Delivery of dietary triglycerides to *Caenorhabditis elegans* using lipid nanoparticles: Nanoemulsion-based delivery systems

Daniel Colmenares, Quancai Sun, Peiyi Shen, Yiren Yue, D. Julian McClements,
and Yeonhwa Park *

Department of Food Science, University of Massachusetts,
102 Holdsworth Way, Amherst, MA 01003, **USA**

*To whom correspondence should be addressed

Department of Food Science, University of Massachusetts

102 Holdsworth Way, Amherst, MA 01003

Telephone: (413) 545-1018, Fax: (413) 545-1262; e-mail: ypark@foodsci.umass.edu

ABBREVIATIONS

C. elegans, *Caenorhabditis elegans*; CLA, conjugated linoleic acid; SOR, surfactant-to-oil ratio.

ABSTRACT

The nematode *Caenorhabditis elegans* is a powerful tool for studying food bioactives on specific biochemical pathways. However, many food bioactives are highly hydrophobic with extremely low water-solubilities, thereby making them difficult to study using *C. elegans*. The purpose of this study was to develop nanoemulsion-based systems to deliver hydrophobic molecules in a form that could be ingested by *C. elegans*. Optical microscopy showed that oil-in-water nanoemulsions with a range of particle diameters (40 to 500 nm) could be ingested by *C. elegans*. The amount of lipid ingested depended on the size and concentration of the nanoparticles. Fatty acid analysis showed incorporation of conjugated linoleic acid and there was a significant reduction in the fat levels of *C. elegans* when they were incubated with nanoemulsions containing conjugated linoleic acid, which suggested that this hydrophobic lipid was successfully delivered to the nematodes. The incorporation of hydrophobic molecules into nanoemulsion based-delivery systems may therefore enable their activities to be studied using *C. elegans*.

KEYWORDS: *Caenorhabditis elegans*; *C. elegans*; Conjugated linoleic acid; CLA; nanoemulsion; delivery systems.

1. Introduction

Caenorhabditis elegans, a nematode, is currently used as an *in vivo* model organism (Pellettieri, Reinke, Kim & Seydoux, 2002). The genome of *C. elegans* has approximately 72% similarity to humans (Zhang, Zou, Ding, Wang, Wu & Liang, 2013), due to the fact that there is a common descending evolutionary line that shares ancestors in the animal phylogeny between *C. elegans* and humans. *C. elegans* are simple hermaphrodites with a short life cycle (3 days for adulthood), have a large brood size (approximately 300 progeny), are relatively easy to handle, and have transparent bodies that allow one to easily observe their internal organs. The mouth of an adult *C. elegans* has six symmetrical lips surrounding its opening (McGhee, 2013) and forms a circular cavity of 1 to 3 μm from where food is transported to the pharynx. In laboratory settings, *C. elegans* ingests dietary *Escherichia coli* OP50 by pumping them into the mouth and concentrating them in the pharynx prior to passage into the intestine (Ashrafi, 2007, Solis & Petrascheck, 2011). This pumping action occurs regardless of food presence; however, the pumping rate is modulated by food availability (Ashrafi, 2007). The use of *C. elegans* for the study of the digestion, absorption, and bioactivity of hydrophobic compounds is currently limited because of their specialized feeding behaviour (selectivity for *E. coli* OP50) and the hydrophilic nature of their growth media leading to low solubility (McGhee, 2013). However, the permanent pumping-in reflex in the *C. elegans* pharynx and the application of emulsion-based delivery systems may overcome these limitations (Ashrafi, 2007, Chauhan, Orsi, Brown, Pritchard & Aylott, 2013).

Oil-in-water nanoemulsions (radius < 100 nm) and emulsions (radius > 100 nm) consist of small oil droplets coated by a surfactant layer, which are dispersed in an aqueous medium (McClements, Decker & Weiss, 2007, McClements, Decker & Park, 2009). These emulsion-

based delivery systems are relatively easy to prepare using standard laboratory equipment. The particle size of an emulsion-based delivery systems designed to feed *C. elegans* should be smaller than 1000-3000 nm (the dimensions of the nematode mouth) to be ingested. Ingestion may occur through two different mechanisms: (i) voluntary ingestion or (ii) non-voluntary ingestion of substrates simultaneously with ingestion of *E. coli OP50* bacteria, due to the continuous pumping action of the pharynx (McGhee, 2013, Shtonda & Avery, 2006). Therefore, the purpose of the present study was to establish an emulsion-based method to deliver triglycerides into the digestive system of *C. elegans*.

