Understanding *Drosophila suzukii* characteristics to implement novel pesticide delivery for environmentally-friendly population control

Calum T. J. Ferguson

School of Biological Sciences & School of Chemical and Process Engineering

University of Leeds

A thesis submitted for the degree of

Doctor of Philosophy

1st May 2018

IP

The candidate confirms that the work submitted is his own, except where work which has formed part of jointly-authored publications has been included. The contribution of the candidate and the other authors to this work has been explicitly indicated below. The candidate confirms that appropriate credit has been given within the thesis where reference has been made to the work of others.

Refereed Journal Articles

1. C. T. J Ferguson, T. L. O'Neill, N. Audsley, and R. E. Isaac. The sexually dimorphic behaviour of adult drosophila suzukii: elevated female locomotor activity and loss of siesta is a post-mating response. Journal of experimental biology, 218(23): 3855-3861, 2015. 94
2. C. T. J Ferguson, A. A. Al-Khalaf, R. E. Isaac, and O. J. Cayre. pH-responsive polymer microcapsules for targeted delivery of biomaterials to the midgut of *Drosophila suzukii* PlosOne, (*Under review*)
3. C. T. J Ferguson, J. D. Behra, N. J. Warren, R. E. Isaac, and O.J.Cayre. double-stranded RNA complexation with well-defined block copolymers, enhancing stability and uptake for use in pest control. (*In preparation*)
4. C. T. J Ferguson, T. L. O'Neill, and R. E. Isaac. Climatic stress dictates the dispersion of *Drosophila suzukii*. (*In preparation*)

Acknowledgements

I would like to take this opportunity to thank both of my supervisors, Elwyn Isaac and Olivier Cayre, for their support and guidance throughout this PhD. I would like to acknowledge the EU and the Dropsa project for providing funding for this research.

I would also like to thank my friends and family for being related to me and/or dealing with my amazing sense of humour.

Cheers.

Calum

Abstract

Drosophila suzukii is an economically important fruit fly pest, which can have a devastating impact on soft and stone fruit industries. To combat this pest, novel methods for the delivery of bioinsecticides have been investigated along with research into the locomotor activity of *D. suzukii* under semi-natural conditions.

Bioinsecticides have been proposed as safer environmentally friendly alternatives to existing chemical insecticides. However, they are subject to degradation and therefore require protection. This study has developed two new methods for peptide/protein and double-stranded RNA (dsRNA) based bioinsecticide delivery.

Model protein/peptide based bioinsecticides have been encapsulated in pH-responsive poly(2-vinylpyridine) (P2VP) microcapsules, providing protection and triggered release. The P2VP microcapsules were stable at pH > 6, but underwent rapid dissolution at pH < 4.2. *In vivo* studies showed that the natural acidity of a midgut region (pH < 3) of *D. suzukii* also induced the breakdown of responsive P2VP microcapsules to release their cargo.

dsRNA mediated RNA interference (RNAi) can be utilised for insect pest control. However, its wide-scale use is currently limited by its rapid degradation upon ingestion. Well-defined diblock copolymers have been produced by RAFT polymerisation, and their complexation with dsRNA has been investigated. Phenotypic observations showed that upon complexation of the dsRNA, around a 75 % increase in mortality was achieved compared to naked dsRNA. Feeding of the complex to *D. melanogaster* had no adverse effects, demonstrating the selective nature of this complexed dsRNA.

Understanding the behaviour of *D. suzukii* might be important in the design of effective control strategies. There is a post-mating effect on the behaviour of adult females, which is due to the transferal of sex peptide, increasing the activity of mated females. Raising the temperature can also cause an increase in locomotor activity in *D. suzukii*, as the fly looks to escape the hot conditions.

Abbreviations

A	Afternoon
ACVA	4,4'-azobis(4-cyanovaleric acid)
AIBN	2,2-Azobis(2-methylpropionitrile)
ASNP	Functionalised silica particles
ASTs	Allatostatins
ATRP	Atom transfer radical polymerization
BMA	Butyl methacylate
Bps	Base pairs
BSA	Bovine serum albumin
Bt	Bacillus thuringiensis
CDQ	Carbon quantum dots
CPDT	2-(2-Cyanoprop-2-yl)-S-dodecyltrithiocarbonate
CTA	Chain transfer agent
Da	Dalton
DD	Constant darkness
DDT	Dichlorodiphenyltrichloroethane
DEnM	*Drosophila* environment monitor
Dp	Degree of polymerisation
dsRNA	Double-stranded RNA
DSS	2,2-dimethyl-2-silapentane- 5-sulfonate sodium salt
EDTA	Ethylenediaminetetraacetic acid
EDX	Energy dispersive x-ray spectroscopy

FNP	Fluorescent nanoparticle
GFP	Green fluorescent protein
GNA	Galanthus nivalis agglutinin
HLB	Hydrophilic-lipophilic balance
ICK	Inhibitor cystine knot
IPA	Isopropyl alcohol
$IPEC$	Interpolyelectrolyte complex
LAM	Locomotor activity monitor
$LAMs$	Less-activated monomers
LD	Light Dark
Lx	Lux
$MAMs$	More-activated monomers
$miRNAs$	MicroRNAs
MMA	Methyl methacrylate
$mmol$	Millimoles
mV	Millivolts
nm	Nanometer
NPs	Polymeric nanoparticles
$P2VP$	Poly(2-vinylpyridine)
PAA	Propylacrylic acid
PAC	Poly-alkyl-cyano-acrylate
$PBANs$	Pheromone biosynthesis activating neuropeptides
PCL	Poly-ϵ-caprolactone
$PDMAEMA$	Poly(2-(dimethylamino) ethylmethacrylate)
$PDMA$	Polydimethylacetamide
PEG	Polyethylene glycol
PEI	Polyethyleneimine
$PEMV$	Pea enation mosaic virus
PEO	Poly(ethylene oxide)
PFT	Pore-forming toxin
$PGPMA$	Poly[N- (3-guanidinopropyl) methacrylamide]
$PHPMA$	Poly(N-(2-hydroxypropyl) methacrylamide)
$piRNAs$	PIWI-interacting RNAs

PKs	Pyrokinins
PLA	Polylactic acid
$PLGA$	Poly-δ,l-lactide-co-glycolide
$PMPC$	Polymethacryloyloxyethyl phosphorylcholine
$PNIPAM$	Poly(N-isopropylacrylamide)
$PQDMAEMA$	Quaternised poly(2-(dimethylamino) ethylmethacrylate)
PVA	Poly(vinyl acetate)
PVP	Poly(vinyl pyrrolidone)
$RAFT$	Reversible addition-fragmentation chain transfer
$RdRP$	RNA polymerase
RH	Relative humidity
$RISC$	RNA-induced silencing complex
RNA	Ribonucleic acid
$RNAi$	RNA interference
RT	Room temperature
SEM	Scanning electron microscope
$s.e.m$	Standard error of the mean
$SFRP$	Stable free radical polymerisation
SID	Systemic interference defective
$siRNA$	Small-interfering RNAs
SKs	Sulfakinins
$SPDm$	*D. melanogaster* sex peptide
$SPDs$	*D. suzukii* sex peptide
$SS-rich$	Disulfide-rich
Tc	Toxin complex
TEM	Transmission electron microscopy
TFA	Trifluoroacetic acid
$TMOF$	Trypsin modulating oostatic factor
V	Volts
$Vips$	Vegetative insecticidal proteins
$vol.\%$	Volume percent
W/O	Water-in-oil
$W/O/W$	Water-in-oil-in-water
$wt.\%$	Weight percent

Contents

List of Figures

Chapter 1

Introduction

Chapter One is separated into multiple sections. Initially, I will explain the motivation behind this research, introducing the invasive pest that this project is tasked to control. Secondly, I will introduce biologically based pesticides (biopesticides) covering their origins, modes of actions and the drawbacks preventing their widescale use. Finally, I will present a literature survey on the current methods of delivery of two classes of biopesticides and draw similarities to delivery of biological species for biomedical applications.

1.1 *Drosophila suzukii* emergence and economic importance

Drosophila suzukii, is an Asiatic pest that has come to the forefront of research with its emergence in North America and Europe and more recently South America.[1–7] Unlike most other fruit flies, *D. suzukii* can puncture the skin of ripening fruit. This ability is facilitated by an ovipositor which is much larger and sharper than those of other *Drosophila* species. Moreover, *D. suzukii's* ovipositor is modified with toothlike bristles on the lateral side that aid in puncturing ripe fruit for egg laying.[8] After puncturing the skin of ripe soft and stone fruit *D. suzukii* deposit their eggs into the oviposition scar.[4] Eggs hatch into larvae which progress through three larval instar stages within the fruit, sustaining themselves on the ripe flesh, causing serious economic damage. Furthermore, due to the rupturing

Figure 1.1: Adult male *D. suzukii* or spotted wing drosophila.

of the skin, the fruit becomes susceptible to secondary pathogens as well as other fruit fly pests.[9–11]

Serious damage was first detected in 1916 in Japan on ripening cherries, with *D. suzukii* classified as the cause by Matsamura in 1931.[12] It is, however, unknown whether *D. suzukii* originates from Japan or was introduced to that country at the turn of the 20th century as the species is also found in other areas of East Asia.[4] *D. suzukii* has a very high ability to disperse, and by 1980 it was observed on the Hawaiian island of Oahu. This dispersion is both active and passive, through infected fruit and other man-made pathways. By 2008, *D. suzukii* had emerged on both the mainland USA and Mediterranean Europe and was spreading across both continents (Figure 1.3).[4] Facilitated by commercial activity and the high reproductive output of *D. suzukii*, these invasions have been relatively quick and further spreading of *D. suzukii* is predicted.

The fruit host range of *D. suzukii* is very broad; stone fruit and thin-skinned berries are particularly susceptible to infestation with the preferred host plant changing with geographic location. This broad host range enables *D. suzukii* to propagate throughout a large geographic area. *D. suzukii* can also infest several non-crop plants including ornamental and hedge plants.[13] If these are in close vicinity to crop plants, they may enable re-infestation and an area-wide management scheme may be needed.

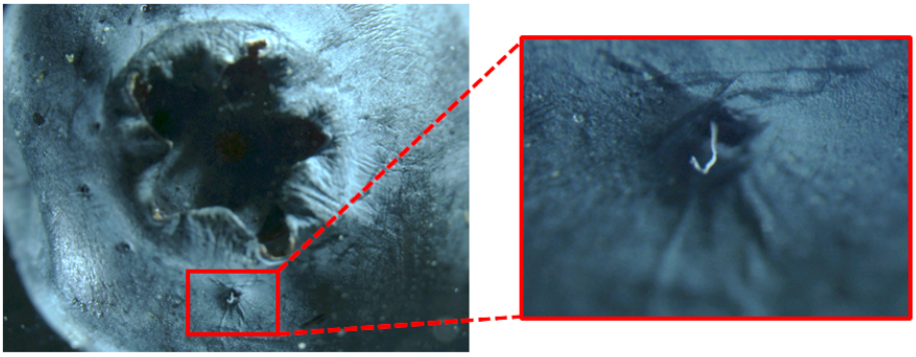


Figure 1.2: Blueberry infested with *D. suzukii* larvae. Blueberry with multiple oviposition sites where *D. suzukii* eggs have been deposited into the fruit.

Figure 1.3: Spread of *D. suzukii* in Europe per year since its invasion in 2008. Reproduced from[4].

In Europe the population of *D. suzukii* is low during early spring but increases rapidly with the ripening of cherries, an early fruit crop. The population increases over the summer and reaches a peak in late autumn; with this increase in population density the levels of infestation increase dramatically, leading to up to 100 % loss of crops for caneberries and strawberries.[9] The economic implication has been estimated by a number of authors and varies widely depending on the geographic area and assumptions made during the calculations. In the eastern-most states of the USA, where *D. suzukii* was first found in the Americas, it is estimated that the economic impact on the soft and stone fruit industry ranges from 0.5 - 2.6 billion dollars annually.[1,10] In Europe, the area of Torintino in Northern Italy has been investigated by multiple authors and may be used as a case study for Mediterranean Europe.[5,14,15] It has been estimated that for this one area of Northern Italy the annual economic impact is between 3 - 4 million Euro annually, when you take into account damage inflicted, increase in labour and pesticide costs.[5]

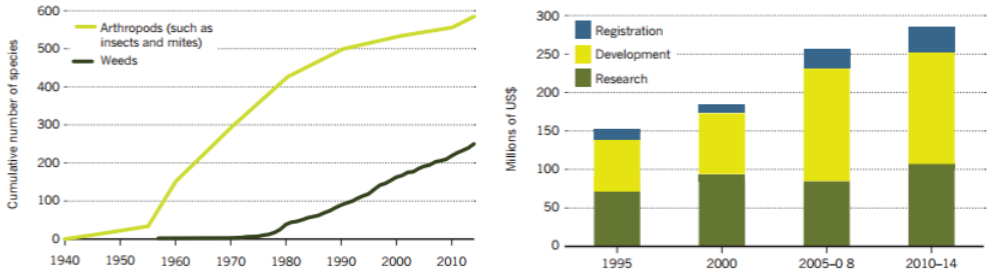
To mitigate against the losses incurred by the invasion of *D. suzukii*, growers are implementing many costly control measures. The most common methods of controlling losses are through the use of chemical insecticides, harvesting of all fruit (including damaged fruit to reduce possible hosts) and through netting of crops.[16–19] The economic gain of such systems is very dependent on the specific conditions of each case (e.g. crop, environment and equipment available), and as a result control strategies must be implemented on a case by case basis.

1.2 Limitations of chemical insecticides

Crop protection strategies are vital to sustaining the world population; it is estimated that without the use of chemical pesticides there would be a 35 - 40 % reduction in global food production, leading to an increase in costs and reduced food security.[20,21] Further to this, global population is increasing at a projected rate of 70 million people per year, and this increase in global population puts further stress on food production. It is estimated that by 2050, we will need to produce 50 - 70 % more food globally, without increasing the total cultivated

land.[22,23] Additionally, the amount of agricultural land available for food production may decrease due to increased demand for non-food commodities such as biofuel.[23] We are dependent on the use of chemical pesticides to maintain and increase global food production. There are, however, many negative aspects to the use of and dependency on chemical pesticides.

Our arsenal of effective chemical insecticides is diminishing due to the rate of pesticide resistance in insect species far exceeding the current rate of discovery (Figure 1.4).[24,25] High rates of resistance are a direct result of overuse of chemical insecticides, producing selection pressure, which leads to mutations and resistance. 586 different arthropod species have been shown to be resistant to at least one synthetic insecticide, with more than 15,000 unique cases of insecticide resistance.[24,26]

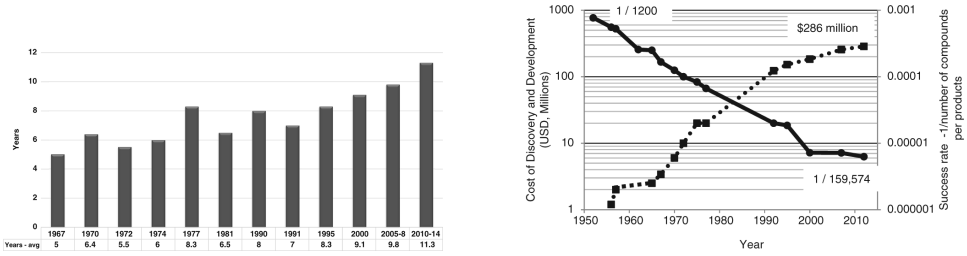


(a) (b)

Figure 1.4: (a) Rate of resistance in insects over the last 80 years. (b) Research and development into new pesticides over 25 years. Reproduced from[24].

Insect resistance is not a new problem facing farmers, with the first report of resistance to the synthetic insecticide DDT in 1948.[27] The most efficient method for minimising the rate of insect resistance is through the use of an integrated pest management system, where chemical insecticides with different modes of action are rotated or alternative control measures are used. However, with the ever-diminishing tool box of effective insecticides, resistance is increasing due to limited rotation between insecticides. On the other hand, the rate of discovery of new insecticides is not increasing, especially for chemicals with new modes of action to combat resistance. This is due to several different factors, including

the increased cost in discovery, stricter regulatory laws and reduced industrial research.[26] The average time it takes to get from the discovery of the active ingredient to a product on the shelf has increased over the past 50 years from 5 to 11.3 years (Figure 1.5a). This increase in time can be linked to the increase in regulatory requirements needed to take a product to market. Along with increased regulatory requirements, the cost of discovery has increased and rate of discovery of new active has decreased dramatically (Figure 1.5b), leading to fewer companies investing in the discovery of novel compounds.[26]



(a) (b)

Figure 1.5: (a) Estimation for the time it takes to go from novel active discovery to product. (b) Cost and success of novel compound development. Reproduced from[26].

In addition to the increasing rate of resistance and reducing rate of novel compound discovery, there is a growing concern with the environmental impact of chemical insecticides. Recently, there has been a dramatic reduction in seasonal trappings of arthropod species, decreasing by 76 % over the last 27 years in Germany.[28] The source of this decrease in insect biomass is debated, but a common hypothesis is that the reduction is due to increased use of synthetic insecticides. The reduction in arthropods observed may not be solely due to the acute toxicity of insecticides, but sub-lethal effects may lead to reduced fitness and death. These sub-lethal effects include impacts on learning performance, behaviour, and neurophysiology.[29] Around 80 % of wild plant species require insect pollinators and therefore a reduction in pollinator population is extremely detrimental to the ecosystem. In the USA, it is estimated that the eco-services

provided by insect pollinators is worth over 57 billion dollars annually.[30] The effect of synthetic insecticides on bees has been widely researched due to their capacity to pollinate and the general decrease of species richness over the past 50 years.[31] The decrease in bee population is thought to be down to three main causes: habitat loss through the reduction in wild flora, increased exposure to a myriad of new parasites and pathogens, and increased exposure to a cocktail of pesticides.[31] In a three year study (2012 - 14), the pollen collected by honey bees in Germany was analysed for pesticide contamination; 62 % of all pollen samples were contaminated with at least one pesticide, with 38 % containing multiple different contaminants.[32] The effect of these pesticide residues often does not lead to acute toxicity, but non-lethal exposure can lead to behavioural changes. For example, thiamethoxam (a neonicotinoid) causes high indirect mortality rates in bees through bee homing failure, where the insect can no longer find its way back to the hive, leading to a risk of colony collapse.[33]

Synthetic insecticides may also have toxicity to mammalian populations. Insecticides commonly kill the target pest by targeting its nervous system, interfering with chemical neurotransmission. Most synthetic pesticides are not highly selective and have neurotoxic effects in mammals.[34] These effects on the mammalian nervous system may be acute or contribute to chronic neurodegenerative disorders such as Parkinson's disease.[34]

A greater understanding and concern over the ecotoxicity of synthetic pesticides has led to an increase in regulation.[26] Numerous older products have had their registrations cancelled over the past several decades as they no longer meet the current eco-safety standards. This has resulted in a reduction in the available compounds, leading to increased reliance on a small number of insecticides. It is vital for the continued increase in global crop production that alternative control measures to insect pests are found. Crop protection is required whilst maintaining wild insect biodiversity and satisfying current safety guidelines. Additionally, the cost of goods must be low enough to make them economically viable, especially in developing countries.

1.3 Bioinsecticides, an alternative to synthetic insecticides

With the recent restrictions on the use of chemical pesticides, for both *D. suzukii* and other pest species, there is interest in increasing the use of biologically based pesticides. Biopesticides cover a wide range of substances that are derived from natural sources, and can be classified as protein/peptide based (derived from venoms, microbes, endogenous regulators or plant sources)[35–38] or nucleic acid based (double-stranded RNA (dsRNA)).[39–41] Biopesticides can have a number of positive attributes compared to their chemical alternatives, such as specificity for a target pest,[42] biodegradability with non-toxic residues,[38] and easily integrated with most other plant protection measures. In the following section I will investigate the sources of several classes of biopesticides, along with their properties, their current uses and the physical characteristics that are currently limiting wide-scale use. The economic factors and the rate of control will not be covered, although these are often limiting factors in the use of biopesticides.

1.4 Peptide/protein biopesticide toxins

Protein and peptide based bioinsecticides occur naturally and can be isolated from a number of different sources including predatory/parasitoid venom-derived toxins, endogenous neuropeptides and hormones, plant protein toxins, pathogenic microbes, and microbial toxins.[37] In this section I will detail the sources of peptide/protein biopesticide toxins, examples of use and their limiting physical characteristics.

1.4.1 Predatory/parasitoid venom-derived toxins

Venom-derived biopesticides are naturally available from a number of different arthropods including spiders, scorpions, wasps, mites and cone snails. Venoms have evolved within these species for paralysing prey and predators, and are composed of a mixture of salts, protein and peptidic toxins. Proteomic studies have shown that individual spider venoms may contain over 1,000 different small

peptide toxins (1 - 10 kDa).[36] There are over 40,000 species of venomous spider that conservatively contain more than 250 peptides per venom, giving over 10 million bioactive peptides, most of which will show insecticidal properties.[35,36] Therefore, there is an almost limitless supply of potential bioinsecticides available from spider venoms. As the venom aims to induce paralysis in the prey, the vast majority of the insecticidal peptide toxins target the nervous system of insects.[36]

Venoms have also been shown to contain a number of larger proteins ($>$ 30 kDa), which are mostly enzymes.[35] It has been hypothesised that their role is to degrade the extracellular matrix and cell membrane, allowing penetration of the toxins into the prey's central and motor nervous system. Individually they do not show insecticidal properties, but they can aid in the uptake of active species such as peptidic toxins. Apart from enzymes, spider venoms may contain high molecular weight (Mw) proteins known as latrotoxins, 110 - 140 kDa. These are of specific interest because of their phylum specificity. The European black widow spider contains a number of different latrotoxins which are individually specific to either vertebrates, crustaceans or insects. Insect-specific latrotoxins have potential for bioinsecticide use; however, due to their complex mode of action and difficulty of production they have not yet been exploited.

For a protein biopesticide to be used widely, it must be delivered orally in an aqueous environment. Typically, venoms are not applied orally; instead they are injected into the prey and as a result a large percentage of the discovered peptide toxins that show insecticidal behaviour via injection are not orally active. This is due to two main barriers: the degradation of the peptide toxin in the gut juices by proteases, and the poor epithelial penetration of peptide toxins.

The exception to this is the over 200 disulfide-rich (SS-rich) peptide toxins that have been found to be both stable in the gut juices of a number of pest species. These SS-rich peptides make up the majority of spider venom, contributing to the majority of the insecticidal activity. Although the structure of the majority of peptide toxins has not been deciphered, the majority that have been discovered conform to one structural class, named the inhibitor cystine knot (ICK).[43] This ICK motif has evolved to provide the small peptide toxins with high chemical, thermal and biological stability. Importantly, this modification provides high resistance to proteases, the main degrader of peptides.[43] ICK peptides have

shown extended stability in gastric juices for over 12 h. However, these stable peptide toxins have had limited use as commercial bioinsecticides due to their poor penetration of the epithelial layer and systemic uptake into the pest.[43]

1.4.2 Endogenous neuropeptides and hormones

Endogenous regulators produced by insect neurosecretory cells have lately been intensely studied as possible bioinsecticides. These insect neuropeptides and hormones regulate the crucial physiological and behavioural processes in insects, potentially providing receptor targets and species specific biopesticides.[37,38] Through the investigation of insect neurohormonal regulation, several classes of neuropeptides and hormones have been determined, including trypsin modulating oostatic factor (TMOF), hormone biosynthesis activating neuropeptides (PBANs), pyrokinins (PKs) sulfakinins (SKs) and allatostatins (ASTs).

TMOF is a decapeptide that stops the production of trypsin and chymotrypsin enzymes in the midgut epithelial cells. Specifically, the hormone binds to the TMOF receptor on the haemolymph side of the gut, greatly reducing the levels of trypsin. Inhibition has been demonstrated in the mosquito *Ades aegypti* (where the TMOF peptide was discovered) along with a number of significant pest species, including Dipteran.[44] However, the poor water solubility of TMOF and varying enzyme suppression has limited its use.

The physiological functions of PKs and PBANs are to control pheromone biosynthesis, to stimulate intestinal contraction, and to regulate the secretion and control of digestive enzymes.[45,46] This class of peptide range from 8 - 33 residues long and have a common C-terminal pentapeptide active portion Phe-Xxx-Pro-Arg-Leu-NH_2, used for receptor recognition. The peptide bond between the Pro and Arg residues is susceptible to hydrolysis by tissue-bound peptidases in insects' guts, and as a result is not active orally.[46]

SKs function as neurotransmitters in the central nervous system. They have been shown to modulate the contraction of the gut, crop and heart. Furthermore, SKs have been shown to inhibit food uptake and reduce the production of digestive enzymes. SKs have a prominent tyrosine sulphate residue, which is required

for its insecticidal properties. However, this residue is extremely susceptible to hydrolysis, especially under acidic conditions.[38]

ASTs have been shown to inhibit the production of juvenile hormone, which plays a main role in growth and reproduction, allowing the control of insect maturation and egg production. Furthermore, peptides of this family have been shown to modulate muscle activity, inhibit of vitellogenin production and stimulate carbohydrate metabolism. Again, ASTs have not been utilised as a bioinsecticide due to their instability in the environment and rapid degradation by insect peptidases.[38]

1.4.3 Pathogenic microbes and microbial toxins

Over 90 species of bacteria have been discovered that infect insect pests,[47] producing a large number of insect specific peptide toxins.[37] This section will cover some of the predominant bioinsecticides produced from bacteria.

Bacillus thuringiensis (Bt) is the most extensively studied and most widely used bioinsecticide, accounting for around 80 % of the sprayable bioinsecticide market.[37,48] Bt is primarily active against the larval stages of insects, killing through the disruption of the midgut tissue, followed by septicaemia.[49] The disruption of the midgut tissue is achieved through the production of an array of different insecticidal protein factors, including parasporal Cry δ-endotoxins, vegetative insecticidal proteins (Vips) and phospholipases. Of these, the parasporal Cry δ-endotoxins are most commonly utilised and are highly selective. They belong to the pore-forming toxin (PFT) class of bacterial toxins. During sporulation, crystalline inclusions are formed which contain δ-endotoxins. These inclusions are solubilised on ingestion, releasing the δ-endotoxin. Upon proteolytic activation, this then undergoes conformational changes and inserts into the gut membrane of a pest.[49–51] These δ-endotoxins have been widely adopted as an alternative to conventional chemical pesticides through the use of a classical spray approach and expression in transgenic plants. However, a number of cases of insect resistance to δ-endotoxins of Bt have been documented, with enhanced gut digestion of the toxins being postulated as one mechanism of resistance.[44]

Increased resistance and UV instability have led to research into other families of insecticides and modified delivery of δ-endotoxins.[48]

Along with the Cry δ-endotoxins, Bt produces a range of Vips that can be used to control pests that are less susceptible to degradation. This protein type consists of three classes, Vip1 and Vip2 (which are coleopteran specific) and Vip3 (which is lepidopteran specific).[52] Vips have insecticidal activity, sharing no sequence homology with δ-endotoxins and have a separate mode of action. For example, Vip3A an 88 kDa protein is proteolytically activated in a similar manner to Cry toxins but does not bind to the same receptors on the brush border membrane, and therefore supports a unique mode of action.[53]

Insecticidal proteins can also be obtained from many insect-active bacteria, including *Xenorhabdus nematophilus*, *Serratia entomophila* and *Photorhabdus luminescens*, which produce toxins called Toxin complex (Tc).[54,55] Tc is the major component of a high Mw fraction secreted during the stationary phase of bacterial growth, containing a number of different toxins secreted by *P. luminscens* (each approximately 1 MDa). These proteins belong to one of three distinct classes, with at least one subunit from each of the three classes combining to form a complex with potent insecticidal activity. *Per os* application of Tc displays similar histopathology to Bt toxins, with apical swelling and bleeding of large cytoplasmic vesicles by the columnar cells, leading to the eventual extrusion of cell nuclei in vesicles into the gut lumen.[54] The mode of action of Tc compounds has recently been discerned, whereby the bacteria use a special syringe-like mechanism to perforate the host cell membrane and inject a deadly enzyme into the host cytosol.[56] Although this class of proteins has shown insect selectivity, the production of these large proteins is costly and as a result the delivery of these compounds has only been investigated through the use of transgenic plants.

1.5 RNA interference

RNA interference (RNAi) is a cellular process by which gene expression and translation are inhibited by the selective degradation of targeted messenger RNA (mRNA). This RNAi machinery is found in all eukaryotic cells and serves as a defence mechanism against viral infections.[57] RNAi in animals was discovered by

Fire *et al.*, who worked with the nematode *Caenorhabditis elegans* and discovered that ingestion of sense or anti-sense RNA could have an effect, but that double-stranded RNA (dsRNA) enabled more efficient RNAi.[39,58] The RNAi mechanism can also lead to the down regulation of microRNAs (miRNAs), resulting in translational repression which controls development.[59]

Since the discovery of the RNAi machinery, it has been exploited as a powerful tool in functional genomics for discerning the function of newly discovered genes.[25,57,60,61] Small non-coding RNA molecules can cause specific knockdown of gene expression, and the resulting phenotypic observations can be used to determine gene function. RNAi-based pest control works through the suppression of a gene vital for the survival of the pest.

1.5.1 RNAi mechanism

The mechanism of RNAi is now well defined, with the pathways between insect species differing in several ways, with only a small number of the key steps well conserved. The dsRNA is taken into the cell by dsRNA specific channels and/or endocytosis. Upon entry into the cytoplasm, the long dsRNA is recognised and processed into small-interfering RNAs (siRNA; 20 - 25 nucleotides).[57] Of these, siRNA is of interest to regulate expression of specific genes. dsRNA is processed by the RNase-III enzyme Dicer into siRNA duplexes. These fragments are then incorporated into the multi-protein RNA-induced silencing complex (RISC), where one of the duplex strands is eliminated and the other is used as a guide strand. This complex then cleaves single-stranded RNA with a complementary sequence, through the RNase H-like domain of the Argonaut protein (Figure 1.6). This RNAi machinery is found in eukaryotic cells and is used to defend against viral dsRNA, where upon processing the RISC complex corresponds to the viral RNA, leading to its breakdown. With the introduction of a dsRNA sequence that is gene specific, we can utilise this machinery to cleave targeted messenger RNAs (mRNA) produced within the cell.[60]

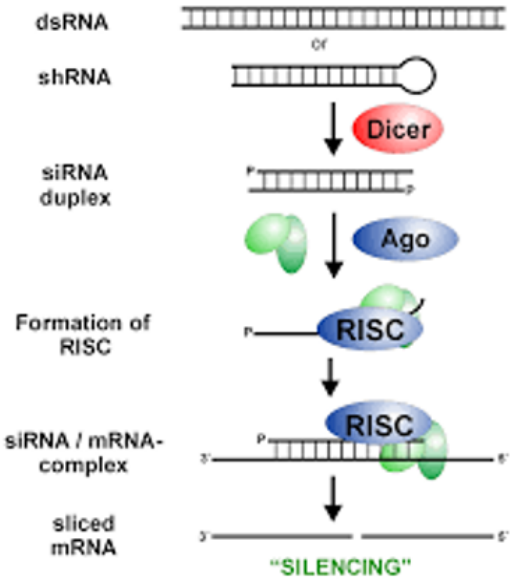


Figure 1.6: Simplified RNAi mechanism.

1.6 RNAi variation between insect orders

The efficiency of RNAi is not equal across all insect taxa, due to the variation of many factors. Firstly, oral application of dsRNA results in exposure to extracellular nucleases in the gut and harsh pH environments, which change across taxa. Secondly, the cellular uptake across different insect groups varies widely. Thirdly, the production of secondary dsRNA molecules is only observed in certain species. Finally, the level of transfer of dsRNA between cells is not conserved across taxa. In the following sections I will summarise the main differences found across insect orders and the implications they have.

1.6.1 dsRNA degradation in the insect gut

To date, only a small number of arthropods have had RNAi induced through oral treatment with dsRNA. The success is spread across a number of different insect orders, including *Lepidoptera, Diptera, Hymenoptera, Hemiptera, Coleoptera* and *Isoptera.*[62] However, it has been shown that other species within the orders

described do not show RNAi through feeding of dsRNA, but do via injection. This variation may be partially due to differences in the degradation rates of dsRNA in the gut juices.[63,64]

Wang *et al.* investigated the degradation rate of dsRNA by targeting the homologous chitinase gene in 4 different insect orders: *Blattodea, Coleoptera, Orthoptera* and *Lepidoptera*.[65] This study looked at the degradation in both the gut juices and in the haemolymph. Degradation of 95, 72.6, 24.7 and 9 % in the haemolymph after 1 h and 88, 80, 17 and 16 % in insect midgut juices after 10 mins were found for *Spodoptera litura* (Lepidoptera), *Locusta migatoria* (Orthoptera), *Zophobas atratus* (Coleoptera) and *Periplaneta americana* (Blattodea) respectively.[65] Furthermore, they found a negative correlation between the concentration of dsRNA in the haemolymph and degradation rate after *per os* application, showing that the degradation in the midgut impacts on the uptake into the haemocoel. There was a direct link between degradation of dsRNA and levels of mRNA depletion in the various insects. For injection, depletion levels of 20, 76, 78 and 82 % and for ingestion 1, 5, 29 and 47 % were observed after 72 h for *S. litura, L. migatoria, Z. atratus* and *P. americana* respectively.[65] As a result, it is important to consider what is causing the differing degradation rates across species and ways to protect ingested dsRNA.

It has been demonstrated in various laboratories that upon ingestion into the gut of arthropods, dsRNA is subject to varying degrees of degradation. The midgut juices of the silk moth *Bombyx mori* contain 3 main degrading enzymes: proteases to digest food, lipase to protect against nucleopolyhedrovirus and a DNA/RNA non-specific nuclease. This DNA/RNA non-specific nuclease is often designated dsRNase due to the high activity against dsRNA.[66] This ribonuclease has subsequently been found in a number of other species, not only in the midgut but in the haemocoel of insects and appears to be important in dsRNA degradation in insects.[62,65,67,68]

Another nuclease, Eri-1, found in the nematode *C. elegens*, along with a homolog in the pea aphid *Acyrthosiphon pisum* induce a reduction in RNAi. Homologs in *D. melanogaster* and *Tribolium castaneum* do not have an effect on RNAi.[68,69]

1.6.2 dsRNA cellular uptake pathways

At present, there are two main routes of dsRNA uptake into cells: via a transmembrane protein Systemic Interference Defective (SID) and/or via endocytosis.[57,60,70] Following the discovery of the RNAi machinery in *C. elegans*, significant research has been made into dsRNA uptake mechanisms and the systemic spread. It has been shown for *C. elegans* that the SID-1 channel is essential for uptake and systemic RNAi. SID-1 is a multi-span transmembrane protein that functions as a multimer, transporting dsRNA passively into cells but is not a vital component for the export.[70] However, dsRNA must be larger than 50 base pairs (bps) to be disseminated throughout the body of *C. elegans*.[60,70]

Orthologs of the SID-1 protein have been identified in a number but not all of insects. Furthermore, some SID-like orthologs do not appear to aid the uptake of dsRNA. They have greater sequence similarity to the CHUP-1 receptor in *C. elegans*, a cholesterol transporter with no known involvement in RNAi. *D. melanogaster*, a model species and close relation of *D. suzukii*, lack's robust systemic RNAi, with no SID gene orthologs , but dsRNA can be taken up into cells.

Endocytosis is another route of entry into the cell for dsRNA; a mechanism first observed in *D. melanogaster* S2 cells and has since been shown to be evolutionarily conserved. The uptake of dsRNA by endocytosis in *D. melanogaster* has been confirmed by two main tests. Firstly, blocking of endocytosis by pharmaceutals has led to poor dsRNA uptake. Secondly, fluorescently tagged dsRNA was shown to be associated with intracellular vesicles, suggesting receptor-mediated endocytosis. This mechanism of uptake is mediated by the scavenger receptors Eater and SR-C1.[71] These receptors have previously been shown to play an important role in phagocytosis of bacterial pathogens, and it is believed that the mechanism is similar. Saleh *et al.* investigated the uptake of dsRNA in great detail, screening over 7,000 genes for involvement in dsRNA uptake; of these, 23 were shown to be directly or indirectly involved in endocytosis, confirming this as a main route of uptake.[72]

In *D. melanogaster*, uptake can proceed by endocytosis, however, the rate and efficacy of this transfection is related to factors such as electrostatic interac-

tions between anionic dsRNA and anionic cell membranes. Therefore, there is an electrostatic barrier preventing uptake by the gut epithelial cells.

1.6.3 Systemic dsRNA amplification and dissemination

In some animals RNAi can be regenerated by RNA polymerase (RdRP), amplifying the signal which can then spread from tissue to tissue.[57] For *C. elegans*, Dicer-processed siRNAs are utilised as a template by RdRP to produce secondary siRNA.[73] However, no insects have been shown to possess a RdRP-related protein or use this method of amplification.[57]

RNAi has been shown to not be systemic in *D. melanogaster* but is systemic in the beetle *Tribolium*, although the main genes involved in amplification are found in both species. There are however, several variations in the number of key genes, which may explain the differences observed.[74] Specifically, one of the key proteins Ago is duplicated in *Tribolium*, while *D. melanogaster* only has the *AGO-2* gene, which may account for the observed difference in RNAi response.

No long-lasting systemic RNAi occurs in *D. melanogaster*, and therefore RNAi mediated knockdown may be limited to the area of delivery. As a result, it is sensible for control of *D. suzukii* to target knockdown in the midgut cells, the site of entry after oral application. There are multiple targets in the gut cells that are proven to be critical for survival, with the vATPase proton pump being commonly studied.[25,59] This vATPase proton pump is used to acidify tissues in order to activate proteases.

1.7 Protein & peptide bioinsecticide delivery in agrochemicals

There are multiple sources for potential biopesticides based on proteins and peptides, but their physical properties sometimes limit their wide-scale use. The most successful biopesticide is Bt toxin; this is in part due to its physical features. Bt is delivered in a crystalline form, which provides protection on the leaf surface and upon ingestion in the insect gut, where it is dissolved to release the active δ-endotoxins.[37,48,49] In order to increase the potency and efficiency

of bioinsecticides, a number of different delivery systems have been investigated in the agrochemical industry. This section will explore some of these delivery methods.

1.7.1 Protein and peptide conjugation

The stability of peptide and protein bioinsecticides may be increased through the conjugation of the active protein/peptide with a secondary species such as another protein, or with a synthetic polymer such as polyethylene glycol. This fusion can provide increased protection from proteases. Furthermore, depending on the nature of the conjugated species, some targeting and/or increased uptake can be achieved. Here both fusion strategies will be detailed, drawing similarities between the two approaches and highlighting drawbacks.

Plant lectin fusion

Plant lectins are carbohydrate-binding proteins that bind with avidity and specificity to sugar moieties and have been utilised to protect a number of different insecticidal proteins. The fusion of proteins does not interfere with the inhibitory activity or binding efficiency of either species. Moreover, the insecticidal activity was enhanced compared to the application of a mixture of the separate proteins at the same concentration.[75,76]

Some lectins, such as the snowdrop lectin (*Galanthus nivalis* agglutinin, GNA), have a small amount of insecticidal activity specific towards lepidopteran insects. Although the mechanism of this toxicity is still unclear, it appears to involve specific binding to the gut epithelium.[77] The fusion of GNA to insecticidal peptides increased uptake of the active species into the insect's circulatory system because of GNA's ability to easily cross the epithelium.[78] Furthermore, GNA is resistant to proteolysis in insect gut juices, providing protection by association for a conjoined protein.[79,80]

These fusion proteins were produced through the ligation of cDNA coding for the specific insecticidal peptide and the lectin. The protein was then expressed and purified from yeast cells. Fitches *et al.* amplified the cDNA coding for a spider toxin (SF11), which was then inserted into an expression vector, pGAPZαA.

The resulting SFI1pGAPZαA plasmid was ligated with GNA cDNA and transformed into *Pichia pastoris* yeast X33 cells for protein production.[80] Through this recombinant methodology, GNA has been fused to several toxins, including spider venoms,[80,81] scorpion toxins,[82] Bt toxin Cry1Ac and *Allium sativum* agglutinin.[83] The fusion of peptide toxins to plant lectins significantly increased the potency of the toxin in *Lepidoptera*, resulting in 100 % mortality.[83] In some cases, although the lectin is resistant to peptidases, the linker between it and the peptide toxin is not, leading to cleavage and subsequent degradation of the toxin.[84] The wide-scale application of this approach is limited by the cost of production and purification.

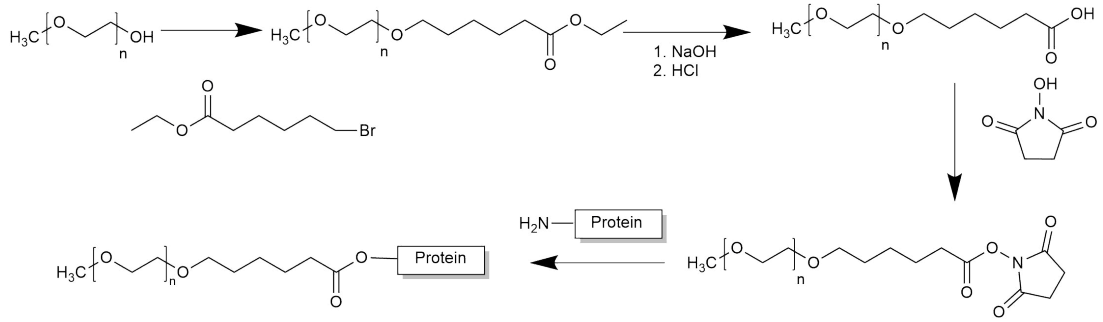
1.7.2 Plant virus coat proteins

Plant viruses are often vectored by sucking pests, where the virus is taken up by the pest after feeding on sap of infected plants, and is transported into the insect haemocoel from the gut. It is then transferred to the salivary glands, where it is passed into another plant substrate in saliva during subsequent feeding.[84,85] This ability to transfer from the gut to the haemocoel is of interest for its potential to deliver insect peptide toxins. This transfer can be receptor-mediated, as shown for the Pea enation mosaic virus (PEMV), which uses gut cell receptors for efficient uptake. This can give specificity for the transport of the peptide toxin into pests containing the required receptor.[84] A recombinant protein synthesis approach, similar to that described for making the plant lectin protein conjugates, has been used to fuse the coat of luteoviruses to insect peptide toxins.[86] This approach showed a significant increase in the mortality of 4 aphid pests compared to the toxin alone. However, the field use of fused virus coat proteins is also limited by production costs. In addition, this method is specific to aphids since the virus receptor is only found in this group of insects. For the control of plant sucking pests such as aphids, the delivery vector must be present inside the plant, which is not achievable with current agrochemical application technology.

Protein lipophilic-polyethylene glycol (PEG) polymers

Transport of proteins across biological membranes can be increased and proteolytic degradation can be reduced by conjugation to polyethylene glycol(PEG). This technique of PEGylation has been used extensively in therapeutics to increase protein transport across the digestive tract. The conjugation of a model human protein (insulin) was used to demonstrate that a similar methodology could be used in insects.[87–89]

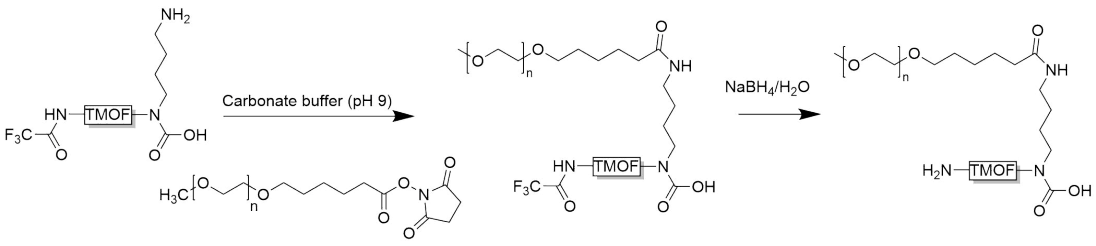
The synthesis of these conjugated proteins to linear PEG proceeds by first transforming the PEG into a carboxylic alternative. This carboxylic acid is then activated using *N*-hydroxysuccinimide and reacted with the N-terminal of the protein/peptide, as seen in Scheme 1.1. Through this method, a 7-fold increase in uptake of insulin into the insect haemocoel was achieved.[87] This method was used for the delivery of the insecticidal peptide TMOF, conjugation of which led to accumulation of the peptide in the haemolymph of larval tobacco budworm, *Heliothis virescens*.[89] Initial investigations suggested that this technique may increase delivery and control of mosquito larvae.[89] However, later studies showed that conjugation through the N-terminal of TMOF lead to inactivation of the peptide toxin.[44,90]



Scheme 1.1: Conjugation of linear PEG to the N-terminus of proteins for enhanced stability. Adapted from[87].

In order to conserve the functionality of TMOF, conjugation cannot be undertaken at the N-terminus. Addition of a lysine group to the C-terminus of TMOF, and a trifluoroacetic acid (TFA) protecting group to the N-terminus,

allowed for a similar synthetic route to be used (Scheme 1.2).[44,90] TMOF-K conjugated to PEG decreased the rate of degradation by leucine aminopeptidase, with an inverse relationship between PEG Mw and rate of degradation.[90] *In vivo* testing of TMOF-K showed a reduction in toxicity against larval *Aedes aegypti* compared to TMOF. However, TMOF-K conjugated with methyl(ethylene glycol)$_7$-O-propionyl led to an increase in oral toxicity (6 and 10-fold compared to TMOF and TMOF-K).[44]



Scheme 1.2: Conjugation of linear PEG to lysine at the C-terminus of TMOF. Adapted from[44,90].

PEGylation of the binary toxin (BinAB) from *Lysinibacillus sphaericus* provided increased protection of the toxin against both trypsin and proteinase K, enhancing the toxicity of the larvicide 6-fold. Moreover, the PEGylation enhanced the thermal stability of the toxin by 3 - 5 °C.[91] The PEGylation in this case was achieved using a similar method to couple at the N-terminus, but used an isocyanate functionalised PEG to conjugate. This demonstrated that PEGylation of peptides is not limited to just TMOF, and that this approach may be used to increase the stability and uptake of other insecticidal peptides.

1.8 Protein & peptide delivery for use in therapeutics

The protection and delivery of biopesticides is a new area of research, with few systems having been developed for efficient delivery. Similarities between the delivery of biopesticides to the administration of therapeutic drugs can be made as similar barriers to uptake are found. The delivery of proteins and peptides for

use in therapeutics generally concentrates on the delivery of the active species over a long-time period. Delivery vectors are designed so that they can flow through the circulatory system of a patient, releasing their contents at a constant rate. In a few cases, typically targeting cancer cells, a triggered release mechanism is used, stimulating burst release of the drug at its target site. For efficient biopesticide delivery, a large concentration of the peptide/protein need to be delivered to the main site of uptake in the pest species in a short period of time. Furthermore, the polymer carriers used in therapeutics need to be safely metabolised and excreted from the body. For pest management systems, the polymers used do not need to be degraded within the pest and some level of polymer toxicity is acceptable, which broadens the range of polymers available for use in crop protection. The acceptable size of the delivery vector also differs between the two targets. Therapeutic vectors tend to be in the nano range to increase cellular uptake and minimise any immunological response. Whereas for pest delivery, the mouth parts of the pest determine the maximum particle size, typically in the micron range.

This section will explain the problems encountered in the field of therapeutic protein drug delivery, and will compare these issues with those encountered in protein biopesticide delivery. Examples of protein drug vectors will be discussed, and their potential evaluated for use in pest control.

With the advent of DNA recombinant technologies, the availability of peptide and protein-based drugs for therapeutics has increased dramatically. Using DNA technologies, proteins can be produced on a large scale by expression in vectors including bacteria and yeast.[92,93] This advance in technology avoids the need for extraction and purification of proteins from animal sources. Therapeutic proteins are being increasingly used in treatment of cancer, inflammatory diseases and as vaccines.[92,94,95] Protein-based drugs show considerable promise due to their low toxicity, widespread applicability, high specificity and activity at relatively low concentrations.[92,95–97]

Currently there are around 130 FDA approved products using therapeutic protein technologies, with more in development.[93] However, the wide spread use of protein/peptide drugs is limited by the delicate tertiary structure that

needs to be maintained for their activity. These proteins often have poor stability and are degraded either chemically or proteolytically. They can also unfold or aggregate, leading to loss of activity and often the elicitation of an immune response.[92,98,99] Oral application of protein drugs is particularly challenging due to high proteolytic activity and acidic conditions in the stomach. Furthermore, the bioavailability of protein drugs applied orally is limited due poor absorption through biological membranes. Consequently, the majority of protein drugs are injected into the body, avoiding the digestive tract.[92] Fast renal clearance and hepatic metabolism leads to short circulatory system retention times upon injection, limiting the therapeutic effect. To combat these factors, drug delivery systems have been developed to enable the use of needle-free administration, by protecting the active therapeutic from premature degradation, increasing the uptake profile across biological membranes and extending the circulation time of protein drugs.[92]

The following sections will discuss a variety of different delivery systems used for oral, nasal and injected applications. Polymer hydrogels and nanoparticles of varying sizes and compositions are often used. In most cases, not all aspects of the systems are appropriate for the delivery of biopesticides, but component parts may be incorporated into a biopesticide vehicle. Notably, the mechanism of release from both hydrogels and nanoparticles is sustained over long periods of time, which may not be optimum for delivery to insect pests due to their short digestive times.

1.8.1 Polymeric hydrogels for peptide/protein delivery

Hydrogels are a network of hydrophilic polymers that are cross-linked, either physically or chemically, and have the ability to retain a large amount of water (over 50 %) whilst maintaining structure. Since the first biomedical use, they have been applied in numerous biomedical applications including drug delivery.[92,94–96,100–103] Hydrogels for therapeutic applications are in two forms; injectable gels that do not circulate through the body, or discrete spheres that move through the circulatory system. Hydrogels provide a number of advantages over

other delivery systems, for example: controllable size from nanometres to micrometres; biocompatibility, enabling prolonged circulation in the blood stream; surface chemistry suitable for bioconjugation; mild chemical and temperature conditions used for formation; and biodegradability for sustained release.[92,102] Furthermore, the release rate from hydrogels can be tuned by altering the degree and type of cross-linking. Hydrogels can be segregated into two different classes depending on whether the polymer precursors are natural or synthetic.

