Electronic Supplementary Material (ESI) for RSC Advances.
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Supplementary information

Synthesis of γ-Fe_2O_3 NPs:

1. Weigh in 8.89 g $FeCl_3$ * 6 H_2O (Sigma-Aldrich n. cat: 31232) and 3.28 g $FeCl_2$ * 4 H_2O (Sigma-Aldrich n. cat: 44939) and dissolve it in 380 mL H_2O (in a 500 mL Erlenmeyer flask) on a magnetic stirrer for 30 min. While stirring add slowly 1.5 mL of 37% HCl to dissolve iron salts completely.

2. Add slowly and under strong stirring 25 mL of 25% NH_4OH to iron solution: a black precipitate will appear. Stir for additional 10 min.

3. Remove flask from magnetic stirrer and let particles precipitate by placing a permanent magnet (NdFeB-magnet) under the flask and wait until all the NPs are collected to the bottom (that happens after 10-20 min.). Be careful working with NdFeB-magnet since their magnetic field is very strong.

4. Remove the supernatant (aqueous media) by decantation and wash twice the NPs with 100 mL of H2O MilliQ, leaving them 10 min on magnetic stirrer for each washing. During the last washing steps, transfer the suspension into a smaller Erlenmeyer flask (100 mL).

5. After the transfer, add 40 mL of 2 M HNO_3 and heat up to 90 °C for a 5 min. The suspension will turn color from black to dark brown.

6. Isolate particles again with the magnet and add 60 mL of 0.34 M solution of $Fe(NO_3)_3$ * 9 H_2O (Sigma-Aldrich n. cat: 216828). Heat up to 90 °C and stir for 30 min.

7. Cool down to RT and remove supernatant (NdFeB-magnet).

8. Add 50 mL of H_2O (can be more) on the particles and stir properly. The particles will now “dissolve” leading to a magnetic fluid containing a well dispersion of iron oxide nanoparticles.

9. Filter NPs through 0.2 µm syringe filters for removal of larger aggregates. If dispersion cannot be filtered well, add more water to dilute the ferrofluid and use new filter.

10. Pipette into a glass bottle and store at 4 °C. If needed the IONPs solution can be dialyzed to remove any Iron salts still present after the last steps.

TEM analysis of γ-Fe_2O_3 NPs, NP-APTES and NP-DAAO

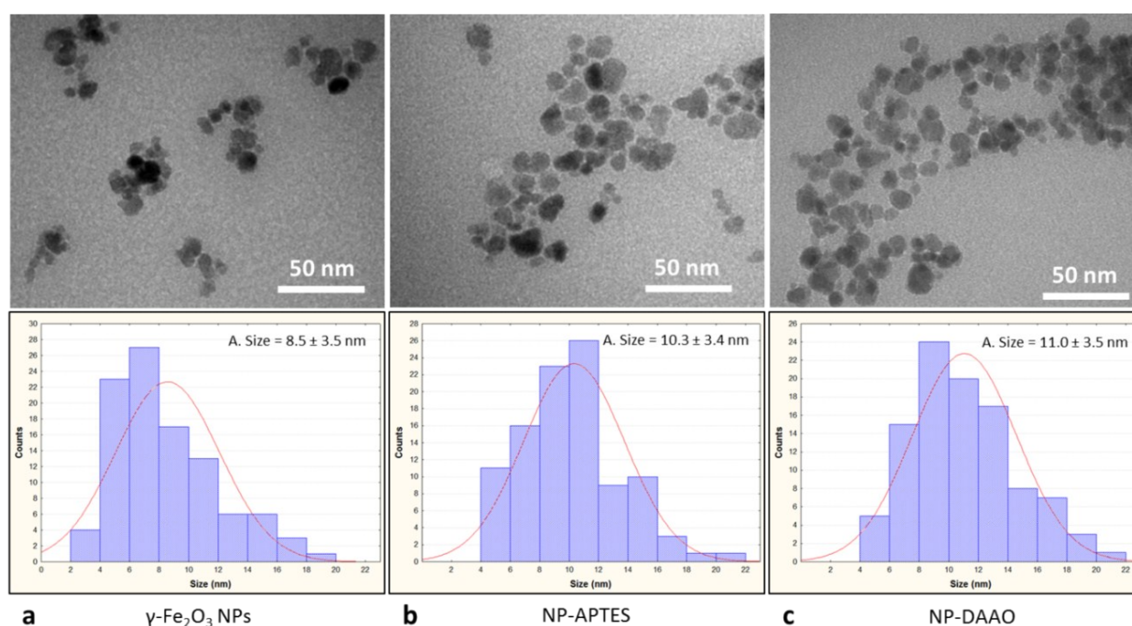

Fig. 1: TEM analysis of γ-Fe_2O_3 NPs (a), NP-APTES (b) and NP-DAAO (c). Each distribution analysis was performed counting 100 NPs.

