Semi-quantitative analysis for the uptake of fluorescently labeled nanocrystalline cellulose in zebrafish embryos *(Danio rerio)*

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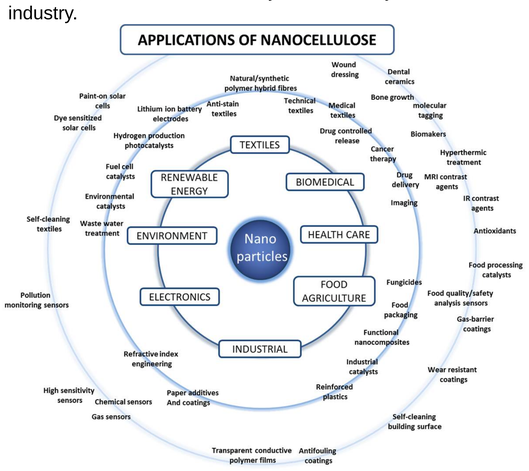
Abstract

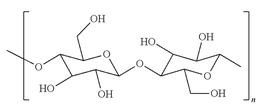
Uptake and biodistribution of nanomaterials in an *in vivo* model system are important considerations for nanotoxicology. The use of fluorophore labeling can help with identifying particle location within a specimen and enable particle tracking. This study aimed to quantify the uptake of rhodamine labeled carboxylated nanocrystalline cellulose (CNC) throughout development in the model vertebrate, *Danio rerio*. CNC was selected for initial investigation because it elicits low toxicity in zebrafish embryos and is increasingly important in commerce. Similar to unlabeled CNC, no significant mortality or developmental abnormalities were observed at the highest dose tested. Fluorescent microscopy images of control embryos and embryos exposed to 500ppm rhodamine labeled CNC were taken on days 1- 5. Integrated density (ID) was used as a surrogate measure for uptake. The experiment was repeated using embryos with and without intact chorions. On day 1, embryos exposed without a chorion had an ID value 3 times higher than those with the chorion intact. On day 2, the chorion off embryos had an ID value 1.43 times that of the chorion on embryos. There was a linear increase in uptake over the first 4 days, and then an exponential increase on day 5, possibly due to the onset of mouth gaping. Confocal microscopy was used to identify heavily concentrated locations within the embryos that seem to be associated with the integumentary system. Our data demonstrates that carboxylated CNC was taken up both dermally and orally by the embryos depending on the stage of development. In addition, the chorion, in some cases, does pose a barrier for these particles prior to hatching which is important when assessing toxicity within the first 72 hours of development.

Background

- Nanocrystalline cellulose (CNC) is a strong, lightweight pseudo plastic with a high aspect ratio.
- Due to its abundance in nature and its high tensile strength and stiffness, nanocellulose is widely used in many realms of industry.



- CNC can be easily altered to have specific surface chemistry. Changes in surface chemistry are known to alter the uptake and toxicity of other nanomaterials suggesting a need to determine if the same holds for CNC.



- CNC is considered to have very low toxic potential. However, questions have been raised about whether the CNC is taken up by organisms and has an opportunity to elicit toxic responses.

Objective

To better understand the uptake and potential toxicity of CNC in zebrafish and asses the role of the chorion as a protective barrier to exposure.

Hypotheses

1.) The addition of Rhodamine B to the surface of CNC will not alter the toxicity of the material. If supported, this will allow for the use of Rhodamine B labeling to study uptake.

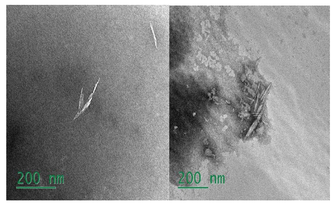
2.) Developing zebrafish will take up CNC dermally and orally at different rates. It is thought that the onset of mouth gaping will increase the rate of uptake.

3.) The chorion can pose as a protective barrier that will slow the rate of particle uptake. If supported, the chorion should be considered in future toxicity testing with embryos.

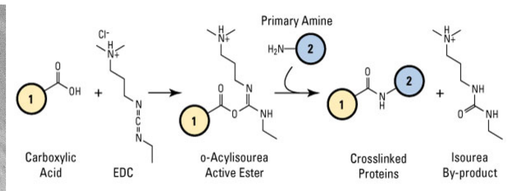
Materials and Methods

Materials:

- Nanocell™ carboxylated nanocrystalline cellulose (CNC) was obtained from Bio-vision Technology Enterprise Inc. Novia Scotia, Canada
- Rhodamine B tagged by Dr. John Simonsen, OSU Wood Sciences by TEMPO oxidation.

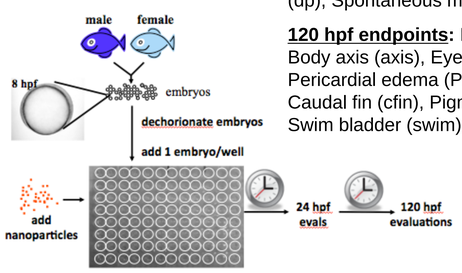


TEM images of Rhodamine B labeled CNC were provided by Dr. John Simonsen



EDC (carbodiimide) crosslinking reaction scheme. Carboxyl-to-amine crosslinking with the popular carbodiimide, EDC. Reaction used to label CNC with Rhodamine B.

