International Journal on Magnetic Particle Imaging
Vol 8, No 1, Article ID 2209001, 5 Pages

Infinite Science Publishing

Research Article

Encapsulation in human and murine erythrocytes of the Synomag®-D-PEG-OMe tracer for MPI application

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Received 03 March 2022; Accepted 30 June 2022; Published online 03 September 2022

Abstract

Recently, the potential of red blood cells (RBCs) loaded with superparamagnetic iron oxide (SPIO)-based nanoparticles as new blood-pool tracer material for the Magnetic Particle Imaging (MPI) has been investigated. It was shown that the encapsulation of SPIO-based contrast agents in the RBCs increase the circulation time in blood of these nanomaterials. However, not all iron oxide nanoparticles are eligible to the encapsulation into RBCs, depending on several factors such as dispersant agent nature, nanoparticle size and synthesis protocol. Therefore, we have recently started a program to identify those nanoparticles that can be potentially loaded with our method into RBCs. The goal is to produce biocompatible SPIO-RBCs carriers that can be used as new intravascular magnetic susceptible agents in biomedical applications, such as MRI and MPI. Here, we report the *in vitro* results obtained by using the Synomag®-D-PEG-OMe nanoparticle suspension (micromod Partikeltechnologie GmbH) with both human and murine red blood cells. MPS analysis showed that human Synomag®-D-PEG-OMe-loaded RBCs produced a signal that is weaker respect to the remarkable signal of ferucarbotran loaded-RBCs prepared at the same condition, but it is to be noted that the encapsulation efficiency of Synomag®-D-PEG-OMe into cells is lower compared to ferucarbotran nanoparticles.

I. Introduction

New biomedical strategies have been developed to improve the sensitivity and resolution of *in vivo* diagnostic imaging modalities for early and accurate disease detection and for therapeutic monitoring in a number of pathological conditions such as cardiovascular disorders. Several contrast agents are available to discriminate between healthy and pathological tissues; however, especially with reference to the circulatory system, their use is hampered by their limited circulation in the bloodstream. Several attempts to overcome these limits have only marginally solved the problem and the application of SPIO-based contrast agents continues to be limited by the uptake of macrophages abundantly present in the liver and spleen. Encapsulation of SPIO

nanoparticles into red blood cells (RBCs) has been suggested to increase the blood circulation time of nanoparticles [1]. The RBCs, thanks to their properties to be reversibly opened under hypotonic conditions without losing their natural features and functionalities, could represent extraordinary intravascular carriers for drugs, biologics and other therapeutic agents [2]. Recently, the suitability of different SPIO nanoparticle [3] for the encapsulation into human and murine RBCs was investigated in order to produce SPIO-RBCs carriers that can be used as new blood-pool tracer agents for Magnetic Resonance Imaging (MRI) and Magnetic Particle Imaging (MPI) [4–6]. The use of RBCs as a diagnostic tool is related to urgent identification of nanomaterials potentially suitable for a cardiac vitality evaluation, tissue perfusion, detection of bleeding sources and brain diseases, such as stroke [7]. In this field, we have started a program to identify those nanoparticles potentially eligible for loading into red blood cells in order to produce SPIO-RBCs carriers that can be used as new intravascular magnetic susceptible agents in biomedical applications. Here, we report the preliminary results obtained applying the RBC loading procedure to the Synomag®-D-PEG-OMe nanoparticles. This nanoparticle suspension that was provided from micromod Partikeltechnologie GmbH, is composed of iron oxides dextran-coated and functionalized with PEG 25000-OMe. It was recently demonstrated that Synomag®-D-PEG-OMe provided excellent MPI properties, with a mean signal increase by a factor of 2 compared to Perimag®-COOH and by a factor of 4 compared to the clinically approved ferucarbotran (Resovist®). It was shown that Synomag®-D-PEG-OMe is a longer-circulating tracer considering that its blood half-life is 64 min versus 27 min of Perimag®-COOH. Moreover, we previously evidenced that Perimag®-COOH nanoparticles were suitable for loading into human RBCs [8] but not for murine RBCs (data not shown); in fact, the use of Perimag®-COOH nanoparticles leads to the complete lysis of murine cells. It is known that native properties of human and murine RBCs can be damaged by the use of some type of nanoparticles during loading procedure; a low cell recovery, or even a possible irreversible damage can occur when cell integrity and biological properties are altered. This result discriminates the suitability of an iron oxide nanomaterial for cell encapsulation without affecting the cell viability. Here, we report the first characterization of Synomag®-D-PEG-OMe nanoparticles in term of efficiency of encapsulation in human and murine RBCs and their effect on native properties of cells.

