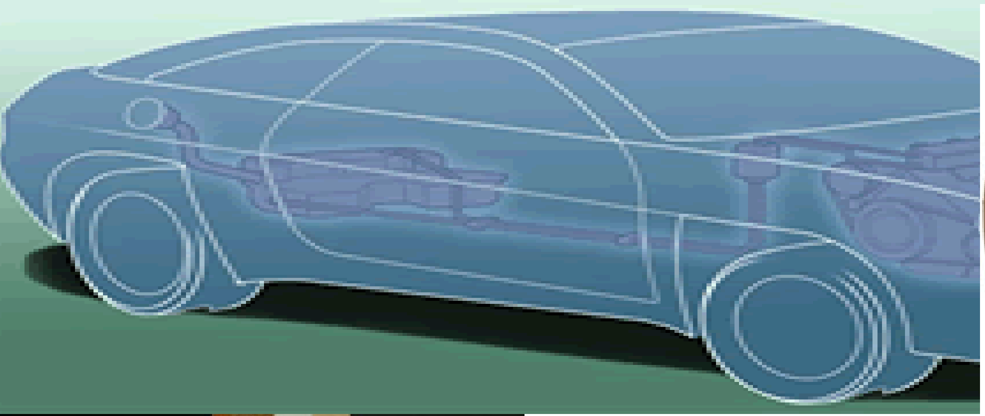
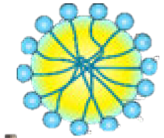





Safety and Regulatory Aspects of Cellulose Nanomaterials: Challenges and Needs

Jo Anne Shatkin, Ph.D.

Vireo Advisors, LLC
Raising the Bar on Sustainability in Innovation




24 August 2023

UMaine CNM Researchers Meeting

Orono, ME

U.S. Endowment for Forestry and Communities

USDA Forest Service U.S. Department of Agriculture

AGENDA

1. Update on Food Safety Study/Alliance
2. Functionalized CN Toolbox: synthesis, characterization and safety testing
3. Characterization challenges and ideas
4. Safety demonstration needs

NANO COLLABORATORS

U.S. Endowment for Forestry and Communities
OECD
European Commission
ALBERTA INNOVATES
Alberta-Pacific Forest Industries Inc.
HARVARD T.H. CHAN SCHOOL OF PUBLIC HEALTH
Health Canada Santé Canada
OSU Oregon State University
AGENDA 2020 TECHNOLOGY ALLIANCE
UC DAVIS
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Environment and Climate Change Canada
National Institute for Occupational Safety and Health NIOSH
UNIVERSITY OF GEORGIA
NIST
UBC
VIRGINIA TECH
NATIONAL INSTITUTE FOR OCCUPATIONAL HEALTH
Division of the National Health Laboratory Service
THE UNIVERSITY OF IOWA
National Institute for Public Health and the Environment
Ministry of Health, Welfare and Sport
UNIVERSITY OF BIRMINGHAM
BAYLOR UNIVERSITY
NanoCASE
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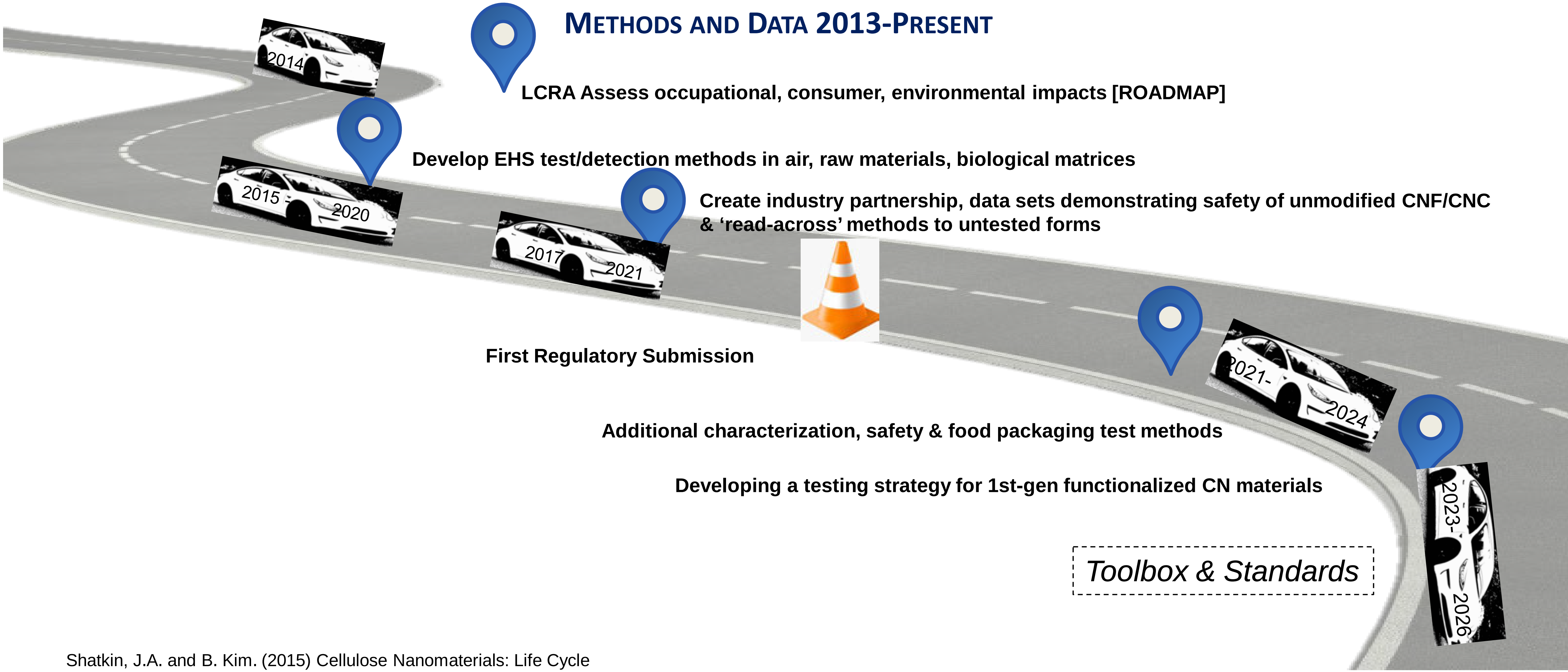
Environmental Health and Safety EHS ROADMAP – Cellulose Nanomaterials

Life-Cycle Risk Assessment (LCRA)

Methods and Data 2013-Present



Shatkin, J.A. and B. Kim. (2015) Cellulose Nanomaterials: Life Cycle Risk Assessment, and Environmental Health and Safety Roadmap

Environmental Science: Nano, (2):477-499. DOI: 10.1039/C5EN00059A

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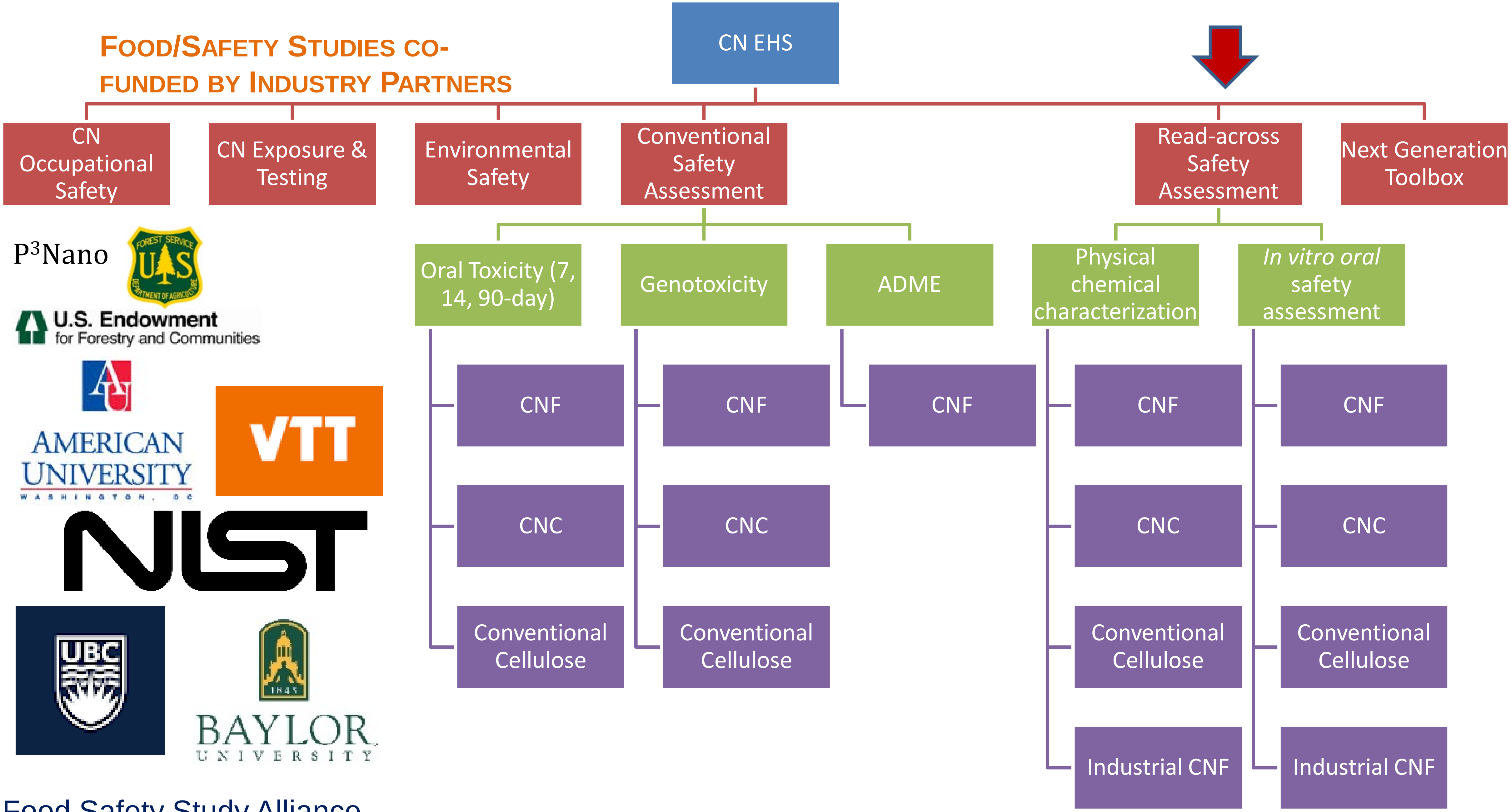
Food/Safety Studies co-funded by Industry Partners



