Surface reactivity measurements as required for grouping and read-across: An advanced FRAS protocol

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Abstract. Oxidative stress is a widely accepted paradigm associated with different adverse outcomes of particulate matter, including nanomaterials. It has frequently been identified in in vitro and in vivo studies and different assays have been developed for this purpose. Here we describe a newly developed multi-dose protocol of the FRAS assay (Ferric Reduction Ability of Serum). The purpose of this SOP is the measurement of the surface reactivity of nanomaterials under physiological conditions. Antioxidative components as present in human blood serum (HBS) serve as reporter molecules. The assay separates the oxidative damage from the read-out of the reporter molecules. The results show significantly enhanced repeatability with better sensitivity towards low reactivity, enabling application of FRAS both to a rough grouping by reactive vs. passive nanomaterials and further to substantiation of read-across by enhanced resolution of the similarity between different nanoforms of the same substance.

1. Introduction
The French nano-inventory highlights the diversity of substances in nanoform, which are dominated by conventional fillers and pigments produced in multi-k-ton/y of various nanoforms per substance.[1] Therefore, alternative testing approaches with reduced costs and high predictivity suitable for high throughput screening and prioritization are urgently needed. In parallel, extensive research efforts are targeted to understanding modes of action of Engineered Nano-Materials (ENMs), which may also support the development of new predictive assays and which are also needed to establish NM grouping.[2] Oxidative stress is a widely accepted paradigm associated with different adverse outcomes of ENMs. It has frequently been identified in in vitro and in vivo studies [2,3] and different assays have been developed for this purpose.[4-11]

Previously, we have investigated a representative panel of 32 ENMs including functionalized amorphous silica (6 nanoforms), zirconium dioxide (4), silver (4), titanium dioxide (3), zinc oxide (2), multiwalled carbon nanotubes (3), barium sulfate and AlOOH. Surface reactivities of all were studied in a cell-free system by electron spin resonance (ESR) spectroscopy. NRK-52E cells were treated with all NP, analyzed for viability (WST-1 assay) and intracellular ROS production (DCFDA assay). Carbonylated proteins were analyzed on 1D and 2D immunoblots and identified by matrix assisted laser desorption time-of-flight mass spectrometry.[3] We found overall an excellent match of ESR vs. in vitro carbonylation.

To complement the grouping concepts by several complementary protocols to assess the extrinsic property of surface reactivity, we explore here an optimization of the FRAS assay (Ferric Reduction...
Ability of Serum). The principle of the FRAS assay is well established to assess oxidative damage [4] including its application to ENM surface reactivity [5], and has shown potential to separate active from passive ENMs [6-8].

2. Methods and Materials

2.1. FRAS assay principle

In short, the antioxidative components as present in human blood serum (HBS) serve as reporter molecules.[4,5] The assay separates the oxidative damage from the read-out of the reporter molecules, as schematically shown in Figure 1: (1), HBS is incubated with nanomaterials, which are then (2) removed by centrifugation. Afterwards (3) an aliquot of that pre-incubated HBS is mixed with a solution containing a Fe3+ complex and is again incubated. The ability of the pre-incubated HBS to reduce Fe3+ to Fe2+ is detected optically by a color change from transparent to blue. Any damage to anti-oxidative species in HBS by nanomaterials during step (1) will reduce the blue color in the final step (3).

2.2. FRAS assay optimization summary

The individual steps of the protocol were not / not all / not sufficiently described in earlier publications.[5-8] We optimized them for high sensitivity & increased statistical significance, and found the following elements to be essential at each of the respective steps:

1. Optimized step ENM@serum incubation time is 3 h at 37°C. The use of stock solutions is strongly disadvised because the dilution of serum by de-ionized water create a substantial signal. Instead, HBS is given directly on ENM powder. Despite potential trade-offs by the denaturation of proteins, we found short (1 min bath) sonication highly beneficial to enhance reproducibility, presumably by making the ENM surface accessible to the antioxidants
   a. Tested incubation time from 60 to 180 min – preferred 180 min for enhanced sensitivity towards low reactive ENM.
   b. Sonication preferred, otherwise visibly large (>µm) agglomerates can remain even after the incubation time, leaving doubts on the biologically accessible surface area.
   c. For screening purposes established ENM mass dose range 0.75 g/L, 2 g/L, 5.5 g/L, 15 g/L, 40 g/L (which extends to both sides from the previous default of 10 g/L)
   d. Triplicate testing of dose response, then averaging of fit parameters.

