International Conference on Nanotechnology for Renewable Materials

U.S. Endowment for Forestry and Communities

USDA Forest Service U.S. DEPARTMENT OF AGRICULTURE

# Safer by Design Toolbox to Advance Functionalized Cellulose-based Nanomaterials

**Yueyang (Brian) Zhang,** Kimberly Ong, James Ede, Ana Diges, You-Lo Hsieh, Mengzhe Guo, Ben Pingrey, Christie Sayes, James Liu, Amanda Sevcik, Clancy Collom, Nicole Stark, Robert Moon, Jo Anne Shatkin

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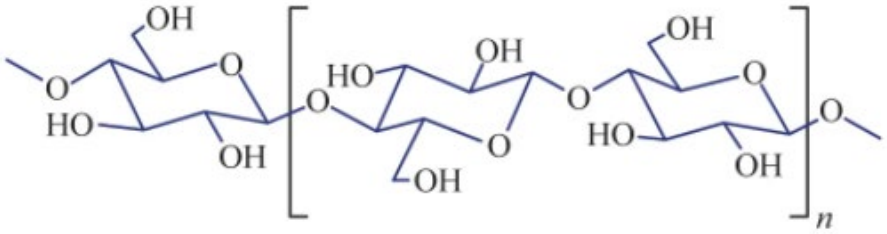
# Cellulose

1. Most abundant biopolymer




cellulose

2. Wood as source material

3. Applications

4. Cellulose-based nanomaterials (CNs)
   - Hydrophilic
   - Free hydroxyl group for surface modifications

# Cellulose Nanomaterials Food Safety Study

1. CNs behave similarly to conventional cellulose and raise no safety concerns when used as a food ingredient at 4% of diet.

2. Baseline measurements for examining potential impact of future functionalization on toxicity.

| 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 |

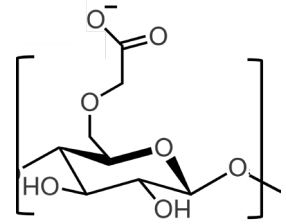
[1] Ong, K.J et al. (2020)
[2] Pradhan et al. (2020)
[3] Ede et al. (2020)

# Surface Modifications and Applications

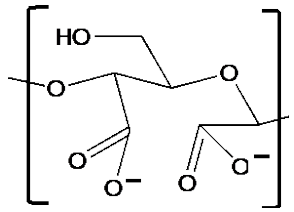
**Table 1.** Survey results - CN surface modifications and applications with high commercial potential.

| Material | % | Functional group | % | Applications | % |
|---|---|---|---|---|---|
| CNC | 46% | TEMPO oxidized | 27% | Packaging | 35% |
| CNF | 46% | Carboxylated | 20% | Polymer and composite | 26% |
| Hairy cellulose | 4% | Alkylated | 13% | Metal ion absorbant | 13% |
| Chitin | 4% | Cationic | 10% | Coating | 9% |
| | | Sulfated | 10% | Cosmetics | 4% |
| | | Carboxylmethylated | 7% | Others | |
| | | Others | | | |

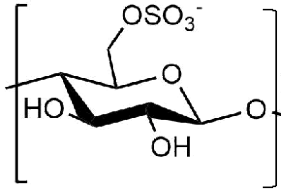
* TEMPO: 2,2,6,6-tetramethylpyperidine-1-oxyl




**TEMPO**




**C2/C3 Carboxy**




**Sulfated**

# Key Questions

How changing the surface chemistry of CNs affects safety and regulatory acceptance?

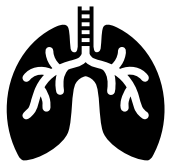
# Overarching Goal

Development of safer-by-design toolbox and life cycle risk assessment (LCRA) for the production of safe functionalized CNs.

P$^3$Nano

# Tool Box Methods & Data Development

**Methods and data to evaluate safety of CNs forms:**



**Inhalation**

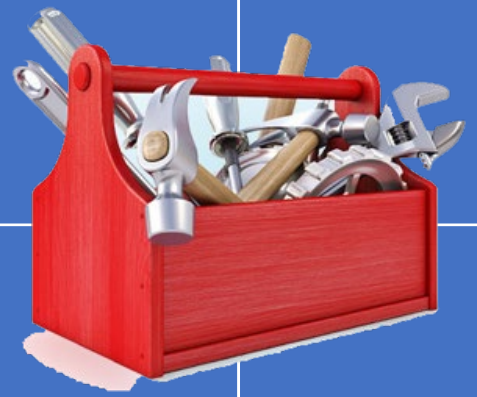
**Oral**

**Dermal**

**Env.**

ATS Safety Testing Methods

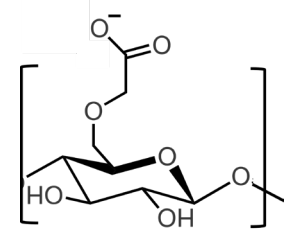
Physical Chemical Characterization Methods



Database of Safety Data

Database of Physical and Chemical Data

**1st generation of modified CNs:**

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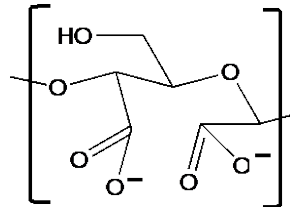


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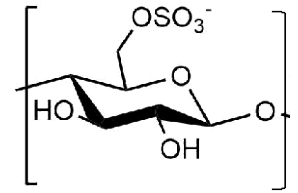


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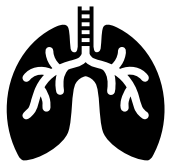

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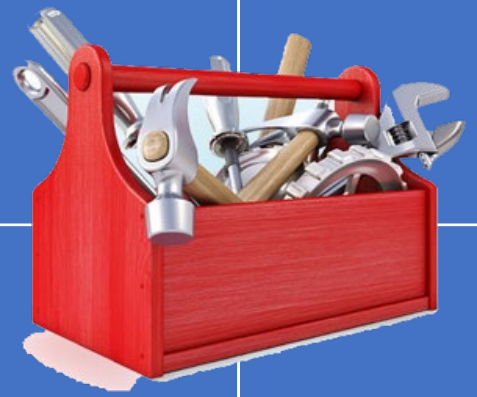

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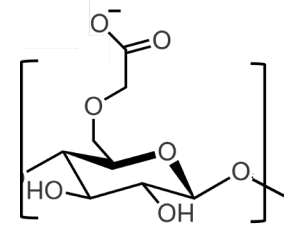


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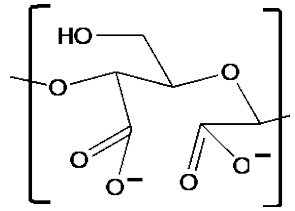


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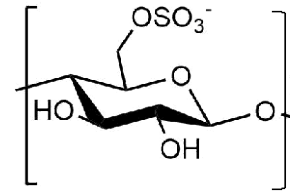


**Sulfated**

# Tool Box Methods & Data Development

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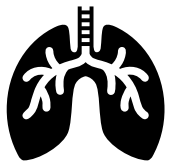

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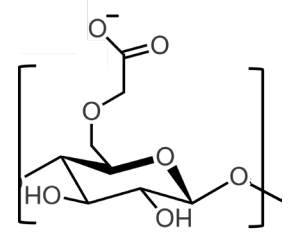


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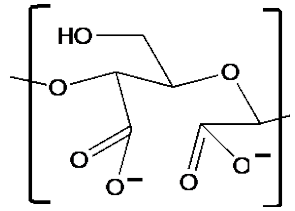


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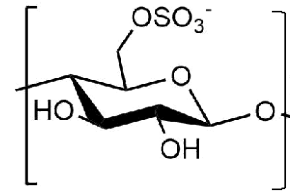


**Sulfated**

# Life-Cycle Risk Assessment (LCRA) Development

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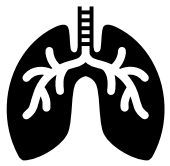
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Database of Safety Data

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LCRA

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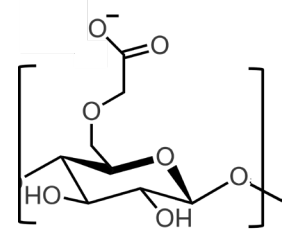

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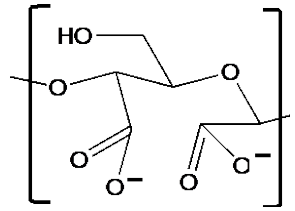

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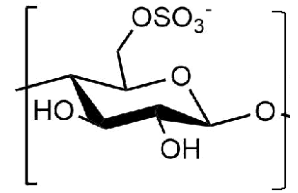


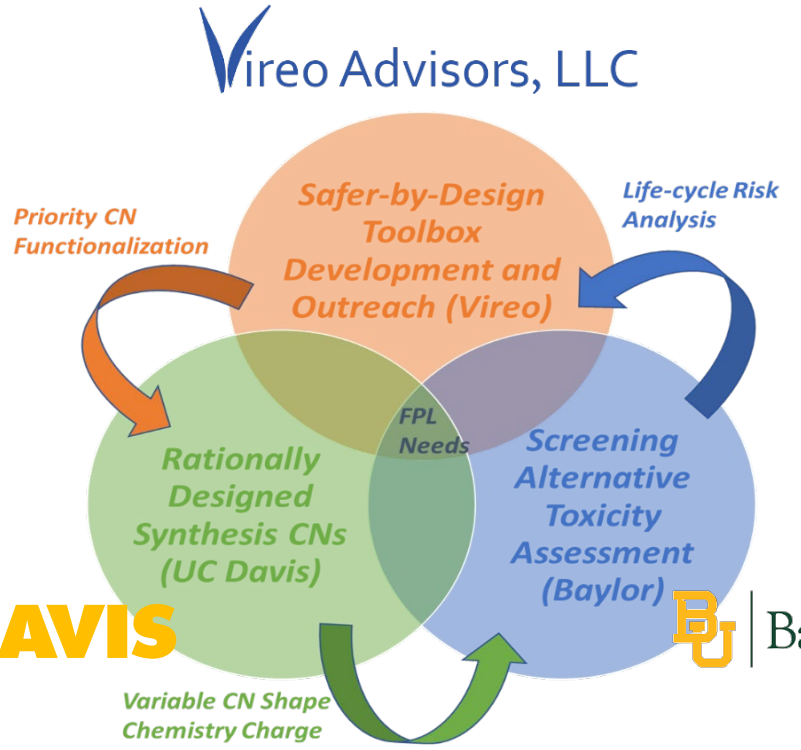
**Sulfated**

# Interdisciplinary Approach to Safer by Design Toolbox

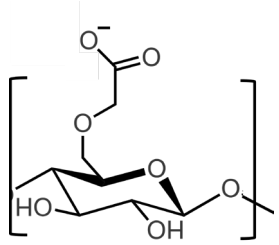
1. Synthesis of CNs with selected surface chemistries and in different geometric dimensions and aspect ratios.