Food Safety Study Alliance

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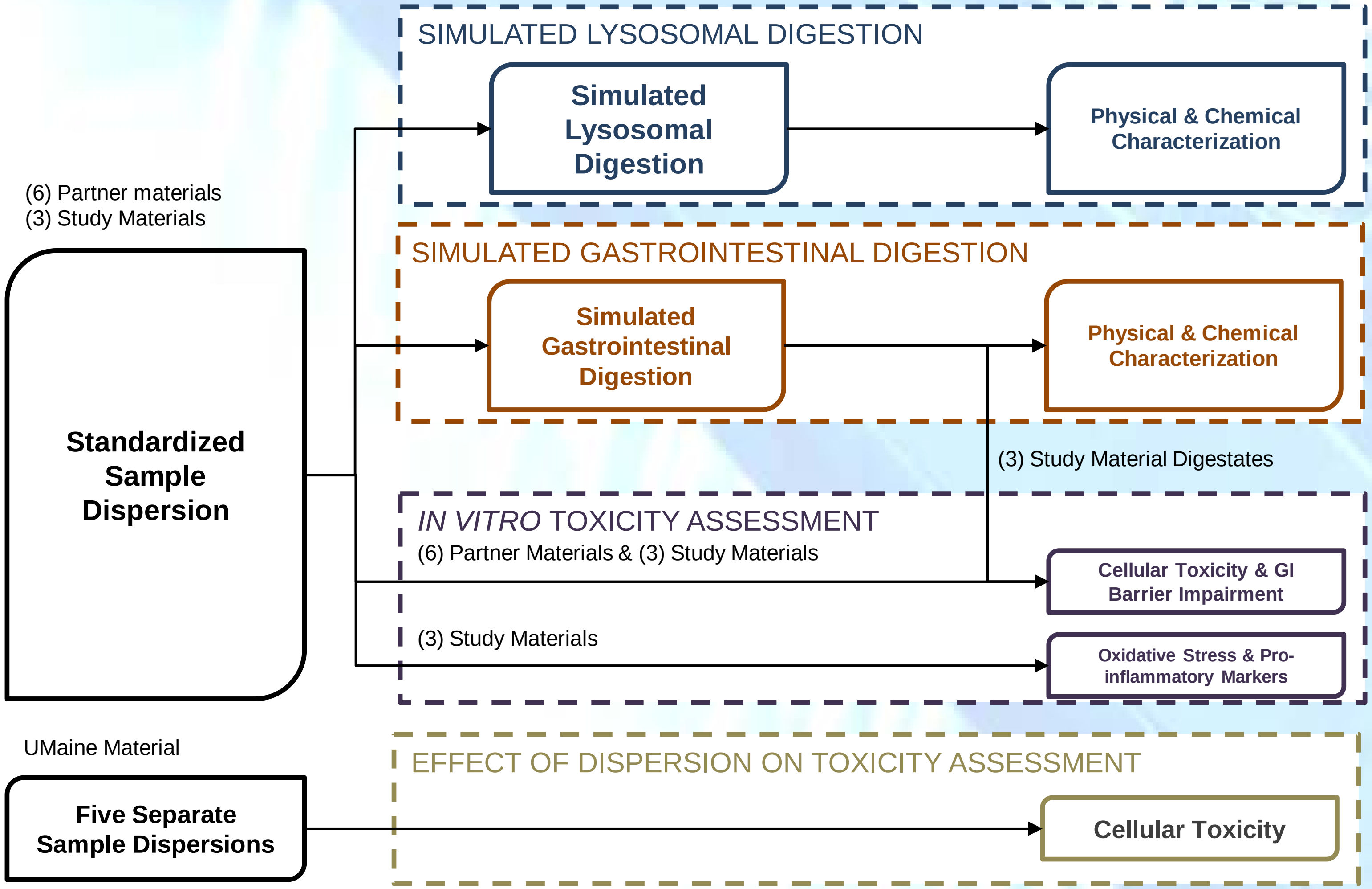
STRATEGY: ALTERNATIVE TESTING STRATEGIES



Ede, J.D., Ong, K.J., Mulenos, M.R., Pradhan, S., Gibb, M., Sayes, C.M., Shatkin, J.A. (2020). *Toxicology Research*, 9(6): 808-822.

Pradhan S.H., Mulenos M.R., Steele L.R., Gibb M., Ede J.D., Ong K.J., Shatkin J.A., and Sayes C.M. (2020). *Toxicology Research*, 9(3): 290-301.

Cellulose Nanomaterials Food Safety Study

Animal Studies [1,3]		Cell-based Studies [2,3]	
Study	Result	Endpoint	Result
Acute Oral Rat Toxicity			
7-day Oral Toxicity (OECD TG 407)	NO ADVERSE EFFECTS	Cytotoxicity In Co-Culture Model	NO ADVERSE EFFECTS
14-day Oral Toxicity (OECD TG 407)	NO ADVERSE EFFECTS	Barrier Integrity Over 7-days	NO ADVERSE EFFECTS
Sub-chronic Oral Rat Toxicity		Oxidative Stress	NO ADVERSE EFFECTS
90-day Oral Toxicity (OECD TG 408)	NO ADVERSE EFFECTS	Inflammation	NO ADVERSE EFFECTS

- **CNC & CF behave similarly to conventional cellulose and raises no safety concerns when used as a food ingredient at 4% of diet;**
- **GRAS status (FDA) allows use in food and food contact applications.**
- **CNs behave similarly to conventional cellulose - supporting evidence for use in food;**
- **Baseline measurements for examining potential impact of future functionalizations on toxicity.**

[1] Ong, K.J et al. (2020)

[2] Pradhan et al. (2020)

[3] Ede et al. (2020)

Toxicokinetics rat study – Results (ADME) with U Maine PDC MFC in Commercial Lab

Mass balance

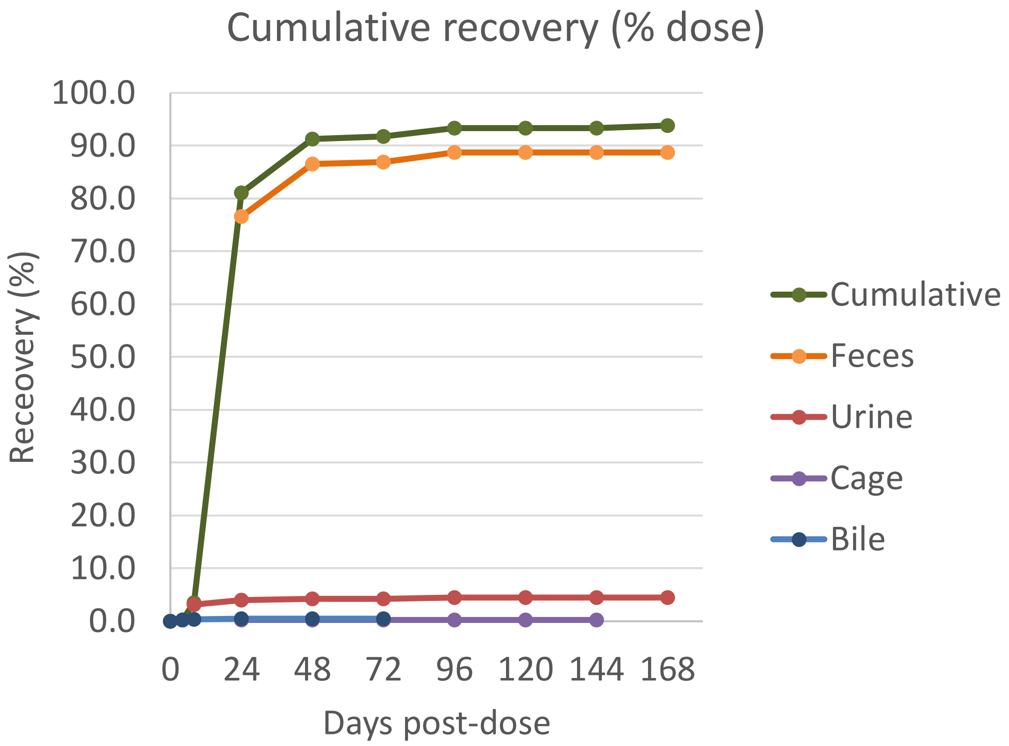
Cumulative recovery
= 93.5 ± 7.6%

Feces – 88.8%

Urine – 4.4%

Cage – 0.2%

Bile – 0.4%



Manuscript in Preparation

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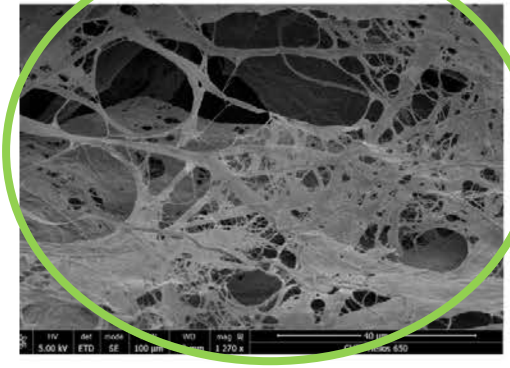
MFC is similar to cellulose used in food

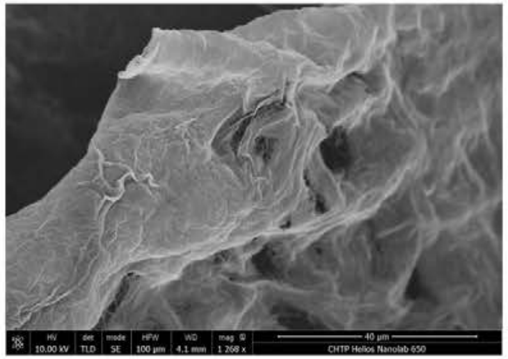
2. Size/Morphology: Micro Scale SEMs

Ref MFC

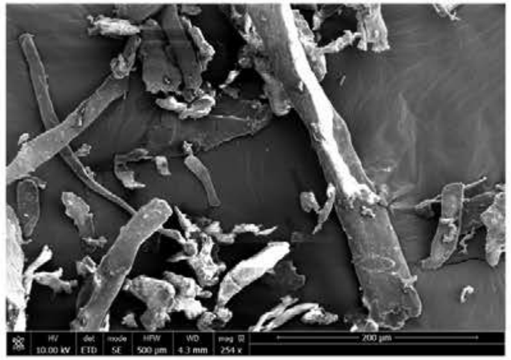


MFC Ketchup

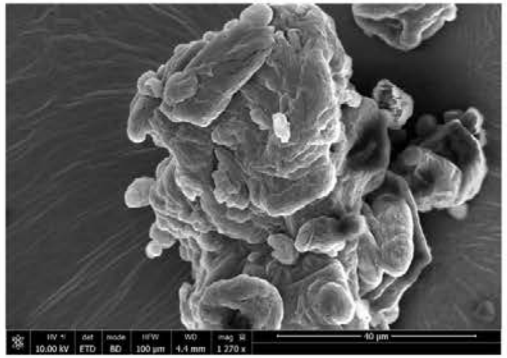
MFC Nata de Coco



Commercial Cellulose



Commercial MCC



Ref MFC is from U Maine PDC.