2. Centrifugal extraction to remove ENM and yet to retain antioxidants works fine with 150 minutes at 14,000 g. When transferring ENM-free serum from centrifugation tubes into the FRAS reagent, the reproducibility of the volume of 100 µL translates directly into the final reproducibility of the assay.
   a. Tested 2000 rpm, found no increase of antioxidants, hence no issues with inadvertent antioxidant depletion by ENM separation.
b. Pipettes tested: plastics, coated plastic “Ultra Low Retention” tips, glass with “positive displacement” pipettes using a Teflon plug (strongly recommended).

c. Tested fresh pipette for each vial – no effect.

3. Handling of the FRAS reagent in the dark is absolutely essential. The doubled dilution of 0.1 mL HBS into 2 mL FRAS reagent enhances the sensitivity to low reactive ENM, because it ensures that the final state of Fe oxidation is limited by the antioxidant concentration and not by the Fe-TPTZ concentration.

a. Optimized serum@FRAS reaction time at room temperature is 60 min ± 1 min precision.

b. Tested under ambient lab conditions vs. no light during handling.

c. Tested pre-wetting of pipette tips in water / in HBS, found no influence, tested glass vs. plastic vials and pipette tips, found strong influence.

d. Tested range of reaction times: from 60 to 120 min, with 5 minute deliberate variations, or in reversed order – only reaction time counts.

The following sections 2.2.1 to 2.2.3 document the exact materials, disposables, equipment used in our labs, marking these as “recommended” and highlighting in bold those that we now know to be critical.

2.2.1. Recommended materials:

• sodium acetic trihydrate: Sigma Aldrich BioUltra, purity: ≥99.5%, CAS number: 6131-90-4, EC number: 204-823-8, Product number: 71188

• glacial acetic acid: Alfa Aesar, purity ≥99.7%, CAS number: 64-19-7, EC number: 200-580-7, Product number: 36289

• 2,4,6-tri(2-pyridyl)-s-triazine (TPTZ): Sigma Aldrich, purity: ≥98%, CAS number: 3682-35-7, RC number: 222-965-9, Product number: T1253

• 1M HCl: Riedel-De Haen, concentration: 1M, CAS number: 7647-01-0, Product number: 35328

• FeCl₃.6H₂O: Sigma Aldrich, purity: ≥98-102%, CAS number: 10025-77-1, EC number: 231-729-4, Product number: 44944

• human blood serum (HBS): Sigma Aldrich, P2918-100ml (frozen in 2 g aliquots)

Note that HBS is of human origin and requires appropriate risk management. It is anticipated that the source of human blood serum has an influence on the absolute FRAS

2.2.2. Recommended disposables:

• Pipette: Eppendorf Reference, 100-1000 µL, with plastic tips

• Disposal glass Pasteur pipettes with pipetting aid: volac, (product number D810, 150 mm); Brand accu-jet pro

• 100 mL glass bottles: Kavalier Simax, product number: 2070 M, EAN: 8593419604089, code: 1632414321100, diameter: 56 mm, height: 100,00 mm, GL: 45

• 30 mL glass vials: chemoline, product number 50-2030

• 10 mL glass vials: Max Wiegand, product number 4382810)

• PTFE stirrers: Polygon, length:15 mm diameter: 6 mm  [glass stirrers would be preferable but could not be obtained at this small dimensions required for low volumes]

