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Tailoring superparamagnetic nanoparticles for entrapment into red blood cells

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Summary. — The aim of the work here described is the synthesis of magnetic nanoparticles (MNPs) for the inclusion into human Red Blood Cells (RBCs) and as potential contrast agent for magnetic resonance imaging (MRI). Synthesis, characterization and preliminary *in vitro* tests are the main topics. Superparamagnetic iron oxide nanoparticles and manganese/zinc ferrite nanoparticles have been synthesized, by using wet-chemistry techniques such as co-precipitation and thermal decomposition methods. For biomedical application, a suitable surface coating must be grafted to MNPs surface, to preserve the colloidal stability even in physiological condition. Therefore, a biomimetic coating based on non-modified dextran was grafted to MNPs for ensuring optimal stability and low toxicity for the inclusion in living RBCs.

1. – Introduction

During the last decades, MNPs have aroused an exponentially grown of interest for applications in several fields of nanomedicine and bio-imaging, especially in cancer treatment. At the nanoscale, their unique magnetic properties make them useful as efficient contrast agent for magnetic resonance imaging (MRI), as carrier for drug delivery, as actuator for therapeutic hyperthermia protocols [1, 2]. Anyway, the obtained nanoparticles often show a low stability in simulated biofluids; therefore, a proper surface coating with a biocompatible agent is very important for enhancing the stability of the nanoparticles [3, 4]. Nanocomposites with narrow distribution, high magnetization and suitable for a stable biofunctionalization are expected as a result of this research activity.

2. – Experimental work

2˙1. *Co-precipitation method.* – Iron oxide NPs and zinc/manganese ferrite NPs were synthesized by co-precipitation method modifying the standard according to Massart's procedure [5]. Firstly, a mixture of iron chloride salts was dissolved in 10 ml of deionized water and stirred at 40 °C for 5 minutes. A hydroxide ammonium solution was added by

drop wise to the stirring mixture. The resulting black solution was continuously stirred for 3 hours at 40 °C. After that, the nanoparticles were washed several times by magnetic sorting with water and resuspended in 10 ml of a sodium citrate solution (0.5 M) and reacted for 1 hour at 80 °C. Then, the sample was ultrasonicated for 30 min and washed with acetone and water and finally resuspended in 5 ml of water. For the additional dextran shell, an aqueous solution of polysaccharide was added to the nanoparticle suspension, reacted at 70 °C for 30 min, ultrasonicated and washed by concentrator tubes with water. The so-obtained nanoparticles were dispersed in PBS for RBC encapsulation experiments [6]. For the preparation of zinc-manganese ferrite NPs, $ZnCl_2$ and $MnCl_2$ were introduced to the initial metal precursor mixture, without any further modification.

2˙2. *Thermal decomposition method.* – Highly monodispersed nanoparticles were synthesized via thermal decomposition of organometallic acetylacetonate precursors. The precursors were mixed with oleic acid, and benzyl ether under a flow of nitrogen and heated together until 100 °C and degassed at this temperature for 1 h. The solution was then heated to the reflux temperature (280 °C) at a rate of 9 °C/min with vigorous magnetic stirring and kept at this temperature for 1 hour. The mixture was then washed twice, at room temperature, with acetone and ethanol, centrifuged and resuspended in chloroform. The resulting hydrophobic surface was coated with a layer of dextran, according to the procedure described in ref. [7], with no modifications.

2˙3. *Encapsulation into RBCs.* – The encapsulation of MNPs into the erythrocytes [8] was carried out with highly concentrated suspensions of iron oxide and Zn/Mn ferrite NPs. Human blood was collected from healthy volunteers into heparinized tubes. RBCs were isolated by centrifugation at 1400 g at 4 °C for 10 min from freshly drawn blood. The serum and buffy coat were removed and the packed cells were washed three times with Hepes buffer and then resuspended in the same buffer at a 70% hematocrit. 1 ml of RBCs (70%) was dialyzed in the presence of 4 mg/Fe NPs suspension, for 75 min using a tube with a 12–14 kDa cut-off in 50 vol of a dialysis buffer. Resealing of RBCs was obtained by adding 0.1 vol of PIGPA. The loaded RBCs were recovered by centrifugation at 400 g and washed four times with Hepes buffer to remove unentrapped magnetic particles. All these procedures were performed at 4 °C under sterile conditions.

2˙4. *Characterizations.* – The particle size distribution of MNPs was determined using a Malvern ZetaSizer Nano ZSP. The surface morphology of the nanoparticles and the unloaded and loaded erythrocytes were studied using Transmission Electron Microscope (TEM) JEOL JEM 1011. The cells were then post-fixed in 1% OsO_4, dehydrated with ethanol, embedded in epoxy resin and thin sections were obtained by ultramicrotome. Magnetic resonance imaging was performed using a preclinical 1 tesla desktop MRI scanner (Bruker ICON). All samples were imaged by a T2 multislice multiecho (MSME) sequence with a TR of 3000 ms, multiple TE from 12 to 90 ms, and a flip angle of 180 ° and a T2-RARE sequence with a TR of 2500 ms, a TE of 60 ms and a flip angle of 180 °. The metal concentrations were measured by elemental analysis, using an inductively coupled plasma atomic emission spectrometer (Varian 720 ICP-OES).