Toxicity Testing:

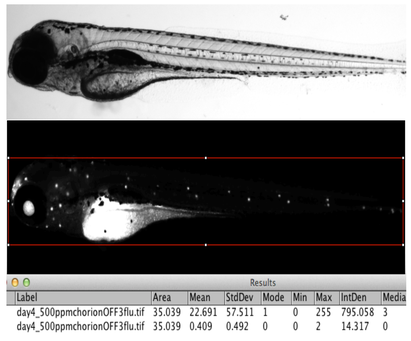


24 hpf endpoints: Mortality (mort), Developmental progression (dp), Spontaneous movement (sm) Notochord (nc)

120 hpf endpoints: Mortality (mort), Yolk sac edema (YSE), Body axis (axis), Eye, Snout, Jaw, Otic vessicle (otic), Pericardial edema (PE), Brain, Somites, Pectoral fin (pfin), Caudal fin (cfin), Pigmentation (pig), Circulation (circ),Trunk, Swim bladder (swim), Motility (touch response, tr)

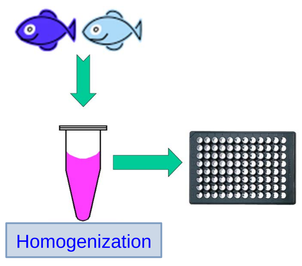
Quantifying Uptake:

Image analysis:



- Embryos were exposed to 0, 100 and 500 ppm Rhodamine B labeled CNC.
- The chorion of the fish was either left on or manually removed before exposure.
- The fish were imaged everyday for 5 days with an inverted Zeiss microscope.
- Before imaging, the fish were rinsed and anesthetized. If still in the chorion, they were manually removed.
- ImageJ was used to measure the relative intensities of the images

Embryo Homogenate:

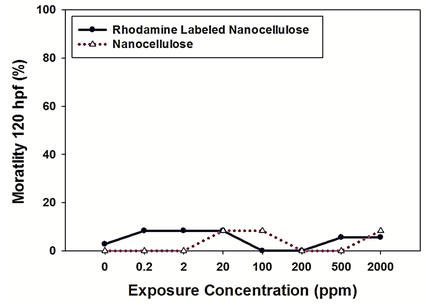


- Zebrafish were exposed to 0 and 500 ppm Rhodamine B labeled CNC.
- The chorion of the fish was either left on or manually removed before exposure.
- The fish were homogenized on days 1-5 after thorough rinsing and manual chorion removal.
- The homogenate was loaded into a 96 well clear-bottom black plate and scanned using a spectofluorometer.

  (excitation = 540nm, emission = 625nm)
- Fluorescence units were converted to concentration (ppm) using a calibration curve.

Results

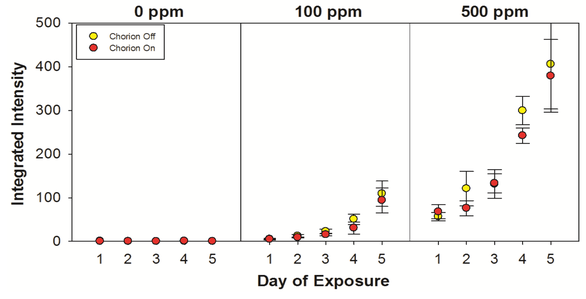
Toxicity:



Overall toxicological impact from CNC to zebrafish was low with no significant impact from the Rhodamine B labeling.

The occurrence of all other sublethal malformations was insignificant.

Image Analysis:



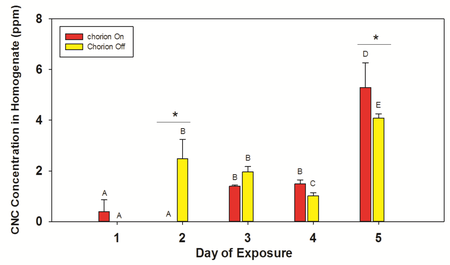
Exposure concentration significantly impacted intensity (uptake).

Overall, chorion on and off exposures had different intensity (uptake) across exposure concentrations.

Overall, significant impact of exposure duration on intensity (uptake).

Uptake of CNC occurred during zebrafish development, both dermally and orally, in a concentration dependent manner.

Homogenate:

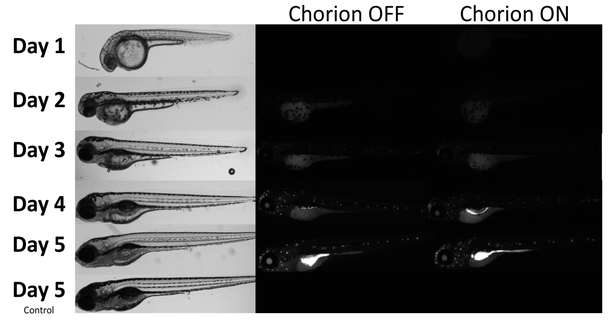


The chorion, in some cases, did block a significant amount of the CNC from entering the embryo before hatching occurred.

* indicates significant difference between chorion On/Chorion Off for given day.

Letters indicate significance at p<0.05

Images for Days 1-5 (fish exposed to 500 ppm CNC):



Conclusion

CNC elicited no toxic responses in early developing zebrafish although the CNC was readily taken up by the fish, both dermally and orally, throughout the exposure period.

Acknowledgments

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