2. Adapt non-animal testing protocols to assess cytotoxic response and inflammatory potential of surface-modified CNs.

3. Develop safer-by-design toolbox and life cycle risk assessment (LCRA).



# Surface Functionalization



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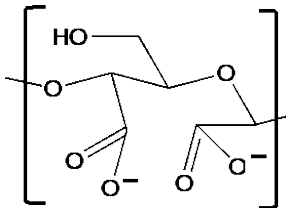
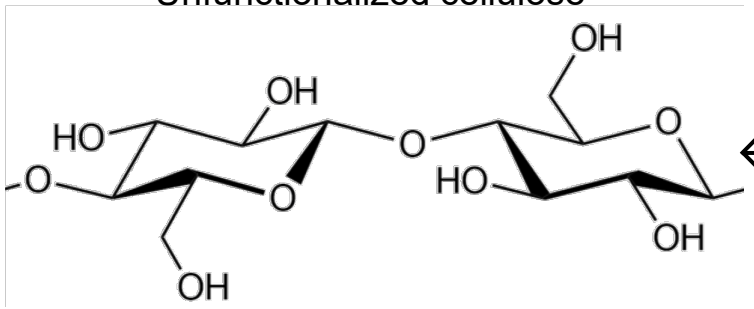


C2/C3 Carboxy

Unfunctionalized cellulose



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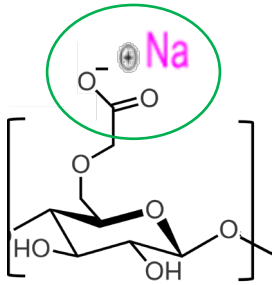
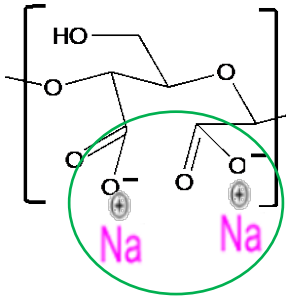


TEMPO-CNFs

Two charge levels

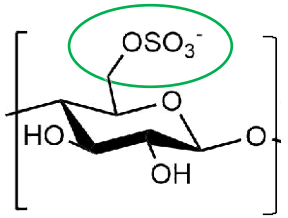
**Periodate Chlorite**



PC-CNFs

Different L/W ratios

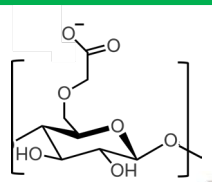
**$HSO_3Cl$**



SCNFs

Three charge levels

# Synthesis of TEMPO-Oxidized Cellulose Nanofibrils (TCNFs)

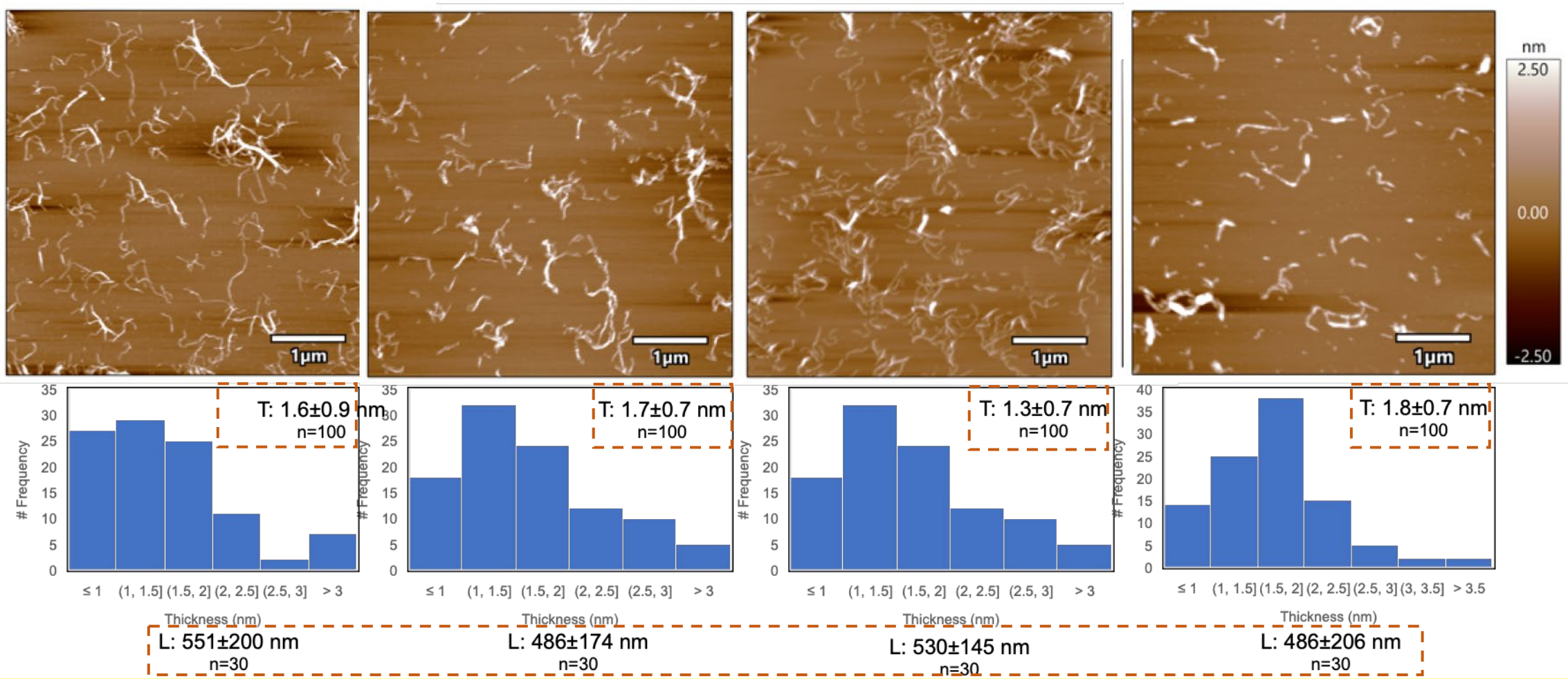



**TCNF3.30**
3 mmol NaClO/g, 30 min blending

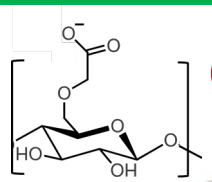
**TCNF5.10**
5 mmol NaClO/g, 10 min blending

**TCNF5.30**
5 mmol NaClO/g, 30 min blending

**TCNF8.30**
8 mmol NaClO/g, 30 min blending



# Synthesis of TEMPO-Oxidized Cellulose Nanofibrils (TCNFs)

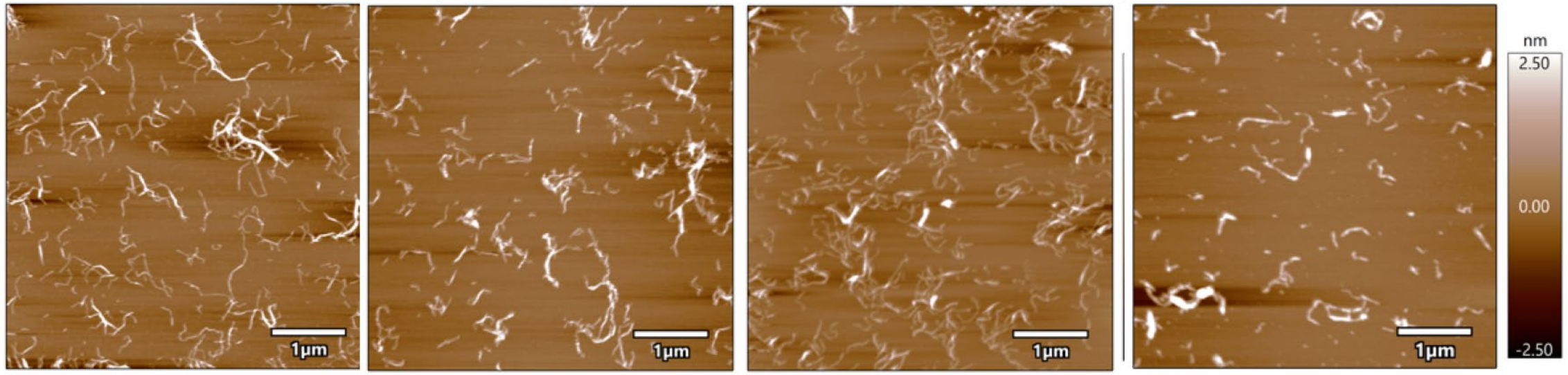


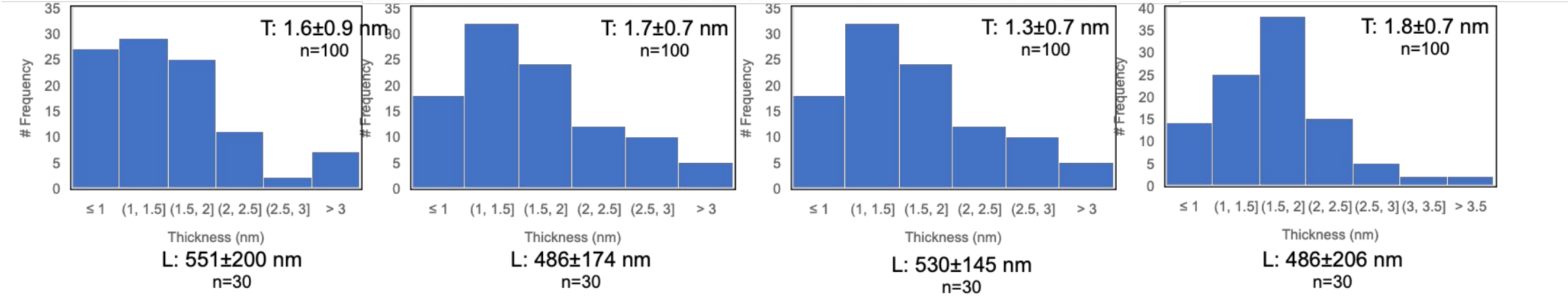
**TCNF3.30**
3 mmol NaClO/g, 30 min blending
Charge: 1.10 mmol/g;
Yield : 82.1%