MFC has a morphology composed of fibers with varying lengths and widths that form a complex webbed and entangled network.

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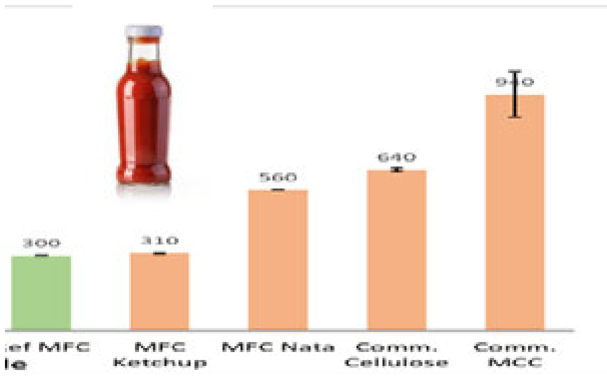
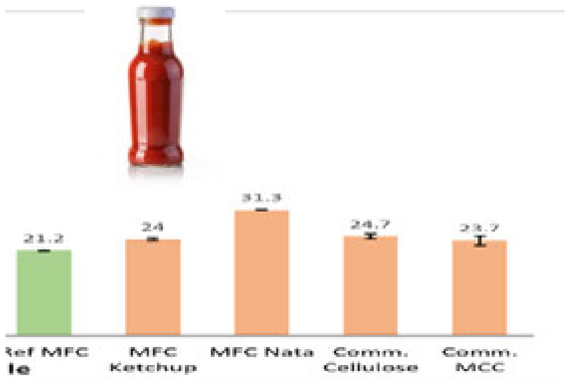
MFC is similar to celluloses long used in food

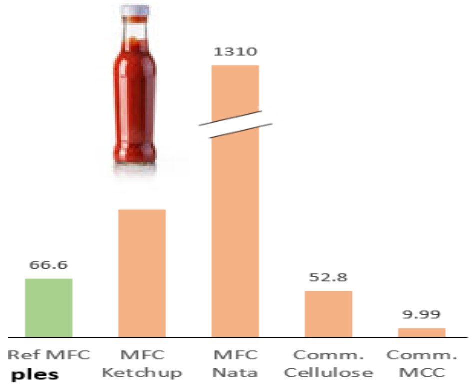
2. Size/Morphology: Micro Scale



Dynamic image analysis: Average fiber length (μm)



Dynamic image analysis: Average fiber width (μm)



Laser diffraction: Dv(50) (μm)

Ref MFC is from U Maine PDC, in **green**.

The average fiber lengths and widths of MFC are in the range of celluloses already used in food (**in orange**).

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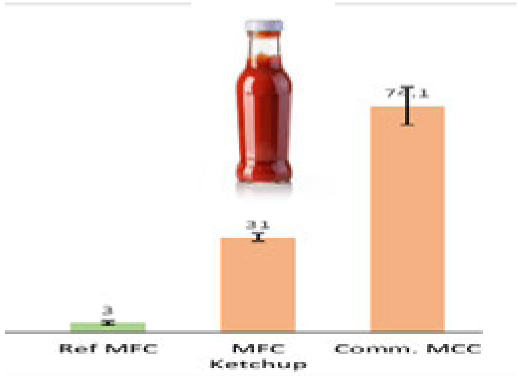
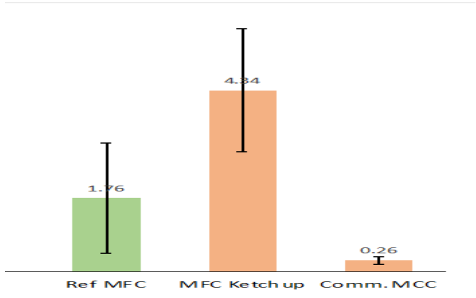
MFC is similar to cellulose in food

3. Size/Morphology: ***Finest Fraction***

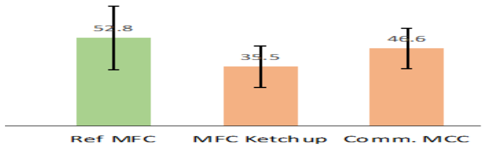
- Centrifugation protocol to isolate smallest fibers and fibrils (wt. %)
- Atomic force microscopy to characterize average fiber length and width in finest fraction
- MFC has a lower percentage of fine fibers than cellulose already used in/present in food.
- The finest fraction of MFC has fibers and fibrils with similar lengths and widths to cellulose already used in/present in food.



Weight % finest fraction



Average fiber length (μm) finest fraction



Average fiber width (nm) finest fraction

VTT

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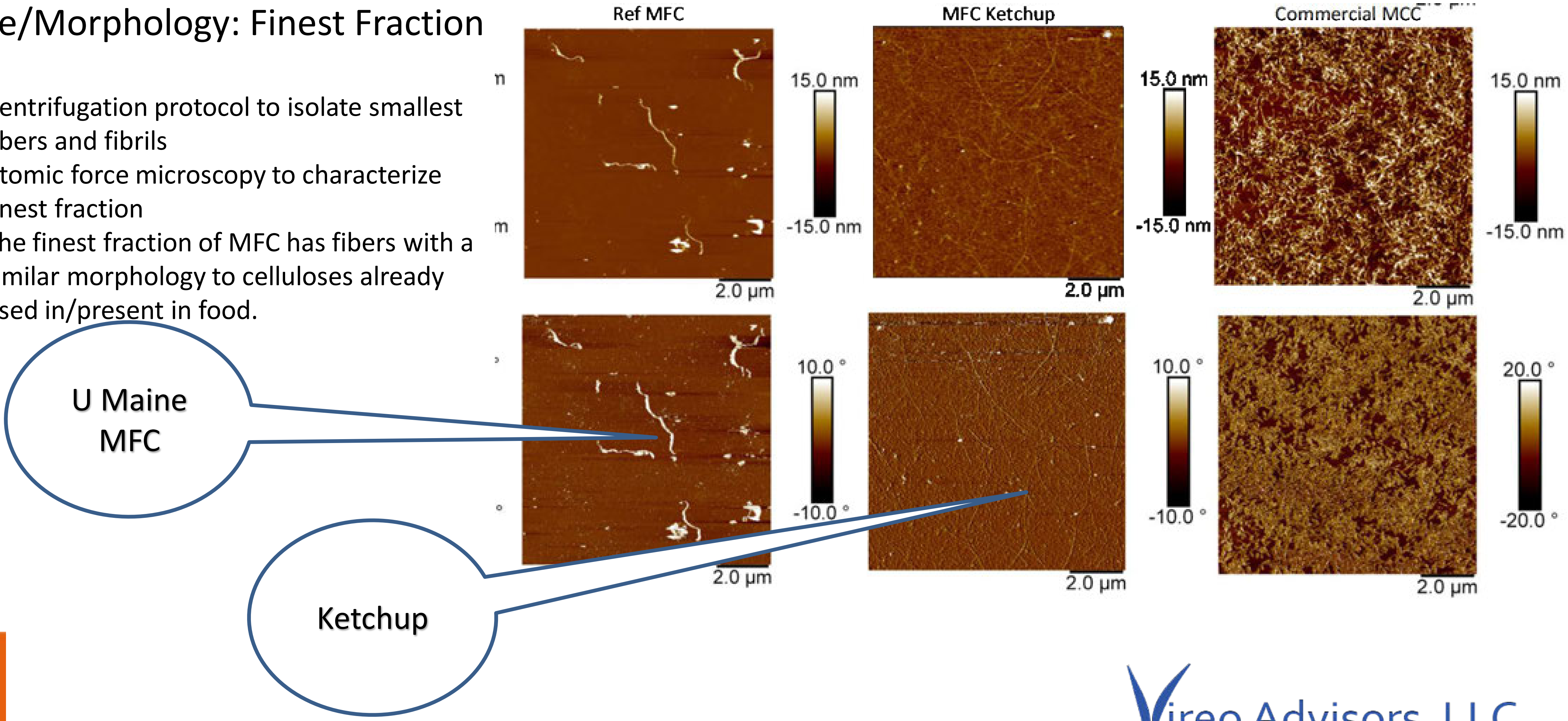
AFM Shows the isolated finest fraction similar to celluloses from fruit fiber long used in food

3. Size/Morphology: Finest Fraction

- Centrifugation protocol to isolate smallest fibers and fibrils
- Atomic force microscopy to characterize finest fraction
- The finest fraction of MFC has fibers with a similar morphology to celluloses already used in/present in food.