2. Materials and methods

2.1. Materials

Crude cold-pressed flax seed oil and corn oil were purchased from a local store. The triglyceride form of conjugated linoleic acid (CLA, Clarinol® G-80) was provided by Lipid Nutrition (Channahon, IL). This contained 78% of CLA (about 1:1 ratio of cis-9,trans-11 and trans-10,cis-12 isomers), 0.8% oleic acid, and 7.3% saturated fatty acids. Trioctadecadienoin (trilinolein, the TG form of linoleic acid) was purchased from Nu-Chek Prep (Elysian, MN). *N2 (wild type) C. elegans* and *Escherichia coli OP50* were purchased from the Caenorhabditis Genetics Center, University of Minnesota (Minneapolis, MN). The method used to prepare the S-complete medium used to culture *C. elegans* has been described previously (Solis & Petrascheck, 2011). All other chemicals were either from Fisher Scientific (Pittsburg, PA) or Sigma-Aldrich (Saint Louis, MO).

2.2. Methods

2.2.1. Nanoemulsion preparation

Sudan red (amethoxybenzenazo- β -naphthol) and Nile red (9-diethylamino-5-benzo [α] phenoxazinone) were dissolved in methanol, diluted in water and homogenized for 24 hours until complete dissolution. The dye solution was mixed with the oil phase, at a concentration of 1 mg/ml, and then the solvent (water and methanol) was removed by evaporation. 1 ml nanoemulsions were prepared by mixing oil (flax seed oil, corn oil, trilinolein, or CLA-TG form with or without dye), surfactant and S-complete medium, and then sonicated (Sonicator 505, Fischer Scientific) for 6-8 min (O'Sullivan, Murray, Flynn & Norton, 2015). Particle size was determined using a dynamic light scattering instrument (Malvern® Zetasizer Nano ZS, Malvern, UK) (McClements, 2007). In a preliminary study, both Tween 80 (polyoxyethylene-sorbitan monooleate) and Tween 20 (polyoxyethylene-sorbitan monolaurate) consistently delivered stable nanoemulsions at surfactant-to-oil ratios between 0.5 – 2.0 for oil concentrations of 0.1-100 mM. There were no major changes in appearance or particle size of the nanoemulsions with either Tween 80 or Tween 20 for 6 weeks at room temperature (data not shown). For experiments in Fig. 4 and 5, emulsions were prepared without dye.

2.2.2. Determination of nanoemulsion ingestion by *C. elegans*

C. elegans cultures were prepared according to standardized methods (Solis & Petrascheck, 2011, Stiernagle, 2006). 100 mM oil nanoemulsions with four different particle sizes were prepared. Each emulsion was diluted in S-complete buffer to obtain a series of nanoemulsions with four concentrations (100, 10, 1 and 0.1 mM). Aliquots (200 μ l) of these nanoemulsions were dispersed over the surface of individual agar plates containing a lawn of *E. coli* OP50 and

were air-dried overnight. L1 state *C. elegans* were then transferred to these plates and grown for 72 hours. Treatment plates containing nanoparticles with Sudan red were used for optical microscopy, and treatment plates with Nile red were used for confocal microscopy.

After 72 hours, adult worms were transferred to tubes with 1 ml of 20 mM sodium azide (NaN_3) solution, centrifuged (3,000 rpm for 3 min), and rinsed five times with NaN_3 solution. The concentration of worms was adjusted to 150 worms per 100 μl by addition of NaN_3 solution. Tubes were centrifuged, and supernatant were used as a blank and the remaining with worms were used to measure the fluorescence in triplicate after transfer into a 96-well plate with SpectraMax spectrophotometer (Molecular Devices, Sunnyvale, CA) at 520 nm excitation and 600 nm emission. The value of relative fluorescence units per worm (RFU/worm) was calculated as the difference between the RFU of the sample and RFU of the blank, divided by the number of worms. Microscopic observations were done on immobilized worms by optical microscopy (worms with Sudan red) and confocal microscopy (worms with Nile red). Confocal microscopy was done with an Eclipse 80i SOP, Nikon (Tokyo, Japan) microscope and optical microscopy using an Olympus 40SZ microscope (Tokyo, Japan).