**TCNF5.10**
5 mmol NaClO/g, 10 min blending
Charge: 1.42 mmol/g;
Yield : 84.2%

**TCNF5.30**
5 mmol NaClO/g, 30 min blending
Charge: 1.42 mmol/g;
Yield : 93.5%

**TCNF8.30**
8 mmol NaClO/g, 30 min blending
Charge: 1.48 mmol/g;
Yield : 100%



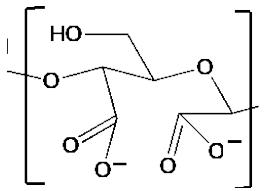

L: 551±200 nm
n=30

L: 486±174 nm
n=30

L: 530±145 nm
n=30

L: 486±206 nm
n=30

# PCCNF by Periodate-Chlorite Oxidation

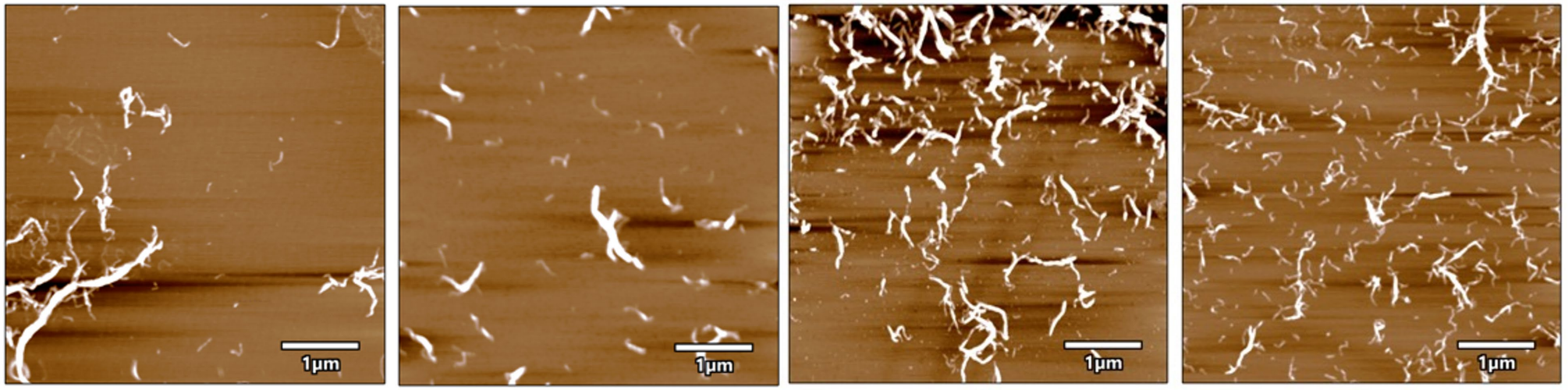


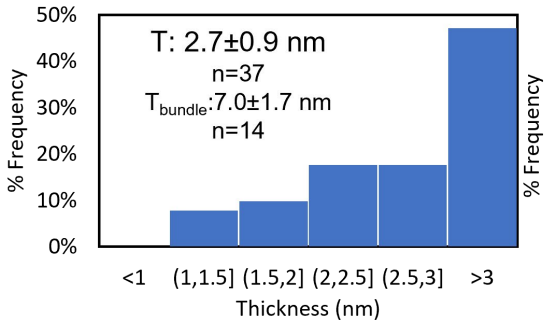

**PC-CNF0.5-0.5H**
3.08 mmol NaIO4/g;
6.16 mmol $NaClO_2$/g
30 min blending
Charge: 0.72 mmol/g;
Yield : 49.3 %

**PC-CNF0.5-9H**
3.08 mmol $NaIO_4$/g;
6.16 mmol $NaClO_2$/g
30 min blending
Charge: 0.82 mmol/g;
Yield : 78.0 %

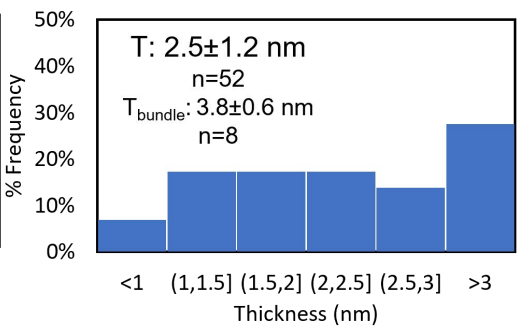
**PC-CNF0.5-12H**
3.08 mmol $NaIO_4$/g;
6.16 mmol $NaClO_2$/g
30 min blending
Charge: 0.91 mmol/g;
Yield : 92.8 %

**PC-CNF0.75-6H**
4.62 mmol $NaIO_4$/g;
6.16 mmol $NaClO_2$/g
30 min blending
Charge: 1.04 mmol/g;
Yield : 99+ %

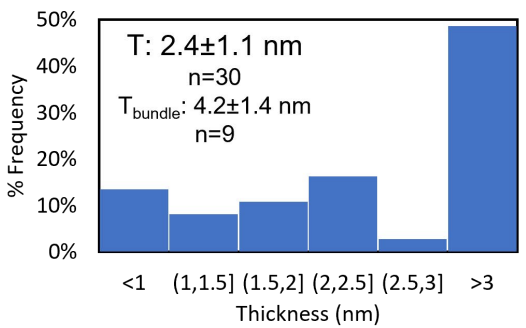


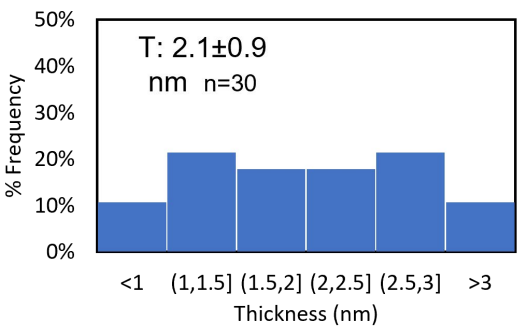

L: 452±162 nm
n=30




L: 533±275 nm
n=30

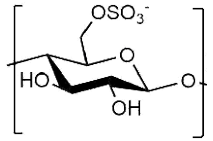



L: 381±150 nm
n=30




L: 344±170 nm
n=30

# Sulfation of CNF via Chlorosulfonic Acid ($HSO_3Cl$)

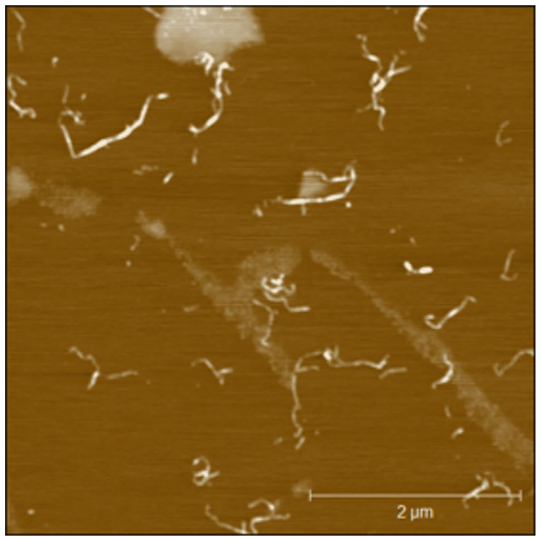


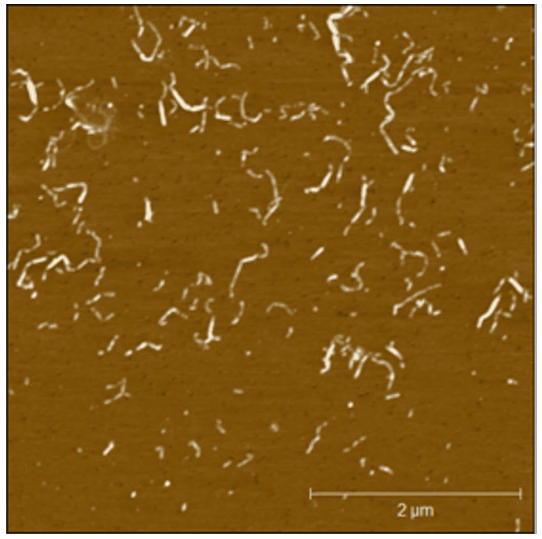

**SCNF1_30**
1 mmol/g $HSO_3Cl$ & 30 min,
30 min blending
Charge: 1.49 mmol/g sulfate
Yield: 99%

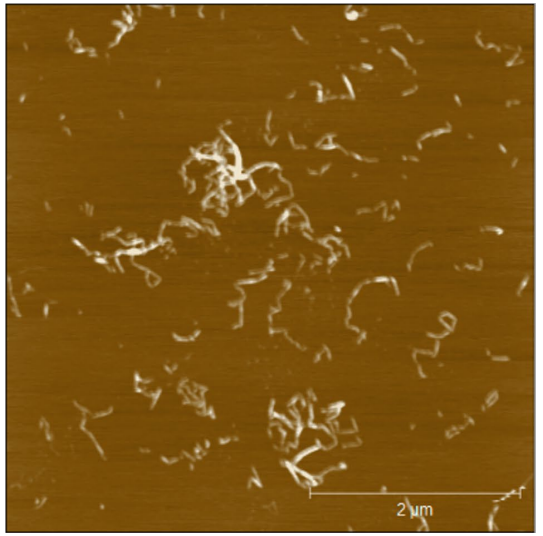
**SCNF1_25_45**
1.25 mmol/g $HSO_3Cl$ & 45 min,
30 min blending
1.84 mmol/g sulfate
Yield: 99%

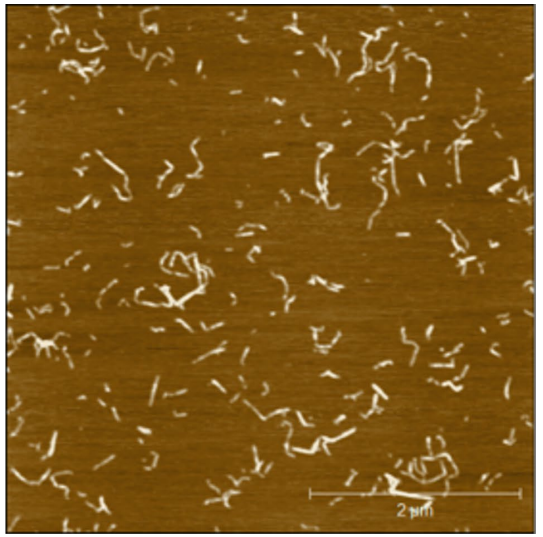
**SCNF1.5_60_5**
1.5 mmol/g HSO3Cl, 60 min,
5 min blending
Charge: 2.23 mmol/g;
Yield: 99 %

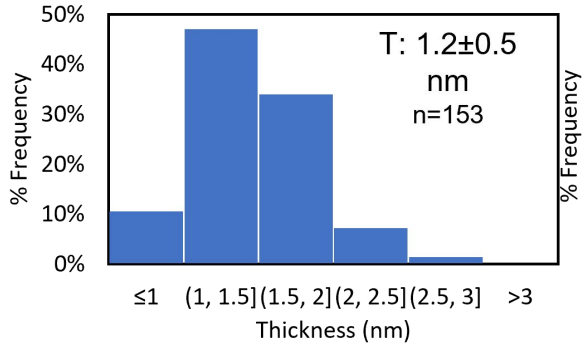
**SCNF1.5_60**
1.5 mmol/g HSO3Cl, 60 min,
30 min blending
Charge: 2.23 mmol/g;
Yield: 99 %