DLS and DCS details

NP size measurements by DLS were carried out in water. Measurements were performed at 25°C using a Malvern Zetasizer Nano ZS90. DLS results are reported as the average of at least 3 runs, each containing 13 individual measurements.

DCS experiments were performed with a CPS Disc Centrifuge DC24000. The analyzer measures particle size distribution using centrifugal sedimentation within an optically clear spinning disk that is filled with fluid. During the measurements, 8−24% sucrose gradient was used in water at 19000 rpm, and the NP concentration was 75 μg/mL.

NP-DAAO yield and activity recovery

The immobilization yield was calculated as the percentage of the ratio between the *immobilized enzymatic activity* of DAAO on NP-DAAO (IA) and the *starting enzymatic activity* of DAAO added to the conjugation reaction (SA):

$$Yield\ (\%) = \frac{IA}{SA} \times 100$$

The IA refers to the difference between the SA and the DAAO enzymatic activity retained by the supernatant at the end of conjugation reaction.
Finally, to describe how much enzymatic activity is retained by the immobilized DAAO, it was calculated the Activity Recovery as the percentage of the ratio between the *observed enzymatic activity* of the DAAO loaded on NP-DAAO (OA) and the SA:

$$Activity\ recovery\ (\%) = \frac{OA}{SA} \times 100$$

This parameter estimates the total immobilization process.

Cell culture details

RPMI1640 medium was employed to maintain SKOV-3 and HCT116 cell lines as adherent cells while DMEM medium was used for U87 cell line. Every cell line was kept at 37°C in a humidified 5% CO_2 atmosphere. Both RPMI1640 and DMEM medium were supplemented with 10% fetal bovine serum, 1% l-glutamine and 1% penicillin/streptomycin solution, DMEM medium was supplemented with also 1% sodium pyruvate. Cells were passaged as needed using a solution at 0.25% trypsin–EDTA.

Cell viability

Cell viability was determined as ATP content by using the CellTiter-Glo Luminescent Cell Viability Assay (Promega) according to the manufacturer’s instruction. In detail, 200 μl of cell suspension containing 10000 cells were seeded into 96-well assay plates and cultivated for 24 h at 37 °C in 5% CO_2 to equilibrate and become attached prior the treatment. Cells were then exposed to increasing amounts of free DAAO, bared γ-Fe_2O_3 NPs, NP-APTES NPs and NP-DAAO for 24 h. Following the treatment, plates were equilibrated for 30 min at room temperature and 100 μl of CellTiter-Glo Reagent was then added to each well. Plates were shaken for 2 min and left at room temperature for 10 min prior to the recording of luminescent signals using the Infinite F200 plate reader (Tecan Group, Männedorf, Switzerland). For all cell lines, experiments were performed in triplicate. Cell viability, expressed as ATP content, was normalized against control values.

DAAO and NP-DAAO activity

The activity of NP-DAAO was determined by measuring the absorbance increase accompanying the H_2O_2-induced oxidation of o-dianisidine. One DAAO unit corresponds to the amount of enzyme that converts 1 µmol of substrate per min at 25°C and at 0.253 mM oxygen concentration. The standard assay mixture contained 890 µL of 100 mM D-Ala in 100 mM sodium pyrophosphate buffer, pH 8.5, 100 µL 3.2 mg/mL o-dianisidine in water, 10 µl of 0.4 mg/mL horseradish peroxidase in 100 mM sodium pyrophosphate buffer, pH 8.5, and 10 µL of 40 µg/mL NP-DAAO (stock sol. was 4 mg/mL) in the same buffer. The reaction was initiated by the addition of the enzyme and the absorbance increase was monitored at 440 nm (~13 $mM^{-1}cm^{-1}$) for 1 min using an UV-Vis V-560 Spectrophotometer (Jasco, Cremello, Italy).