• Centrifuge tubes: Beckman Coulter, Centrifuge Tubes Ultra-Clear, 11 x 60 mm, product number 344062

• Brand Transferpettor with 100 µL glass tips

• Quartz cuvettes: Helma Analytics, High Precision Cell made of Quartz SUPRASIL, light path 10 mm, 104-10-40
2.2.3. Recommended equipment:
- Millipore Advantage A10 → DI water
- Balance: Mettler AT250
- Ultrasoundation bath: Sonorex Digital 10p (320 W electrical power)
- Shaker: IKA Vibrax VXR (set to level 200), equipped with home-made extension for 10 mL vials (see photo below)
- Heating chamber: mytron WB 55
- Stirrer: Thermo Scientific, Variomag Poly 15
- Centrifuge: Beckman Coulter OPTIMA-XL80K with swing-out rotor SW60-TI
- UV/Vis spectrometer: Perkin Elmer Lambda 35 equipped with automatic linear cell changer for 8 samples (the automatic changer is not ultimately required, but makes it much easier to keep the timing identical for all doses.)

2.3. Optimized multi-dose protocol of the FRAS assay

2.3.1. Preparation of FRAS reagent:
As the following solutions cannot be stored, they have to be prepared freshly before every experiment.

Prepare S1:
- Weigh 0.2021 g of sodium acetic trihydrate to a 100 mL glass bottle.
- Add 50 g DI water.
- Add 1.060 mL (2 x 0.53 ml) of glacial acetic using a pipette.
- Add DI water to achieve a total of 100 g.

Prepare S2:
- Weigh 0.0946 g of TPTZ to a 30 mL glass vial.
- Add 15 g DI water.
- Add 1.2 mL (2 x 0.6 mL) of 1M HCl using a pipette.
- Add DI water to achieve a total of 30 g.
- Put S2 to an ultrasonication bath and sonicate for 30 minutes with maximum power at room temperature.

Prepare S3:
- Weigh 0.1635 g of FeCl₃.6H₂O to a 30 mL glass vial.
- Add DI water to achieve a total of 30 g.

Prepare FRAS reagent:
- Weigh 50 g of S1, 5 g of S2, and 5 g of S3 to a 100 mL glass bottle.
- Store FRAS reagent in the dark while shaking slowly.

2.3.2. Test protocol:

**Make sure to only use glass equipment to avoid interferences with plastic parts.**

1. Unfreeze 6 x 2 g human blood serum (HBS) at 37°C for 30 minutes.

**STEP (1) INCUBATION:**
2. Prepare powder nanomaterial dispersions in concentrations of 0 (blank), 0.75, 2, 5.5, 15, and 40 g/L by first weighing the powder nanomaterial to six 10 mL glass vials and then adding 1.5 g of unfrozen HBS to each glass vial by using glass Pasteur pipettes.
3. Add a PTFE stirrer to each bottle, close bottles with caps.
4. Put the nanomaterial dispersions to an ultrasonication bath and sonicate for 1 minute with maximum power at room temperature.
5. Incubate dispersions **at 37°C for 3 hours in the dark** while stirring constantly at 400 rpm.

**STEP (2) SEPARATION:**
6. Transfer nanomaterial dispersions completely to six centrifuge tubes.
7. Centrifuge at 14,000 g (11,900 rpm in our centrifuge) **for 150 minutes**. Earlier implementations of the FRAS assay [8] specify the same g-number for twice 20 minutes. We found this procedure less effective due to convection and re-diffusion issues. We checked that this condition does not deplete the antioxidants from serum (FRAS signal remains the same with only 2000rpm)
8. During centrifugation, label 18 10 ml glass vials with blank A, C1A, C2A, C3A, C4A, C5A, blank B, C1B, C2B, C3B, C4B, C5B, blank C, C1C, C2C, C3C, C4C, C5C.
9. Add 2.00 g ± 0.002 g of FRAS reagent to each glass vial by using a glass Pasteur pipette. **Make sure to avoid any direct light during aliquotation of FRAS reagent.** Put each glass vial to the dark directly after the addition of FRAS reagent.
10. Label six 10 ml glass bottles with blank, C1, C2, C3, C4, C5.