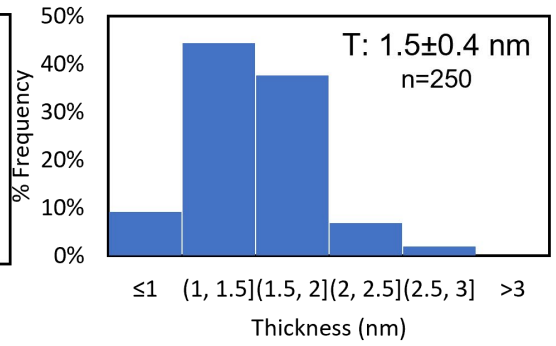



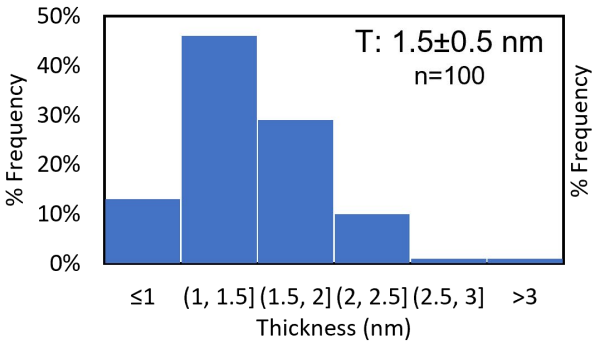



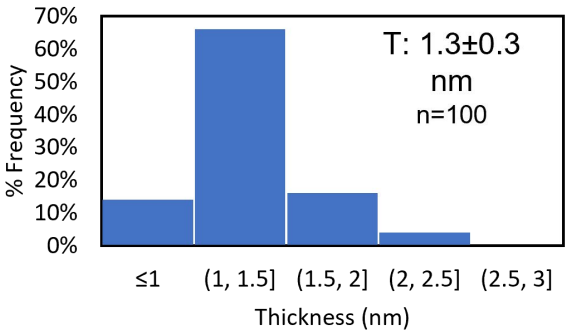

L: 690±330 nm
n=166




L: 580±290 nm
n=175




L: 501±295 nm
n=100

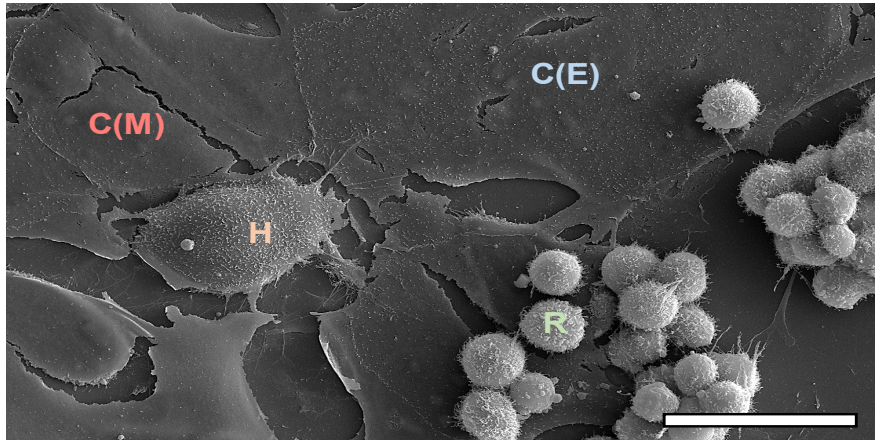



L: 365±194 nm
n=100

# Alternative Testing Strategies (ATS)

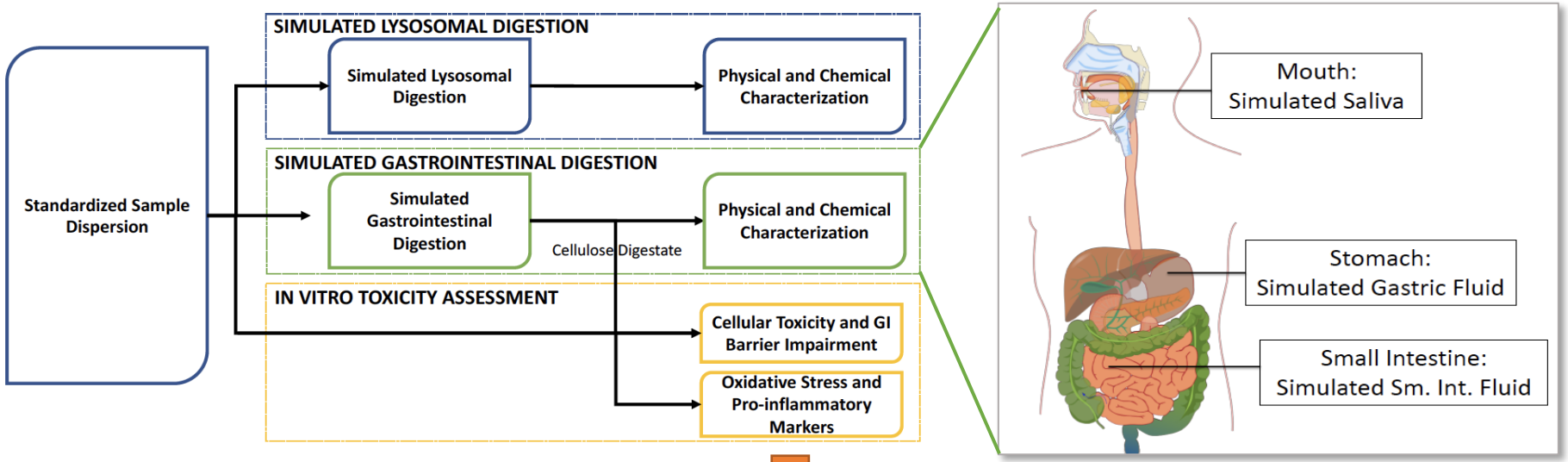
- Reducing and replacing animal testing with ATS.

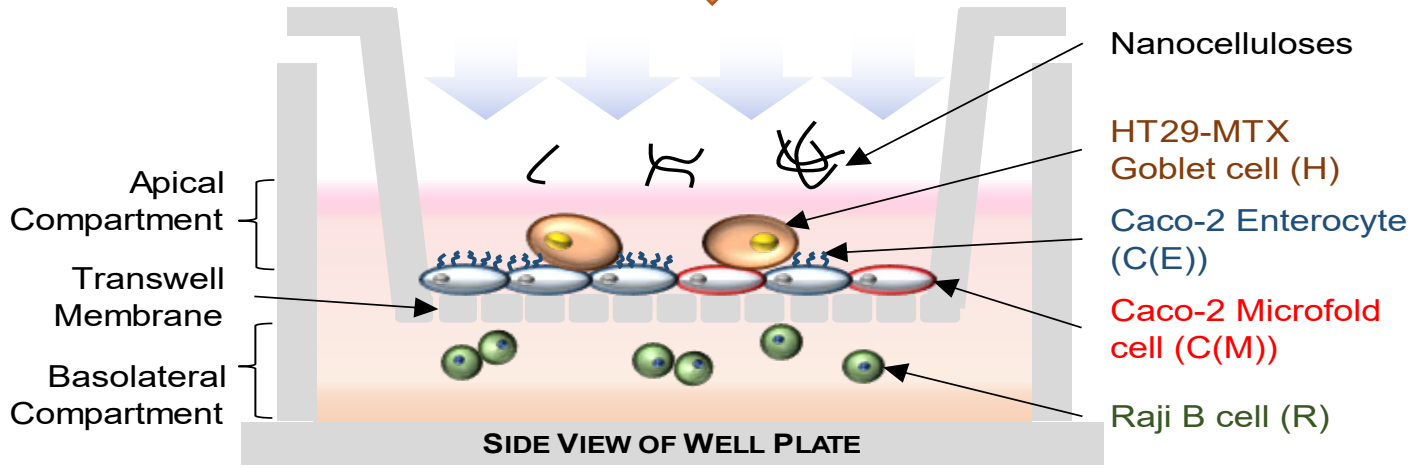
- ATS developed for safety assessment
  - Simulated oral exposure: gastrointestinal model
  - Simulated inhalation exposure: alveolar lung model
  - Dermal exposure: dermal and epidermal cells
  - Environmental toxicity: zebrafish




| | | |
|---|---|---|
| **Mode(s)-of-action** | *Gut cell co-culture model* | Cell death, inflammation, oxidative stress |
| **Structural endpoints** | *Gut cell co-culture model* | Cellular morphology, barrier resistance |
| **Molecular pathways** | *Gut cell co-culture model* | RNA-seq analyses |
| | *Zebrafish model* | |
| **Developmental endpoints** | *Zebrafish model* | Coagulated eggs; somite formation; detached tail; heartbeat |

# Simulated Oral Exposure (Gut on a Chip)




EFSA (2018). Guidance on risk assessment of the application of nanoscience and nanotechnologies in the food and feed chain: Part 1, human and animal health. *EFSA Journal*.

Ede *et al.* (2020). Physical, chemical and toxicological characterization of sulfated cellulose nanocrystals for food-related applications using *in vivo* and *in vitro* strategies. *Toxicology Research* **9**(6):808.

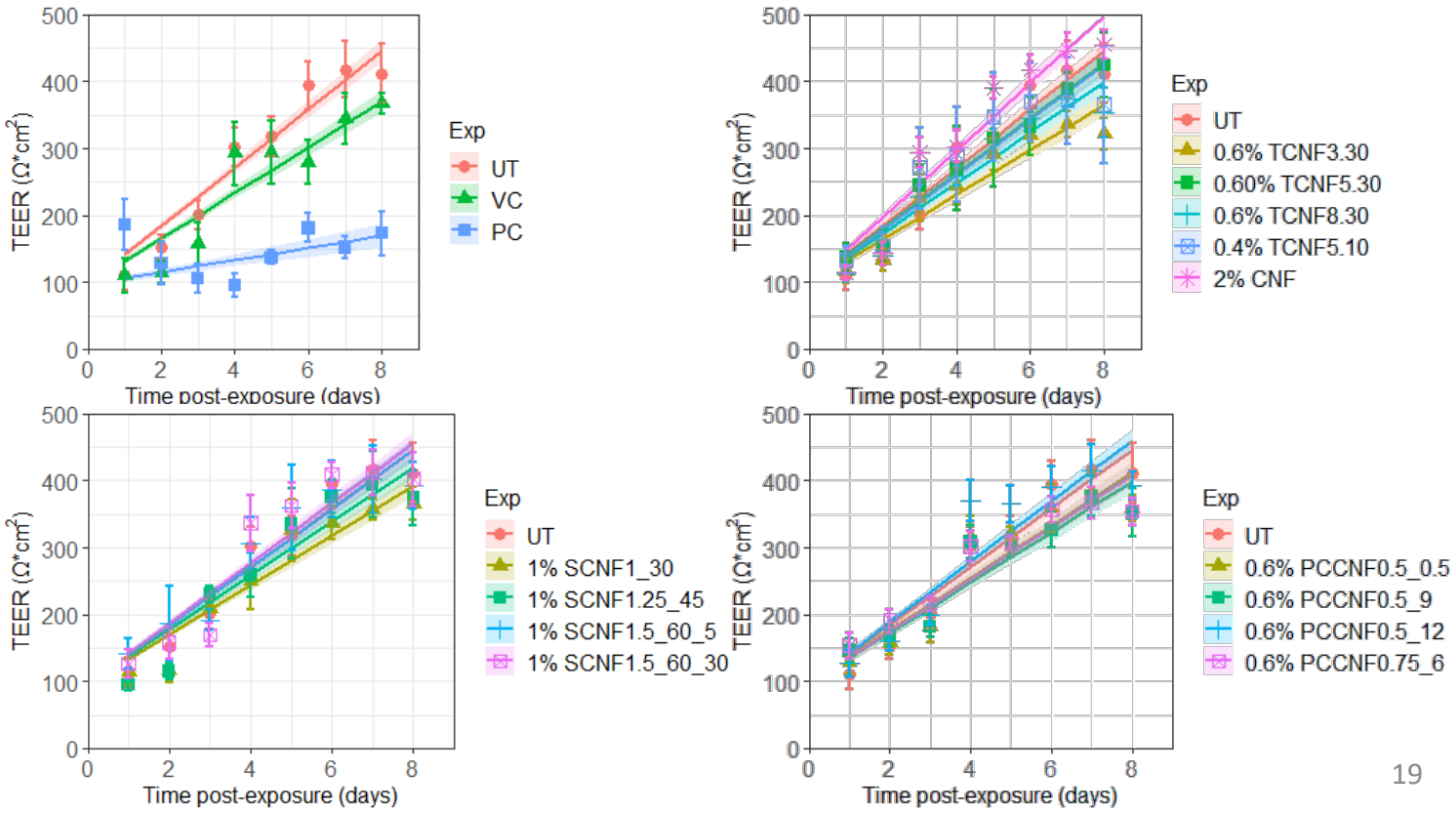
# Gastrointestinal Model Barrier Integrity (TEER)