Natural polymer hydrogels

Natural polymer hydrogels are extracted from a number of natural sources and can be chemically modified after or during extraction. Cross-linking of these natural substances can bestow different physical characteristics. There are three main natural polymers that are used for protein delivery in therapeutics; these are gelatin, chitosan and alginate.[94,95,102] Other natural based polymers have been investigated with varied success, as documented in the review by Lee *et al.*.[94]

Gelatin is generally obtained from bovine skin or bone through acid or base extraction. It is a single stranded polymer that easily gels upon changing temperature. The release from gelatin is dependent on the enzymatic degradation of the polymer, which is a function of cross-linking density and the chemical nature of the cross-linking. To modify the release rate of proteins and to alter the mechanical properties of the hydrogels, chemical modifications have been investigated. For example, styrene-derivatized gelatin gels formed using photo-cross-linking with camphorquinone gave a longer release profile and reduced cytotoxicity compared to non-modified gelatin.[104]

Chitosan is the second most abundant polymer in nature after cellulose.[105] This cationic polymer is extracted from the outer shell of crustaceans in the form of chitin, which is processed to chitosan through deacetylation. Commonly, α-chitosan is produced, and its structure is shown in Figure 1.7a. This polysaccharide linear co-polymer consists of 2-amino-2-deoxy-D-glucose (D-glucosamine) and 2-acetamido-2-deoxy-D-glucose (N-acetyl-D-glucosamine) units, similar in structure to cellulose. The degree of deacetylation commonly varies between

70 - 95 %, with the Mw ranging from 10 to 1,000 kDa.[105] Chitosan is biodegradable and has many positive attributes that make it suitable for use in protein drug delivery.[106] Chitosan is a bioadhesive and can adhere to the mucosa of the gastric system, increasing the retention time by adhesion to the mouth, stomach and small intestine mucosa. The mechanism for this adhesion is mainly through ionic interactions, where the positively charged amino groups in chitosan and the negatively charged mucosa layer interact.[107] The degree of mucoadhesion is a function of the deacetylation and the Mw of chitosan; both factors increase the number of amino groups for adhesion.[108,109] In addition to mucoadhesion, it has been reported that chitosan enhances penetration by opening epithelial tight junctions. This enhanced penetration is due to the positive charge of the chitosan interacting with the cell membrane, specifically with tight junction-associated proteins.[110] Chitosan is a weak polybase with amino functionality that dissolves at low pH and is insoluble at high pH. This dissolution at low pH is a result of protonation of the amino groups leading to charge repulsion between chains. This release mechanism is suitable for release in the human stomach; however, most drug delivery systems target uptake in the intestine. Chemical modifications must be made to chitosan for delivery to the intestine, including thiolation[111–113] and quaternisation[114,115] of the amino groups, and alteration of the alcohol groups to carboxymethyl groups.[116] These modifications allow for control of the swelling and response of the polymer with varying pH.

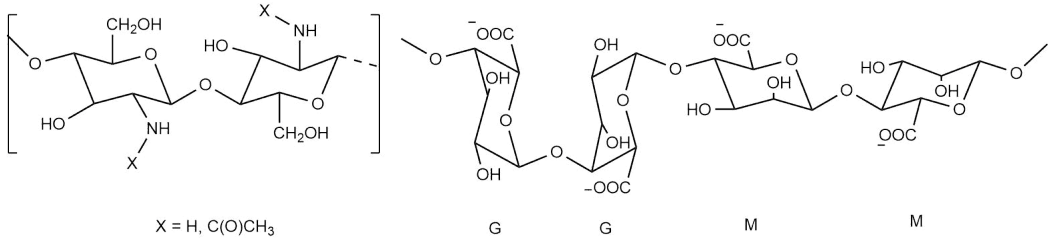


Figure 1.7: Structure of chitosan (a) and alginate (b) with β-D-mannuronic (M) and α-L-guluronic acid residues (G). Adapted from[95].

Alginate is a biopolymer obtained from several species of brown algae, including *Laminaria hyperborean* and *Ascophyllum nodosum*. Alginate exists in each of these species with a mixture of different cations, and accounts for roughly 40 % of the dry weight.[117] It is a linear polysaccharide that contains varying amounts of β-D-mannuronic acid and α-L-guluronic acid residues (Figure 1.7). Upon the addition of divalent cations, the polymer chains adopt an egg-box structure formed by chelation between the α-L-guluronic acid residues of different chains (Figure 1.8).[95] Release of proteins from alginate beads occurs by two mechanisms: diffusion through pores in the network, and degradation of the polymeric network. The resulting release profile for proteins is slow, and is a function of the protein Mw as well as charge.[95,118] The production of alginate-based protein drug carriers is severely limited by the entrapment efficiency upon formation, due to leaching from alginate beads. To mitigate this, modifications have been made in order to better cross-link the alginate chains.

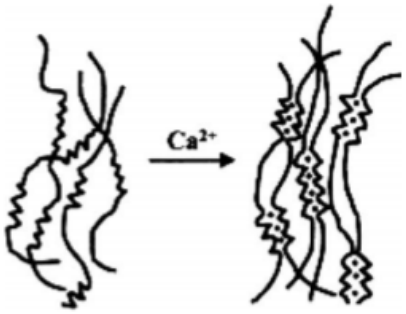


Figure 1.8: Alginate gelation upon addition of Ca^{2+}. Reproduced from[95].

Synthetic polymer hydrogels

Synthetic polymer based hydrogels have a number of advantages compared to their natural based analogues. Synthetic hydrogels can be designed to be responsive to external stimuli, and can be synthesised in a controlled manner, regulating the Mw distribution. This extra functionality can be used to develop more targeted delivery strategies whilst maintaining low toxicity and biodegradability. Numerous different synthetic polymer systems have been developed for

protein delivery, most notably based on poly(vinyl alcohol), poly(ethylene oxide), poly(vinyl pyrrolidone) and poly(N-isopropylacrylamide) (Figure 1.9).[94]

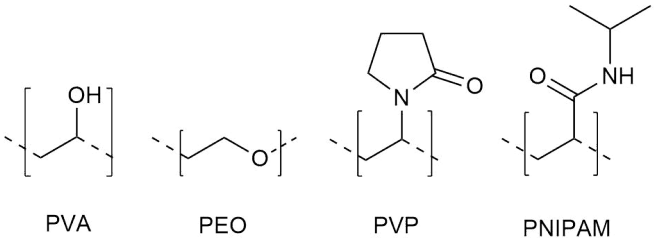


Figure 1.9: Common polymers used for the formation of synthetic hydrogels. Adapted from[94].

Poly(vinyl alcohol), PVA, is produced through free radical polymerisation of vinyl acetate, and subsequent hydrolysis of the acetate groups to yield PVA. PVA can be cross-linked via chemical or physical methods through the addition of cross-linking agents or via a freeze-thaw methodology.[119,120] The formation of PVA hydrogel delivery systems using a water-in-oil emulsion/cyclic freezing-thawing procedure led to an encapsulation efficiency of over 95 %. Release of the model protein bovine serum albumin (BSA) was temperature dependent and at 37 °C around 100 % release took 30 h.[121] Further advances have been made through the combination of PVA with other biodegradable polymers to enable self-assembly with protein drugs, avoiding the need for solvents or surfactants.[122]

Poly(ethylene oxide), PEO, is chemically similar to poly(ethylene glycol) and both can be photo cross-linked through modification of the polymer ends with acrylates or methacrylates. PEO is FDA approved for several medical uses and is one of the most commonly used polymer in therapeutics.[120] Smart hydrogels have been produced through the formation of block copolymers of PEO with a number of secondary blocks, giving thermally reversible hydrogels or gels with different degradation pathways.[123,124]

Poly(vinyl pyrrolidone), PVP, is FDA approved to be non-antigenic and biocompatible, and has been investigated as a possible carrier for protein delivery in hydrogels. Through combinations with poly(alanine), thermogelling biomaterials

can be produced, where micelles are formed and aggregate upon increasing temperature.[92] PVP hydrogel magnetic nanospheres have also been produced that release drugs passively, and can be aggregated at a specific tissue for targeted delivery of proteins.[125]

Limitations of the use of hydrogel delivery systems for biopesticide delivery

The use of hydrogels as delivery vectors for proteins and peptides in therapeutics has been an extensive area of research for the last 25 years. Hydrogels can be produced using of both natural and synthetic polymers, to deliver protein and peptides in the body. Typically, these are introduced into the body by injection, and avoid the harsh conditions of the stomach. For delivery of proteins and peptides for insecticidal use, delivery must be orally administered. Moreover, delivery vehicles need to be sprayed onto the crop, which is likely to result in the drying of the sprayed substance. Dehydration of a hydrogel may lead to the loss of the encapsulated cargo. Furthermore, the typical mechanism for release of cargo is via degradation of the carrier, which typically takes over 10 h to release the contents. Release on this timescale would result in only a fraction of the cargo being released upon ingestion by the insect. Hydrogels may be an appropriate delivery system for therapeutic delivery of protein and peptides, but are not suitable for biopesticide delivery.

1.8.2 Polymeric nanoparticles for peptide/protein delivery

Polymeric nanoparticles (NPs) are solid particles in the size range of 10 - 1,000 nm. These NPs can be used for delivery of peptide and protein drugs by providing protection from enzymatic and hydrolytic degradation.[126,127] This protection can be by encapsulation within a nanocapsule, or through embedding into the polymer matrix of a nanosphere (Figure 1.10).[127] These NPs pass from the gastrointestinal tract into the main body of the patient, where they release their contents. NPs tend to be used in preference to microparticles due to their greater surface area, leading to greater epithelial uptake. The increased stability in bio-

logical fluids of polymeric nanoparticles is resulting in a shift to more prominent use compared to liposomal or hydrogel systems.[128]

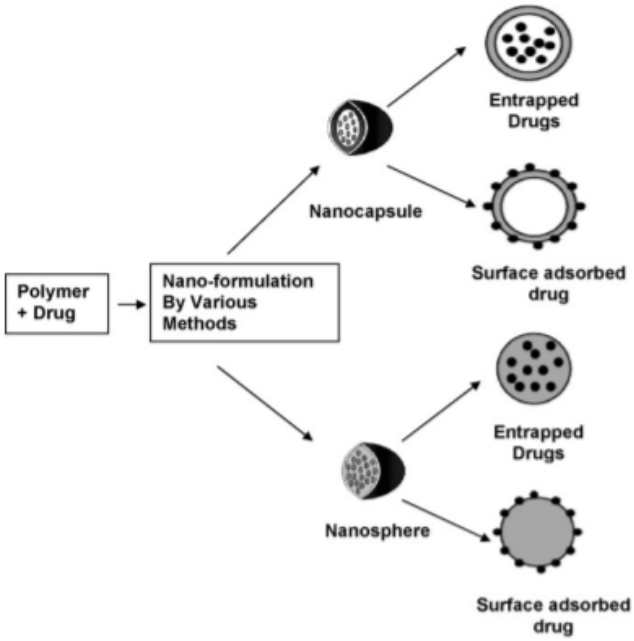


Figure 1.10: Polymer nanoparticle formation. Reproduced from[127].

Nanoparticles can be made by a number of different methods, dependent on the physical properties of the required polymer (Table 1.1).[126,129] Extensive research has been undertaken into many aspects of NP design synthesis and modification. This review will concentrate on the aspects relevant for biopesticide delivery; the protection provided by polymer NPs, the release rate and mechanism of release. For details on the formation of NPs for protein and peptide delivery,[128,130,131] and for surface modifications that increase the targeting of NPs, several reviews are available.[126,129]

The release rate of a protein drug from a polymer particle or capsules is dependent on four key factors: (i) the rate of diffusion through the matrix or polymer shell, (ii) matrix/shell erosion rate, (iii) distribution of the drug within the carrier, and (iv) desorption of the surface-bound/adsorbed drug.[128] Release of a uniformly distributed protein from a polymer NP can be dominated by

Table 1.1: Preparation methods for NPs of the most common polymers for forming polymeric matrices. Adapted from[129].

Polymers	Methods of nanoparticle preparation
Poly(alkyl cyanoacrylate) Poly(alkyl methacrylate) Poly(styrene) Poly(vinylpyridine)	Monomer polymerization
Poly(ϵ-caprolactone) Poly(lactic acid) Poly(lactic-co-glycolic acid) Poly(methacrylate)	Nanoprecipitation
Poly(ϵ-caprolactone) Poly(lactic acid) Poly(lactic-co-glycolic acid) Poly(β-hydroxybutyrate) Ethyl cellulose	Solvent evaporation
Cellulose acetate phthalate Poly(alkyl methacrylate) Ethyl cellulose Poly(lactic acid) Poly(lactic-co-glycolic acid)	Salting out

either diffusion or degradation. When diffusion dominates, there is initially a period of rapid release as the surface-bound protein is released from the large NP surface. This rate then decreases, and is controlled by the rate of diffusion through the matrix.[128,132,133] When degradation dominates, a constant rate of release is observed, corresponding to the degradation of the polymer particle.

Capsules are a reservoir-type drug delivery system, where the drug core is coated with polymer. The drug is contained in a single location, so if diffusion dominates, then a constant release of drug is observed, following first-order kinetics.[134] The rate of this release is controlled by partitioning of the drug into the shell and continuous phase, therefore dilution of the continuous phase increases the rate of release. Furthermore, the Mw of the encapsulated polymer affects the rate of dissolution, as the diffusion times are affected.[135] If release is dominated by degradation, either biodegradation or a triggered burst release of the contents is observed.

Non-toxic synthetic biodegradable polymers for therapeutic delivery

The requirement for a non-toxic biodegradable polymer for therapeutic delivery of proteins and peptides has limited the range of polymers available for use. There are four major polymer types that are most often used for therapeutic delivery: poly-δ,l-lactide-co-glycolide (PLGA), polylactic acid (PLA), poly-ϵ-caprolactone (PCL), and poly-alkyl-cyano-acrylate's (PAC) (Figure 1.11).[127]

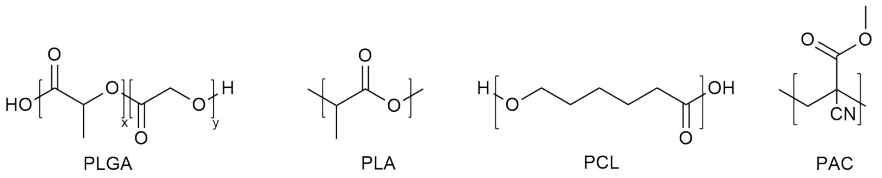


Figure 1.11: Polymers commonly used for nanoparticle formation. Adapted from[127].

PLGA and PLGA-based systems have been the focus of many protein-controlled release systems, due to their biocompatibility and variable release rates from hours

to months.[136] Although PLGA is extensively used, it has some drawbacks including unfavourable conditions for proteins during synthesis and acid-catalysed degradation of the polymer resulting in protein denaturing.[136,137]

For the encapsulation of peptides or proteins, three methods are commonly used: water-in-oil-in water emulsion technique, phase separation, and spray drying (as demonstrated by Figure 1.12).[136] The use of multiple emulsion templates to form capsules (in the micron range) can often lead to denaturing of the protein, as it unfolds at the interface. In order to mitigate this, surfactants can be used in the formation of the multiple emulsions, resulting in competition for the interface and reducing protein denaturation.[136,138] A multiple emulsion templated synthesis could be utilised for biopesticide delivery, due to the high loading capacity and burst release mechanism of the capsule.

PLGA microcapsules have been shown to encapsulate model proteins to high efficiency levels of over 70 %.[139] The release from PLGA microcapsules could be modified by the inclusion of surfactant into the oil phase of the multiple emulsion, producing different surface morphologies.[140] The addition of excess hydrophilic surfactant has led to a porous structure, which facilitated quick release.[136] Research has been undertaken to improve entrapment efficiency and the release profile from PLGA-based microcapsules by adding another polymer species, as reviewed by Mundargi *et al.*.[136]

PLA based nanoparticles (in nano size range) formed from a multiple emulsion template (with a small amount of internal water) and solvent evaporation showed poorer encapsulation efficiency compared to PLGA of around 40 %, with a release rate of BSA of 70 % over 30 days.[141] The entrapment efficiency for hormones of a similar Mw to BSA was significantly higher, at 70 % when an ABA copolymer of PLA-PEG-PLA was used, with the release rate of the drug a function of the PEG content.[142]

PCL is easily degraded by non-enzymatic hydrolysis, and has been investigated as a safe polymer for protein drug delivery.[127] Degradation of PCL is substantially slower than both PLA and PLGA, providing a longer release profile.

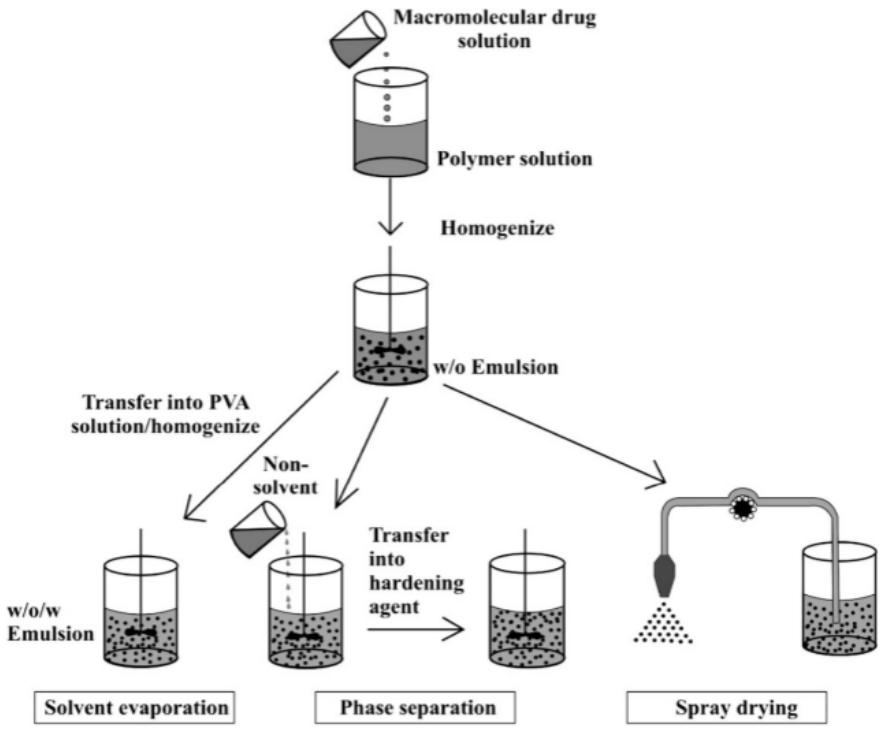


Figure 1.12: Methods for the production of PLGA delivery systems based on an initial W/O emulsion. This primary emulsion can be processed into PLGA delivery systems by three main methods solvent evaporation, phase separation or by spray drying. Reproduced from[136].

1.8.3 Responsive polymers for therapeutic delivery

For both hydrogels and solid polymer systems, the release rate of loaded peptide or protein is often slow, measured in days rather than minutes. In many cases, this sustained period of release is advantageous, but for biopesticide delivery, a quicker release rate is required. Responsive or smart systems can be employed, which respond to an external stimuli to trigger release of the drug. Polymeric delivery systems can be triggered by a number of factors, such as temperature, pH, light, ionic strength, magnetic fields and biomaterials (e.g. enzymes).[143–146] The present project is specifically interested in release triggered by changes in pH, and this section will concentrate on this. There are two main mechanisms for pH-responsive release, through ionisable residues whose ionisation depends on solution pH, or through acid-cleavable cross-linking units.[96,143,147]

Ionisable residues may be acidic or basic, and (de)protonate depending on the pH environment, leading to a change in the charge of the polymer. This newly charged polymer may undergo a phase transition, becoming soluble in the continuous phase and causing swelling or dissolution.[143,146] pH-responsive polymers can be produced with a pKa between pH 1 and 14, where polymers with basic units are cationic at low pH and neutral at high pH. Acidic functionalised polymers are neutral at low pH but become anionic at neutral to basic conditions. Acidic polymers are typically formed with carboxylic functionality, but can also be produced with sulfonic, phosphoric and boronic acids (Figure 1.13).[143] Basic polymers are based on nitrogen functionality, and can contain amines, morpholino, pyrrolidine, piperazine, pyridine or imidazole groups (Figure 1.14). The choice of responsive residue is dependent on the pH change that is needed to induce release. Furthermore, the pH-responsiveness can be tuned through the inclusion of non-responsive monomers, with differences in hydrophobicity in the polymer chain altering the response to pH.[146]

Drug release can also be triggered through the degradation of covalent bonds of pH-liable linkers (Figure 1.15). This degradation can involve deconjugation of the drug/polymer, or breakdown of the cross-linker in a hydrogel or solid polymer particles.[143,147] Hydrogels are typically cross-linked (as previously explained) and acid-degradable linkers can be used to trigger release from these hydrogels.

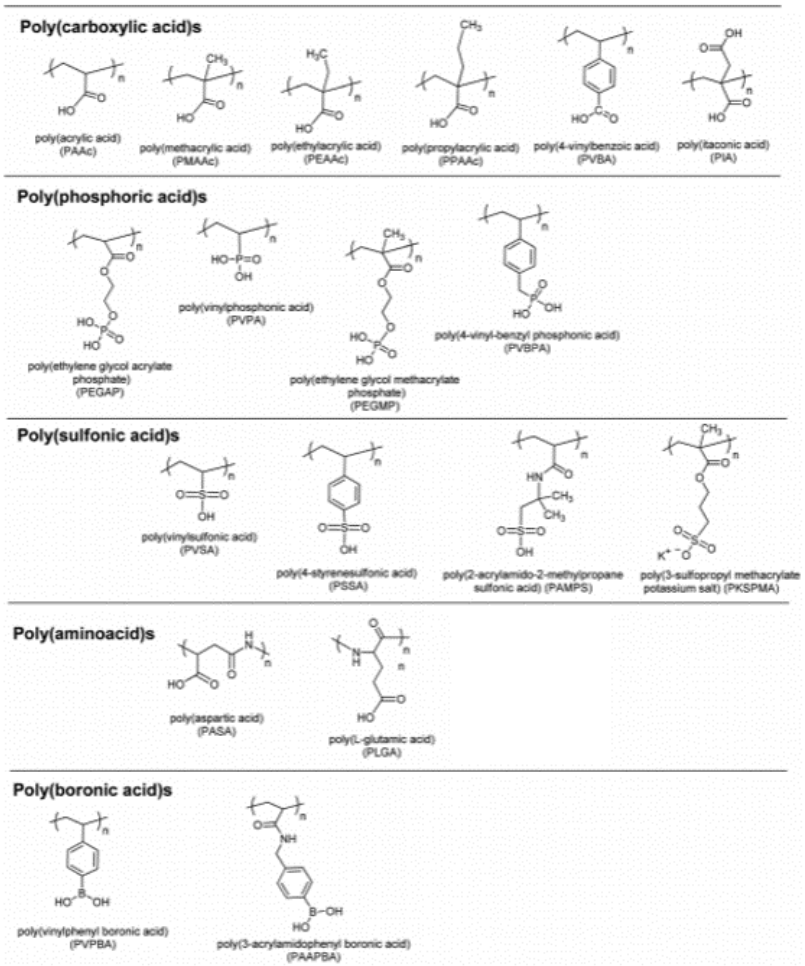


Figure 1.13: Acidic polymer residues. Reproduced from[143].

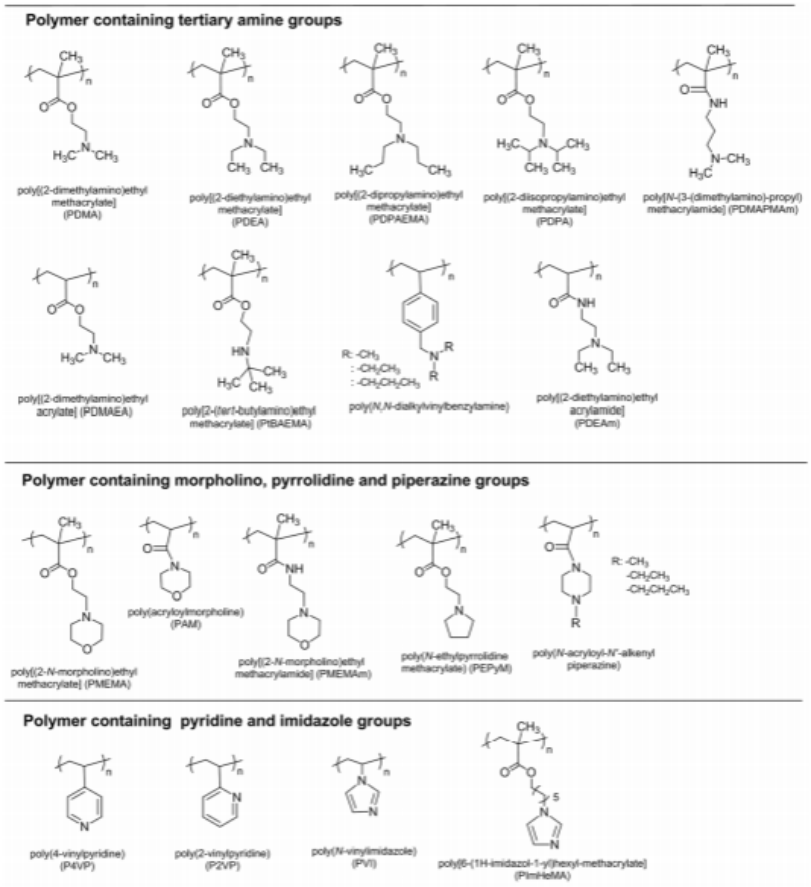


Figure 1.14: Basic polymer residues.Reproduced from[143].

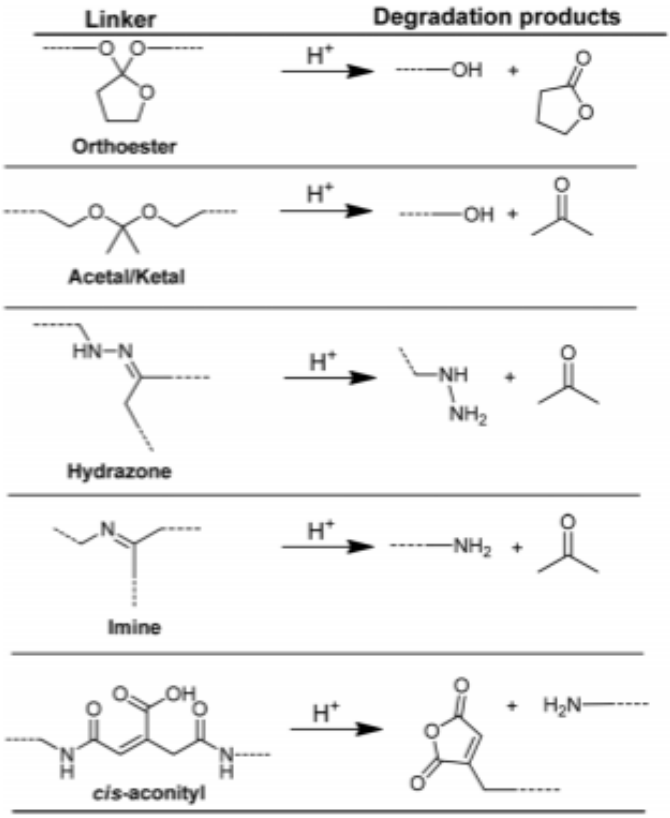


Figure 1.15: Acid labile covalent bonds found in responsive cross-linkers. Reproduced from[143].

Therapeutic pH-responsive polymer systems have become of interest due to the pH differences in several key targets. Tumour tissue has a pH of 6.5 - 7.2 and lysosomes of cancer cells have a pH of 4.5 - 5, both lower than the normal physiological pH of 7.4.[148] These differences have led to the formation of many different polymer systems with a release that is triggered by this reduction in pH.

1.8.4 Use of therapeutic systems for biopesticide delivery

Common biodegradable non-toxic polymers used for protein and peptide delivery in therapeutics do not possess the release rates required for biopesticide delivery. However, the mechanisms used to form the micro/ nanocapsules are of interest. A multiple emulsion templated synthesis allows for the entrapment of proteins and peptides either in a solid or aqueous phase. The separation of inner water phase from continuous aqueous phase by an oil layer provides a barrier and maintains encapsulation efficiency. The composition of this oil layer creates flexibility in the final capsule's composition. This oil layer may consist of a polymer and solvent that is to be extracted, or it may consist of a monomer that is polymerised to form the polymer shell. Furthermore, the ratio of inner water to oil can be altered, varying the polymer shell thickness and changing the release rate from these capsules.

To increase the rate of protein release from polymer microcapsules, a triggered release mechanism may be utilised. pH-triggered release of protein and peptide biopesticides may be induced by the changes in gut pH of the pest. *D. melanogaster*, a close relation to *D. suzukii*, has an acidic section in its midgut.[149] If such a region exists in *D. suzukii*, pH-triggered release may be used, by selection of an appropriate pH-responsive polymer.

1.9 dsRNA complexation in agrochemicals

The use of dsRNA as a potential bioinsecticide is a new field of investigation, since their use has so far been limited by rapid degradation and poor uptake. To combat the degradation and poor uptake of the nucleic acid, different delivery vectors have been investigated. Unlike protein and peptides, nucleic acids are anionic, and

this negative charge may be used in order to complex the dsRNA. The following section will discuss recent approaches that have been used to complex and deliver dsRNA to insects.

1.9.1 Liposomes

While investigating new gene targets for efficient control of insect pest species, it was found that the success of oral application to induce RNAi varied depending upon the species. The seminal paper by Whyard *et al.* showed that feeding naked dsRNA to several species had no effect on gene suppression. To address this issue, they used a common transfection agent to increase the uptake of the dsRNA. This transfection agent was the commercially available Lipofectamine 2000, a cationic liposome that increases transfection of nucleic acid by overcoming the electrostatic repulsion between the nucleic acid and the cell membrane.[25,150] Lin *et al.* showed control of the German cockroach (*Blattella germanica*) through oral delivery of dsRNA, using another commercially available GenJet Plus liposome transfection agent. They demonstrated that complexation of dsRNA with liposome could also significantly reduce degradation by the midgut juices in *ex vivo* studies. Furthermore, this method increased mortality through oral application compared to naked dsRNA.[151]

Recently, Zhang *et al.* have compared three commercially available lipid transfection agents: Lipofectamine 2000, DMRIE-C and Cellfectin.[152] All three of the lipid agents were able to significantly enhance dsRNA-induced suppression of gene expression by feeding in a tick species at the larval, nymph and adult life stages. Differences between the transfection agents were only observed at the larval stage, where a significantly higher knockdown was observed for lipofectamine.[152]

To improve thermal stability and tolerance to dilution, Gudlur *et al.* recently developed peptide vesicles, based on amphiphilic peptides.[153] Hydrophobic forces and hydrogen bonding sustain lipid bilayer assemblies, which can entrap dye molecules. Avilaa *et al.* utilised these branched amphiphilic peptide capsules to facilitate dsRNA uptake through an oral application to both a beetle and an aphid species.[154] This method increased the mortality of *T. castaneum* by about 45 % and increased the rate of mortality in *A. pisum* by a factor of 4.[154]

Application of dsRNA in the field will be by spraying the complexes onto the crop surface, where they will dry. Liposomes need to be suspended in liquid, typically water, to avoid disassembly and collapse, and are therefore unsuitable for use as a dsRNA delivery vector in the field.

1.9.2 Polymeric based dsRNA delivery

As previously detailed, polymer nanoparticles can be utilised for delivery of biomaterials due to their stability, ease of surface modification, ease of preparation and, in some cases, biodegradability.[155,156] dsRNA loaded nanoparticles, unlike peptides and proteins, are formed though electrostatic interactions of the anionic dsRNA and a cationic species; this dsRNA-polymer complex has been termed either an interpolyelectrolyte complex (IPEC) or a polyplex.[157]

Chitosan nanoparticles have been extensively investigated as a delivery vector for biomaterials, including dsRNA and protein and peptides (as previously covered). Chitosan nanoparticles form through electrostatic driven self-assembly, where at a pH < 6, the amine residues on the chitosan are positively charged and interact with the anionic phosphate backbone of the dsRNA.[156] Zhang *et al.* produced dsRNA/chitosan polyplexes that ranged from 100 - 200 nm in diameter.[155] This complexation of the dsRNA lead to sustained entrapment with approximately only 6 % of the dsRNA released into water over 24 h. Moreover, feeding of dsRNA complexes targeting chitin production in third instar *Anopheles gambiae* larvae significantly reduced the transcript levels by 50 - 60 %, and the chitin quantity in the pest was significantly reduced compared to control nanoparticles containing non-targeting dsRNA coding for GFP (green fluorescent protein). However, Zhang *et al.* did not test the effect the nanoparticles had on dsRNA stability, and did not compare the efficacy of the complex with control insects fed naked dsRNA. It has been shown that mosquito larvae are very susceptible to dsRNA without complexation, where RNAi can be induced by soaking the insect and by ingestion of the uncomplexed dsRNA.[40] It is therefore unclear what effect the complexation of dsRNA with chitosan has on RNAi.

Kumar *et al.* further investigated the use of chitosan/dsRNA polyplexes, looking at their uptake profile and comparing them with engineered bacterial

cells and bacterially-expressed dsRNA.[158] In this system, an 85 % entrapment efficiency was achieved, and again the diameter ranged from 100 to 200 nm, dependent on the concentration of polymer used. Uptake and knockdown efficiency was investigated using an Sf21 cell line expressing GFP. Chitosan/dsRNA complexes effectively suppressed fluorescence, demonstrating their ability enter the cell cytoplasm. Furthermore, a chitosan/dsRNA complex targeting the *vg gene* of *A. aegypti*, increased the mortality rate compared to engineered bacterial cells and purified bacterially-expressed *vg*dsRNA.[158]

Das *et al.* investigated the use of chitosan-based nanoparticles, along with functionalised silica particles (ASNP) and carbon quantum dots (CQD).[159] Chitosan/dsRNA nanoparticles were produced using the same method as Zhang *et al.*; silica was functionalised with amine groups using (3-aminopropyl)triethoxysilane, and carbon quantum dots were produced using the microwave method, stabilised with polyethyleneimine (PEI). Each of these delivery systems utilised amine functionality to complex dsRNA. Chitosan-based delivery systems complexed the dsRNA at a lower ratio of 10:1 vector:dsRNA compared to both ASNPs (30:1) and CQDs (20:1). *In vivo* testing of these vectors and analysis of gene silencing showed that dsRNA complexed with either chitosan or CQDs led to gene suppression and mortality. However, no effect was observed using the functionalised silica nanoparticles. The mortality rate for PEI coated CQDs was significantly faster than for chitosan based vehicles. However, it is unclear whether this increased RNAi was due to the complexation of dsRNA to PEI or the combination of the PEI and CQD.[159]

More elaborate polymeric delivery systems have also been developed. He *et al.* utilised a core shell polymer nanoparticle for the complexation of dsRNA, where the core of the nanoparticle comprised of a fluorophore allowing for easy detection. This fluorophore was conjugated to amino-functionalised brush-like chains (Figure 1.16).[160] *In vivo* testing of dsRNA complexed with the fluorescent nanoparticle (FNP) in *D. melanogaster* showed that the complex could be ingested and taken into the gut cells. Furthermore, FNP/dsRNA complexes targeting a chitinase-like gene, CHT10, retarded larval growth compared to both naked dsRNA and FNP complexed to a non-coding dsRNA.[160]

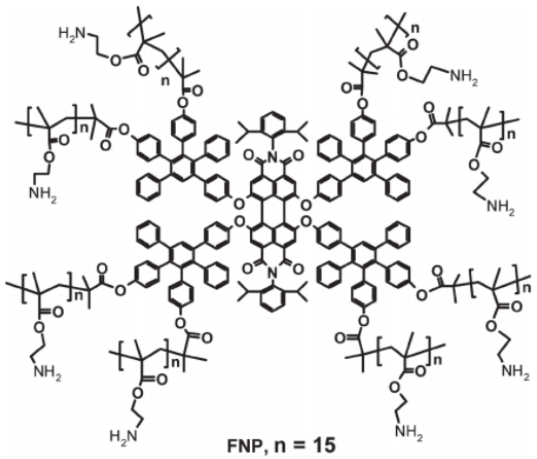


Figure 1.16: Fluorescent nanoparticle for dsRNA conjugation. Reproduced from[160].

Recently, guanidinium-functionalised cationic polymers have been synthesised by Parsons *et al.* in order to complex dsRNA, enabling RNAi which is effective in the cotton pest *Spodoptera frugiperda*.[161] Poly[N- (3-guanidinopropyl) methacrylamide] (PGPMA, Figure 1.17) cationic polymer was synthesised by RAFT polymerisation (explained in Section 1.11) in order to complex dsRNA. This homopolymer was selected due to its high pKa (12.5), which means the polymer maintains its cationic charge in the alkaline gut of the *Lepidopteran* cotton pest species. Additionally, PGPMA can form multiple hydrogen bonds with the phosphoric dsRNA backbone, increasing the binding and therefore protection. Light scattering of these complexes revealed that they are rod-like in nature, a hydrodynamic radius of around 320 nm at pH 7.4 and about 240 nm at pH 10. Parsons *et al.* proposed that this reduction in hydrodynamic radius was due to deprotonation of the PGPMA at the higher pH; however, this was far below the reported pKa.[161] PGPMA-dsRNA complexes showed improved transfection and knockdown compared to naked dsRNA, with over 90 % gene suppression in *Lepidopteran* cell lines. *In vivo* testing showed an increase in knockdown compared to naked dsRNA, although this gene silencing was reduced compared to Sf9 cell lines ($>$ 80 %). Gene suppression did not translate to high larval mortality, with only about 30 % of the larvae dead after 30 days, suggesting that the knockdown

may be transient/partial or that the gene product may not be vital to survival.

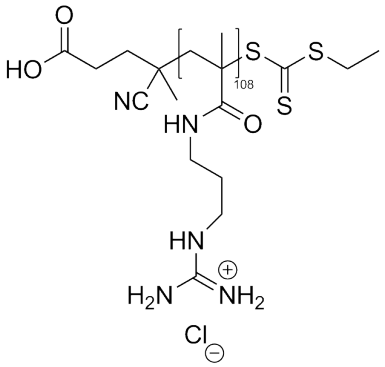


Figure 1.17: Poly[N-(3-guanidinopropyl)methacrylamide synthesised by RAFT polymerisation for dsRNA complexation. Modified from[161].

The use of RNA based gene suppression is not limited to pest control and has also been implemented in therapeutics. Commonly in therapeutic applications siRNA is used due to its higher specificity compared to dsRNA, commonly used in pest control. However, the complexation and delivery of both nucleic acids are similar, it is therefore prudent to consider the research that has been conducted in therapeutics and consider how similar systems could be used in pest control.

1.10 siRNA complexation for therapeutic use

Since the discovery by Fire *et al.* that dsRNA can silence gene expression in the nematode *C. elegans*,[39] and subsequent observations that small interfering RNA duplexes (siRNAs) can induce sequence-specific gene suppression in mammalian cells, RNA-based therapeutics have become widely researched.[162,163] In the following decade, siRNA was developed and progressed to Phase I and II clinical trials, with more than 30 clinical trials involving 21 different gene targets and 14 different diseases taking place.[164,165] However, siRNA-based drug systems are not widely implemented due to four main concerns: (i) off-target silencing/effects, (ii) triggered immune response in the patient, (iii) limited delivery of the nucleic acid duplexes into the cytoplasm of target cells, and (iv) rapid degradation of

siRNAs in the serum.[164,166] The concerns regarding controlled delivery and limiting siRNA degradation are analogous to problems associated with dsRNA for use in pest control. Therefore, it is prudent to consider the delivery mechanisms used for biomedical applications.

Due to the wide range of tissue targets for siRNA in the body, delivery systems can vary greatly. Typically, delivery of siRNA focuses on three main criteria: increasing the stability in the serum, increasing cell penetration, and enabling specific tissue targeting.[166] This section will review some of the polymeric-based delivery systems that have been used in therapeutics, starting with the early systems before moving to more complicated architectures currently under investigation.

1.10.1 Polyethyleneimine based delivery systems

Prior to the discovery of RNA mediated gene suppression, polyethyleneimine (PEI) had been implemented in gene therapy through the complexation and delivery of DNA.[167–169] Polyethyleneimine is formed from ethylenimine by either anionic or cationic polymerisation, and can be linear or branched (Figure 1.18).

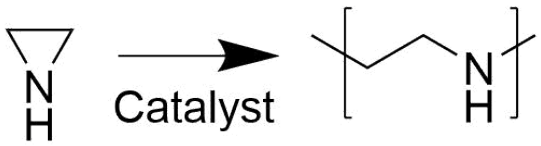


Figure 1.18: Polyethyleneimine formation.

Urban-Klein *et al.* demonstrated the ability of linear low Mw PEI to complex siRNA.[170] Complexation of the siRNA with PEI reduced degradation in serum, with 70 % of the complexed siRNA intact after 4 h, whereas all the naked siRNA was degraded within 1 h. *In vivo* studies using a subcutaneous mouse tumour model resulted in tumour reduction after injection with PEI-complexed siRNA which contrasted with a lack of effect with naked siRNA.[170] Grayson *et al.* extended this research and investigated the biophysical characteristics of siRNA-PEI complexes, along with the cytotoxicity of PEI.[171] The diameter, zeta

potential, binding efficacy and transfection efficiency were investigated for three PEI-siRNA complexes (25 KDa branched-PEI, 22 KDa linear-PEI and 800 Da branched-PEI). The effect of charge ratio on the complexes was also investigated for each of the PEIs. The charge ratio is quoted as a ratio of nitrogen (cationic atom on PEI) to phosphorus (anionic species in siRNA backbone), N:P.

At all N:P ratios, the 25 KDa branched-PEI based system had the smallest effective diameter, followed by 22 KDa linear-PEI, with the smallest Mw of PEI producing the largest complexes. However, there was large error attributed to these measurements, with much variability between measurements.[171] Changing the N:P ratio from 1:1 to 8:1 did not affect the particle size for samples produced from the 25 K branched-PEI, whereas with increasing concentration of the 800 Da branched-PEI, the complex diameter increased dramatically from 200 to 800 nm. The zeta potential of 25 K branched-PEI became positive at the lowest N:P ratio (8:1), suggesting superior binding at low N:P ratios compared to the lower Mw PEIs. The relative binding affinity of all three complexes were similar when the N:P ratio was greater than 6. At an N:P ratios of less than 6, the linear polymer had poorer binding, but again large variability was observed. Gene expression in HR5-CL11 cells was only significantly suppressed using 25 KDa branched-PEI at an N:P ratio of at least 8:1.

Sajeesh *et al.* used PEI to provide effective delivery of dsRNA.[172] The long dsRNA was synthesised by conjugating multiple small siRNAs modified with a thiol at each end, allowing for reduced disulfide bridges. In this study, it was again shown that large branched PEI was better at complexing dsRNA compared to smaller alternative structures. 25 K branched-PEI dsRNA complexes provided strong RNAi mediated gene silencing, highlighting the possibility of using PEI for the delivery of dsRNA in the agrochemical industry.

A series of papers by Shim and Kwon reported modifications to PEI for controlled delivery of siRNA, where PEI was ketalized, enabling disassociation via hydrolysis.[173–175] The addition of the ketal functionality aids with escape from the endosomal compartment after endocytosis into the cell. Modified linear-PEI/siRNA showed higher levels of gene suppression than unmodified linear-PEI/siRNA, but was still outperformed by higher Mw branched PEI/siRNA complexes. Confocal microscopy demonstrated that the siRNA could be dissociated

from the modified linear PEI and localised in the cytoplasm, whereas unmodified PEI complexes did not efficiently unpackage.[174]

PEI-based systems may be suitable for the delivery of dsRNA in pest control, in order to increase cellular uptake and reduce degradation of the RNA, either on the crop or in the insect gut. However, complexation of siRNA with PEI gives complexes of variable size, which may not be suitable due to restrictions in the mouth parts of some pests and due to the size restriction of endocytosis of 200 nm into cells.[176,177] Larger Mw branched PEI can produce smaller complexes that would be more suitable, but the variability between batches is an area of concern.

1.10.2 Well-defined polymers for RNA complexation

With the advent of controlled radical polymerisation techniques, the production of well-defined polymers has enabled more complicated architectures for RNA complexation. With the introduction of stable free radical polymerisation (SFRP), atom transfer radical polymerization (ATRP) and reversible addition-fragmentation chain transfer polymerisation (RAFT), rational design of new polymeric vectors is possible.[157] The design of polymeric vehicles typically involves a complexing moiety, which in the case of RNA loading is a cationic polymer. The delivery vehicle also contains a stabilising water-soluble block that allows enhanced water solubility of the vehicle and reduces degradation. Finally, a targeting moiety may be included in the polymer design to aid in the specific uptake of the vehicle into target cells.[178] I am specifically interested in the use of RAFT polymerisation for the production of well-defined polymers for the complexation of dsRNA. This section will highlight different polymeric systems produced by RAFT polymerisation that have been used to successfully complex siRNA.

The simplest RNA polymeric vehicle is a cationic homopolymer, which can complex RNA through electrostatic interactions.[157] Poly(2-(dimethylamino) ethyl methacrylate) (PDMAEMA) has been extensively studied for the complexation of nucleic acids, initially concentrating on DNA condensation,[179–181] but more recently RNA condensation.[182–184] PDMAEMA effectively complexes nucleic acids,

and can increase cell transfection rates by charge cancelling. Due to close proximity to the cell membrane, polyplexes are taken into cells through endocytosis, but the lack of fusogenic activity often results in the degradation of the polyplexes, as the endosomes can fuse with lysosomes, resulting in no RNAi activity.[185] Endosomal disruption and escape can be used to decrease polyplex degradation through the proton sponge effect, where, once in the endosome, the polymer absorbs protons that are pumped into the organelle. This leads to a swelling of the polymer and a build-up of ions, generating an osmotic pressure that can lead to endosome disruption.[186] PDMAEMA-based polyplexes have been used to transfect many cell types. However, the efficiency of this delivery is often low with a large portion of the polyplexes retained in endosomes.[185,187] Moreover, complexation often leads to a reduction in water solubility, increasing excretion and addition of excess cationic polymer can lead to undesirable aggregation with salts and proteins in the body serum.[184,188]

A common solution to these limiting factors is the addition of a secondary hydrophilic moiety. This hydrophilic block can comprise of either a linear/brush polyethylene glycol (PEG), poly(N-(2-hydroxypropyl) methacrylamide) (PHPMA), polydimethylacetamide (PDMA) or polymethacryloyloxyethyl phosphorylcholine (PMPC) group (Figure 1.19), which extend from the complexed RNA, providing a steric barrier. Historically, PEG-based systems have been used to stabilise complexes for therapeutic delivery, and more recently PHPMA has become extensively studied for use in therapeutic siRNA delivery systems.[189–193] The increased interest in PHPMA as the stabilising block is due to its ease of polymerisation through controlled radical polymerisation techniques, lack of an immune response and FDA approval.

Recently, Jackson *et al.* investigated the effect three water soluble polymer blocks had on pharmacokinetics of siRNA targeting tumours.[194] Comparison of linear PEG, brush-PEG and PMPC at varying Mw showed that high Mw linear PEG and PMPC (both 20 kDa) stabilised siRNA in simulated serum, and effectively blocked the absorption of proteins to the surface of the complex. Larger Mw PEG and MPC extended circulation time 5-fold compared to smaller Mw species (5 kDa). *In vivo* testing showed that PMPC based systems outperformed all

others in both gene silencing and tumour cell uptake of dsRNA compared to linear and brush PEG. Furthermore, high Mw PMPC blocks outperformed smaller PMPC blocks, demonstrating the improved stability induced by increasing the length of the water soluble block.[194]

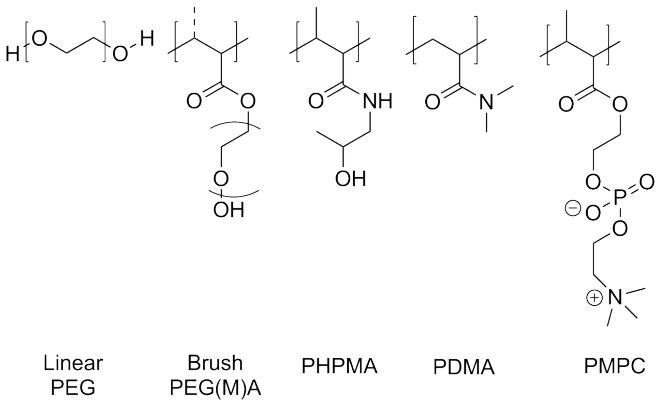


Figure 1.19: Common polyplex hydrophilic stabilising blocks utilised for siRNA delivery.

Another significant barrier to delivery of siRNA is the release from endosome compartments after cellular uptake. To address this, Convertine *et al.* produced polymeric micelle delivery vectors as a new class of siRNA carriers.[195,196] The micellar vector contained a DMAEMA block to condense siRNA, and a second block comprising of a copolymer of propylacrylic acid (PAA), butyl methacylate (BMA) and DMAEMA, to form a pH-responsive micellar vector. Upon reduction in pH in the endosome, the DMAEMA in the second block becomes protonated and the PAA deprotonates, forming a zwitterionic block. Along with the BMA units, this interferes with the endosome membrane, aiding release.[196] This methodology resulted in a 66 % increase in mRNA suppression and a 3-fold increase in siRNA cellular uptake compared to commercially available liposomal-based delivery systems.[195]

Gary *et al.* investigated three different vectors (Figure 1.20) for complexing siRNA with well-defined polymers. siRNA complexation was achieved at a lower polymer concentration for both the homo and diblock polymers compared to the triblock. The micellar-based vector also had reduced cellular uptake compared to

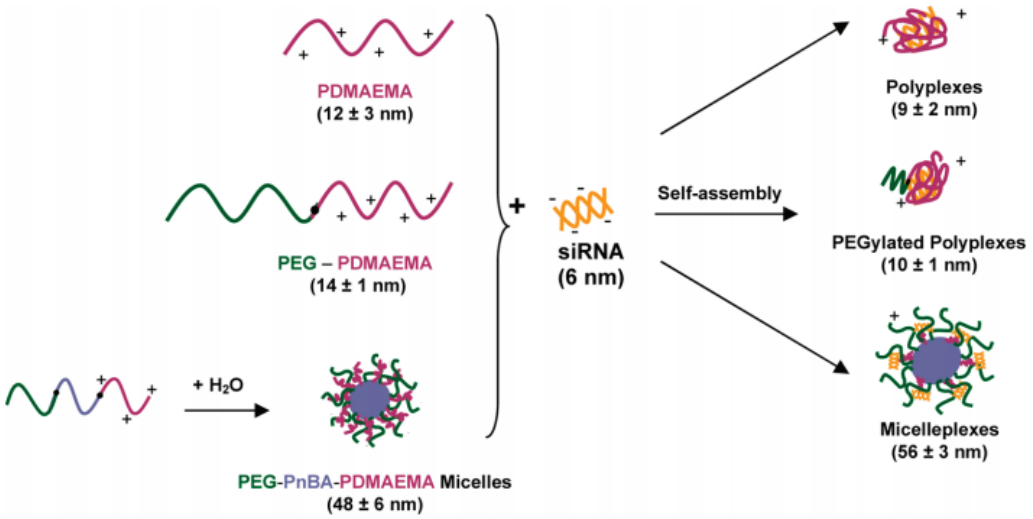


Figure 1.20: Possible routes for siRNA complexation. Reproduced from[184].

the other delivery systems, including Lipofectamine 2000. However, the micellar system outperformed the other polymeric systems at gene silencing, due to the different mechanism of internalisation.[184] The micelleplexes accumulated more effectively, and a greater number were retained in tumour tissues compared to delivery by the other systems. Gary *et al.* conclude that this micelleplex-type carrier architecture is a useful platform for potential theranostic and tumour-targeting applications.

The three systems outlined for siRNA delivery may be useful for dsRNA delivery in pest control. With increasing research into therapeutic siRNA delivery, the vectors have become increasingly complex, from polydisperse PEI to well-defined homopolymers to diblocks and triblock micellar complexes. I have attempted to synthesise diblock copolymers for the complexation of dsRNA, as homopolymer complexes will become coated and have reduced uptake into the pest. Furthermore, the micellar-type delivery vectors formed previously were not investigated, as endosomal escape is not generally considered a limiting factor in dsRNA delivery in insects.[197]

1.11 RAFT polymerisation

Reversible addition-fragmentation chain-transfer (RAFT) polymerisation has been utilised to produce well-defined polymers for the delivery of bioinsecticides, as described previously. In this section, I will briefly explain the mechanism of RAFT polymerisation. For a more detailed explanation of the mechanism and overall considerations for the use of RAFT polymerisation refer to reviews by Keddie[198] and Perrier.[199]

RAFT polymerisation is one type of reversible deactivation radical polymerisation that enables the formation of new polymeric materials with narrow molecular mass distributions and defined architecture.[200] Radical polymerisation enables the use of less stringent conditions than those employed in cationic or anionic polymerisation, and enables the use of a broader range of functional monomers.