**STEP (3) ANALYSIS:**
11. After centrifugation, carefully recover the liquid part of samples (approximately 0.5 mL) by using disposal glass Pasteur pipettes. Make sure to recover no solids: neither particles from the sediment nor floating components. Add the removed supernatants to the six labelled 10 ml glass bottles by disposing the first and the last drop.

**STEP (3) ANALYSIS:**
12. Take glass vials blank A, C1A, C2A, C3A, C4A, C5A containing the FRAS reagent out of the dark and add 100 µl of the supernatant of blank to blank A, C1 to C1A, C2 to C2A, C3 to C3A, C4 to C4A, C5 to C5A by using a specific pipette (Brand Transferpettor, 100 µL glass tip) according to a strict time schedule depending on the time required for UV/Vis detection (absorption at 500-700 nm). Such a time schedule is crucial to ensure that a reaction time of 60 ± 1 min. for each sample is achieved. In our case detection by UV/Vis takes approximately 45 seconds per sample (approximately 4 minutes in total) for a wavelength range of 500-700 nm resulting in the time schedule below. If using another UV/Vis detector, the time schedule has to be adapted accordingly in dependence on the detection duration of the used spectrometer. Start timer directly after adding the supernatant of blank to FRAS reagent of blank A and afterwards carefully follow the time schedule. Put each sample to the shaker in the dark, directly after adding the supernatant to the vial.
13. Accordingly, take glass vials filled with FRAS reagent (blank B, C1B, C2B, C3B, C4B, C5B) out of the dark and add 100 µl of the supernatant blank to blank B, C1 to C1B, C2 to C2B, C3 to C3B, C4 to C4B, C5 to C5B by strictly following the time schedule.
14. Accordingly, take glass vials filled with FRAS reagent (blank C, C1C, C2C, C3C, C4C, C5C) out of the dark and add 100 µl of the supernatant blank to blank C, C1 to C1C, C2 to C2C, C3 to C3C, C4 to C4C, C5 to C5C by strictly following the time schedule.
Table 1 Recommended timing scheme

| sample | time A in min | time B in min | time C in min |
|--------|---------------|---------------|---------------|
| blank  | 0:00          | 4:45          | 9:28          |
| C1     | 0:45          | 5:25          | 10:09         |
| C2     | 1:26          | 6:05          | 10:50         |
| C3     | 2:07          | 6:46          | 11:30         |
| C4     | 2:47          | 7:28          | 12:11         |
| C5     | 3:27          | 8:08          | 12:51         |

15. Take glass vials blank A, C1A, C2A, C3A, C4A, C5A out of the dark and transfer each sample to a quartz cuvette by using glass Pasteur pipettes. Put these 6 cuvettes to the automatic linear cell changer of the UV/Vis detector and measure absorption of samples at 500-700 nm. Make sure that this step is performed accurately timed ensuring that all samples are measured exactly after 60 minutes of reaction time.

16. Prepare and measure samples of series B and C accordingly.

2.3.3. Calibration to Trolox-equivalent units (TEU):

Trolox (C₁₄H₁₈O₄ or 6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid, $M_{\text{Trolox}} = 250.29 \text{ g mol}^{-1}$) is a water-soluble analog of vitamin E. It is an antioxidant like vitamin E and it is routinely used in biological or biochemical applications to reduce oxidative stress or damage – and to measure antioxidant capacity of foods, beverages and supplements.[7,9] We introduce the quantities:

