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Optimizing the Delivery of a GFP Gene via a Biocompatible Nanocarrier in Daphnia and a Chironomid

Alexandra R. Job

OPTIMIZING THE DELIVERY OF A GFP GENE *VIA* A BIOCOMPATIBLE NANOCARRIER IN *DAPHNIA* AND A CHIRONOMID

Alexandra Job

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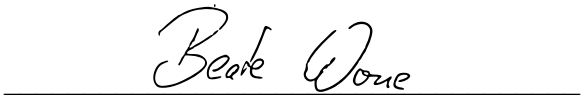
find it satisfactory and recommend that it be accepted.

Bernie Wone, Ph.D.
Advisor of the Thesis



Beate Wone, M.S.
Thesis Advisory Committee Member

Dallas Doane, M.A.
Thesis Advisory Committee Member

ABSTRACT

OPTIMIZING THE DELIVERY OF A GFP GENE *VIA* A BIOCOMPATIBLE NANOCARRIER IN *DAPHNIA* AND A CHIRONOMID

Alexandra Job

Advisor: Dr. Bernie Wone

Current pest control methods impose risks including pest resistance to insecticides, bioaccumulation of the insecticide, and ecosystem impact. There is a need for a better and more sustainable method of pest control in order to protect the environment and the populations reliant on it. Currently, an alternative method of pest control uses RNA interference (RNAi), that exploits heterologous protein expression to disable the insect pest, delivered by the use of nanoparticles. Nanocarriers show great promise in this method of use, but concerns of cytotoxicity, biodegradability, and transfer to off target organisms require careful consideration. This study focuses on optimizing a biocompatible nanoparticle-mediated gene delivery system in a chironomid species and *Daphnia* spp. (*Daphnia)* to induce transient GFP expression. A weak GFP expression was detected in the head and thoracic regions of *Daphnia*, but additional trials with a few changes in protocol are expected to yield more conclusive results. The successful expression of GFP would provide insight on the molecular mechanisms and interactions involved in nanoparticle-mediated gene delivery, as well as broaden understanding of a biocompatible nanocarrier's use with insects.

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1. Introduction

1.1. Introduction

Utilizing nanoparticles as a means to deliver molecular cargo has important applications in many fields. These applications include delivering of drugs in biomedicine (Baddar et al., 2020), genome editing in basic biological and applied research (Miyata et al., 2019, Izuegbunam et al., 2021), delivering short-interfering RNA (siRNA) to induce RNA interference (RNAi) in insect pest control (Baddar et al., 2020), delivering insecticide in pest control (Baddar et al., 2020), and limiting the spread of diseases transmitted by insect vectors (Dhandapani et al., 2019). Expanding on the current understanding of nanoparticle-mediated gene delivery and its extensive applications in the near future has the potential for solving a wide variety of modern issues. This study focuses on optimizing the use of a biocompatible nanoparticle-mediated gene delivery system for various applications, including in the field of sustainable pest control.

For context, pests are characterized as organisms that feed on, damage, or destroy agricultural products including crops and/or livestock. Entire human populations are dependent on the efficiency of food production and its balance with the surrounding area where the health of the environment is already heavily impacted. Traditional pest control methods, biological or chemical control, have been a topic of concern due to varying degrees of harm that they could impose on the environment (Nicolopoulou-Stamati et al.,

2016). Introducing a natural predator of a pest, also known as biological pest control, likely has limitations when applied to a larger scale. Inserting large numbers of a new species into an established ecological environment has the potential to push the ecosystem out of balance (Taghavi et al., 2013). Chemical control methods utilizing natural or synthetic insecticides, have numerous concerns caused by the active ingredients being used. For example, two common concerns with synthetic chemical pesticides are that pests become resistant to insecticides and that off-target organisms are harmed (Baddar et al., 2020). Bioaccumulation, chemicals increasing in concentration in organisms further up on the food chain, is another issue that has potential to damage ecosystems in a variety of ways, which ultimately harms human health (Nicolopoulou-Stamati et al., 2016). These examples represent just a fraction of the concerns regarding traditional pest control methods, which demonstrates the need to find more sustainable pest control methods to minimize risk to the ecosystem.

One alternative method of pest control of current interest is using RNA interference (RNAi). RNAi is a biological process where its function is controlling gene expression within an organism. The use of double stranded RNA (dsRNA) in RNAi allows for targeted genes to be silenced or downregulated, which could be an advantageous strategy in finding a more sustainable method of pest control (Baddar et al., 2020). Most importantly, the expression of RNAi is transient. Reducing or temporarily eliminating the expression of essential genes in pests could lessen their negative impact on agricultural products that are important for the food supply chain. However, the proteins and cofactors required for RNAi are located in the cytoplasm. In order for RNAi to be utilized as an effective pest control method, the desired genetic sequence has to be

able to reach the machinery necessary for the RNAi process to take place (Baddar et al., 2020).

