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Development of a biopolymer nanoparticle-based method of oral toxicity testing in aquatic invertebrates

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ABSTRACT

Aquatic toxicity testing generally focuses on the water absorption/dermal route of exposure to potential toxic chemicals, while much less work has been done on the oral route of exposure. This is due in part to the difficulties of applying traditional oral toxicity testing to aquatic environments, including the tendency for test chemicals to dissolve into water. The use of biopolymer nanoparticles to encapsulate test chemicals onto food to prevent dissolution is one solution presented herein. The biopolymers zein and chitosan were explored for their previously known nanoparticle-forming abilities. Nanoparticles containing the test chemical rhodamine B were formed, applied as films to coat food, and then fed to the test organism, the freshwater amphipod *Hyalella azteca*. In feeding trials both zein and chitosan nanoparticles showed a significantly lower release rate of rhodamine B into water than food dyed with rhodamine B without biopolymer nanoparticles. Zein nanoparticles also showed better retention ability than chitosan nanoparticles. Both kinds of nanoparticles showed no significant effect on the survival, growth, or feeding behavior of *H. azteca*. Thus these biopolymers may be an effective system to encapsulate and deliver chemicals to aquatic invertebrates without interfering with common toxicity assessment endpoints like survival and growth.

1. Introduction

Organisms in an aquatic environment have several possible routes of exposure to chemical stressors. These include dermal uptake from contaminated water or sediment, diffusion into the body across gills, and oral exposure from eating contaminated food or sediment (Walker et al., 2012). Uptake across skin or gills from contaminated water is most often tested due to the lack of easily accessible methods for examining the other possible exposure pathways (Carbonell et al., 2000). The current methods of testing oral toxicity have several shortcomings. First, chemicals mixed into hand-made diets may undergo chemical changes during the food's processing. Second, the chemical used to make contaminated food is not bound to the food in any way and can dissolve from the food into the water. Lastly, while dissolution of the chemical into the water may not be of high concern when dealing with highly hydrophobic chemicals (Warlen et al., 1977; Pickford et al., 2003), more hydrophilic chemicals could dissolve more easily and are therefore difficult to study.

The inability to easily and effectively perform tests of oral toxicity on aquatic organisms remains a problem in modern toxicological testing. Current testing of oral toxicity is most often done either through slow feeding of contaminated food to research organisms to ensure all food is eaten or through gavage in which the contaminated food is delivered directly into the stomach through a tube (Bjerregaard et al., 2007; Lefebvre et al., 2007; Sung and Ye, 2009). The former is time consuming. The latter is stressful to the organism and potentially interferes with detection of responses to the chemical due to the organism's physiological response to handling and the anesthetization often required before performing gavage. Monitoring ad libitum feeding or performing gavage also requires the use of large research organisms. Given these current difficulties and requirements, examples of exploration of oral toxicity to aquatic organisms are few (e.g., Allner et al., 1999; Grinwis et al., 2000; Palace et al., 1996), and direct comparisons of oral toxicity to other routes of aquatic exposure are even rarer (but see Pickford et al., 2003 and Gutierrez-Praena et al., 2011). The majority of aquatic toxicity studies focus on dissolved chemicals only, but an understanding of all the possible routes of exposure to a chemical is needed to fully determine the chemical's potential toxicity.

A solution proposed in the present study consists of an aquatic oral toxicity test based on biopolymer nanoparticles that can encapsulate a chemical and then form a film on food while not confounding the effects of the chemical itself. Biopolymers are polymers from natural sources, such as zein from corn, chitosan

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from arthropod exoskeletons, and carrageenan from red seaweed. Currently biopolymers are most widely used in the food industry as thickening and emulsifying agents, packaging and coating materials, and film-forming agents, among other uses (Siracusa et al., 2008; Stephen et al., 2006). Biopolymers also show promise in the medical and pharmaceutical fields as materials for drug delivery, wound dressings, and tissue scaffolds (Rinaudo, 2008). Biopolymer nanoparticles are particles generally between 1 and 100 nm that display novel properties different from those of the same biopolymer at larger scales (Cushen et al., 2012). Biopolymer nanoparticles have been used to encapsulate and deliver a variety of chemicals, including nutrients and drugs (Liu et al., 2005; Luo et al., 2012, 2013; Parris et al., 2005).