3. – Results and discussion

The TEM analysis (fig. 1(a), (b)) confirms that the MNPs obtained by co-precipitation method have a non-regular shape with a mean size of 12 nm. The distribution was quite broad, but it is noteworthy that in the described protocol no magnetic size selection was applied to sort a specific population. Regarding the MNPs prepared by thermal decomposition method, quasi-cubic nanoparticles were obtained, with a diameter of 15nm and

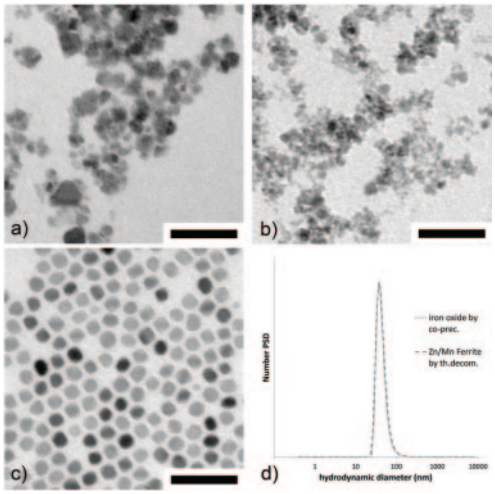

Fig. 1. – TEM images (scale bars of 50 nm) of iron oxide NPs (a) and Zn/Mn ferrite NPs (b) obtained by co-precipitation method and thermal decomposition method (c). In DLS measurement (d), the dashed line represents the Zn/Mn ferrite NPs by thermal decomposition and the dotted line represents the iron oxide by co-precipitation method.

a very good monodispersibility (fig. 1(c)). The DLS analysis yielded a hydrodynamic size between 20 and 25 nm for the MNPs by thermal decomposition and 25–30 nm for the NPs by co-precipitation method (fig. 1(d)) which depends on the inorganic core, the surface modification and the hydration layer. These values confirm that the obtained nanoparticles were mainly monodispersed. At the end of the loading procedure, the MNP-loaded RBCs were analyzed by TEM and compared to unloaded RBCs. Final cell recovery was calculated after the evaluation of the number of the total intact cells before and after the loading procedure. Data showed a cell recovery of MNP-loaded RBCs ranging from 40% to 60% with respect to 65% of control cells (UL-RBCs). The addition of MNP did not lead to any cell damage (*e.g.*, cell lysis or agglutination), as confirmed also by TEM analysis. Relevant biological parameters, as mean corpuscular volume, mean hemoglobin concentration and mean corpuscular hemoglobin concentration were not significatively affected by the MNP loading procedure. The presence of encapsulated MNPs into the RBCs was investigated firstly by MRI (fig. 2) and elemental analyses. The techniques confirmed that a stable interaction between MNPs and RBCs was achieved, observing some dark spots in T2-weighted sequences, confirming the expected behavior of the MNPs. Anyway, the resolution was not sufficient to determine the localization of the nanoparticles into the cells. TEM images were acquired to evaluate the cell integrity and the MNP distribution inside the cells and on RBC surface. In comparison to the unloaded RBC control sample (fig. 3(a)), the presence of nanoparticles was evidenced in all MNPs-loaded RBCs samples, both inside the cells and also in proximity of the internal cell membrane (fig. 3(b) and (c)).

4. – Conclusion and future perspective

In our work we focused the attention on the possibility to encapsulate superparamagnetic nanoparticles, with different chemico-physical characteristics, into human erythrocytes through the open membrane pores without affecting cell viability. TEM analysis confirmed that the NPs are distributed inside the cells but also attached to the membrane. Anyway, even if TEM observations demonstrated that the amount of NPs entrapped in

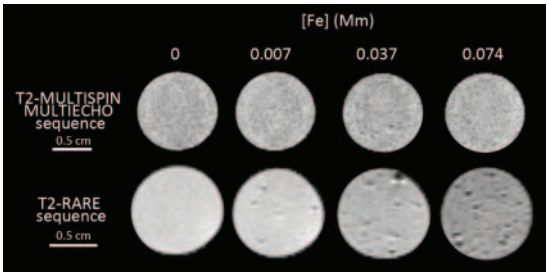

Fig. 2. – MRI image of MNPs-loaded red blood cells, dispersed in agarose phantoms. The dark spots were induced by the presence of magnetic nanoparticles and were proportional to the iron content measured for each experimental point. The R2 of the included MNP was of 117 $\mathrm{mM}^{-1} \cdot \mathrm{s}^{-1}$.

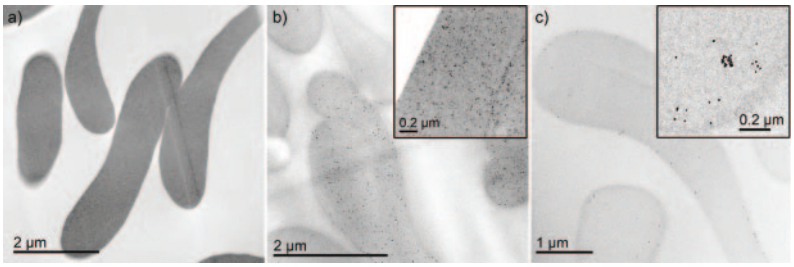

Fig. 3. – TEM images of the unloaded RBC (a), RBCs loaded with the nanoparticles obtained by co-precipitation method (b) and by thermal decomposition (c). For panel b and c, the insets represent some RBC portions with a higher encapsulation rate, acquired with higher magnification.

the cells was low, a significant contrast in MRI was obtained. Moreover, the MNPs entrapment depends on the intrinsic properties of the nanomaterials used, such as size, surface coating and nature of the dispersant. The immediate perspective of this work is to improve the stability of the nanoparticle suspensions for enhancing the encapsulation rate into the RBCs and to deeply characterize their magnetic behavior in related applications. The goal is to test the erythrocytes loaded with selected MNPs as intravascular contrast agents for some diagnostic applications, as MRI and Magnetic Particle Imaging (MPI), and also for magnetic hyperthermia and laser-assisted photo-ablation.

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