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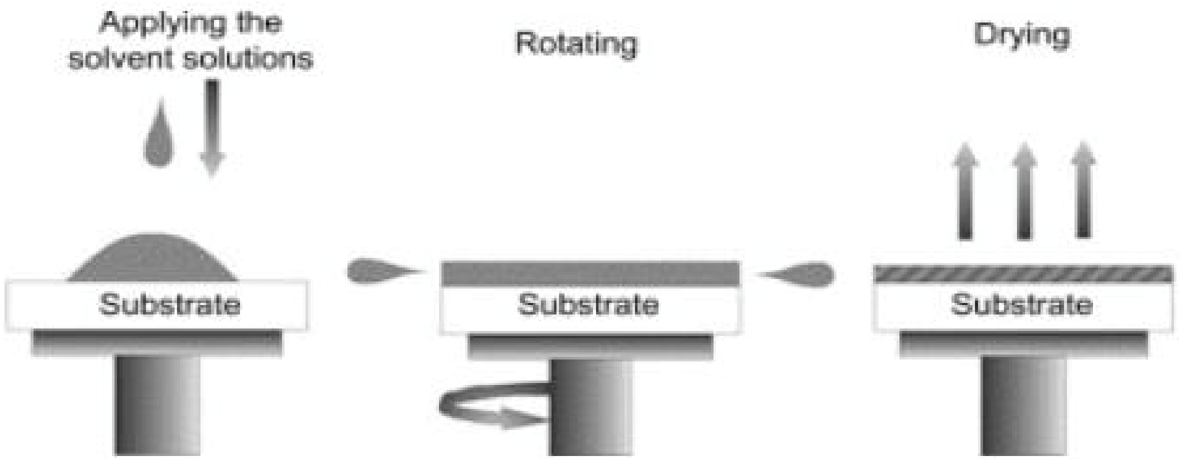
Detection of discrete fibrils in extract

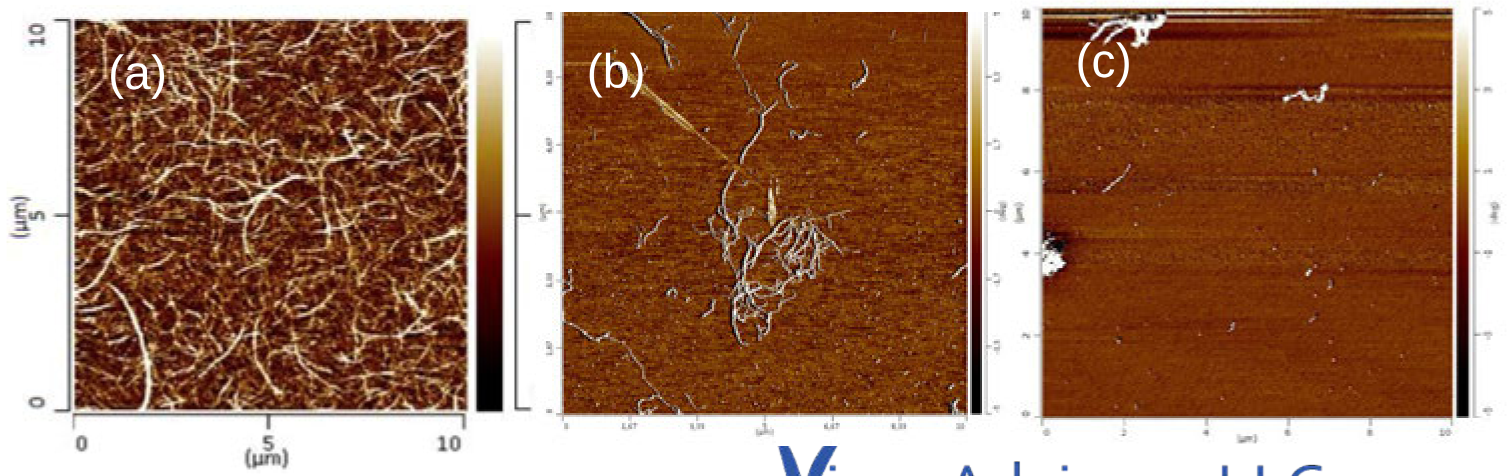
Atomic Force Microscopy (AFM)

- Centrifuge sample to separate smallest size fraction
- Spincoat
- Image with AFM
- Goal is to identify discrete fibrils in extract



Validation of method (representative MFC, these are not notified materials)

a) Representative image of MFC (finest fraction not separated)
b) Image of 0.15 wt% MFC, finest fraction separated – fibrillar material detected
c) Image of 1:5 dilution of 0.15 wt% MFC, finest fraction separated

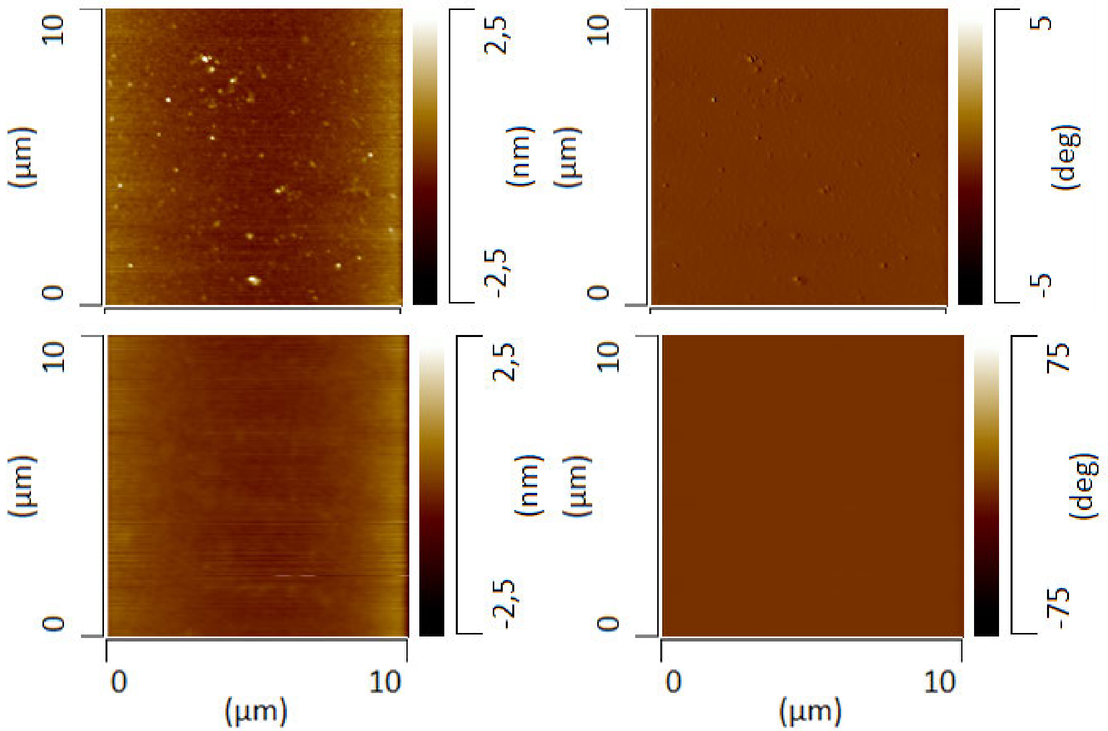


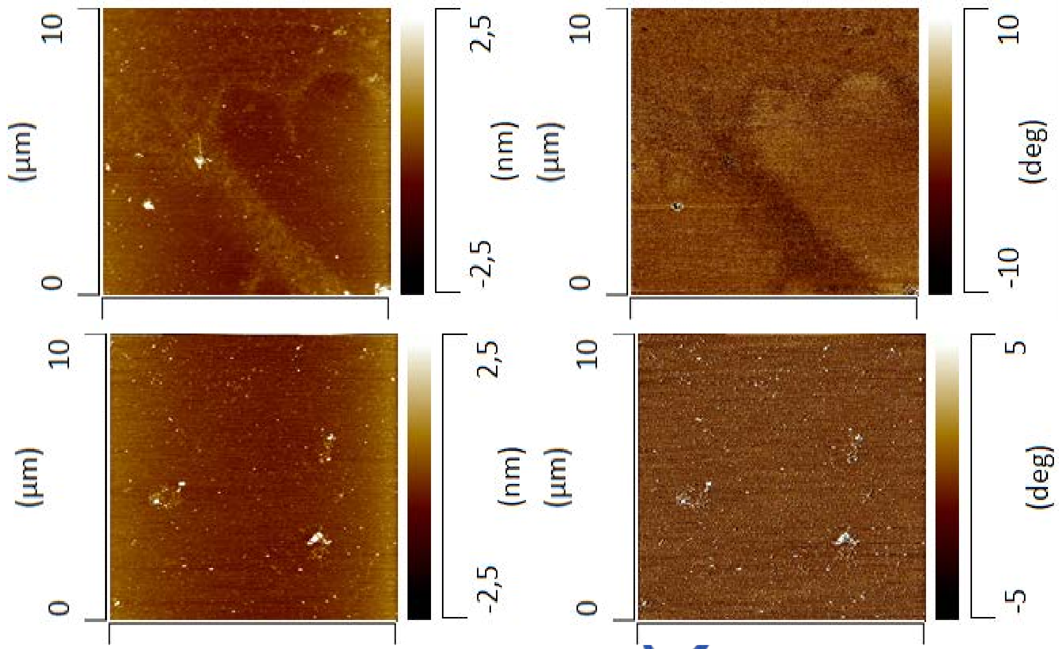
AFM Images of industrial material food contact extracts

Goal is to evaluate whether the extract contains any individual, discrete fibers

- ***Results***: None of the extracts contain discrete microfibrils. Extracts are composed of aggregated, tangled MFC, with no presence of discrete fibres or microfibrils in any of the extracts

(Representative images – no fibrillar material detected)





Food Safety Study Materials

In Animal Tests

Novel forms

- UMaine CNF (cake)
- Hardwood CNC (powder)