- **Experiment:**

1. The barrier integrity was measured by using transepithelial/endothelial resistance (TEER).

2. Caco-2 and HT29 co-cultures were exposed for 4 h.

3. Decreased resistance is an indication of decreased barrier integrity.



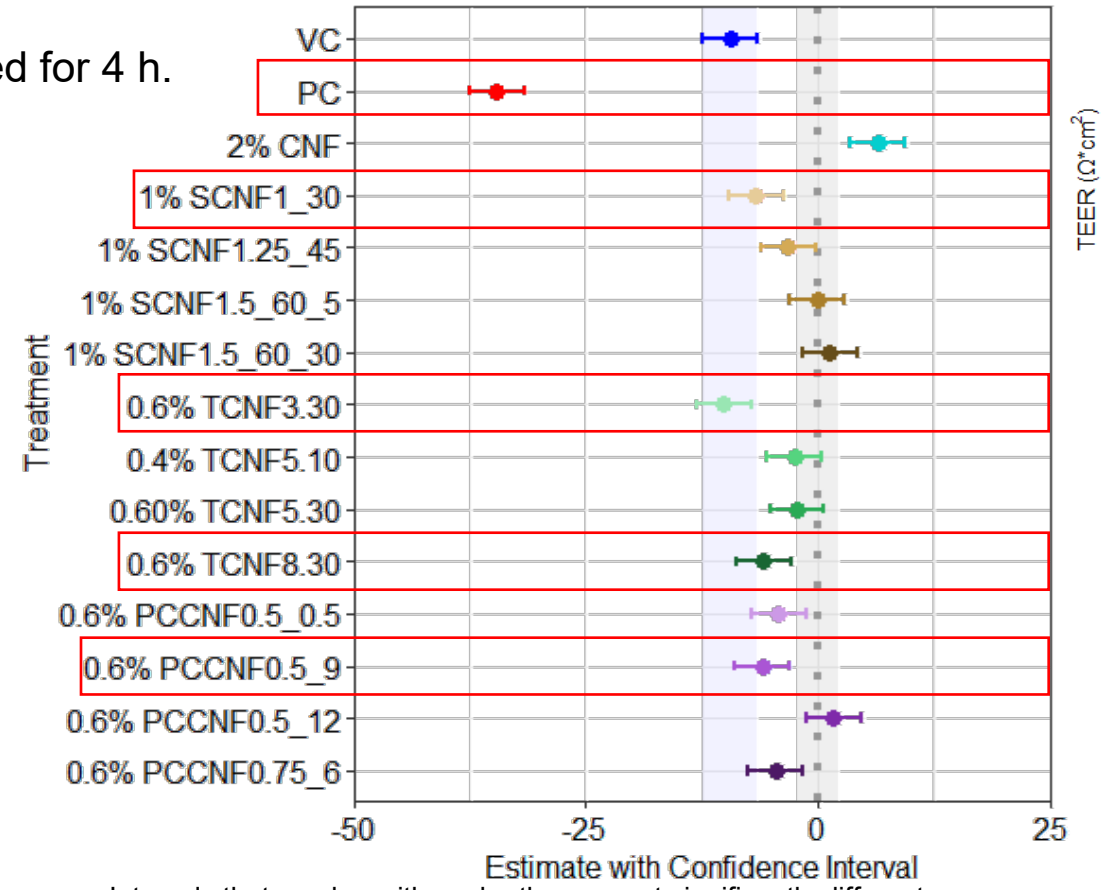
# Gastrointestinal Model Barrier Integrity (TEER)

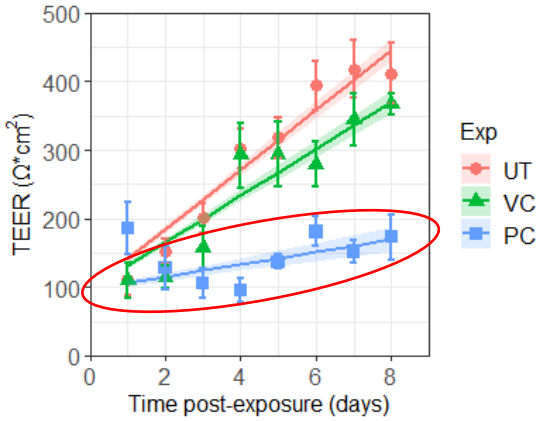
- **Experiment:**

1. The barrier integrity was measured by using transepithelial/endothelial resistance (TEER).
2. Caco-2 and HT29 co-cultures were exposed for 4 h.
3. Decreased resistance is an indication of decreased barrier integrity.

- **Conclusion:**

1. The positive control reduced the most resistance.
2. SCNF1_30, TCNF3.30, TCNF8.30, PCCNF0.5_9 had significantly lower TEER over 8 days post-exposure as compared to UT. However, they were not significantly different from the VC.




- Intervals that overlap with each other are not significantly different.
- Intervals that overlap with UT interval (gray region around dotted line) are not significantly different.

# Effects of Cellulose Type and Concentration

- **Questions:**

1. Does cellulose type affect response?

2. Does concentration have effects on response within the same cellulose type?

- **Experiment:**

1. GI tri-culture model (Caco-2, Raji B and HT29) was exposed to different cellulose types at various concentrations for 15 min or 4 h.
2. Endpoints:
    - Cell death – LDH assay
    - Inflammation - Interleukin 6 (IL-6)
    - Oxidative stress - Glutathione Reductase (GR) activity

# Cell Death (by LDH) vs. Cellulose Type

- **Method:**

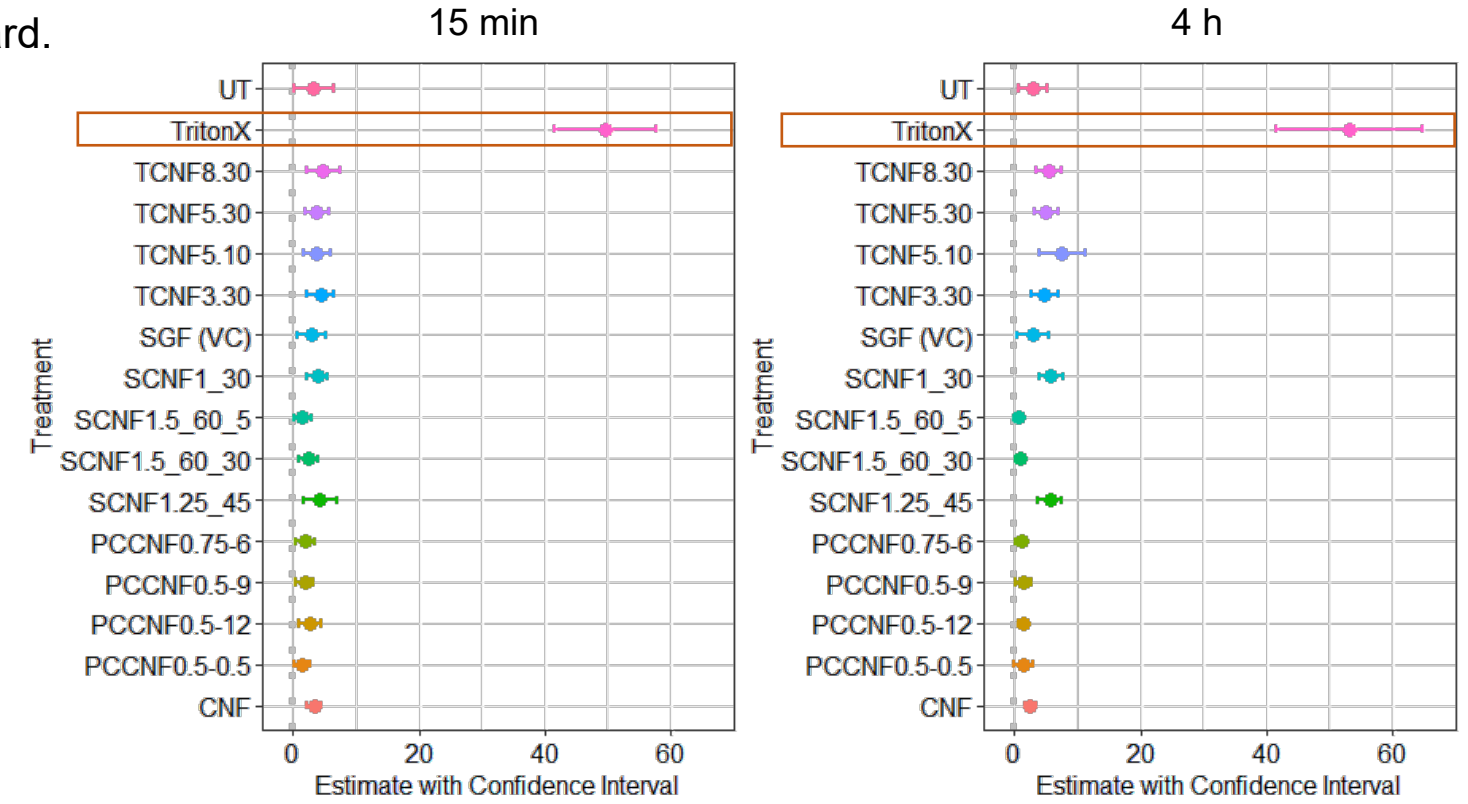
1. Estimated means with confidence intervals of LDH activity vs. various cellulose treatments.

2. All measurements were normalized to LDH standard.

- **Conclusion:**

1. At 15 min and 4 h exposure, TritonX (positive control) triggered significantly higher cell death as compared to all other groups.