$\text{Abs} = \text{Absorption [-]}$

$l = \text{light path of cuvette [cm]}$

$kE = \text{extinction coefficient of TPTZ induced by 1 Mol antioxidant [TEU]}$

$d = \text{dilution factor 0.048 (0.1 ml HBS in 2 ml FRAS reagent)}$

Performing the FRAS protocol step (3 ANALYSIS), replacing the HBS serum by a concentration series of Trolox from 0.001 to 0.1 g/L, we obtain a FRAS absorption signal that can be linearly fitted with $\text{Abs}_{\text{Trolox}} = kE \times l \times d \times c_{\text{Trolox}} \text{ [mM]} + b \ (R^2 = 0.9998)$. The offset absorption $b = 0.075$ at zero antioxidants determines the maximum detectable oxidative damage. The linear fit factor $kE \times l \times d = 2.654 \text{ / (mM Trolox)}$ calibrates the absorption signal per „Trolox-Equivalent Unit“ (TEU) antioxidant molarity (mM TEU) for our specific implementation of the FRAS assay. Removing the dilution factor and the optical path length, the extinction coefficient of TPTZ per Trolox calibration is $kE = 55.76 \text{ mM TEU/cm}$ for our FRAS reaction time of 60 minutes.

The earlier study by Hsieh et al [6] found a value of 4.18 / mM/L TEU at unspecified factors l and d. Assuming that the protocol in [6], [7] and [8] is identical, the description in [8] “1-10 mg typical quantities; 1.0 mL serum at 37 °C for 90 min, separation of the ENM, transfer of 100 μL of the supernatant to 1.0 mL of working FRAS reagent.” indicates a dilution factor of 0.091. Assuming further identical values of cuvette depth $l = 1$ cm and 60 min FRAS reaction time, considering our slightly increased damage by longer incubation but halved signal by dilution, their value of 4.18 / mM TEU is very consistent with our value of 2.65 / mM TEU.

2.3.4. Data analysis:

1. Calculate Biological Oxidative Damage (BOD) for A1-A5, B1-B5, C1-C5, according to the following equation, shown as an example for sample A. in mM TEU: (x= 1 to 5)

$$BOD \equiv \frac{\text{Abs}(\text{blank A}) - \text{Abs (Ax)}}{1 + kE \times d} \text{ [in mM TEU]}$$

The denominator is determined by calibration with Trolox, see section 2.3.3.

2. Convert the nanomaterial doses to surface metrics

$\text{Dose_x [m²/L]} = c_x [g/L] \times \text{BET [m²/g]}$

$\text{BET} = \text{BET surface of nanomaterial [m²/g]}$
c_x = concentration of nanomaterial [mg/mL]  [note: mg/mL = g/L, no conversion required]

3. Convert to the maximum oxidative damage in the two relevant metrics (both max at same x)
   a. Mass-based max damage \( m\text{BOD} = \max \{\text{BOD} (c_x) / c_x \} \) [nmol TEU /mg]
   b. Surface-based max damage \( s\text{BOD} = \max \{\text{BOD} (dose_x) / dose_x \} \) [nmol TEU /m²]

4. Plot each series A, B, C independently. Use log (Dose) on x-axis and log (BOD) on y-axis.

5. Fit each series A, B, C independently with a linear regression curve, giving slope and offset for
   series A, B, C.  [note: for reactive materials, the linear fit is not appropriate and should be
   replaced by sigmoidal fit, but most ENM remain at very low damage, hence benchmark dose
   approaches or related sigmoidal metrics are not easily applicable]

6. Calculate from these three values the average and standard deviation for:
   a. Slope [mM TEU / m²]
   b. Offset [mM TEU]

3. Results

3.1. Repeatability of blank measurements (= in the absence of ENM)

By performing the complete protocol as described in section 2.3, but without adding ENM in step 2.3.2-
2, we assess the repeatability of the protocol and eliminate sources of variability. As demonstrated by
Figure 3, the scatter is reduced by nearly an order of magnitude (from 8 to 1 µM TEU), and the average
remaining signal from blanks is reduced from 6 to 2 µM TEU.