2.2.3. Fatty acid composition assay

1 ml of 100 mM CLA nanoemulsions (mean particle diameters 150 nm) was dispersed over plates. The assay included a Tween 80 treatment (88 mg/ml) group as a control. After overnight drying, *C. elegans* L1 larvae were transferred to the prepared plates and left for 60 hours. Worms were transferred to centrifuge tubes and washed five times with distilled water, centrifugation at 2,500 rpm for 3 min. The worm pellets were suspended in 500 μl of distilled water and sonicated for 2 minutes prior to extraction. Total lipids were extracted as previously described (Folch, Lees

& Sloane Stanley, 1957). Fatty acid methyl esters (FAME) were prepared by treatment with 3N methanolic HCl at 55-60°C for 20 minutes (Park, Albright, Cai & Pariza, 2001). Final fatty acid methyl esters in hexane were used for GC/MS analysis.

FAME analysis was carried out using either 6890N Agilent GC equipment coupled to a 5973 Agilent mass selective detector (Santa Clara, CA) or Shimadzu GC/MS-QP2010 SE (Tokyo, Japan). *Oven conditions*: initial temperature 50 °C; temperature increase: 20 °C/min to 190 °C, held for 30 min, increase 2 °C/min to 220 °C and held for 130 minutes. *Other conditions*: injector temperature 250 °C; detector temperature 250 °C; carrier gas Helium, split ratio: 3:1. *Column*: Supelcowax 10 (fused silica), 100 m x 0.25 mm x 0.25 µm. The fatty acid methyl esters were identified by comparing with fatty acid standards (Sigma-Aldrich, St. Louis, MO, or Nu-Chek Prep, Elysian, MN) or by their mass spectra, which were further compared to the American Oil Chemists' Society mass spectra data or the NIST Mass Spectral library.

2.2.4. Determination of biological effects of emulsion

Stock emulsions containing the triglyceride form of CLA or linoleic acid at 100 mM were prepared with Tween 20 as an emulsifier at surfactant-to-oil ratios of 1:1. Average particle sizes of these emulsions were ~100 nm. Then, these emulsions were diluted with culture media for final concentrations of 100 µM. Blank treatments contained the same concentration of Tween 20 without any additional fat. *C. elegans* were treated with emulsions starting from the L1 stage for 3 days at 20°C. At the end of the treatment, *C. elegans* were collected, washed twice with M9 buffer, dissolved in 0.05% Tween 20 solution, and sonicated prior to quantify triglyceride and protein contents using commercial assay kits (Infinity™ Triglycerides Reagent, Thermo

Scientific, and Bio-Rad DC protein assay kit, respectively) according to the manufacturers' instructions. Triglyceride content was normalized relative to the measured protein concentration.

2.3. Statistical analyses

Data were expressed as mean $\pm$ S.E. and analyzed with Statistical Analysis System (SAS Institute, Cary, NC). Differences between groups were assessed with one-way (data in Fig. 4) or two-way ANOVA (Data in Fig. 1) followed with Tukey's multiple-range test. Significance of differences was defined at the $P < 0.05$ level.

3. Results and discussion

3.1. General

C. elegans is typically grown in hydrophilic aqueous-based media, which makes it difficult to study highly hydrophobic compounds due to their low water-solubility (Horikawa & Sakamoto, 2010). The current study therefore aimed to develop a lipid nanoparticle suspension for delivery of hydrophobic compounds, and to confirm the ingestion of the lipid nanoparticles by the *C. elegans*.