Conventional/GRAS

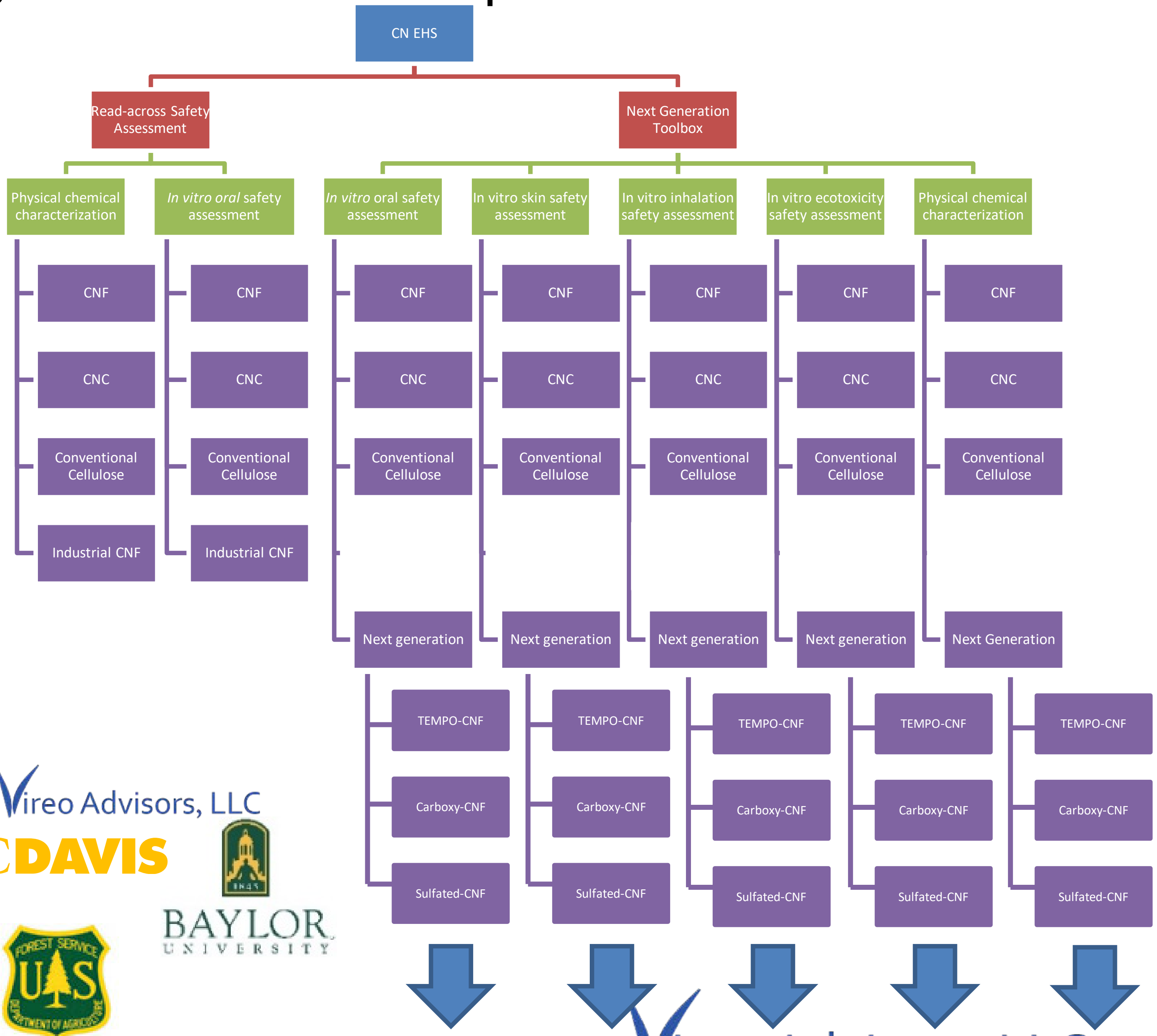
- Commercial Cellulose (SolkaFloc)

PChem/Alternative Tests

- UMaine CNF
- Hardwood CNC
- 6 industrial CMF/CNF
- Conventional cellulose

Testing strategy allows “read across” from animal to alternative testing

Safer by Design Toolbox Development



Functionalization Collaborators

James Ede, Angel Precious Eger, Brian Zhang — Vireo Advisors, LLC
You-Lo Hsieh, Mengzhe Guo, Ben Pingrey — UC DAVIS
Christie Sayes, Amanda Zevcik, Clancy Collom — BAYLOR UNIVERSITY
Nicole Stark, Robert Moon, Forest Products Lab — Forest Service U.S. Department of Agriculture

P3Nano — U.S. Endowment for Forestry and Communities

Vireo Advisors, LLC

Tool Box Development Goals

1. Generate standardized safety methods and data sets for CNs
   - Methods development
   - Working toward standard test methods & regulatory acceptance

2. ‘Read-across’ toxicity testing strategy for industrial and functionalized forms of CNs

1. Continue to develop ‘Safer-by-Design’ Toolbox for next generation CN materials
   - Commercially-relevant forms
   - Promote CN safety and regulatory acceptance for applications in food, food contact, cosmetics, *etc*.

Interdisciplinary approach to Safer by Design Toolbox

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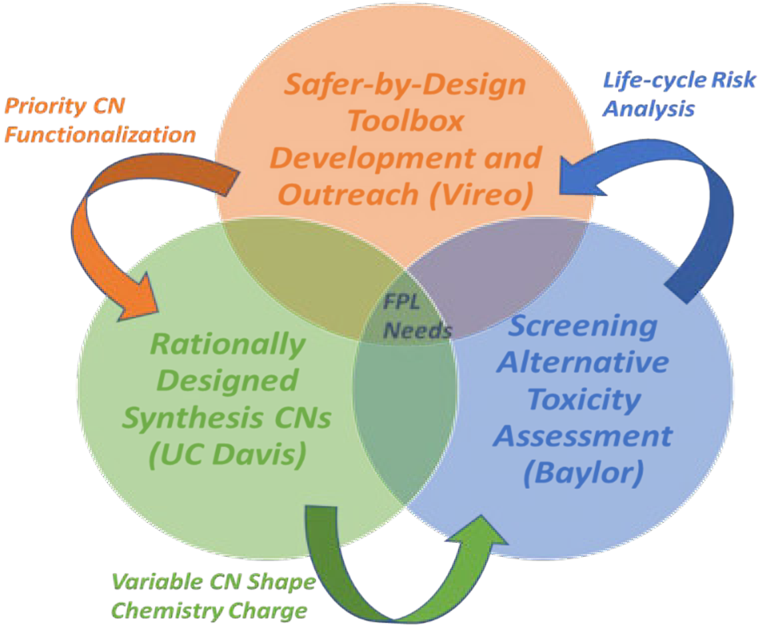

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Baylor University

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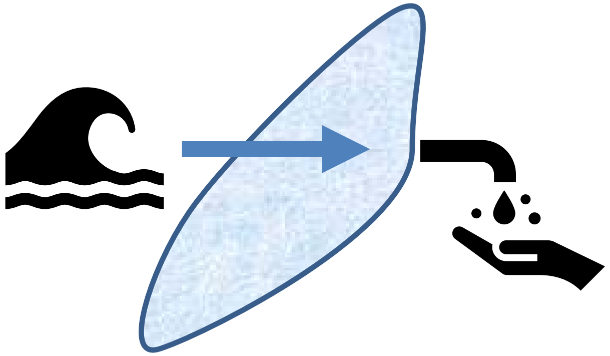
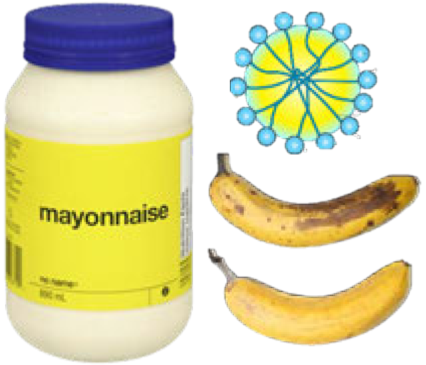
Life Cycle Risk Analysis
Application of Toolbox to Demonstrate Safety of Priority Commercial CN Forms and Applications

LCRA





CS1: Carboxylated CNF Water Filtration Membranes	**CS2.1**: Carboxylated CNF Food Packaging	**CS2.2**: Sulfated CNF Food Packaging	**CS3**: Carboxylated CNF Food Additive

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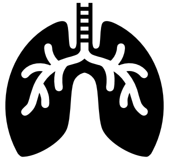
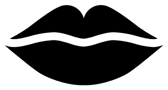
Toolbox Methods & Data Development

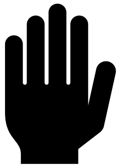
Methods and data to evaluate safety of CNs forms:



Inhalation



Oral



Dermal

Env.

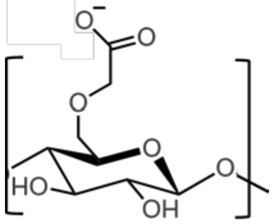
ATS Safety Testing Methods

Physical Chemical Characterization Methods

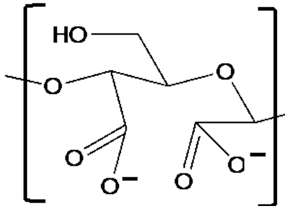
Database of Safety Data

Database of Physical and Chemical Data

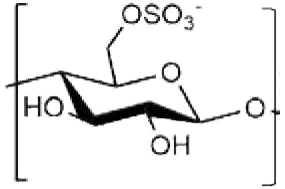
1st generation of modified CNs:



TEMPO



C2/C3 Carboxy



Sulfated

Life Cycle Risk Analysis

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1. **Status of Toolbox Development**
2. **Overview of Toolbox (About Toolbox; Experimental Overview)**

	STATUS	Complete	In Progress	On hold
		FUNCTIONALIZATION TOOLBOX STATUS UPDATE		
Worksheet	TASK	DESCRIPTION	STATUS	Comment
1.1	Experimental Overview		Complete	
2.1	Methods of Pchem. Characterization			
	Task 1	Characterization of Pristine CNFs	Complete	
	Task 2	Characterization of Simulated digested CNFs	In Progress	
	Task 3			
2.2	Methods of Oral tox. Characterization			
	Task 1	Simulated digestion protocol	Complete	
	Task 2	Co-culture model cell culture set-up	Complete	
	Task 3	Toxicity Assessment	Complete	
2.3	Methods of Inhalation tox. Characterization			
	Task 1		Not Started	
	Task 2		Not Started	
	Task 3		Not Started	
2.4	Methods of Dermal tox. Characterization			
	Task 1		Not Started	
	Task 2		Not Started	
	Task 3		Not Started	
2.5	Methods of Environmental tox. Characterization			
	Task 1		Not Started	
	Task 2		Not Started	
	Task 3		Not Started	
3.1	Pchem Database			
	Task 1	Identification of physical properties using atomic force microscopy	Complete	
	Task 2	[illegible] functionalized and one unmodified CN before and after simulated digestion	In Progress	Confirm HDD, DLZP data for SCNFc; SCNFd, PCCNFa,PCCNFc, PCCNFd
3.2	Oral tox Database			
	Task 1	Viability assay (% cytotoxicity) 15min/4hr	Complete	
	Task 2	Oxidative stress assay (GR) 15min/4hr	Complete	
	Task 3	Pro-inflammation (IL-6) 15min/4hr	Complete	
	Task 4	Cellular barrier integrity (TEER) 15min/4hr	Complete	
3.3	Inhalation tox Database			
	Task 1		Not Started	
	Task 2		Not Started	

