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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

environmental chamber. Shelves holding the flasks were covered with black plastic to hold conditions consistent with the biopolymer retention efficacy experiment. Each flask began with 10 amphipods. An additional five sets of 10 amphipods (50 amphipods total) were collected, dried in a 60 °C oven overnight, and weighed to obtain a mean initial mass. Food (1.5 mg) was delivered every other day for two weeks. At the end of two weeks, amphipods were collected from the flasks. The percent surviving amphipods was recorded. Amphipods were then dried in a 60 °C oven overnight and weighed to obtain mean final masses. Mean change in mass was calculated as the mean initial mass subtracted from the mean final mass from each replicate of each treatment. Percent survival was arcsine square root transformed and analyzed using ANOVA. Mean change in amphipod mass was analyzed using ANOVA.

2.5. Feeding behavior of H. azteca

Tests of feeding frequency were performed with the three food types used to assess fitness effects on amphipods: food with no biopolymer nanoparticles, food coated in zein nanoparticles, and food coated in chitosan nanoparticles. The nanoparticles were not encapsulating any chemical. Each experimental unit consisted of a circular plastic cup filled with 200 mL of dechlorinated tap water. Cups were not aerated during the experiment. Fifteen replicates of each treatment were performed. Food (1.5 mg) was placed in the center of each cup. One amphipod was then placed in each cup. The amphipods behavior was then tracked over time at 0, 0.5, 1, 2, 3, 4, 5, 6, 7, 8, and 24 h, giving 11 observations of each cup. Behaviors were defined as either feeding (amphipod observed on or holding food) or not feeding (amphipod visibly not eating or not near food). Feeding frequencies were calculated as the number of times an amphipod was observed to be feeding divided by the total number of observations of that amphipod. Given a non-normal distribution of feeding frequencies (Shapiro–Wilk, $p < 0.05$), analysis was performed using the non-parametric Kruskal–Wallis analysis of variance.

3. Results and discussion

3.1. Biopolymer retention efficacy

A significant interaction between day and biopolymer restricted comparisons to those between measurements made on the same day only. Comparison of treatments with the same type of food but differing in the presence or absence of amphipods revealed no significant differences on any day ($p > 0.05$) (Fig. 1). Thus either the nanoparticles were robust enough to resist releasing their encapsulated test chemical while being fed upon or feeding truly had no effect on the nanoparticles regardless of their robustness. Given the lack of an amphipod feeding effect, replicates of each food type with and without amphipods were combined (Fig. 2). Slopes of the regression lines for each food type represented the rate of change in fluorescence of water samples, and therefore the rate of change in water concentration of rhodamine B. Slopes of all three regression lines were significantly different from zero (Table 1). ANOVA revealed that at least one slope was significantly different from the others (Table 2). Pairwise contrasts between the slopes of the food types showed the rate of change in water fluorescence of the zein nanoparticle treatment to be significantly less than those of both other food types, and that of chitosan was significantly lower than that of the treatment with no nanoparticles (Table 2). Reduced release of the encapsulated chemical means that tank cleanings to remove uneaten food that disturb research animals could be less frequent.

While encapsulation of substances by biopolymer nanoparticles to prevent dissolution is proving to be a promising and effective method, certain quality control aspects must still be optimized to ensure this system performs optimally. Previous trials of this nanoparticle-based delivery system performed with a different batch of nanoparticle-coated food showed far less difference between treatments and lower retention of rhodamine B, though zein nanoparticles were still marginally the best. These differences in retention may be due to differences in encapsulation efficiency of the nanoparticles. Ultra-centrifugation may be used to quantify encapsulation efficiency of the nanoparticles. Previous work with this method has shown the encapsulation efficiency of