Another potential new route for a sustainable pest control method could be utilizing heterologous protein expression to produce proteins that reduce the harmful effect of pests. An example of this would be the expression of genes encoding for insecticidal proteins (Zhao et al., 2008). Heterologous protein expression is also useful for other types of research. Inducing exogenous expression can provide insight to interactions between proteins and DNA, molecular signals and events, or gain-of-function and loss-of-function experiments (Miyata et al., 2019).

Whether delivering dsRNA for RNAi or delivering plasmid DNA for heterologous protein expression, there are multiple barriers to get past in insects' molecular anatomy and cellular environments. Some of the obstacles that genetic material must get past includes endonucleases, endosomal entrapment and degradation, and varying cellular environments of insect species (Baddar et al., 2020). One way to overcome these difficulties is through protecting and delivering the genetic material by conjugation with a nanocarrier (Khan et al., 2016).

Recently, a nano-biomimetic gene carrier was developed for plants (Izeugbunam et al. 2021). This nanocarrier utilizes arginine-functionalized nanohydroxyapatite (R-nHA) nanoparticles for delivering a plasmid DNA in a variety of plants (Izuegbunam et al., 2021). Hydroxyapatite is an abundant form of mineralized calcium phosphate (CaP) found in nature (Baddar et al., 2020), so it is biocompatible to living systems. In that study, Izuegbunam et al. (2021) conjugated R-nHAs with plasmid DNA and these

conjugates were able to pass through the plant's cell walls and plasma membranes for transient expression of a reporter gene. Because these R-nHA complexes were small enough in size to traverse across the plant cell walls, I hypothesize that these complexes could be effective in delivering a green fluorescent protein gene (GFP) as these R-nHAs are expected to traverse the exoskeleton of chitin in *Daphnia* and chironomids (Shahzad and Manzoor 2018).

This research investigates the use of R-nHA nanoparticles to deliver a plasmid DNA encoded with a GFP gene in *Daphnia* and a chironomid with the goal of inducing transient GFP expression. For this study, expression of GFP would be proof-of-concept of utilizing R-nHA as biocompatible nanocarriers for gene delivery in insects to induce transient protein expression. Findings for this study will help develop more sustainable pest control methods that use RNAi approaches.

1.2. Background

The organisms used to study were *Daphnia* and chironomid larvae. *Daphnia*, commonly known as water fleas, are extremely small crustaceans typically 1 to 5 mm in size (Ebert, 2005). Found on every continent on Earth, typically in bodies of freshwater, *Daphnia* are frequently used in research studies thanks to the advanced understanding of their genetics and physiology. In recent years, there has been an abundance of progress from genomic sequencing and observational reports of studies using *Daphnia*, making this species incredibly useful for research (Kato et al., 2012). The benefit of utilizing *Daphnia* for this study is supplemented by the fact that *Daphnia* are filter feeders. As explained by Ebert (2005), they have an extremely efficient method of consuming particles in the water that allows them to secure floating particles as small as bacteria. *Daphnia* typically consume particles with a diameter of 1 μm to 50 μm, but they can take in particles 20 μm greater depending on their size. The trait of filter feeding such small particles is advantageous for the design of this study. The gut of the *Daphnia* is the area where expression of GFP would be most likely to be present (Figure 1; Shahzad and Manzoor, 2018). Due to their mechanism of feeding, this is where the nanoparticles with the plasmid DNA could passively enter into the organism. *Daphnia* also have a nuchal organ that is located dorsally, behind the eye in the head (Figure 1). This organ has been shown to participate in the role of osmoregulation, specifically Na^+, K^+, H^+, Cl^-, NH_4^+, and Ca^{2+} ions (Morris and O'Donnell, 2019). Functioning in ion transport between the *Daphnia* and the environment at earlier life stages, the nuchal organ then begins to secrete proteins necessary to form the exoskeleton, utilizing Ca^{2+} to assist in calcification (Morris and O'Donnell, 2019). Because of this osmoregulation role of the organ, the

nuchal organ is another prospective area to expect GFP expression. The exoskeleton of *Daphnia* is mainly composed of chitin, and referred to as a carapace (Ebert, 2005). Incubating *Daphnia* in spring water with a suspended complex of nanoparticles and plasmid DNA allows for maximal contact and absorption of the gene to provide proof-of-concept of the efficiency of delivery. However, autofluorescence of chitin must be considered when interpreting results (Schür et al., 2019).