In contrast, the HBS used here has a total antioxidant capacity of 366 µM TEU, as derived from the
Trolox calibration and the absorption signal induced by the blank measurements (0.97). For comparison,
a value of 530 mM TEU is given by Hsieh et al. for their HBS.[7] We believe the unit mM to be a typo
(correct: µM), as all other values and units are consistent with ours. The difference thus represents the
natural spread of HBS sources. As the FRAS assay detects only relative changes, this difference is not
critical to the comparison of oxidative damage results.
3.2. Positive control: Mn2O3

Based on earlier report of both FRAS assay [7] and cytochrome c assay [10] and band gap analysis [11], Mn2O3 is recurring as the most reactive, yet insoluble material. Hence, we chose to establish Mn2O3 as positive control. We use the same batch as in [10], sourced from Skyspring Nanomaterials, with primary particle diameters ranging from 20 to 100 nm (TEM) and a specific surface area of 19.9 m²/g (BET).[10] As expected, Mn2O3 induces maximum antioxidant damage already at low dose (Figure 4, red line), and levels off into a plateau at higher doses. The maximum sBOD of 1610 ± 4 nmol TEU/m² (equivalent to mBOD of 32.0 ± 0.09 nmol TEU/mg) is reached at a surface dose of 244.5 m²/L (corresponding to mass concentration of 4.9 g/L), generating a BOD of 156,680 ± 435 mM TEU.

The same substance in a different grade (BET = 7.4 m²/g) was studied earlier. The simple BOD, dose and standard deviation were not reported, but the mBOD was reported as 104 ± 5 nmol TEU/mg and sBOD as 20,555 nmol TEU/m².[7,8] The mBOD value is a factor 5 higher than ours (the sBOD value a factor 13, but marked by Hsieh et al. as potentially influenced by uncertainty of the BET value). As we observe a plateau at high dose, interpreted as maximal damage of all antioxidants that are susceptible to the specific reaction pathway induced by Mn2O3, dose is probably not the origin of the quantitative discrepancy.

Despite the quantitative disagreement between the two implementations of FRAS, both agree that Mn2O3 is among the most reactive materials, of which it is the only one with vanishingly low solubility. Both also agree on excellent reproducibility of Mn2O3 reactivity (<1% standard deviation in our triplicates). Mn2O3 is thus chosen as our positive control, as established also for the cytochrome-c [10] and bandgap assays.[11]

3.2.1. Limit of Detection. At low reactivity, Hsieh et al. note their limit of statistical relevance for mBOD at 1.7 nmol TEU/mg, probably determined at their concentration of 10 g/L (corresponding to 17 µM TEU). In comparison, our blank signal of 2 ± 1 µM TEU allows an estimate for the limit of detection at blank + three standard deviations, resulting in 5 µM TEU. Together with the extended concentration range up to 40 g/L, our limit of statistical relevance for mBOD is around 0.12 nmol TEU/mg. This is more than an order of magnitude improvement already for single-dose triplicates. Additionally, the dose-dependence provides highly relevant information.

At high reactivity, the limit is ultimately the complete consumption of the HBS antioxidant capacity of 366 µM TEU, which can be converted into an upper detection limit of mBOD, assuming a material that damages all antioxidants at the lowest routine dose of 0.75 g/L. This results in a maximum mBOD of 488 nmol TEU/mg detectable by screening ENMs with the routine dose range, which is well above the value of the positive control Mn2O3. Thus, the reactivity range from below 1% to above 100% of the control material is detectable.