3.2. Ingestion of nanoemulsion-based delivery systems

Initially, *C. elegans* were incubated with nanoemulsion-based delivery systems containing dyed flaxseed oil, and then the increase in fluorescence intensity of the nematodes was measured (Figure 1). A direct correlation between the fluorescence intensity of the nematodes and the initial oil concentration of the nanoemulsions was observed, especially at oil concentrations above 10 mM (significant overall effects of concentration at $P < 0.0001$). In addition, the

fluorescence intensity of the nematodes increased with increasing nanoparticle size (significant overall effects of particle size at $P=0.0005$), which suggests that the amount of lipid ingested was higher for larger particles.

Figures 2 and 3 shows representative microscopy images of nematodes treated with nanoemulsions containing different concentrations or sizes of lipid particles. Consistent with the results in Fig. 1, there was a direct correlation between Nile red intensity and oil concentration (Fig. 2A-2D) or particle size (Fig. 2E-2H). Similar results were obtained when Sudan red was used (Fig. 3). The intensity of dye observed in the pharynx (particularly shown well in Fig. 2B, 3A, & 3F) and in the gut (Fig. 2A, 2E, 3A, 3E, & 3F) is direct evidence that the hydrophobic dyes in the nematodes were from ingested lipid nanoparticles rather than simple diffusion of dyes into their body.

The increase in particle uptake with increasing particle diameter may be attributed to a number of factors. First, *C. elegans* may have actively preferred to ingest larger particles rather than smaller ones. Second, *C. elegans* may have retained larger particles more effectively than small ones after ingestion. This may have occurred because small particles are highly susceptible to Brownian motion, and may therefore have rapidly diffused out of the mouth, whereas larger ones may have been physically trapped after ingestion. Third, the smaller particles may have diffused through the pores in the network of biopolymer molecules in the agar gel, thereby reducing the amount available at the gel surfaces for ingestion by the worms. Conversely, the larger particles may have remained on the surfaces of the agar gels, where the worms could readily ingest them. It should be noted that triglyceride particles with diameters greater than 500 nm became physically unstable upon contact with the agar surfaces leading to the visible separation of an oily layer (data not shown) and were therefore unsuitable for use. Overall, our

results suggest that oil concentrations from 10 – 100 mM and particle diameters from 200 – 500 nm were the most suitable for ensuring ingestion of a high amount of triglycerides by *C. elegans*.

3.3. Effect of dietary triglycerides on fatty acid composition

The fluorescent intensity and confocal fluorescence microscopy measurements indicated that the lipid nanoparticles were ingested by *C. elegans*, but they gave no clear evidence that these particles were digested and/or absorbed by *C. elegans*. The observed increase in fluorescence intensity in the nematodes may have been because the fat droplets were digested and absorbed, or it may simply have been because the fat droplets remained physically present within the gastrointestinal tract. Thus, in a follow-up study, we incorporated a hydrophobic bioactive lipid (conjugated linoleic acid, CLA) into the nanoemulsion-based delivery systems. The term “CLA” actually refers to a group of geometric and positional isomers of fatty acids with unique biological activities, particularly the ability to significantly reduce body fat in various animals, including *C. elegans* (Dilzer & Park, 2012, Martorell et al., 2012, Park, Albright, Liu, Storkson, Cook & Pariza, 1997). In our study, the CLA was delivered as a triglyceride because this molecular form is most likely to be used in commercial foods, and because it has a low water-solubility. Table 1 shows the fatty acid composition of control *C. elegans*. Twenty-six fatty acid methyl esters were identified in the wild type *C. elegans* fed *E. coli* OP50. The main components were 23.8% carbocyclic fatty acids (C17:delta and C19:delta), 23.2% vaccenic acid, 11c-C18:1, 13.54% unsaturated C20:n isomers, 8.5% monomethyl branched-chain fatty acids (C15:iso and C17:iso), and also linoleic, oleic, palmitic and stearic acids. No CLA was detected in the control worms. Thus, nanoemulsions containing CLA (as TG form) were prepared to determine the absorption of CLA from nanoparticles to *C. elegans*. The average particle diameter of the CLA

nanoemulsions was 150 nm, and CLA was detected in the CLA-treated *C. elegans* (Fig. 4). The gut transit time for *C. elegans* is about 1-2 min, and several washing steps prior to fat extraction have been reported be enough to eliminate any remaining gut contents (McGhee, 2013). Thus, we believe that the CLA detected in the *C. elegans* was present within the worms' body, and not from any remaining nanoemulsion in the gut.