Cytotoxicity studies

Three different DAAO and NP-DAAO concentrations were evaluated: 3.5, 7 and 14 mU.
The 3.5 mU of NP-DAAO correspond to 146 ng of bared NP and NP-APTES, so 7 mU correspond to 292 ng and 14 mU correspond to 583 ng.

NP-DAAO system incubation at different temperature with human serum

We investigated the NP-DAAO activity at different time and incubation conditions with or without addition of human serum (hS) 10%. The results reported in Fig. 2 show that DAAO and NP-DAAO are sensible to temperature: increasing the incubation temperature, all the samples slowly lose their activity suggesting a decrease of DAAO integrity. However the presence of hS at 10% seems to counteract the activity loss of both the samples, this suggesting that hS plays a key role in preventing the DAAO unfolding process.

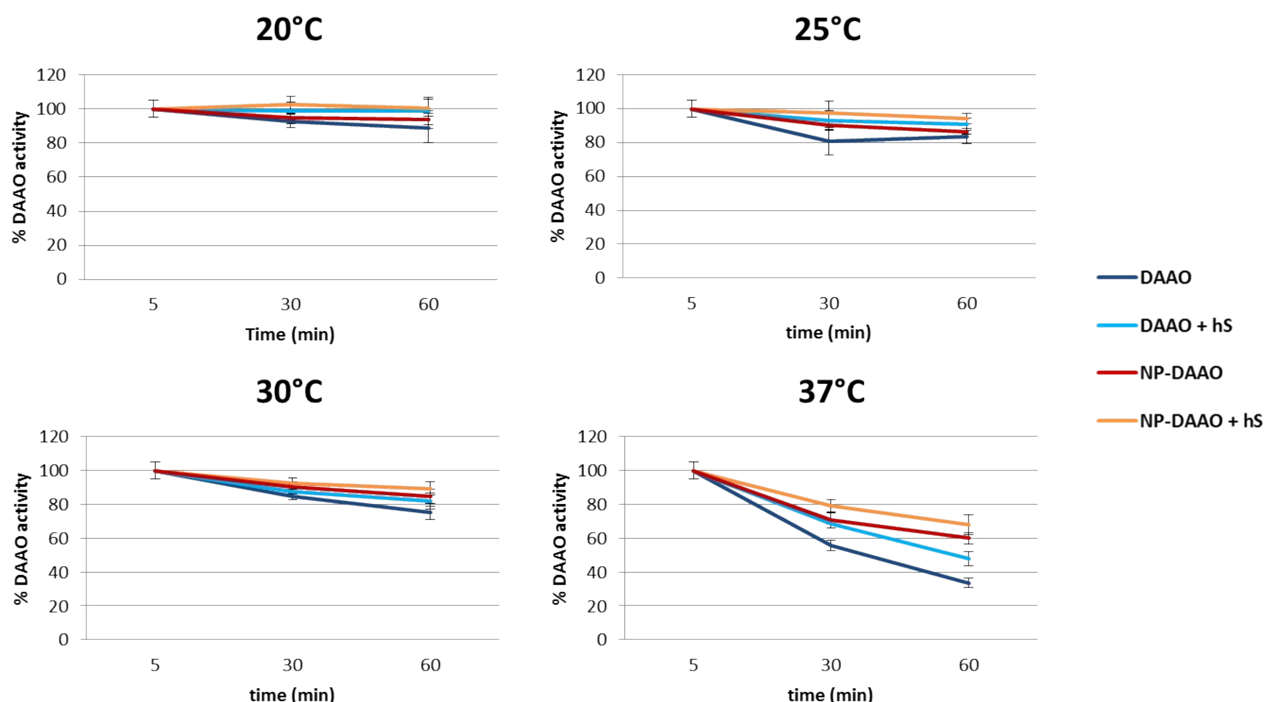

Fig. 2: DAAO and NP-DAAO activity with or without human serum (hS) when incubated at different temperatures.

During the hS incubation was prepared a solution containing 890 µL of 100 mM sodium pyrophosphate buffer pH 8.5, 100 µL of hS and 10 µL of NP-DAAO (stock sol. 4 mg/mL).