This consideration confirms again that the FRAS assay is more sensitive than DCFH, especially for ENMs with low to moderate oxidative damage potential, and may serve as a more biologically relevant substitute for acellular ROS measurements of ENMs.[6,8]

3.3. First application to compare nanoforms of the same substance

3.3.1. Comparing CeO2, OECD NM211 vs. NM212, we find that both have a significant reactivity with a clear dose response (Figure 4, blue lines). Both remain far below the positive control, and would not have been detected without the improved protocol. Within the statistically relevant range of BOD > 5 µM TEU, the curves a very close, so that for each individual dose, the sBOD values are very similar, as expected for surface-induced reactions. (In contrast, the NM211 has higher mBOD values than NM212 due to the higher BET surface of NM211). The slope of the dose response is the same or steeper for NM211, consistent with the increased share of unsaturated Ce³⁺ by the smaller particles. All this is very plausible, and any further interpretation is beyond the scope of the present protocol-centered contribution.
We note that the same CeO$_2$ NM212 shows only borderline reactivity in a macrophage assay,[13] and that it has not been possible to determine significant reactivity by the cytochrome-c assay, ascorbic acid depletion, glutathione depletion, whereas Mn$_2$O$_3$ was significantly reactive on all of these.[10] This evidences the sensitivity of the advanced FRAS assay to weakly reactive ENM.

3.3.2. Comparing ZnO, OECD NM110 vs. NM111, we find significant oxidative damage. This was expected from several other assays.[2,7,8,10,11,13] For the surface-coated NM111 we find significant suppression of reactivity as compared to the naked NM110. Most notably, the oxidative damage at lowest doses is above Mn$_2$O$_3$, but remains below Mn$_2$O$_3$ at higher doses. A crossing of dose-response curves points to different reaction pathways – in this case attributed to the contribution of Zn$^{2+}$ ions, which will remain relevant at doses down to the steady-state solubility far below our lowest dose of 0.75 g/L. We note that ZnO NM110 is used as benchmark “dissolving ENM” in the ECETOC grouping scheme.[3] All this is very plausible, and any further interpretation is beyond the scope of the present protocol-centered contribution.

Any dose can be formally converted to a mBOD, with values for the naked ZnO NM110 ranging from 3 to 44 nmol TEU /mg (sBOD ranging from 239 to 3700 nmol TEU /m²). This is fully consistent with the single-dose value of mBOD of 12 ± 3 nmol TEU /mg (sBOD 340 ± 40 nmol TEU /m²) determined by Hsieh et al.[7]

4. Conclusion and Outlook

We developed the FRAS assay further to become a protocol to determine dose-dependent oxidative damage by nanomaterials dispersed in physiological medium, with statistical relevance also for intermediate and weakly reactive materials. Already for single-dose determination of oxidative damage, our advanced protocol achieves a limit of statistical relevance for mBOD around 0.1 nmol TEU/mg. This constitutes an improvement by more than an order of magnitude. Further, we propose to establish Mn$_2$O$_3$ as positive control material, in accord with other recent assays of oxidative stress. Thus, the range from below 1% to above 1000% of the control material is measurable with our routine dose range. Thus, single-dose screenings can group materials by properties of concern (here: surface reactivity), which we currently define as “sBOD more than 10% of Mn$_2$O$_3$ reactivity”. [3] Additionally, the dose-response relationship may point to differences of oxidative stress mechanisms, such as the contributions by dissolved ions, and serve to substantiate read-across.

To benchmark FRAS against other measures of surface reactivity and against toxicological studies, the same silica, titania, zincoxide families as before[2] and families of organic DPP pigments, metal-organic CuPhthalocyanins and iron oxide will be investigated by the present multi-dose protocol of the FRAS assay. For most of these materials, in vivo inhalation and macrophage assay results are available for benchmarking the proposed groupings/screenings.[3,12,13]

Based on the outcome of this study we currently develop a comprehensive testing strategy for assessing the oxidative stress potential of ENMs, which combines acellular methods (ESR, FRAS) and fast in vitro screening approaches, as well as a more involved detailed redox proteomics approach. This allows for screening and prioritization in a tier of “system-depending properties” and, if required, also for unraveling mechanistic details down to compromised signaling pathways. This approach appears highly useful within an ENM grouping strategy.

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