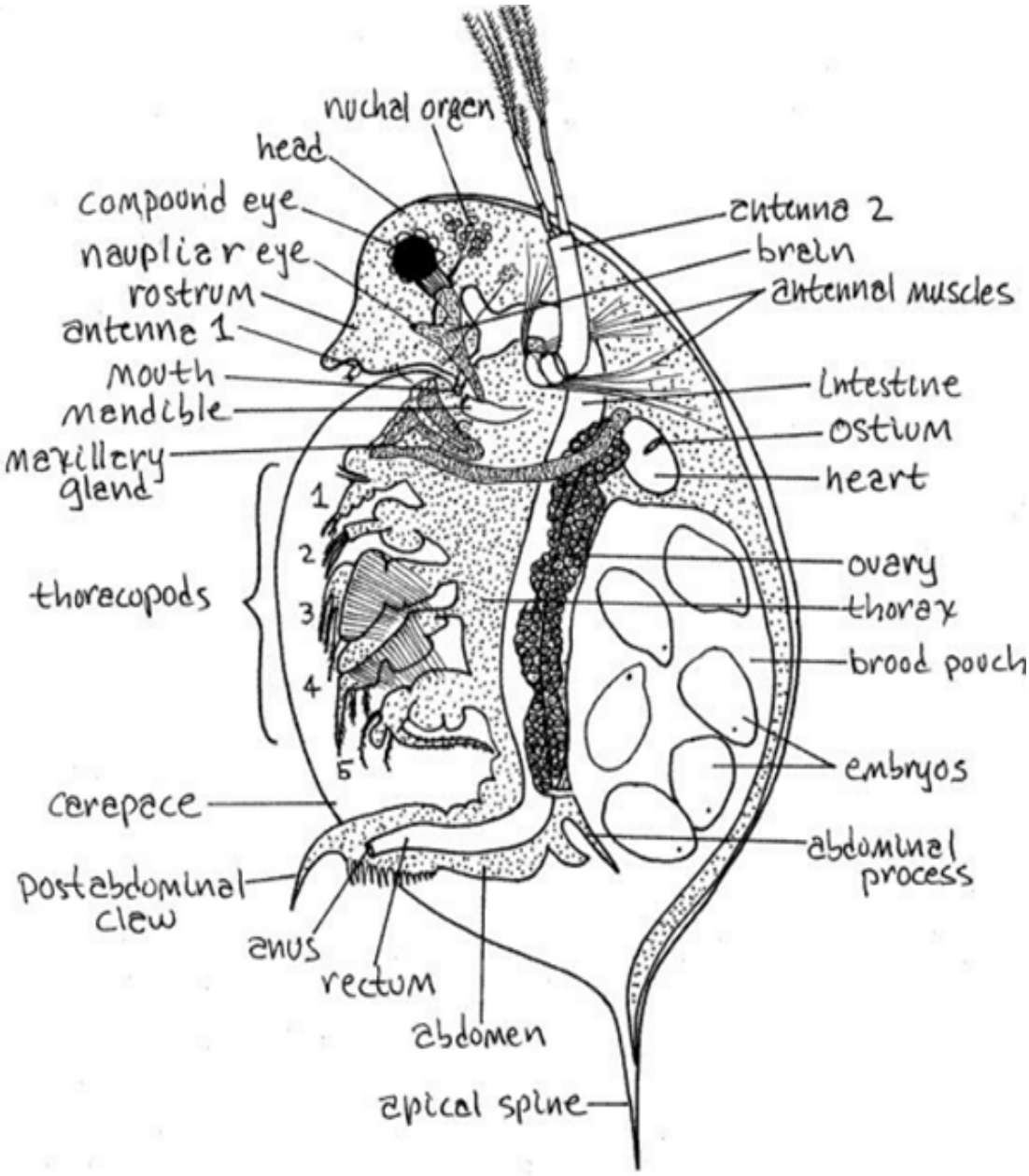

Figure 1: Anatomy of *Daphnia*. Female *Daphnia* (from Freeman and Bracegirdle 1971).

Chironomid larvae, adults known as midges, were also chosen for use in this study. The desiccation tolerant abilities of some midges have been extensively studied by Miyata et al. (2019). They have identified and isolated a 121 promoter in a cell line of chironomids to improve heterologous gene expression (Miyata et al., 2019). Hence, this

promoter with a GFP (AcGFP1) will be used in the current study. Larval chironomids found locally in South Dakota bodies of water will be used in this study to broaden the applications of the results beyond a single insect species and life stage.

Green fluorescent protein (GFP) is an excellent protein to study heterologous protein expression and as a reporter gene for delivery in insects. Joon Cha et al. (1997) describes GFP as a bioluminescent protein, originally from jellyfish, that emits a characteristic green light. This protein is favorable for its use in research due to the fact that it can independently fluoresce when exposed to UV light, without the need for any additional molecules such as cofactors or substrates. The expression of GFP can be seen visually, thereby providing a marker that the GFP gene transfection process was effective.

A study done by Cha et al. (1997) also focused on the timeline of GFP expression in *Trichoplusia* spp. insect larvae. Looking at the correlation between green light intensity and the mass of the GFP, it was found that maximal expression occurred around 3 days after infecting the insect larvae. After this time period, protease activity begins to rapidly increase which leads to a loss of GFP being expressed (Joon Cha et al., 1997). Due to this finding, it was decided to incubate the *Daphnia* and chironomids for 3 days before checking for GFP expression. The effectiveness of *in vivo* transient expression of a green fluorescent protein gene using a biocompatible nanocarrier is explored in this study to provide insight on size exclusion in insect exoskeleton capability of plasmid DNA delivery *via* nanoparticles.