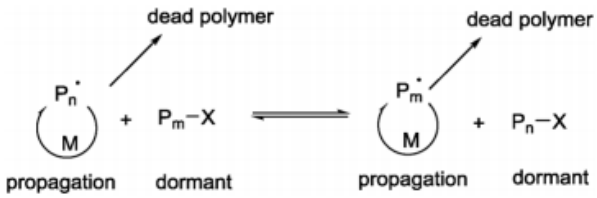


Figure 1.21: Degenerative chain transfer. Reproduced from[198].

RAFT polymerisation proceeds through degenerative chain transfer between active and dormant states (Figure 1.21). This polymerisation is mediated through the addition of a RAFT agent, which enables partitioning between active and dormant propagating polymer radical states, through a reversible activation/ deactivation equilibrium.[198] This is different from other controlled radical polymerisations techniques and requires a source of radicals to proceed. RAFT agents contain thiocarbonylthio functionality, which swaps between growing chains during the polymerisation process. After polymerisation the RAFT thiocarbonylthio will form an end group on most polymer chains, allowing for the growth of another polymer block (Figure 1.22).

The control of polymer chain dispersity and Mw is maintained by the rapidity of the equilibrium rate compared to polymerisation rate, where $k_{addP} \gg k_p$ and

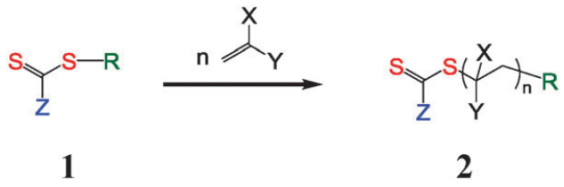


Figure 1.22: RAFT polymerisation general outcome. Reproduced from[198].

$k_\beta \gg k_p$ (Figure 1.23). This rate difference results in all the chains growing at the same speed, due to the fact that their activity depends on length, with longer chains entering a dormant state. RAFT polymerisation does not require much process development; however, the RAFT agent must be chosen depending on the target polymer and monomers to be used.

Monomers can be divided into two groups, more-activated monomers (MAMs) or less-activate monomers (LAMs), depending on how readily they react in a free radical process. MAMs have a double bond conjugated to the vinyl group, whereas LAMs have the vinyl bond adjacent to oxygen, nitrogen, halogen, sulfur lone pairs, or saturated carbons.[199] General guidelines for the selection of both R and Z groups can be seen in Figure 1.24, with a detailed explanation by Keddie *et al.*.[198,199,201]

Finally, for controlled diblock formation, consideration must be given to the first formed block, which should be chosen such that it is a good homolytic leaving group with respect to that of the second block.[200,202] The leaving group ability for each monomer class is as follows: methacrylates $\sim$ methacrylamides $\gg$ styrenes $\sim$ acrylates $\sim$ acrylamides $>$ *N*-vinyl heteroaromatics $>$ vinyl amides $\sim$ vinyl esters. Furthermore, polymers are typically better leaving groups than monomers of the same class, due to increased steric hindrance.[198] With consideration of all the factors outlined, RAFT agent selection can be undertaken to produce targeted diblock copolymers with low polydispersity and well-defined structure.

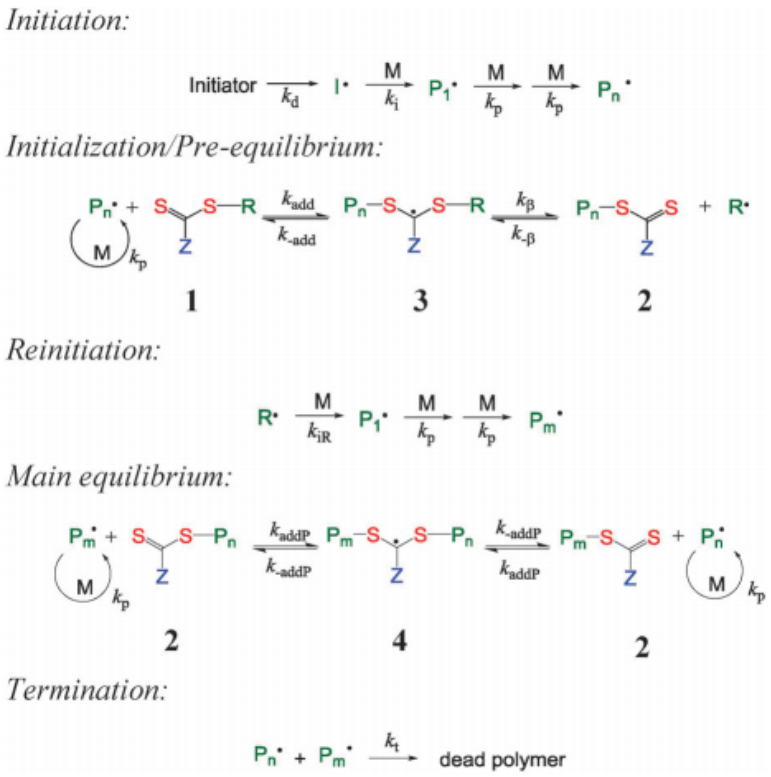


Figure 1.23: RAFT polymerisation mechanism. Reproduced from[198].

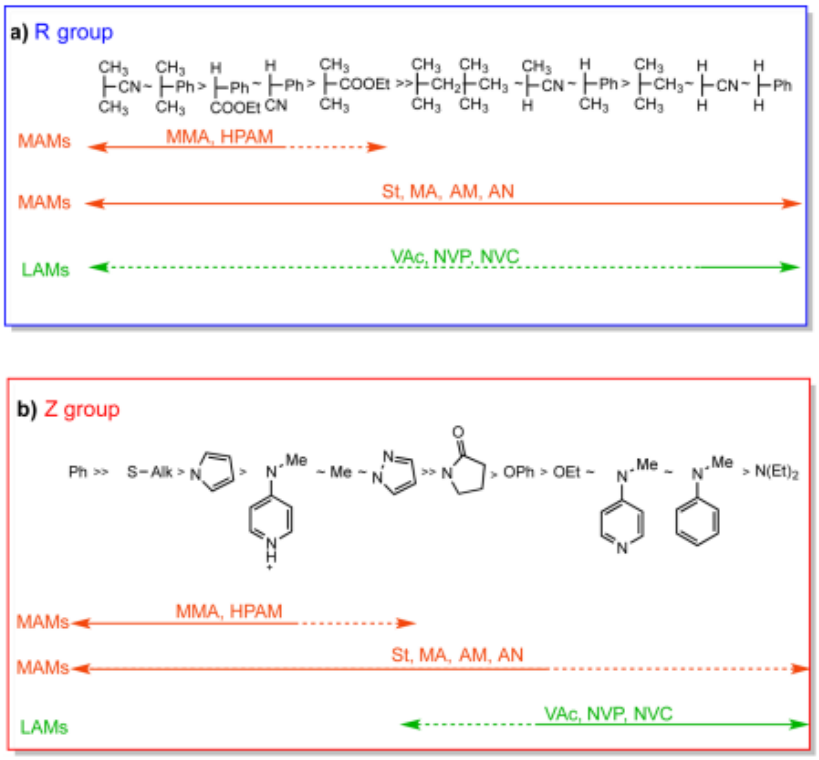


Figure 1.24: RAFT agent R and Z group selection is dependent on monomer choice. Solid lines indicate good control, dashed indicate partial control. (MMA = methyl methacrylate, HPMA = *N*-(2-hydroxypropyl)methacrylamide, St = styrene, MA = methyl acrylate, AM = acrylamide, AN = acrylonitrile, VAc = vinyl acetate, NVP =*N*-vinylpyrrolidone, and NVC = N-vinylcarbazole). Reproduced from[198,199].

1.12 Aims

1.12.1 Delivery of protein/peptide bioinsecticides

As I have discussed, the use of protein/peptide bioinsecticides is currently limited by the lack of an appropriate delivery method. I aim to develop a vector for the transport of a protein/peptide species to the main site of uptake into the pest's body, the midgut. I investigate the intrinsic changes in pH across *D. suzukii's* gut, and look to exploit these changes to trigger release from pH-responsive microcapsules.

1.12.2 Delivery of dsRNA bioinsecticides

RNAi, mediated by dsRNA, has the potential to be a powerful new tool in pest management. The wide-scale implementation of this technique is often limited by the rapid degradation of dsRNA by extra cellular nucleases and poor gut cellular uptake.[64] In order to protect and deliver dsRNA, I aim to produce discrete diblock copolymers that can complex the dsRNA, protecting it from degradation and increasing cellular uptake. Furthermore, I look to determine what effect altering the complexing block length would have on dsRNA complexation.

1.12.3 Development of pest management strategies through better understanding the pest

Along with the delivery of biopesticides to control *D. suzukii*, I aim to gain a better understanding of its locomotor behaviour. I look to investigate the effect of mating status and temperature on the activity, allowing for the determination of likely geographic dispersal and the production of more efficient control strategies.

Chapter 2

pH-responsive polymer microcapsules for targeted delivery of biomaterials to the midgut of *Drosophila suzukii*

2.1 Overview

In this chapter, I will cover the design and development of a novel delivery method for protein/peptide biopesticides. This chapter is based on a paper of the same name that is currently under review at PLOS One. This paper has been expanded with the inclusion of a section on emulsion stability not included in the submitted manuscript.

This study has developed a novel method for the encapsulation of large peptide biopesticides, providing protection of these delicate species upon foliar application and upon ingestion. Furthermore, I have engineered these microcapsules to release their contents upon changes in environmental pH. Specifically, these microcapsules respond to the intrinsic pH changes across the gut of the economically important pest, *D. suzukii*, resulting in the release of the encapsulated cargo, as exemplified in Figure 2.1.

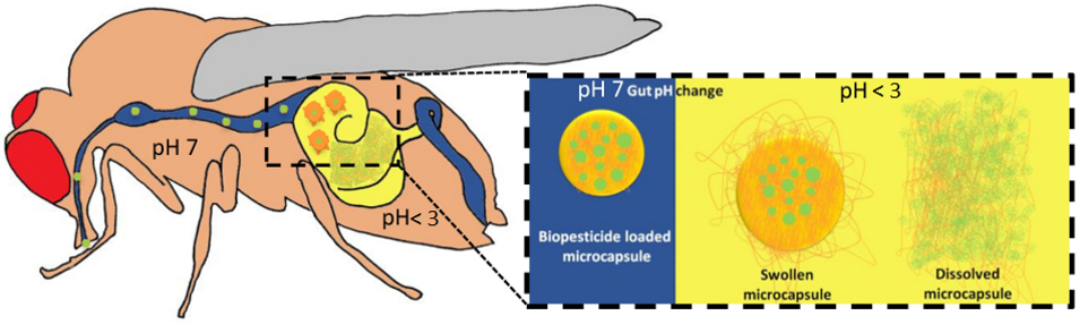


Figure 2.1: Graphical abstract demonstrating triggered release of biomaterials in the midgut of adult *D. suzukii* from loaded pH-responsive microcapsules, induced by the changes in gut pH.

2.2 Abstract

Drosophila suzukii (or spotted wing *Drosophila*) is an economically important pest which can have a devastating impact on soft and stone fruit industries. This invasive pest targets ripe fruit to lay its eggs, leading to extensive crop loss in temperate regions across the globe. Biological pesticides are being sought as alternatives to synthetic chemicals to control this invasive pest, but many biopesticides are subject to degradation and therefore require protection during the process of delivering it to the insect. This study identified a sharp change in pH of the adult midgut from neutral to acidic (pH $<$ 3), which was then exploited to develop pH-responsive poly(2-vinylpyridine) (P2VP) microcapsules. These microcapsules were designed to respond to the change in midgut pH through dissolution of their polymeric shells thus leading to release of their cargo for uptake into the insect. To encapsulate water-soluble biological species in an aqueous continuous phase, a multiple emulsion template was used as a precursor for the synthesis of pH-responsive P2VP microcapsules with a fluorescent (FITC-dextran) cargo. The water-soluble agent was initially separated from the aqueous continuous phase by an oil barrier (the 2VP monomer), which was subsequently polymerised. The P2VP microcapsules were stable at pH $>$ 6 but underwent rapid dissolution at pH $<$ 4.2. *In vivo* studies showed that the natural acidity of the midgut of *D. suzukii* also induced the breakdown of the responsive P2VP

microcapsules to release FITC-dextran which was taken up into the body of the insect and accumulated in the renal tubules.

2.3 Introduction

The invasive and polyphagous pest, *Drosophila suzukii*, is native to South East Asia, but has recently attracted much attention because of its economic impact on the soft and stone fruit industries in North America, Europe and more recently South America.[1–7] The economic damage is inflicted by the adult female laying eggs in ripening fruit by virtue of a well-developed serrated ovipositor that cuts the fruit skin for egg deposition.[8] Current control strategies rely heavily on chemical pesticides, but their use is limited by the need to avoid chemical residues on the fruit post-harvest.[203–206] Moreover, our current arsenal of insecticides is likely to shortly no longer be sufficient, due to environmental concerns and the increasing frequency of resistance. As a result, it is important to consider alternatives to existing synthetic pesticides.[207,208] Biopesticides derived from natural sources with new modes of action, such as peptide/protein toxins,[42,49,207] neuropeptides,[41,209–211] insect hormones[38,212–214] and double-stranded RNA (dsRNA)[25] have been proposed as alternatives, either individually or as part of an integrated pest management strategy. Biopesticides have several positive attributes such as specificity for a target pest,[42] low environmental persistence,[38] non-toxic residues to both mammals and arthropods, and easy integration with other plant protection measures.

Unfortunately, many biopesticides are subject to degradation on storage, field application[49,215] and in the insect's midgut when taken up *per os*.[38,216] Specifically, the degradation of protein/peptide biopesticides can occur due to hydrolysis overtime in storage, degradation due to UV exposure on the crop substrate or breakdown induced by the proteases produced by the pest. Many biopesticides are also water soluble and can be easily washed from crop surfaces, resulting in reduced efficacy.[215] For instance, spider venoms contain more than a thousand peptide toxins, many of which have insecticidal activity. The majority however are not active *per os*, due to poor uptake and rapid degradation in the pest's digestive tract.[35,208] When these peptides are orally active, a post-translational

modification often provides increased stability, where after the peptide has been synthesised within the cell it is modified. Such modifications include the addition of other moieties such as glycans shielding the active species, or by cross-linking by disulphide bonds which leads to folding of the peptide/protein increasing resistance to proteases.[35,43,208] Recently, the instability of ingested biopesticides and their lack of epithelial penetration have been addressed in several ways, including fusion of peptides to plant lectins,[84] co-delivery with inhibitors to reduce gut protease activity,[217,218] attachment to plant virus coat proteins,[84] development of stable mimetic analogues,[38,46,219] and conjugation to polyethylene glycol (PEG) polymers.[44,87,211]

With advances in biotechnology, numerous proteins and peptides have also been developed for therapeutic purposes, such as protein-based vaccines[96,97] and immunoregulatory peptides.[220,221] Similarly to peptide biopesticides, their short half-life and poor uptake profile has limited their use. Instead of the fusion approach, where the actives species is covalently linked to another peptide or protein to provide increased protection and enhanced delivery of biopesticides, therapeutic proteins tend to be encapsulated in polymer microcapsules or embedded into nanoparticles or microgels.[94,96,103,220,222–224] However, in this form bioactives do not remain active as there is a polymeric barrier between the active species and its target and a release mechanism is therefore required.

Commonly, polymeric carriers are made from two classes of polymers, biodegradable or acid-degradable polymers. The biodegradable polymers are based on poly(lactic acid) (PLA), poly(lactic acid-glycolic acid) (PLGA) and chitosan, and are slowly degraded with the assistance of enzymes within the body.[96,220,224] This degradation typically leads to a slow sustained release profile of the bioagent, over several days.[94,220,223,224] Acid-degradable protein-loaded vehicles tend to be either microgels or nanoparticles that are cross-linked with an acid-labile acetal/ketal linkage that breaks down in mildly acidic conditions.[96,97] This method has been successfully implemented to trigger degradation and release within the acidic phagosomes of antigen-presenting cells, disrupting the phagosomes by an osmotic pressure mechanism, which releases the loaded cargo.[96] This mechanism of release is faster, compared to that achieved with biodegradable polymer based

vehicles, with 80 % of the loaded protein released after 5 h. However, this release rate would likely be too slow for good biopesticide efficacy, as the midgut retention time is typically less than 1 h.[225]

In the present study I have developed microcapsules to deliver and protect a water soluble bioinsecticide into the intestine of adult *D. suzukii* (Figure 2.1). It is known that in the related fruit fly *D. melanogaster* the pH of the intestinal lumen is mostly alkali apart from a central "stomach" region in the midgut which has a pH < 4.[149] This change in pH might be exploited as an acid trigger to rapidly release water soluble bioactive compounds such as insecticidal peptides and protein toxins from pH-responsive microcapsules. A similar acid region is likely also to occur in the midgut of *D. suzukii*, which might then be exploited to efficiently deliver biological insect control agents. I have built on previous work[226,227] using a water-in-oil-in-water emulsion as a template for the synthesis of pH-responsive polymer microcapsules. The middle oil-phase in the water-in-oil-in-water emulsion precursors consist of a pH-responsive monomer, subsequently polymerised to produce a shell of low porosity for large molecular weight biopesticides. Using a fluorescent dextran as a model cargo demonstrated that these microcapsules can deliver and release their load in the acid region of the adult intestine for improved uptake into the body of the insect.

2.4 Materials and methods

2.4.1 Insects

D. suzukii (an Italian strain) were maintained on a standard *Drosophila* diet (oatmeal, 7.5 %; molasses, 5 %; agar, 8.4 %; yeast, 8.4 %; methyl paraben, 0.35 % in water) at 25 °C in a 12:12 light-dark cycle. Females (5-10 days old) were used for the experiments apart from the particle ingestion study, which used both males and females.

2.4.2 Materials

Bromocresol purple, bromophenol blue, Sudan III ($\geq$ 85 % purity), the hydrophilic polymer emulsifier poly(vinyl alcohol) (PVA, $\geq$ 99 % hydrolysed, 65,000

Da), FITC-dextran (average Mw 10,000 Da), 2-vinylpyridine (2VP, 97 % purity), methyl methacrylate (MMA, $\geq$ 99 % purity), Span 80, Brij 30, alumina and all buffer chemicals were purchased from (Sigma-Aldrich Company Ltd.) 2,2-Azobis(2-methylpropionitrile) (AIBN) was purchased from DuPont Chemicals. The monomers MMA and 2VP were purified using basic alumina prior to use. Milli-Q water was used at all times.

2.4.3 pH profile of the intestine of *D. suzukii*

pH-sensitive dyes were used to determine the pH environment of the gut lumen of adult females, via incorporation of the dye into the standard *Drosophila* diet. The following dyes were used separately at the concentrations given: 0.5 % (w/w) bromocresol purple (pKa 6.3) and 0.5 % (w/w) bromophenol blue (pKa 3.8). Flies were transferred to the dye-containing diet at 7-8 days of adult life. They were allowed to feed for at least 24 hours before observations were made, allowing for sufficient dye to be ingested. Observations were made by chilling the flies on ice, the intestine tissues were removed which contained the pH responsive dye, and images captured by optical microscopy (Leica EZ4 W). OM images were then analysed to determine the pH of the gut based on the colour changes of the pH dye. As the gut pH only remains stable for a few minutes post dissection, optical micrographs were taken a few seconds after dissection.

2.4.4 Synthesis of solid polymerised methyl methacrylate (PMMA) particles for ingestion limit determination

A polydisperse range of solid PMMA particles was produced by suspension polymerisation. In this process, an oil-in-water emulsion was first produced, where the dispersed oil phase contained the dissolved initiator and was subsequently polymerised, yielding solid polymer particles. In a typical synthesis, the oil phase was comprised of: monomer, MMA (10 ml); a thermal initiator, AIBN (126 mg); and a lipophilic dye, Sudan III (75 mg). This oil phase was emulsified using a high-shear mixer, 2 min 15,000 rpm, (Ultra-Turrax, IKA T25) into an aqueous phase (50 ml) containing a hydrophilic emulsifier, PVA (1.5 g). The emulsion was

placed in a 250 ml round bottom flask and degassed with nitrogen for 30 min before polymerisation was undertaken at 70 °C for 4 h while maintaining stirring at 300 rpm. The resulting particles were cleaned to remove any unreacted monomer by multiple centrifugation and re-dispersion steps, into PVA solution.

2.4.5 Particle Ingestion

Dye containing particles were fed to adult flies of both sexes at an age of 7-8 days. The particle suspension was mixed into standard *Drosophila* diet, and the flies were kept on this diet in a plastic petri dish before observations were made. After two days of feeding, the whole intestine was dissected in water and images were taken by optical microscopy (Leica M165FC). Excreted material was analysed by first re-dispersing the faeces deposited on the surface of the petri dish in a small volume of water. The dispersed particles were then observed by optical microscopy (Olympus BX51), and the particle diameters of 700 excreted particles were measured using ImageJ software. This analysis was undertaken by first thresholding the optical micrographs to clearly define the edges of the excreted particles, from this the diameters were determined and converted to a volume % in 1 μm bin intervals.

2.4.6 Synthesis of FITC-dextran loaded microcapsules from a multiple emulsion template

To encapsulate water-soluble biomaterials in an aqueous continuous phase a W/O/W multiple emulsion template precursor was used, where the middle oil phase comprised of a monomer and initiator that could be polymerised to form a polymer shell. The water-in-oil-water (W/O/W) emulsion was produced via a two-step process. First, a water-in-oil (W/O) emulsion was prepared as follows. An aqueous solution of FITC-dextran (4 ml, 7 mg/ml) was injected into an oil phase composed of monomer (2VP or MMA) (10 ml), Span 80 (0.5 g), Brij 30 (1.1 g) and AIBN (128 mg). The mixture was emulsified using a homogenizer (Ultra-Turrax, IKA T 25) for 5 min at 15,000 rpm at room temperature, producing a milky W/O emulsion. The FITC-dextran containing W/O emulsion was then

added to an aqueous PVA solution (100 ml, 3 wt.% PVA) and homogenised mildly by inverting the container three times, producing a multiple emulsion. The formation of the multiple emulsion was confirmed by optical microscopy (Olympus BX51). This emulsion was subsequently polymerised in a 250 ml round-bottom flask equipped with a magnetic stirrer, reflux condenser and nitrogen inlet system. After degassing with nitrogen for 30 min, polymerisation was carried out at 70 °C for 20 h whilst agitating at a speed of 300 rpm, to ensure the maximum amount of toxic monomer was polymerised. This yielded microcapsules, which were characterised by optical microscopy. The size distribution of the microcapsules was determined by light scattering (Mastersizer 2000), where ten measurements were taken and averaged plotted on a semi log scale of size against vol. %. Microcapsules were cleaned by dialysis (BioDesignDialysis Tubing, 14,000 Mw, Fisher Scientific) over a 5-day period to remove unreacted monomer and FITC-dextran ($\sim$ 20 %) from the continuous phase. The pH-responsive microcapsules were also analysed using a Hitachi SU8230 scanning electron microscope (SEM). The microcapsules were diluted and deposited on a SEM stub and allowed to air-dry overnight. The dry microcapsules were then sputter-coated with a 4 nm layer of platinum before insertion in the SEM chamber, where they were analysed at 2 kV.

2.4.7 Emulsion stability and entrapment efficiency

For the successful formation of responsive microcapsules, the multiple emulsion precursor must be stable, maintaining structure during the polymerisation process. Therefore, he stability of the primary W/O emulsion was monitored via droplet size (Malvern Zetasizer ZS) analysis as a function of time while visual observations were made in parallel. To optimise the emulsion stability, I investigated the effect of surfactant ratio, surfactant concentration, shear type and water volume fraction on emulsion stability. W/O emulsions were formed as previously described with the omission of the thermal initiator. Droplet sizes were monitored by first diluting the emulsion with the appropriate continuous phase to limit multiple scattering events.

The entrapment efficiency of two different primary emulsions (containing FITC dextran as a model species) with differing emulsion stabilities were investigated. Each of these emulsions had the same composition but used different methods of shear, producing differing average emulsion droplet diameters. These primary emulsions were then further emulsified into a multiple emulsion, which could cream for 24 h in a separating funnel. The lower phase, which contained the continuous phase was collected and analysed using a 96-well plate (Corning Life Sciences) and a FLUOstar Omega (BMG LABTECH GmbH) with $\lambda excitation$ at 485 nm and $\lambda emission$ set at 520 nm to detect the FITC-dextran in the continuous phase.

2.4.8 pH-dependent release of FITC-dextran from multiple emulsion templated microcapsules

The equivalent pKa of the synthesised responsive microcapsules was determined by monitoring light transmission using a UV/VIS spectrometer (Agilent 89090A UV/VIS, 500 nm) through a microcapsule suspension at varying pHs. Upon dissolution of the capsules, light transmission through the suspension increases. Solutions varying in pH from 2.8 to 6 were produced from concentrated HCl. The microcapsule suspension light transmission was measured by diluting the microcapsules (50 μl) into each pH solution (2 ml), inverting the samples three times and measuring the transmission. Data was fitted using OriginPro 9 to a sigmoidal curve.

To further analyse the *in vitro* dissolution of the responsive microcapsules, continuous video recording was undertaken as the pH of the continuous phase decreased. On a microscope slide a drop of citric acid was introduced to the side of a microcapsule sample, gradually decreasing in the surrounding pH. This was achieved by bringing together two separate coverslips containing the responsive microcapsule suspension and 1 M citric acid solution, respectively. Microcapsules were imaged over time as the surrounding pH decreased, and still images from this video were subsequently reported.

The *in vitro* release of FITC-dextran from the responsive microcapsules was determined by fluorescence spectroscopy in different pH environments (pH 3.8

and 6). W/O/W templated microcapsules (2 ml) were added to solutions of differing pH (pH 3.8 and 6; 20 ml), where the low pH conditions were produced using a citric acid buffer. At selected time points, samples (1 ml) were taken and passed through a 0.22 μm syringe filter to remove any undissolved capsules and solid material. The filtrate was acidified to pH 1.5 (0.1 ml of 0.1 M HCl added to 0.2 ml of filtrate) to dissolve any remaining polymer. The resulting samples were analysed using a 96-well plate (Corning Life Sciences) and a FLUOstar Omega (BMG LABTECH GmbH) with $\lambda excitation$ at 485 nm and $\lambda emission$ set at 520 nm. The collected data was normalised against standard solutions of FITC-dextran in the relevant buffers, subjected to the same processing steps as above to filter and acidify.

In vivo release of cargo from the pH-responsive P2VP and non-responsive PMMA microcapsules was investigated by depositing microcapsules containing FITC-dextran on to the intact surface of an organic blueberry substrate. Flies could feed on the microcapsules for 48 h before being immobilised on ice and dissected in water to reveal the distribution of the ingested FITC-dextran, which was captured by optical fluorescence microscopy (Leica M165FC). The presence of microcapsules was investigated in the crop, midgut and in the faeces. Investigation of the microcapsules in the foregut prior to the pH transition was not possible due to the short retention time in this tissue.

2.5 Results

I have investigated a novel method for delivery of biomaterials to control the invasive pest *D. suzukii*. Initially, the changes in pH of the pest's intestine that can be exploited to trigger release. Secondly, the maximum size of particles that the pest can ingest was investigated. Thirdly, a method to encapsulate a fluorescently tagged, water soluble model bioagent using a multiple-emulsion template was investigated. Fourthly, the triggered release from these microcapsules *in vitro* was monitored. Finally, pH-responsive microcapsules were fed to adult *D. suzukii* to study their *in vivo* release properties.

2.5.1 Identification of an acidic region of the midgut of adult *D. suzukii*

After 24 h feeding on the pH-indicator dyes, the dissected gut showed clear and reproducible staining. Bromocresol purple (pKa 6.3) staining showed that much of the foregut and anterior midgut had a pH between 5.2 and 5.8, as demonstrated by the dark staining (Figure 2.2a). A region of yellow staining (Figure 2.2a) was observed in the middle of the midgut showing it is acidic, pH $<$ 5.2. The posterior midgut was stained purple with a pH $<$ 5.8. The inclusion of bromophenol blue (pKa 3.85) in the diet showed that this central region of the midgut was strongly acidic with a pH $<$ 3 (Figure 2.2b).

2.5.2 Particle ingestion size limit of adult *D. suzukii*

To investigate the maximum particle size that can be ingested by adult *D. suzukii* flies, solid PMMA particles containing the red dye (Sudan III) were produced via suspension polymerisation. Due to the random shear method used to produce these particles, the resulting samples were inherently polydisperse with a size distribution ranging from 1 to 200 μm (Figure 2.3a and d), which makes them ideal to determine the size threshold for ingestion. These particles are clearly visible in the dissected midgut of *D. suzukii* 48 h after feeding on a diet containing these particles (Figure 2.3b). However, due to the opacity of the gut tissue, unfortunately I was unable to accurately measure the particle size in the gut lumen. I was able to measure the size of the excreted particles in the faeces after resuspension in water (Figure 2.3c).

There was a dramatic difference in the particle size distribution before ingestion and after passage through the intestine (Figure 2.3d). The excreted particles have an upper size limit of around 15 μm. Particles smaller than 5 μm contained a small amount of dye and were difficult to detect in the insect's excrement, as a result we do not see the full particle distribution.

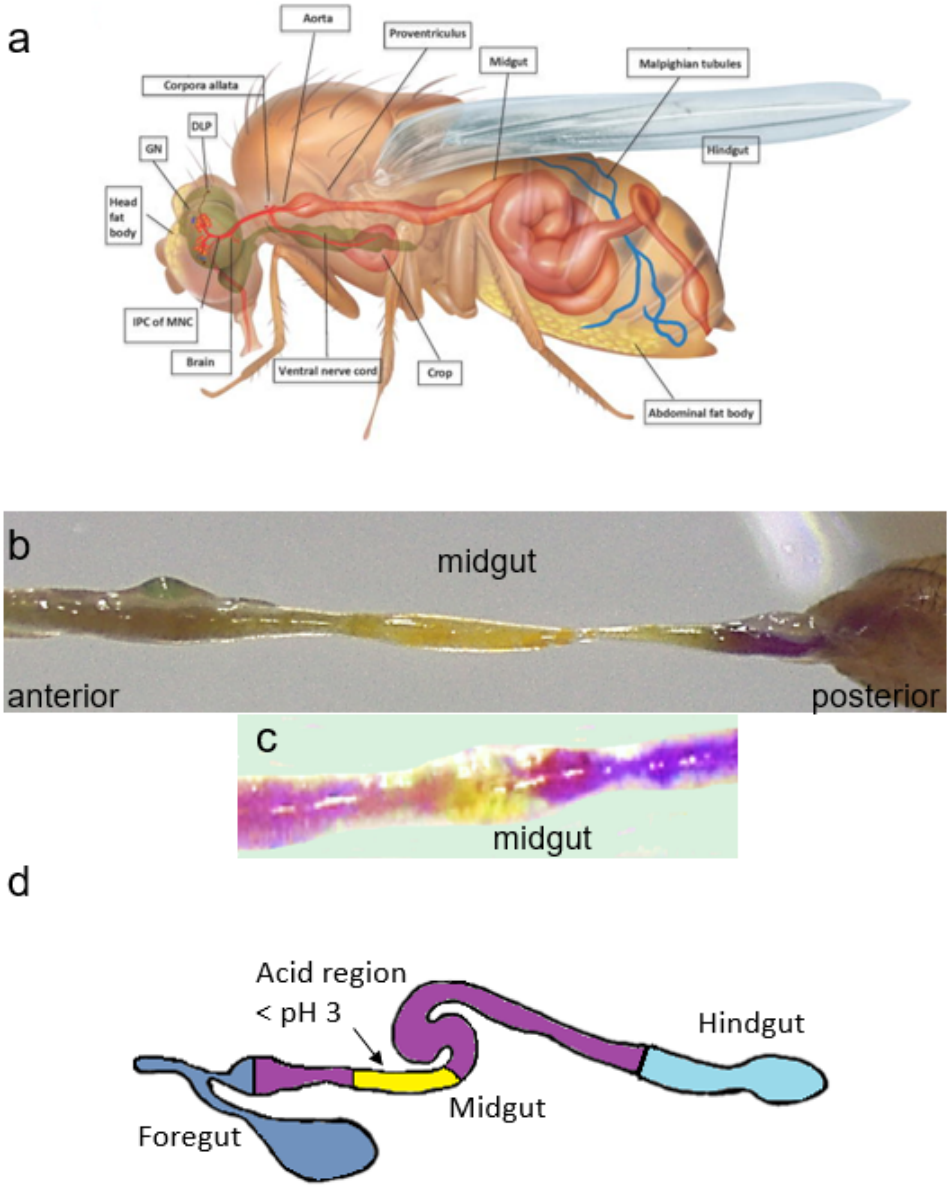


Figure 2.2: (a) Generic anatomy of *Drosophila* adapted from[228]. The pH changes of the midgut of adult *D. suzukii* (7-8 days old) fed for 24 h on standard *Drosophila* diet containing either (b) bromocresol purple (0.5 w/w %) or (c) bromophenol blue (0.5 w/w %). In (b) purple indicates pH $>$ 5.8 and yellow pH $<$ 5.2. In (c) yellow, pH $<$ 3 and purple/blue, pH $>$ 4.6. (d) Diagram showing the central region of the midgut has a pH of less than 3. All scale bars are 200 μm.

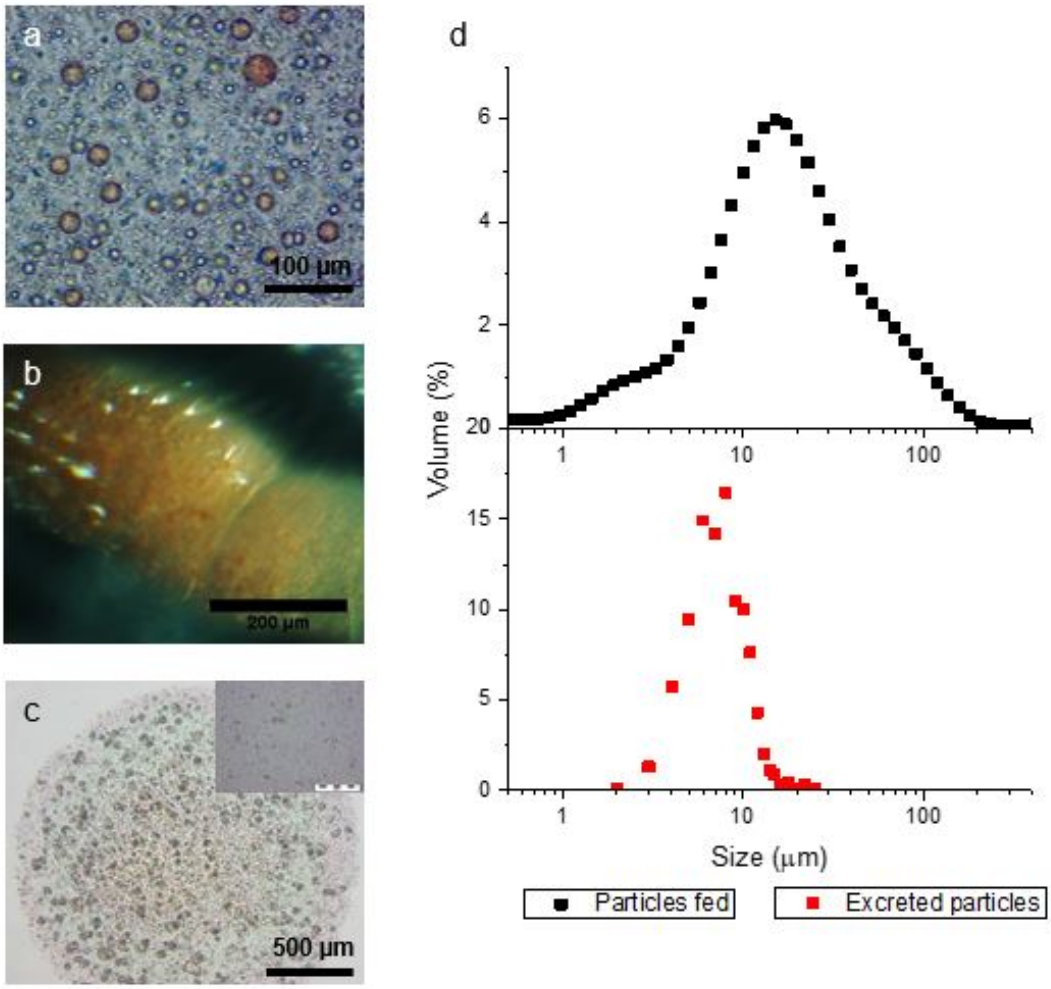


Figure 2.3: The size range of particles ingested by adult *D. suzukii*. (a) A micrograph showing polydisperse suspension of PMMA particles dyed red with Sudan III ranging in size from $\sim$ 1 - 200 μm. (b) Dissected midgut of *D. suzukii* coloured red from ingested dyed particles, opacity of gut hinders particle size determination. (c) Solid polymer particles in the fly excrement. The micrograph shows polymer particles resuspended from the excrement allowing particle size to be measured. (d) Comparison of the particle size in the diet and in the excrement showed that the maximum particle size ingested was $\sim$ 15 μm. Particles $\sim$ 5 μm were below the detection limit.

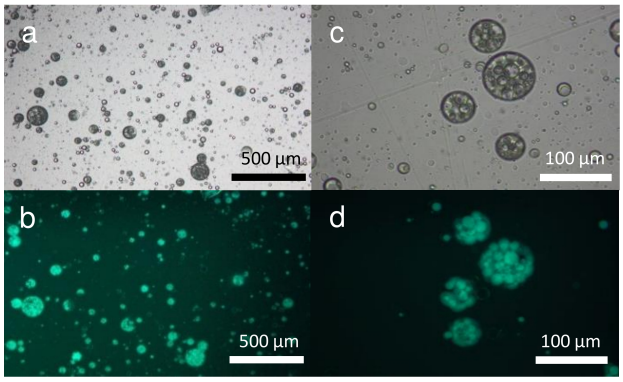


Figure 2.4: Multiple emulsion template containing FITC-dextran (10 kDa).(a and c) Bright field images.(c and d) Images captured using the fluorescent microscope. The water droplets in the oil contain FITC-dextran that is detected in discrete pockets of fluorescence corresponding to the inner water droplets.

2.5.3 W/O/W templated synthesis of pH-responsive microcapsules

The preparation of water-core microcapsules through polymerisation of multiple emulsion templates was achieved using a methodology similar to that of Kim *et al.*.[226] Firstly, a stable multiple emulsion was obtained by using a surfactant system comprising of a mixture of Brij 30 and Span 80 in the organic phase (see methods section for further details). Multiple water droplets were incorporated into the secondary emulsion oil droplets, as observed by optical microscopy (Figure 2.4a-d). The presence of the inner water droplets was easily determined by incorporating FITC-dextran into the inner water phase. Indeed, most of the fluorescent material appears to have been incorporated in the multiple emulsion inner droplets (Figure 2.4b and d).

Subsequent polymerisation of the multiple emulsion template yielded solid microcapsules with encapsulated FITC-dextran (Figure 2.5a-d). These microcapsules appear to have diffuse fluorescence, compared to the pockets of fluorescence observed in the original emulsion, indicating that the inner water droplets had likely coalesced during the polymerisation process. Dialysis was used to remove non-incorporated FITC-dextran and ensure that the microcapsules used in deliv-

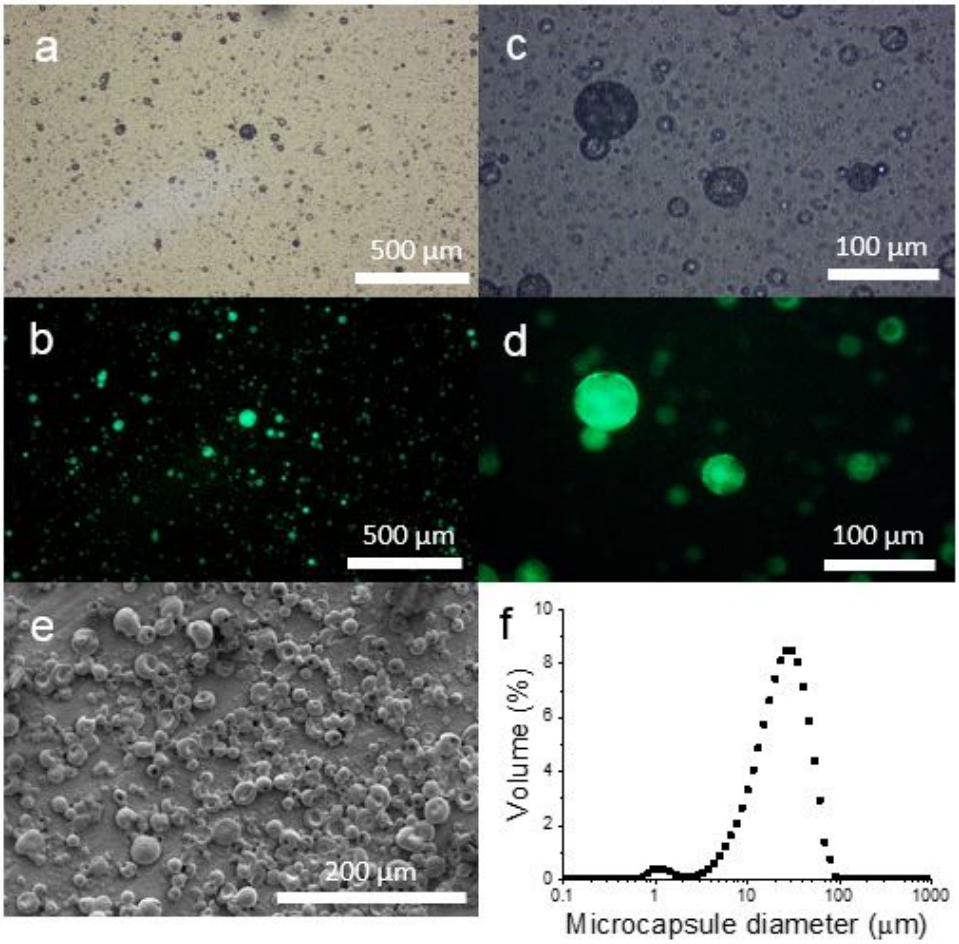


Figure 2.5: Polymerised multiple emulsion template yielding solid P2VP microcapsules (a-d) Optical and fluorescent microscopy of microcapsules formed from polymerisation of the multiple emulsion template oil phase. (e) Scanning electron micrograph of the loaded microcapsules (f) Particle size diameter, expressed as vol. %, of the microcapsules ranging from approximately 2 - 100 μm. The smaller peak around 1 μm is a by-product formed of solid polymer particles containing no active species.

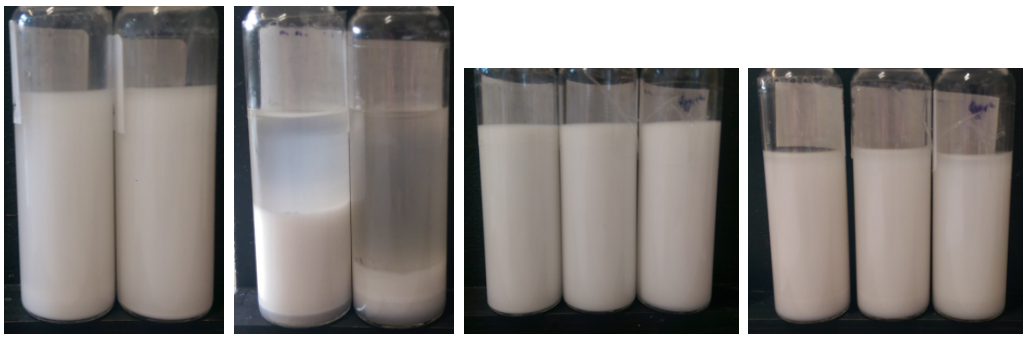
ering dextran to the insects was localised only within the microcapsules. SEM showed that all microcapsules had collapsed under vacuum (Figure 2.5e), probably because of loss of the internal water during sample preparation. The method of emulsification produces polydisperse multiple emulsions, and as a result most of the microcapsules ranged in size from $\sim$ 2 - 100 μm as shown in Figure 2.5f. A small proportion (by volume) had a diameter of less than 1 μm possibly due to the undesired formation of single oil-in-water emulsion droplets during the emulsification process.

2.5.4 Emulsion optimisation for efficient encapsulation

Several different variables that contribute to the stability of the multiple emulsion precursor were investigated. This optimisation was undertaken subsequently to the production of microcapsules used for *in vivo* testing. Emulsion stability significantly affects the entrapment efficacy within the microcapsules and therefore an attempt to optimise the stability was undertaken. First the primary emulsion stability was investigated, through visual observations and light scattering. The primary emulsion was monitored over a 24 h period at room temperature, to study the stability of the emulsion during the synthesis process. However, this does not fully represent the conditions during polymerisation as the increased temperature and the changing nature of the oil phase will alter the stability of the emulsion droplets, which was not investigated.

The stability of the primary emulsion was found to vary when changing the average droplet size, induced by differing the shear type and duration of the emulsification process. Two different methods were used to create the emulsion, an Ultra-turrax high shear mixer and an ultrasonic probe.

After emulsification using an Ultra-turrax high speed mixer for 1 or 5 min both samples are equivalent at t = 0 h; milky and homogeneous. However, after t = 24 h large amount of settling of the emulsion droplets along with some phase separation (Figure 2.6a & b) was noticed. Conversely, with the use of an ultrasonic probe no change in the appearance of the samples after 24 h was observed (Figure 2.6c & d).



(a) (b) (c) (d)

Figure 2.6: Effect of applied shear in emulsification process on emulsion stability. Water-in-oil emulsions formed through use of an Ultra turrax high speed mixer at t = 0 h (a) and t = 24 h (b). 1 min emulsification (left) 5 min emulsification (right). Samples formed from an ultrasonic probe t = 0 h (c) and t = 24 h (d), 30 s, 1 min and 2 min emulsification (left to right).

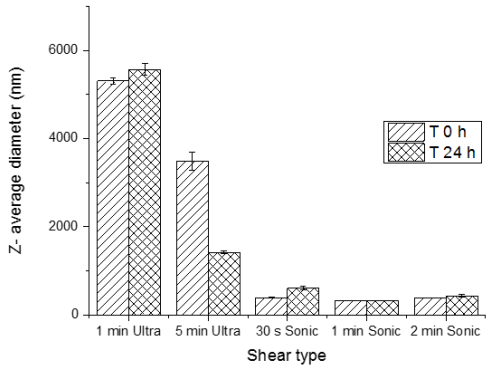
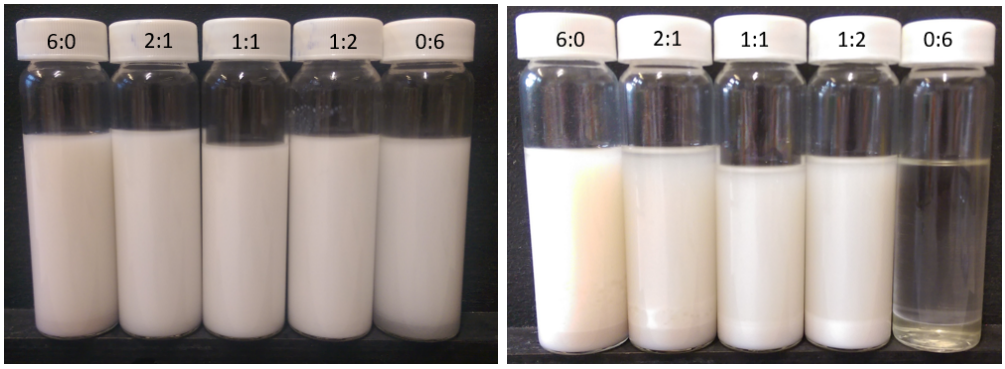


Figure 2.7: Water-in-oil droplet size as a function of time and shear type. Z-average droplet diameter size (mean±s.e.m; n=30).

The initial droplet size for emulsions produced using an Ultra-turrax for 1 min had a large average droplet size of 5 μm. Shearing the droplet for 5 min resulted in a reduction in the average droplet size to 3.8 μm, shear times longer than 5 min did not reduce the droplet size further. After 24 h the droplet size of the 1 min sample had increased. Conversely, a large reduction in the droplet size was observed for 5 min shear (Figure 2.7). However, there was a large amount of coalescence of water droplets observed. During the droplet size measurements, the sample settles if the emulsion droplets are too large, lowering the average diameter.

Droplet sizes produced by the ultrasonic probe are substantially smaller ($\sim$ 300nm) than those produced by Ultra-turrax (Figure 2.7). No significant differences in the initial droplet sizes were observed when the duration of shear was altered. After 24 h a small amount of settling was observed in the sample sheared for 30 s. The droplet size increased over 24 h when the sample was sheared for 30 s indicating coalescence. The droplet sizes for samples sheared greater than 30 s did not significantly increase.



(a) (b)

Figure 2.8: Emulsion stability as a function of surfactant ratio (Span 80:Brij 30)in oil phase. All samples were produced using an ultra-sonic probe for 1 min and the total surfactant concentration was 6 vol.% of the oil phase. (a)The initial emulsion appears milky at t = 0 h. All samples were homogeneous except in the case of the sample with only Brij 30. (b) After t = 24 h phase separation or settling can be observed.

Along with the mechanism of emulsification the influence of the ratio of surfactants in the system, at a constant volume fraction (6 vol.%). The surfactant ratios tested were 6 vol.% Span 80 (6:0); 4 vol.% Span 80 and 2 vol.% Brij 30 (2:1); 3 vol.% Span 80 and 3 vol.% Brij 30 (1:1) ; 2 vol.% Span 80 and 4 vol.% Brij 30 (1:2); and 6 vol.% Brij 30 (0:6).The primary emulsions were formed by the use of an ultrasonic probe for 1 min, producing a milky emulsion in each of the samples. The sample containing only Brij 30 started to phase separated within minutes of formation (Figure 2.8a). After 24 h full phase separation of the oil and water was observed when the surfactant system was comprised fully of Brij 30. A small amount of phase separation was present in the 6:0 & 2:1 samples. Whereas, the 1:1 and 1:2 samples had settled after 24 h but were easily resuspended upon gentle inversion. From droplet size analysis the 0:6 sample produced a large initial average droplet size and varied to a large degree during the measurement process. Droplet size could not be determined after 24 h as the sample had fully phase separated. All other samples produced a small initial average droplet size, between 200 - 300 nm, with a small increase after 24 h in the droplet size for both the 2:1 and 1:1 samples.