Comparison among the new NP-DAAO system and the previous one

The **DLS** and **DCS** analysis of our new NP-DAAO system compared to the one reproduced following the procedure of *Cappellini et al.*[1] are reported in Tab. 1.

Tab. 1: Comparison between the DCS and DLS analysis of the new NP-DAAO (light green) and the previous NP-DAAO (light red) systems).

Sample	IONPs		NP-APTES		NP-DAAO	
Method	*New*	*Previous*	*New*	*Previous*	*New*	*Previous*
Peak (nm)^{DCS}	34.5 ± 0.2	176 ± 0.5	24 ± 0.2	83.5 ± 0.4	76 ± 2	351 ± 3.5
Z-Average (nm) ^{DLS}	38.3 ± 0.4	1585 ± 81.1	47.0 ± 0.2	142.9 ± 2.4	185.1 ± 6.1	2043 ± 192.6
Polydispersity Index (PDI) ^{DLS}	0.191	0.353	0.123	0.197	0.279	0.690

The **DAAO conjugation efficiency** of the two different NP-DAAO systems is compared in Tab. 2. The ratio among DAAO conjugated onto the new NP-APTES and previous NP-APTES is about 7.1. This represents a clear increase in the amount of DAAO conjugated.

Tab. 2: Comparison between the new and the previous NP-DAAO system.

NP-DAAO System	μg DAAO / mg NPs	% Yield	% Activity recovery	U/mg NPs
Previous	62.5	100	90 ± 2	3.5 ± 0.1
New	446	100	91 ± 2	24 ± 1

The last aspect that was compared between these two systems was their **intrinsic cytotoxicity** (Fig. 3). The cytotoxicity exerted on the SKOV-3 cell line by the new NP-DAAO is extremely low if compared to previous NP-DAAO. However, when D-Ala is added both the systems show similar results though the new NP-DAAO at 3.5 mU is less toxic than previous NP-DAAO. That is due to the higher intrinsic toxicity of the previous NP-DAAO that can be added to the oxidative stress caused by the H_2O_2 production.
These results obtained on SKOV-3 cell line are almost identical to U87 and HCT-116 cell lines (data not shown).

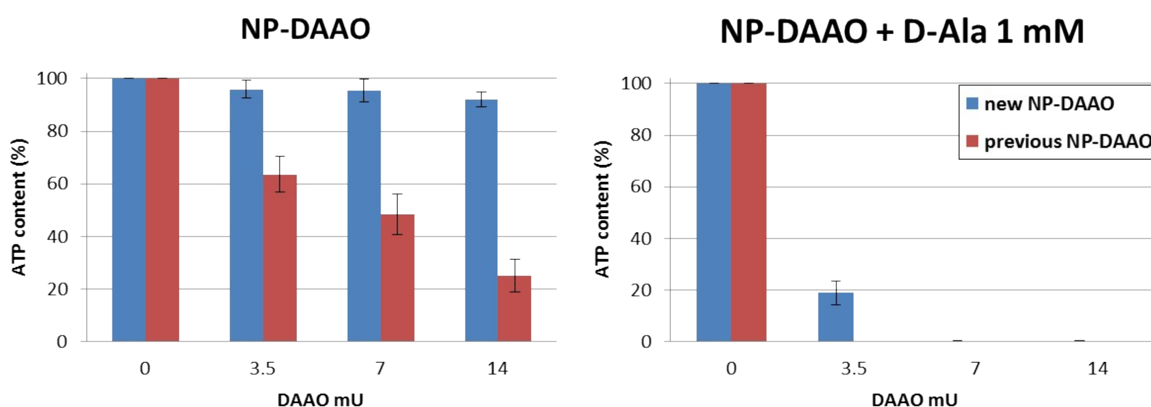

Fig. 3: Cytotoxicity of both the new NP-DAAO and the previous NP-DAAO systems on SKOV-3 cell line. Cytotoxicity was performed in presence / absence of the substrate D-alanine (D-Ala) 1 mM.

References

1 F. Cappellini, C. Recordati, M. De Maglie, L. Pollegioni, F. Rossi, M. Daturi, R. Gornati and G. Bernardini, Future Sci. OA, 2015, 1, 4