Optimising the use of commercial LAL assays for the analysis of endotoxin contamination in metal colloids and metal oxide nanoparticles

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Supporting Figure 1. Interference of Fe₃O₄ NPs with the different LAL assays’ readouts

The interference of Fe₃O₄ NPs was assessed with the readout of pNA at 405 nm (panel A), with the readout of azo dye (generated by adding diazo reagents to the released pNA) at 540 nm (panel B), and with fluorescence at excitation/emission 380/440 nm (panel C). In panels A and B, readings were performed in the absence of NPs (black circles) and in the presence of increasing NP concentrations: 0.5 (red squares), 1 (green triangles), 5 (violet diamonds) and 10 nM (blue hexagons). Data are the mean ± SD of triplicate determinations within one test representative of three performed. In panel C, the dotted line represents the positive fluorescence control (generated by 1 EU/ml endotoxin in the fluorogenic assay), shaded areas showing the SD above and below the mean. * p <0.05 vs. control; ** p <0.01 vs. control.
Supporting Figure 2.

Supporting Figure 2. Optical density and auto-fluorescence of NPs

The optical density at 405 nm (panels A, D, and G) and at 540 nm (panels B, E, and H), and the autofluorescence at excitation/emission 380/440 nm (panels C, F, and I) were assessed for Au NPs (panels A, B, and C), Ag-SC NPs (panels D, E, and F) and Fe₃O₄ NPs (panels G, H, and I) and their solvents. Red symbols, NPs; black symbols, solvents. Data are the mean ± SD of triplicate determinations within one test representative of three performed. In panels C, F, and I the dotted lines represents the positive fluorescence control (generated by 0.5 EU/ml endotoxin in the fluorogenic assay), shaded areas showing the SD above and below the mean.

* \( p < 0.05 \) vs. control; ** \( p < 0.01 \) vs. control.
Supporting Figure 3. Endotoxin detection and recovery in Fe$_3$O$_4$ NPs with different LAL assays

Endotoxin in Fe$_3$O$_4$ NPs was measured with the traditional chromogenic assay (black circles), the modified chromogenic assay (blue squares), and the fluorogenic assay (red triangles), in the absence (panel A) or in the presence of 0.5 EU/ml of exogenously added endotoxin (panel B). Endotoxin recovery rates (panel C) are calculated based on results of panels A and B. Data are the mean ± SD of duplicate determinations within one test representative of 2 performed. The horizontal dotted lines represent the endotoxin value measured in the absence of NPs (0.515 EU/ml, mean of 10 data, panel B; 100%, panel C), shaded areas showing the SD above and below the mean. * $p < 0.05$ vs. control; ** $p < 0.01$ vs. control.
Supporting Table 1. Determination of free ions in NP solution by ICP-MS

| Nanoparticles | Mass at synthesis (mg/l) | Free ions in solution | % |
|---------------|--------------------------|-----------------------|---|
| Au NPs        | 32.8                     | 1.7                   | 3 |
| Ag-SC NPs     | 27.0                     | <0.3                  | <1|
| Fe₃O₄ NPs     | 83.8                     | 7.1                   | 9 |
## Supporting Table 2. SWOT analysis of different LAL assays for use with NPs

| ASSAY SWOT | Gel-clot | Turbidimetric | Chromogenic | Fluorogenic |
|------------|----------|---------------|-------------|------------|
| **Strengths** | Easy, no special equipment needed, cheap | Quantitative, highest sensitivity, cheaper vs. chromogenic | Quantitative, high sensitivity (highest for kinetic test), kinetic test is automatic, one step, easy, end-point test can use two different wavelengths | Very specific, high sensitivity |
| **Weaknesses** | Semi-quantitative, low sensitivity, low specificity, variability, subjectivity | Low specificity, it may be interfered with by NPs at high optical density, end-point test is not commercially available, kinetic test needs special equipment and longer time | High cost, low specificity, likely to be interfered with by NPs with absorbance at or close to 405 or 540 nm, end-point test is less sensitive than kinetic assay, kinetic test needs special equipment and longer time | High cost, requires special equipment |
| **Opportunities** | In the case of doubt or dispute with other tests, the final decision is usually made based on the gel-clot technique, although not clear if this may also apply to NPs | Can be applied to NPs that interfere with the chromogenic assay, by using them at low optical density. High sensitivity allows to increase NP maximal dilution, which will also decrease possible interference with the assay | Can be used with NPs having high optical density, after appropriate controls. High sensitivity allows to increase NP maximal dilution, which will also decrease possible interference with the assay | May be used for NPs that do not have auto-fluorescence |
| **Threats** | Not precise, prone to mistakes due to subjective performance, misleading results may be obtained with NPs | May be interfered with by most NPs, in particular at high concentrations, due to their turbidity | Specificity and NP interference must be controlled | NPs may significantly interfere with enzyme reaction, or quench fluorescence |

**CAUTION:** NPs may adsorb and modify enzyme and substrate, thereby interfering with the assay development.

NPs can also adsorb endotoxin and alter its detection in the assay.