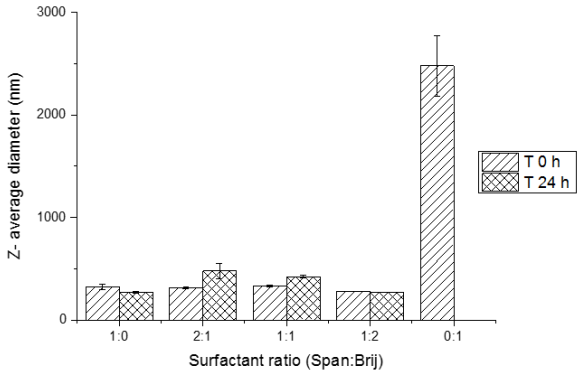
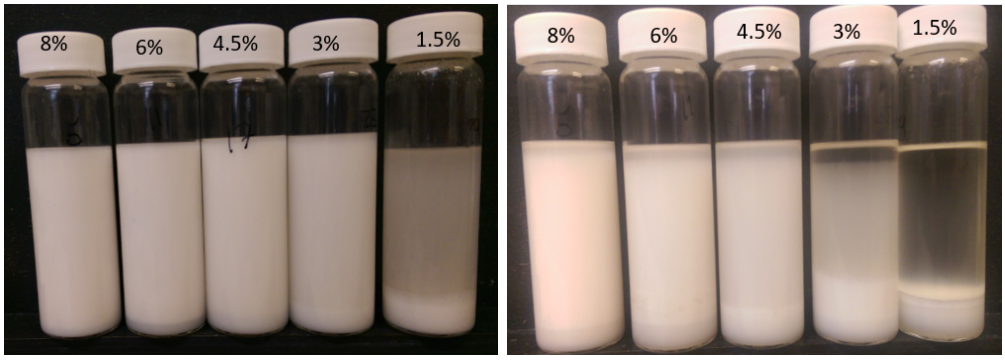


Figure 2.9: Water droplet diameter as a function of surfactant ratio in the oil phase. Z-average droplet diameter size (mean±s.e.m; n=30) at t = 0 h and t = 24 h. All emulsions were formed by ultrasonic probe for 1 min. The total surfactant concentration was kept constant at 6 vol.% of the oil phase.

1:2 Span:Brij surfactant system produced a stable emulsion for 24 h and was therefore used to determine the minimum surfactant concentration required to form a stable emulsion. The total surfactant concentration in the oil phases was varied from 8 to 1.5 vol.%, whilst maintaining the same ratio of surfactants. The emulsion with 1.5 vol. % surfactant was poorly formed and its droplet diameter could not be determined. Emulsions containing 3 to 8 vol.% surfactant produced homogeneous emulsions initially. The emulsion formed with a surfactant concentration of 8 vol.% remained unchanged after 24 h with no obvious settling or phase separation. Emulsions formed with a surfactant concentration of 6, 4.5 and 3 vol.% settled after 24 h (Figure 2.10). Analysis of emulsions formed with 8 vol.% surfactant showed very little increase in the average droplet diameter over 24 h. Conversely, when the surfactant concentration was reduced a large increase in average droplet diameter was observed (Figure 2.11).



(a) (b)

Figure 2.10: Effect of surfactant concentration on emulsion stability. (a) Initial emulsions formed using an ultrasonic probe for 1 min. At a surfactant concentration of 1.5 vol.% the emulsion was very unstable. (b) After t = 24 h sedimentation was observed in all samples except the sample formed with a surfactant concentration of 8 vol.%.

The volume fraction of water in the inner phase was investigated as a possible route of producing microcapsules with different polymer shell thicknesses. Emulsions were formed using an ultrasonic probe to form a small initial emulsion (200

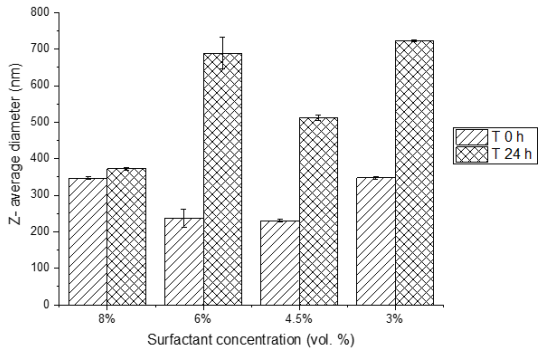


Figure 2.11: Average emulsion droplet diameter as a function of surfactant concentration. Z-average droplet diameter size (mean±s.e.m; n=30) at t = 0 h and t = 24 h. All emulsions were formed by ultrasonic probe for 1 min. The surfactant ratio was kept constant at 1:2 Span:Brij.

- 300 nm), at 20 vol.% water a cloudy emulsion was formed, whereas, at lower water concentrations a translucent solution was formed (Figure 2.12a). After 24 h the 20 vo.% sample had decreased in turbidity but was still opaque; whilst the lower water volume fractions were translucent (Figure 2.12b).

The initial emulsions (W/O), containing FITC-dextran, was used as the dispersed phase in the second stage of emulsification, allowing visualisation of the emulsion morphology. Localised florescence in multiple emulsions formed with an inner water volume fraction of 40 and 20 vol.% was observed. Strong background fluorescence was present in the continuous phase when the volume fraction of the inner water phase was 20 vol.% (Figure 2.12c & d). Oppositely, with the inner water fraction of 10 vol.% the entrapped material is significantly reduced, and the continuous phase is bright (Figure 2.12e). No multiple emulsions were formed when the inner water phases volume fraction was set to 5 vol.%, with only oil droplets formed.

To investigate the effect of primary emulsion size and stability on microcapsule loading and entrapment the cargo retention of two samples was investigated. The two samples were formed with the same chemical composition but with the method of shear changing, producing a different average droplet diameter. The

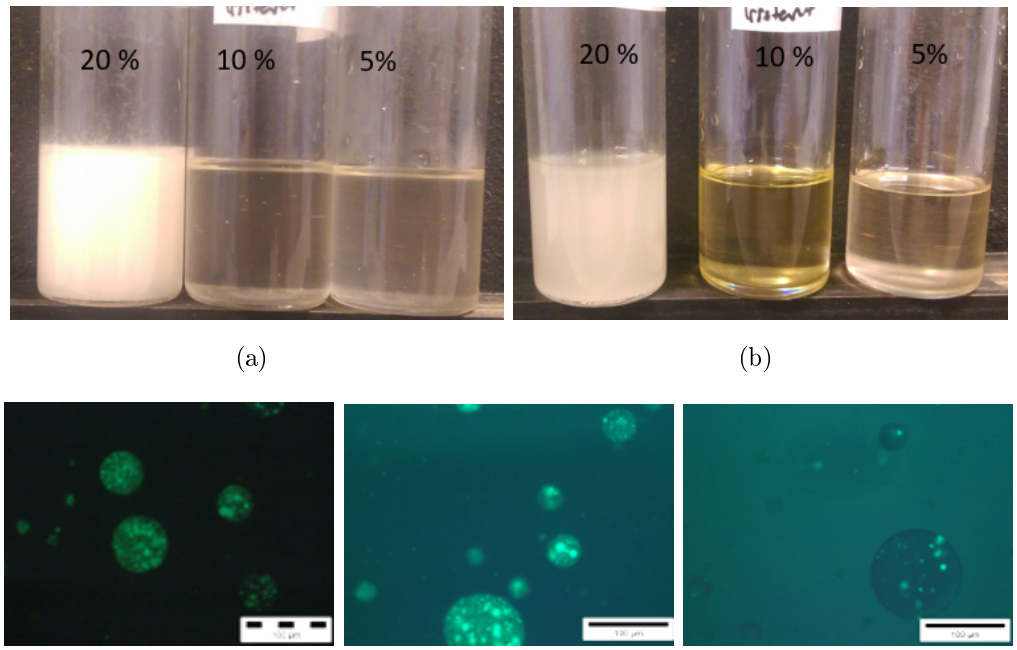
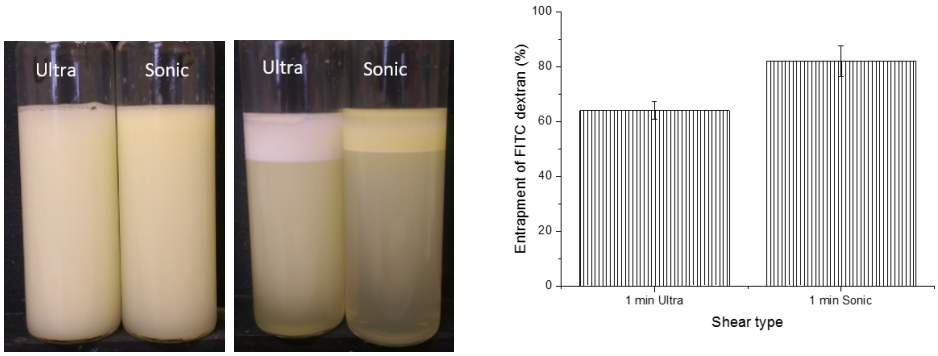


Figure 2.12: Emulsion stability at lower inner water phase volume fraction. Primary emulsion at t = 0 h (a) and at t = 24 h (b). W/O/W emulsion formed with an inner water volume fraction of previously shown 40 vol.% (c) along with reduced water content of 20 vol.% (d) and 10 vol.% (e). Secondary emulsions formed with 5 vol.% internal water showed no visible FITC-dextran droplets in the oil droplets.



(a) (b) (c)

Figure 2.13: Entrapment efficiency as a function of shear type and emulsion stability. Multiple emulsion containing FITC-dextran t = 0 h (a) and t = 24 h (b). Entrapment efficiency as a function of different shear types shown as a percentage.

samples were then allowed to cream, and the lower portion was extracted and analysed for presence of FITC-dextran by fluorescence. Both initial multiple emulsions at t = 0 h are milky yellow in colour, due to the inclusion of FITC-dextran into the inner water phase (Figure 2.13a). After 24 h the samples had creamed, and clear differences in the colour of the creamed layer were observed. The emulsion formed using an Ultra-turrax mixer had a pale creamed layer, whereas, the emulsion formed using an ultrasonic probe had a yellow creamed layer (Figure 2.13b). Analyses of FITC-dextran in the continuous phase and comparison with theoretical values showed that the Ultra-turrax sample contained $\sim$ 60 % of the payload. Whereas, the sample produced using an ultrasonic probe retained $\sim$ 80 % of the FITC-dextran after 24 h (Figure 2.13c). Note that is allows for a comparison of different emulsion stabilities but does not correspond to the true entrapment efficiency of the microcapsules.

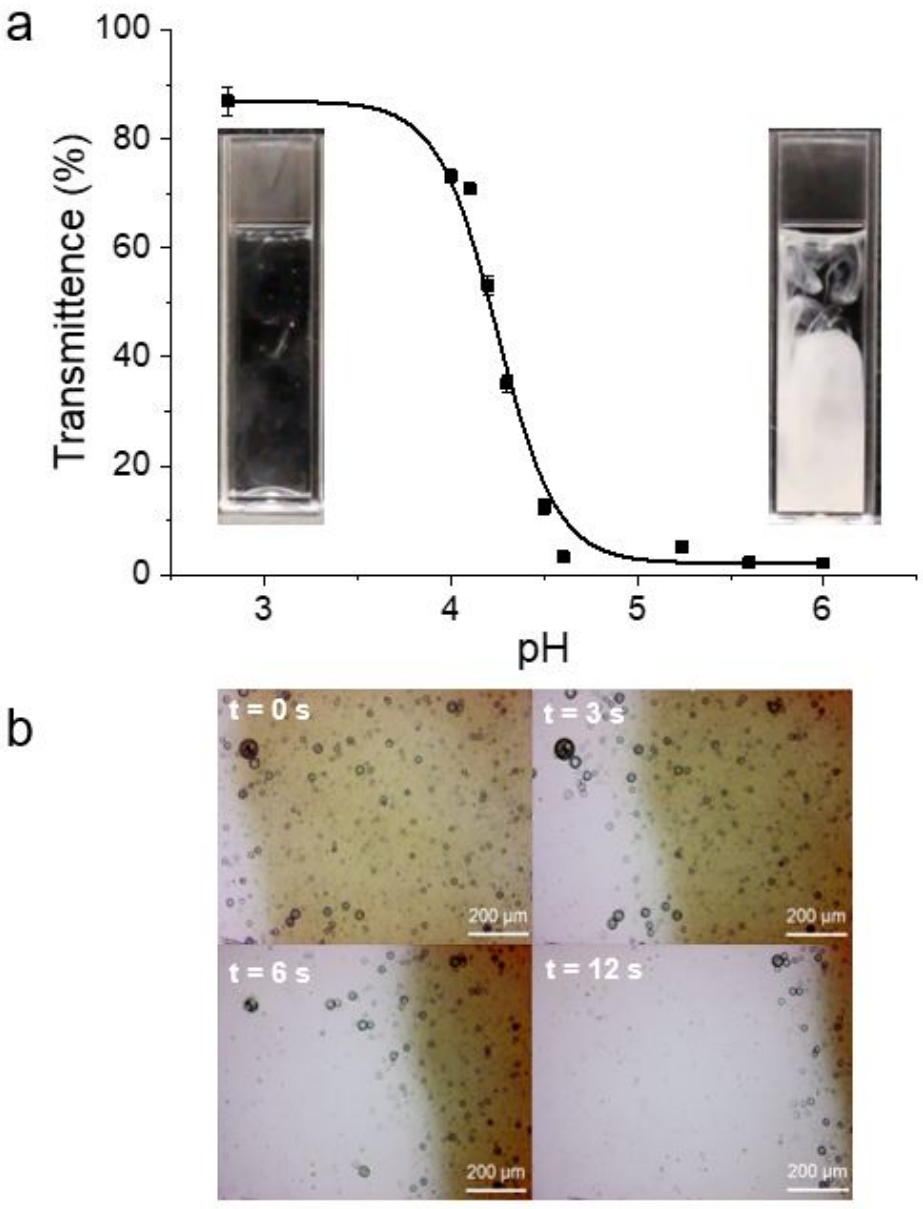


Figure 2.14: pH-dependence of P2VP microcapsule dissolution. (a) Determination of the apparent pKa of the microcapsules undertaken by monitoring light transmission (at 500 nm) as a function of pH. Data was fitted to a sigmoidal curve using OriginPro 9.1 software. (b) Still frames taken at 0, 3, 6 and 12 s from a video recording showing the effect of reducing pH on the P2VP microcapsules (see methods section) by introducing citric acid. The reduction in pH leads to swelling and rapid dissolution of the microcapsules.

2.5.5 *In vitro* release study of responsive microcapsules

The release profile of the encapsulated FITC-dextran under different pH conditions was investigated. Initially, the pH required for dissolution of the microcapsules was determined by measuring light transmission through the microcapsule suspension as a function of pH (Figure 2.14a). At pH greater than 4.5, transmission was low due to light scattering from the microcapsules. At a pH less than 4, a transmission of $\sim$ 85 % was measured, which indicated that the polymer chains of the microcapsules had become protonated and disentangled, resulting in dissolution of the microcapsule shell. At intermediate pH between 4 and 4.5, a sharp change in transmission was observed. A microcapsule apparent 'pKa' was estimated to be 4.2 ± 0.1 by fitting a sigmoidal curve to the data in Figure 2.14a, which is consistent with the pKa previously reported for P2VP polymers of 3.8 - 4.5.[229,230] The inserted images in Figure 2.14a show the appearance of the samples when microcapsules were added to pH 3 and 6 solutions. At pH 3 the solution was translucent with little evidence of undissolved microcapsules, in contrast at pH 6 the suspension became opaque due to light scattering from the microcapsules.

To further investigate the microcapsule dissolution, light microscopy was used to observe and video-record changes in microcapsule morphology over time as the pH was gradually reduced by the introduction of citric acid at one edge of the observed sample area (Figure 2.14b). As the acid was introduced onto the microscope slide, a pH gradient was induced with the the pH decreasing in the images from left to right over time. The microcapsules underwent an initial period of swelling followed by full dissolution. After 6 s, around a third of the microcapsules had disappeared, mostly the smaller ones, while the larger microcapsules fully dissolved within 15 s.

The release profile of FITC-dextran overtime from the microcapsules as a function of pH, using *in vitro* tests simulating the regional pH environments of *D. suzukii's* intestine was investigated. These *in vitro* tests were undertaken to determine if triggered release from the microcapsules could be induced by the changes in the pest's gut pH. Moreover, it was important to look at both the release at near neutral pH and at a pH analogous to the pest's midgut.

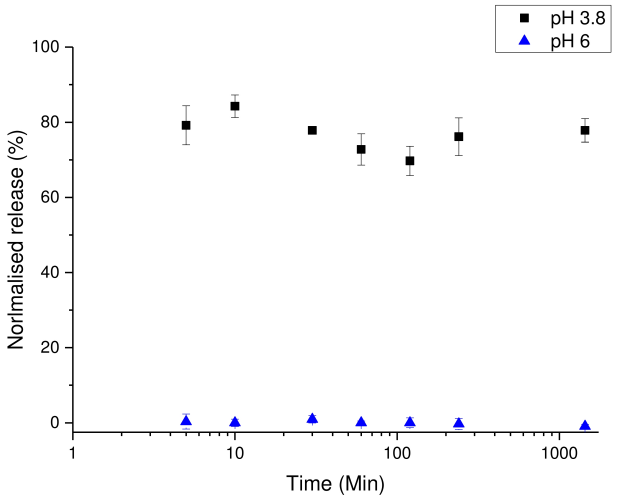


Figure 2.15: *In vitro* release of FITC-dextran from responsive microcapsules in different pH buffers. Microcapsules in a pH 6 environment showed no release over 24 h as the pH was above the pKa of the microcapsules. At the lower pH of 3.8 around 80 % of the encapsulated FITC-dextran was released within the first 5 min. It is assumed that the unaccounted 20 % is lost during the production of the microcapsules.

At pH 3.8 the encapsulated FITC-dextran (80 % of the total dextran incorporated within the inner emulsion template water phase) is released within the first 5 min (Figure 2.15). An earlier observation was not possible because of the time required to separate the continuous phase from the microcapsules, after 5 min there was no significant additional release. It is believed that the remaining 20 % FITC-dextran was lost during the formation of the microcapsules, and during dialysis used to remove unreacted monomer. At pH 6, which is above the pKa of the polymer, FITC-dextran was not released from the microcapsules over the same period. Release induced by low pH (less than 3) but not at neutral pH was further investigated *in vivo*, using the natural pH changes in the pest's intestine.

2.5.6 *In vivo* release

Triggered release of FITC-dextran from pH-responsive microcapsules could be induced *in vitro* at a pH of 3.8, with no release at a pH of 6. The triggered release of FITC-dextran was investigated *in vivo* from pH-responsive P2VP microcapsules and non-responsive PMMA microcapsules. The inclusion of non-responsive PMMA microcapsules acted as a negative control to determine if release was induced by another factor.

Insects were first fed control non-responsive PMMA fluorescent microcapsules. They were found to have intact microcapsules in the crop, midgut and the faeces (Figure 2.16a-c). In contrast, intact microcapsules were only found in the crop of flies fed with the pH-responsive P2VP microcapsules (Figure 2.16d). Fluorescence was seen in the midgut and in the excrement of these flies, but this was diffuse and clearly not contained in microcapsules (Figure 2.16e and f). Fluorescence was also present in the haemolymph, that was released into the bathing solution used during dissection of the tissues (Figure 2.16f), showing that the FITC-dextran had been taken across the gut wall into the haemocoel of the P2VP microcapsule fed flies. The excretory Malpighian tubules from flies fed the control microcapsules (Figure 2.16c) appeared dull with limited background fluorescence, whereas *per os* delivery of P2VP microcapsules led to bright fluorescence accumulating in the tubules (Figure 2.16f) indicating the removal and concentration of FITC-dextran from the haemolymph.

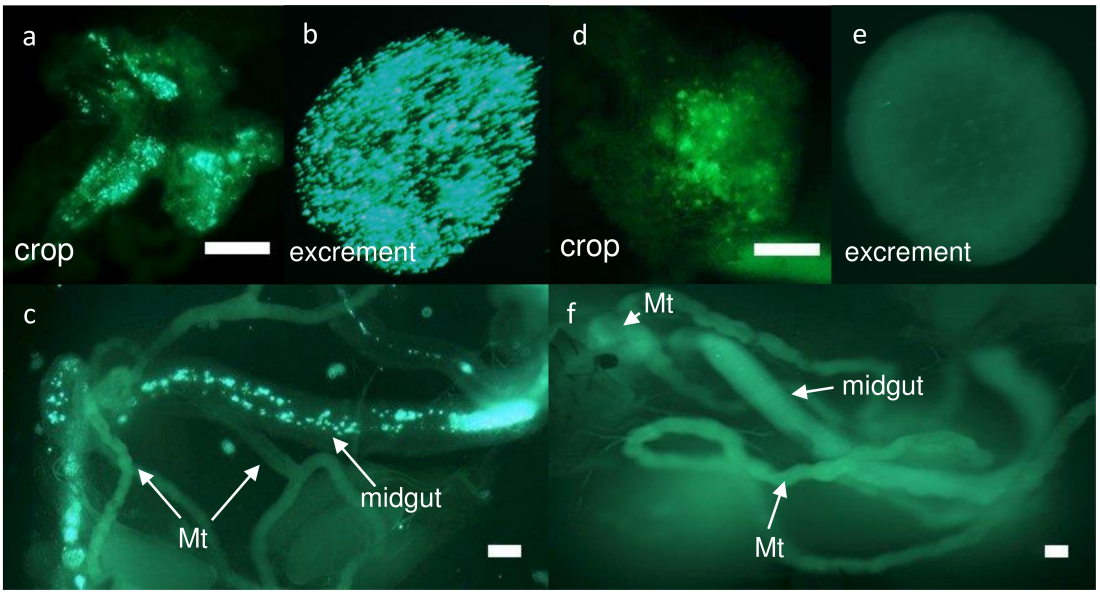


Figure 2.16: *In vivo* release of FITC-dextran from pH-responsive P2VP (d-f), but not from control non-responsive PMMA (a-c) microcapsules fed to adult *D. suzukii*. Intact fluorescent PMMA microcapsules in the crop (a), excrement (b) and the lumen of the midgut (c). Intact pH-responsive P2VP microcapsules in the crop (d), but none in the excrement (e) and midgut (f). Dispersed fluorescence in the posterior midgut shows that the P2VP microcapsules have released their contents. Fluorescence was also seen in the haemolymph released from the body during dissection. Strong fluorescence accumulated in the Malpighian tubules demonstrating uptake of FITC-dextran from the haemolymph. All scale bars 200 μm.

2.6 Discussion

The aim of this work was to develop microcapsules suitable for the protection and delivery of water soluble biological control molecules to the gut of the fruit pest *D. suzukii*. To achieve a multiple-emulsion templated synthesis was used to encapsulate a model biological species (FITC-dextran) in pH-responsive microcapsules. The gut of adult *D. suzukii* is divided into three distinct regions: the foregut, the midgut and the hindgut. The pH of the foregut and initial sections of the midgut were above or equal to pH 5.8 and this was followed by a reduction in pH to < 3 in the central or 'stomach' section of the midgut. These changes in pH are very similar to those reported for *D. melanogaster*, a phylogenetically close relation and model species.[149,231] This sharp reduction in pH was exploited for a triggered release of cargo from the pH-responsive microcapsules at the main site of nutrient uptake by the insect.[197,232,233] To form these triggerable microcapsules, the pH-responsive monomer 2VP was chosen to be polymerised into the microcapsule shell as the pKa of non-crosslinked linear P2VP chains is in the region of 3.8 - 4.7, depending on chain length.[230] In acidic conditions (pH < 3.8), it is expected that the protonation of the tertiary amines on the polymer chains would lead to dissolution of the entangled linear polymer chains and dissociation of the microcapsule shells, thus enabling full release of the contents.[229]

Before developing pH-responsive microcapsules, it was necessary to understand what microcapsule size could be ingested by the insects. Indeed, microcapsules used for the oral delivery of biopesticides should be below the size limit for particle ingestion, which can differ greatly between species. For example, the Mediterranean fruit fly *Ceratitis capitata* filters food through modified mouthparts which results in only particles of less than 0.5 μm diameter being readily ingested and passed into the intestine.[176] In contrast the decorous black fly *Simulium decorum*, which is of a similar body size, can ingest much larger particles up to a maximum of 400 μm.[234] Therefore, it was initially necessary to determine the size limit for ingested particles for the adult *D. suzukii* to ensure that the microcapsules were designed for the appropriate size range. Our results showed that particles larger than 15 μm are unlikely to be ingested.

The method used to produce pH-responsive microcapsules utilised uncontrolled shear to produce both the initial and secondary emulsions and as a result the microcapsules produced were polydisperse in the size range 2 - 100 μm with a secondary peak evident at 0.5 - 2 μm, which is likely the result of nucleation and limited growth of solid P2VP particles. Indeed, during the secondary emulsion step, inevitably a small quantity of oil-in-water emulsion droplets (not containing any inner water droplets) form along with the targeted multiple emulsion droplets. The quantity of this by-product is small and as a result did not interfere with our investigations. Only a portion of the microcapsules produced were smaller than 15 μm, and therefore further product development is desirable. More controlled emulsification techniques, such as membrane emulsification, which would decrease the multiple emulsion template size and polydispersity.[235] However, a sufficient proportion of our developed microcapsules could be ingested by the insects.

It is important for successful delivery of biopesticides that we can efficiently encapsulate the material limiting waste, especially considering the current high costs of bioinsecticides. One factor controlling the efficiency of encapsulation is the stability of the emulsion template. A stable emulsion precursor allows for the cargo to be retained in the inner water phase during the synthesis of the microcapsules. Several different variables that impact emulsion stability were investigated with the emulsion droplet size monitored over time.

Initially, the effect of shear on emulsion stability was investigated and it was found that the smaller emulsion droplet size provided by the high shear of an ultrasonic probe produces a more stable water-in-oil emulsion, compared to that formed by a high-speed mixer, under consistent physio/chemical conditions. This smaller emulsion has a higher stability for several reasons, small emulsion droplets are less likely to settle over time which reduces the rate of coalescence (a result of fewer contacts between drops) increasing emulsion stability. Moreover, the distribution of droplet sizes produced by an ultrasonic probe is narrower and as a result Ostwald ripening is reduced.[236] Microcapsules produced for investigating release *in vitro* and *in vivo* used the less stable larger emulsion droplets. Future work will use smaller diameter droplet primary emulsions that had a greater entrapment efficacy.

The stability of the primary emulsion is a function of the characteristics of the surfactant system used. Both long and short chain surfactants (Span 80 and Brij 30 respectively) were used to stabilise the emulsion. To gain an understanding of how the ratio of these surfactants and therefore the hydrophilic-lipophilic balance (HLB) affected primary emulsion stability, the surfactant system was varied at a constant total surfactant concentration allowing for the determination of the optimum combination. These surfactants were selected as typically they can be used to form stable water-in-oil emulsions with HLB values of 4.3 and 9.7 allowing through combination to cover a wide range of the typical optimum range for W/O emulsions (3-7).[237,238] When the surfactant system was comprised fully of Brij 30 rapid phase separation was observed. This poor stability was expected as the HLB value for Brij 30 was higher than the typical range required for stabilising a W/O emulsion.

With the addition of a long chain surfactant the HLB reduces to 7.8 (1:2 s Span 80:Brij 30) and a stable emulsion is formed. Surprisingly, this is not within the typical HLB range required for stabilising W/O emulsions (3-7), however, the 2VP oil phase is comprised of a more polar oil than is typically studied. Increasing the lipophilicity of the surfactant system (by increasing the ratio of long chain surfactant compared to short chain) destabilised the system leading to phase separation.

The surfactant system is critical for emulsion stability, with excess surfactant having a detrimental effect on entrapment of an active species. Excess surfactant in the system can lead to loss of entrapment via reverse micellar transport, where reverse surfactant micelles can actively transport the hydrophilic cargo across the oil phase into the continuous phase. To minimise the transportation of the active through the oil phase the critical surfactant concentration needed to form a stable emulsion was investigated. Moreover, reducing the surfactant concentration to the minimum decreases the cost of the production of the microcapsules and enhances stability by minimising depletion flocculation effects. For each of the different surfactant concentrations tested a constant surfactant ratio of Span:Brij of 1:2 was maintained. Surfactant concentrations of less than 6 vol.% are unstable over 24 h with settling and coalescence, leading to an increase in droplet size. On the other hand, at a surfactant concentration of 8 vol.% a stable emulsion is formed

with little change to the emulsion droplet size over 24 h. However, this large surfactant concentration may lead to reverse micellar transport of the cargo. The microcapsules initially produced had a surfactant concentration of 6 % and formed microcapsules with a large amount of material encapsulated, this shows that poor emulsion stability over 24 h does not necessarily lead to poor entrapment. Indeed, the initiation of polymerisation of the W/O/W multiple emulsion will alter the oil phase with rapid polymer chain growth leading to hindered diffusion.

Along with the emulsion stability of the microcapsules the volume fraction of the inner water phase was varied, altering the thickness of the polymer shells. Increasing the thickness of the polymer shell decreases the diffusion rate through it helping retain contents with in the microcapsules. However, due to the relatively high-water solubility of the 2VP monomer, the lower volume fraction of the inner water content led to poor entrapment as it became solubilised. Moreover, with the reduction in inner water volume fraction the osmotic pressure which may disrupt the multiple emulsion template.

The effect primary emulsion stability had on the entrapment of FITC-dextran over 24 h was investigated, to determine if a stable primary emulsion could increase entrapment efficiency. Two different primary emulsions were selected with the same chemical composition but prepared using two different methods. Over 24 h there was a link between primary emulsion stability and the retention of an active species within the multiple emulsion. Although I have investigated extensively the stability of the initial emulsion there are many other factors that lead to the breakdown of multiple emulsion (such as coalescence of oil droplets, expulsion of the internal droplets or by passage of water through the oil phase by diffusion) which will affect cargo retention prior to polymerisation.[239]

In order to demonstrate the potential of our microcapsules to carry and deliver water-soluble bioagents to the insect gut, FITC-dextran (10 kDa) was used as a model biological encapsulate of a similar size to known venom derived insecticidal proteins.[36] Multiple emulsion templated synthesis lead to encapsulation of the water soluble dextran within the inner core of the microcapsules, in a water continuous phase. The use of a W/O/W multiple emulsion template allows for the encapsulation of over 80 % of the dextran within the polymer microcapsule in this case. The remaining 20 % of the dextran was lost during the formation of the

multiple emulsion template into the continuous phase, which could be recycled to prevent waste in an industrial process.

To investigate the physical response of the microcapsules to changes in pH, a series of *in vitro* and *in vivo* experiments were conducted. The microcapsules dissolve in a suspension upon a reduction in pH with an estimated pKa of 4.2, which is consistent with literature values for the pKa of P2VP.[229,230]

To further investigate microcapsule dissolution at low pH, the microcapsules were observed using an optical microscope as they were introduced to a low pH environment. Swelling and subsequent dissolution of the microcapsules was observed as the pH of the continuous phase around the microcapsules decreased to < 4.2. Dissolution of the microcapsules is a favourable mechanism for the release of large molecules, compared to diffusion through a swollen shell due to (a) a more rapid release with dissolution and (b) reduced adsorption of the active onto or within the polymer shell. As a result, it is important to consider the composition of the oil phase. The polymer was designed not to be cross-linked (which increases the microcapsules physical strength), where upon a reduction in pH the polymer chains become fully solubilised. *In vitro* experiments also showed at pH 3.8 (below the pKa for the polymer), that full dissolution of the microcapsules occurred. Conversely, at pH 6, no release of FITC-dextran was observed over the course of the experiment, establishing that the dextran molecules were retained within the microcapsules at a pH above their pKa. Optical microscopic observations made in parallel showed no visible changes in the microcapsules at pH 6.

Further investigation was undertaken to determine if the *in vitro* release could be replicated *in vivo*, elicited by the intrinsic changes in the gut pH of *D. suzukii*. A *per os* study investigation was undertaken that compared the release of FITC-dextran from responsive P2VP microcapsules and non-responsive PMMA control microcapsules in the insect gut. The inclusion of non-responsive control microcapsules in our study showed that the release of fluorescence was not triggered by factors other than the gut pH, such as rupture by the mouthparts of the pest. In both cases, discrete microcapsules were observed in the crop of *D. suzukii* flies, showing that they were not broken by the mouthparts. Note that the food stored in the crop had not yet reached the acidic midgut, thus both the responsive and

non-responsive microcapsules remained intact in this part of the insect. Dissection of the intestine was undertaken to avoid damaging the tissue so that any fluorescence seen in the haemolymph and renal tubules must have been actively taken up from the gut lumen. Control non-responsive PMMA microcapsules were easily identified in the lumen of the acidic midgut, demonstrating that they remain unaltered by the reduction in pH. In contrast, a band of soluble non-encapsulated fluorescence was observed in the midgut of the insect when fed the responsive P2VP microcapsules, indicating that the microcapsule contents had been released. Moreover, the renal (Malpighian) tubules were brightly fluorescent, in the case of the P2VP pH-responsive microcapsules, thus showing that the FITC-dextran had crossed the gut wall and transferred to the haemolymph before being excreted via the tubules. All these observations confirmed that the responsive microcapsules successfully dissolved in the midgut and released the FITC-dextran, which subsequently was taken up into the fly's body in a manner typical to other foods. Finally, comparison of the excrement showed striking differences between flies that were fed the control and responsive microcapsules, respectively. Control non-responsive PMMA microcapsules were seen clearly in the excrement and appear to be unaltered by the digestive juices of *D. suzukii*. In contrast, there were no intact pH-responsive P2VP microcapsules in the excrement. Instead fluorescence was seen evenly distributed throughout the faeces, confirming that they had been degraded during passage through the intestine releasing their soluble FITC-dextran cargo.

2.7 Conclusion

This study has developed and tested pH-responsive microcapsules suitable for the protection and delivery of water-soluble biopesticides. I have exploited the sharp change in pH from neutral to acid that occurs in the midgut of adult *D. suzukii* to trigger the release of a cargo in a region of the intestine critical for nutrient adsorption.[197,232,233] These microcapsules offer a smart technology to deliver water soluble control agents such as peptide hormones and peptide toxins that are normally unstable to the spotted wing drosophila, *D. suzukii*.

Chapter 3

Double-stranded RNA complexation with well-defined block copolymers for enhanced stability and uptake for use in pest control

3.1 Overview

This chapter will discuss a new method for protection and delivery of dsRNA as a bioinsecticide. The data presented here is taken from a manuscript due to be submitted to Biomacromolecules, titled *Double-stranded RNA complexation with well-defined block copolymers, enhancing stability and uptake for use in pest control*. Data from the supplementary and data not presented in the manuscript have been added to form this chapter.

This study details the synthesis of diblock copolymers which are used to complex dsRNA, protecting it from extracellular nucleases and enabling uptake (Figure 3.1). Diblock copolymers were designed to contain a complexing and water-soluble stabilising block and were synthesised using RAFT polymerisation. The effect of complexing diblock length and the effect of the charge ratio on dsRNA condensation was investigated. The lead diblock copolymer was then tested *in*

vivo with phenotypic observations of naked and complexed dsRNA in *D. suzukii* and a close relation but non-target species *D. melanogaster*.

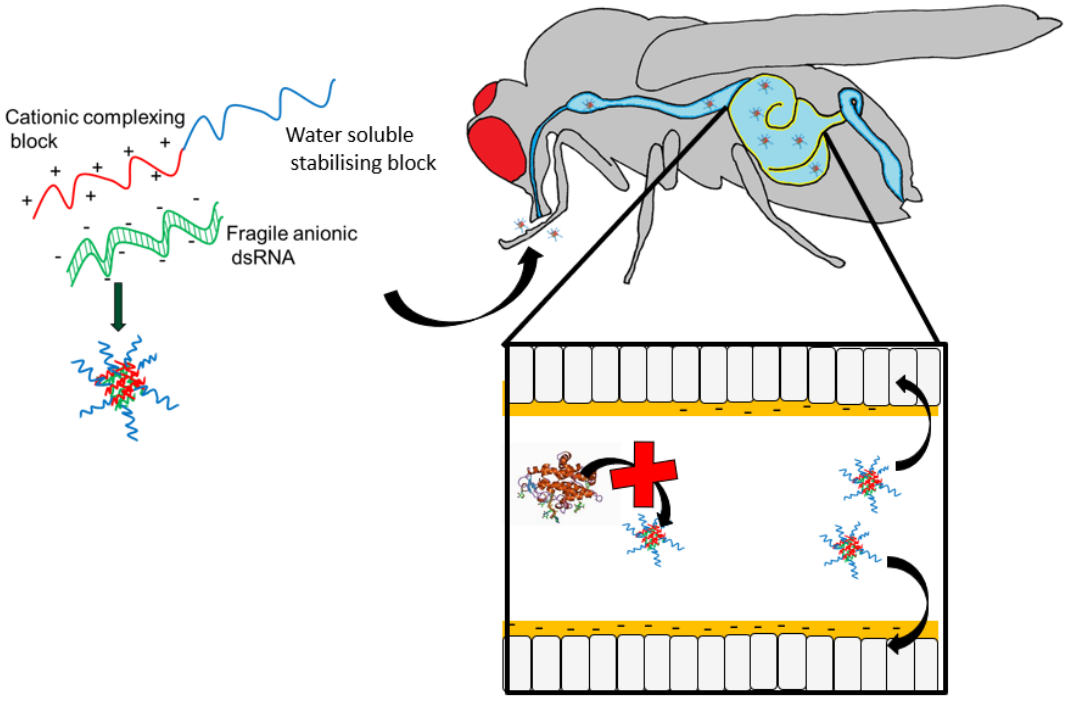


Figure 3.1: dsRNA delivery abstract demonstrating the complexation of dsRNA, protecting it from degradation and enabling uptake. Diblock copolymers were synthesised containing a water soluble stabilising block and a cationic complexing block. This cationic block can complex the anionic dsRNA to from a polyplex (dsRNA:polymer complex). This complexation retards the degradation of dsRNA by extra cellular nucleases in the insect's gut. Furthermore, through charge cancelling increased cellular uptake is induced into the gut cells.

3.2 Abstract

Safer alternatives to chemical pesticides are in great demand due to increased insect resistance to synthetic insecticides and the growing concern of their ecotoxicity. Biological alternatives such as double-stranded RNA (dsRNA) have the

potential to be used as a safe replacement because they can be used to target specific species without damaging beneficial insect populations. However, their use is hindered by their rapid degradation and poor uptake profile within an insect pest's body. For dsRNA-based solutions to be developed, there is a need to protect it during transport to the target species and to simultaneously facilitate delivery to the insects. For these purposes, the anionic nucleic acid can be complexed with a copolymer to prevent degradation and to enhance dsRNA uptake when ingested by the insects. In this study I have produced well-defined polyhydroxypropylacrylamide -*block*- poly2(dimethylamino) ethyl methacrylate (PHPMA -*b*- PDMAEMA) diblock copolymers using RAFT polymerisation, where the PDMAEMA block was subsequently quaternised thus, enabling complexation with the negative dsRNA. Here the water soluble PHPMA block acts as a steric stabiliser for the complexes formed. I have shown that varying the length of the complexing block, allows for the control of the dsRNA-polymer complex particle size and that varying the size of the complexing polymer block alters the complexation rate. Through *in vivo* testing I have shown that the dsRNA complexed outperformed naked dsRNA, where the naked dsRNA had no statistical impact on pest survival, while the complexed version had a 75 % increase in pest mortality. Furthermore, I have demonstrated that this method can specifically target one species, with no detrimental effects observed when the complexed dsRNA was fed to a close non-pest relation.

3.3 Introduction

As a result of the world's population expanding at an ever-increasing rate, there is a need for increased food production.[22] Crop protection strategies are one vital part of sustaining the necessary increase in food production and it is estimated that without the use of chemical pesticides global food production would be reduced by 35 - 40 %, leading to an increase in food costs and security.[20,21] However, our arsenal of effective insecticides is diminishing due to increasing pesticide resistance in insect species far exceeding the current rate of new pesticide discovery.[24,25] Furthermore, the environmental impact chemical insecticides have on beneficial insects such as honeybees[33] along with toxicity to mammals

including humans is of great concern.[34,240] Alternatives to chemical insecticides are thus in great demand, with the use of biological-based pesticides, which have increased selectivity and lower environmental toxicity in particular, hold great promise in providing a potential solution for this paramount challenge.

RNA interference (RNAi) is one such biopesticide alternative that has long been established as a powerful tool for reverse genetics through specific knockdown of genes in insects and observations of the phenotypic response.[39,241–243] RNAi typically involves specific mRNA cleavage that prevents protein expression in the targeted species. By targeting a specific protein that is vital to the insect's survival, it is possible to induce sufficient knockdown for insect death, which can be used to control the pest species population. Recently, the use of double-stranded RNA (dsRNA) mediated gene silencing has been explored for its potential as a new class of bioinsecticides.[25,197] Varying success has been achieved through the oral application of 'naked' dsRNA, where RNAi is only induced in a handful of species. Furthermore, large differences in the efficiency of the use of dsRNA has been observed between oral application and injection, where a species may have a strong RNAi response by injection but a weak response upon feeding. The poor efficiency encountered with feeding naked dsRNA has been attributed to two main factors: a) rapid degradation of the dsRNA in the gut of the pest due to the presence of extracellular nucleases[244] and b) poor penetration into the gut cells from the pest's intestine, due to repulsive electrostatic interactions.[25] It is believed, however, that the main barrier to successful RNAi is the rapid degradation of the ingested nucleic acid. dsRNA degradation occurs upon ingestion into the intestine of pest species where specific RNase nucleases target and rapidly degrade the nucleic acid.[63,197]

To combat the rapid degradation and poor gut cellular transfection of naked dsRNA in insect pests a protection and delivery mechanism may be used. Typically, cationic liposomes are used for increased transfection of dsRNA in many laboratories investigating different RNAi gene targets in pest species.[25,152] Synthetic and natural cationic homopolymers have recently been investigated for the complexation of dsRNA for enhanced protection and uptake for pest control.[156,161,245] The delivery of RNA has been extensively researched in the field of therapeutics where similar barriers to use are found. It is prudent, therefore,

to investigate these systems to gain a better understanding of what may be used to deliver dsRNA in pest control.

RNAi has been effectively used in therapeutics as a method for treating diseases arising from genetic defects, degenerative or viral sources. Short-interfering RNA (siRNA) is used in therapeutics as its shorter sequence produces fewer off target effects and does not invoke and immune response but it has a reduced stability as compared to the larger dsRNA used for pest. dsRNA, a precursor to siRNA, is indeed chosen as a biopesticide due to its increased stability compared to siRNA. Similarly to agrochemical use, degradation by endogenous enzymes and poor cellular uptake have hindered the implementation of RNAi in therapeutics. Therefore, the development of efficient delivery methods is a key challenge to enable siRNA protection and delivery to the cytoplasm of targeted cells within the patient.

A wide range of both viral and non-viral carriers have been produced to efficiently protect siRNA from degradation in the bloodstream and to deliver it to a specific target tissue of the patient.[246,247] I am interested in implementing similar systems utilised for therapeutics in a new field of pest control. Of particular interest is the stabilisation of RNA via condensation with cationic homopolymers, producing polyplexes/ interpolyelectrolyte complexes (IPECs), where degradation is significantly reduced. However, these cationic polyplexes tend to be polydisperse in size and often become bound to negative proteins in serum and are easily excreted, reducing the RNAi effect.[184,188,246] The addition of hydrophilic surface groups enables the control of polyplex size, due to steric stabilisation reducing the aggregation. Moreover, the addition of the hydrophilic surface groups on the polyplexes aids in the water solubility of the complexes, increasing retention time within the body increasing the uptake profile.[194] Due to the screening of the polyplex charge and stabilising corona upon the addition of a hydrophilic group prevents protein aggregation to the surface is reduced in serum.[194]

In this work, I design copolymers that are capable of complexing with the larger dsRNA to prevent degradation and enable delivery to the gut cells of an economically significant invasive pest, *Drosophila suzukii*. Here, I have used reversible addition-fragmentation chain transfer (RAFT) polymerisation to produce

well- defined diblock copolymers, which consist of a polyhydroxypropylacrylamide (PHPMA) block and a poly[2-(dimethylamino)]ethyl methacrylate (PDMAEMA) block. The PHPMA block, commonly used in therapeutics, provides solubility in water and steric stabilisation for the formed complexes.[189–191] I have investigated the effects of varying the length of the complexing block on complexation efficiency. I have also investigated the effect of charge ratio on complexation by varying the nitrogen to phosphorus ratio (N/P) for each of the diblocks. Finally, the ability of the diblock copolymer/dsRNA complex to deliver the biopesticide to the targeted species was investigated through *in vivo* studies, where I determined the increased population control efficacy of the dsRNA when complexed.

3.4 Materials and method

3.4.1 Materials

Amino-2-propanol (93 %), 4,4'-azobis(4-cyanovaleric acid) (ACVA, $\geq$ 75 %), methacryloyl chloride ($\geq$ 97.0 %), anhydrous dichloromethane ($\geq$ 99.8 %), 2-(dimethylamino)ethyl methacrylate (98 %), Isopropyl alcohol (IPA, $\geq$ 98 %), iodomethane (99.5 %), tetrahydrofuran ($\geq$ 99.9 %), agarose powder, Tris (hydroxymethyl) aminomethane (Tris, $\geq$ 99.8 %), ethylenediaminetetraacetic acid (EDTA, $\geq$ 99 %), dimethylformamide (DMF,$\geq$ 99.8 %), sodium chloride (NaCl, $\geq$ 99 %) and acetic acid ($\geq$ 99.5 %) were all purchased from Sigma Aldrich. 2-(2-Cyanoprop-2-yl)-S-dodecyltrithiocarbonate (CPDT, $>$ 98 %) was purchased from Strem chemicals. Ethidium bromide was purchased form Fischer Scientific. Gel red was purchased from Biotium. dsRNA ladder was purchased from New England Biolabs. dsRNA targeting the *vha26* subunit of the vATPase proton pump was synthesised by agroRNA (4.68 μg/μl), sequence specific to *D. suzukii* as previously reported.[61]

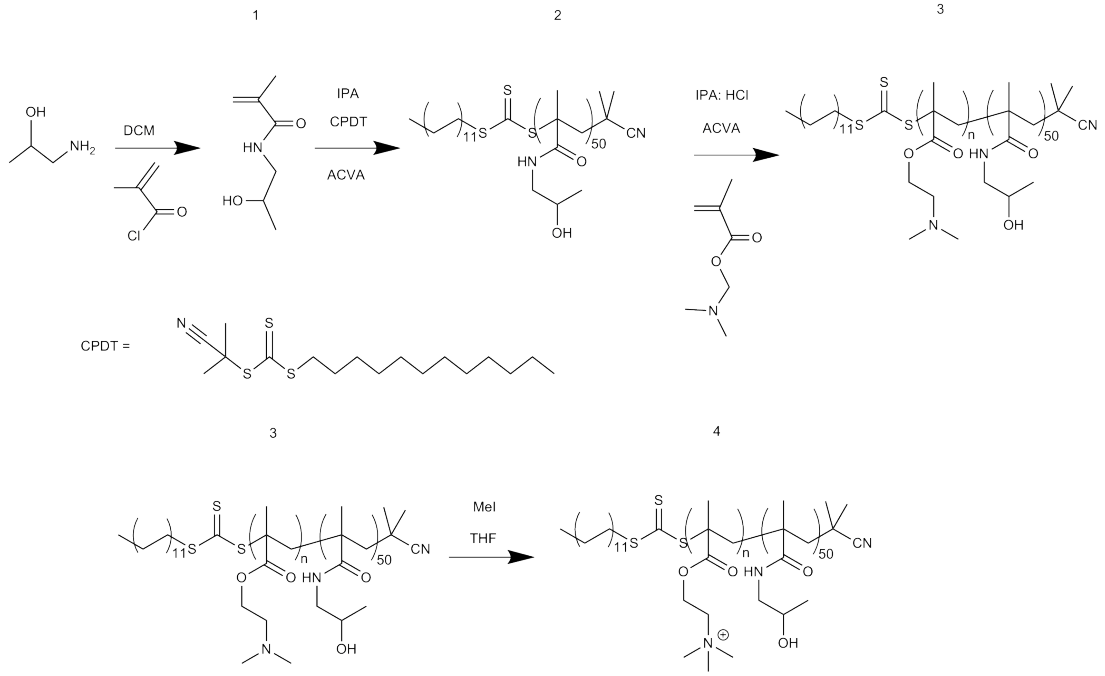
3.4.2 Insects

D. suzukii (an Italian strain) were maintained on a standard *Drosophila* diet (oatmeal, 7.5 %; molasses, 5 %; agar, 8.4 %; yeast, 8.4 %; methyl paraben, 0.35

% in water) at 25 °C in a 12:12 light-dark cycle. Mated Females and Males (5-10 days old) were used for *in vivo* testing.

3.4.3 Synthesis of PHPMA-*b*-PQDMAEMA for complexation with dsRNA

Diblock polymers of PHPMA-*b*-PQDMAEMA were synthesised by first producing a macroRAFT agent of PHPMA and subsequently adding a second block of PDMAEMA of various number of units. This diblock was then selectively quaternised to produce PHPMA-*b*-PQDMAEMA with varying lengths of complexing block (Scheme 3.1).



Scheme 3.1: Synthesis route for PHPMA-*b*-PDMAEMA by RAFT polymerisation and subsequent quaternisation.Forming PHPMA-*b*-PQDMAEMA with varying complexing block degrees of polymerisation.

Synthesis of PHPMA Macro RAFT (Compound 2)

Hydroxypropylmethacrylamide (Compound 1) was prepared as has been previously reported (^{1}H- NMR Figure A.1).[248] Kinetic studies were undertaken prior to the large-scale synthesis of Compound 2 to determine the likelihood of formation of dead polymer chains which limits the second chain extension step. 0.1 ml samples were taken every 30 min and quenched in DMF, each sample was analysed by SEC-GPC and ^{1}H-NMR relating the loss of vinyl peaks to calculate conversion.

In a typical synthesis the water soluble HPMA monomer (20 g, 140 mmol) was dissolved in 50 ml of IPA. CPDT (0.48 g, 1.39 mmol) RAFT agent was dissolved into the solution along with a thermal initiator ACVA (0.078 g, 0.28 mmol). The reaction mixture was degassed using N_2 for 30 min and subsequently heated to 70 °C for 2 h. The reaction was quenched by exposure to air and dilution into Milli-Q water and subsequently dialysed to remove unreacted monomer and impurities ($<$ 3,500 Mw). The purified solution was freeze dried to yield the PHPMA macroRAFT agent (Compound 2).

Synthesis of PHPMA-*b*-PDMAEMA (Compound 3)

PHPMA macroRAFT agent (Compound 2, 2 g, 0.252 mmol) was dissolved in 9.5 ml IPA and to this solution ACVA was then added (0.0141 g, 0.0504 mol). DMAEMA was added at varying concentrations, depending on the targeted Degree of polymerisation (Dp; Dp = 125, 4.75 g, 30.24 mmol. Dp = 500, 19.812 g, 126 mmol. Dp = 1000, 39.6 g, 252 mmol). The solution was then acidified through dropwise addition of concentrated 37 % HCl to pH 5.5. The solution was degassed using N_2 for 30 min and subsequently heated to 70 °C for 24 h to yield Compound 3. The conversion of DMAEMA was monitored by ^{1}H- NMR by observing the loss of the vinyl peaks and through comparison of the PHPMA macro to the PDMAEMA that was grown off it.

pH-titrations of PHPMA-*b*-PDMAEMA diblocks

pH-titrations of each of the diblock was undertaken by dissolving them in 1M HCl at 2 wt.%. The pH was monitored while adding either 0.1 or 0.01 M NaOH in 10

- 100 μl increments. The resulting data was plotted as moles of NaOH added vs pH. Differentiation of this allowed for the accurate determination of the points of inflection.