For applications to pest control, traditional methods for delivering naked dsRNA/siRNA such as microinjection, soaking, and oral feeding have too many limitations to be efficient when applied to a large-scale agricultural area (Yan et al., 2021). However, the use of nanoparticles can improve the efficiency of transfection. Nanoparticles are characterized by having a size within the range of 1 to 100 nanometers and can help protect and promote delivery of nucleic acids for use in RNAi (Yan et al., 2021) or in heterologous protein expression.

Khan et al. (2015) reported on the optimization of calcium phosphate (CaP) nanoparticles through adjustment of the precipitation and transfection conditions and found that CaP nanoparticles significantly improved plasmid DNA delivery of an enhanced GFP gene. Using particles that are naturally found in the environment gives a higher likelihood for biocompatibility and potential large-scale use in the case of pest control. While CaP is biodegradable, hydroxyapatite, a mineralized form of CaP, is more abundantly found in nature (Baddar et al., 2020). The study done by Baddar et al. (2020) analyzed the efficacy of using hydroxyapatite nanoparticles. Hydroxyapatite nanoparticles have been successfully used to improve delivery of siRNA, transfection, and drugs that are small molecules. Using polymer coated hydroxyapatite nanocarriers for dsRNA delivery intended for RNAi gene silencing, Baddar (2020) found naked dsRNA did not reduce gene expression at all, but an excessive number of nanoparticles caused a cytotoxic response. There was a maximal decrease of gene expression, 35%, using a nanoparticle to dsRNA ratio of 5:1, which is important for applications in delivering RNA for use in RNAi. Recently, there has been additional research reported for use of hydroxyapatite nanocarriers in delivering plasmid DNA.

Izuegbunam et al. (2021) synthesized rod shaped nanohydroxyapatite (nHA) and arginine-functionalized them (R-nHA) to use for delivery of plasmid DNA (pDNA) in a variety of plants. The nHA and R-nHA nanoparticles, reported to be about 10 nm in diameter, were conjugated with pDNA in varying mass ratios. It was found that the optimal ratio of pDNA:R-nHA occurred at 1:100 and 1:200, which provided maximum efficiency of conjugation (Izuegbunam et al., 2021). Maximal conjugation efficiency between the positively charged nanoparticles and the negatively charged pDNA is advantageous in that it offers improved protection of the pDNA from enzymatic degradation (Yan et al., 2021). The findings from the study done by Izuegbunam et al in 2021 provided a basis for the decision to use R-nHA nanocarriers with plasmid DNA encoding a GFP gene for transfection in *Daphnia* and chironomid larvae.

2. Methodology

2.1. Extraction of plasmid DNA

For *Daphnia*, the pCS-EF1a1-H2B-eGFP plasmid was chosen (Figure 2; Kato et al., 2012), whereas for chironomid larve the pPv121-AcGFP1-Pv121-ZeoR plasmid was chosen (Figure 3). These plasmids utilized a strong constitutive genus-specific promoter that was found to promote heterologous gene expression in *Daphnia* (Kato et al., 2012) and a chironomid (Miyata et al. 2019). These plasmids were kindly provided by Kato et al. (2012) and Miyata et al. (2019), respectively. Plasmids were transformed into NEB 10 beta competent *E. coli* cells (New England Bio Labs, Ipswich, MA, USA) and grown overnight for plasmid extraction. Plasmids were extracted using QIAprep Spin Miniprep Kit (Qiagen, Hilden, Germany) following the manufacturer's protocol, where the plasmid DNA was eluted with 50 µl of molecular grade water.