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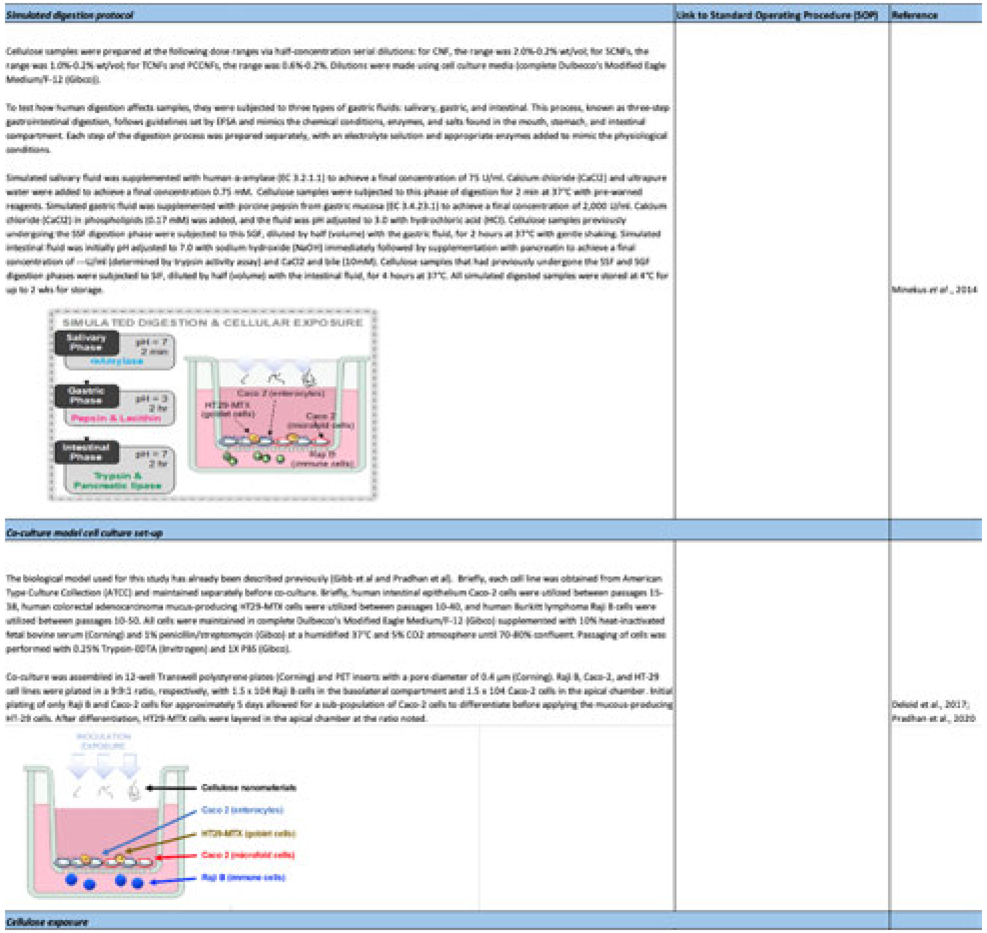
1. Status of Toolbox Development
2. Overview of Toolbox (About Toolbox; Experimental Overview)
3. **Synthesis and Physical Chemical Characterization Methodologies**
4. **Oral, Dermal, Inhalation Toxicity Methodologies**



Simulated digestion protocol	Link to Standard Operating Procedure (SOP)	Reference
Cellulose samples were prepared at the following dose ranges via half-concentration serial dilutions: for CNF, the range was 2.0%-0.2% wt/vol; for SCNFs, the range was 1.0%-0.2% wt/vol; for TCNFs and PCCNFs, the range was 0.6%-0.2%. Dilutions were made using cell culture media (complete Dulbecco's Modified Eagle Medium/F-12 (Gibco)). To test how human digestion affects samples, they were subjected to three types of gastric fluids: salivary, gastric, and intestinal. This process, known as three-step gastrointestinal digestion, follows guidelines set by EFSA and mimics the chemical conditions, enzymes, and salts found in the mouth, stomach, and intestinal compartment. Each step of the digestion process was prepared separately, with an electrolyte solution and appropriate enzymes added to mimic the physiological conditions. Simulated salivary fluid was supplemented with human α-amylase (EC 3.2.1.1) to achieve a final concentration of 75 U/ml. Calcium chloride (CaCl2) and ultrapure water were added to achieve a final concentration 0.75 mM. Cellulose samples were subjected to this phase of digestion for 2 min at 37°C with pre-warmed reagents. Simulated gastric fluid was supplemented with porcine pepsin from gastric mucosa (EC 3.4.23.1) to achieve a final concentration of 2,000 U/ml. Calcium chloride (CaCl2) in phospholipids (0.17 mM) was added, and the fluid was pH adjusted to 3.0 with hydrochloric acid (HCl). Cellulose samples previously undergoing the SSF digestion phase were subjected to this SGF, diluted by half (volume) with the gastric fluid, for 2 hours at 37°C with gentle shaking. Simulated intestinal fluid was initially pH adjusted to 7.0 with sodium hydroxide (NaOH) immediately followed by supplementation with pancreatin to achieve a final concentration of ~ U/ml (determined by trypsin activity assay) and CaCl2 and bile (10mM). Cellulose samples that had previously undergone the SSF and SGF digestion phases were subjected to SIF, diluted by half (volume) with the intestinal fluid, for 4 hours at 37°C. All simulated digested samples were stored at 4°C for up to 2 wks for storage.		Minekus *et al.*, 2014
Co-culture model cell culture set-up		
The biological model used for this study has already been described previously (Gibb et al and Pradhan et al). Briefly, each cell line was obtained from American Type Culture Collection (ATCC) and maintained separately before co-culture. Briefly, human intestinal epithelium Caco-2 cells were utilized between passages 15-38, human colorectal adenocarcinoma mucus-producing HT29-MTX cells were utilized between passages 10-40, and human Burkitt lymphoma Raji B-cells were utilized between passages 10-50. All cells were maintained in complete Dulbecco's Modified Eagle Medium/F-12 (Gibco) supplemented with 10% heat-inactivated fetal bovine serum (Corning) and 1% penicillin/streptomycin (Gibco) at a humidified 37°C and 5% CO2 atmosphere until 70-80% confluent. Passaging of cells was performed with 0.25% Trypsin-EDTA (Invitrogen) and 1X PBS (Gibco). Co-culture was assembled in 12-well Transwell polystyrene plates (Corning) and PET inserts with a pore diameter of 0.4 µm (Corning). Raji B, Caco-2, and HT-29 cell lines were plated in a 9:9:1 ratio, respectively, with 1.5 x 104 Raji B cells in the basolateral compartment and 1.5 x 104 Caco-2 cells in the apical chamber. Initial plating of only Raji B and Caco-2 cells for approximately 5 days allowed for a sub-population of Caco-2 cells to differentiate before applying the mucous-producing HT-29 cells. After differentiation, HT29-MTX cells were layered in the apical chamber at the ratio noted.		DeLoid et al., 2017; Pradhan et al., 2020
Cellulose exposure		

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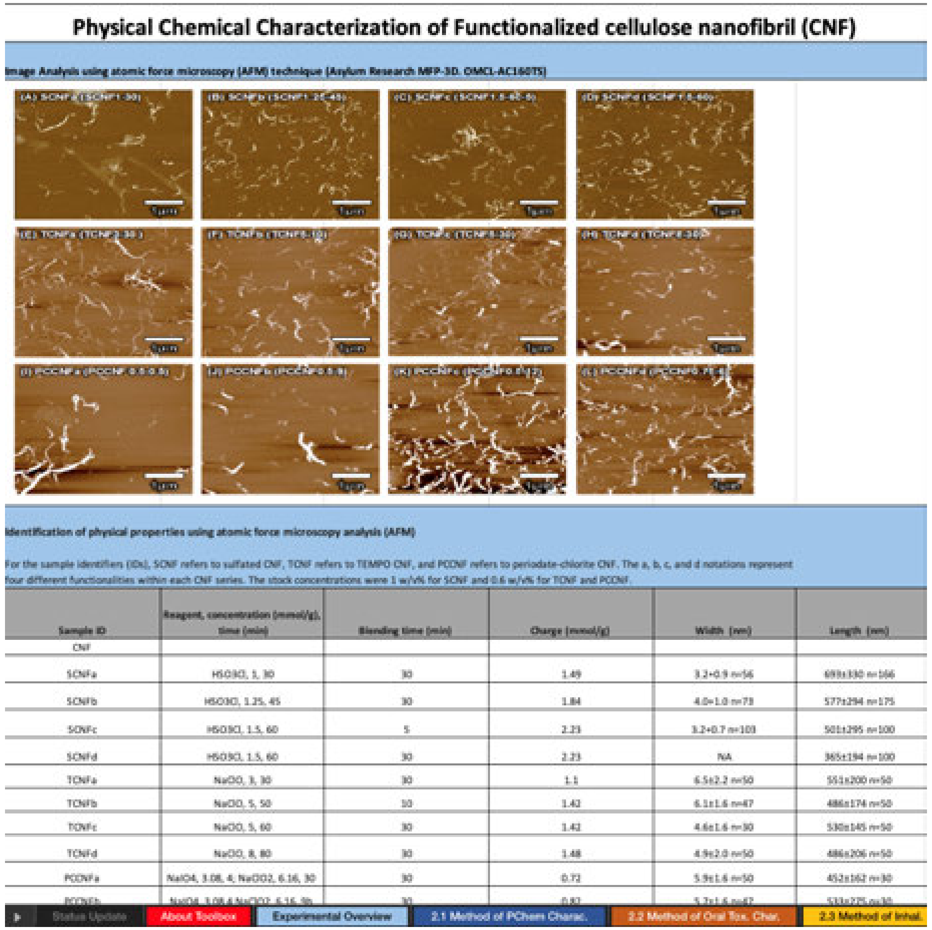
1. Status of Toolbox Development
2. Overview of Toolbox (About Toolbox; Experimental Overview)
3. Synthesis and Physical Chemical Characterization Methodologies
4. Oral, Dermal, Inhalation Toxicity Methodologies
5. **Physical Chemical Database**



Physical Chemical Characterization of Functionalized cellulose nanofibril (CNF)

Image Analysis using atomic force microscopy (AFM) technique (Asylum Research MFP-3D. OMCL-AC160TS)

