Nanofibrillated cellulose: results of *in vitro* and *in vivo* toxicological assays

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Hazard assessment approach for NFC

Methodology

• *In vitro* assays for cytotoxicity, genotoxicity, and immunotoxicity
• A nematode model for systemic effects and neurotoxicity
• Pharyngeal aspiration study with mice for pulmonary immunotoxicity and genotoxicity
NFC samples studied

NFC-VTT
• Masuko grinder (5 passages)
• No pre-treatment, no bioside
• Bacteria

NFC-CTP/VTT
• Masuko grinder (5 passages)
• Enzymatic pre-treatment, bioside added to the pulp
• Bacteria

NFC-TE/VTT
• Masuko grinder (3 passages)
• TEMPO-mediated oxidation as a pre-treatment, no bioside
• Bacteria and yeast
NFC-TE/CTP samples studied

NFC-TE/CTP B1
- Lab scale high-pressure homogenizer
- TEMPO-mediated oxidation as pre-treatment
- Dialysed to remove traces of reactant
- No biocide added
- Bacteria

NFC-TE/CTP B2 ± biocide Busan 1009
- Pilot scale high-pressure homogenizer
- TEMPO-mediated oxidation as pre-treatment
- Washed four times but might contained residues of reagents (TEMPO, sodium bromide and sodium hypochlorite)
- Without biocide: bacteria, yeast and mold
- With biocide: some bacteria
Cytotoxicity

• Highest Tolerated Dose (HTD) assay
  • Qualitative morphological changes in human cervix carcinoma HeLa cells under an inverted phase contrast microscope
  • Comparison with untreated cells and evaluation on a scale ranging from 0 to 4
  • Only marginal effects seen at top doses (\(\rightarrow 2 \text{ mg/ml}\)) of NFCs, probably as a result of mechanical influence

• Total Protein Content (TPC) assay
  • A quantitative test measuring growth inhibition of HeLa cells, total protein content as the indicator of the cells’ viability
  • Based on reaction of fluorescamine with compounds containing primary amino groups to produce fluorescent products.

• None of the NFCs indicated a cytotoxic effect

• Boar Sperm Motility Assay (BSM)
  • A qualitative test particularly suitable for testing of suspensions
  • Boar spermatozoa’s movement inhibition indicates mitochondrial or membrane damage

• No toxic effects seen
Immunotoxicity – pulmonary inflammation

*In vitro studies*

- Macrophages exposed to several doses of nanocelluloses in cell culture
- **Cytotoxicity** assessed by photometric and luminometric methods
- Macrophage activation assessed by measuring the *expression of essential cytokines*
  - at the mRNA level by real-time quantitative PCR
  - at the protein level by ELISA

*In vivo studies on NFC-TE/CTP B2 + biocide*

- Material for *in vivo* studies chosen by the Consortium
- Mice exposed by pharyngeal aspiration
- Inflammatory parameters analysed
Macrophages

- act as the first line of defense against pathogens and particles in the lungs
- ingest pathogens and particles and break them down
- release proinflammatory cytokines which promote inflammatory processes
  - TNF-alpha, IL-1beta, and IL-6 determined in the present study

In the immunotoxicological part of the present study, human macrophages derived from blood monocytes of were used

Figures: Wikimedia Commons
Cytotoxicity in human macrophages

- LDH release from cells expressed as percentage of total LDH, measured photometrically
- ATP in cells expressed as relative luminescence (percent of unexposed control), measured luminometrically
- Human monocyte-derived macrophages exposed for 6 h
- Cell culture supernatants collected
- Cytotoxicity measured by lactate dehydrogenase (LDH) leakage and decrease of ATP (luminescent cell viability assay)
- Roridin A (RoA) and silica used as positive controls

NFCs were not cytotoxic to macrophages at doses up to 300 µg/ml

Ilves et al., in preparation
mRNA expression of pro-inflammatory cytokines IL-1β and TNF-α in exposure to NFCs

- Primed and unprimed human monocyte-derived macrophages exposed for 6 h
- Cell culture supernatants collected, mRNA measured by PCR
- NFC-TE/VTT slightly increased IL-1-β and TNF-α mRNA (due to bacteria & yeast?)

Ilves et al., in preparation
Secretion of pro-inflammatory cytokines IL-1β and TNF-α in exposure to NFCs

- Primed and unprimed human monocyte-derived macrophages exposed for 6 h
- Cell culture supernatants collected, pro-inflammatory cytokines measured by ELISA
- All NFCs slightly induced TNF-α and, in LPS-primed samples, IL-1-β

Ilves et al., in preparation
mRNA expression of pro-inflammatory cytokines IL-1β and TNF-α in exposure to NFC-TE/CTP B2 biocide

- Primed and unprimed human monocyte-derived macrophages exposed for 6 h
- Cell culture supernatants collected, mRNA measured by PCR
- No significant effects observed

Ilves et al., in preparation
Secretion of pro-inflammatory cytokines IL-1β and TNF-α in exposure to NFC-TE/CTP B2 ± biocide

- Primed and unprimed human monocyte-derived macrophages exposed for 6 h
- Cell culture supernatants collected, pro-inflammatory cytokines measured by ELISA
- **No effects observed**
Genotoxicity – identifying possible carcinogens

**In vitro studies**

- Human bronchial epithelial BEAS 2B cells exposed to several doses of nanocelluloses in cell culture
- **Cytotoxicity** (cell count) utilized for dose finding
- **DNA damage** examined by the Comet assay
- **Oxidative DNA damage** studied by the enzyme-modified Comet assay
- **Chromosome damage** assessed by the micronucleus assay
- **Cell cycle delay** studied by the cytokinesis-block proliferation index

