**Surface bio-interactions stand at the base of the short-term nano CuO-induced cell oxidative stress: insights for a safe(r)-by-design approach**

**ADDITIONAL FILE 3**

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***1. Copper cytochemistry***

Intracellular copper ion dissolution was determined by a cytochemical method using rhodanine. After exposure to 10 mg/ml and 25 mg/ml CuO NPs for 6h. Cells were rinsed, formalin fixed, and incubated with 0.12 g/l rhodanine (p-Dimethylaminobenzylinene-rhodanine) alcoholic solution. Abundantly rinsing was done and nuclei were counterstained with haematoxylin. Slides were mounted in a glycerol-based medium and immediately observed under the light microscope (Axioplan - Zeiss).

Results from this test are summarised in Additional Figure 4.

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Additional Figure 4. Cytochemistry of Cu2+ by Rhodanine staining in A549 exposed to nCuO for 6h. a) negative control (unexposed cells); b) positive control (10 mg/ml cCuO-BSA-exposed cells, 24h); c, e) 10 mg/ml and 25 mg/ml cCuO-exposed cells; d, f) 10 mg/ml and 25 mg/ml sCuO-exposed cells. Red/orange spots testify for the intracellular release of Cu++ (b, c, e).