## SUPPLEMENTARY INFORMATION

for:

## Optimization of magnetic nanoparticles for engineering erythrocytes as theranostic agents

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| Sample  | T of iron salts solution | T of<br>NH <sub>4</sub> OH<br>solution | DLS size<br>(nm) | PDI   |  |
|---------|--------------------------|----------------------------------------|------------------|-------|--|
| MNPs 1  | 25°C                     | 25°C                                   | 68.01            | 0.246 |  |
| MNPs 2  | 25°C                     | 30°C                                   | 35.89            | 0.569 |  |
| MNPs 3  | 25°C                     | 40°C                                   | 37.99            | 0.214 |  |
| MNPs 4  | 25°C                     | 60°C                                   | 50.99            | 0.421 |  |
| MNPs 5  | 30°C                     | 30°C                                   | 24.07            | 0.283 |  |
| MNPs 6  | 40°C                     | 40°C                                   | 45.22            | 0.227 |  |
| MNPs 7  | 60°C                     | 60°C                                   | 37.43            | 0.286 |  |
| MNPs 8  | 30°C                     | 25°C                                   | 43.60            | 0.275 |  |
| MNPs 9  | 40°C                     | 25°C                                   | 32.48            | 0.220 |  |
| MNPs 10 | 60°C                     | 25°C                                   | 38.46            | 0.282 |  |

*Table S1*. List of the magnetic nanoparticles (MNPs) obtained by variation of solutions temperature.

*Table S2.* List of iron oxide nanoparticles obtained by stabilizing the co-precipitated particles with different concentrations of sodium citrate solution.

| Sample  | Citrate<br>solution<br>concentration | DLS<br>size<br>(nm) | PDI   |  |
|---------|--------------------------------------|---------------------|-------|--|
| MNPs 11 | 0.5 M                                | 29                  | 0.327 |  |
| MNPs 12 | 1 M                                  | 35                  | 0.340 |  |
| MNPs 13 | 2M                                   | 45                  | 0.272 |  |

**Table S3**. List of MNPs obtained varying the nucleation step duration, modifying the ammonium hydroxide rate addition, ranging from 30 seconds to 10 minutes. The standard procedure considers 2 minutes as optimal step duration, confirmed by the TEM and DLS analysis.

| Sample  | Nucleation time | DLS size<br>(nm) | PDI   |
|---------|-----------------|------------------|-------|
| MNPs 23 | 30 sec          |                  |       |
| MNPs 24 | 1 min           |                  |       |
| MNPs 25 | 2 min           | 51.14            | 0.197 |
| MNPs 26 | 4 min           | 169.4            | 0.242 |
| MNPs 27 | 10 min          |                  |       |



*Figure S1. TEM images of MNPs 25 (a) and MNPs 26 (b) obtained respectively with 2 and 4 minutes of nucleation step time. All scale bars correspond to 100 nm.* 



**Figure S2**. TEM analysis of Zn-Mn Ferrite nanoparticles obtained by varying the precursors ratio in the synthesis, as reported in Table 3. Zn-Ferrite (MNPs 33) and Mn-Ferrite (MNPs 38) nanoparticles are reported respectively in panel a) and b); MNPs 35 and 36 are reported respectively in panel c) and d). All scale bars correspond to 50 nm.



*Figure S3. DLS analysis of dextran-coated nanoparticles, MNPs 29 and MNPs 40, after longstorage (1 year) at room temperature.* 



**Figure S4.** TEM images of slices of human RBCs loaded with **a**) MNPs 20<sub>DX-filt.0.22</sub> and **b**) MNPs 20<sub>DX-filt.0.1</sub>.

## Ferucarbotran<sup>®</sup> contrast agent (Gold Standard)

Ferucarbotran<sup>®</sup>, consisting of iron oxides (28 mg Fe/ml) carboxydextran coated with a size of 57 nm, was obtained from Prof. Ikiro Kato (Meito Sangyo Co., Ltd. Nagoya Research Lab., Japan). This contrast agent was previously evaluated for the encapsulation into human and murine red blood cells (RBCs) following the same procedure of hypotonic dialysis, isotonic resealing and reannealing applied for the new MNPs suspensions as reported in ref.<sup>1</sup>

The Fe concentrations incorporated into human and murine RBCs obtained at the end of loading procedure were estimated by NMR relaxation measurements using a dose-response curve (**Figure S5**) generated by adding known amounts of Ferucarbotran<sup>®</sup> contrast agents to human or murine RBC samples.

The values of  $(1/T1_c-1/T1_0)$  or  $(1/T2_c-1/T2_0)$  were plotted versus the concentration of Ferucarbotran<sup>®</sup> and were fitted by least squares method to a straight line, the slope of which is the longitudinal (*r1*) or transversal (*r2*) relaxivity.



*Figure S5. NMR* dose-response curves generated by adding known iron concentrations (ranging from 0 to 12 mM Fe) of Ferucarbotran suspension to human RBCs at 44% Ht (**a** and **b**) and murine RBCs (**c** and **d**). The longitudinal r1 and transverse r2 relaxivities were reported for human (r1=1.3003; r2=87.228) and murine (r1=1.2128; r2=84.976) RBCs.

**Table S4**. Data obtained after the loading of MNPs  $29_{DX-filt 0.1}$  and MNPs  $40_{DX-filt 0.1}$  nanoparticles with **human** RBCs by using 11.2 mg Fe/ml RBC70%. Values are expressed as means  $\pm$  SD of three similar experiments.

| Sample           | T1<br>(ms)         | T2<br>(ms)       | Fe<br>[mM]    | MCV<br>(fl)      | MCH<br>(pg)    | MCHC<br>(g/dl)   | Cell<br>recovery<br>(%) |
|------------------|--------------------|------------------|---------------|------------------|----------------|------------------|-------------------------|
| UL-RBCs          | $2069 \pm \! 15.6$ | $62.1\pm\!\!1.3$ | /             | $77.5\pm\!\!0.7$ | $25.2 \pm 0.6$ | $34.3 \pm 1.3$   | $72 \pm 1.2$            |
| L3-MNPs 29       | $481.5\pm\!123$    | <5               | $1.5 \pm 0.5$ | $72.2 \pm 3.7$   | $21.8 \pm 3.2$ | 29.4 ±2.1        | $54.9 \pm 7.3$          |
| L3-MNPs 40       | $196.5\pm77.2$     | <5               | 2 ±0.9        | $73.5\pm\!\!1.7$ | $21.4 \pm 3.3$ | $28.6\pm\!\!4.2$ | $46.5\pm\!\!0.7$        |
| L3-Ferucarbotran | 110±25             | <5               | 6.81±1.43     | 70.8±2.3         | 19.9±1.4       | 29.1±3.1         | 66.5±3.6                |



Figure S6. TEM images of slices of non-dialyzed human RBCs.



*Figure S7. TEM images of human Ferucarbotran*<sup>®</sup>*-loaded RBCs obtained by using 11.2 mg/ml RBCs 70% Ht during the dialysis step of loading procedure. (a, b) whole-Ferucarbotran*<sup>®</sup>*-loaded RBCs; (c, d) slices of Ferucarbotran*<sup>®</sup>*-loaded -RBCs.* 

## References

 Antonelli, A.; Szwargulski, P.; Scarpa, E. S.; Thieben, F.; Cordula, G.; Ambrosi, G.; Guidi, L.; Ludewig, P.; Knopp, T.; Magnani, M. Development of Long Circulating Magnetic Particle Imaging Tracers: Use of Novel Magnetic Nanoparticles and Entrapment into Human Erythrocytes. *Nanomedicine* 2020, 15, 739-753.