2. No cellulose treatment induced significant cell death when compared to untreated cells (UT) at 15 min and 4 h.

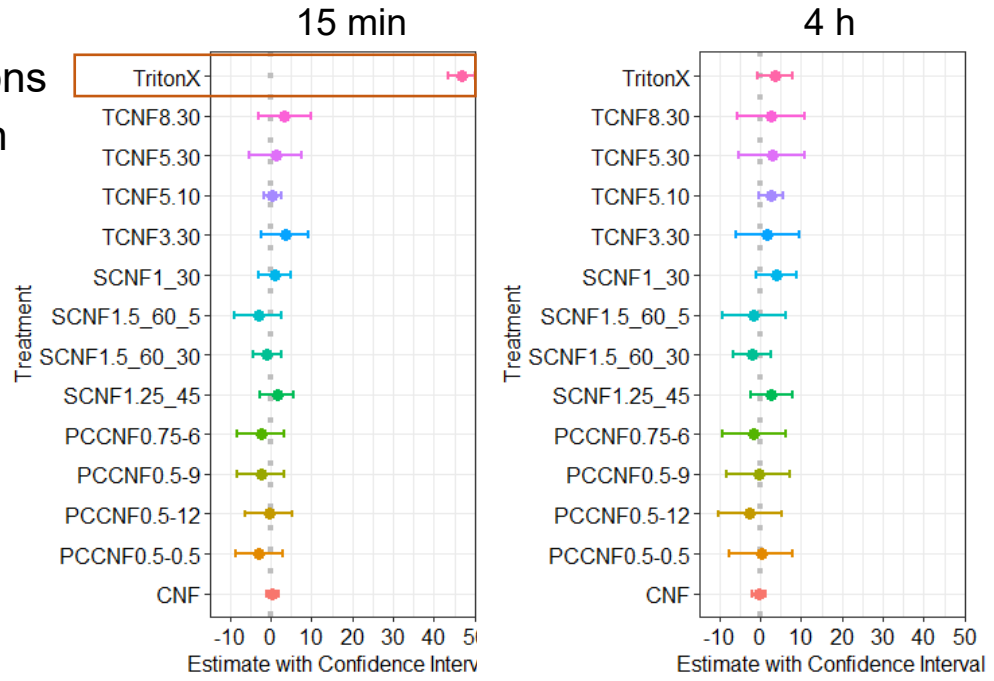



- Intervals that overlap with each other are not significantly different.

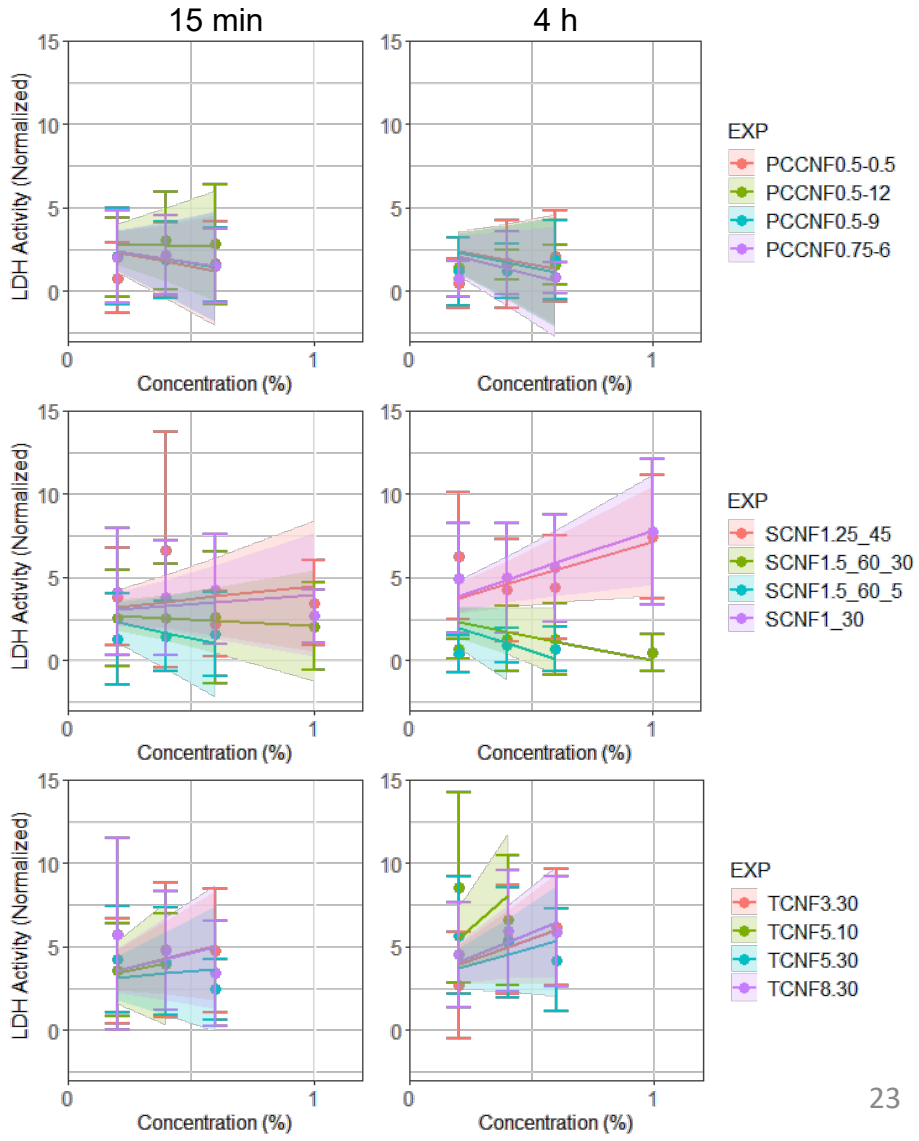
# Cell Death (by LDH) vs. Concentration

- **Method:**
  1. Estimated slopes with confidence intervals of LDH activity vs. various treatment concentrations.
  2. All measurements were normalized to LDH standard.

- **Conclusion:**
  1. All treatment concentrations had no effect on cell death except TritonX at 15 min.




- Intervals that overlap with each other are not significantly different.
- Intervals that contain 0 are not significantly different from control group.



# Inflammation (IL-6) vs. Cellulose Type

- **Method:**

1. Estimated means with confidence intervals of IL-6 (interleukin 6) vs. various cellulose treatments.

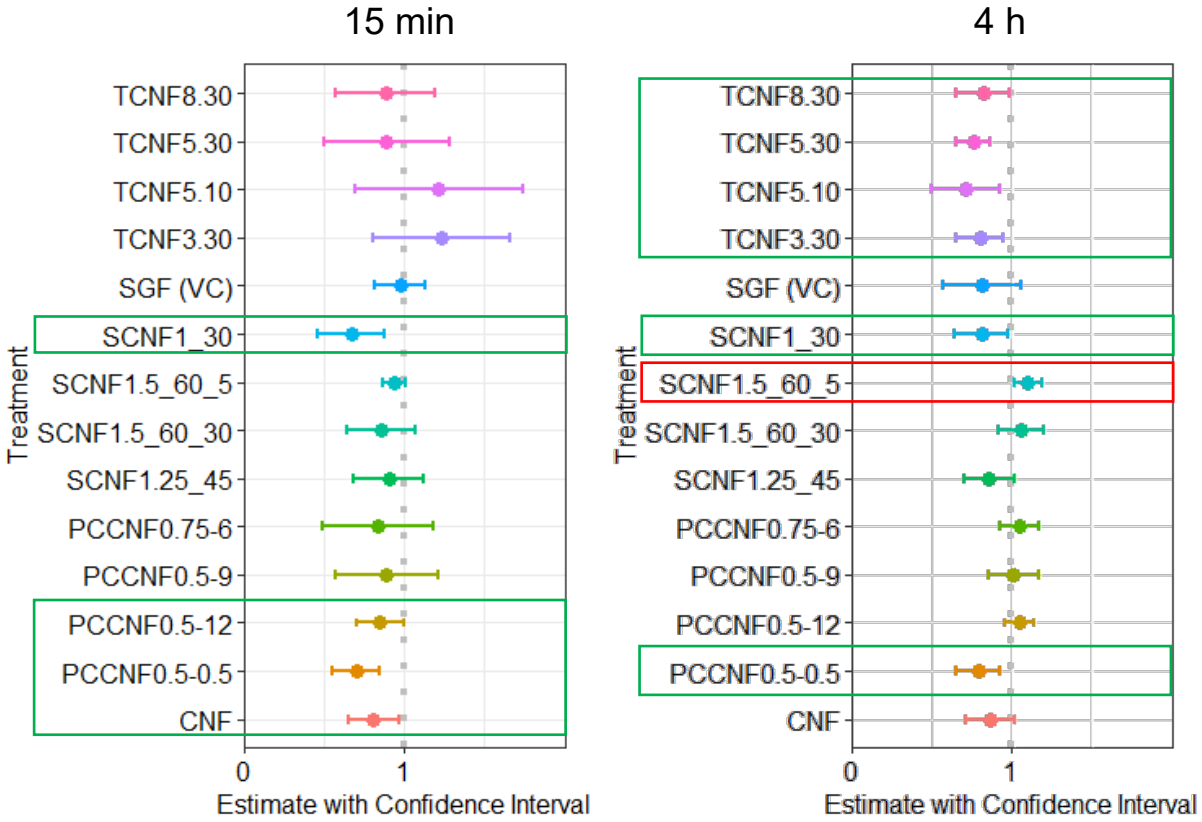
2. All measurements were normalized to untreated cells (UT).