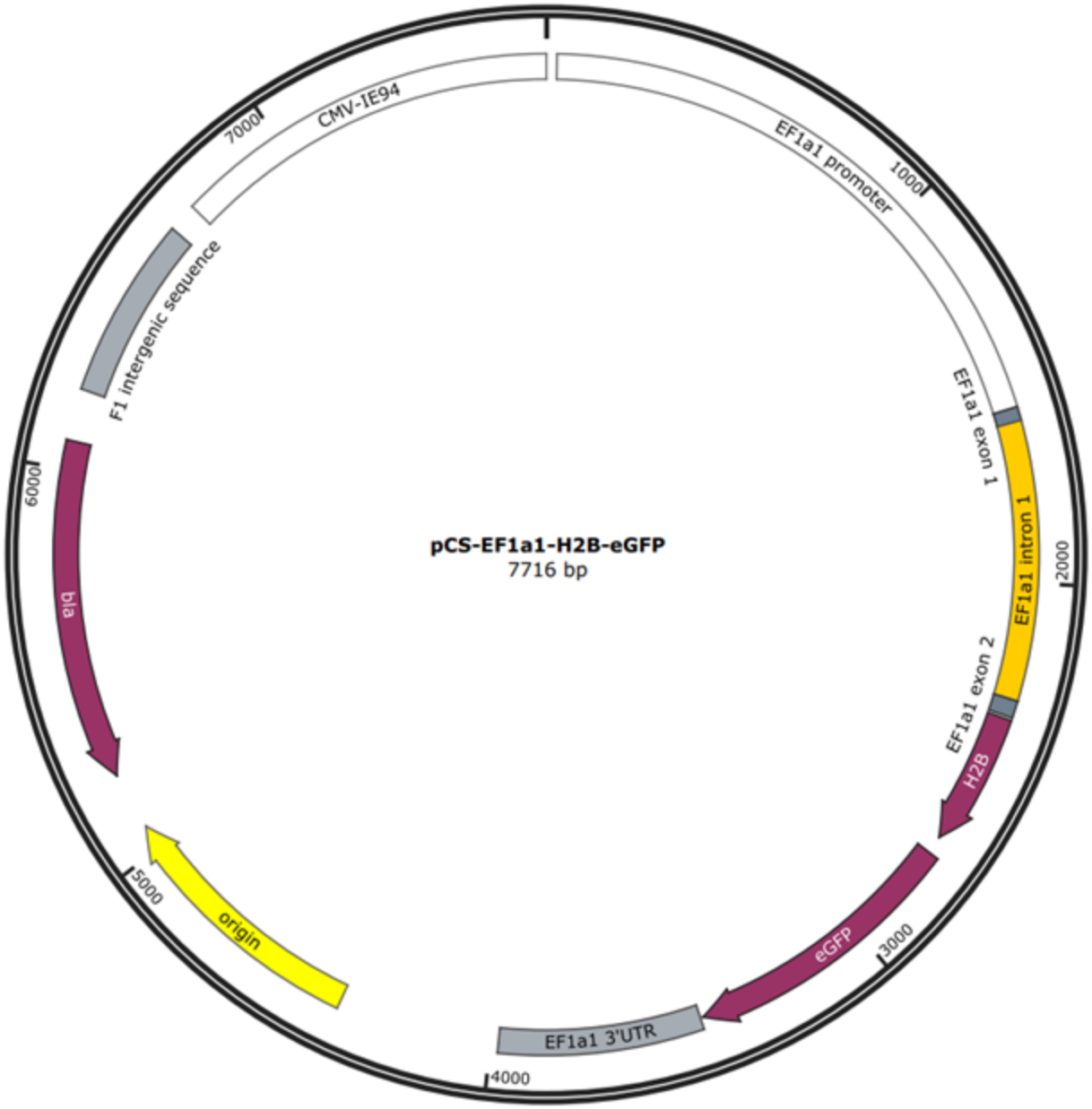

Figure 2: Plasmid map of pCS-EF1a1-H2B-eGFP used for *Daphnia.*

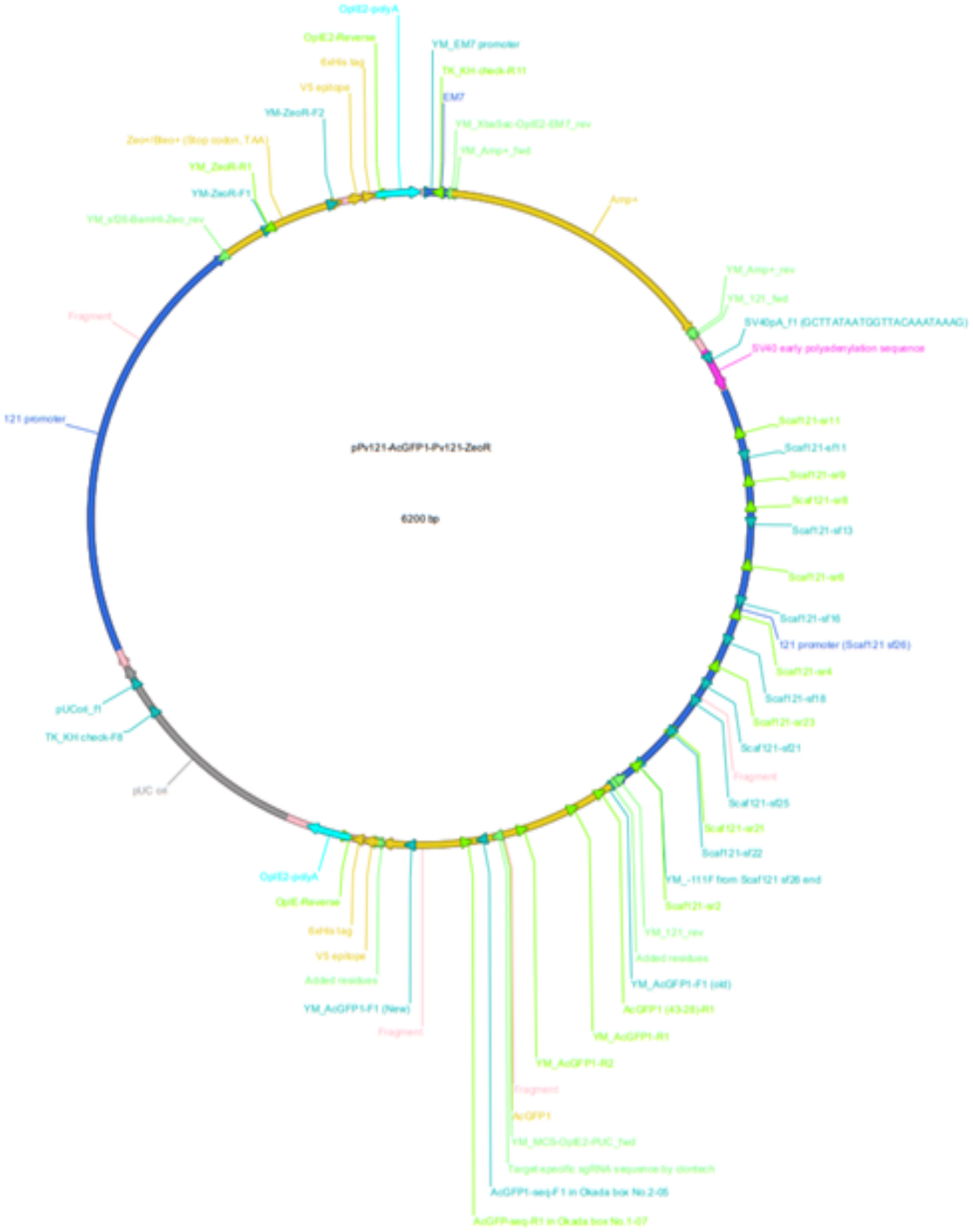

Figure 3: Plasmid map of pPv121-AcGFP1-Pv121-ZeoR used for chironomid larvae.

2.2. pDNA and nanoparticle conjugation

Arginine-functionalized nanohydroxyapatite (R-nHA) nanoparticles were synthesized following the procedure of Izuegbunam, et al (2021). To conjugate the two molecules, the R-nHA were sonicated on ice for 10 minutes. Using the pDNA that had previously been extracted and stored in -20 °C, mass ratios of 1µg:100µg, 2µg:200µg, and 3µg:300µg of pDNA:R-nHA were tested by transferring volumes of plasmid DNA on the basis of their concentration of GFP. The conjugates were vortexed and then incubated for one hour and 30 min at 37 °C, shaking at 200 rpm. The conjugates were vortexed every 30 min throughout the incubation period. At the end of the incubation, the conjugates were spun for 10 min at 10,000 rpm and resuspended in spring water (see below).

2.3. Culture conditions and transfection

The cultures of *Daphnia* and chironomids were obtained locally and kept at room temperature throughout the course of the experiment. For transfection, the conjugates were resuspended in pH 7 spring water. To begin, the supernatants were removed and discarded after being centrifuged. The pellet was resuspended using 10 mL of spring water at pH 7. The solution was vortexed well before being added to a test dish. Control dishes contained 10 mL of pH 7 spring water alone. *Daphnia* and chironomids were transferred and kept in dishes that contained solution with their corresponding selected plasmid DNA suspended or in control dishes with spring water. Five to ten organisms were used in each condition, and the samples were inspected for expression after 3 d. GFP was examined using a Leica DMRA2 fluorescence microscope and imaged using a

Leica DFC3000 G camera (Leica Microsystems Inc., Buffalo Grove, IL). The organisms were fed a small amount of food every other day.

3. Results and Discussion

3.1. Daphnia

In the head region of *Daphnia*, it appears that there is tentative GFP expression of the nuchal organ (Figure 4). Control images (Figure 4A and C) show autofluorescence and the organ was not visible, whereas there is a faint structure present in images of the GFP transfected *Daphnia* (Figure 4B and D). Green dotted patches are seen in the faint circular structure identified as the nuchal organ. Due to its role in ion transport for osmoregulation and membrane protein secretion, it is plausible to be observing GFP expression in this active of a tissue. However, further repeating the experiment is needed for this preliminary finding.