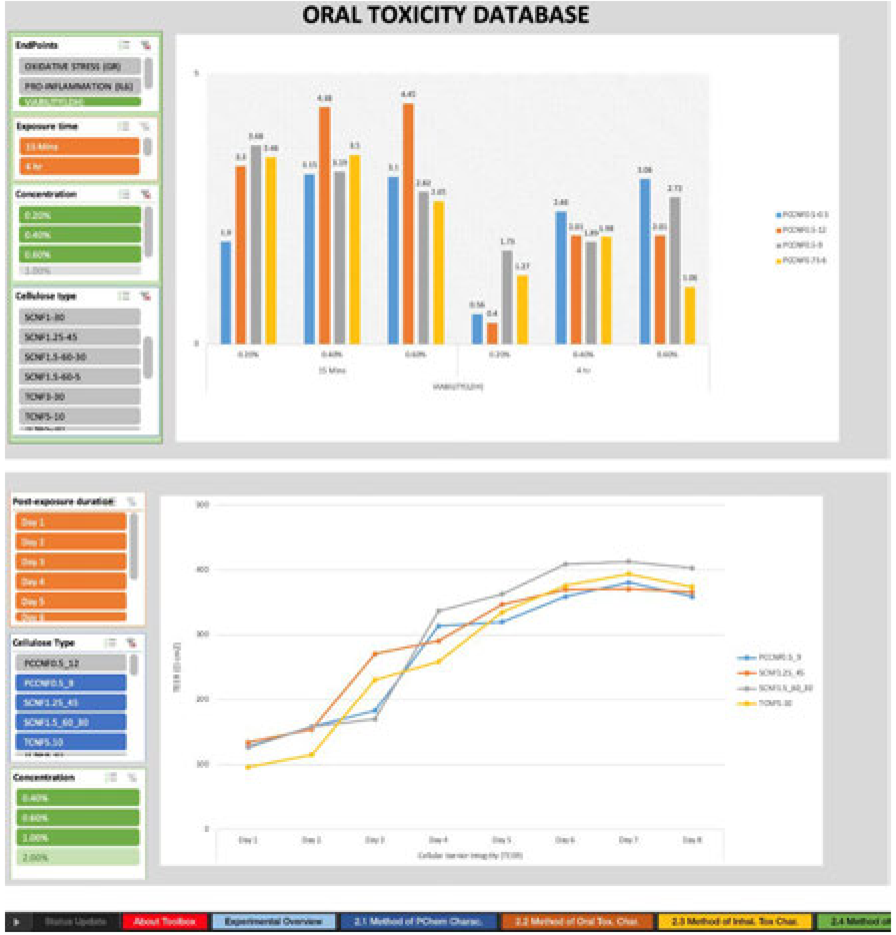
Identification of physical properties using atomic force microscopy analysis (AFM)

For the sample identifiers (IDs), SCNF refers to sulfated CNF, TCNF refers to TEMPO CNF, and PCCNF refers to periodate-chlorite CNF. The a, b, c, and d notations represent four different functionalities within each CNF series. The stock concentrations were 1 w/v% for SCNF and 0.6 w/v% for TCNF and PCCNF.

Sample ID	Reagent, concentration (mmol/g), time (min)	Blending time (min)	Charge (mmol/g)	Width (nm)	Length (nm)
CNF					
SCNFa	HSO3Cl, 1, 30	30	1.49	3.2±0.9 n=56	693±330 n=166
SCNFb	HSO3Cl, 1.25, 45	30	1.84	4.0±1.0 n=73	577±294 n=175
SCNFc	HSO3Cl, 1.5, 60	5	2.23	3.2±0.7 n=103	501±295 n=100
SCNFd	HSO3Cl, 1.5, 60	30	2.23	NA	365±194 n=100
TCNFa	NaClO, 3, 30	30	1.1	6.5±2.2 n=50	551±200 n=50
TCNFb	NaClO, 5, 50	10	1.42	6.1±1.6 n=47	486±174 n=50
TCNFc	NaClO, 5, 60	30	1.42	4.6±1.6 n=30	530±145 n=50
TCNFd	NaClO, 8, 80	30	1.48	4.9±2.0 n=50	486±206 n=50
PCCNFa	NaIO4, 3.08, 4, NaClO2, 6.16, 30	30	0.72	5.9±1.6 n=50	452±162 n=30
PCCNFb	NaIO4, 3.08 4 NaClO2, 6.16, [illegible]	30	0.82	5.7±1.6 n=47	533±275 n=30

Status Update | About Toolbox | Experimental Overview | 2.1 Method of PChem Charac. | 2.2 Method of Oral Tox. Char. | 2.3 Method of Inhal.

1. Status of Toolbox Development
2. Overview of Toolbox (About Toolbox; Experimental Overview)
3. Synthesis and Physical Chemical Characterization Methodologies
4. Oral, Dermal, Inhalation Toxicity Methodologies
5. Physical Chemical Database
6. **Safety Database**
   1. **Oral Toxicity**
   2. **Dermal Toxicity (In Progress)**
   3. **Inhalation Toxicity (In Progress)**



Video Toolbox Demonstration

	STATUS	Complete	In Progress	On Hold	Not Started	Overdue

FUNCTIONALIZATION TOOLBOX STATUS UPDATE

Worksheet	TASK	DESCRIPTION	STATUS	Comment	START DATE	DATE COMPLETED
1.1	**Experimental Overview**		Complete			
2.1	**Methods of Pchem. Characterization**					
	Task 1	Characterization of Pristine CNFs	Complete		MM/DD/YY	MM/DD/YY
	Task 2	Characterization of Simulated digested CNFs	In Progress		MM/DD/YY	MM/DD/YY
	Task 3	Task description here…			MM/DD/YY	MM/DD/YY
	Task 4				MM/DD/YY	MM/DD/YY
	Task 5				MM/DD/YY	MM/DD/YY
2.2	**Methods of Oral tox. Characterization**					

Status Update | About Toolbox | Experimental Overview | Method of PChem Cha…

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Regulatory Definitions for Nanomaterials

- European Commission (EC) adopted new regulatory definition of a nanomaterial in 2022; replacing/updating the 2011 definition

1) It consists of solid particles

2) **50 % or more of its constituent particles** fulfil at least one of the following conditions:

- One or more **external dimensions of the particle** are in the **size range 1 nm to 100 nm**
- The particle has an elongated shape, such as a rod, fibre or tube, where two external dimensions are smaller than

1 nm and the other dimension is larger than 100 nm

- The particle has a plate-like shape, where one external dimension is small- er than 1 nm and the other dimensions are larger than 100 nm

The EC NM definition specifies one additional property, the volume specific surface area (VSSA), which can be used to demonstrate that a given particulate material is **not** a nanomaterial. The corresponding exclusion criterion is a VSSA of less than 6 m^2/cm^3.

- Determining if MFC meets this definition is difficult; complex morphology makes measuring external dimensions difficult; no consensus on terminology for MFC (e.g. external vs internal dimensions)

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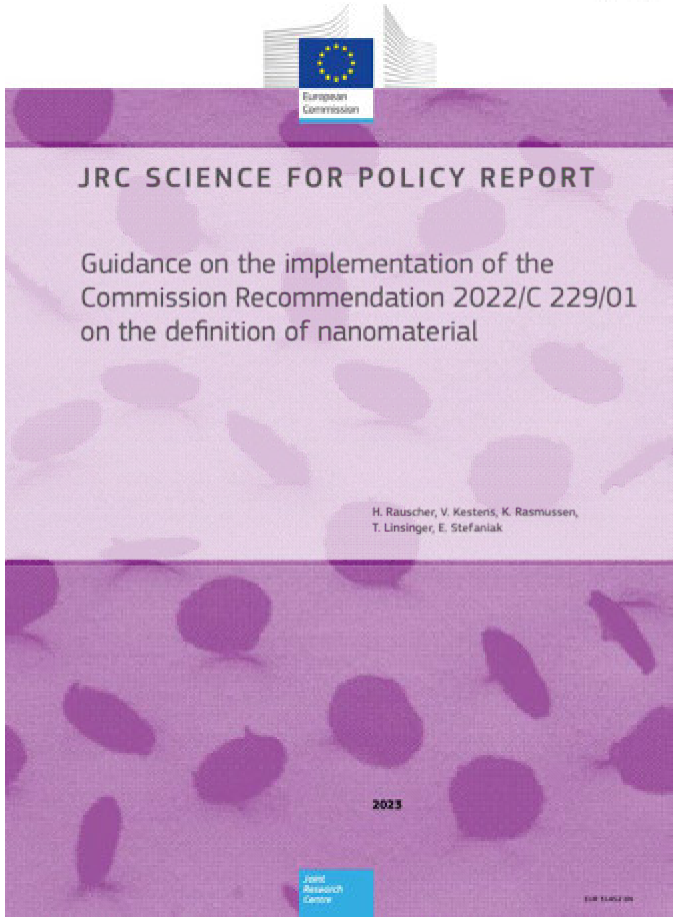
Regulatory Definitions for Nanomaterials

- Joint Research Centre (JRC) released 2023 guidance on implementing EC Recommended Definition of Nanomaterial
- Clarifies concept of external dimension for complex morphologies and Includes guidance on measuring **number-based external dimensions of particulates**
  - Minimum Feret diameter: minimum distance between two parallel tangents
  - Maximum inscribed circle: diameter of largest circle that fits inside



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Regulatory Definitions for Nanomaterials