**In vivo studies on NFC-TE/CTP B2 + biocide**

- One NFC material chosen for *in vivo* studies by the Consortium
- Mice exposed by pharyngeal aspiration
- DNA damage and oxidative DNA damage studied in bronchoalveolar lavage (BAL) cells
Cytotoxicity of NFC-TE/CTP B2 ± biocide in human bronchial epithelial BEAS 2B cells

- **Double staining:** Propidium iodide stains dead and dying cells, Hoechst stains cell nuclei
- Cell count in fluorescence microscope

Cytotoxicity of the NFCs in BEAS 2B cells was generally low

Hannukainen *et al.* in preparation
DNA damage detection by the comet assay

- DNA with strand breaks wanders out of the nucleus in electrophoresis.
- The proportion of DNA in "comet tail" reflects the amount of DNA damage.
- Oxidative DNA damage visualized by turning oxidative DNA adducts to strand breaks by a specific enzyme (FPG).
- Analysis in fluorescence microscope using a semiautomatic interactive software.
DNA damage induced *in vitro* by NFCs

- **NFC/VTT**
  - No increase in DNA damage
  - Slight increase in oxidative DNA damage (FPG vs Buffer)

- **NFC-TE/CTP**
  - Slight increase in DNA damage in the comet assay
  - Slight increase in oxidative DNA damage at one dose
DNA damage induced *in vitro* by NFCs

- Slight Increase in DNA damage in the Buffer and FPG series, **but not in ordinary Comet assay**
- No increase in oxidative DNA damage

- Slight increase in DNA damage in the comet assay and Buffer series
- No increase in oxidative DNA damage
DNA damage by NFC-TE/CTP B2 ± biocide

- No increase in DNA damage
- No increase in oxidative DNA damage

NFC-TE/CTP B2 without biocide

- Increase in DNA damage in the Buffer series, but not in ordinary Comet assay
- No increase in oxidative DNA damage
Micronucleus assay with NFCs

Micronuclei reflect structural and numerical alterations of chromosomes

- **Acridine orange staining:**
  - nuclei (DNA) are green,
  - cytoplasm (RNA) is red

- Analysis in fluorescence microscope

Human bronchial epithelial BEAS 2B cells in fluorescence microscope after treatment with NFC-VTT (250 µg/cm²).

Photo: Kati Hannukainen and Hilkka Järventaus, FIOH
No induction of micronuclei by NFCs in BEAS 2B cells

- No effect on cell cycle length either (data not shown)

Hannukainen et al., in preparation
Nematode toxicity assay *in vivo*

- Transgenic line (P_{dat-1::GFP}) of *Caenorhabditis elegans* expressing
- Green Fluorescent Protein in its dopaminergic neurons
- Bright fluorescence of ganglia indicates functional nervous system
- NFC-TE/CTP dose 0.5 mg/ml (biocide)
- Single walled carbon nanotubes used as fibre control, DMSO (5%) as a positive control
- 24-h follow-up for viability, behavior and reproduction
- **The NFCs were not toxic to C. elegans**

Photo: Prof. Garry Wong, University of Eastern Finland

Photo: Jadiya et al. 2011
In vivo study in mice

- Female C57BL/6 mice
- NFC-TE/CTP B1 tested
- Exposure by pharyngeal aspiration
- Single dose: 20, 40, 80 and 200 µg/mouse (in PBS)
- Biocide Busan 1009 tested separately at the same dose as in the NFC
- Negative and positive controls
- Samples collected 16 h after dosing (acute effect)
  - Bronchoalveolar lavage fluid
  - Lungs
  - Blood
- Analysis of pulmonary inflammation and DNA damage
In histological samples of the lungs, NFC was seen in or near bronchioles. No dramatic tissue changes were observed.
NFC-TE/CTP B2 induced mRNA of pro-inflammatory cytokine IL-6
NFC-TE/CTP B2 induced influx of inflammatory cells to mouse lungs

**macrophages**

- Neg control
- Biocide
- 10
- 40
- MWCNT 10

**neutrophils**

- Neg control
- Biocide
- 10
- 40
- MWCNT 10

**lymphocytes**

- Neg control
- Biocide
- 10
- 40
- MWCNT 10

**eosinophils**

- Neg control
- Biocide
- 10
- 40
- MWCNT 10
No DNA damage in mouse bronchoalveolar lavage (BAL) cells after pharyngeal aspiration of NFC-TE/CTP B2

DNA damage after pharyngeal aspiration of NFC

DNA in tail (%)

NFC mg/mouse

DNA damage in biocide-treated mice

DNA in tail (%)

Control

Biocide

Hannukainen et al. in preparation
Conclusions – toxicity of NFCs

*In vitro*
- Low or no cytotoxicity
- Slight induction of proinflammatory cytokines in macrophages with or without LPS priming *in vitro*
- Slight DNA damage in human bronchial epithelial cells – similarly to a number of other "inert" nanomaterials
- Marginal induction of oxidative DNA damage *in vitro* (some NFCs)
- No increase in chromosome damage (micronuclei)

*In vivo* (NFC-TE/CTP B2, pharyngeal aspiration, mice)
- No DNA damage in bronchoalveolar lavage cells
- Pulmonary inflammation

→ Possibly due to the particulate / bacteria in the NFC
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