Another region of *Daphnia* containing active tissues is the thoracic cavity (the respiratory structures). The metabolic nature of how *Daphnia* function physiologically to obtain nutrients through diffusion and particle uptake translates to a likelihood of GFP expression around the respiratory structures, where *Daphnia* respire. The thoracic region of *Daphnia* is shown in Figure 5. The control organism expectedly exhibits some autofluorescence due to chitin (Figure 5A) but it appears to exhibit less fluorescence than the GFP transfected organism (Figure 5B). Therefore, referencing Figure 5, the thoracic cavity of *Daphnia* exhibited tentative GFP expression. This preliminary finding would be plausible due to the nature of functioning in this area, active uptake of oxygen. Previous research has shown that the permeability of the membrane surrounding the upper gut region of *Daphnia* has been assessed and reported to exclude particles greater that 327 nm (Schür et al., 2019), which would theoretically allow uptake of the rod-shaped R-

nHAs with a diameter of around 15 nm and a length of around 55 nm (Izuegbunam et al., 2021) used in this study. Suspending conjugated pDNA:R-nHA directly in spring water containing the *Daphnia* allows for the conjugate to be filtered through the respiratory structures similar to how typical particles are collected by *Daphnia* for nutrients.

As expected, autofluorescence of the exoskeleton was observed in *Daphnia* due to chitin (Schür et al., 2019). Chitin has been shown to fluoresce around 460 nm in animal cells (Roshchina, 2012), so analysis *via* the use of a fluorescent microscope excites chitin as well as any GFP. Autofluorescence could be seen as a confounding variable when analyzing imaged results, so further analysis to minimize the effect of autofluorescence would be beneficial to increase confidence in the fluorescence being due solely to GFP expression (Schür et al., 2019). Utilizing a microscope able to emit a more precise wavelength could help in excluding autofluorescence and only exciting GFP, improving the baseline expectation of autofluorescence from imaging more control organisms, or using an alternative reporter molecule could be options for yielding more specific results.

For future trials, it is likely that there will be additional alterations in the current protocol. When preparing the conjugates, shaking, vortexing, and centrifugation before resuspension in water were utilized procedures. However, it is likely that this could have damaged the plasmid and nanoparticle conjugate, leading to less expressive results. To avoid shearing the plasmid or disputing the particle and plasmid, less harsh methods of conjugation will be explored in future trials to improve transfection and expression efficiency.

3.2. Chironomids

For chironomid larvae, we were unable to see any GFP expression. It is expected that the phenotypic coloration of chironomids at the larval stage creates the need for further steps to analyze results. The red coloration of the organism's exterior would inhibit accurate analysis of GFP expression through the use of a fluorescent microscope alone, so initial analysis methods were insufficient to view results. Important areas of analysis for chironomid larvae include the more active tissues found in the digestive tract. To improve imaging and assessment, an additional trial utilized a cryo-microtome to better view the organisms' interior. After freezing the organisms, the cryo-microtome was used to make longitudinal sections of chironomids, with a thickness of 16 μm. These sections were then applied to slides for analysis under a microscope but yielded inconclusive results due to the method of freezing. Flash freezing with liquid nitrogen is recommended for future analysis to improve structural clarity.

4. Conclusions

Biocompatible nanoparticle-mediation and insecticidal gene delivery show promise in the field of pest control. Expanding the current body of knowledge on the molecular mechanisms, optimization of gene delivery systems, safety, and environmental impact is necessary before being considered for mass production. In the stages of preliminary research, GFP as a reporter molecule shows promise for improving understanding of gene delivery via the use of biocompatible R-nHAs. Further research utilizing analysis methods that accommodate for species specific variables identified in

this study will be beneficial for yielding conclusive results beyond these preliminary findings.