Quaternisation of PHPMA-*b*-PDMAEMA

$PHPMA_{53}$-*b*-$PDMAEMA_n$ (1 g, 1 equivalent, n = 120, 0.037 mmol. n = 475, 0.0121 mmol. n = 950, 0.00636 mmol) was dissolved in 40 ml THF, forming reverse micelles (observed by DLS). Iodomethane was added at varying concentrations depending on DMAEMA block length (2 equivalent per DMAEMA unit. n = 120, 1.27 g, 8.96 mmol. n = 475, 1.63 g 11.49 mmol. n = 950, 1.715 g, 12.08 mmol) and the resulting reaction mixture was stirred at room temperature for 4 h. The product was purified through multiple centrifugation and resuspension in pure THF followed by removal of residual THF by vacuum drying, yielding a brown solid.

Polymer characterisation

All polymers were analysed by gel permeation chromatography (GPC) to determine molecular weight and polydispersity. The eluent was DMF with LiBr (0.1 mol), and a flow rate of 1.0 ml/min was used. The sample was diluted to 0.5 mg/ml in DMF and filtered through 0.22 μm syringe filters (Sartorius Minisart RC, hydrophilic). 100 μl of sample was injected into the GPC system for analysis. The molecular weights were calibrated against poly(methyl methacrylate) standards.

^{1}H NMR spectrum of block copolymers was obtained by a Bruker 500 MHz NMR spectrometer. Chemical shifts for ^{1}H NMR spectra were reported relative to (2,2-dimethyl-2-silapentane- 5-sulfonate sodium salt) (DSS, δ 0.015 ppm) were MeOD was used as the solvent.

3.4.4 Agarose gel electrophoresis assay

The complexation of dsRNA with charged diblocks was determined by 2 % agarose gel electrophoresis. Known amounts of the synthesised block copolymers were dissolved in 1x TAE buffer and mixed with 1 μg dsRNA, yielding various charge

ratios based on number of nitrogen atoms of the polymer to phosphorus atoms of the dsRNA (N/P; 17 μl). The complexed dsRNA was left to incubate at room temperature for 48 h to ensure the complexation had completed. 3 μl of a gel loading dye which increases the solution density and makes the sample sink into the well was added to each polyplex solution, and 5 μl of the resulting polyplex solution was loaded on a 2 % gel containing 1 μg/ml of gelRed (intercalating dye that enables visualisation of nucleic acid). The samples were run against Mw standards in the form of a dsRNA ladder (2 μl). The samples were electrophoresed in 1x TAE buffer at 100 V for 30 min. The dsRNA bands were then visualized under a UV transilluminator at a wavelength of 365 nm and compared against dsRNA Mw standards.

3.4.5 Ethidium bromide exclusion assay

A standard ethidium bromide exclusion assay was used to determine the complexation of PHPMA-*b*-PQDMAEMA with dsRNA.[175] 1 μg of dsRNA and desired amounts of the polymer (to vary N/P ratios) in 99 μl of 1x TAE buffer were incubated at room temperature for 1 or 48 h to allow complexation. 1 μl of ethidium bromide (1 mg/ml) was added to the solution. The samples were analysed using a FLUOstar Omega (BMG LABTECH GmbH) with λex set at 320 nm and λem set at 600 nm. The fluorescence was normalised against samples of dsRNA and ethidium bromide with no polymer present, with no fluorescence observed in polymer only samples.

3.4.6 Transmission electron microscopy and energy-dispersive X-ray spectroscopy

Samples were prepared in a similar manner to the agarose gel electrophoresis samples, where the N/P ratio of 50 was chosen for all diblocks. A 2 μl aliquot of the sample was deposited onto a carbon-coated copper TEM grid and air dried for 2 min at room temperature. Grids were then negatively stained with uranyl acetate. Micrographs were acquired using a FEI Tecnai TF20 instrument operated at 200 kV. Energy-dispersive X-ray spectroscopy was undertaken on the polyplexes and compared to areas of the grids with no polyplexes present.

3.4.7 Polyplex charge determination

The electrophoretic mobility of the polyplexes with varying N/P ratios was measured using a Malvern Zetasizer ZS. Where solutions were made following the same procedure as for agarose gel electrophoresis in 1x TAE buffer (dielectric constant 77.876 & dynamic viscosity 0.981 mPas).[249]

3.4.8 *In vivo* testing of dsRNA protection and delivery

Third-instar *D. suzukii* larvae were starved for 3 h, on moist filter paper preventing desiccation, before initiation of feeding assay. RNAi was evaluated by placing the insects (5 larvae per replicate with a minimum of 4 replicates investigated) on standard *Drosophila* oatmeal diet containing either polymer, naked or complexed dsRNA at a dsRNA concentration of 0.67 mg/g of diet. The polymer concentration was tested at N/P = 50, either complexed or on their own. The complexation was undertaken in a similar manner as for the agarose gels and left to incubate for 30 min before adding it to the wet diet, creating a slurry. The insects were transferred onto the dsRNA containing diet and monitored for 2 weeks, allowing sufficient time for the larvae to pupate and then eclose to adult flies.

3.4.9 Statistical analysis

The significance of the differences in the survival of *D. suzukii* after ingestion of dsRNA compared to complexed dsRNA was analysed by a one-way ANOVA through comparison of means. A post-hoc Dunnett's test was performed to compare the means. Analysis was undertaken using Prism GraphPad.

3.5 Results and discussion

Herein, I describe for the first time the use of well-defined diblock copolymers, consisting of a complexing and a stabilising block, to complex dsRNA for bioinsecticidal use. This approach allows for the preparation of a size-controlled dsRNA carrier (through complexation of the RNA nucleic acid moieties with the positively charged PQDMAEMA block) that is stabilised sterically by the presence

of the second hydrophilic (PHPMA) block. The following sections describe the different steps conducted from the design and synthesis of the polymer to the *in vivo* testing of the dsRNA efficacy.

3.5.1 Design and synthesis of diblock polymers

PHPMA-*b*-PDMAEMA diblock copolymers were synthesised to complex dsRNA using RAFT polymerisation. The PDMAEMA block has previously been used extensively for the complexation of nucleic acids, typically for therapeutic applications.[191,195,247,250] In these cases, the general approach involved undertaking the complexation of the polymer with the RNA in acidic conditions where the tertiary amine becomes protonated. In the work here, I have quaternised the PDMAEMA block of the diblock copolymer to induce a permanent charge, thus ensuring complexation over a complete pH range.

Synthesis of PHPMA-*b*-PQDMAEMA with varying lengths of PQDMEMA complexing block was undertaken according to Scheme 3.1.PDMAEMA has extensively been used for siRNA complexation in the therapeutic industry, where the siRNA is 20-25 base pairs (bps) in length, whereas dsRNA in this study was 222 bps. To investigate the role of the PDMAEMA block on complexation I varied its length whilst keeping the length of the hydrophilic PHPMA block constant.

Initially, a PHPMA macro-Chain Transfer Agent (macroCTA) was produced in isopropyl alcohol using CPDT as the RAFT agent. The polymerisation of HPMA was first undertaken at 20 wt. % solids producing a polymer with low polydispersity and a 60 % conversion after 6 h (Figure 3.2 a). Analysis of the polymerisation by chromatography over time revealed multiple polymer peaks at high retention time during the early stages of polymerisation. These peaks correspond to monodisperse low molecular weight polymer and where observed by both the RI and UV detectors (Figure 3.2 e & f). RI detection revealed multiple small molecular weight polymer peaks that where found up to the solvent front and the UV detector confirmed the position of these low Mw peaks and showed that they continued past the solvent front. Due to the low polydispersity and the small Mw of these peaks I believe that we are seeing monomeric addition

to the polymer chain during the early stages of polymerisation. Indeed, due to the high retention time and proximity to the monomer peak in UV we may be observing monomer-CTA, the dimer, trimer peaks which eventually merge with increasing chain length. This is the first time such small Mw polymer peaks have been shown for the early stages of RAFT-polymerisation. This system may be used to gain a more fundamental understanding of the process by studying the relative intensities of these peaks over time and in differing environments.

An attempt to increase the polymerisation rate was made by running the reaction for a higher monomer content increasing from 20 to 50 wt. %, which resulted in a linear increase in conversion over 2.5 h to about 75 % as compared to a slightly lower conversion ($\sim$ 70 %) reached in 360 min for the lower monomer content case. This linear reaction growth rate was then followed by a plateau region showing non-linear chain extension and therefore loss of chain fidelity (Figure 3.2 a & c). To obtain a PHPMA macroCTA with full chain fidelity the reaction was therefore quenched before reaching this plateau after 2 h. The resulting PHPMA macroCTA possessed the following characteristic Mn = 9,500 g/mol; PDI = 1.14; Dp = 53. The synthesis of PHPMA macroCTA quenched before 2 h also lead to good blocking off but had high amounts of monomer wastage (Figure 3.3). PHPMA synthesis in IPA allows for the use of a broader range of RAFT agents as compared to similar synthesis under aqueous conditions,[251] thus allowing for a larger choice of monomers to be investigated for the second block addition. Furthermore, the rate of polymerisation appears to be much faster in IPA compared to other commonly used alcohols,[252] and allows for a higher conversion to be achieved whilst maintaining chain fidelity.

The PHPMA macroCTA was further used to prepare 3 PHPMA-*b*-PDMAEMA diblock copolymers with varying DMAEMA degree of polymerisation, namely 120, 475 and 950 (Table 3.1), as measured by ^{1}H-NMR (Figure A.3, Figure A.4 and Figure A.5). The presence of amines in a RAFT polymerisation process has been shown in the past to affect the control over the reaction, thus acidic conditions were used in the synthesis of the second block to ensure protonation of the DMAEMA monomer tertiary amine, which is known to mitigate this effect.[253] The Dp for each of the diblock copolymers was confirmed by ^{1}H-NMR with comparison of the DMAEMA monomer vinyl peaks and PDMAEMA peak. The

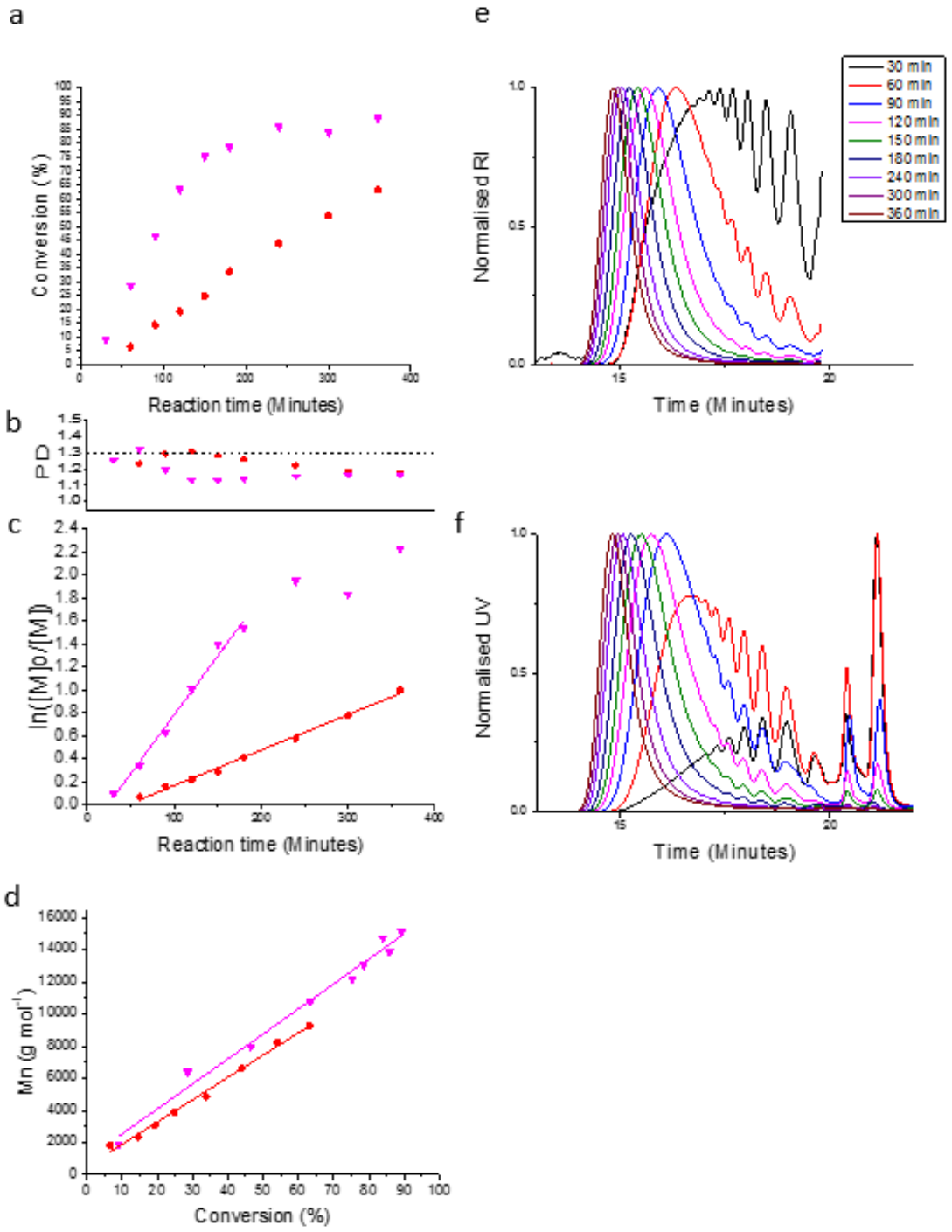


Figure 3.2: PHPMA macroCTA synthesis and kinetic studies. (a) Polymerisation of HPMA in IPA at differing solids content (20 wt. %, red & 50 wt. %, pink). (b) Polymer polydispersity over the reaction time. (c) First order kinetics of HPMA polymerisation over time. (d) Linear growth of the polymer chain as a function of ^{1}H- NMR conversion. SEC-GPC traces of HPMA polymerisation time in RI (e) and UV (f).

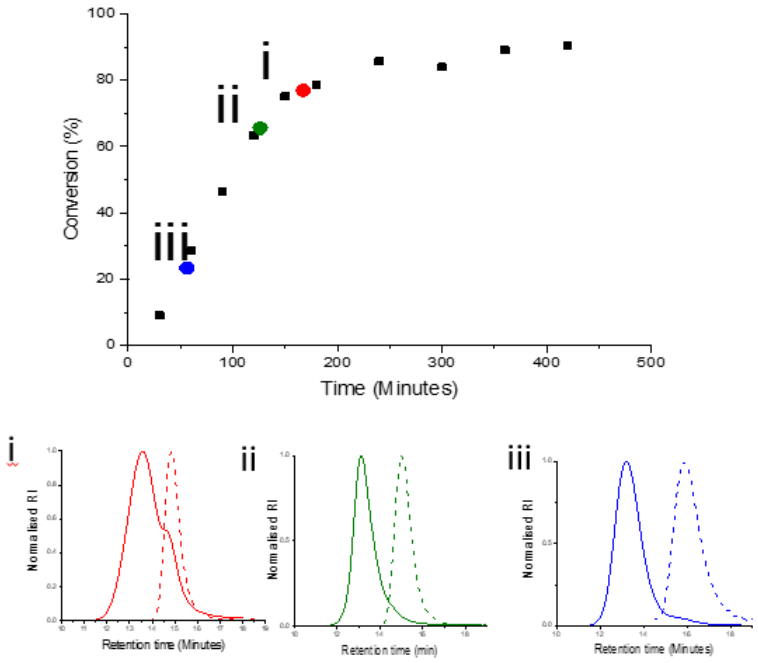
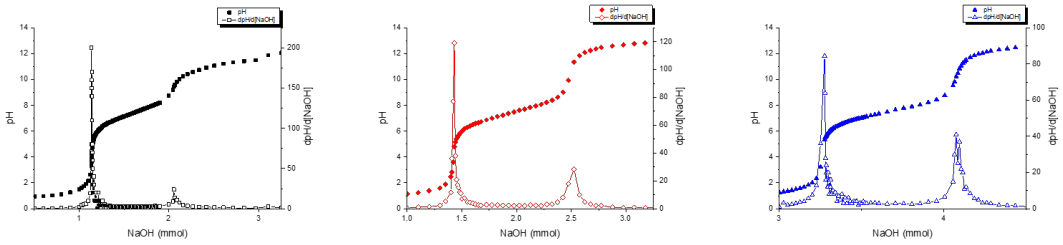


Figure 3.3: DMAEMA blocking off from PHPMA macroCTA's formed at different time points. (i) MacroCTA formed after 175 min polymerisation, dead chains were produced as shown by blocking off. (ii) MacroCTA formed after 125 min with good chain extension after blocking off. (iii) MacroCTA formed after 55 min with good chain extension after blocking off.

Table 3.1: Main characteristics of the synthesised PHPMA-*b*-PDMAEMA diblock copolymers subsequently used for complexation of dsRNA.

PHPMA (Dp)	PDMAEMA (Dp) (Dp)	Conversion by ^{1}H-NMR	Mn (g/mol)	PD (Mn/Mw)
53	0 (macroCTA)	53 %	9,500	1.14
53	120	96 %	19,175	1.11
53	475	95 %	41,054	1.19
53	950	95 %	70,634	1.35

conversion was also confirmed by comparison with the initial PHPMA macroCTA block, confirming the actual Dp for each polymer. The RAFT agent could not be detected in the diblock copolymer and was therefore not used to determine the Dp. Further characterisation was undertaken via pH titrations (Figure 3.4) to determine the pKa of the synthesised diblock copolymers. As expected, two clear jumps in pH were identified while adding base to solutions of the diblock copolymers, corresponding to the start and completion of the PDMAEMA block protonation, respectively.



(a) (b) (c)

Figure 3.4: pH-titrations of PHPMA-*b*-PDMAEMA diblocks and the differentiated sigmoidal graph.

To further ensure that the diblock copolymers can efficiently complex the anionic dsRNA, the PDMAEMA block was permanently quaternised through methylation, a permanent modification using a method described in the literature.[254] However, to minimise the participation of the free alcohol group on the water soluble PHPMA block in this reaction, the polymer was first dissolved in THF, where it forms reverse micelles consisting of a core of PHPMA blocks and a corona of PDMAEMA blocks that are significantly more soluble in the solvent. These conditions ensure that the availability of the PHPMA alcohol moieties to take part in the reaction is significantly reduced. ^{1}H-NMR was then used to characterise the conversion of the tertiary amine into a quaternary amine (Figure 3.5), which appeared to be complete. Because this method of quaternisation used here uses a stoichiometric reaction, it can be used to specifically control the charge density of the complexing block. However, in this report I

only consider the effect of maximum quaternisation while future publications will cover the effect of charge density on dsRNA complexation characteristics. From this point on, all diblock copolymers used are fully quaternised and refer to as PHPMA-*b*-PQDMAEMA.

3.5.2 Complexation of dsRNA with PHPMA-b-PQDMAEMA

With the successful synthesis of PHPMA-*b*-PQDMAEMA diblock copolymers with varying PQDMAEMA block lengths complexation of these polymers with dsRNA was investigated. The composition of the purchased dsRNA was designed to target a subunit of the vATPase proton pump, the most widely studied insecticidal RNAi target.[25,61] The invasive fruit fly pest *D. suzukii* was targeted due to its emergence in Europe and North America, where it is causing significant economic damage to the soft and stone fruit industries. For each of the diblock copolymers, a series of conditions for the complexation process were investigated in which the ratios of charges between the dsRNA and the PQDMAEMA block of the polymers were varied. Commonly, the charge ratio is represented as a ratio of nitrogens (the cationic species of the polymer) to phosphorus (anionic species on the dsRNA backbone), where varying the N/P ratio varies the charge ratio. By comparing results from the same charge ratios but for different polymers, one can specifically probe the effect of the diblock copolymer chain length on the complexation with dsRNA.

Transmission electron microscopy was used to investigate the size and the morphology of the polyplexes formed (Figure 3.6). The average diameter for the diblock:dsRNA polyplexes was: 950 block 142±16 nm (n = 60), 475 block 40±2 nm (n = 100), 120 block 39±2 nm (n = 100). The larger quantity of the PHPMA on the smaller diblock led to smaller complexes due to increased steric hindrance. There was a large difference in the particle sizes between the largest diblock and the other smaller diblocks. Previously it has been shown that using polyethyleneimine to complex nucleic acids the opposite trend is true, where the larger Mw branched polymers produced smaller polyplexes compares to low Mw alternatives.[170,174,175] The difference between the systems is the inclusion of a

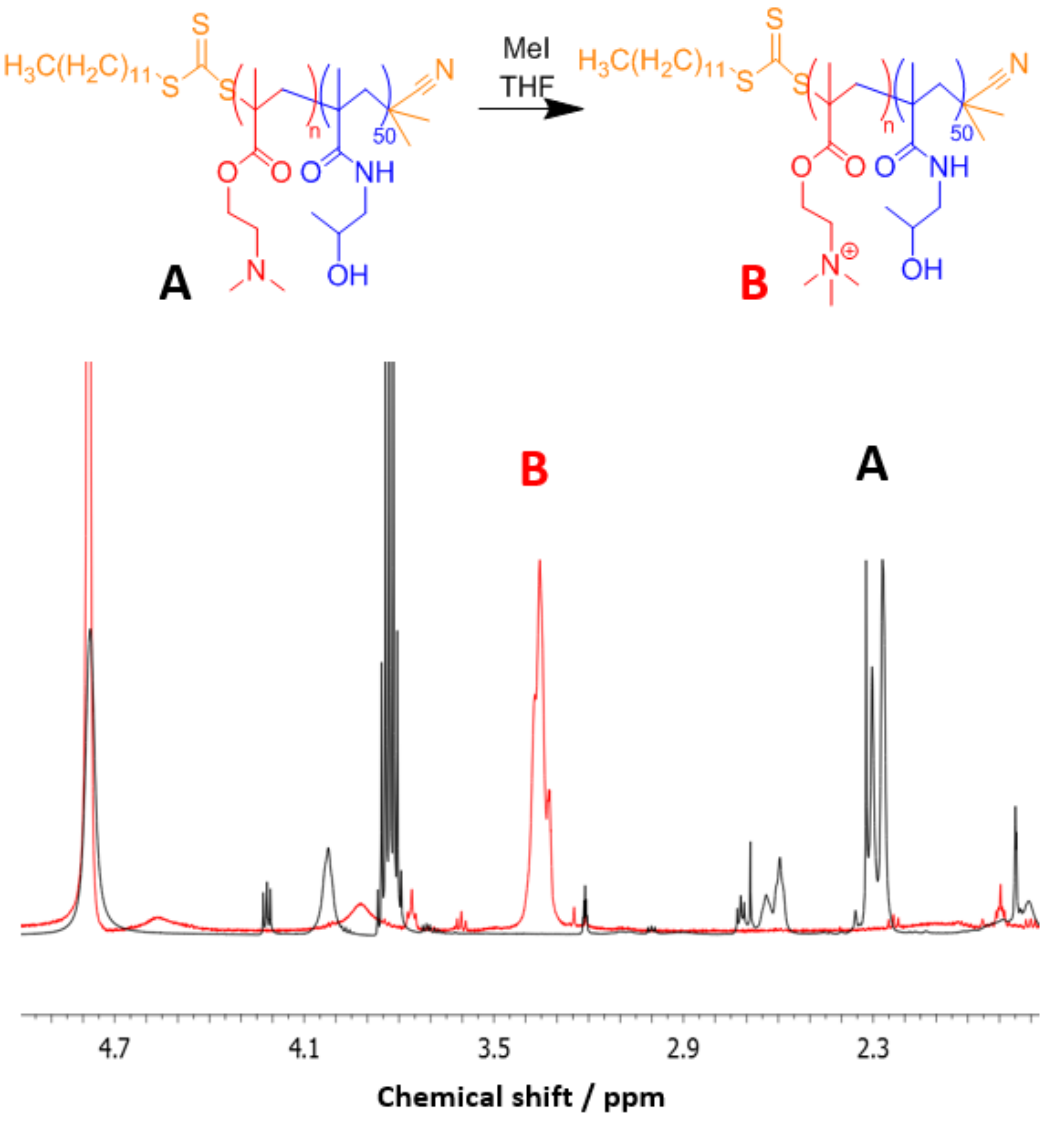


Figure 3.5: ^{1}H-NMR confirming the quaternisation of PHPMA-*b*-PDMEAMA (black) to PHPMA-*b*-PQDMEAMA (red).

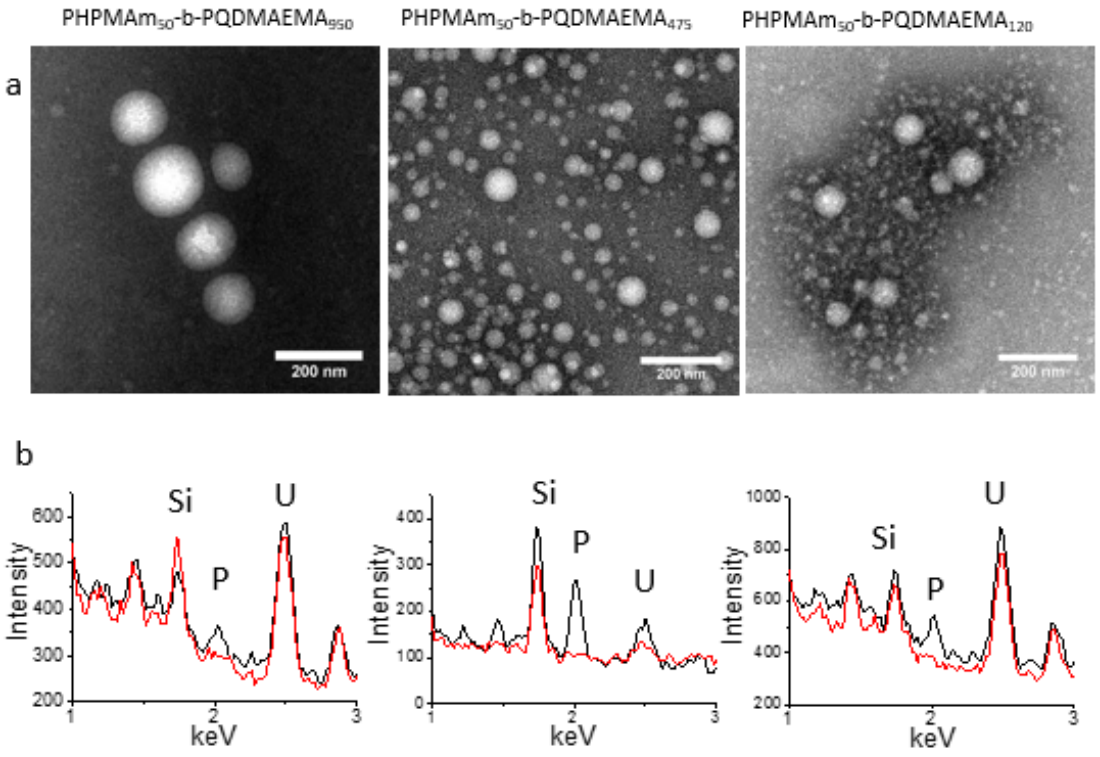


Figure 3.6: Transmission Electron Microscopy (a) and corresponding EDX analysis (b) of diblock copolymer/dsRNA polyplexes with differing complexing block lengths. A constant charge ratio of N/P of 50 was used in all samples. EDX analysis (b) shows a peak present for phosphorus in the polyplexes (black), which is absent form the background TEM carbon grid (red).

PHPMA stabilising block that controls the number of polymer chains that can aggregate together and therefore polyplex size.

I am only aware of one other attempt to complex dsRNA with a well-defined polymer for pesticide delivery applications. In this case the homopolymer poly[N-(3-guanidinopropyl)methacrylamide] (PGPMA) was used by Parsons *et al.* for delivery to the crop pest *Spodoptera frugiperda*.[161] Complexes formed from PGPMA and dsRNA were shown to have a hydrodynamic radius of 220 to 340 nm depending on pH, which is significantly larger than the complexes formed in the work presented here. Complexation of dsRNA with diblock copolymers has not yet been demonstrated for pest control, however, therapeutic siRNA (a shorter sequence than dsRNA) has been complexed with similar diblock copolymers. Gary *et al.* demonstrated that the complexation of siRNA with PEG-*b*-PDMAEMA diblock copolymers resulted in a polyplex with a diameter of 10 nm, significantly smaller than the complexes formed in this work.[184] The noted difference in the size of the complexes formed is likely due to the molecular weight of the RNA used in these studies as the dsRNA used in this study is about 10 times larger than siRNA.

The control of polyplex size is an important factor for efficient pest control as large complexes are less likely to be ingested by some pest species, where a filtering mechanism in the mouthparts prevents uptake of large particles greater than 500 nm in diameter.[176] Furthermore, the mechanism of uptake from the gut tract into the gut cells can be dependent on particle size with, for example, clathrin mediated endocytosis limited to less than 200 nm.[177]

In this study, to visualise the formed polyplexes, samples were negatively stained with uranyl acetate, the effectiveness of this stain differed between the samples with the longer polymer chains staining better than the shorter ones. Variation in the staining efficiency of the polyplex samples may be associated with the hydrophilicity of the samples, with the smallest diblock containing significantly more hydrophilic PHPMA blocks compared to the larger diblocks which are therefore harder to stain. The differences in the staining efficiency may also be due to the presence of free polymer in the system not involved in bonding the dsRNA. Indeed, through the investigation of the zeta potential of the polyplexes

as a function of N/P ratio the charge levelled off for each of the diblock polymers, with the smallest Mw diblock levelling off first (Figure 3.7).

When observing the polyplexes via TEM, well-defined spherical particles were evident with some apparent polydispersity. To confirm that the structures observed contained dsRNA energy-dispersive X-ray spectroscopy was undertaken to identify the presence of phosphorus from the dsRNA backbone. For each of the polyplexes the background intensity at 2 keV corresponding to the presence of phosphorus was low. This was verified by first taking a spectrum of an area without visible polyplexes (Figure 3.6). The presence of phosphorus in the polyplexes was then observed for each of the diblock polyplex samples confirming the complexation of the dsRNA. Due to the small concentrations of dsRNA used compared to polymer, elemental mapping could not be undertaken and therefore, aggregation numbers and dsRNA orientation cannot be obtained.

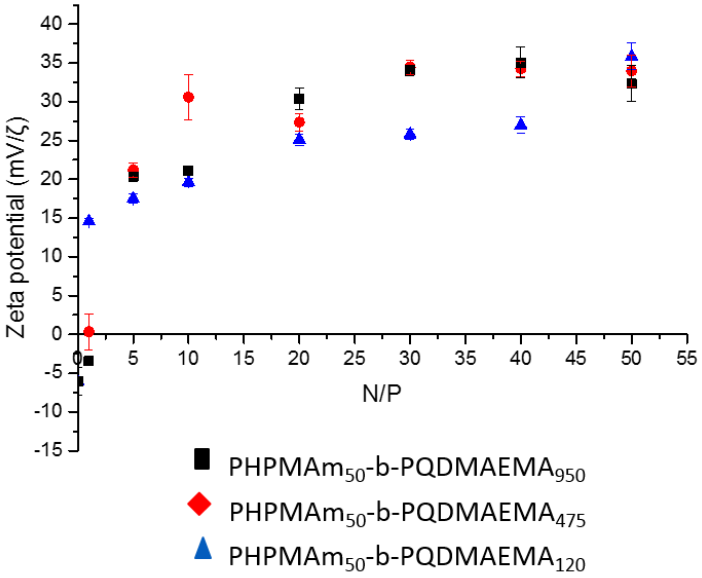


Figure 3.7: Analysis of PHPMA-*b*-PQDMAEMA:dsRNA complexation after 48 h at varying charge ratio for each of the three polymer diblocks. Average zeta potential (mean±s.e.m; n=30) of complexes with increasing polymer concentration, polyplexes become cationic.

For efficient uptake into the gut cells of the target pest the polyplexes must

travel beyond the anionic gut cell membrane. Therefore, it was important to understand the evolution of the net charge of the polyplexes as a function of the ratio of polymer to dsRNA used in the complexation process (Figure 3.7). As expected, these measurements demonstrated that the dsRNA was negatively charged, corresponding to the charge associated with the negative phosphorus backbone. Upon addition of each of the cationic diblocks a gradual charge reversal was observed with the largest initial increase in electrophoretic mobility measured for the smallest diblock copolymer.

The zeta potential for the $PQDMAEMA_{120}$ block increased to about 15 mV at N/P = 1 followed by a gradual increase in the zeta potential with increasing polymer concentration up to around 35 mV for N/P = 50. Less of an initial increase in zeta potential was observed for $PQDMAEMA_{475}$, increasing to around 0 mV at N/P = 1. The zeta potential subsequently jumped to around 35 mV by N/P = 10 with no further increase observed with increasing polymer concentration. For $PQDMAEMA_{950}$ the initial increase was lower than the other diblocks to about - 3 mV, followed by a plateau at N/P = 20, around 35mV. The charging of the polyplexes is due to excess cationic polymer associating with the dsRNA, where not all the PQDMAEMA block is complexed to dsRNA and instead extends away from the inner core into the corona. The levelling off of the polyplex charge for each of the diblock copolymers indicates that with increasing polymer concentration no more polymer chains can associate with the dsRNA, leaving free polymer in solution.

To investigate the presence of free dsRNA after the formation of polyplexes an agarose gel electrophoresis assay was undertaken. The complexations were left to incubate for 48 h at RT as gels performed after half an hour had free dsRNA at higher polymer concentrations (Figure 3.8). Whereas, gels performed after 48 h showed no change in free dsRNA. The time it takes to reach equilibrium is a function of polymer size with the smallest diblock copolymer complexing at a faster rate compared to the larger diblock copolymers. For $PQDMAEMA_{120}$ the dsRNA was partially retained at N/P of 1 and 5 and movement was fully retarded at polymer concentrations greater than this. At N/P = 1 we see that the dsRNA has not progressed as far down the gel compared to naked dsRNA suggesting that it has increased in mass or has decreased in charge. The increase

in dsRNA mass may be due to the block copolymer associating with the dsRNA but not fully complexing so it can still move down the gel but slower due to its increased size. For PQDMAEMA$_{475}$ the dsRNA was fully complexed at a N/P of 10 or greater, with less complexed at N/P = 5 compared to PQDMAEMA$_{120}$. For PQDMAEMA$_{950}$ the dsRNA was complexed at a N/P of 20 or greater, with a small quantity of the dsRNA visible at N/P = 10. The differences in the critical concentration for dsRNA complexation can been seen to be due to the size of the complexing PDMAEMA block. Smaller diblock copolymers may be able to entangle the long-ridged dsRNA more effectively than the larger diblocks. At lower polymer concentrations there was a bigger change in the zeta potential for the smaller block sizes supporting the idea that they complex better.

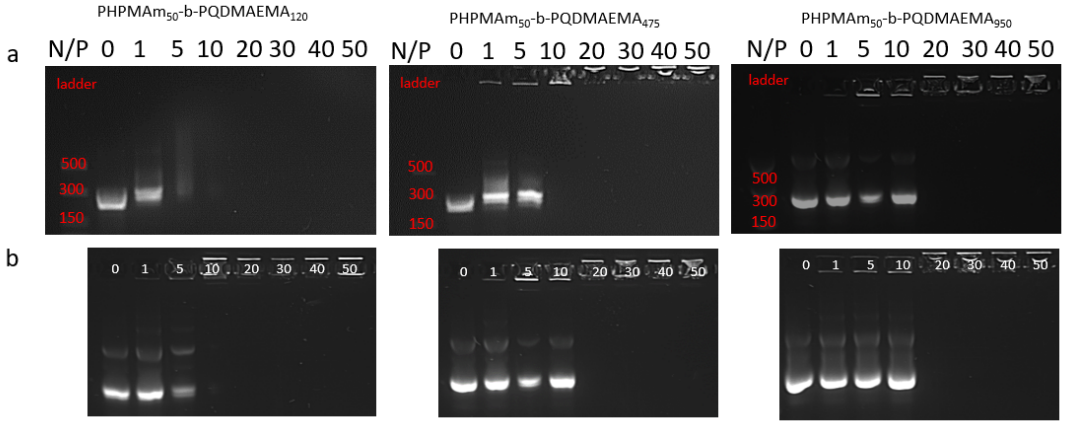


Figure 3.8: Gel electrophoresis of dsRNA complexed with PHPMA$_{53}$-*b*-PQDMAEMA$_{120}$, PHPMA$_{53}$-*b*-PQDMAEMA$_{475}$ and PHPMA$_{53}$-*b*-PQDMAEMA$_{950}$ after 48 h (a) and 30 min (b). Increasing polymer concentration form N/P = 0 - 50.

To further investigate the efficiency in the dsRNA complexation with each of the diblock copolymers an ethidium bromide exclusion assay was undertaken. Once the complexes were formed, ethidium bromide was added to the system and the resulting intercalation of dsRNA was measured via fluorescence. Initially a control experiment was carried out to demonstrate that quaternisation of the diblock copolymer is necessary for complexation with the dsRNA to occur. The

experiment showed that upon mixing addition of ethidium bromide to a solution of both the dsRNA and the non-charged polymer, intercalation of the ethidium bromide with dsRNA was not prevented, thus demonstrating that no complexation occurred between the uncharged polymer and the dsRNA (Figure 3.9a). This phenomenon was observed for all three diblock copolymers. Subsequently, the assay was carried out in the same conditions using the quaternised charged diblock copolymers and a large decrease in fluorescence as compared to experiments using naked dsRNA only was observed. After a 1 h incubation period differences in the fluorescence at N/P equal to or greater than 5 were observed between the largest block and the two smaller diblock copolymers(Figure 3.9b). At a N/P of 5 or greater each of the diblock copolymers behave in a similar manner after 48 h incubation with a 60 % reduction in fluorescence (Figure 3.9c). No difference was observed for the two smaller diblocks between 1 h and 48 h incubation times. However, a reduction in fluorescence was observed for the largest diblock suggesting that it took longer to complex the dsRNA.

At N/P = 1 after 48 h differences were observed between the diblocks with a greater reduction in fluorescence for the smallest diblock copolymer. This highlights the ability of the smallest diblock copolymer to complex the nucleic acid with a higher efficiency compared to the other diblock copolymers at low polymer concentration. The plateau of fluorescence at a N/P of 5 suggests that around 40 % of the dsRNA in the system is still available for intercalation. This may be free dsRNA not contained within the polyplexes, although no free dsRNA was detected in gel electrophoresis at these polymer concentrations. It is more likely that the ethidium bromide can penetrate through the polyplex corona and intercalate with dsRNA on the surface or within the polyplex. No differences between the diblocks were found at high N/P ratios, demonstrating that the PHPMA chains do not prevent the small ethidium bromide from getting to the dsRNA. However, differences may be observed in protection from larger molecules such as degrading enzymes, which are much bulkier and may be hindered by the PHPMA blocks.

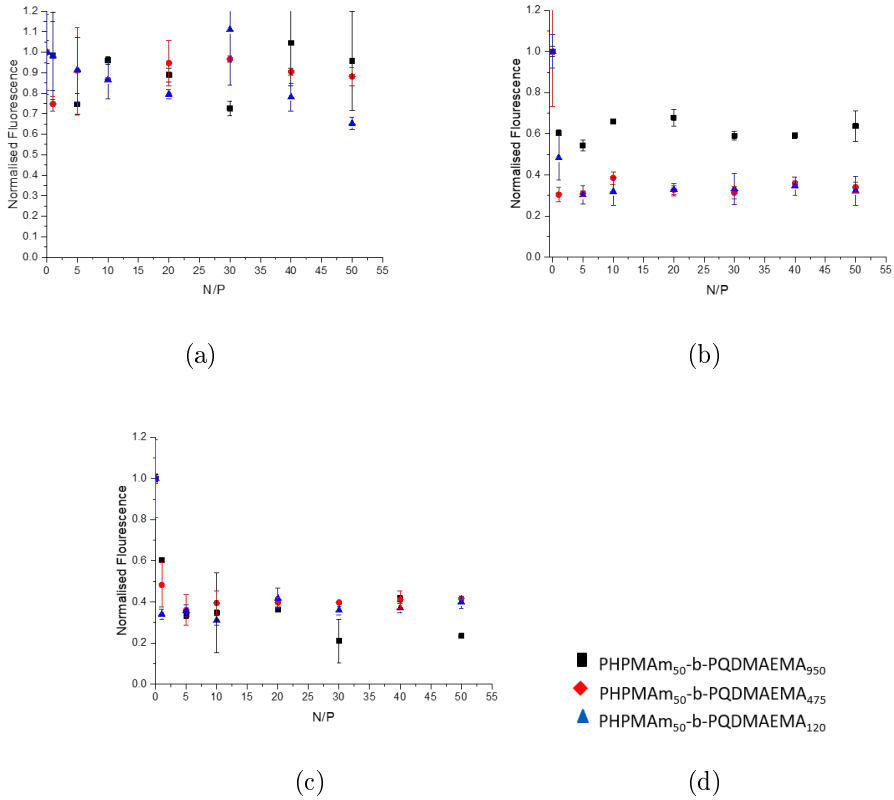


Figure 3.9: Ethidium bromide exclusion assay showing free dsRNA for each of the diblocks. (a) Uncharged polymer shows no complexing with dsRNA. (b) Charged polymer and dsRNA after 1 h incubation. (c) Charged polymer and dsRNA after 48 h incubation.

3.5.3 Enhanced control of *D. suzukii* with dsRNA PHPMA$_{53}$-*b*-PQDMAEMA$_{120}$ polyplexes

Following the successful polymer synthesis and subsequent formation of dsRNA polyplexes, the complexes ability to stabilise and deliver the dsRNA was tested. An *in vivo* feeding bioassay was undertaken to ascertain the effectiveness of this route. Observations were made on the development of *D. suzukii* from a third-instar larval stage through to the pupation of adults. The Vha26 subunit of the vATPase proton pump was selected as the targeted gene as it has been studied by many laboratories. Previously, dsRNA targeting this tissue had been shown to work well when injected into the pest. However, success through oral application feeding 'naked' dsRNA has been limited to only a few species. Specifically, for *D. suzukii* naked dsRNA fed to adults and larvae had no significant effect on the fitness or development.[61] Through the complexation of the dsRNA with a common liposomal transfection agent (lipofectamine 2000) 40 % mortality was observed in larvae. This method of delivery is unfortunately unsuitable for wide-scale use due to the number of processing steps required and the high cost of the transfection agent. Furthermore, liposomal based delivery system is unsuitable for field application of dsRNA for pest control as foliar application leads to drying of the delivery vector on the plant surface, resulting in a collapse of the liposome and breakdown of the carrier. To ascertain the enhanced protection and delivery of complexed dsRNA compared to naked dsRNA *in vivo* testing was undertaken feeding one of the diblock copolymers (PHPMA$_{53}$-*b*-PQDMAEMA$_{120}$) and dsRNA to the target pest *D. suzukii*. The smallest Mw diblock was selected as it showed a better ability to complex the dsRNA compared to the larger diblocks. The pest has poor success rates for development from larvae to adults with $\sim$ 20 % (n = 8) of larvae tested failing to eclose to fully formed adults. Upon feeding naked dsRNA (n = 8) to the larvae it was found that there was no statistical difference between this and the control (p = 0.9668), similar to previous studies.[61] There was no significant effect of the polymer on survival when fed to L3 larvae (n = 8) compared to the control (p > 0.9999). Upon complexation of the dsRNA with PHPMA$_{50}$-*b*-PQDMAEMA$_{120}$ the mortality of the larvae rose to 70 % (n = 12, p = 0.0002). The larvae fed on the complexed dsRNA appeared

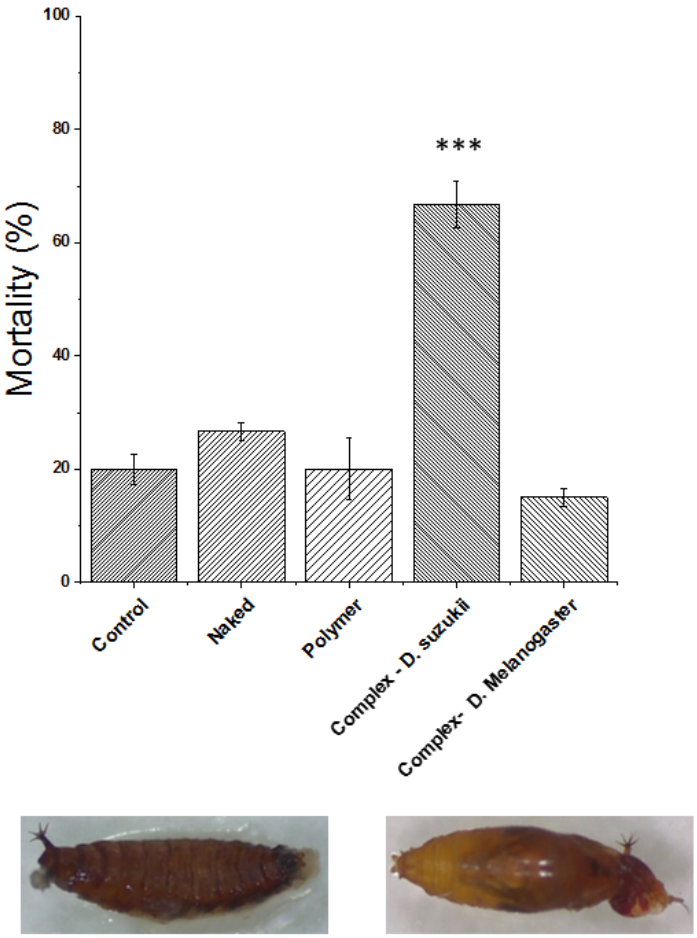


Figure 3.10: *In vivo* study on the effect of the complexes on survival of the target pest *D. suzukii* and a non-target fruit fly and close relation *D. melanogaster*. Complexed dsRNA had a significant effect on the survival of *D. suzukii* ($p = 0.0002$) but not on the control species *D. melanogaster* ($p > 0.9999$). Naked dsRNA or the polymer on its own did not have a significant effect on the survival of the insect ($p = 0.9668$ and $p > 0.9999$ respectively). OM images of malformed larvae and an attempted eclosion of *D. suzukii* after feeding on complexed dsRNA.

to be malformed or failed to eclose properly from their pupal case (Figure 3.10). This indicates that during the transition to pupae and formation of the adult fly there was something limiting their development, prior to the larvae transitioning to the pupal stage they undertake a large increase in feeding, building up an energy store. With significant knockdown of the *Vha26* subunit the pest can no longer process food properly, due to the reduced acidity and poor breakdown of the food in the midgut. This leads to reduced food storage upon pupation, resulting in poor development or failure to eclose form the pupal case. Feeding of the species-specific dsRNA complexed with $PHPMA_{53}$-*b*-$PQDMAEMA_{120}$ to a non-target but close relation *D. melanogaster* had no effect ($n = 4$, $p > 0.9999$). Demonstrating the selectivity of this method to control only the targeted species, whilst causing no damage to a closely related species, producing a more environmentally friendly solution. However, these phenotypic observations do not confirm enhanced RNAi and mRNA expression levels of the target gene would be needed to confirm that the observations are due to enhanced RNAi.

3.6 Conclusion

RNAi technologies have the potential to revolutionise the agrochemical industry for use as environmentally safe biopesticides. Here I have reported, diblock copolymers consisting of a hydrophilic stabilising block and a cationic complexing block that can be used to complex dsRNA for use as a novel biopesticide. Varying polymer molecular weights and charge ratios have been explored for the complexation of dsRNA, looking at polyplex size, charge and efficiency of complexation. Moreover, the ability for such complexes to increase mortality in an economically important pest was investigated compared to naked dsRNA. Increasing the molecular weight of the complexing block led to an increase in the size of the polyplexes produced and led to poorer dsRNA complexation at low polymer concentrations. *In vivo* testing of dsRNA complexes specific to *D. suzukii* led to increased mortality compared to naked dsRNA, which had no significant effect on the pest. This shows the feasibility to employ diblock copolymers to complex insecticidal dsRNA, providing a significant increase in control. Further work will

investigate if the increased control is through enhanced RNAi and look to gain a better understanding of the protection provided.

Chapter 4

Locomotor activity of *D. suzukii* under simulated natural conditions: to develop smart pest management strategies

4.1 Overview

This results chapter is more entomological, considering the locomotor behaviour of *D. suzukii*, and is based on two manuscripts either published or under review. It is important for efficient control of *D. suzukii* that the movement of the pest during the 24 h day is understood to help inform the timing of application of crop protection agents and to predict the likely dispersal of the pest. Therefore, the locomotor behaviour of *D. suzukii* was investigated under simulated environmental conditions, where the temperature and light profiles are altered under constant humidities. Environmental data collected from orchards in Northern Italy during the growing season of 2014 and 2015 was used, when *D. suzukii* pressure was high causing great economic damage. Similarities have been drawn with *D. melanogaster*, a close relation and model non-pest species, to better explain the observations made.

Initially, the circadian rhythms of the pest and how changes in mating affect behaviour were investigated and have been reported in the paper titled "*The sex-*

ually dimorphic behaviour of adult Drosophila suzukii: elevated female locomotor activity and loss of siesta is a post-mating response", published in the Journal of Experimental Biology.[255] Included in this publication is work conducted by Dr. Neil Audsley (Fera Sciences Ltd.) whom undertook some mass spectrometry to demonstrate the transfer of a sex peptide during copulation. For completeness, this data has been included in this chapter and the relevant sections have been acknowledged in the text.

Second, the effect of temperature on ecology and dispersal of *D. suzukii* was investigated to determine areas under threat from invasion of the pest which are reported in a paper titled "*Climatic stress dictates the dispersion of Drosophila suzukii*" for submission to the Journal of Experimental Biology. Due to the close relation between these two papers the data have been combined to form this insect behaviour chapter.

4.2 Introduction

The spotted wing drosophila, *Drosophila suzukii*, is an Asiatic pest that has recently attracted much attention with its emergence in North America, Europe and more recently South America as a major pest of berry and stone fruit.[1–7] Unlike other fruit flies, *D. suzukii* lays eggs in ripening fruit, causing extensive damage leading to serious economic losses.[1,2] This is facilitated by a modified ovipositor, which is larger and sharper than in other fruit flies and well adapted for puncturing the skin of ripening fruit during egg deposition.[8] The speed at which *D. suzukii* populations have invaded and become established in Europe and North America is evidence of successful physiological and behavioural adaptation to new climatic environments either after passive dispersal through fruit imports or by adult migration.[4,9] In Europe *D. suzukii* may be found in hot Mediterranean climates of Italy or Southern France, but it is also devastating crops in cooler regions of Scandinavia and the United Kingdom.[256] However, there are some regions in which *D. suzukii* is not established, typically in dry hot climates such as central Spain.[257] The success and severity of the economic damage inflicted on the fruit industry has recently focused attention on various aspects of the biology of this pest, which might inform the development of effective control strategies.

Specifically, it is important to consider both the dispersal of the pest, its daily activity profile and its chronobiology to develop efficient low pesticide control procedures.

The chronobiology of *D. suzukii* is of particular interest as it is known that insects can display circadian variability in their susceptibility to chemical insecticides.[258–260] It has been shown for both *D. suzukii* and the related *D. melanogaster* that levels of enzymes involved in insecticide metabolism fluctuate with a daily rhythm, suggesting that variation in rates of metabolism during the day can influence toxicity to fruit flies.[261,262] A biological clock might also influence the temporal efficacy of an insecticide by controlling rhythmic locomotor activity of an insect pest, which might determine the timing and extent of contact between the insect and a sprayed surface.