3.4. Bioactivity of nanoemulsion-based delivery systems

To further confirm that *C. elegans* ingested and absorbed lipids from the nanoemulsions, we determined the bioactivity of CLA, *i.e.* its ability to reduce the body fat of *C. elegans*. As a control, we used the triglyceride form of a closely related fatty acid (linoleic acid as trilinolein), which does not exhibit the same biological activity as CLA. The lipid concentration used in this study (100 μ M) was much smaller than those used in the previous section (100 mM), because only a low level of CLA is needed to demonstrate bioactivity. This meant that the lipid droplets could not be visualized using confocal fluorescence microscopy in this part of the study since the intensity levels were too low.

The total body fat of the nematodes was significantly lower when they were incubated with nanoemulsions containing CLA than when they were incubated with ones containing linoleic acid (Fig. 5). In current study, the total body fat of the nematodes was similar for the control sample (no extra fat added) and the nanoemulsion containing CLA (Fig. 5). There are a number of possible reasons for this observation. First, it is possible that nematodes actively avoided ingesting CLA droplets compared to linoleic acid droplets, but this is unlikely since CLA has similar physicochemical properties to linoleic acid. Second, it is possible that the nematodes were unable to digest and/or absorb triglyceride molecules containing CLA. Previous

in vitro studies have shown that triglycerides containing certain CLA isomers are more resistant to digestion by lipase than those containing linoleic acid, however, *in vivo* studies have reported that CLA is digested and absorbed in animal and tissue culture models (Dilzer & Park, 2012, Martin, Sebedio, Caselli, Pimont, Martine & Bernard, 2000, Park & Pariza, 2007, Terpstra et al., 2003). Third, it is possible that the triglycerides in the CLA droplets were digested and absorbed by the nematodes, but that they inhibited the formation of body fat, as has been reported in previous studies using other animal models (Dilzer and Park, 2012). Indeed, in another study using a longer treatment period (7 days), we observed that CLA-treated nematodes had significantly lower levels of fat than the control, and that there were specific biochemical changes indicative of CLA absorption. This data will be reported in a future study that focuses on changes in the biological activity of *C. elegans* after treatment with CLA. Overall, our data suggests that the CLA droplets in nanoemulsion-based delivery systems were digested and/or absorbed by *C. elegans*, supporting their potential application in this model.

A study by Martorell *et al.* (2012) and our current results consistently showed that CLA effectively reduced total body fat compared to the linoleic acid control in this model (Martorell et al., 2012). However, the previous study used ethanol to dissolve free CLA in culture medium. Thus, it is not clear how much CLA was delivered via the gastrointestinal tract in this study (Martorell et al., 2012). Compared to this, the current study shows that nanoemulsion-based delivery systems can be used to deliver hydrophobic triglycerides to *C. elegans* suspended in hydrophilic media and is a more appropriate method to treat and test food-based lipophilic compounds in this model.

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310 **Table 1.** Total fatty acid composition of wild type *C. elegans*.