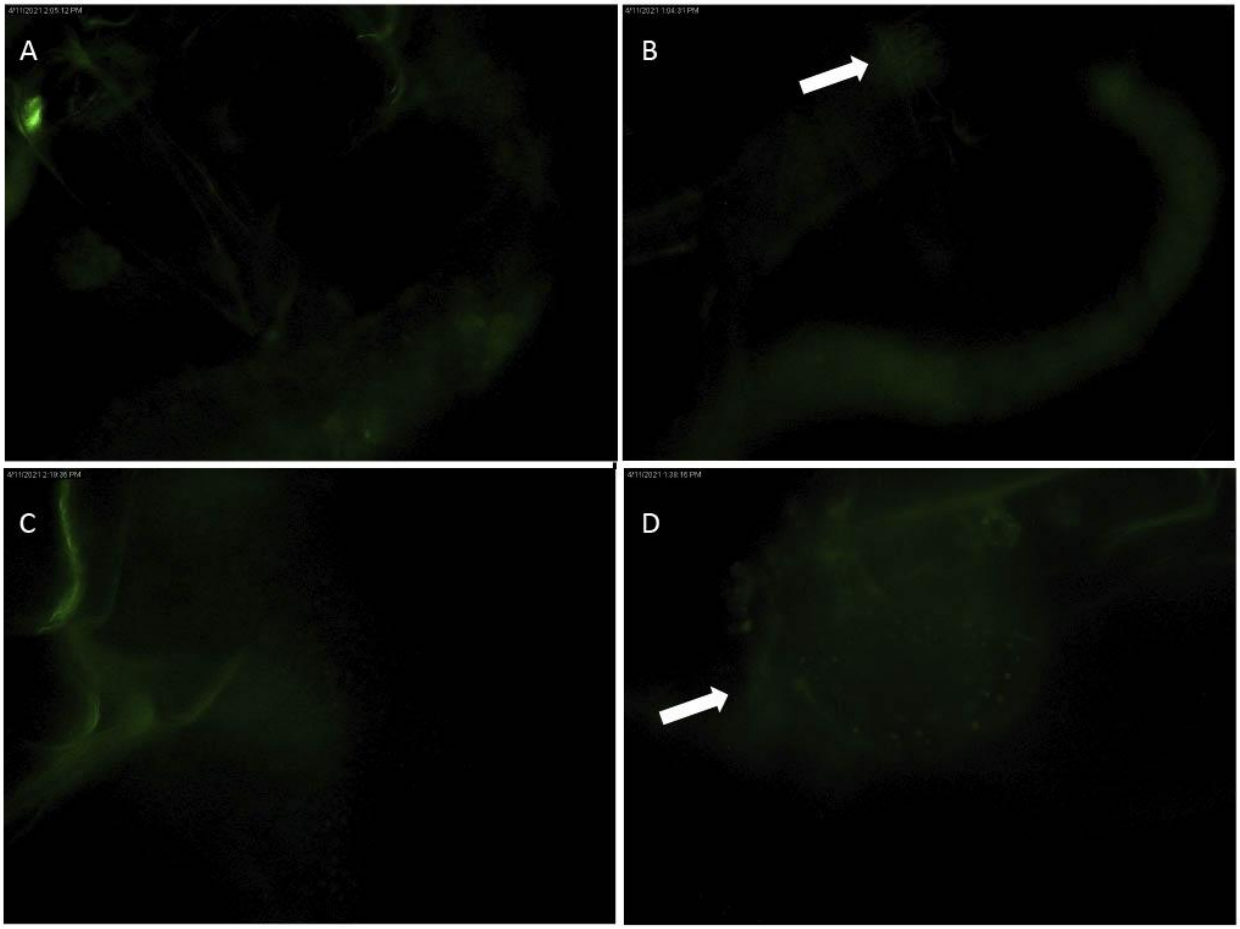

Figure 4: GFP expression in head region of *Daphnia*. (A) Head region of *Daphnia* control at 10X magnification. (B) Head region of *Daphnia* transfected with pDNA:R-nHA at 10X magnification. (C) Head region of *Daphnia* control at 20X magnification. (D) Head region of *Daphnia* transfected with pDNA:R-nHA at 20X magnification. Arrows point to the area of the nuchal organ. Tentative GFP expression exhibited, requires additional testing or alternative analysis to confirm.

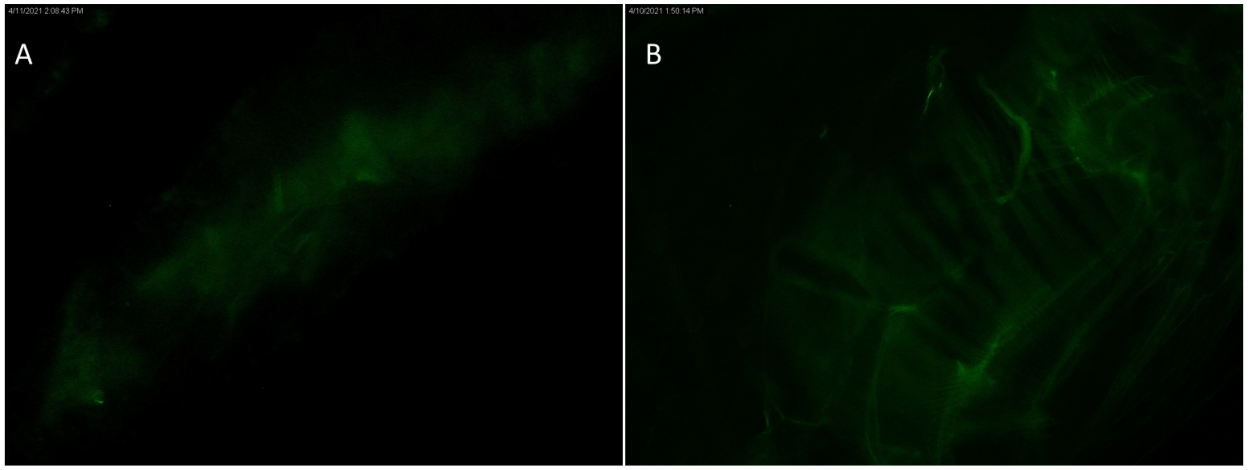

Figure 5: GFP expression in the thoracic region of *Daphnia*. (A) Midgut cavity of *Daphnia* control at 10X magnification. (B) Midgut cavity of *Daphnia* transfected with pDNA:R-nHA at 10X magnification. Tentative GFP expression exhibited, requires additional testing or alternative analysis to confirm.

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