Here we examined the ability of biopolymer nanoparticles on food to retain a chemical while submerged in water and while being fed on by the freshwater amphipod *Hyalella azteca* (Amphipoda: Hyalellidae). By using nanoparticles to prevent dissolution of the chemical, we allow for normal feeding of *H. azteca* without monitored ad libitum feeding or gavage. We also tested for any effects the nanoparticles themselves may have on *H. azteca* in terms of survival, growth, and feeding behavior. The biopolymer nanoparticles must not significantly affect growth or survival in order to prevent confounding of the chemical's effects on these traits. Biopolymer nanoparticles on food also may either attract or repulse amphipods, causing an increase or decrease in the frequency of feeding, respectively. This could be due to changes in the tactile or chemosensory qualities of the food due to the biopolymer nanoparticles that could affect the ability of the amphipod to detect the food. This would result in large variation in how much food, and therefore how much chemical being held by the biopolymer nanoparticles, was ingested.

2. Materials and methods

2.1. Organisms

The freshwater amphipod *H. azteca* was used in all experiments. *H. azteca* is found across North America from the Nearctic through Central America and into northern South America in rivers, ditches, marshes, and wetlands. It was chosen as a test organism because of its widespread distribution, importance in aquatic food webs as a detritivore/shredder and as prey, and previous use in toxicological testing (Environment Canada, 2013; U.S. EPA, 2000). Amphipods originally obtained from Aquatic BioSystems (Fort Collins, CO, USA) were raised in an environmental chamber at 23 °C and a light:dark hour cycle of 16:8. Cultures were maintained at 23 ± 1 °C, 8.1 ± 0.1 mg/L dissolved O_2, and a pH of 8.3 ± 0.2. Amphipods were fed crushed fish food flakes (TetraMin). Amphipods used in experiments were separated by size with a #35 (500 μm) sieve and a #45 (355 μm) sieve as described by U.S. EPA (2000). Amphipods rinsed through the #35 sieve but stopped by the #45 were collected. Amphipods were then held in a separate container without food for 3 days before use in experiments. Amphipods used in all experiments were an average of 9 days old.

2.2. Biopolymers

2.2.1. Zein

Zein is a corn prolamine found in high concentrations in the endosperm of the corn kernel. It is an alcohol-soluble protein rich in nonpolar amino acids and poor in basic and acidic amino acids (Shukla and Cheryan, 2001). Due to the unique solubility of zein, zein nanoparticles can be easily prepared by liquid–liquid phase separation and have already been widely used to encapsulate and deliver hydrophobic nutrients (Luo et al., 2012, 2013; Parris et al., 2005) and drugs (Liu et al., 2005). This ability to encapsulate and deliver chemicals makes zein a good candidate for encapsulation of chemicals and coating of food for oral consumption by aquatic invertebrates.

Zein nanoparticles encapsulating rhodamine B were formed as described by Luo et al. (2011) with slight modifications. Briefly, zein was dissolved in 70 percent isopropyl-aqueous solution at 15 mg/mL. Rhodamine B was dissolved in pure ethanol at 1 mg/mL as stock solution. One mL of rhodamine B was added drop-wise to 7 mL of zein solution with mild stirring for 30 min. This mixture was quickly dispersed into 20 mL of water with vigorous stirring to allow the quick phase separation and formation of zein nanoparticles. The final zein concentration in dispersion was 3.75 mg/mL. The rhodamine B-encapsulated zein nanoparticle dispersion was then carefully dropped onto crushed fish food flakes (TetraMin) placed on a flat aluminum pan. The crushed pieces of food were ensured sufficient contact with the nanoparticle dispersion. The samples were subsequently dried in a vacuum oven (40 °C) overnight.

2.2.2. Chitosan

Chitosan is the *N*-deacetylated form of the polysaccharide chitin. Chitosan is a positively charged polyelectrolyte when dissolved in acidic solutions and has been considered an ideal biomaterial for encapsulation and delivery of drugs/nutrients in food and pharmaceutical sciences (Luo and Wang, 2013, 2014). Chitosan forms nanoparticles via electrostatic interaction with negatively charged molecules, including sodium tripolyphosphate (TPP). This electrostatic interaction holds the polymer strands together to form the nanoparticle. This ability to encapsulate and deliver chemicals makes chitosan a good candidate for encapsulation of chemicals and coating of food for oral consumption by aquatic invertebrates.