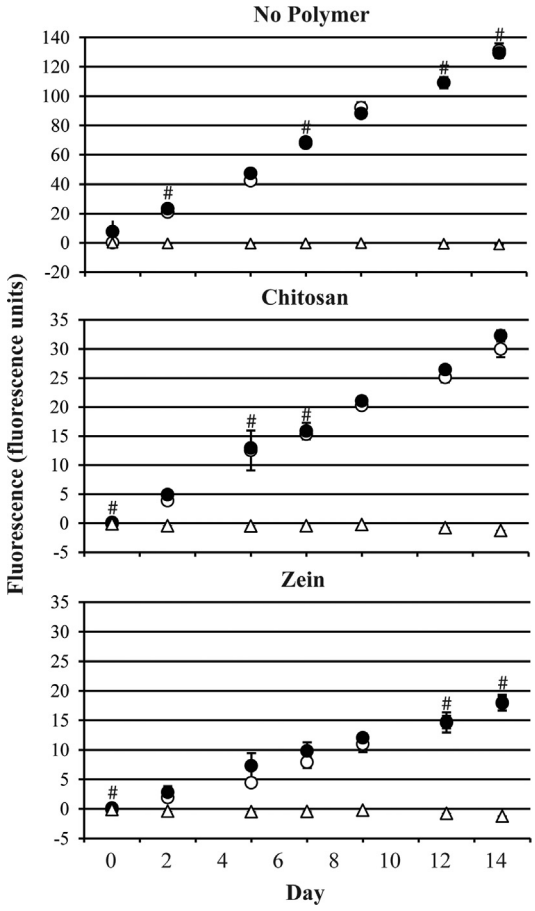

Fig. 1. Mean water sample fluorescences for three food types with and without amphipods. Data points are mean fluorescences with standard error bars ($n=3$ replicates per treatment). Increasing fluorescence indicates increasing concentration of rhodamine B in water samples. Treatments consisting of the same food type but differing in the presence versus absence of amphipods did not have significantly different water fluorescence values for all three food types on every measurement day ($p > 0.05$). Visually overlapping symbols are marked by #. (Open symbol: without amphipods; closed symbol: with amphipods; triangle: dechlorinated tap water).

Table 1
Regression of fluorescence values versus day for three food types. Slopes of regression lines were tested for significant differences from zero.

Food type	Slope	Intercept	Adjusted r^2	p-Value
No polymer	8.99	3.86	0.98	< 0.05*
Chitosan	2.28	0.84	0.95	< 0.05*
Zein	1.27	−0.16	0.91	< 0.05*

* Slopes significantly different from zero ($p < 0.05$).

Table 2
Overall ANOVA of regression line slopes and contrasts between slopes. A significant overall ANOVA revealed at least one slope was significantly different from the other two. Pair-wise comparisons were then performed between all treatments.

Overall ANOVA and paired contrasts		F-value	p-Value
Overall ANOVA		1711.08	< 0.05*
No polymer	Chitosan	1468.6	< 0.05*
No polymer	Zein	764.25	< 0.05*
Chitosan	Zein	51.28	< 0.05*

* All significant differences of $p < 0.05$.

zein nanoparticles for hydrophobic chemicals to be 75–85 percent (Luo et al., 2011). Additionally retention differences may be due to differences in the thickness of the nanoparticle film applied to the food, and thus differences in the number of nanoparticles present. Scanning electron microscopy (SEM) may allow estimation of film thickness on food as well as determination of the effect of film thickness on chemical retention. SEM can also be coupled with focused ion beam microscopy to evaluate the percentage of nanoparticles that successfully encapsulate the test chemical (Wang et al., 2008). Unencapsulated chemical may also be present on the surface of the food which can dissolve into water once submerged. This would inflate the measurement of chemical concentration in the water and artificially decrease the estimation of nanoparticle retention efficacy. Gently washing and then drying the nanoparticle-coated food may help decrease these residues, though any impact this additional processing may have on the nanoparticles would need to be assessed. Using one or more of these methods, a protocol to optimize encapsulation efficiency and standardize the amount of nanoparticle solution applied and final film thickness on food could be developed.

3.2. Nanoparticle delivery efficacy

An important aspect of toxicology not yet addressed using this system is that of delivery efficacy and uptake efficiency of a chemical in an organism. Knowing the actual dose being delivered successfully in the organism that can reach a target site of action is critical. Determination of the encapsulation efficiency of the nanoparticles discussed previously will determine more exactly the dose of a chemical being delivered initially. Analysis of unencapuslated chemical and nanoparticle concentration in an organism's feces will be informative of the breakdown of nanoparticles within the gut and the dose actually free to be absorbed by the organism. In the case of *H. azteca*, gut clearance happens very quickly, up to twice an hour (Hargrave, 1970); but nanoparticles of zein or chitosan may take longer than this to be digested since digestive enzymes are involved in degrading these biopolymers. Thus the actual dose absorbed by the amphipod may be less than the delivered initial dose. This could be compensated for by additional feedings of nanoparticle-coated food, delivering more food per feeding, or with higher concentrations of the chemical during encapsulation by the nanoparticles until the desired dose is delivered. Clearly the former is better suited for tests of chronic toxicity while the latter two are ideal for a more acute test of toxicity. The method of feeding should be chosen to match the desired result. While these issues that may affect the concentration of chemical absorbed by the organism should be addressed, there is little risk of any further complications in processes like distribution or transformation within the organism. Encapsulated chemicals interact with the biopolymers forming the nanoparticles non-covalently, with the main driving forces of encapsulation being hydrophobic interactions and hydrogen bonding. Once the nanoparticles are degraded within the organism, these bonds will break and the unaltered chemical will be released.

