}. Cell interaction, assessed via flow cytometry, showed a significant increase in the presence of Cy3-labelled dsRNA associated with the HEK-293T cells when complexed with both homopolymer and diblock copolymers, in comparison to when Cy3-labelled dsRNA was administered alone. Confocal laser scanning microscopy showed that the fluorescent Q_{110}-*b*-D_{219}/dsRNA polyplexes were perinuclear within the confines of the plasma membrane and not simply adhering to the cell surface, confirming that the flow cytometry data is as a result of cell uptake. A much lower intensity of Cy3 fluorescence was recorded in cells treated with naked dsRNA, and no significant fluorescence was observed in cells treated with polymer alone or when untreated. The more efficient cellular uptake of dsRNA to the cell cytoplasm when delivered as part of a polyplex, in comparison to free dsRNA, is likely due to the positive charge of the polyplexes (when formulated at N/P ratio >1), as electrostatic repulsion resulting from the negative charges of the cell membrane surface has previously been described as a barrier to cell entry for dsRNA.[62,63] Future work could focus on the uptake of dsRNA to endosomes, thus investigating whether the diblock copolymers enhance endosomal uptake and/or escape.

After investigating the *in vitro* delivery of dsRNA using the developed homopolymer and 16eblock copolymers, the effectiveness of the polyplexes for *in vivo* delivery of dsRNA to *D. suzukii* was studied using larval and pupal toxicity as a surrogate readout. Whilst feeding naked dsRNA generates systemic RNAi in some insect species (e.g., hemipterans), other species (e.g., dipterans) are resistant to orally administered dsRNA.[11,16,18,19,21,64,65] In the present study, the larval toxicity assay confirmed a previous report that feeding naked *vha26* dsRNA to *D. suzukii* larvae does not result in significant mortality.[17] The study demonstrated that *D. suzukii*-specific *vha26* dsRNA encapsulated with the cell transfection agent, Lipofectamine 2000, achieved a larval mortality of 42% when administered orally in artificial diet. In contrast, larvae fed a diet treated with polyplexes prepared by complexation of the same *vha26* dsRNA with Q_{110}-*b*-D_{219}, but not with Q_{110}, Q_{110}-*b*-D_{57} or Q_{110}-*b*-D_{89} polymers, resulted in much higher mortality (~70%). These results indicate a more efficient oral delivery of the dsRNA polyplex and a correlation between the length of the diblock copolymer neutral block and the resulting toxicity. Our previous work showed that polyplexes prepared with diblock copolymers of increasing neutral block length resulted in the formation of more compact polyplexes (smaller effective hydrodynamic radii, see Fig. S9).[54] We speculate that the compactness of the Q_{110}-*b*-D_{219}/dsRNA polyplexes facilitates dsRNA uptake by insect cells to elicit a strong RNAi response and larval lethality. This work adds to a body of research that suggests that longer hydrophilic neutral blocks improve the delivery of nucleic acids.[46,66,67] *D. melanogaster* larvae were also fed the Q_{110}-*b*-D_{219}/dsRNA-treated diet to thus demonstrate that there is no off-target toxicity on this species that is closely related to *D. suzukii*.

To conclude, this research demonstrates for the first time the successful delivery of *Drosophila suzukii*-specific dsRNA (*vha26*) for bio-insecticidal application using a hydrophilic diblock copolymer as the protective vector. Our previous work indicated that N/P ratio ≥2 is required for sufficient complexation of homopolymer or diblock copolymer with dsRNA.[54] Agarose gel electrophoresis showed the effective protection of dsRNA against *ex vivo* degradation by *D. suzukii* adult and L3 larvae gut enzymes through polymer complexation. Delivery of Cy3-labelled dsRNA was illustrated at a cellular level in HEK-293T cells through flow cytometry, with confocal microscopy confirming the cytoplasmic delivery through the plasma membrane. *In vivo* orally administered delivery of naked *vha26* dsRNA to *D. suzukii* did not have a significant impact on the survival of L1/2 larvae. However, the oral administration of dsRNA formulated by complexation with the hydrophilic diblock copolymer containing the longest neutral block, Q_{110}-*b*-D_{219} (and thus forming the most compact polyplexes, as illustrated in our previous work[54]), induced high levels of larval mortality of L1/2 *D. suzukii* larvae. These results therefore suggest that hydrophilic diblock copolymers are promising candidates for the complexation, protection and delivery of dsRNA for bio-insecticidal applications, but optimisation of the stabilising, neutral block length is required for successful RNAi *in vivo*.

AUTHOR CONTRIBUTIONS

The manuscript was written by Charlotte E. Pugsley. All authors commented on versions of the manuscript and approved the final manuscript. Olivier J. Cayre, Nicholas J. Warren, R. Elwyn Isaac, Kaat Cappelle and Rosa Dominguez-Espinosa contributed to the study conception and design. Polymer synthesis and characterisation were performed by Charlotte E. Pugsley. Flow cytometry and confocal microscopy were performed by Charlotte E. Pugsley with assistance from Martin Stacey. Biological assays, including agarose gel electrophoresis and *in vivo* feeding, and analysis were performed by Charlotte E. Pugsley.

ACKNOWLEDGEMENTS

The authors would like to acknowledge the support of Dr Elpiniki Kalogeropoulou by way of training for biological assays, and Elizabeth Korevaar for her assistance with statistical analysis. We thank Dr Julia Y. Rho (University of Birmingham) and Prof. Sébastien Perrier (University of Warwick) for valuable discussions during the course of this study.

FUNDING INFORMATION

The authors would like to acknowledge the support of the EPSRC Centre for Doctoral Training in Soft Matter and Functional Interfaces (SOFI CDT – EP/L015536/1) and Syngenta for joint funding of a Ph.D. studentship for Charlotte E. Pugsley. The Cytoflex flow cytometer was acquired with support from the Biotechnology and Biological Sciences Research Council (BB/R000352/1).

CONFLICT OF INTEREST STATEMENT

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

DATA AVAILABILITY STATEMENT

The data that support the findings of this study are available from the corresponding author upon reasonable request.

SUPPORTING INFORMATION

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

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