Dispersal of a pest species and the successfulness of invasion is controlled by three main environmental factors temperature, humidity and winter severity.[263] In the summer insects such as *D. suzukii* are prone to heat stress and desiccation due to their small body size and must adapt to overcome water loss and thermal stress as weather conditions change. Therefore, it is important to investigate the effect of these climatic factors on *D. suzukii's* development, fecundity and survival, to determine likely geographic regions under threat. Numerous studies have been conducted into the overwintering of *D. suzukii* and its thermal tolerance under cold conditions.[263–265] The developmental rate of *D. suzukii* as a function of both low and high temperatures has also been investigated by a number of different groups, with an upper limit for development being $\sim$ 31 °C.[257,263,266] Whereas, for *D. melanogaster*, a phylogenetically close relation, the upper limit for development is around 33 °C two degrees higher.[267] The effect of temperature on the survival of *D. suzukii* has been investigated by several authors concentrating on survival at low constant temperatures.[257,263,266,268] Only recently have studies considered the effects of constant elevated temperatures on *D. suzukii* survival, where it was found that at elevated constant temperatures males tend to have a lower thermal tolerance than females.[269] These studies consider the effect of constant temperature on *D. suzukii*, however, in the field under natural conditions the pest is exposed to fluctuations in both temperature, light and humidity levels within the 24 h day.

Locomotor activity has previously been used to investigate the impact of environmental conditions along with behavioural changes induced by mating for a number of different species, including *D. melanogaster*.[270] Historically, studies into locomotor behaviour have been undertaken using a 12 h light:12 h dark cycle and constant temperature and humidity. For *D. melanogaster*, a classical bimodal distribution of activity is observed with peaks centred around lights on and off.[271] Recently with the advent of more sophisticated incubators, simulation of semi-natural conditions can be undertaken, where both the light intensity and temperature can be altered incrementally to simulate natural conditions. These studies led to the discovery of an unexpected peak of activity for *D. melanogaster*, known as the afternoon peak.[272,273] This effect of the elevated temperature has been dubbed a stress/escape response, where it is proposed that the flies increase their locomotor activity when the temperature exceeds a threshold in order to move to cooler regions, reducing the risk of heat stress and desiccation.[272] It was found through the use of *D. melanogaster* mutants that this afternoon peak of activity is not just a simple escape response, but is an environmentally modulated circadian phenotype.[272,274]

In contrast to the extensive study of locomotor rhythms in *D. melanogaster*, there has only been one report (prior to our work) describing the daily pattern of locomotor activity of *D. suzukii*.[262] This study looked at the ambulation of virgin adults in a changing environment that approximated to local "summer" and "winter" conditions in North America. It showed that most of the activity of male and female flies occurred during daylight hours. Both sexes displayed peaks of activity at dusk, which also extended into the period of total darkness. Over 24 h males appeared to be more active than females.

In this chapter, the locomotor activity of adult *D. suzukii* is reported in several different light/dark and temperature cycles for comparison with previous studies on *D. melanogaster* and the work of Hamby *et al.* on *D. suzukii*.[262,272,274] Two main questions were addressed in this study: firstly, do mated female *D. suzukii* display higher levels of activity compared to males and virgin females when monitored in semi-natural and simulated natural environments as was found for *D. melanogaster*?[270] Secondly, does *D. suzukii* elicit an afternoon peak of activity when the temperature reaches above 30 °C and what is the impact of this on fly

survival. Through comparison with *D. melanogaster* the differences in temperature preference was investigated to better understand likely dispersal behaviour of *D. suzukii*.

4.3 Materials and methods

4.3.1 Insects

Drosophila suzukii (an Italian strain) and *D. melanogaster* (CantonS) were maintained on a standard *Drosophila* diet (oatmeal, 7.5 %; molasses, 5 %; agar, 8.4 %; yeast, 8.4 %; methyl paraben, 0.35 % in water) at 25 °C in a 12LD cycle. Insects were sexed as newly ($\leq$ 4 h) eclosed adults from stock bottles.

4.3.2 Locomotor activity analysis

Effect of mating status

Virgin adults (3-4 days old) were placed in small groups of 10 or 20 and either single sex or equal numbers of males and females in vials (80 mm x 20 mm) containing standard oatmeal/molasses/agar diet. After three days, flies in the mixed sex vials were separated into mated males and mated females. Both virgin and mated flies were then lightly anaesthetised using CO_2 and placed in unisex groups of five in glass vials (100 mm x 16 mm) plugged at one end with 2 % agar containing 5 % sucrose and at the other end with cotton wool. Tubes were placed in an activity monitor (LAM16 Locomotor Activity Monitor, Trikinetics Inc. Waltham, U.S.A.) that uses infrared beams to detect movement as flies move along the glass tube. The number of beam breaks occurring in 5 min time-bins was recorded for each tube and the data was processed using the DAMFileScan application (Trikinetics Inc., Waltham, U.S.A.) and Microsoft Excel, where each replicate (n) corresponds to one tube containing 5 flies. Flies were allowed left in the tubes under the simulated conditions for at least 24 h prior to the start of the experiment allowing them to become entrained in the environmental conditions. Longer entrainment times could not be used due to the short period of action of sex peptide

At the end of some experiments individual mated females were allowed to lay eggs on oatmeal/molasses/agar diet revealing that $>$ 90 % of the females laid fertile eggs and had therefore successfully mated. Quiescence, defined as 5 min time bins with zero beam breaks, was analysed using the BeFly sleep analysis tool provided by Dr Ed Green, University of Leicester. Experiments were conducted using a Sanyo incubator (MIR-253) with a daylight fluorescent tube (Aquardaylight from Aqualine, Barkerfield Rd. Bradford, U.K.) as the light source controlled in an on-off manner by a programmable electronic timer. For studies in semi-natural light conditions, monitors were placed in a secure room (North Yorkshire, U.K., latitude, 54.0582N and longitude, 0.7976W) with illumination by natural light from an east facing sky-light. The weather was a mixture of bright sunlight and fast-moving clouds. For a laboratory simulated natural environment, a controlled programmable environment incubator (Memmert HPP 110, Camlab, Cambridge, U.K.) was used with variable LED illumination and humidity set at 75 % to simulate the Northern Italian weather (provided by Dr. Nicola Mori, University of Padova). The programmed variation of temperature and light levels was confirmed using a DEnM *Drosophila* Environment Monitor (Trikinetics Inc.).

Effect of elevated temperature

Virgin adults (3-4 days old) were placed in small groups of 10 or 20 of either single sex or equal numbers of males and females in vials (80 mm x 20 mm) containing standard oatmeal/molasses/agar diet for three days. After three days, "mated" males in mixed sex vials were separated from females. Activity was monitored using identical set up to the investigation of the effect of mating status. The number of beam breaks occurring in 1 min or h time-bins were recorded for each tube and the data was processed using the DAMFileScan application (Trikinetics Inc.) and Microsoft Excel, where each replicate (n) corresponds to one tube containing 5 flies. No differences were observed between virgin or mated males, so their results were combined. Experiments were conducted using a Memmert HPP 110 incubator (Camlab) where a natural Italian environment was simulated, through the use variable LED illumination and temperature with the humidity

set at a constant 75 % RH. The programmed changes in temperature and light levels was confirmed using a DEnM *Drosophila* Environment Monitor (Trikinetics Inc.).

4.3.3 Peptide extraction (performed by Dr. N Audsley Fera Science LTD.)

Peptides were extracted from male accessory gland and the female uterus by placing five dissected tissues into 10 μl of ice-cold acidic methanol (87 % methanol, 5 % glacial acetic acid). The extraction medium was removed after centrifugation (4 °C, 12,000 g for 20 min) and the supernatant was stored at -20 °C until required for analysis. Females were separated from males after 10 min of copulatory activity and dissected immediately to provide inseminated uteri.

4.3.4 Mass spectrometry (performed by Dr. N Audsley Fera Science LTD.)

Mass spectra were acquired using a Voyager DE STR Matrix assisted laser desorption ionisation mass spectrometer (MALDI - TOF MS) (Applied Biosystems, Warrington, UK) in linear positive mode, using a 20 kV acceleration voltage, over the mass range m/z 1,000 - 10,000 Daltons. Samples were mixed 1μl:1μl with sinapinic acid (10 mg/ml in 30 % acetonitrile 0.1 % trifluoroacetic acid), 1 μl was then pipetted onto a MALDI sample plate and air dried. External calibration was conducted using a calibration mixture (Applied Biosystems) containing angiotensin I, adrenocorticotropic hormone (clip 1-17, 18-39 and 7-38) and bovine insulin. Results are the mean of three independent MS measurements for each sample and all masses are shown as average masses $[M+H]^+$.

4.3.5 Temperature preference determination

A temperature preference chamber was made from a solid aluminium bar (240 mm x 60 mm x 10 mm) which was coated in moist filter paper and was topped with glass. A temperature gradient was established in the chamber ranging from 10 - 34 °C (over 1 cm increments), by placing a hotplate at one end and cooling

the other with ice, replicating previous studies.[275] The temperature was allowed to equilibrate for 1 h before experiments were undertaken. The temperature was monitored after before and after each test using a non-contact digital thermometer (Stanley STHT0-77365).

Twenty five mated adults of mixed sex (*D. suzukii* or *D. melanogaster* Cs, 6 - 8 days old) were lightly anaesthetised using CO_2 and randomly placed along the length of the temperature preference chamber. They were allowed to equilibrate for 30 min, with any flies that failed to move discounted from the experiment. Flies were then counted in each of the different temperature sections (∼1 cm increments) in 1 °C bins.

4.3.6 Survival at elevated temperature

The survival of both *D. suzukii* and *D. melanogaster* males was determined at elevated temperature, using a method previously described,[276] using activity monitors (LAM16 Locomotor Activity Monitor, Trikinetics Inc.) to detect the fly movement as they moved along the glass tube. Experiments were conducted using a Memmert HPP 110 incubator (Camlab) where light levels were set to constant 500 lux, temperature was set to either 32 or 34 °C and with the humidity set at 75 % RH. The programmed temperature and light levels was confirmed using a DEnM *Drosophila* Environment Monitor (Trikinetics Inc.).

4.4 Results

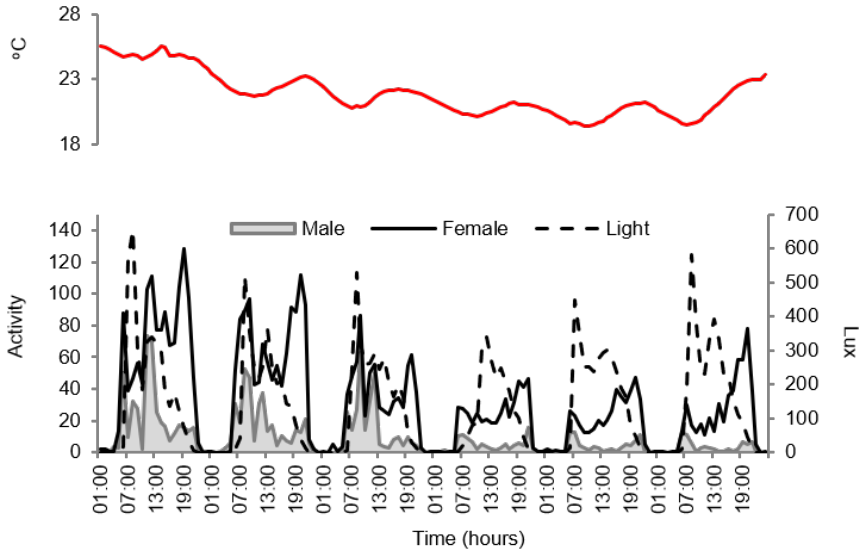
4.4.1 The locomotor behaviour of *D. suzukii* in a semi-natural summer environment is sexually dimorphic

Young male and female flies were kept together in equal numbers for a period of 3 days, to allow sufficient time for mating to occur, before separating and placing into 16 mm diameter tubes for recording locomotor activity using a Trikinetics LAM monitor. The daily activity profiles of male and mated female *D. suzukii* in semi-natural conditions were compared by placing the LAM beneath an East facing skylight to provide natural daily fluctuations of light (maximum lux, 700)

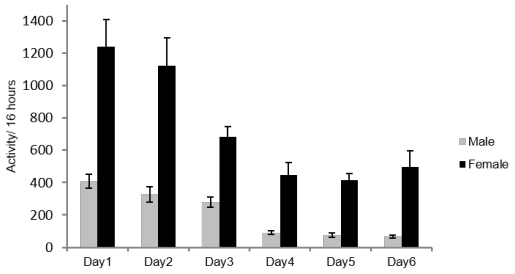
and temperature, ranging between 19 and 26 °C with a minimum at around dawn and a maximum occurring in the late afternoon. The LD cycle approximated to 16 h light - 8 h dark (16L8D) with sunrise at around 05.00 and sunset at around 21.00. Variation in the light levels were observed by shadows in the testing area and clouds obscuring the sun. In comparison to males, females displayed greatly elevated (442 ± 77 %, mean ± s.e.m. for 6 consecutive days) daily locomotor behaviour (Figure 4.1a & b). The female activity began at around 50 min before dawn light had reached the 1 lux sensitivity threshold of the environmental monitor (Figure 4.1a). The maximum afternoon activity of females occurred between 18:00 and 21:00 over the six days of recording, this activity declined rapidly to 50 % by the time light levels had dropped to around 15 lux. This highly significant sexual dimorphic behaviour observed for *D. suzukii* was markedly different from the previous study of Hamby *et al.*,[262] where males were found to be more active than females under summer-like conditions. The main difference between these two studies was the mating status of the insects, where Hamby *et al.* used virgin adults as opposed to the mated flies of the present study. Virgin adults were not used in our testing due to the availability of insects at the time of the experiment. Moreover, due to the logistical barriers for undertaking this semi-field study a move to a lab based technique was undertaken.

4.4.2 Increased activity of females is a post-mating response that disrupts the siesta

The activity of virgin and mated *D. suzukii* of both sexes were compared under laboratory conditions of constant 25 °C and a rectangular 12LD cycle with the light-on level set to 800 lux. In these conditions, virgin and mated males displayed rhythmic morning and evening peaks of activity separated by extended periods of quiescence with few either quantitative or qualitative differences between the behaviour of the two groups (Figure 4.2a & b). Males increased their activity during the "late afternoon" period leading up to a strong and sharp evening peak, occurring around 20 min after lights-off. Switching from a LD regime to constant darkness (DD) resulted in the replacement of the prominent evening (E) peak with a general increase in male activity throughout the subjective day,



(a)



(b)

Figure 4.1: Sexually dimorphic locomotor rhythms of *D. suzukii* in a semi-natural environment. Male and female flies were given the freedom to copulate over a period of 3 days before being separated and placed in a LAM16 locomotor activity monitor for 6 days. (a) Locomotor activity is expressed as the mean number of beam breaks per hour (n = 12). Daily fluctuations in temperature and light levels were also monitored. (b) The daylight period was from the time that the environmental monitors first registered light (05:00) until light levels fell below 1 lx (21:00) and therefore total daylight activity was expressed as the number of beam breaks per 16 h (mean ± s.e.m., n = 11). The variance between mated males and females for each of the 6 days was highly significant (maximum p = 0.019, independent sample t-test).

but with most ambulation occurring just prior to the time of expected lights-off. In the 12 LD cycle, virgin females, but not post-mated females, behaved in a similar manner to the males with a prominent evening activity that peaked just after lights-off and which disappeared in DD conditions (Figure 4.2a). Post-mated females responded to lights-on with a sharp rise in activity that diminished after 1 hour but remained relatively high all morning before rising to a broad peak during the afternoon. The average increase in lights-on activity relative to virgins was 540 ± 125 % (mean ± s.e.m.) for the three days of LD, whereas night-time activity was reduced by 78 ± 1 % (mean ± s.e.m.) (Figure 4.2c). The large drop of activity in the dark was primarily the result of the absence after entering the dark phase of the sharp spike in activity, which was so prominent in virgin females and males. In DD most of the post-mated female activity was restricted to the subjective day and was reduced by around 45 % in comparison to the day-time activity of the LD regime. There appears to be a general trend amongst all groups of flies of a progressive reduction in daily activity during the 12LD cycle.

When the activity data was expressed as quiescence/sleep (5 min periods of inactivity) using parameters established for studying sleep in *D. melanogaster*,[277] the increase in female activity after mating resulted in $>$ 50 % loss of siesta quiescence but had no significant effect on night-time sleep (Figure 4.3 a & c). Much of the remaining day-time quiescence of post-mated females was characterised by a large reduction in the bout length of the inactivity (Figure 4.3 b).

4.4.3 Locomotor behaviour of *D. suzukii* in simulated natural summer conditions

It was of interest to see if the marked difference in the activity of virgin and mated female *D. suzukii* would also occur when fly behaviour was recorded under simulated natural conditions in the laboratory as this would allow greater control of environmental conditions. The first step increase in light levels of our incubator was from 0 to 100 lux and occurred at 05:00 with incremental changes until a peak of 1000 lux was attained at 12:00, before declining back to 0 lux at 21:00. The temperature cycled between 22 °C and 30 °C with the minimum and maximum temperatures occurring at 05:00 and 13:00, respectively (Figure 4.4). In this

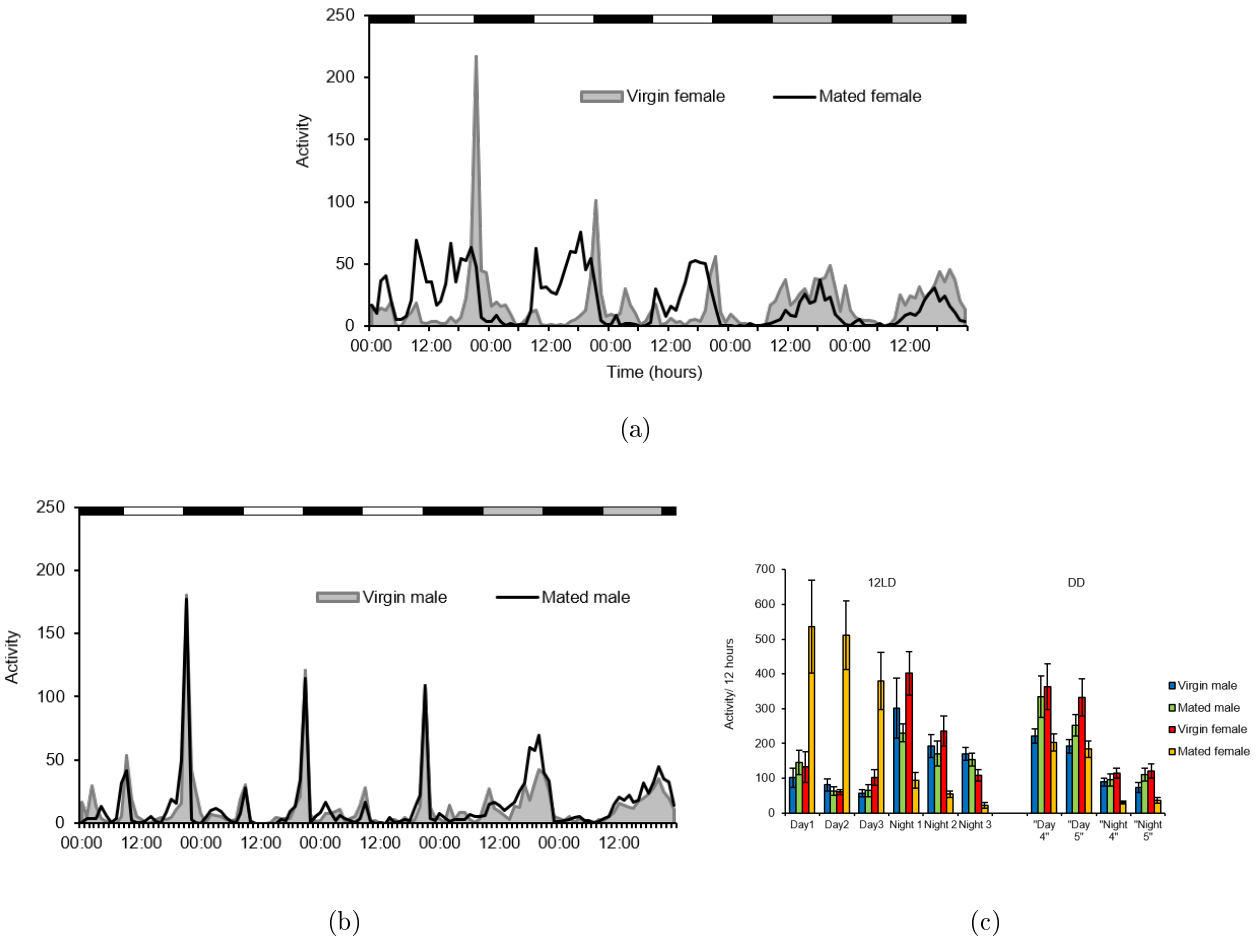


Figure 4.2: Elevated activity is a post-mating response in female *D. suzukii*. Activity profiles of female (a) and male (b) *D. suzukii* maintained on a 12 h light:12 h dark (12LD) cycle. Lights off, black bars; lights on, white bars; subjective day, grey bars. (c) Total activity during the lights-on (day) and lights off (night) periods for three consecutive days in 12LD followed by 3 days in constant dark (DD) expressed as (mean ± s.e.m.) number of beam breaks per 12 h (n = 8). "Day" and "Night" refer to subjective periods during DD. During daylight, the activity of the mated female was significantly higher than that of the other groups ($p < 0.005$) for all 3 days. Night-time activity of the mated females was significantly lower on night 1 and night 2 compared with that of the other groups (maximum $p < 0.003$).

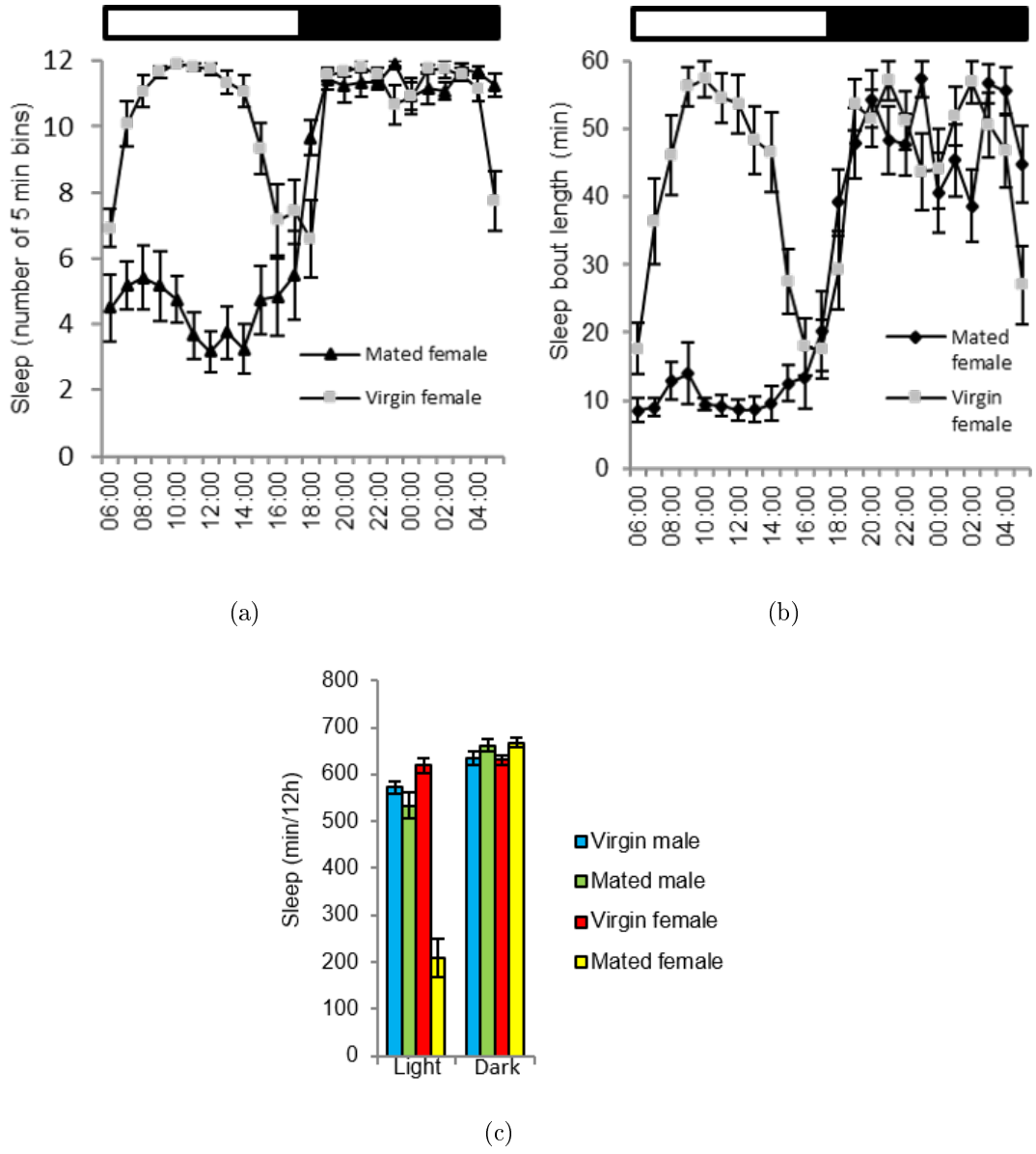
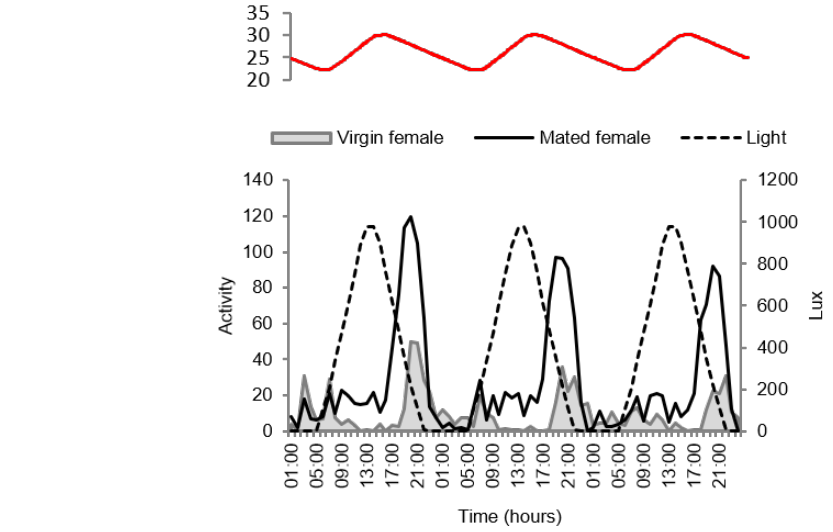


Figure 4.3: Mating disrupts daytime quiescence (siesta) of female *D. suzukii*. (a, c) Mating results in significant loss of daytime quiescence for females compared with males and virgin females (maximum p = 0.000, one-way ANOVA followed by a Bonferroni post hoc test) and a severe reduction in the length of the daytime sleep bout (b) of mated females. Mating has little effect on the siesta and night-time sleep of males. White and black bars in a and b indicate daytime and night-time, respectively. Quiescence or sleep is expressed as either the number of 5 min periods of no activity per hour (a) or the number of 5 min periods of no activity per 12 h of light and dark (c) (mean values for two consecutive days ± s.e.m., n = 16).

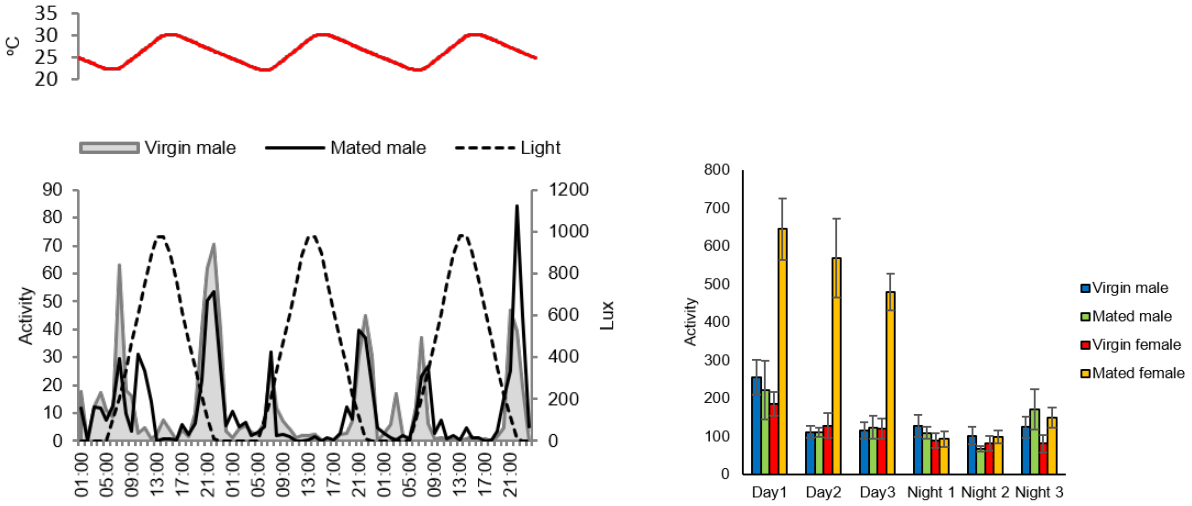
simulated natural LD cycle, both virgin and mated males as well as virgin females experienced an extended siesta period of 6 - 8 h that coincided with both high light and high temperature levels. Locomotor activity increased in the late afternoon, reaching a maximum at 21:00 - 22:00 h (26.6 - 26.0 °C and 105 - 0 Lux) (Figure 4.4 a & b). In contrast, there was a large increase ($>$ 400 %) in day-time (05:00 and 21:00) activity for post-mated females during the first 3 days of recording (Figure 4.4 c), recapitulating the previously seen reduction of the siesta in post-mated females in the 12LD cycle. This elevated afternoon activity peaked earlier than the activity of males and virgin females with a maximum between 18:00 (28.5 °C and 440 Lux) and 20:00 h (27 °C and 170 Lux) (Figure 4.4 a & b). When these experiments were repeated using the same light cycle but with the temperature cycling between 27 °C and 35 °C, all flies died within 24 h.

4.4.4 The *D. suzukii* sex peptide is transferred from the male to the female during copulation

Previously it has been shown that an increase in afternoon activity and loss of siesta in post-mated female *D. melanogaster* is triggered by the male Sex Peptide Dm (SPDm), which is transferred to the female in the seminal fluid.[270] An orthologous Sex Peptide (SPDs) has been isolated from the male accessory glands of *D. suzukii* and shown to be a 41 amino acid peptide (Figure 4.5 a) by N-terminal sequencing and translation of cloned SPDs cDNA.[278] MALDI-MS was used to analyse the peptides in the male accessory glands of *D suzukii* and identified a mass ion of m/z, 5145, which corresponds to the SPDs containing a disulphide link between two cysteines and three 4-hydroxyproline residues (Figure 4.5 b). This molecular ion was absent from extracts of the female reproductive tract of virgin females (Figure 4.5 c), but present in the same tissues taken from copulating females that were separated from their male partner 10 min after initiation of copulation, which normally lasts around 25 min (Figure 4.5 d).[279]



(a)

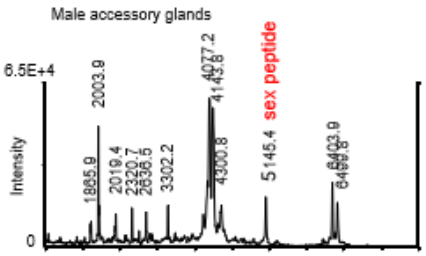


(b) (c)

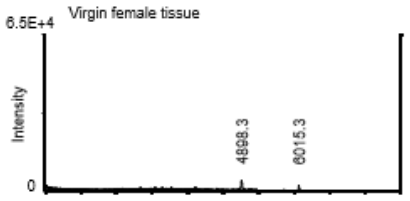
Figure 4.4: Activity of virgin and mated *D. suzukii* in a simulated summer environment. (a & b) Locomotor activity for three consecutive days expressed as the mean number of beam breaks per hour (N=8). Daily fluctuations in temperature and light levels are indicated. (c) Total day (16 h) and night-time (8 h) activity for males and females expressed as the mean ± s.e.m. (n = 8). The daytime activity of mated females was significantly different from that of all other groups ($p = 0.000$) and there was no discernible statistical difference between any of the groups during the night-time ($p > 0.912$) using one-way ANOVA, followed by a Bonferroni *post hoc* test.

D. suzukii SP WEWPWNKQKKPWERPRFPIPNPNPRDKWCRLNLGPGWGGRC
D. melanogaster SP WEWPWNRKPTKFPIPSPNPRDKWCRLNLGPAWGGRC

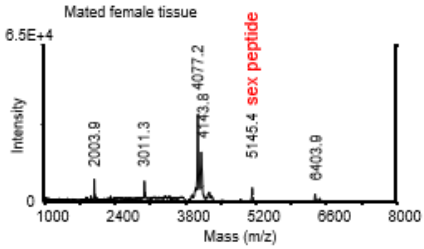
(a)



(b)



(c)



(d)

Figure 4.5: Mass spectra of peptides present in reproductive tissues of *D. suzukii*. Peptides were extracted from five reproductive tissues of *D. suzukii*. (a) The amino acid sequence of male sex peptide from *D. suzukii* (SPDs) aligned with SPDm of *D. melanogaster*. P marks the position of 4-hydroxyproline residues in SPDm. The position of the 4-hydroxyprolines in SPDs has not been determined. (b - d) Mass spectra from the male accessory gland (b), the uterus of virgin females (c) and the uterus of mid-copulation females (d).

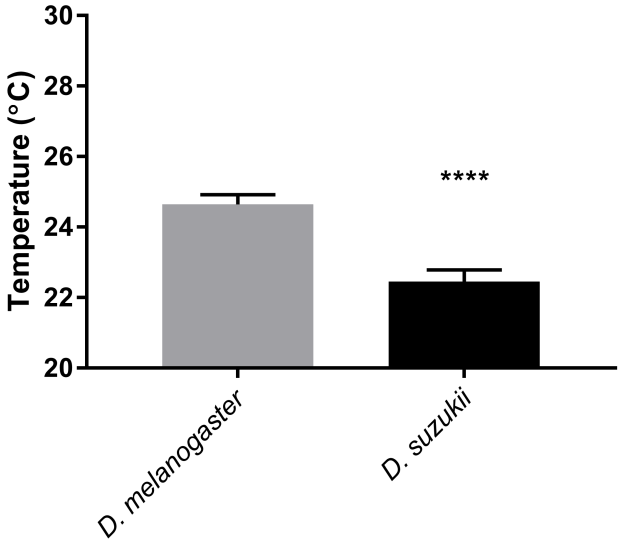
4.4.5 Temperature preference of *D. suzukii* and *D. melanogaster*

Since the arrival of *D. suzukii* in Europe and North America it has been common for the pest to be found in alpine and cooler regions compared to its close relation *D. melanogaster*. To determine if this temperature preference could be simulated under laboratory conditions a simple choice assay was constructed. Previously I have shown that *D. suzukii* are very susceptible to changes in humidity, therefore to avoid complications the bottom of the chamber was covered in filter paper and wetted. It was found that for both species the flies tended to cluster at a given temperature. For each species the test was replicated multiple times (n = 6, 150 flies) and it was found that *D. melanogaster's* mean temperature preference was 24.65 ± 0.28, whereas for *D. suzukii* it was significantly lower at 22.45 ± 0.33 (Figure 4.6a).*D. melanogaster* tended to cluster between 24 - 25 °C; whereas, *D. suzukii* tended to cluster at a significantly (unpaired t-test, $p < 0.0001$) lower temperature 22 - 23 °C (Figure 4.6b).

4.4.6 Increased daytime activity at elevated temperatures

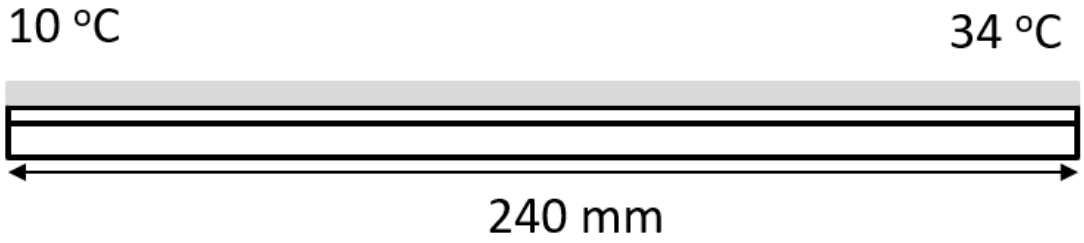
It was of interest to see what effect an increase in the maximum day temperature would have on the locomotor activity of *D. suzukii*. Previously, with a cycling daily temperature from 22 - 30 °C males had a large period of afternoon quiescence, consistent with the siesta period observed in *D. melanogaster*. Furthermore, I have shown with a daily temperature cycle from 22 - 35 °C *D. suzukii* die within 24 h (Section 4.4.3).

I investigated the activity under simulated natural summer conditions, where the first step-increase in light levels of the incubator was from 0 to 50 lx occurring at 05:00 with incremental changes until a peak of 500 lx was attained at 12:00, before declining back to 0 lx at 21:00. Humidity was set to a constant 75 % RH and the temperature was programmed to increase from 22 to 32 °C with the minimum and maximum temperatures occurring at 05:00 and 13:00, respectively (Figure 4.7). This was followed by a period of constant darkness with the same temperature profile. We observed a period of high locomotor morning peak of activity upon lights on (04:00 - 08:00), this is followed by an afternoon (A) peak



(a)

(b)



(c)

Figure 4.6: Temperature preference of *D. suzukii* and *D. melanogaster* (a) average location in temperature preference chamber for both *D. suzukii* and *D. melanogaster* after 30 min expressed as mean ± s.e.m (n = 130). The temperature preference for *D. suzukii* was significantly lower than for *D. melanogaster* (unpaired t-test $p < 0.0001$). (b) Top view of a temperature choice chamber (1 cm per °C) showing the clustering of flies at a certain temperature. (c) Schematic of temperature preference chamber.

in activity, (11:00 - 17:00) and an evening peak upon the lights being switched off (21:00 - 23:00) (Figure 4.7). In the constant darkness regime, the same afternoon peak of activity at 13:00 was observed.

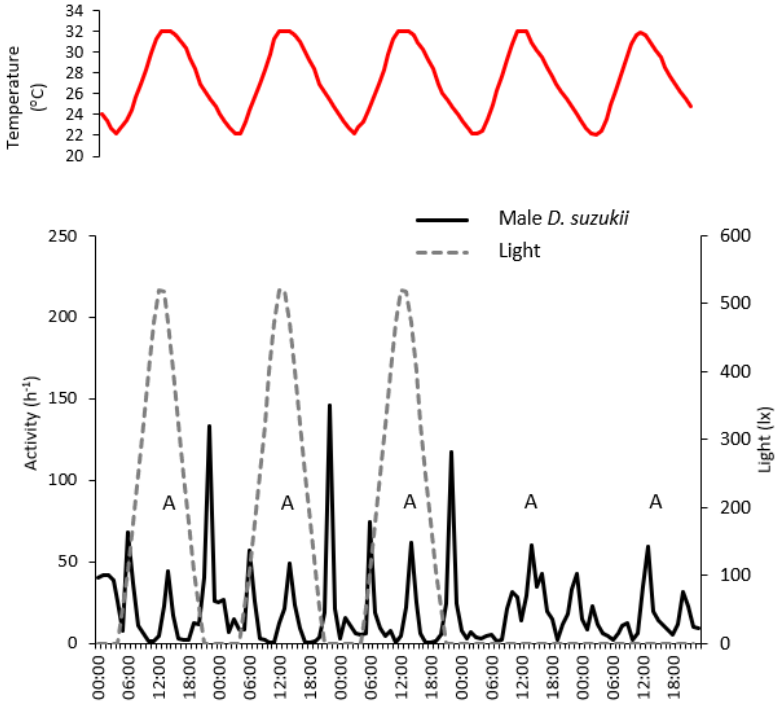


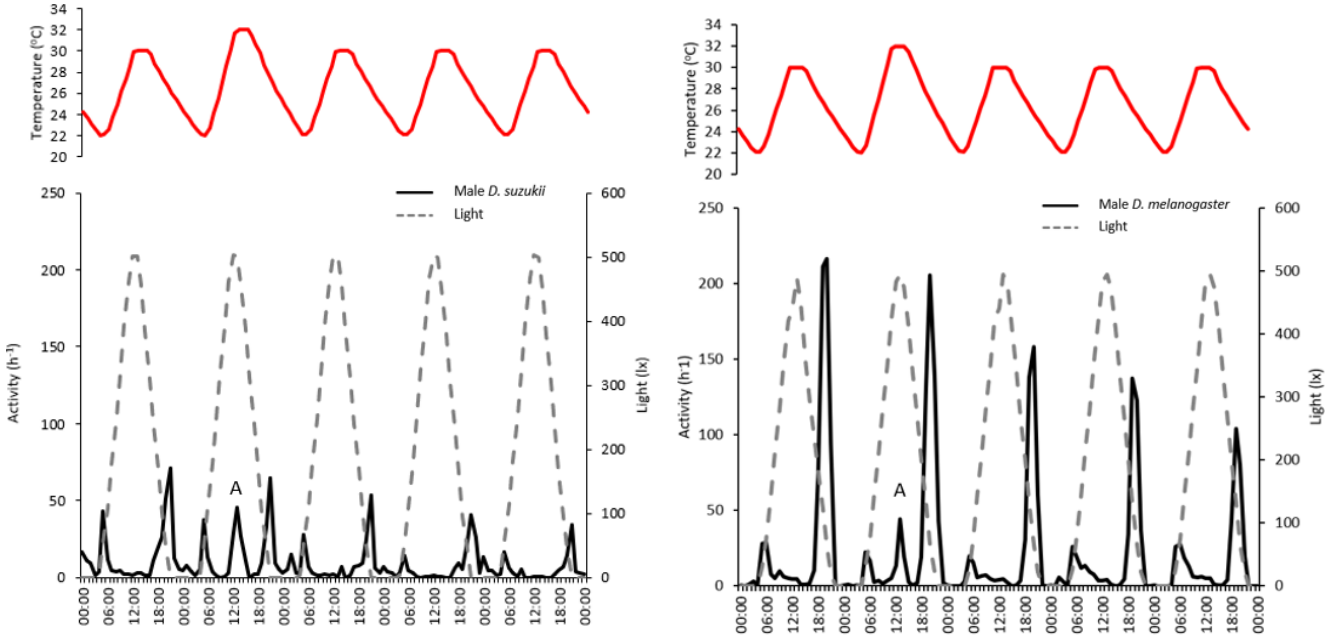
Figure 4.7: Activity of adult male *D. suzukii* in simulated summer environment, where daily maximum temperature varied from 22 - 32 °C. Locomotor activity for three consecutive days expressed as the mean number of beam breaks (N = 36). Daily fluctuations in light levels are indicated (dotted grey line) with humidity kept constant at 75 % RH. An afternoon peak (A) in activity was observed for 3 consecutive days at 13:00. This peak in afternoon activity is also observed when the lights are off at 13:00.

Further investigation was undertaken into the effect elevated temperature on the activity of *D. suzukii* and compared to the response of *D. melanogaster*, where the daily maximum temperature was increased from 30 °C to 32 °C and then back to 30 °C for three consecutive days (Figure 4.8). The humidity was again kept constant at 75 % RH and the light levels varied as previously described. For the

first day (maximum temperature, 30 °C) a bimodal distribution of activity was observed at lights on and off, separated by a siesta period. This was followed by a day where the maximum temperature increased to 32 °C, where both morning and evening peaks were observed as before but once again there was a loss of the afternoon siesta period with the addition of an afternoon peak. Over the subsequent three days (maximum daily temperature, 30 °C) a bimodal activity profile returned with a long afternoon siesta present (Figure 4.8 a & b). The main difference between the response of the two species was the magnitude of the evening activity peak which is two to three times larger for *D. melanogaster* than *D. suzukii*.

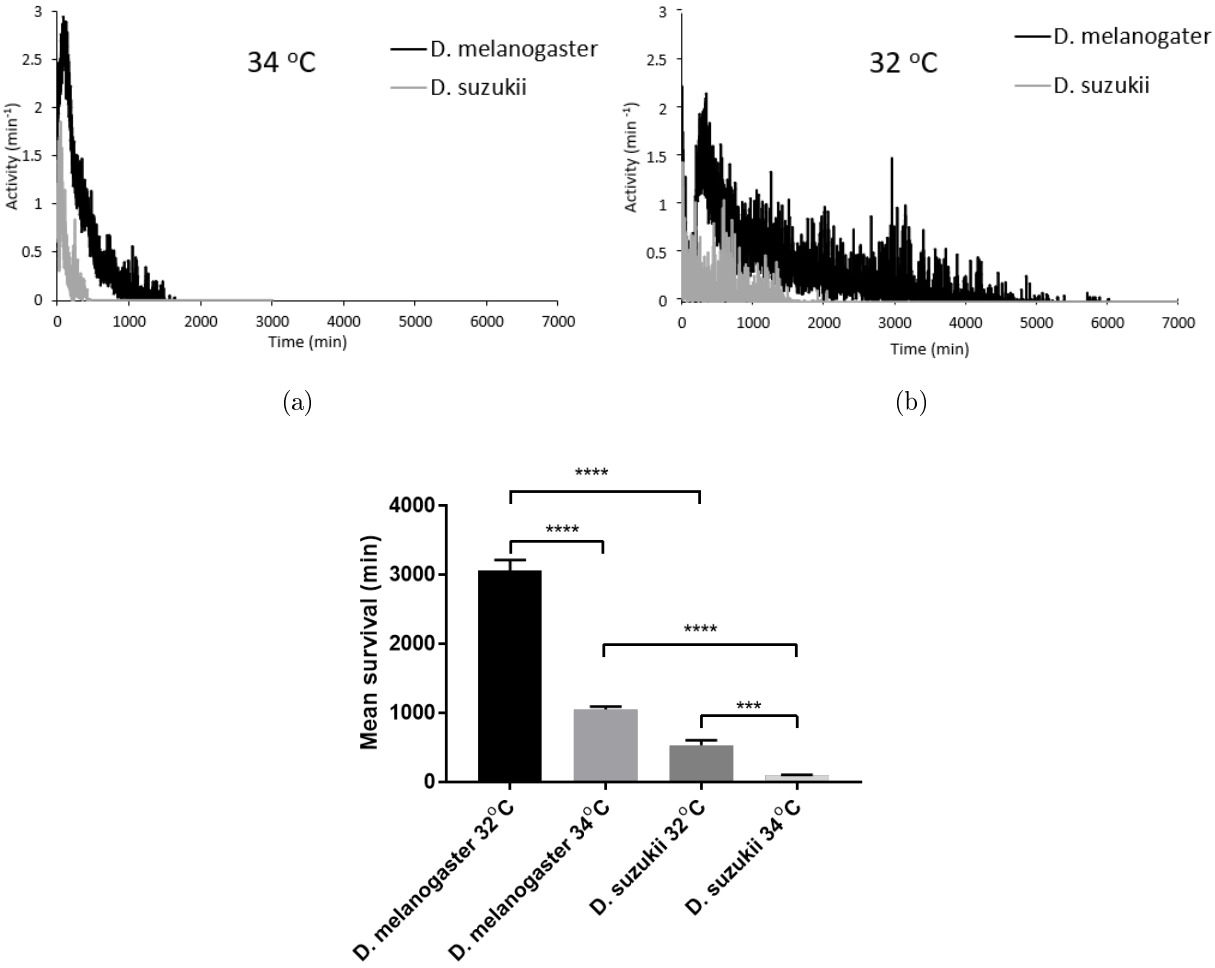
4.4.7 High mortality rates for *D. suzukii* at temperatures *D. melanogaster* can survive

There is clear difference in temperature preference between *D. suzukii* and *D. melanogaster*. To compare the effect of elevated temperature on the two species, a survival test was undertaken where sustained inactivity was used as a surrogate for death. For *D. suzukii* at constant 34 °C a rapid reduction in activity occurred over 2 h with total population mortality occurring within 8 h (n = 82). For *D. melanogaster* there was an initial increase in activity over the first 2 h, followed by a reduction in activity over the next 24 h until full mortality was observed within 25 h (n = 83) (Figure 4.9 a). At 32 °C, 100 % mortality of *D. suzukii* was observed within 31 h (n = 60). For *D. melanogaster* activity decreased due to fly death and all flies had deceased within 96 h (n = 63) (Figure 4.9 b). When the mean survival time for *D. suzukii* and *D. melanogaster* was considered significant differences were observed between the species at both 32 and 34 °C (Figure 4.9 c). For *D. suzukii* at 32 °C there was a mean survival time of around 500 min, this was reduced significantly to around 100 min when the temperature is increased to 34 °C. A similar trend is observed for *D. melanogaster*, but with a significantly longer survival time compared to *D. suzukii*. At 32 °C *D. melanogaster* survived for about 3,000 min which is six times longer than for *D. suzukii*. At 34 °C the survival of *D. melanogaster* decreased to around 1,000 min, but this was still ten times longer than *D. suzukii*.



(a) (b)

Figure 4.8: Afternoon peak of activity is triggered by the elevated temperature in both *D. suzukii* and *D. melanogaster*. Simulated natural temperature varied in a gradient from 22 - 30 °C on the first day then 22 - 32 °C on the second day then 22 - 30 °C for the subsequent three days. (a) Locomotor activity 1 h time bins for *D. suzukii* expressed as the mean number of beam breaks (n = 36). (b) Locomotor activity for *D. melanogaster* expressed as the mean number of beam breaks (n = 24). The appearance of an afternoon peak of activity is indicated "A". Daily fluctuations in light levels are indicated (dashed grey line). Humidity kept constant at 75 % RH.



(a) (b)

(c)

Figure 4.9: Survival of *D. suzukii* and *D. melanogaster* at elevated temperatures (a) Activity of male *D. suzukii* (n = 82) and *D. melanogaster* (n = 83) at 34 °C with constant light 500 lx and humidity 75 % RH. Survival monitored as a function of activity (min $^{-1}$) over time. (b) Activity of *D. suzukii* (n = 60) and *D. melanogaster* (n = 63) at 32 °C with constant light 500 lx and humidity 75 % RH. Activity was measured as the number of beam breaks per minute (min $^{-1}$). (c) Average survival time (expressed as mean ± s.e.m) indicating differences in the survival of each species at each temperature. Death was determined as after the last one-minute time period with activity, the experiment was continued several days after the last beam break. *D. melanogaster* survive significantly longer than *D. suzukii* at both 32 and 34 °C (one-way Anova $p < 0.0001$).

4.5 Discussion

This chapter reports the locomotor behaviour of *D. suzukii* can be affected by external stimuli, such as environmental conditions and by mating status. Knowledge of when a pest is most active provides information on the optimum time for insecticidal spraying. Furthermore, by understanding their preferences to temperature as well as humidity, prediction of the likely movement and dispersal of the pest can be determined, and procedures can be put in place to minimise crop losses. Moreover, better understanding of the likely survival of *D. suzukii* allows for the formation of better prediction of pest pressure for given environmental conditions through the formation of more accurate life tables.[266] These life tables allow for the determination of likely pest pressure and allow agronomists to develop suitable spraying schemes to combat the pest.

Throughout this study LAM16 monitors (Trikinetics Inc.) were used to record the locomotor activity of *D. suzukii* under various environmental conditions. The LAM16 employs multiple infrared beams and detectors held in a thin board to detect insect movement as beam breaks. The monitor has been designed differently from the commonly used DAM2 Trikinetics monitors to reduce the risk of shadows being cast by the plastic housing.