<i>Fatty acid (IUPAC name)</i>	<i>Condensed formula</i>	<i>Fatty acid content (Area %)*</i>		
<i>Saturated fatty acids:</i>				
Dodecanoic acid	C12:0	0.1	±	0.1
Tetradecanoic acid	C14:0	1.5	±	0.1
Hexadecanoic acid	C16:0	5.0	±	0.4
Heptadecanoic acid	C17:0	0.2	±	0.1
Octadecanoic acid	C18:0	4.5	±	0.2
Nonadecanoic acid	C19:0	0.8	±	0.1
Eicosanoic acid	C20:0	0.1	±	0.1
<i>sum of SAFA:</i>		12.1	±	0.4
<i>Monomethyl branched-chain fatty acids:</i>				
9-Methyltetradecanoic acid	C15:iso	4.2	±	0.2
Hexadecanoic acid - 15-methyl	C17:iso	4.3	±	0.1
<i>sum of mmBFAs:</i>		8.5	±	0.2
<i>Carbocyclic fatty acids:</i>				
Cyclopropaneoctanoic acid, 2-hexyl	C17:delta	17.0	±	0.8
Cyclopropaneoctanoic acid, 2-octyl	C19:delta	6.8	±	0.5
<i>sum of carbocyclic FAs:</i>		23.8	±	1.0
<i>Unsaturated fatty acids (except C20:n):</i>				
11-Tetradecenoic acid (Z)	C14:1 (11c)	0.3	±	0.1
11-Hexadecenoic acid (Z)	C16:1 (11c)	3.1	±	0.1
9-Octadecenoic acid (Z)	C18:1 (9c)	5.0	±	0.4
11-Octadecenoic acid (Z)	C18:1 (11c)	23.3	±	0.7
8-Octadecenoic acid (E)	C18:1 (8t)	0.1	±	0.1
9,12-Octadecanoic acid (all Z)	C18:2 (9c,12c)	7.0	±	0.2
10,13-Octadecadienoc acid (all Z)	C18:2(10c,13c)	0.1	±	0.0
9,12,15-Octadecatrienoic acid (all Z)	C18:3 (9c,12c,15c)	0.3	±	0.1
<i>sum of unsaturated FAs (except C20:n):</i>		39.3	±	0.8
<i>Unsaturated C20:n isomers:</i>				
11-Eicosenoic acid (Z)	C20:1 (11c)	0.7	±	0.1
11,14-Eicosadienoic acid (all Z)	C20:2 (11c,14c)	1.5	±	0.3
8,11,14-Eicosatrienoic acid (all Z)	C20:3 (8c,11c,14c)	1.8	±	0.1
11,14,17-Eicosatrienoic acid (all Z)	C20:3 (11c,14c,17c)	0.3	±	0.2
5,8,11,14-Eicosatetranoic acid (all Z)	C20:4 (5c,8c,11c,14c)	0.4	±	0.1
8,11,14,17-Eicosatetranoic acid (all Z)	C20:4 (8c,11c,14c,17c)	2.2	±	0.2
5,8,11,14,17-Eicosapentaenoic acid (all Z), EPA	C20:5 (5c,8c,11c,14c,17c)	6.6	±	0.1
<i>sum of C20:n isomers:</i>		13.5	±	0.3
<i>Un-identified fatty acids:</i>		2.8	±	0.2

311 *Values are mean ± S. E. (n=6).

Figure 1. Relative fluorescence (RFU/worm) as a function of particle size (Dp) and oil concentration. Worms were grown on agar containing flax seed oil nanoparticles of different particle size for 72 hours. Nanoparticles contained 1mg/ml Nile red in oil. Fluorescence was determined by spectrometry at 520nm (excitation) and 600nm (emission). Values are mean $\pm$ standard error (n=4).

Figure 2. Confocal images of worms grown on agar containing nanoparticles. Worms were grown on agar plates containing flax seed oil nanoparticles with different oil concentrations (A-D) and different particle size (E-H). Nile red was used as dye.

Figure 3. Optical images of worms grown on agar containing nanoparticles. Worms were grown on agar plates containing flax seed oil nanoparticles with different oil concentrations (A-D) and different particle size (E-H). Sudan red was used as dye.

Figure 4. GC/MS chromatograms of fatty acid composition from control (A) and CLA-treated (B) *C. elegans*. A chromatogram from standard CLA methyl esters is shown in (C).

Figure 5. Effects of conjugated linoleic acid (CLA) delivered as nanoemulsion on body fat accumulation. *C. elegans* were treated with 100 μ M of emulsions prepared with TG form of CLA or linoleic acid (LA) and Tween 20 for 3 days. A Tween 20 treatment alone was used as a blank. Values are mean $\pm$ standard error (n=3). Means with different letters are significantly different at $P<0.05$.

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Concentration of oil (mM)	100
	10
	1
	0.1

Particle size of the nanomulsion (nm)	300-500
	200-250
	100-150
	40-60

Concentration of oil (mM)	100	A
	10	B
	1	C
	0.1	D

Particle size of the nanomulsion (nm)	300-500	E
	200-250	F
	100-150	G
	40-60	H

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