- **Conclusion:**

1. At 15 min exposure, SCNF1_30, PCCNF0.5-12, PCCNF0.5-0.5 and CNF decreased IL-6.

2. At 4 h exposure, all the TCNFs, SCNF1_30, and PCCNF0.5-0.5 reduced IL-6 while SCNF1.5_60_5 slightly increased IL-6.

3. None of the treatments were significantly different compared to the vehicle control.

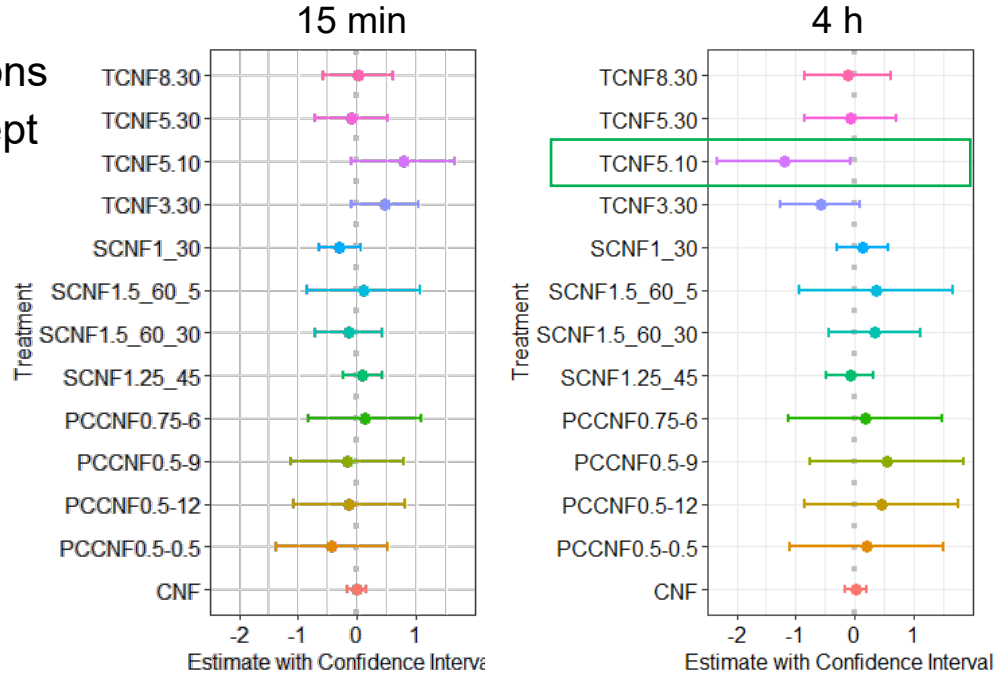


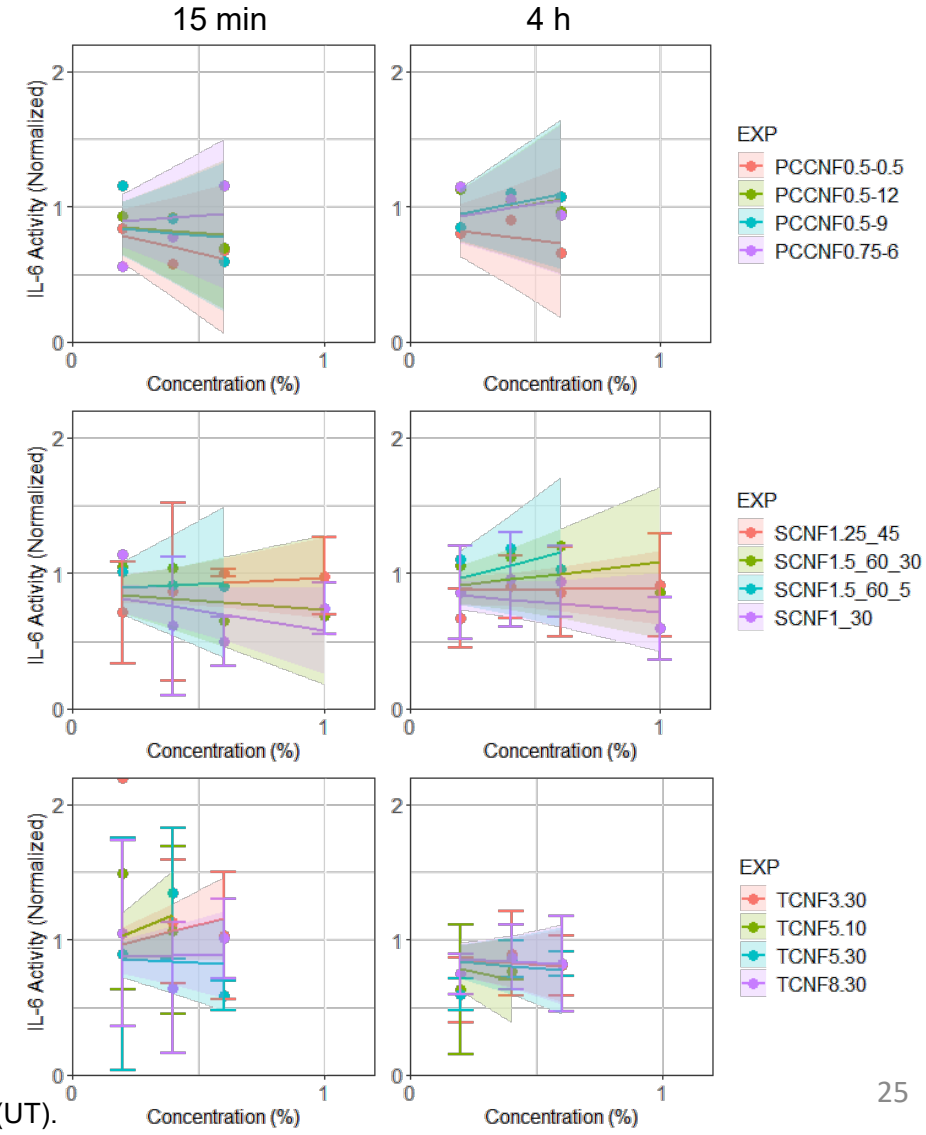

- Intervals that overlap with each other are not significantly different.
- Intervals that contain 1 are not significantly different from control group (UT).

# Inflammation (IL-6) vs. Concentration

- **Method:**
  1. Estimated means with confidence intervals of IL-6 (interleukin 6) vs. various cellulose treatments.
  2. All measurements were normalized to untreated cells (UT).

- **Conclusion:**
  1. All treatment concentrations had no effect on IL-6 except TCNF5.10 at 4 h.




- Intervals that overlap with each other are not significantly different.
- Intervals that contain 0 are not significantly different from control group (UT).

# Oxidative Stress (Glutathione Reductase) vs. Cellulose Type

- **Method:**

1. GR activity was measured for the various cellulose treatments.

2. All measurements were normalized to untreated cells (UT).

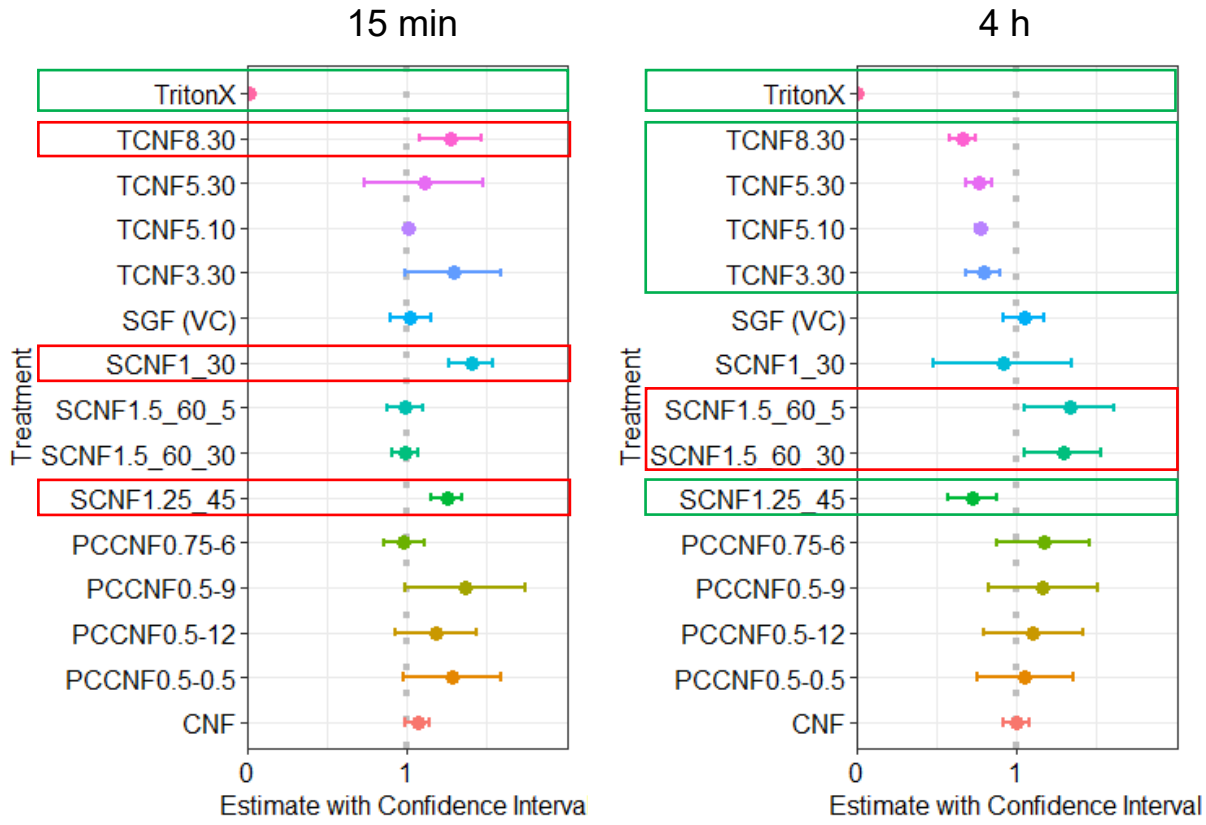
- **Conclusion:**

1. At 15 min and 4 h exposure, TritonX (positive control) significantly inhibited the GR activity as compared to all other groups.

2. At 15 min exposure, TCNF8.30, SCNF1_30 and 1.25_45 induced higher GR activity.

3. At 4 h exposure, all four TCNF and SCNF1.25_45 reduced GR activity.

4. At 4 h exposure, SCNF1.5_60_5 and 1.5_60_30 elevated GR activity as compared to UT but not significantly different from vehicle control.




- Intervals that overlap with each other are not significantly different.
- Intervals that contain 1 are not significantly different from control group (UT).

# Oxidative Stress (Glutathione Reductase) vs. Concentration

- **Method:**

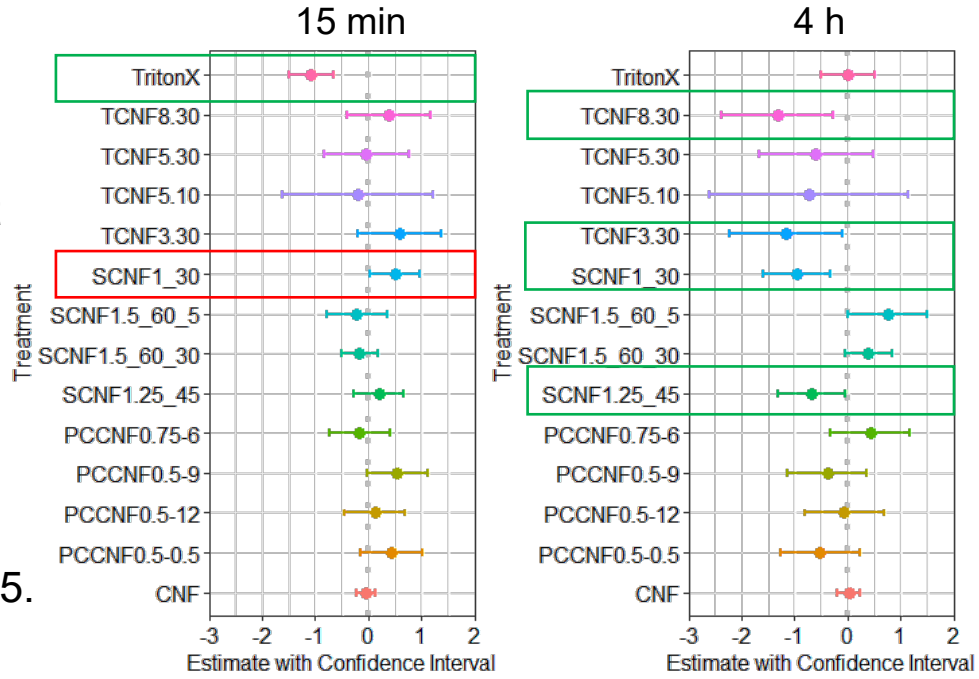
  1. GR activity was measured for the various cellulose treatments.