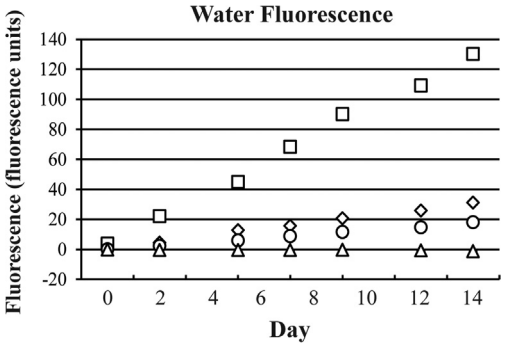

Fig. 2. Mean water sample fluorescence for three food types. Data points represent mean fluorescences with standard error bars ($n=6$ replicates per food type; standard error bars too small to be seen). Increasing fluorescence indicates increasing concentration of rhodamine B in water samples. Both zein nanoparticle-coated and chitosan nanoparticle-coated foods showed significantly lower fluorescence than food with no nanoparticles. (Square: no biopolymer; diamond: chitosan; circle: zein; triangle: dechlorinated tap water).

3.3. Survival and growth of H. azteca

Aquatic toxicity testing using a polymer nanoparticle delivery system has been attempted in at least one other study using nanoparticles of the synthetic polymer polymethacrylate (De Jong et al., 2008). Synthetic polymers or their monomers may themselves be toxic (e.g., Blaschke et al., 2012). The use of biopolymers circumvents this potential for toxicity, as they are derived from natural sources. To our knowledge the present study is the first to attempt to use biopolymers to perform this kind of oral toxicity testing. No significant effect of either kind of biopolymer nanoparticle on survival of amphipods was observed. There was also no significant effect of either biopolymer on mean change in amphipod mass (Table 3). Zein and chitosan were both non-toxic and non-beneficial to the amphipods. Thus the common toxicity assessment endpoints of survival/mortality and growth would not be confounded through the use of the biopolymer nanoparticles.

3.4. Feeding behavior of H. azteca

Biopolymer nanoparticles may potentially change the tactile or chemosensory qualities of food that amphipods use to locate and determine the suitability of food. A change in feeding frequency would have altered the change in mass and survival of amphipods. No significant effect of food type on feeding frequency of amphipods was detected (Table 3), indicating that the biopolymer nanoparticles did not negatively or positively affect qualities of the food that the amphipods may use for locating food or for food quality determination.

Table 3
Effect of food type on amphipod survival, mean change in mass of one amphipod, and feeding frequency. All values are shown as mean ± standard error. Overall ANOVA showed no significant effect of food type on both arcsine (square root) transformed survival and mean change in mass. Kruskal–Wallis analysis of variance showed no significant effect of food type on feeding frequency (i.e., the proportion of time spent feeding).

Parameter	Food type			*F*/*K*-values	*p*-Value
	No polymer	Chitosan	Zein		
Percent survival	72 ± 4	68 ± 7	80 ± 7	1.16	0.35
Mass change (mg)	0.030 ± 0.005	0.019 ± 0.005	0.035 ± 0.007	2.08	0.17
Proportion of time feeding	0.067 ± 0.017	0.065 ± 0.022	0.056 ± 0.007	0.62	0.73

4. Conclusions

Aquatic oral toxicity testing is difficult due in part to the tendency for chemicals to dissolve into water and affect organisms through other routes of exposure. The use of biopolymer nanoparticles to encapsulate chemicals was explored as a potential method to prevent this dissolution. Nanoparticles of the protein biopolymer zein had significantly lower release rates of rhodamine B, and thus a lower daily increase in water sample fluorescence, compared to chitosan biopolymer nanoparticles and to having no nanoparticles. Survival, growth, and feeding behavior of amphipods feeding on biopolymer nanoparticle-coated food were not significantly different from that of amphipods feeding on uncoated food. Given their retention ability and their non-interference with common toxicity measurement endpoints, biopolymer nanoparticles are a promising system for comparison of the oral route of exposure's contribution to a chemical's toxicity to those of other routes.

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