Our initial objective was to record the activity profiles of mated male and female *D. suzukii* in a natural summer environment. Conducting such an experiment outdoors was technically problematic as natural infrared radiation resulted in spurious counts being recorded by the activity monitors. Therefore, the monitors were placed beneath a skylight window in a building located away from any polluting artificial light. In these semi-natural summer conditions, the activity of the mated females greatly exceeded that of mated males, with the most pronounced difference in behaviour occurring during the late afternoon when females were consistently more active than males. Although the intensity of the activity waned with time, the sex difference was sustained for at least 6 days. The only previous study of locomotor activity of adult *D. suzukii* was conducted under "summer" conditions, where a temperature cycle of between 12.2 and 22.2 °C simulated the gradual natural gradients of Watsonville, CA, USA.[262] Changes in light intensity, however, were restricted to two undefined levels, with the lower

intensity occurring in the morning (dawn) and late afternoon (dusk). This study, using virgin males and females, showed that activity was largely restricted to daylight hours, with peaks occurring at "dawn" and "dusk", and indicated that males were more active than females, which is opposite to what was observed in the present study.

As it had been shown previously that mating increased daytime activity of female *D. melanogaster*, it was suspected that the reason for the high female activity relative to male activity seen with *D. suzukii* in our semi-natural conditions resulted from a similar post-mating response.[270] This was confirmed by investigating the activity profiles of mated male and female *D. suzukii* using standard incubator conditions of a 12LD cycle and constant temperature. Here, we observed for virgin and mated males, as well as virgin females, a small peak of morning activity and a late afternoon rise in activity leading to a prominent evening peak. There was a period of quiescence that extended for several hours in the early to middle of the afternoon and was reminiscent of the siesta sleep of *D. melanogaster*. In contrast to male and virgin female *D. suzukii*, mated females were active throughout the daytime, with the evening peak now occurring much earlier in the afternoon. The sharp peak of activity that occurred just after lights off in the 12LD conditions appears to be a startle effect from the sudden switch to complete darkness. This reaction, which was seen in males and virgin females, was much reduced or even blocked in mated females, perhaps resulting from the dampening down of the startle response by SP signalling pathways. This pathway has been well studied for *D. melanogaster* further information can be found in several review articles.[280,281] The induced activity in mated *D. suzukii* females gave rise to a large reduction in daytime quiescence, but not night-time sleep. Furthermore, the mated females could not sustain a daytime sleep-like state for periods much beyond 10 min. Rhythmic activity was maintained when both male and female flies, irrespective of mating status, were placed in continuous dark (DD) after 12LD entrainment, as expected for behaviour under the control of an internal clock. It was very noticeable, however, that the activity was now spread across the whole of the subjective lights-on period, suggesting that afternoon light suppresses activity, reflecting a reluctance to be active in the middle of the day to protect against the afternoon sun. In constant darkness, the

evening peak of activity occurred earlier in the subjective day because of the disappearance of the prominent lights-off peak, consistent with this being a startle response to the sudden switching off of the light.

The behavioural responses by female *D. suzukii* to mating are very similar to what has been observed previously for *D. melanogaster* (OregonR, Dahomey and to a lesser extent, CantonS strains).[270] For this species, the loss of daytime quiescence is elicited by the transfer in the seminal fluid of SPDm, a 36 amino acid pheromone peptide made by the male accessory gland. SPDm elicits a wide variety of female post-mating responses that include rejection of male advances, increase in oviposition, activation of immune defences, elevated food intake and altered food preference, in addition to the effect on sleep.[280] The orthologous SPDs of *D. suzukii* has the same structure as SPDm, although there are five additional amino acids in the central proline-rich region. Some of the functionality of SPDs is also conserved as the synthetic peptide elicits oviposition and loss of male receptivity when injected into female *D. suzukii* as well as female *D. melanogaster*.[278] Unlike SPDm, however, there is no knowledge from amino acid analysis of any post-translational modifications of the amino acid side-chains, e.g. prolyl hydroxylation. Mass spectrometry detected a molecular ion (m/z 5145) corresponding to the predicted SPDs with three 4-hydroxyproline residues, although these post translational modifications have yet to be confirmed by chemical analysis. The detection of the m/z 5145 molecular ion in the reproductive tract of the mated females provides evidence of its transfer from the male, and leads us to conclude that SPDs in the ejaculate is probably responsible for the behavioural changes observed in mated female *D. suzukii*

It has recently been shown that adult male *D. melanogaster* display a mid-afternoon (A) peak of activity, but only when the afternoon temperatures reached above 30 °C.[245,272–274,282] This thermo-sensitive A peak was seen in simulated natural conditions in a controlled environment incubator, where temperatures fluctuated from a low of 17 or 25 °C to a peak of 32 or 35 °C in the afternoon. When the variable light and temperature were out of phase, the A peak coincided with the highest temperature and not peak light levels.[245] Initially, it was hypothesised that this afternoon peak was an experimental artefact produced by the TriKinetics locomotor monitoring system.[15] However, further investigation

through the use of video recordings showed that this afternoon peak was not an artefact. For *D. melanogaster* the afternoon peak requires the internal thermosensitive channel transient receptor potential A1 (TrpA1) and that the magnitude of afternoon activity can be controlled by altering the maximum daily temperature.[274] An early evening peak of activity was recorded for mated female *D. suzukii* (maximum daily temp, 30 °C) at 19:00 which was 6 h after the hottest period (13:00) and is therefore not equivalent to the temperature-sensitive A peak of *D. melanogaster*. The activity of *D. suzukii* was monitored with temperatures cycling between 27 and 35 °C, but high mortality occurred, reflecting the fact that this species is poorly adapted to hot climates.

In order to better understand these differences in thermal responses between *D. suzukii* and *D. melanogaster* their temperature preferences were determined, using similar methodology to what has been used in other laboratories for *D. melanogaster*.[275] This methodology maintains a high relative humidity across the length of the chamber, which is particularly important as *D. suzukii* is sensitive to low humidity.[265] Previously, it has been shown that in this temperature preference test *D. melanogaster* cluster around 24 °C.[275] In the present study *D. melanogaster* also tended to cluster between 24 - 25 °C, averaging at 24.65 °C. *D. suzukii* in comparison clustered at a significantly lower temperature between 22 - 23 °C, averaging at 22.45 °C. This 2 °C difference between the two species is similar to the difference in maximum upper limit for development (30.9 °C for *D. suzukii* and 33 °C for *D. melanogaster*).[263,266,267]

As shown in simulated natural conditions where the afternoon maximum temperature reached 30 °C, *D. suzukii* males experienced a sustained period of quiescence during the middle of the day. By increasing this daily maximum temperature to 32 °C this period of sleep was disrupted and instead an afternoon activity peak was observed. This afternoon behaviour at elevated temperatures was similar to that previously described for *D. melanogaster*.[272] This elevated afternoon activity was confirmed and was a direct response of the increased maximum temperature by including a period of constant darkness, where the afternoon peak of activity was still present. This afternoon peak is therefore a direct result of temperature and not due to the increasing light intensity as has been observed for several other *Drosophila* species.[245]

Daily maximum temperatures tend to fluctuate in natural conditions, therefore the affect these fluctuations had on *D. suzukii* was investigated and compared with *D. melanogaster*. For both species raising the temperature to a maximum of 30 °C did not disrupt the afternoon quiescence period. A daily temperature maximum of 32 °C led to the appearance of an afternoon peak in both species, which was of a similar magnitude. The striking difference between the species occurs in the evening peak which is substantially bigger for *D. melanogaster* than for *D. suzukii*. The days following the 32 °C maximum temperature did not appear to be affected by the previous day's increased locomotor activity. However, the stress created by this loss of siesta may have a longer-term effect not observed in this short-term test.

Periods of elevated temperature lead to increased stress in the insect increasing locomotor activity. The effect of sustained periods of high temperatures were expected to put the flies under extreme stress and lead to mortality. Under conditions of constant 34 °C it was found that both species were poorly adapted to sustained periods of high heat and perish. For males the rate of mortality for *D. suzukii* is substantially greater than for *D. melanogaster*. At 32 °C both species survive for substantially longer but not as long as at ambient temperature and *D. suzukii* perished faster than *D. melanogaster*. This indicates that *D. suzukii* is more susceptible to heat stress than *D. melanogaster* and sustained periods of elevated temperature leads to high mortality rates. The increased daytime activity in response to elevated temperature is probably a defensive adaption to avoid loss of water and overheating. When the environmental temperature exceeds a critical temperature both species increase their activity to move to a cooler region, this may be through the gain in altitude or through seeking a cooler microclimate such as a shaded area under foliage. What is of interest is that it appears that the critical temperature to increase the activity for both species is similar, but that *D. suzukii* cannot withstand as long periods at this elevated temperature so needs to find a cooler region faster. This inability to withstand periods of elevated temperature, even under high humidity, goes some way to explain the lack of *D. suzukii* in hot dry regions of Europe.[257]

4.6 Conclusion

The siesta observed for male and virgin female *D. suzukii* is probably a defensive adaptation for conserving energy, protection from predators and avoiding exposure and loss of water during hot summer afternoons. A recent study has shown that *D. suzukii* mate in the morning and evening of a 16L8D day and are seldom seen copulating in the afternoon, which is consistent with our observation of a siesta for virgin males and virgin females.[283] Mated females, however, are expected to adopt a higher risk lifestyle for the acquisition of nutrients to support egg production, for finding suitable egg laying sites and for reducing competition through increased dispersal. The switch in the afternoon behaviour of the female may be triggered by the transfer of SPDs from the male during copulation. This peptide has an impact on sensory neurons in behaviour-modifying pathways.

The increase in locomotor activity with elevated temperature observed in both *D. suzukii* and *D. melanogaster* is probably a stress avoidance adaptation against thermal stress and water loss. Recently, it has been shown that adult *D. suzukii* are indeed more susceptible to water loss compared to *D. melanogaster* which may explain the poorer survival of *D. suzukii* at temperatures above 30 °C.[265]

Chapter 5

Conclusions and future work

D. suzukii is a serious economic pest that has created large amounts of damage since its invasion of temperate horticultural regions. This thesis has highlighted three main aspects required for the control of this pest. First, I have developed a potential method for the delivery of protein based bioinsecticides. Second, I have developed diblock copolymers that are capable of complexing dsRNA. These complexes induce increased mortality of *D. suzukii* compared to naked dsRNA at the larval stage. Finally, I have observed the pest's locomotor behaviour under different climatic and mating status conditions in order to develop better control strategies.

Historically protein and peptide based bioinsecticides have been limited by their rapid degradation on the crop substrate and upon ingestion by the pest. To combat this I have developed a novel delivery system, where we can encapsulate water-soluble biomaterials in a water continuous phase. This was achieved using a water-in-oil-in-water (W/O/W) multiple emulsion-template, where the oil phase contained a monomer and initiator which could be polymerised to give solid polymer microcapsules. The encapsulation of water soluble biomaterials may lead to a protection of the encapsulate due to the physical polymeric barrier between the active species and degrading enzymes. However, for a biopesticide to be active it needs to be released from the protective microcapsule. Release from these microcapsules was enabled using a triggered mechanism, where the microcapsules responded to an external pH change. This response was engineered so that the microcapsules would rapidly swell with a reduction in pH, corresponding

to the gut pH changes in *D. suzukii*. I have shown through *in vitro* and *in vivo* testing that the microcapsules maintain their cargo at a pH above the ionisation point of the polymer, but rapidly release below this ionisation point. To date, we have demonstrated the encapsulation and triggered release of a model biomaterial. Optimisation of the multiple emulsion template has been undertaken in this research, this should be continued to gain a better understanding of the morphology of the multiple emulsion as the oil phase polymerises.

The next stage in the development of this delivery method will be to encapsulate relevant protein/peptide bioinsecticides. There are many different possible bioinsecticides that may be encapsulated as outlined in Chapter 1, the system that I have demonstrated allows for the specific release of the contents at the main site of uptake in the pest and a wide range of possible bioactives could be investigated. With each different bioactive the encapsulation efficiency over time should be investigated to determine the short and long term storage of these microcapsules. *Ex vivo* testing of the protection provided by the microcapsules should be undertaken in the gut juices of the insect and in the storage liquid of the crop tissue.

RNAi has recently come to the forefront of bioinsecticide research, due to its specificity and eco-safety. However, degradation by extracellular nucleases and poor cellular uptake has limited its use via oral application. To combat this, I have synthesised diblock copolymers, which may increase the stability of the dsRNA and increase the RNAi effect in *D. suzukii*. These diblock copolymers consisted of a complexing quaternised PQDMAEMA block that condensed the dsRNA. A secondary water-soluble PHPMA block was added to provide steric stabilisation. I have shown for the first time that well-defined diblock copolymers can efficiently complex dsRNA to form a polymer:dsRNA polyplex. The size of these polyplexes can be controlled by the ratio of complexing and stabilising blocks. Complexation of dsRNA has increased the mortality in *D. suzukii* by ~ 75 % compared to naked dsRNA. Furthermore, when the complexes were fed to a close relation and model species, *D. melanogaster*, no effect on survival was observed, demonstrating the specificity of this technique. This increased control may be due to the enhanced protection and delivery of the dsRNA upon complexation compared to the easily degraded naked dsRNA. However, future

work is needed to confirm that the phenotypic observations are due to enhanced RNAi after complexation of the dsRNA.

It is vital that monitoring of gene suppression by RT-qPCR is investigated to confirm that the increased mortality is indeed due to enhanced RNAi. This should be undertaken with the comparison in gene expression for larvae fed naked and complexed dsRNA coding for the *vha26* subunit. An accompanying experiment should be set up testing non-coding dsRNA both in the naked and complexed form to show that the effect is due to enhanced RNAi. It is also of interest to better understand the protection provided by the diblock copolymers synthesised. To do this an *ex vivo* study should be undertaken to determine the longevity of dsRNA in the pest's gut juices after complexation and compared to naked dsRNA. This can be undertaken using two main methods, either by incubating the dsRNA in the gut juices in the presence of ethidium bromide to quantitatively measure dsRNA concentration or an agrose gel can be run after the polymer has been decoupled with the dsRNA using SDS. A combination of these methods would allow for the determination of dsRNA concentration over time and investigate how the dsRNA degrades.

The locomotor activity of female *D. suzukii* is significantly altered upon copulation, this change in behaviour is likely to be due to the transferral of sex peptide in the seminal fluid. Previously for the close relation and model species *D. melanogaster* similar behaviour changes have been observed after copulation which are induced due to the transferral of SP. In collaboration with Dr Neil Audsley we have shown that there is an analogue in *D. suzukii* of this SP. This peptide in *D. melanogaster* has been shown to modify the insects behaviour leading to the loss of the afternoon siesta period in mated females. It is hypothesised that this increased activity is needed in order to find oviposition sites and along with extra foraging. In order to confirm that the effect observed in *D. suzukii* is indeed induced by the transferral of SP suppression of the SP gene in males should be induced. Unfortunately, the production of synthetic SP and the introduction into virgin flies would be unlikely to work due to the rapid removal of the peptide that is not co-applied with the sperm. As the mated females create the damage, it is important to understand when the optimum time to spray insecticides is to maximise their effect. Along with the modification of the locomotor

behaviour, the transferral of peptides during copulation can alter other processes in the insect. We have shown that there are several other peptides present in the seminal fluid that are transferred during copulation, and future work will investigate what these are and what effect they have.

D. suzukii has spread rapidly throughout temperate regions of the world due to its suitability to a wide range of climates. I have shown that although this polyphagous pest is found in many regions, it is less well adapted to thermal stresses than its close relation *D. melanogaster*. Increasing the daily maximum temperature above 30 °C results in a loss of the afternoon siesta peak, where instead a period of increased activity is observed. This increased activity does not appear to have an effect on the short term behaviour of the insect. However, long periods of elevated daily maximum temperature are likely to lead to a reduce in fitness of the pest. Therefore it would be of interest to monitor the survival of this pest over longer time periods under simulated natural conditions where the maximum temperature is set to 32 °C. In conjunction with accurate environmental prediction and monitoring an estimate on the likely pressure of the insect pest could be made. This production of life tables for the insect based on true weather conditions would help growers determine the correct spray regimes of insecticides, reducing over-spraying and limiting crop losses. Furthermore, with the increasing computational power and expertise in dispersal modelling this data will aid in enhanced predictive models. The results presented here along with the future experiments described would allow for the maximum upper temperature to be set, allowing a prediction on how long the fly could survive those conditions. These parameter in conjunction with other environmental conditions enables the prediction of likely dispersal patterns of the pest into new geographic areas.

Appendix A

^{1}H-NMR characterisation of polymers used to complex dsRNA

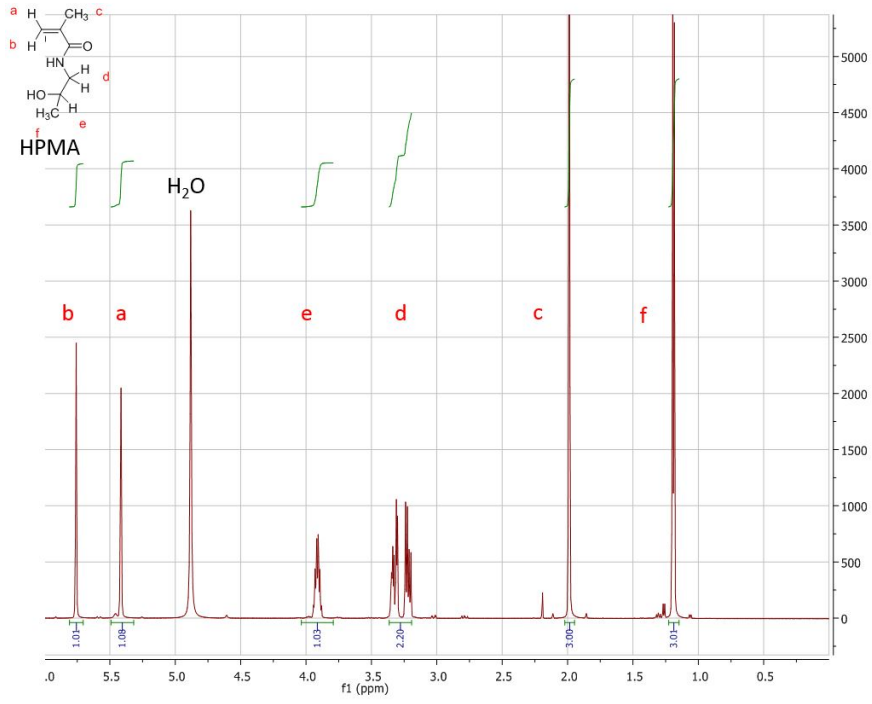


Figure A.1: ^{1}H-NMR of HPMA after purification in cold diethyl ether and vacuum drying

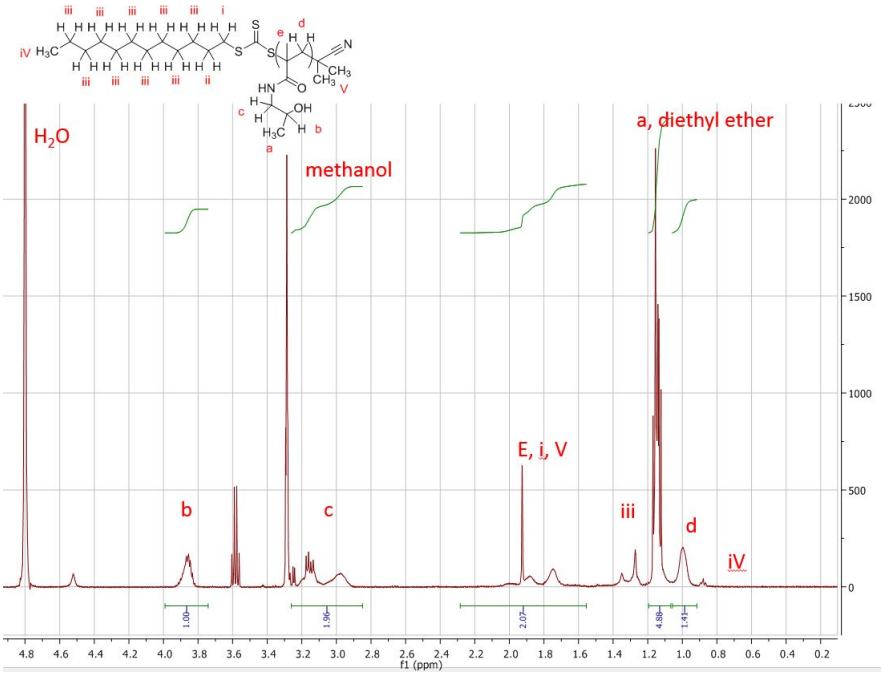


Figure A.2: $PHPMA_{53}$ macroCTA ^{1}H-NMR after dialysis in milli-Q water to remove unreacted monomer and subsequent freeze drying

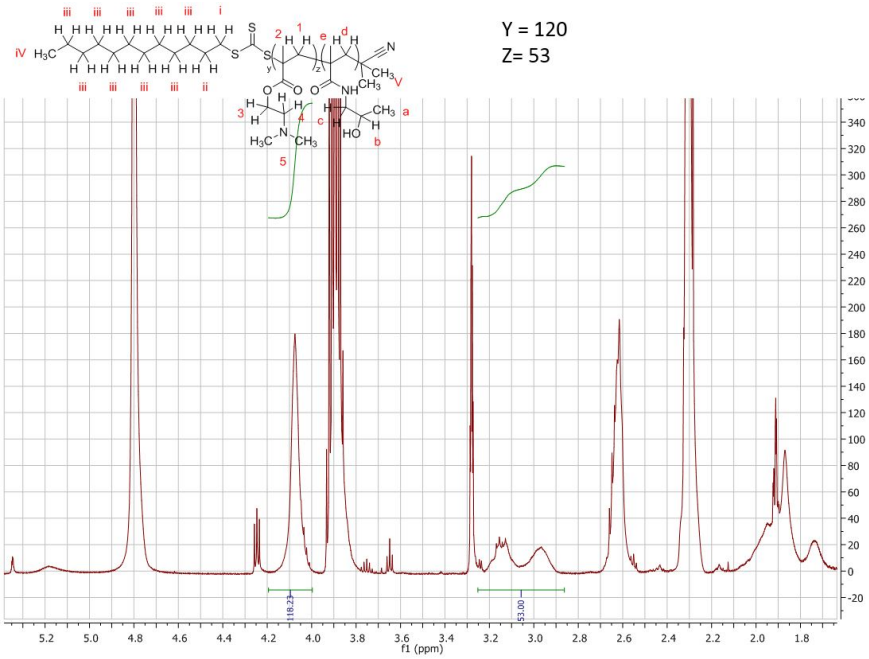


Figure A.3: $PHPMA_{53}$-b-$PDMAEMA_{120}$ ^{1}H-NMR

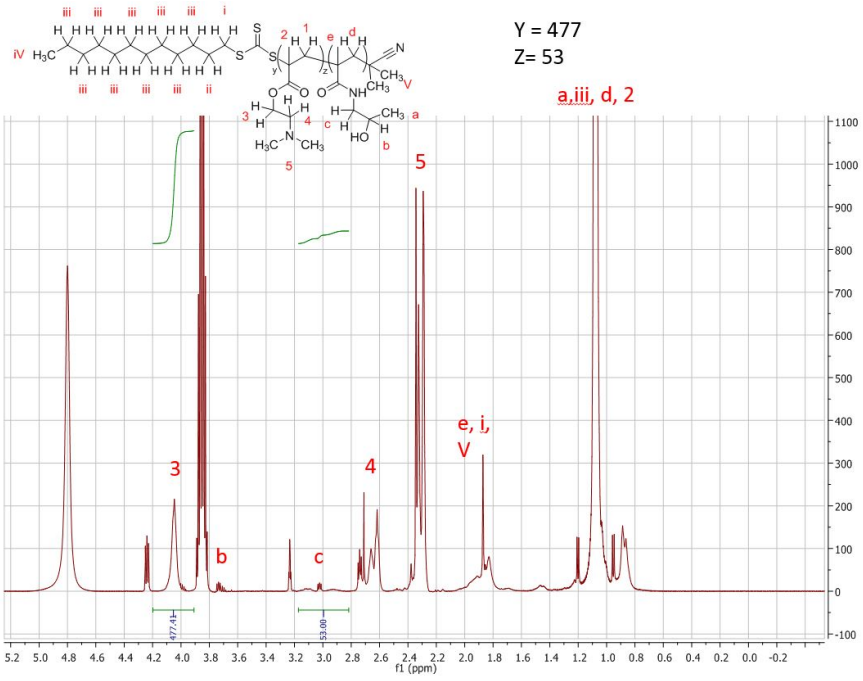


Figure A.4: $PHPMA_{53}$-b-$PDMAEMA_{475}$ ^{1}H-NMR

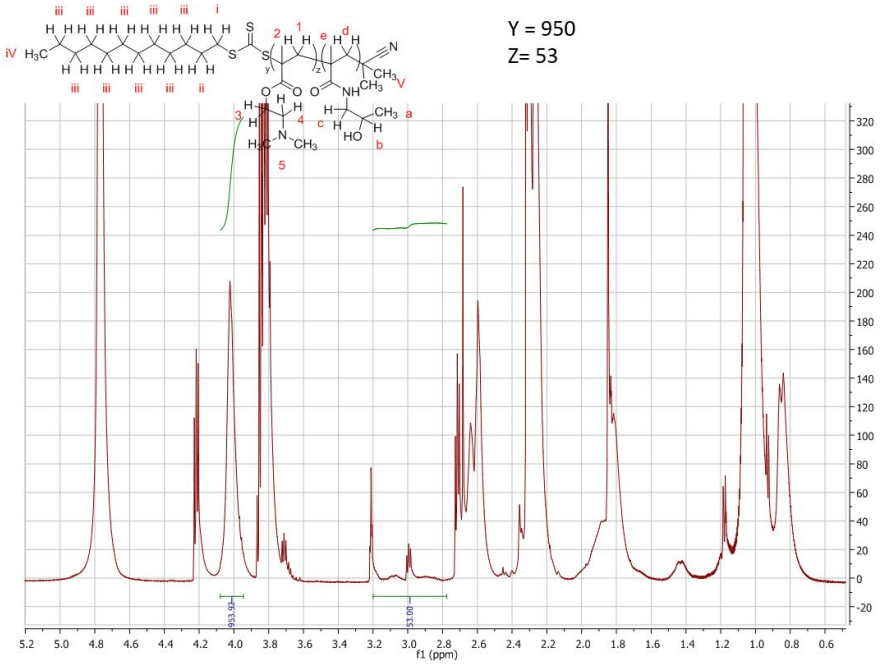


Figure A.5: $PHPMA_{53}$-b-$PDMAEMA_{950}$ ^{1}H-NMR

References

[1] Mark P Bolda, Rachael E Goodhue, and Frank G Zalom. Spotted wing drosophila: potential economic impact of a newly established pest. *Agricultural Res. Econ. Update*, 13:5–8, 2010. 1, 4, 57, 119

[2] Rachael E Goodhue, Mark Bolda, Derek Farnsworth, Jeffrey C Williams, and Frank G Zalom. Spotted wing drosophila infestation of california strawberries and raspberries: economic analysis of potential revenue losses and control costs. *Pest management science*, 67(11):1396–1402, 2011. ISSN 1526-4998. 119

[3] Jana C Lee, Denny J Bruck, Amy J Dreves, Claudio Ioriatti, Heidrun Vogt, and Peter Baufeld. In focus: Spotted wing drosophila, drosophila suzukii, across perspectives. *Pest management science*, 67(11):1349–1351, 2011. ISSN 1526-4998.

[4] Mark K Asplen, Gianfranco Anfora, Antonio Biondi, Deuk-Soo Choi, Dong Chu, Kent M Daane, Patricia Gibert, Andrew P Gutierrez, Kim A Hoelmer, William D Hutchison, et al. Invasion biology of spotted wing drosophila (drosophila suzukii): a global perspective and future priorities. *Journal of Pest Science*, 88(3):469–494, 2015. 1, 2, 3, 119

[5] Giorgio De Ros, Sandro Conci, Tommaso Pantezzi, and Gianluca Savini. The economic impact of invasive pest drosophila suzukii on berry production in the province of trento, italy. *Journal of Berry Research*, 5(2):89–96, 2015. 4

[6] Rosa Gabarra, Jordi Riudavets, Gustavo A Rodríguez, Juli Pujade-Villar, and Judit Arnó. Prospects for the biological control of drosophila suzukii. *BioControl*, 60(3):331–339, 2015.

[7] Maríndia Deprá, Jean Lucas Poppe, Hermes José Schmitz, Daniela Cristina De Toni, and Vera LS Valente. The first records of the invasive pest drosophila suzukii in the south american continent. *Journal of Pest Science*, 87(3):379–383, 2014. 1, 57, 119

[8] Joel Atallah, Lisa Teixeira, Raul Salazar, George Zaragoza, and Artyom Kopp. The making of a pest: the evolution of a fruit-penetrating ovipositor in drosophila suzukii and related species. *Proceedings of the Royal Society B: Biological Sciences*, 281(1781):20132840, 2014. ISSN 0962-8452. 1, 57, 119

[9] A Cini, G Anfora, LA Escudero-Colomar, A Grassi, U Santosuosso, G Seljak, and A Papini. Tracking the invasion of the alien fruit pest drosophila suzukii in europe. *Journal of Pest Science*, 87(4):559–566, 2014. 2, 4, 119

[10] Douglas B Walsh, Mark P Bolda, Rachael E Goodhue, Amy J Dreves, Jana Lee, Denny J Bruck, Vaughn M Walton, Sally D O'Neal, and Frank G Zalom. Drosophila suzukii (diptera: Drosophilidae): invasive pest of ripening soft fruit expanding its geographic range and damage potential. *Journal of Integrated Pest Management*, 2(1):G1–G7, 2011. 4

[11] Kelly A Hamby, Alejandro Hernández, Kyria Boundy-Mills, and Frank G Zalom. Associations of yeasts with spotted-wing drosophila (drosophila suzukii; diptera: Drosophilidae) in cherries and raspberries. *Applied and environmental microbiology*, 78(14):4869–4873, 2012. 2

[12] T Kanzawa. Research into the fruit-fly drosophila suzukii matsumura (preliminary report). *Yamanashi Prefecture Agricultural Experiment Station Report*, 1935. 2

[13] Marc Kenis, Lorenzo Tonina, René Eschen, Bart van der Sluis, Manuel Sancassani, Nicola Mori, Tim Haye, and Herman Helsen. Non-crop plants

used as hosts by drosophila suzukii in europe. *Journal of pest science*, 89 (3):735–748, 2016. 2

[14] Claudio Ioriatti, A Frontuto, A Grassi, G Anfora, and S Simoni. Drosophila suzukii (matsumura): una nuova specie dannosa alle colture dei piccoli frutti. In *CriticitÃă e prospettive delle emergenze fitosanitarie: Firenze*. Polistampa, 2012. 4

[15] Joydeep De, Vishwanath Varma, Soham Saha, Vasu Sheeba, and Vijay Kumar Sharma. Significance of activity peaks in fruit flies, drosophila melanogaster, under seminatural conditions. *Proceedings of the National Academy of Sciences*, 110(22):8984–8989, 2013. 4, 142

[16] Dominique Mazzi, Esther Bravin, Manuela Meraner, Robert Finger, and Stefan Kuske. Economic impact of the introduction and establishment of drosophila suzukii on sweet cherry production in switzerland. *Insects*, 8(1): 18, 2017. 4

[17] Heather Leach, Steven Van Timmeren, and Rufus Isaacs. Exclusion netting delays and reduces drosophila suzukii (diptera: Drosophilidae) infestation in raspberries. *Journal of economic entomology*, 109(5):2151–2158, 2016.

[18] Emanuele Del Fava, Claudio Ioriatti, and Alessia Melegaro. Cost-benefit analysis of controlling the spotted wing drosophila (drosophila suzukii (matsumura)) spread and infestation of soft fruits in trentino, northern italy. *Pest management science*, 2017.

[19] Mary A Rogers, Eric C Burkness, and WD Hutchison. Evaluation of high tunnels for management of drosophila suzukii in fall-bearing red raspberries: Potential for reducing insecticide use. *Journal of pest science*, 89(3):815–821, 2016. 4

[20] Kathleen A Lewis, John Tzilivakis, Douglas J Warner, and Andrew Green. An international database for pesticide risk assessments and management. *Human and Ecological Risk Assessment: An International Journal*, 22(4): 1050–1064, 2016. ISSN 1080-7039. 4, 91

[21] E-C Oerke. Crop losses to pests. *The Journal of Agricultural Science*, 144 (1):31–43, 2006. ISSN 1469-5146. 4, 91

[22] Sukumar Chakraborty and Adrian C Newton. Climate change, plant diseases and food security: an overview. *Plant Pathology*, 60(1):2–14, 2011. 5, 91

[23] József Popp, Károly Pető, and János Nagy. Pesticide productivity and food security. a review. *Agronomy for sustainable development*, 33(1):243–255, 2013. 5

[24] Brooke Borel. When the pesticides run out, 2017. 5, 91

[25] Steven Whyard, Aditi D Singh, and Sylvia Wong. Ingested double-stranded rnas can act as species-specific insecticides. *Insect biochemistry and molecular biology*, 39(11):824–832, 2009. ISSN 0965-1748. 5, 13, 17, 39, 57, 91, 92, 105

[26] Thomas C Sparks and Beth A Lorsbach. Perspectives on the agrochemical industry and agrochemical discovery. *Pest management science*, 73(4):672–677, 2017. 5, 6, 7

[27] RJ Hammerstrom. Insect resistance to insecticides. *Public Health Reports*, 73(12):1126, 1958. 5

[28] Caspar A Hallmann, Martin Sorg, Eelke Jongejans, Henk Siepel, Nick Hofland, Heinz Schwan, Werner Stenmans, Andreas Müller, Hubert Sumser, Thomas Hörren, et al. More than 75 percent decline over 27 years in total flying insect biomass in protected areas. *PloS one*, 12(10):e0185809, 2017. 6

[29] Nicolas Desneux, Axel Decourtye, and Jean-Marie Delpuech. The sublethal effects of pesticides on beneficial arthropods. *Annu. Rev. Entomol.*, 52: 81–106, 2007. 6

[30] John E Losey and Mace Vaughan. The economic value of ecological services provided by insects. *Bioscience*, 56(4):311–323, 2006. 7

[31] Dave Goulson, Elizabeth Nicholls, Cristina Botías, and Ellen L Rotheray. Bee declines driven by combined stress from parasites, pesticides, and lack of flowers. *Science*, 347(6229):1255957, 2015. 7

[32] Simone Tosi, Cecilia Costa, Umberto Vesco, Giancarlo Quaglia, and Giovanni Guido. A 3-year survey of italian honey bee-collected pollen reveals widespread contamination by agricultural pesticides. *Science of the Total Environment*, 615:208–218, 2018. 7

[33] Mickaël Henry, Maxime Beguin, Fabrice Requier, Orianne Rollin, Jean-François Odoux, Pierrick Aupinel, Jean Aptel, Sylvie Tchamitchian, and Axel Decourtye. A common pesticide decreases foraging success and survival in honey bees. *Science*, 336(6079):348–350, 2012. ISSN 0036-8075. 7, 91

[34] Lucio G Costa, Gennaro Giordano, Marina Guizzetti, and Annabella Vitalone. Neurotoxicity of pesticides: a brief review. *Front Biosci*, 13(4): 1240–1249, 2008. 7, 92

[35] Glenn F King and Margaret C Hardy. Spider-venom peptides: structure, pharmacology, and potential for control of insect pests. *Annual review of entomology*, 58:475–496, 2013. 8, 9, 57, 58

[36] Monique J Windley, Volker Herzig, Sławomir A Dziemborowicz, Margaret C Hardy, Glenn F King, and Graham M Nicholson. Spider-venom peptides as bioinsecticides. *Toxins*, 4(3):191–227, 2012. 9, 86

[37] Paul A Whetstone and Bruce D Hammock. Delivery methods for peptide and protein toxins in insect control. *Toxicon*, 49(4):576–596, 2007. ISSN 0041-0101. 8, 10, 11, 17

[38] Szymon Chowański, Milena Kudlewska, Paweł Marciniak, and Grzegorz Rosiński. Synthetic insecticides–is there an alternative? *Polish Journal of Environmental Studies*, 23(2), 2014. 8, 10, 11, 57, 58

[39] Andrew Fire, SiQun Xu, Mary K Montgomery, Steven A Kostas, Samuel E Driver, and Craig C Mello. Potent and specific genetic interference by

double-stranded rna in caenorhabditis elegans. *nature*, 391(6669):806–811, 1998. 8, 13, 43, 92

[40] Steve Whyard, Cassidy NG Erdelyan, Alison L Partridge, Aditi D Singh, Nigel W Beebe, and Rupert Capina. Silencing the buzz: a new approach to population suppression of mosquitoes by feeding larvae double-stranded rnas. *Parasites & vectors*, 8(1):96, 2015. 40

[41] Guy Smagghe, Kamran Mahdian, Pawel Zubrzak, and Ronald J Nachman. Antifeedant activity and high mortality in the pea aphid acyrthosiphon pisum (hemiptera: Aphidae) induced by biostable insect kinin analogs. *Peptides*, 31(3):498–505, 2010. 8, 57

[42] Erich Y. T. Nakasu, Sally M. Williamson, Martin G. Edwards, Elaine C. Fitches, John A. Gatehouse, Geraldine A. Wright, and Angharad M. R. Gatehouse. Novel biopesticide based on a spider venom peptide shows no adverse effects on honeybees. *Proceedings of the Royal Society B: Biological Sciences*, 281(1787), 2014. doi: 10.1098/rspb.2014.0619. 8, 57

[43] Natalie J Saez, Sebastian Senff, Jonas E Jensen, Sing Yan Er, Volker Herzig, Lachlan D Rash, and Glenn F King. Spider-venom peptides as therapeutics. *Toxins*, 2(12):2851–2871, 2010. 9, 10, 58

[44] Laura A Jeffers, Hongyan Shen, Sayed Khalil, Brooke W Bissinger, Alan Brandt, T Brent Gunnoe, and R Michael Roe. Enhanced activity of an insecticidal protein, trypsin modulating oostatic factor (tmof), through conjugation with aliphatic polyethylene glycol. *Pest management science*, 68 (1):49–59, 2012. 10, 11, 20, 21, 58

[45] Orna Ben-Aziz, Irina Zeltser, and Miriam Altstein. Pban selective antagonists: inhibition of pban induced cuticular melanization and sex pheromone biosynthesis in moths. *Journal of insect physiology*, 51(3):305–314, 2005. 10

[46] Ronald J Nachman, Peter EA Teal, and Allison Strey. Enhanced oral availability/pheromonotropic activity of peptidase-resistant topical amphiphilic

analogs of pyrokinin/pban insect neuropeptides. *Peptides*, 23(11):2035–2043, 2002. 10, 58

[47] Muhammad Sarwar. Microbial insecticides-an ecofriendly effective line of attack for insect pests management. *International Journal of Engineering and Advanced Research Technology*, 1(2):4–9, 2015. 11

[48] B Ratna Kumari, R Vijayabharathi, S Srinivas, and S Gopalakrishnan. Microbes as interesting source of novel insecticides: A review. *African Journal of Biotechnology*, 13(26), 2014. 11, 12, 17

[49] Alejandra Bravo, Supaporn Likitvivatanavong, Sarjeet S Gill, and Mario Soberón. Bacillus thuringiensis: a story of a successful bioinsecticide. *Insect biochemistry and molecular biology*, 41(7):423–431, 2011. 11, 17, 57

[50] Juan J Estruch, Gregory W Warren, Martha A Mullins, Gordon J Nye, Joyce A Craig, and Michael G Koziel. Vip3a, a novel bacillus thuringiensis vegetative insecticidal protein with a wide spectrum of activities against lepidopteran insects. *Proceedings of the National Academy of Sciences*, 93 (11):5389–5394, 1996.

[51] Mi Kyong Lee, Frederick S Walters, Hope Hart, Narendra Palekar, and Jeng-Shong Chen. The mode of action of the bacillus thuringiensis vegetative insecticidal protein vip3a differs from that of cry1ab δ-endotoxin. *Applied and Environmental Microbiology*, 69(8):4648–4657, 2003. 11

[52] Xiumei Yu, Aiping Zheng, Jun Zhu, Shiquan Wang, Lingxia Wang, Qiming Deng, Shuangcheng Li, Huainian Liu, and Ping Li. Characterization of vegetative insecticidal protein vip genes of bacillus thuringiensis from sichuan basin in china. *Current microbiology*, 62(3):752–757, 2011. 12

[53] Mi Kyong Lee, Paul Miles, and Jeng-Shong Chen. Brush border membrane binding properties of bacillus thuringiensis vip3a toxin to heliothis virescens and helicoverpa zea midguts. *Biochemical and biophysical research communications*, 339(4):1043–1047, 2006. 12

[54] Michael Blackburn, Elena Golubeva, David Bowen, and Richard H Ffrench-Constant. A novel insecticidal toxin from photorhabdus luminescens, toxin complex a (tca), and its histopathological effects on the midgut of manduca sexta. *Applied and Environmental Microbiology*, 64(8):3036–3041, 1998. 12

[55] Michael J Landsberg, Sandra A Jones, Rosalba Rothnagel, Jason N Busby, Sean DG Marshall, Robert M Simpson, J Shaun Lott, Ben Hankamer, and Mark RH Hurst. 3d structure of the yersinia entomophaga toxin complex and implications for insecticidal activity. *Proceedings of the National Academy of Sciences*, 108(51):20544–20549, 2011. 12

[56] Christos Gatsogiannis, Felipe Merino, Daniel Prumbaum, Daniel Roderer, Franziska Leidreiter, Dominic Meusch, and Stefan Raunser. Membrane insertion of a tc toxin in near-atomic detail. *Nature structural & molecular biology*, 2016. 12

[57] Thais Barros Rodrigues and Antonio Figueira. Management of insect pest by rnaiâĂŤa new tool for crop protection. *RNA INTERFERENCE*, page 371, 2016. 12, 13, 16, 17

[58] Su Guo and Kenneth J Kemphues. par-1, a gene required for establishing polarity in c. elegans embryos, encodes a putative ser/thr kinase that is asymmetrically distributed. *Cell*, 81(4):611–620, 1995. 13

[59] Hao Zhang, Hai-Chao Li, and Xue-Xia Miao. Feasibility, limitation and possible solutions of rnai-based technology for insect pest control. *Insect science*, 20(1):15–30, 2013. 13, 17

[60] Jeffrey G Scott, Kristin Michel, Lyric C Bartholomay, Blair D Siegfried, Wayne B Hunter, Guy Smagghe, Kun Yan Zhu, and Angela E Douglas. Towards the elements of successful insect rnai. *Journal of insect physiology*, 59(12):1212–1221, 2013. 13, 16

[61] Clauvis Nji Tizi Taning, Olivier Christiaens, Nick Berkvens, Hans Casteels, Martine Maes, and Guy Smagghe. Oral rnai to control drosophila suzukii. *Journal of Pest Science*, 89(3):803–814, 2016. 13, 94, 105, 114

[62] Niels Wynant, Dulce Santos, Rik Verdonck, Jornt Spit, Pieter Van Wielendaele, and Jozef Vanden Broeck. Identification, functional characterization and phylogenetic analysis of double stranded rna degrading enzymes present in the gut of the desert locust, schistocerca gregaria. *Insect biochemistry and molecular biology*, 46:1–8, 2014. 14, 15

[63] Daniel RG Price and John A Gatehouse. Rnai-mediated crop protection against insects. *Trends in biotechnology*, 26(7):393–400, 2008. 15, 92

[64] Rayssa Almeida Garcia, Leonardo Lima Pepino Macedo, Danila Cabral do Nascimento, François-Xavier Gillet, Clidia Eduarda Moreira-Pinto, Muhammad Faheem, Angelina Maria Moreschi Basso, Maria Cristina Mattar Silva, and Maria Fatima Grossi-de Sa. Nucleases as a barrier to gene silencing in the cotton boll weevil, anthonomus grandis. *PloS one*, 12(12): e0189600, 2017. 15, 54

[65] Kangxu Wang, Yingchuan Peng, Jian Pu, Wenxi Fu, Jiale Wang, and Zhaojun Han. Variation in rnai efficacy among insect species is attributable to dsrna degradation in vivo. *Insect Biochemistry and Molecular Biology*, 77: 1–9, 2016. 15

[66] Yuji Arimatsu, Eiji Kotani, Yukio Sugimura, and Toshiharu Furusawa. Molecular characterization of a cdna encoding extracellular dsrnase and its expression in the silkworm, bombyx mori. *Insect biochemistry and molecular biology*, 37(2):176–183, 2007. 15

[67] Jisheng Liu, Luc Swevers, Kostas Iatrou, Hanneke Huvenne, and Guy Smagghe. Bombyx mori dna/rna non-specific nuclease: expression of isoforms in insect culture cells, subcellular localization and functional assays. *Journal of insect physiology*, 58(8):1166–1176, 2012. 15

[68] Olivier Christiaens, Luc Swevers, and Guy Smagghe. Dsrna degradation in the pea aphid (acyrthosiphon pisum) associated with lack of response in rnai feeding and injection assay. *Peptides*, 53:307–314, 2014. 15

[69] Jeremy M Kupsco, Ming-Jing Wu, William F Marzluff, Roopa Thapar, and Robert J Duronio. Genetic and biochemical characterization of drosophila snipper: A promiscuous member of the metazoan 3' hexo/eri-1 family of 3' to 5' exonucleases. *Rna*, 12(12):2103–2117, 2006. 15

[70] Hanneke Huvenne and Guy Smagghe. Mechanisms of dsrna uptake in insects and potential of rnai for pest control: a review. *Journal of insect physiology*, 56(3):227–235, 2010. 16

[71] Johanna Ulvila, Mataleena Parikka, Anni Kleino, Raija Sormunen, R Alan Ezekowitz, Christine Kocks, and Mika Rämet. Double-stranded rna is internalized by scavenger receptor-mediated endocytosis in drosophila s2 cells. *Journal of Biological Chemistry*, 281(20):14370–14375, 2006. 16

[72] Maria-Carla Saleh, Ronald P van Rij, Armin Hekele, Amethyst Gillis, Edan Foley, Patrick H O'Farrell, and Raul Andino. The endocytic pathway mediates cell entry of dsrna to induce rnai silencing. *Nature cell biology*, 8(8): 793–802, 2006. 16

[73] Titia Sijen, Jamie Fleenor, Femke Simmer, Karen L Thijssen, Susan Parrish, Lisa Timmons, Ronald HA Plasterk, and Andrew Fire. On the role of rna amplification in dsrna-triggered gene silencing. *Cell*, 107(4):465–476, 2001. 17

[74] Yoshinori Tomoyasu, Sherry C Miller, Shuichiro Tomita, Michael Schoppmeier, Daniela Grossmann, and Gregor Bucher. Exploring systemic rna interference in insects: a genome-wide survey for rnai genes in tribolium. *Genome biology*, 9(1):R10, 2008. 17

[75] K Zhu-Salzman, H Koiwa, RA Salzman, RE Shade, and J-E Ahn. Cowpea bruchid callosobruchus maculatus uses a three-component strategy to overcome a plant defensive cysteine protease inhibitor. *Insect molecular biology*, 12(2):135–145, 2003. 18

[76] Maria Lígia R Macedo, Caio FR Oliveira, and Carolina T Oliveira. Insecticidal activity of plant lectins and potential application in crop protection. *Molecules*, 20(2):2014–2033, 2015. 18

[77] Elaine Charlotte Fitches. *The mechanisms of action of insecticidal lectins from snowdrop (GNA) and jackbean (concanavalin a) ontomato moth larvae*. PhD thesis, Durham University, 1998. 18

[78] Elaine Fitches, Neil Audsley, John A Gatehouse, and John P Edwards. Fusion proteins containing neuropeptides as novel insect contol agents: snowdrop lectin delivers fused allatostatin to insect haemolymph following oral ingestion. *Insect Biochemistry and Molecular Biology*, 32(12):1653–1661, 2002. 18

[79] Elaine Fitches, Stephen D Woodhouse, John P Edwards, and John A Gatehouse. In vitro and in vivo binding of snowdrop (galanthus nivalis agglutinin; gna) and jackbean (canavalia ensiformis; con a) lectins within tomato moth (lacanobia oleracea) larvae; mechanisms of insecticidal action. *Journal of Insect Physiology*, 47(7):777–787, 2001. 18

[80] Elaine Fitches, Martin G Edwards, Christopher Mee, Eugene Grishin, Angharad MR Gatehouse, John P Edwards, and John A Gatehouse. Fusion proteins containing insect-specific toxins as pest control agents: snowdrop lectin delivers fused insecticidal spider venom toxin to insect haemolymph following oral ingestion. *Journal of Insect Physiology*, 50(1):61–71, 2004. 18, 19

[81] Sheng Yang, Prashant Pyati, Elaine Fitches, and John A Gatehouse. A recombinant fusion protein containing a spider toxin specific for the insect voltage-gated sodium ion channel shows oral toxicity towards insects of different orders. *Insect biochemistry and molecular biology*, 47:1–11, 2014. 19

[82] Nghia Pham Trung, Elaine Fitches, and John A Gatehouse. A fusion protein containing a lepidopteran-specific toxin from the south indian red scorpion (mesobuthus tamulus) and snowdrop lectin shows oral toxicity to target insects. *BMC biotechnology*, 6(1):18, 2006. 19

[83] Sunita Tajne, Dayakar Boddupally, Vijayakumar Sadumpati, Dashavantha Reddy Vudem, and Venkateswara Rao Khareedu. Synthetic fusion-protein containing domains of bt cry1ac and allium sativum lectin (asal) conferred enhanced insecticidal activity against major lepidopteran pests. *Journal of biotechnology*, 171:71–75, 2014. 19

[84] Bryony C Bonning and Nanasaheb P Chougule. Delivery of intrahemocoelic peptides for insect pest management. *Trends in biotechnology*, 32(2):91–98, 2014. ISSN 0167-7799. 19, 58

[85] Saskia A Hogenhout, El-Desouky Ammar, Anna E Whitfield, and Margaret G Redinbaugh. Insect vector interactions with persistently transmitted viruses. *Annu. Rev. Phytopathol.*, 46:327–359, 2008. 19

[86] Bryony C Bonning, Narinder Pal, Sijun Liu, Zhaohui Wang, S Sivakumar, Philip M Dixon, Glenn F King, and W Allen Miller. Toxin delivery by the coat protein of an aphid-vectored plant virus provides plant resistance to aphids. *Nature biotechnology*, 32(1):102, 2014. 19

[87] Laura A Jeffers, Hongyan Shen, Brooke W Bissinger, Sayed Khalil, T Brent Gunnoe, and R Michael Roe. Polymers for the stabilization and delivery of proteins topically and per os to the insect hemocoel through conjugation with aliphatic polyethylene glycol. *Pesticide biochemistry and physiology*, 115:58–66, 2014. 20, 58

[88] R Roe and Alan Brandt. Polymer conjugates of insecticidal peptides or nucleic acids or insecticides and methods of use thereof, June 12 2003. US Patent App. 10/285,398.