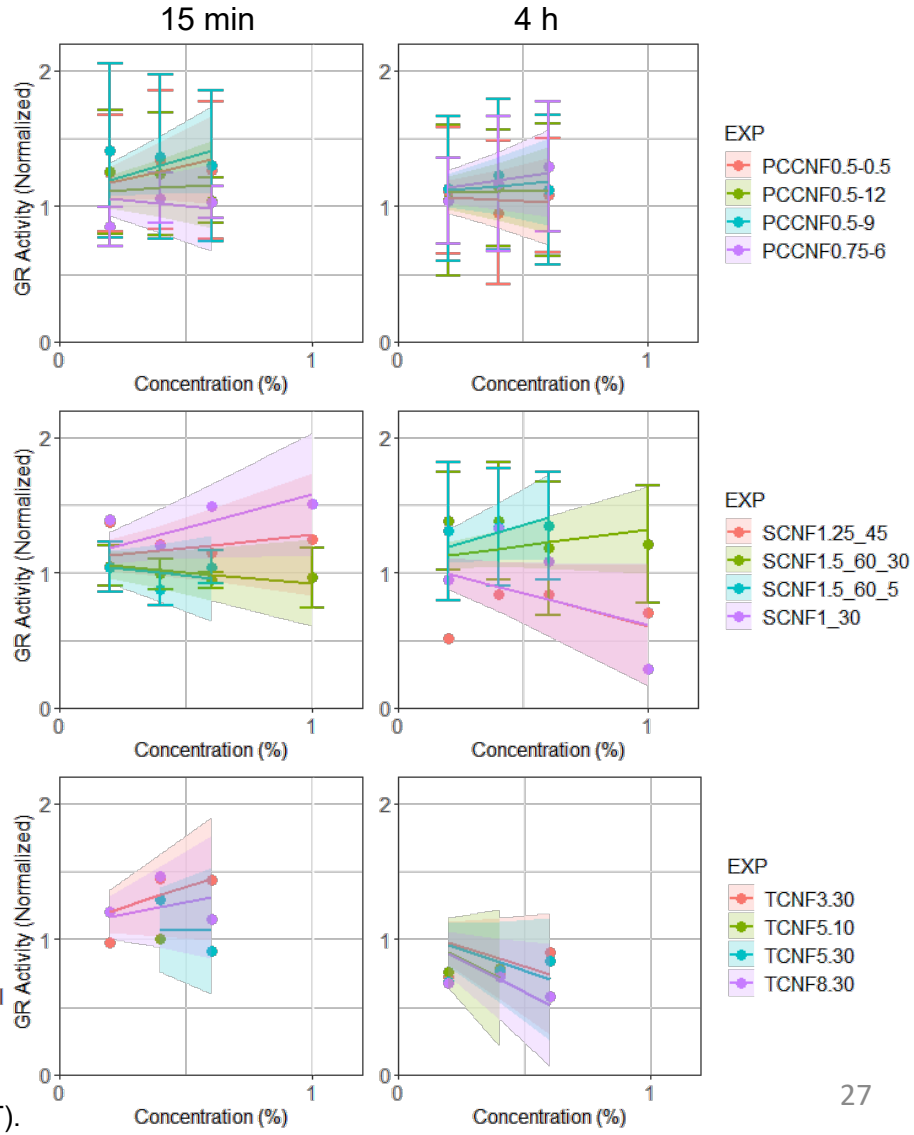
  2. All measurements were normalized to untreated cells (UT).

- **Conclusion:**

  1. At 15 min exposure, all treatment concentrations had no effect on cell death except Triton and SCNF1_30.

  2. At 4 h exposure, GR activity decreased as cellulose concentration increased in TCNF8.30, TCNF3.30, SCNF1_30 and SCNF1.25_45.




- Intervals that overlap with each other are not significantly different.
- Intervals that contain 0 are not significantly different from control group (UT).

# Life-Cycle Risk Assessment (LCRA) Development

Raw Material → Product Manufacture → Product Application → Product Use → Re-use/ Recycling → Disposal

**1.** Identify and Characterize Potential Hazards

**2.** Assess Exposure Potential and Risk

**3.** Prioritize Data Gaps

**4.** Address Data Gaps (E.g., Toxicity Studies)

**Output:** **Safety Demonstration**

# Summary

1. Synthesis of CNs with selected functionalized groups
   - All materials have been successfully synthesized.
2. Generate standardized safety methods and data sets
   - TEER, LDH, IL-6 and GR.
3. Build ‘Safer-by-Design’ Toolbox for next generation CN materials
   - Targeting 2023 for Toolbox rollout.
4. Future steps
   - Apply toolbox to select safe form of CNs in food packaging applications.
   - Additional toxicological assays and physiochemical characterization to further develop the toolbox.

# Publications to Date

1.Ede, J.D.; Ong, K.J.; Mulenos, M.R.; Pradhan, S.; Gibb, M.; Sayes, C.M.; Shatkin, J.A. Physical, chemical, and toxicological characterization of sulfated cellulose nanocrystals for food-related applications using *in vivo* and *in vitro* strategies. *Toxicology Research*, 2020 December. https://doi.org/10.1093/toxres/tfaa082

2.Ong, K.J, Ede, J.D., Pomeroy-Carter, C.A., Sayes, C.M., Mulenos, M.R., Shatkin, J.A. (2020) A 90-day dietary study with fibrillated cellulose in Sprague-Dawley rats. *Tox Rep* 7: 174-182. DOI: 10.1016/j.toxrep.2020.01.003 .

3.Patel, I., Woodcock, J., Beams, R., Stranick, S.J., Nieuwendaal, R., Gilman, J.W., Mulenos, M.R., Sayes, C.M., Salari, M., DeLoid, G., Demokritou, P., Harper, B., Harper, S., Ong, K.J., Shatkin, J.A., Fox, D.M. (2021). Fluorescently Labeled Cellulose Nanofibers for Environmental Health and Safety Studies. *Nanomaterials*, 11: 1015. https://doi.org/10.3390/nano11041015

4.Pradhan, S, George, M, Steele, L, Gibb, M, Ede, JD, Ong, KJ, Shatkin, J, Sayes, CM. 2020. Physical, chemical and toxicological characterization of fibrillated forms of cellulose using an in vitro gastrointestinal digestion and co-culture model. *Toxicology Research* 9(3): 290-301. https://doi.org/10.1093/toxres/tfaa026 .

5.Gibb M, Pradhan S.H., Steele L.R, Mulenos M.R, Lujan H, Ede J.D, Ong K.J, Shatkin J.A., Sayes C.M. (2020) Characterization of an Intestinal Co-culture System: Seeding Density Matters. FASEB J. Submitted. Ong, KJ, Ede, JE. Cassidy A. Pomeroy-Carter, CA, Christie M. Sayes, CM, Marina R. Mulenos, MR, and JA Shatkin. 2020. A 90-day dietary study with fibrillated cellulose in Sprague-Dawley rats. *Toxicology Research*, 9(6): 808-822. https://doi.org/10.1093/toxres/tfaa082

6.Ede JD…Shatkin JA et al (2019) Risk Analysis of Cellulose Nanomaterials by Inhalation: Current State of Science, *Nanomaterials. 9(3):337. https://doi.org/10.3390/nano9030337* .

7.Roberts R…Shatkin JA (2018) Collection of airborne ultrafine cellulose nanocrystals by impinger with an efficiency mimicking deposition in the human respiratory system. *J Occup Environ Hygiene* DOI: 10.1080/15459624.2018.1540876

8.Foster EJ…Ong, KJ…Shatkin JA, et al. (2018) Review: Current characterization methods for cellulose nanomaterials. Chem Soc Rev. 47(8):2609-2679. DOI: 10.1039/C6CS00895J

9.DeLoid et al. (2017). An integrated methodology for assessing the impact of food matrix and gastrointestinal effects on the biokinetics and cellular toxicity of ingested engineered nanomaterials. *Particle and Fibre Toxicology* 2017 14:40.

10.Ong KJ, Shatkin JA et al.(2017). Establishing the safety of novel bio-based cellulose nanomaterials for commercialization. *NanoImpact, (6): 19-29. https://doi.org/10.1016/j.impact.2017.03.002*

11.Shatkin, J.A., Ong, K.J., Ede, J.D., Wegner, T.H., Goergen, M. (2016). Toward Cellulose Nanomaterial Commercialization: Knowledge Gap Analysis for Safety Data Sheets According to the Globally Harmonized System. *TAPPI Journal*. 15:425.

12.McClements, D.J., DeLoid, G., Pyrgiotakis, G., Shatkin, J.A., Xiao, H., Demokritou, P. (2016). "The role of the food matrix and gastrointestinal tract in the assessment of biological properties of ingested engineered nanomaterials (iENMs): State of the science and knowledge gaps". *NanoImpact,* July 2016. (3-4); 47-57. http://dx.doi.org/10.1016/j.impact.2016.10.002

13.Shatkin J.A. and G. Oberdörster. 2016. Comment on Shvedova et al. (2016), “Gender differences in murine pulmonary responses elicited by cellulose nanocrystals”. *Particle Fibre Toxicol* 13:59 https://doi.org/10.1186/s12989-016-0170-4

14.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

# Acknowledgement

P³Nano, the U.S. Endowment for Forestry and Communities and the USDA US Forest Service Forest Product Laboratory (FPL)

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

Mitacs Elevate

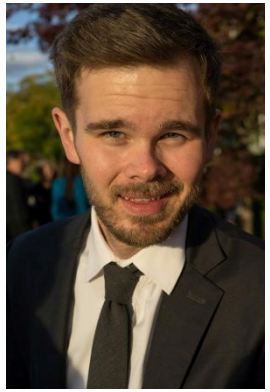
TAPPI committee

The Vireo Team

# 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.

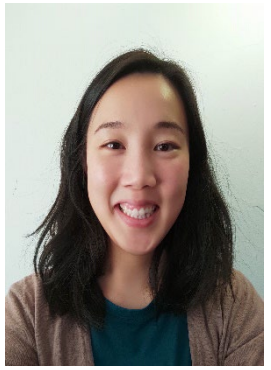
**Dr. Shaun Clancy** is a chemist with over 30 years experience in the chemicals industry, directing programs in health, safety, and regulatory affairs in major corporations. He is ANSI Co-Chair and participates in ISO TC229 and other international safety committees.

**Fiona Case** is a content writer with more than 20 years experience covering scientific innovations in foods, personal and home care products, sustainability, and computer-aided materials design.

**Patricia Hodgkinson** is Vireo's research consultant with a background in Food Policy law.

**Leslie Hockman** has industry experience working for a commercial manufacturer of cellulose nanomaterials and is Vireo's administrator.

**Yueyang (Brian) Zhang** is a toxicologist and a post doctoral fellow at University of Alberta and a MITACS Fellow with Vireo.



**Wei Ng** is an intern and is currently pursuing her PhD in Biological and Biomedical Science from Yale University.

**Angel Precious-Egere** is an intern and is pursuing a master's degree with a focus on antimicrobial nanotechnology.

**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.

**Tatiana von Rheinbaben** is a fellow and a M.S. Environmental Engineering and Science graduate of Stanford University.

# Thank you


## Yueyang (Brian) Zhang, Ph.D.

**Brian@VireoAdvisors.com**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WWW.VIREOADVISORS.COM/BLOG