Chitosan nanoparticles encapsulating rhodamine B were prepared according to our previous study with slight modifications (Luo et al., 2010). Chitosan was dissolved in 1 percent acetic acid at 5 mg/mL. TPP was dissolved in pure water at 6 mg/mL. Rhodamine B was dissolved in pure ethanol at 1 mg/mL as stock solution. One milliliter of rhodamine B was added drop-wise to 6 mL of chitosan solution with mild stirring for 30 min. Then 1 mL of TPP solution was added drop-wise into the chitosan/rhodamine solution with mild stirring for another 30 min. Chitosan/TPP nanoparticles formed spontaneously when chitosan and TPP mixed together. The final chitosan concentration in nanoparticle dispersion was 3.75 mg/mL. The chitosan nanoparticle-coated food was prepared similarly to the zein nanoparticle-coated food as described above.

2.3. Biopolymer retention efficacy

The two chosen biopolymers, zein and chitosan, were tested for their ability to retain a chemical inside nanoparticles once nanoparticle-coated food was submerged in water. Rhodamine B dye was used as a test chemical for its non-toxicity at concentrations easily detected via fluorescence. The three food types used as treatments were food dyed with rhodamine B with no biopolymer nanoparticles, food coated with rhodamine B-containing zein nanoparticles, and food coated with rhodamine B-containing chitosan nanoparticles. Each food type treatment was also performed with and without amphipods to examine the effect of biopolymer shearing via amphipod feeding activity on rhodamine B release. Three replicates of each treatment were performed along with three replicates consisting only of dechlorinated tap water with no food to serve as an absolute control. Each experimental unit consisted of a 250 mL glass Erlenmeyer flask filled with 200 mL dechlorinated tap water with an air bubbler in a 23 °C environmental chamber. All treatments receiving amphipods began with 10 amphipods. Shelves holding the flasks were covered with black plastic to block light, which degrades rhodamine B. Each flask received food (1.5 mg) every other day for 14 days. Retention efficacy of the biopolymers was assessed as the amount of rhodamine B released, measured as the fluorescence of water samples from the flasks. Every other day two 1 mL water samples were removed from each flask and placed in centrifuge tubes. Fresh dechlorinated tap water (2 mL) was then added back to each flask. Samples were spun in a centrifuge for 30 s at 2000 rpm to precipitate heavy particles. Two hundred microliters were then pipetted from each water sample into a 96-well plate. Two wells were also loaded with 0.052 M rhodamine B as a standard for comparison of rhodamine B fluorescence, and two wells were loaded with fresh dechlorinated tap water. Plates were then measured for rhodamine B fluorescence (540 nm excitation, 625 nm emission) using a SpectraMax M2 Multi-mode Microplate Reader (Molecular Devices LLC, USA) and SoftMax Pro Microplate Data Acquisition and Analysis Software (Molecular Devices LLC, USA). Appropriate blanks for each treatment were run concurrently with the treatments described above and read during fluorescence measurement. Fluorescence values for treatments receiving the same food type but differing in the presence versus absence of amphipods were compared using repeated measures ANOVA. Where there was no significant effect of amphipod feeding, replicates receiving the same food type were combined. Fluorescence values were regressed against day for each food type, and slopes of regression lines were compared using ANOVA and paired contrasts. All statistical procedures were performed in SAS version 9.3 (SAS Institute Inc., USA).

2.4. Survival and growth of H. azteca

Survival and growth of amphipods were measured to assess any fitness effects the biopolymers may have on the amphipods. Food was coated with either zein or chitosan nanoparticles with no additional encapsulated chemical and then fed to amphipods. Food was made following the same procedures described above, except zein was used at an initial concentration of 15 mg/mL and a final concentration of 3.75 mg/mL, the same as chitosan. An additional treatment of amphipods fed uncoated food was used as a control. Five replicates of each treatment were performed. Each experimental unit consisted of a 250 mL glass Erlenmeyer flask filled with 200 mL of dechlorinated tap water with an air bubbler in a 22 °C