[89] Laura A Jeffers and R Michael Roe. The movement of proteins across the insect and tick digestive system. *Journal of insect physiology*, 54(2): 319–332, 2008. ISSN 0022-1910. 20

[90] Hongyan Shen, Alan Brandt, Brooke E Witting-Bissinger, T Brent Gunnoe, and R Michael Roe. Novel insecticide polymer chemistry to reduce the enzymatic digestion of a protein pesticide, trypsin modulating oostatic

factor (tmof). *Pesticide biochemistry and physiology*, 93(3):144–152, 2009. 20, 21

[91] Mahima Sharma, Ramesh S Hire, Ashok B Hadapad, Gagan D Gupta, and Vinay Kumar. Pegylation enhances mosquito-larvicidal activity of lysinibacillus sphaericus binary toxin. *Bioconjugate chemistry*, 28(2):410–418, 2017. 21

[92] Tina Vermonden, Roberta Censi, and Wim E Hennink. Hydrogels for protein delivery. *Chemical Reviews*, 112(5):2853–2888, 2012. 22, 23, 24, 28

[93] Nafees N Malik. Drug discovery: past, present and future, 2008. 22

[94] Kuen Yong Lee and Soon Hong Yuk. Polymeric protein delivery systems. *Progress in polymer science*, 32(7):669–697, 2007. ISSN 0079-6700. 22, 23, 24, 27, 58

[95] Meera George and T Emilia Abraham. Polyionic hydrocolloids for the intestinal delivery of protein drugs: alginate and chitosanâĂŤa review. *Journal of controlled release*, 114(1):1–14, 2006. 22, 24, 25, 26

[96] Niren Murthy, Mingcheng Xu, Stephany Schuck, Jun Kunisawa, Nilabh Shastri, and Jean MJ FrÃľchet. A macromolecular delivery vehicle for protein-based vaccines: acid-degradable protein-loaded microgels. *Proceedings of the National Academy of Sciences*, 100(9):4995–5000, 2003. ISSN 0027-8424. 23, 34, 58

[97] Stephany M Standley, Young Jik Kwon, Niren Murthy, Jun Kunisawa, Nilabh Shastri, Steven J Guillaudeu, Lana Lau, and Jean MJ FrÃľchet. Acid-degradable particles for protein-based vaccines: enhanced survival rate for tumor-challenged mice using ovalbumin model. *Bioconjugate chemistry*, 15 (6):1281–1288, 2004. ISSN 1043-1802. 22, 58

[98] Mark C Manning, Kamlesh Patel, and Ronald T Borchardt. Stability of protein pharmaceuticals. *Pharmaceutical research*, 6(11):903–918, 1989. 23

[99] Mark Cornell Manning, Danny K Chou, Brian M Murphy, Robert W Payne, and Derrick S Katayama. Stability of protein pharmaceuticals: an update. *Pharmaceutical research*, 27(4):544–575, 2010. 23

[100] Kwangwoo Nam, Junji Watanabe, and Kazuhiko Ishihara. Modeling of swelling and drug release behavior of spontaneously forming hydrogels composed of phospholipid polymers. *International journal of pharmaceutics*, 275(1-2):259–269, 2004. 23

[101] Gert W Bos, John JL Jacobs, Jan Willem Koten, Sophie Van Tomme, Theo Veldhuis, Cornelus F van Nostrum, Willem Den Otter, and Wim E Hennink. In situ crosslinked biodegradable hydrogels loaded with il-2 are effective tools for local il-2 therapy. *European Journal of Pharmaceutical Sciences*, 21(4):561–567, 2004.

[102] Jung Kwon Oh, Ray Drumright, Daniel J Siegwart, and Krzysztof Matyjaszewski. The development of microgels/nanogels for drug delivery applications. *Progress in Polymer Science*, 33(4):448–477, 2008. 24

[103] Alexander V Kabanov and Serguei V Vinogradov. Nanogels as pharmaceutical carriers: finite networks of infinite capabilities. *Angewandte Chemie International Edition*, 48(30):5418–5429, 2009. ISSN 1521-3773. 23, 58

[104] H Okino, Y Nakayama, M Tanaka, and T Matsuda. In situ hydrogelation of photocurable gelatin and drug release. *Journal of Biomedical Materials Research Part A*, 59(2):233–245, 2002. 24

[105] George AF Roberts. Structure of chitin and chitosan. In *Chitin chemistry*, pages 1–53. Springer, 1992. 24, 25

[106] Twana Mohammed M Ways, Wing Man Lau, and Vitaliy V Khutoryanskiy. Chitosan and its derivatives for application in mucoadhesive drug delivery systems. *Polymers*, 10(3):267, 2018. 25

[107] Ioannis A Sogias, Adrian C Williams, and Vitaliy V Khutoryanskiy. Why is chitosan mucoadhesive? *Biomacromolecules*, 9(7):1837–1842, 2008. 25

[108] Ping He, Stanley S Davis, and Lisbeth Illum. In vitro evaluation of the mucoadhesive properties of chitosan microspheres. *International journal of pharmaceutics*, 166(1):75–88, 1998. 25

[109] Claus-Michael Lehr, Joke A Bouwstra, Etienne H Schacht, and Hans E Junginger. In vitro evaluation of mucoadhesive properties of chitosan and some other natural polymers. *International journal of Pharmaceutics*, 78 (1-3):43–48, 1992. 25

[110] Nicolaas GM Schipper, Susanne Olsson, Janet A Hoogstraate, Kjell M Vårum, Per Artursson, et al. Chitosans as absorption enhancers for poorly absorbable drugs 2: mechanism of absorption enhancement. *Pharmaceutical research*, 14(7):923–929, 1997. 25

[111] Andreas Bernkop-Schnürch, Margit Hornof, and Davide Guggi. Thiolated chitosans. *European Journal of Pharmaceutics and Biopharmaceutics*, 57 (1):9–17, 2004. 25

[112] Margit D Hornof, Constantia E Kast, and Andreas Bernkop-Schnürch. In vitro evaluation of the viscoelastic properties of chitosan–thioglycolic acid conjugates. *European journal of pharmaceutics and biopharmaceutics*, 55 (2):185–190, 2003.

[113] Krum Kafedjiiski, Alexander H Krauland, Martin H Hoffer, and Andreas Bernkop-Schnürch. Synthesis and in vitro evaluation of a novel thiolated chitosan. *Biomaterials*, 26(7):819–826, 2005. 25

[114] Giuseppina Sandri, Silvia Rossi, Maria Cristina Bonferoni, Franca Ferrari, Ylenia Zambito, Giacomo Di Colo, and Carla Caramella. Buccal penetration enhancement properties of n-trimethyl chitosan: influence of quaternization degree on absorption of a high molecular weight molecule. *International journal of pharmaceutics*, 297(1-2):146–155, 2005. 25

[115] Dewald Snyman, Josias H Hamman, and Awie F Kotze. Evaluation of the mucoadhesive properties of n-trimethyl chitosan chloride. *Drug development and industrial pharmacy*, 29(1):61–69, 2003. 25

[116] Lingyun Chen, Zhigang Tian, and Yumin Du. Synthesis and ph sensitivity of carboxymethyl chitosan-based polyampholyte hydrogels for protein carrier matrices. *Biomaterials*, 25(17):3725–3732, 2004. 25

[117] David Byrom. *Biomaterials: novel materials from biological sources*. Springer, 1991. 26

[118] Olav Smidsrød, Gudmund Skja, et al. Alginate as immobilization matrix for cells. *Trends in biotechnology*, 8:71–78, 1990. 26

[119] Mehrdad Hamidi, Amir Azadi, and Pedram Rafiei. Hydrogel nanoparticles in drug delivery. *Advanced drug delivery reviews*, 60(15):1638–1649, 2008. 27

[120] Jeanie L Drury and David J Mooney. Hydrogels for tissue engineering: scaffold design variables and applications. *Biomaterials*, 24(24):4337–4351, 2003. 27

[121] Jia Kui Li, Nuo Wang, and Xue Shen Wu. Poly (vinyl alcohol) nanoparticles prepared by freezing–thawing process for protein/peptide drug delivery. *Journal of Controlled Release*, 56(1-3):117–126, 1998. 27

[122] Thomas Kissel, Armin Breitenbach, Tobias Jung, and Walter Kamm. Self-assembling colloidal carriers for protein delivery, September 9 2003. US Patent 6,616,944. 27

[123] Kang Moo Huh and You Han Bae. Synthesis and characterization of poly (ethylene glycol)/poly (l-lactic acid) alternating multiblock copolymers. *Polymer*, 40(22):6147–6155, 1999. 27

[124] AT Metters, KS Anseth, and CN Bowman. Fundamental studies of a novel, biodegradable peg-b-pla hydrogel. *Polymer*, 41(11):3993–4004, 2000. 27

[125] Ding Guowei, Kamulegeya Adriane, XingZai Chen, Chen Jie, and Liu Yinfeng. Pvp magnetic nanospheres: Biocompatibility, in vitro and in vivo bleomycin release. *International journal of pharmaceutics*, 328(1):78–85, 2007. 28

[126] T Jung, W Kamm, A Breitenbach, E Kaiserling, JX Xiao, and T Kissel. Biodegradable nanoparticles for oral delivery of peptides: is there a role for polymers to affect mucosal uptake? *European Journal of Pharmaceutics and Biopharmaceutics*, 50(1):147–160, 2000. 28, 29

[127] Avnesh Kumari, Sudesh Kumar Yadav, and Subhash C Yadav. Biodegradable polymeric nanoparticles based drug delivery systems. *Colloids and Surfaces B: Biointerfaces*, 75(1):1–18, 2010. 28, 29, 31, 32

[128] Kumaresh S Soppimath, Tejraj M Aminabhavi, Anandrao R Kulkarni, and Walter E Rudzinski. Biodegradable polymeric nanoparticles as drug delivery devices. *Journal of controlled release*, 70(1-2):1–20, 2001. 29, 31

[129] Anne des Rieux, Virginie Fievez, Marie Garinot, Yves-Jacques Schneider, and Véronique Préat. Nanoparticles as potential oral delivery systems of proteins and vaccines: a mechanistic approach. *Journal of controlled release*, 116(1):1–27, 2006. 29, 30

[130] Ya-Ping Li, Yuan-Ying Pei, Xian-Ying Zhang, Zhou-Hui Gu, Zhao-Hui Zhou, Wei-Fang Yuan, Jian-Jun Zhou, Jian-Hua Zhu, and Xiu-Jian Gao. Pegylated plga nanoparticles as protein carriers: synthesis, preparation and biodistribution in rats. *Journal of controlled release*, 71(2):203–211, 2001. 29

[131] ML Hans and AM Lowman. Biodegradable nanoparticles for drug delivery and targeting. *Current Opinion in Solid State and Materials Science*, 6(4): 319–327, 2002. 29

[132] JM Rodrigues Jr, H Fessi, C Bories, F Puisieux, et al. Primaquine-loaded poly (lactide) nanoparticles: physicochemical study and acute tolerance in mice. *International journal of pharmaceutics*, 126(1-2):253–260, 1995. 31

[133] Massimo Fresta, Giovanni Puglisi, Gaetano Giammona, Gennara Cavallaro, Norberto Micali, and Pio M Furneri. Pefloxacine mesilate-and ofloxacin-loaded polyethylcyanoacrylate nanoparticles: Characterization of the colloidal drug carrier formulation. *Journal of pharmaceutical sciences*, 84(7): 895–902, 1995. 31

[134] Pilar Calvo, José L Vila-Jato, and María J Alonso. Comparative in vitro evaluation of several colloidal systems, nanoparticles, nanocapsules, and nanoemulsions, as ocular drug carriers. *Journal of pharmaceutical sciences*, 85(5):530–536, 1996. 31

[135] MJAM Tobio, R Gref, A Sanchez, R Langer, and MJ Alonso. Stealth pla-peg nanoparticles as protein carriers for nasal administration. *Pharmaceutical research*, 15(2):270–275, 1998. 31

[136] Raghavendra C Mundargi, V Ramesh Babu, Vidhya Rangaswamy, Pradip Patel, and Tejraj M Aminabhavi. Nano/micro technologies for delivering macromolecular therapeutics using poly (d, l-lactide-co-glycolide) and its derivatives. *Journal of Controlled Release*, 125(3):193–209, 2008. 32, 33

[137] Fabienne Danhier, Eduardo Ansorena, Joana M Silva, Régis Coco, Aude Le Breton, and Véronique Préat. Plga-based nanoparticles: an overview of biomedical applications. *Journal of controlled release*, 161(2):505–522, 2012. 32

[138] Chuanyun Dai, Bochu Wang, and Hongwei Zhao. Microencapsulation peptide and protein drugs delivery system. *Colloids and Surfaces B: Biointerfaces*, 41(2-3):117–120, 2005. 32

[139] Paolo Giunchedi, Bice Conti, Ida Genta, Ubaldo Conte, and Giovanni Puglisi. Emulsion spray-drying for the preparation of albumin-loaded plga microspheres. *Drug development and industrial pharmacy*, 27(7):745–750, 2001. 32

[140] Feirong Kang and Jagdish Singh. Effect of additives on the release of a model protein from plga microspheres. *Aaps Pharmscitech*, 2(4):86–92, 2001. 32

[141] H Gao, YN Wang, YG Fan, and JB Ma. Synthesis of a biodegradable tadpole-shaped polymer via the coupling reaction of polylactide onto mono (6-(2-aminoethyl) amino-6-deoxy)-β-cyclodextrin and its properties as the new carrier of protein delivery system. *Journal of controlled release*, 107 (1):158–173, 2005. 32

[142] Junko Matsumoto, Yuichiro Nakada, Kazuo Sakurai, Tomomi Nakamura, and Yoshiteru Takahashi. Preparation of nanoparticles consisted of poly (l-lactide)–poly (ethylene glycol)–poly (l-lactide) and their evaluation in vitro. *International journal of pharmaceutics*, 185(1):93–101, 1999. 32

[143] G Kocak, C Tuncer, and V Bütün. ph-responsive polymers. *Polymer Chemistry*, 8(1):144–176, 2017. 34, 35, 36, 37

[144] Carolina De las Heras Alarcón, Sivanand Pennadam, and Cameron Alexander. Stimuli responsive polymers for biomedical applications. *Chemical Society Reviews*, 34(3):276–285, 2005.

[145] Adam E Smith, Xuewei Xu, and Charles L McCormick. Stimuli-responsive amphiphilic (co) polymers via raft polymerization. *Progress in polymer science*, 35(1-2):45–93, 2010.

[146] Yong Qiu and Kinam Park. Environment-sensitive hydrogels for drug delivery. *Advanced drug delivery reviews*, 53(3):321–339, 2001. 34

[147] Niren Murthy, Yi X Thng, Stephany Schuck, Ming C Xu, and Jean MJ Frechet. A novel strategy for encapsulation and release of proteins: hydrogels and microgels with acid-labile acetal cross-linkers. *Journal of the American Chemical Society*, 124(42):12398–12399, 2002. 34

[148] Dirk Schmaljohann. Thermo-and ph-responsive polymers in drug delivery. *Advanced drug delivery reviews*, 58(15):1655–1670, 2006. 38

[149] Shubha Shanbhag and Subrata Tripathi. Epithelial ultrastructure and cellular mechanisms of acid and base transport in the drosophila midgut. *Journal of Experimental Biology*, 212(11):1731–1744, 2009. ISSN 0022-0949. 38, 59, 83

[150] Brian Dalby, Sharon Cates, Adam Harris, Elise C Ohki, Mary L Tilkins, Paul J Price, and Valentina C Ciccarone. Advanced transfection with lipofectamine 2000 reagent: primary neurons, sirna, and high-throughput applications. *Methods*, 33(2):95–103, 2004. 39

[151] Yu-Hsien Lin, Jia-Hsin Huang, Yun Liu, Xavier Belles, and How-Jing Lee. Oral delivery of dsrna lipoplexes to german cockroach protects dsrna from degradation and induces rnai response. *Pest management science*, 73(5): 960–966, 2017. 39

[152] Yuting Zhang, Jie Cui, Yongzhi Zhou, Jie Cao, Haiyan Gong, Houshuang Zhang, and Jinlin Zhou. Liposome mediated double-stranded rna delivery to silence ribosomal protein p0 in the tick rhipicephalus haemaphysaloides. *Ticks and tick-borne diseases*, 2018. 39, 92

[153] Sushanth Gudlur, Pinakin Sukthankar, Jian Gao, L Adriana Avila, Yasuaki Hiromasa, Jianhan Chen, Takeo Iwamoto, and John M Tomich. Peptide nanovesicles formed by the self-assembly of branched amphiphilic peptides. *PLoS One*, 7(9):e45374, 2012. 39

[154] LA Avila, R Chandrasekar, KE Wilkinson, J Balthazor, M Heerman, J Bechard, S Brown, Y Park, S Dhar, GR Reeck, et al. Delivery of lethal dsrnas in insect diets by branched amphiphilic peptide capsules. *Journal of Controlled Release*, 273:139–146, 2018. 39

[155] X Zhang, J Zhang, and KY Zhu. Chitosan/double-stranded rna nanoparticle-mediated rna interference to silence chitin synthase genes through larval feeding in the african malaria mosquito (anopheles gambiae). *Insect molecular biology*, 19(5):683–693, 2010. 40

[156] Mallikarjuna R Joga, Moises J Zotti, Guy Smagghe, and Olivier Christiaens. Rnai efficiency, systemic properties, and novel delivery methods for pest insect control: what we know so far. *Frontiers in physiology*, 7:553, 2016. 40, 92

[157] DeeDee Smith, Andrew C Holley, and Charles L McCormick. Raft-synthesized copolymers and conjugates designed for therapeutic delivery of sirna. *Polymer Chemistry*, 2(7):1428–1441, 2011. 40, 46

[158] D Ramesh Kumar, P Saravana Kumar, M Rajiv Gandhi, Naif Abdullah Al-Dhabi, M Gabriel Paulraj, and S Ignacimuthu. Delivery of chitosan/dsrna

nanoparticles for silencing of wing development vestigial (vg) gene in aedes aegypti mosquitoes. *International journal of biological macromolecules*, 86: 89–95, 2016. 41

[159] Sumistha Das, Nitai Debnath, Yingjun Cui, Jason Unrine, and Subba Reddy Palli. Chitosan, carbon quantum dot, and silica nanoparticle mediated dsrna delivery for gene silencing in aedes aegypti: a comparative analysis. *ACS applied materials & interfaces*, 7(35):19530–19535, 2015. 41

[160] Bicheng He, Yuan Chu, Meizhen Yin, Klaus Müllen, Chunju An, and Jie Shen. Fluorescent nanoparticle delivered dsrna toward genetic control of insect pests. *Advanced Materials*, 25(33):4580–4584, 2013. 41, 42

[161] KH Parsons, MH Mondal, CL McCormick, and AS Flynt. Guanidinium-functionalized interpolyelectrolyte complexes enable rnai in resistant insect pests. *Biomacromolecules*, 2018. 42, 43, 92, 108

[162] Sayda M Elbashir, Jens Harborth, Winfried Lendeckel, Abdullah Yalcin, Klaus Weber, and Thomas Tuschl. Duplexes of 21-nucleotide rnas mediate rna interference in cultured mammalian cells. *nature*, 411(6836):494, 2001. 43

[163] Natasha J Caplen, Susan Parrish, Farhad Imani, Andrew Fire, and Richard A Morgan. Specific inhibition of gene expression by small double-stranded rnas in invertebrate and vertebrate systems. *Proceedings of the National Academy of Sciences*, 98(17):9742–9747, 2001. 43

[164] Katrin Tiemann and John J Rossi. Rnai-based therapeutics–current status, challenges and prospects. *EMBO molecular medicine*, 1(3):142–151, 2009. 43, 44

[165] John C Burnett, John J Rossi, and Katrin Tiemann. Current progress of sirna/shrna therapeutics in clinical trials. *Biotechnology journal*, 6(9): 1130–1146, 2011. 43

[166] Thu Nguyen, Ellen M Menocal, Jens Harborth, and Johannes H Fruehauf. Rnai therapeutics: an update on delivery. *Current opinion in molecular therapeutics*, 10(2):158–167, 2008. 44

[167] Manfred Ogris, Peter Steinlein, Sebastian Carotta, Sylvia Brunner, and Ernst Wagner. Dna/polyethylenimine transfection particles: influence of ligands, polymer size, and pegylation on internalization and gene expression. *Aaps Pharmsci*, 3(3):43, 2001. 44

[168] Dagmar Fischer, Thorsten Bieber, Youxin Li, Hans-Peter Elsässer, and Thomas Kissel. A novel non-viral vector for dna delivery based on low molecular weight, branched polyethylenimine: effect of molecular weight on transfection efficiency and cytotoxicity. *Pharmaceutical research*, 16(8): 1273–1279, 1999.

[169] Antoine Kichler. Gene transfer with modified polyethylenimines. *The journal of gene medicine*, 6(S1), 2004. 44

[170] B Urban-Klein, S Werth, S Abuharbeid, F Czubayko, and A Aigner. Rnai-mediated gene-targeting through systemic application of polyethylenimine (pei)-complexed sirna in vivo. *Gene therapy*, 12(5):461, 2005. 44, 105

[171] Amy C Richards Grayson, Anne M Doody, and David Putnam. Biophysical and structural characterization of polyethylenimine-mediated sirna delivery in vitro. *Pharmaceutical research*, 23(8):1868–1876, 2006. 44, 45

[172] S Sajeesh, Tae Yeon Lee, Sun Woo Hong, Pooja Dua, Jeong Yong Choe, Aeyeon Kang, Wan Soo Yun, Changsik Song, Sung Ha Park, Soyoun Kim, et al. Long dsrna-mediated rna interference and immunostimulation: a targeted delivery approach using polyethyleneimine based nano-carriers. *Molecular pharmaceutics*, 11(3):872–884, 2014. 45

[173] Min Suk Shim and Young Jik Kwon. Acid-responsive linear polyethylenimine for efficient, specific, and biocompatible sirna delivery. *Bioconjugate chemistry*, 20(3):488–499, 2009. 45

[174] Min Suk Shim and Young Jik Kwon. Controlled cytoplasmic and nuclear localization of plasmid dna and sirna by differentially tailored polyethylenimine. *Journal of Controlled Release*, 133(3):206–213, 2009. 46, 105

[175] Min Suk Shim and Young Jik Kwon. Controlled delivery of plasmid dna and sirna to intracellular targets using ketalized polyethylenimine. *Biomacromolecules*, 9(2):444–455, 2008. 45, 98, 105

[176] Pablo A Coronado-Gonzalez, S Vijaysegaran, and Alan S Robinson. Functional morphology of the mouthparts of the adult mediterranean fruit fly, ceratitis capitata. *Journal of Insect Science*, 8(1):73, 2008. ISSN 1536-2442. 46, 83, 108

[177] Joanna Rejman, Volker Oberle, Inge S Zuhorn, and Dick Hoekstra. Size-dependent internalization of particles via the pathways of clathrin-and caveolae-mediated endocytosis. *Biochemical journal*, 377(Pt 1):159, 2004. 46, 108

[178] Helmut Ringsdorf. Structure and properties of pharmacologically active polymers. In *Journal of Polymer Science: Polymer Symposia*, volume 51, pages 135–153. Wiley Online Library, 1975. 46

[179] Tomáš Reschel, Čestmir Koňák, David Oupickỳ, Leonard W Seymour, and Karel Ulbrich. Physical properties and in vitro transfection efficiency of gene delivery vectors based on complexes of dna with synthetic polycations. *Journal of Controlled Release*, 81(1-2):201–217, 2002. 46

[180] Ye-Zi You, Devika Soundara Manickam, Qing-Hui Zhou, and David Oupickỳ. Reducible poly (2-dimethylaminoethyl methacrylate): synthesis, cytotoxicity, and gene delivery activity. *Journal of Controlled Release*, 122(3):217–225, 2007.

[181] Rachel A Jones, Maree H Poniris, and Mark R Wilson. pdmaema is internalised by endocytosis but does not physically disrupt endosomes. *Journal of controlled release*, 96(3):379–391, 2004. 46

[182] Won-Ho Kong, Dong-Kyung Sung, Yong-Ho Shim, Ki Hyun Bae, Philippe Dubois, Tae Gwan Park, Jin-Hoon Kim, and Soo-Won Seo. Efficient intracellular sirna delivery strategy through rapid and simple two steps mixing involving noncovalent post-pegylation. *Journal of Controlled Release*, 138 (2):141–147, 2009. 46

[183] Anja Schallon, Christopher V Synatschke, Valérie Jérôme, Axel HE Müller, and Ruth Freitag. Nanoparticulate nonviral agent for the effective delivery of pdna and sirna to differentiated cells and primary human t lymphocytes. *Biomacromolecules*, 13(11):3463–3474, 2012.

[184] Dana J Gary, Hoyoung Lee, Rahul Sharma, Jae-Sung Lee, Youngwook Kim, Zheng Yun Cui, Di Jia, Valorie D Bowman, Paul R Chipman, Lei Wan, et al. Influence of nano-carrier architecture on in vitro sirna delivery performance and in vivo biodistribution: polyplexes vs micelleplexes. *ACS nano*, 5(5): 3493–3505, 2011. 46, 47, 49, 93, 108

[185] Arjen M Funhoff, Cornelus F van Nostrum, Gerben A Koning, Nancy ME Schuurmans-Nieuwenbroek, Daan JA Crommelin, and Wim E Hennink. Endosomal escape of polymeric gene delivery complexes is not always enhanced by polymers buffering at low ph. *Biomacromolecules*, 5(1):32–39, 2004. 47

[186] Yong Woo Cho, Jong-Duk Kim, and Kinam Park. Polycation gene delivery systems: escape from endosomes to cytosol. *Journal of Pharmacy and Pharmacology*, 55(6):721–734, 2003. 47

[187] Nicolaas Zuidam, George Posthumab, Else de Vries, Daan Crommelin, Wim Hennink, and Gert Storm. Effects of physicochemical characteristics of poly (2-(dimethylamino) ethyl methacrylate)-based polyplexes on cellular association and internalization. *Journal of drug targeting*, 8(1):51–66, 2000. 47

[188] Haijuan Zou, Zhongjuan Wang, and Min Feng. Nanocarriers with tunable surface properties to unblock bottlenecks in systemic drug and gene delivery. *Journal of Controlled Release*, 214:121–133, 2015. 47, 93

[189] Amir K Varkouhi, Twan Lammers, Raymond M Schiffelers, Mies J Van Steenbergen, Wim E Hennink, and Gert Storm. Gene silencing activity of sirna polyplexes based on biodegradable polymers. *European Journal of Pharmaceutics and Biopharmaceutics*, 77(3):450–457, 2011. 47, 94

[190] Charles W Scales, Faqing Huang, Na Li, Yulia A Vasilieva, Jacob Ray, Anthony J Convertine, and Charles L McCormick. Corona-stabilized interpolyelectrolyte complexes of sirna with nonimmunogenic, hydrophilic/cationic block copolymers prepared by aqueous raft polymerization. *Macromolecules*, 39(20):6871–6881, 2006.

[191] Noura H Abd Ellah, Sarah J Potter, Leeanne Taylor, Neil Ayres, Mona M Elmahdy, Gihan N Fetih, El-Sayed A Ibrahim, and Giovanni M Pauletti. Safety and efficacy of amine-containing methacrylate polymers as nonviral gene delivery vectors. *journal of Pharmaceutical Technology and Drug Research*, 3(1):2, 2014. ISSN 2050-120X. 94, 100

[192] Noura Abd Ellah, Leeanne Taylor, Weston Troja, Kathryn Owens, Neil Ayres, Giovanni Pauletti, and Helen Jones. Development of non-viral, trophoblast-specific gene delivery for placental therapy. *PloS one*, 10(10): e0140879, 2015.

[193] David Valade, Cyrille Boyer, Thomas P Davis, and Volga Bulmus. Synthesis of sirna polyplexes adopting a combination of raft polymerization and thiolene chemistry. *Australian journal of chemistry*, 62(10):1344–1350, 2009. 47

[194] Meredith A Jackson, Thomas A Werfel, Elizabeth J Curvino, Fang Yu, Taylor E Kavanaugh, Samantha M Sarett, Mary D Dockery, Kameron V Kilchrist, Ayisha N Jackson, Todd D Giorgio, et al. Zwitterionic nanocarrier surface chemistry improves sirna tumor delivery and silencing activity relative to polyethylene glycol. *ACS nano*, 11(6):5680–5696, 2017. 47, 48, 93

[195] AJ Convertine, C Diab, M Prieve, A Paschal, AS Hoffman, PH Johnson, and PS Stayton. ph-responsive polymeric micelle carriers for sirna drugs. *Biomacromolecules*, 11(11):2904–2911, 2010. ISSN 1525-7797. 48, 100

[196] Anthony J Convertine, Danielle SW Benoit, Craig L Duvall, Allan S Hoffman, and Patrick S Stayton. Development of a novel endosomolytic diblock copolymer for sirna delivery. *Journal of controlled release*, 133(3):221–229, 2009. 48

[197] Luc Swevers and Guy Smagghe. *Use of RNAi for control of insect crop pests*, pages 177–197. Springer, 2012. ISBN 9400738722. 49, 83, 88, 92

[198] Daniel J Keddie. A guide to the synthesis of block copolymers using reversible-addition fragmentation chain transfer (raft) polymerization. *Chemical Society Reviews*, 43(2):496–505, 2014. 50, 51, 52, 53

[199] Sébastien Perrier. 50th anniversary perspective: Raft polymerization: A user guide. *Macromolecules*, 50(19):7433–7447, 2017. 50, 51, 53

[200] Graeme Moad, Ezio Rizzardo, and San H Thang. Radical addition–fragmentation chemistry in polymer synthesis. *Polymer*, 49(5):1079–1131, 2008. 50, 51

[201] Daniel J Keddie, Graeme Moad, Ezio Rizzardo, and San H Thang. Raft agent design and synthesis. *Macromolecules*, 45(13):5321–5342, 2012. 51

[202]) YK Chong, Julia Krstina, Tam PT Le, Graeme Moad, Almar Postma, Ezio Rizzardo, and San H Thang. Thiocarbonylthio compounds [sc (ph) s-r] in free radical polymerization with reversible addition-fragmentation chain transfer (raft polymerization). role of the free-radical leaving group (r). *Macromolecules*, 36(7):2256–2272, 2003. 51

[203] Andrew G. S Cuthbertson, Debbie A Collins, Lisa F Blackburn, Neil Audsley, and Howard A. Bell. Preliminary screening of potential control products against drosophila suzukii. *Insects*, 5:488–498, 2014. ISSN 2075-4450. 57

[204] Lauren M Diepenbrock, Danielle Olivieri Rosensteel, Jesse A Hardin, Ashfaq A Sial, and Hannah J Burrack. Season-long programs for control of drosophila suzukii in southeastern us blueberries. *Crop Protection*, 81:76–84, 2016.

[205] Tim Haye, Pierre Girod, AGS Cuthbertson, XG Wang, KM Daane, KA Hoelmer, Catherine Baroffio, JP Zhang, and Nicolas Desneux. Current swd ipm tactics and their practical implementation in fruit crops across different regions around the world. *Journal of pest science*, 89(3):643–651, 2016.

[206] DG Pfeiffer, JC Bergh, DL Frank, CRR Hooks, CS Walsh, KS Yoder, M Rahan, JB Kotcon, JF Derr, RS Chandran, et al. 2016 spray bulletin for commercial tree fruit growers. *Spray Bulletin for Commercial Tree Fruit Growers*, 2016. 57

[207] Travis Glare, John Caradus, Wendy Gelernter, Trevor Jackson, Nemat Keyhani, Jürgen Köhl, Pamela Marrone, Louise Morin, and Alison Stewart. Have biopesticides come of age? *Trends in biotechnology*, 30(5):250–258, 2012. ISSN 0167-7799. 57

[208] Margaret C Hardy, Norelle L Daly, Mehdi Mobli, Rodrigo AV Morales, and Glenn F King. Isolation of an orally active insecticidal toxin from the venom of an australian tarantula. *PLoS one*, 8(9):e73136, 2013. ISSN 1932-6203. 57, 58

[209] Gerd Gäde and Graham J Goldsworthy. Insect peptide hormones: a selective review of their physiology and potential application for pest control. *Pest management science*, 59(10):1063–1075, 2003. 57

[210] Ronald J Nachman. Mimetic analogs of pyrokinin neuropeptides for pest insect management. In *Biopesticides: State of the Art and Future Opportunities*, pages 83–94. ACS Publications, 2014.

[211] Ronald J Nachman, Mohamad Hamshou, Krzysztof Kaczmarek, Janusz Zabrocki, and Guy Smagghe. Biostable and peg polymer-conjugated insect pyrokinin analogs demonstrate antifeedant activity and induce high mortality in the pea aphid acyrthosiphon pisum (hemiptera: Aphidae). *Peptides*, 34(1):266–273, 2012. 57, 58

[212] Angela B Lange, Ronald J Nachman, Krzysztof Kaczmarek, and Janusz Zabrocki. Biostable insect kinin analogs reduce blood meal and disrupt ecdysis in the blood-gorging chagasâĂŹ disease vector, rhodnius prolixus. *Peptides*, 80:108–113, 2016. 57

[213] Aliza Hariton Shalev and Miriam Altstein. Pheromonotropic and melanotropic pk/pban receptors: Differential ligand–receptor interactions. *Peptides*, 63:81–89, 2015.

[214] Qirui Zhang, Ronald J Nachman, and David L Denlinger. Diapause hormone in the helicoverpa/heliothis complex: A review of gene expression, peptide structure and activity, analog and antagonist development, and the receptor. *Peptides*, 72:196–201, 2015. 57

[215] Md Nuruzzaman, Mohammad Mahmudur Rahman, Yanju Liu, and Ravi Naidu. Nanoencapsulation, nano-guard for pesticides: a new window for safe application. *Journal of agricultural and food chemistry*, 64(7):1447–1483, 2016. ISSN 0021-8561. 57

[216] Eliahu Zlotkin, Lena Fishman, and Jeffrey P Shapiro. Oral toxicity to flesh flies of a neurotoxic polypeptide. *Archives of insect biochemistry and physiology*, 21(1):41–52, 1992. 57

[217] Joel Atallah, Lisa Teixeira, Raul Salazar, George Zaragoza, and Artyom Kopp. The making of a pest: the evolution of a fruit-penetrating ovipositor in drosophila suzukii and related species. *Proceedings of the Royal Society of London B: Biological Sciences*, 281(1781):20132840, 2014. 58

[218] Larry L Murdock and Richard E Shade. Lectins and protease inhibitors as plant defenses against insects. *Journal of Agricultural and Food Chemistry*, 50(22):6605–6611, 2002. ISSN 0021-8561. 58

[219] Ronald J Nachman, Peter EA Teal, Orna Ben Aziz, Michael Davidovitch, Pawel Zubrzak, and Miriam Altstein. An amphiphilic, pk/pban analog is a selective pheromonotropic antagonist that penetrates the cuticle of a heliothine insect. *Peptides*, 30(3):616–621, 2009. 58

[220] Takahiro Morita, Yumi Sakamura, Yuji Horikiri, Takehiko Suzuki, and Hiroyuki Yoshino. Protein encapsulation into biodegradable microspheres by a novel s/o/w emulsion method using poly (ethylene glycol) as a protein micronization adjuvant. *Journal of controlled release*, 69(3):435–444, 2000. ISSN 0168-3659. 58

[221] James E Talmadge. The pharmaceutics and delivery of therapeutic polypeptides and proteins. *Advanced drug delivery reviews*, 10(2-3):247–299, 1993. ISSN 0169-409X. 58

[222] Mariko Morishita and Nicholas A Peppas. Is the oral route possible for peptide and protein drug delivery? *Drug discovery today*, 11(19):905–910, 2006. ISSN 1359-6446. 58

[223] VR Sinha and Aman Trehan. Biodegradable microspheres for protein delivery. *Journal of Controlled Release*, 90(3):261–280, 2003. ISSN 0168-3659. 58

[224] A Vila, A Sanchez, M Tobio, P Calvo, and MJ Alonso. Design of biodegradable particles for protein delivery. *Journal of Controlled Release*, 78(1): 15–24, 2002. ISSN 0168-3659. 58

[225] Shan Jiang, Choon Peng Teng, Wee Choo Puah, Martin Wasser, Khin Yin Win, and Ming-Yong Han. Oral administration and selective uptake of polymeric nanoparticles in drosophila larvae as an in vivo model. *ACS Biomaterials Science & Engineering*, 1(11):1077–1084, 2015. 59

[226] Jin-Woong Kim, Ji-Young Ko, Jung-Bae Jun, Ih-Seop Chang, Hak-Hee Kang, and Kyung-Do Suh. Multihollow polymer microcapsules by water-in-oil-in-water emulsion polymerization: morphological study and entrapment characteristics. *Colloid and Polymer Science*, 281(2):157–163, 2003. 59, 68

[227] Takayuki Takei, Kaoru Ikeda, Hiroyuki Ijima, Koei Kawakami, Masahiro Yoshida, and Yasuo Hatate. Preparation of polymeric microcapsules enclosing microbial cells by radical suspension polymerization via water-in-oil-in-water emulsion. *Polymer bulletin*, 65(3):283–291, 2010. ISSN 0170-0839. 59

[228] Marc Tatar, Stephanie Post, and Kweon Yu. Nutrient control of drosophila longevity. *Trends in Endocrinology & Metabolism*, 25(10):509–517, 2014. 66

[229] Damien Dupin, Syuji Fujii, Steven P Armes, Paul Reeve, and Steven M Baxter. Efficient synthesis of sterically stabilized ph-responsive microgels of controllable particle diameter by emulsion polymerization. *Langmuir*, 22 (7):3381–3387, 2006. ISSN 0743-7463. 79, 83, 87

[230] Syuji Fujii, Shiori Kameyama, Steven P Armes, Damien Dupin, Motomichi Suzaki, and Yoshinobu Nakamura. ph-responsive liquid marbles stabilized with poly (2-vinylpyridine) particles. *Soft Matter*, 6(3):635–640, 2010. 79, 83, 87

[231] Gayle Overend, Yuan Luo, Louise Henderson, Angela E Douglas, Shireen A Davies, and Julian AT Dow. Molecular mechanism and functional significance of acid generation in the drosophila midgut. *Scientific reports*, 6, 2016. 83

[232] Yiorgos Apidianakis and Laurence G Rahme. Drosophila melanogaster as a model for human intestinal infection and pathology. *Disease models and mechanisms*, 4(1):21–30, 2011. 83, 88

[233] Bruno Lemaitre and Irene Miguel-Aliaga. The digestive tract of drosophila melanogaster. *Annual review of genetics*, 47:377–404, 2013. ISSN 0066-4197. 83, 88

[234] MM Chance. The functional morphology of the mouthparts of blackfly larvae (diptera: Simuliidae). *Quaestiones entomologicae*, 6(2):245–284, 1970. ISSN 0033-5037. 83

[235] Goran T Vladisavljević and Richard A Williams. Manufacture of large uniform droplets using rotating membrane emulsification. *Journal of colloid and interface science*, 299(1):396–402, 2006. ISSN 0021-9797. 84

[236] Peter W Voorhees. The theory of ostwald ripening. *Journal of Statistical Physics*, 38(1-2):231–252, 1985. 84

[237] Paul DT Huibers and Dinesh O Shah. Evidence for synergism in nonionic surfactant mixtures: enhancement of solubilization in water-in-oil microemulsions. *Langmuir*, 13(21):5762–5765, 1997. 85

[238] H Kunieda and K Shinoda. Phase behavior in systems of nonionic surfactant/water/oil around the hydrophile-lipophile-balance-temperature (hlb-temperature). *Journal of Dispersion Science and Technology*, 3(3):233–244, 1982. 85

[239] A.T Florence and D Whitehill. Some features of breakdown in water-in-oil-in-water multiple emulsions. *Journal of Colloid and Interface Science*, 79(1):243 – 256, 1981. ISSN 0021-9797. doi: https://doi.org/10.1016/0021-9797(81)90066-7. 86

[240] Lester G Sultatos. Mammalian toxicology of organophosphorus pesticides. *Journal of Toxicology and Environmental Health, Part A Current Issues*, 43(3):271–289, 1994. ISSN 0098-4108. 92

[241] Gregory J Hannon and John J Rossi. Unlocking the potential of the human genome with rna interference. *Nature*, 431(7006):371–378, 2004. ISSN 0028-0836. 92

[242] Ying-Bo Mao, Wen-Juan Cai, Jia-Wei Wang, Gao-Jie Hong, Xiao-Yuan Tao, Ling-Jian Wang, Yong-Ping Huang, and Xiao-Ya Chen. Silencing a cotton bollworm p450 monooxygenase gene by plant-mediated rnai impairs larval tolerance of gossypol. *Nature biotechnology*, 25(11):1307–1313, 2007. ISSN 1087-0156.

[243] Subba Reddy Palli. Rna interference in colorado potato beetle: steps toward development of dsrna as a commercial insecticide. *Current opinion in insect science*, 6:1–8, 2014. ISSN 2214-5745. 92

[244] Michelle E Powell, Hannah M Bradish, John A Gatehouse, and Elaine C Fitches. Systemic rnai in the small hive beetle aethina tumida murray (coleoptera: Nitidulidae), a serious pest of the european honey bee apis mellifera. *Pest management science*, 73(1):53–63, 2017. ISSN 1526-4998. 92

[245] Antara Das, Todd C Holmes, and Vasu Sheeba. dtrpa1 modulates afternoon peak of activity of fruit flies drosophila melanogaster. *PloS one*, 10(7): e0134213, 2015. 92, 142, 143

[246] Kathryn A Whitehead, Robert Langer, and Daniel G Anderson. Knocking down barriers: advances in sirna delivery. *Nature reviews Drug discovery*, 8(2):129–138, 2009. ISSN 1474-1776. 93

[247] DeeDee Smith, Andrew C Holley, and Charles L McCormick. Raft-synthesized copolymers and conjugates designed for therapeutic delivery of sirna. *Polymer Chemistry*, 2(7):1428–1441, 2011. 93, 100

[248] L Šprincl, J Exner, O Štěrba, and J Kopeček. New types of synthetic infusion solutions. iii. elimination and retention of polyâĂŘ[nâĂŘ(2âĂŘhydroxypropyl) methacrylamide] in a test organism. *Journal of Biomedical Materials Research Part A*, 10(6):953–963, 1976. ISSN 1097-4636. 96

[249] Shou-Shing Hsieh, Hung-Chun Lin, and Chih-Yi Lin. Electroosmotic flow velocity measurements in a square microchannel. *Colloid and polymer science*, 284(11):1275–1286, 2006. ISSN 0303-402X. 99

[250] Cyrille Boyer, Joann Teo, Phoebe Phillips, Rafael B Erlich, Sharon Sagnella, George Sharbeen, Tanya Dwarte, Hien TT Duong, David Goldstein, and Thomas P Davis. Effective delivery of sirna into cancer cells and tumors using well-defined biodegradable cationic star polymers. *Molecular pharmaceutics*, 10(6):2435–2444, 2013. ISSN 1543-8384. 100

[251] Charles W Scales, Yulia A Vasilieva, Anthony J Convertine, Andrew B Lowe, and Charles L McCormick. Direct, controlled synthesis of the non-immunogenic, hydrophilic polymer, poly (n-(2-hydroxypropyl) methacrylamide) via raft in aqueous media. *Biomacromolecules*, 6(4):1846–1850, 2005. 101

[252] Huaizhong Pan, Jiyuan Yang, Pavla KopecÌŇkovaÌĄ, and JindrÌŇich KopecÌŇek. Backbone degradable multiblock n-(2-hydroxypropyl)

methacrylamide copolymer conjugates via reversible addition- fragmentation chain transfer polymerization and thiol- ene coupling reaction. *Biomacromolecules*, 12(1):247–252, 2010. 101

[253] Graeme Moad, YK Chong, Almar Postma, Ezio Rizzardo, and San H Thang. Advances in raft polymerization: the synthesis of polymers with defined end-groups. *Polymer*, 46(19):8458–8468, 2005. 101

[254] Vural Bütün, Steven P Armes, and Norman C Billingham. Selective quaternization of 2-(dimethylamino) ethyl methacrylate residues in tertiary amine methacrylate diblock copolymers. *Macromolecules*, 34(5):1148–1159, 2001. 104

[255] Calum TJ Ferguson, Tara L O'Neill, Neil Audsley, and R Elwyn Isaac. The sexually dimorphic behaviour of adult drosophila suzukii: elevated female locomotor activity and loss of siesta is a post-mating response. *Journal of experimental biology*, 218(23):3855–3861, 2015. 119

[256] Omar Rota-Stabelli, Mark Blaxter, and Gianfranco Anfora. Drosophila suzukii. *Current biology: CB*, 23(1):R8, 2013. ISSN 1879-0445. 119

[257] Andrew Paul Gutierrez, Luigi Ponti, and Daniel T Dalton. Analysis of the invasiveness of spotted wing drosophila (drosophila suzukii) in north america, europe, and the mediterranean basin. *Biological Invasions*, 18 (12):3647–3663, 2016. 119, 120, 144

[258] Charles L Cole and Perry L Adkisson. Daily rhythm in the susceptibility of an insect to a toxic agent. *Science*, 144(3622):1148–1149, 1964. 120

[259] Bartosz Piechowicz, Kinga Stawarczyk, and Michał Stawarczyk. Circadian changes in susceptibility of young honeybee workers to intoxication by pyrethroid, carbamate, organophosphorus, benzoyl urea and pyridine derivative insecticides. *Journal of Plant Protection Research*, 52(2):286–289, 2012.

[260] Erik Shipp and John Otton. Orcadian rhythms of sensitivity to insecticides in musca domestica (diptera, muscidae). *Entomologia Experimentalis et Applicata*, 19(2):163–171, 1976. 120

[261] Louisa A Hooven, Katherine A Sherman, Shawn Butcher, and Jadwiga M Giebultowicz. Does the clock make the poison? circadian variation in response to pesticides. *PLoS One*, 4(7):e6469, 2009. 120

[262] Kelly A Hamby, Rosanna S Kwok, Frank G Zalom, and Joanna C Chiu. Integrating circadian activity and gene expression profiles to predict chronotoxicity of drosophila suzukii response to insecticides. *PLoS One*, 8(7): e68472, 2013. 120, 121, 126, 140

[263] Geraldine D Ryan, Lisa Emiljanowicz, Francesca Wilkinson, Melanie Kornya, and Jonathan A Newman. Thermal tolerances of the spotted-wing drosophila drosophila suzukii (diptera: Drosophilidae). *Journal of economic entomology*, 109(2):746–752, 2016. ISSN 1938-291X. 120, 143

[264] Daniel T Dalton, Vaughn M Walton, Peter W Shearer, Douglas B Walsh, Janet Caprile, and Rufus Isaacs. Laboratory survival of drosophila suzukii under simulated winter conditions of the pacific northwest and seasonal field trapping in five primary regions of small and stone fruit production in the united states. *Pest management science*, 67(11):1368–1374, 2011. ISSN 1526-4998.

[265] Selim Terhzaz, Lucy Alford, Joseph GC Yeoh, Richard Marley, Anthony J Dornan, Julian AT Dow, and Shireen A Davies. Renal neuroendocrine control of desiccation and cold tolerance by drosophila suzukii. *Pest management science*, 2017. ISSN 1526-4998. 120, 143, 145

[266] Samantha Tochen, Daniel T Dalton, Nik Wiman, Christopher Hamm, Peter W Shearer, and Vaughn M Walton. Temperature-related development and population parameters for drosophila suzukii (diptera: Drosophilidae) on cherry and blueberry. *Environmental Entomology*, 43(2):501–510, 2014. 120, 140, 143

[267] Daniel Ludwig and Raymond M Cable. The effect of alternating temperatures on the pupal development of drosophila melanogaster meigen. *Physiological Zoology*, 6(4):493–508, 1933. ISSN 0031-935X. 120, 143

[268] Hirotoshi Kinjo, Yasuhisa Kunimi, and Madoka Nakai. Effects of temperature on the reproduction and development of drosophila suzukii (diptera: Drosophilidae). *Applied entomology and zoology*, 49(2):297–304, 2014. ISSN 0003-6862. 120

[269] Thomas Enriquez and Hervé Colinet. Basal tolerance to heat and cold exposure of the spotted wing drosophila, drosophila suzukii. *PeerJ*, 5:e3112, 2017. ISSN 2167-8359. 120

[270] R Elwyn Isaac, Chenxi Li, Amy E Leedale, and Alan D Shirras. Drosophila male sex peptide inhibits siesta sleep and promotes locomotor activity in the post-mated female. *Proceedings of the Royal Society of London B: Biological Sciences*, page rspb20091236, 2009. 121, 131, 141, 142

[271] Trevor Lewis and Lionel Roy Taylor. Diurnal periodicity of flight by insects. *Ecological Entomology*, 116(15):393–435, 1965. 121

[272] Stefano Vanin, Supriya Bhutani, Stefano Montelli, Pamela Menegazzi, Edward W Green, Mirko Pegoraro, Federica Sandrelli, Rodolfo Costa, and Charalambos P Kyriacou. Unexpected features of drosophila circadian behavioural rhythms under natural conditions. *Nature*, 484(7394):371, 2012. ISSN 0028-0836. 121, 142, 143

[273] Pamela Menegazzi, Taishi Yoshii, and Charlotte Helfrich-FÃűrster. Laboratory versus nature: the two sides of the drosophila circadian clock. *Journal of Biological Rhythms*, 27(6):433–442, 2012. ISSN 0748-7304. 121

[274] Edward W Green, Emma K O'Callaghan, Celia N Hansen, Stefano Bastianello, Supriya Bhutani, Stefano Vanin, James Douglas Armstrong, Rodolfo Costa, and Charalambos P Kyriacou. Drosophila circadian rhythms in seminatural environments: summer afternoon component is not an artifact and requires trpa1 channels. *Proceedings of the National*

Academy of Sciences, 112(28):8702–8707, 2015. ISSN 0027-8424. 121, 142, 143

[275] Omer Sayeed and Seymour Benzer. Behavioral genetics of thermosensation and hygrosensation in drosophila. *Proceedings of the National Academy of Sciences*, 93(12):6079–6084, 1996. ISSN 0027-8424. 125, 143

[276] Caroline S Gough, Grace M Fairlamb, Petra Bell, Ronald J Nachman, Neil Audsley, and R Elwyn Isaac. Peptidergic control in a fruit crop pest: The spotted-wing drosophila, drosophila suzukii. *PloS one*, 12(11):e0188021, 2017. ISSN 1932-6203. 125

[277] Karen S Ho and Amita Sehgal. Drosophila melanogaster: an insect model for fundamental studies of sleep. *Methods in enzymology*, 393:772–793, 2005. 128

[278] T Schmidt, Y Choffat, M Schneider, P Hunziker, Y Fuyama, and E Kubli. Drosophila suzukii contains a peptide homologous to the drosophila melanogaster sex-peptide and functional in both species. *Insect biochemistry and molecular biology*, 23(5):571–579, 1993. 131, 142

[279] Santosh Revadi, Sébastien Lebreton, Peter Witzgall, Gianfranco Anfora, Teun Dekker, and Paul G Becher. Sexual behavior of drosophila suzukii. *Insects*, 6(1):183–196, 2015. 131

[280] Frank W Avila, Laura K Sirot, Brooke A LaFlamme, C Dustin Rubinstein, and Mariana F Wolfner. Insect seminal fluid proteins: identification and function. *Annual review of entomology*, 56:21–40, 2011. 141, 142

[281] E Kubli. Sex-peptides: seminal peptides of the drosophila male. *Cellular and Molecular Life Sciences CMLS*, 60(8):1689–1704, 2003. 141

[282] Antara Das and Vasu Sheeba. *Temperature Input for Rhythmic Behaviours in Flies: The Role of Temperature-Sensitive Ion Channels*, pages 405–424. Springer, 2017. 142

[283] Qing-Cai Lin, Yi-Fan Zhai, Cheng-Gang Zhou, Li-Li Li, Qian-Ying Zhuang, Xiao-Yan Zhang, Frank G Zalom, and Yi Yu. Behavioral rhythms of drosophila suzukii and drosophila melanogaster. *Florida entomologist*, 97 (4):1424–1433, 2014. 145