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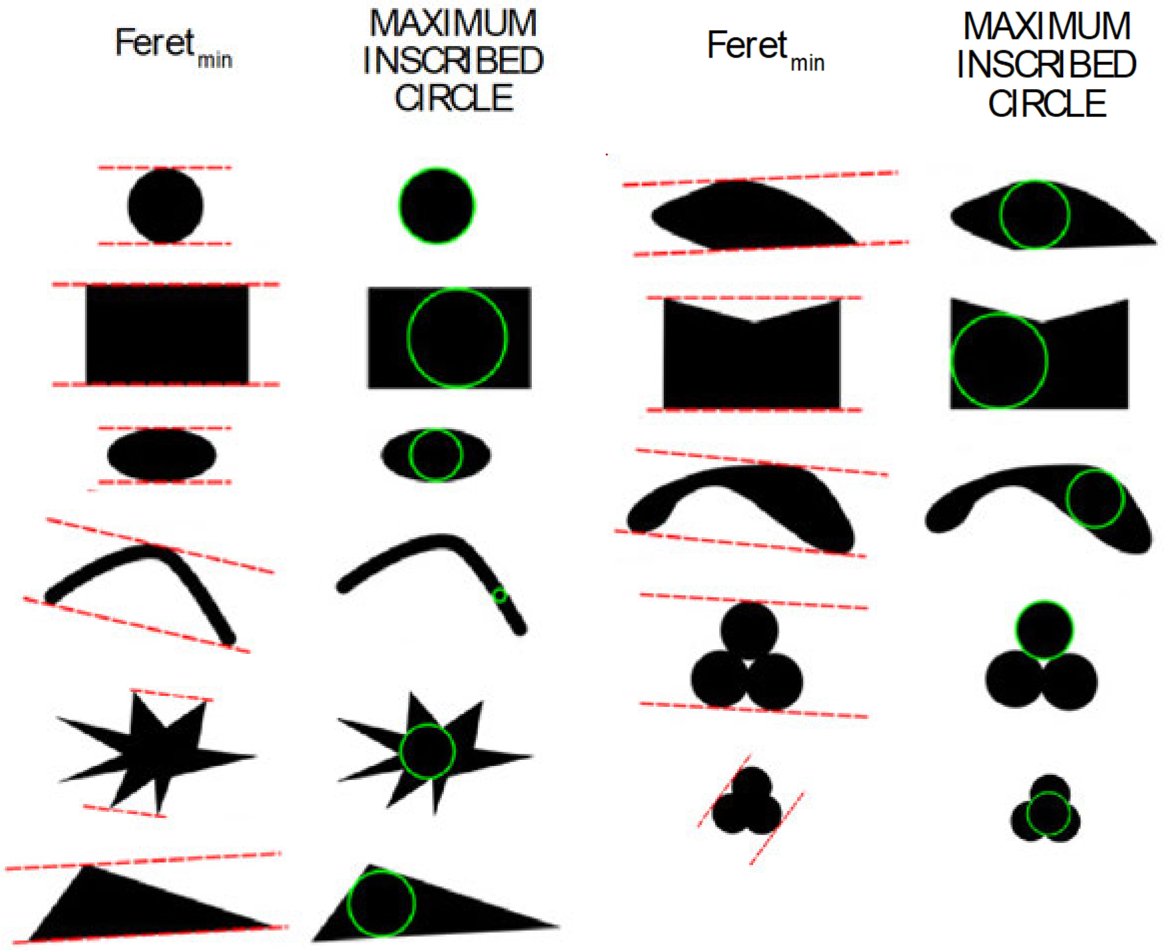
- Clarifies concept of external dimension for complex morphologies and Includes guidance on measuring number-based external dimensions of particulates
  - **Minimum Feret diameter: minimum distance between two parallel tangents**
  - **Maximum inscribed circle: diameter of largest circle that fits inside 'envelop' of particle**



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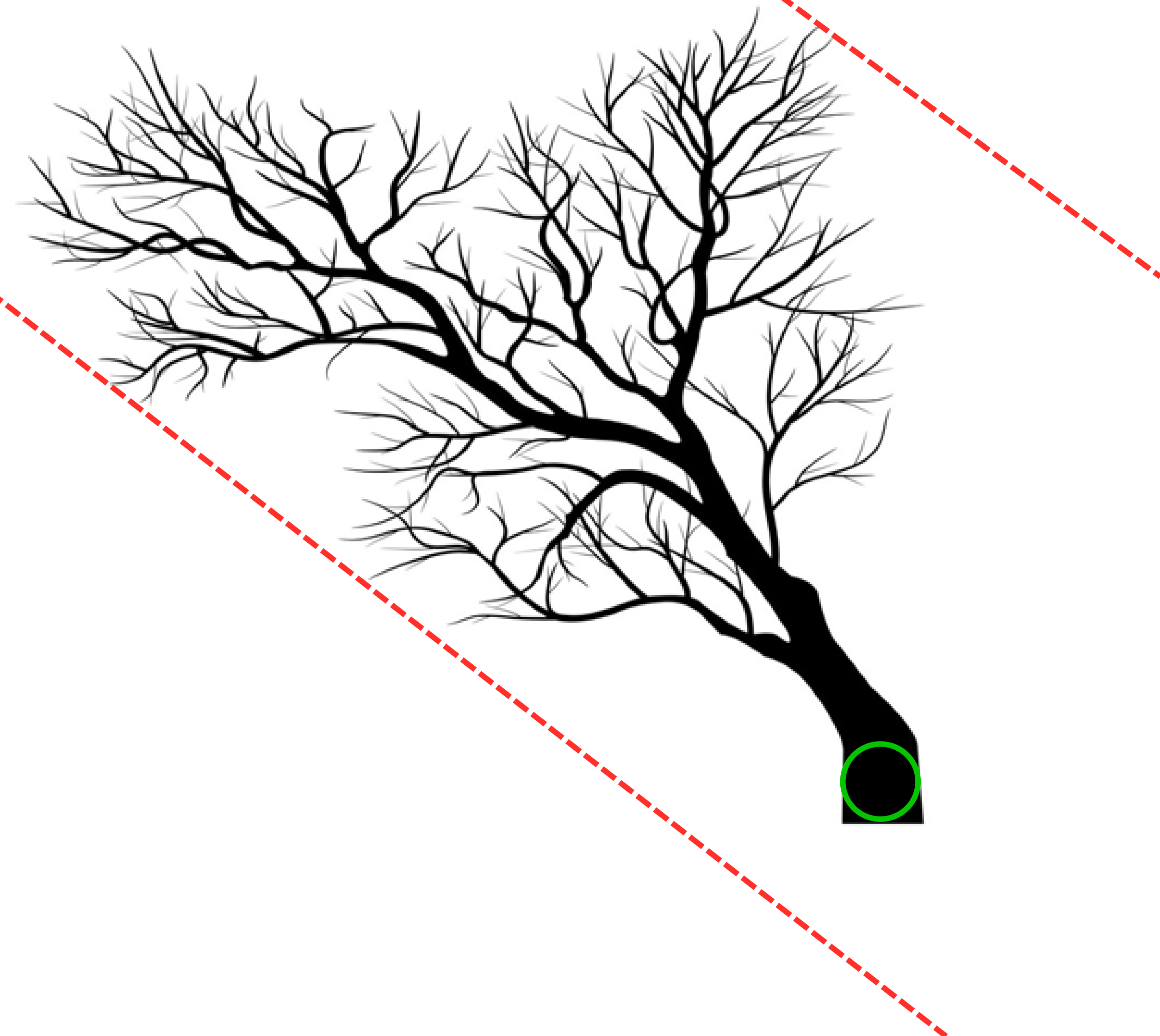
Regulatory Definitions for Nanomaterials

- Joint Research Centre (JRC) released 2023 guidance on implementing EC Recommended Definition of Nanomaterial
- Clarifies concept of external dimension for complex morphologies and Includes guidance on measuring number-based external dimensions of particulates
  - Minimum Feret diameter: minimum distance between two parallel tangents
  - Maximum inscribed circle: diameter of largest circle that fits inside
- **Approach for determining if MFC meets EC Recommended definition?**
  - **TEM/SEM/AFM + manual measurements**



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Next Steps

Safety & Functionalization Work

Safety

1. Regulatory submissions
2. Publish ADME Study & PCHEM data
3. Standardize characterization methods

Functionalization Work

1. Publish methods and data sets
2. Publish Safety findings
3. Data structure for toolbox
4. Additional synthesis methods & characterization
5. Standardize characterization methods

Needs

Technical

- Advancing read across/grouping
- Standards development
  - Characterization
  - Toxicity methods for multi-scale materials
- Support to address outstanding questions

Regulatory

- Acceptance of alternative methods
- Understanding of impact/importance to forest bioeconomy
- Soft advocacy

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Publications to Date

1. Seydibeyoğlu MÖ, Dogru A, Wang J, Rencheck M, Han Y, Wang L, Seydibeyoğlu EA, Zhao X, Ong K, Shatkin JA, Shams Es-haghi S, Bhandari S, Ozcan S, Gardner DJ. Review on Hybrid Reinforced Polymer Matrix Composites with Nanocellulose, Nanomaterials, and Other Fibers. *Polymers*. 2023; 15(4):984. https://doi.org/10.3390/polym15040984

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P3Nano

FOREST SERVICE U S DEPARTMENT OF AGRICULTURE

The partners of the Alliance for the Food Safety Acceptance of Fibrillated and Crystalline Celluloses

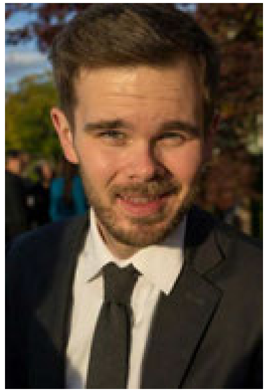
The Vireo Team

USDA Forest Service
U.S. DEPARTMENT OF AGRICULTURE

The Vireo Team

Dr. Jo Anne Shatkin is an expert in novel product safety and environmental and health policy issues, with over 20 years experience leading projects in risk analysis, safety and regulatory policy work including numerous publications.

She is founder and president of Vireo Advisors in Boston, Massachusetts.



Dr. James D. Ede is a toxicologist experienced in testing strategies for novel materials, including molecular, biochemical and cellular techniques, and is experienced in life cycle risk assessment.

Dr. Kimberly J. Ong is a biologist and environmental scientist. Dr. Ong is an expert in developing protocols specific for novel material testing to improve reliability for risk and exposure assessment and is experienced in regulatory analysis for novel products.

Dr. Shaun Clancy is a chemist with over 30 years experience in the chemicals industry, directing programs in health, safety, and regulatory affairs in major corporations. He is ANSI Co-Chair and participates in ISO TC229 and other international safety committees.

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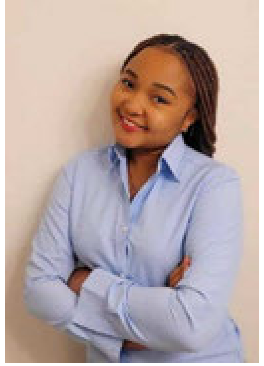

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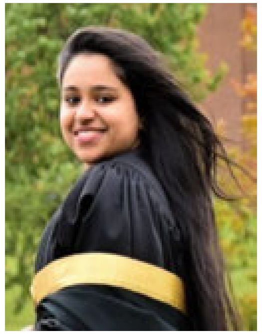
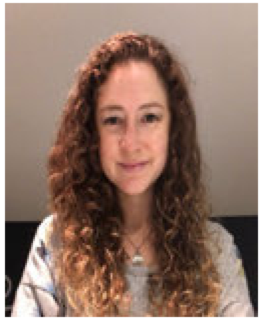
Lauren Payne is a MPH student at Boston University and Vireo Intern with a business background.

Kora Kukk is a fellow and a UMaine graduate with an M.S. in Biomedical Engineering.



Padmapriya Srinivasan is an intern and is pursuing a Bachelors of Science degree from Mount Allison University.



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Thank you!

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Check out our blog at:

www.VireoAdvisors.com/blog