II. Materials and methods

Synomag®-D-PEG-OMe nanoparticles were provided by micromod Partikeltechnologie GmbH (Synomag®-D, surface: PEG 25000-OMe. Lot number; 101-20-104-03, iron concentration of 19.7 mg Fe/ml, 70 nm particle hydrodynamic size). Ferucarbotran nanoparticle suspension (iron concentration of 28 mg Fe/ml, 54 nm particle hydrodynamic size, was provided from Prof. Ichiro Kato (Meito Sangyo, Co. Ldt., Nagoya, Japan). To characterize the efficiency of Synomag®-D-PEG-OMe encapsulation into cells, three independent loading experiments were performed. Essentially, 1 ml of RBCs at 70 % hematocrit was dialyzed in the presence of different iron concentrations (2.8, 5.6, 11.2 and 22.4 mg Fe) following the procedure previously reported [8, 9]. Following the same procedure unloaded red blood cells (UL-RBCs) were prepared, with the exception that they were dialysed in absence of magnetic material. The encapsulation of magnetic nanoparticles in murine RBCs was performed as previously reported [10]. The mean corpuscular volume (MCV), mean hemoglobin concentration (MCH) and mean corpuscular hemoglobin concentration (MCHC) of Synomag®-D-PEG-OMe-loaded RBCs were evaluated with an automated hemocytometer (Model MICROS O.T, Horiba ABX Diagnostics, Italy). The final cell recovery was also determined by measurement of total intact RBCs before and after loading. Unloaded (UL-RBCs) and Synomag®-D-PEG-OMe-loaded RBCs were observed by Transmission Electron Microscopy (TEM), analyzing whole-RBCs, dropped on copper grid and embedded into a thin polysaccharide film, or 70 nm slices of epoxy resin-embedded RBCs, in order to investigate the cell integrity and the presence of iron oxides in the cell cytoplasm [11]. The Fe concentration in human and murine Synomag®-D-PEG-OMe-loaded RBCs was determined by NMR relaxation measurements using a dose-response curve generated by adding known amounts of Synomag®-D-PEG-OMe contrast agent to human and murine RBC samples as previously reported [8, 9]. The concentration range studied was 0 to 12 mM Fe with at least 9 concentration levels. The longitudinal (T1 and transverse (T2) relaxation times of these samples at 44 % of hematocrit were measured at magnetic field strength of 9.4 Tesla at 37°C using an AC-400 NMR-Bruker spectrometer. The *in vitro* stability of Synomag®-D-PEG-OMe-loaded RBCs was evaluated in comparison to control cells (unloaded and not dialysed RBCs) and ferucarbotran-loaded RBCs. All samples resuspended at a hematocrit of 0.5 % in Hepes buffer were plated in petri dishes and incubated at 37°C. At various time intervals the samples were removed and packed by centrifugation at 400 g for 10 min. The supernatants were recovered and centrifuged at 12000 g before spectrometric measurements at 540 nm. The amounts (mg) of hemoglobin released from all samples in the corresponding supernatants were compared. MPS analyses of 10 μL Synomag®-D-PEG-OMe-loaded RBCs were performed and compared to free- and immobilized-Synomag®-D-PEG-OMe at the same concentration as reported in [8].

10.18416/ijmpi.2022.2209001

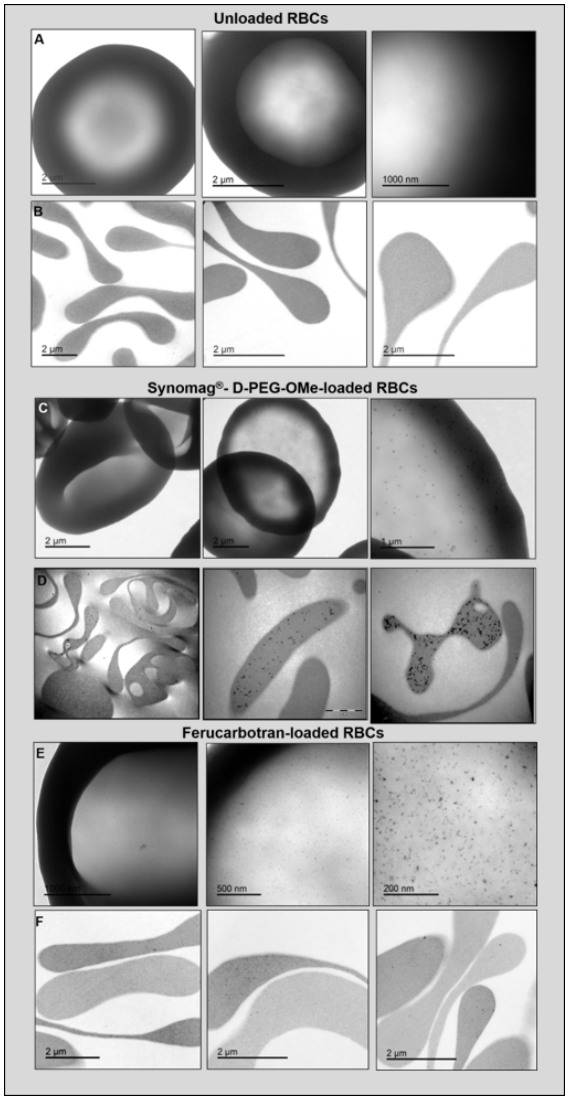

Figure 1: TEM images of whole-RBCs (A,C,E) and sectioned-RBCs (B,D,F) of human UL-RBCs (A, B), Synomag®-D-PEG-OMe- (C, D) and ferucarbotran-loaded RBC (E, F) samples prepared by using 11.2 mg Fe/ml RBC 70 %.

III. Results

The data evidenced that Synomag®-D-PEG-OMe iron oxide nanoparticles are suitable for encapsulation into human and murine RBCs (Table 1, 2, 3). Representative images reported in Figure 1 show the entrapment of Synomag®-D-PEG-OMe nanoparticles into human RBCs in comparison to results obtained with control cells (unloaded RBCs, Figure 1 A and B) and with the reference standard, ferucarbotran nanoparticles (E and F). Incorporated Synomag®-D-PEG-OMe iron oxides appear within RBCs both in dispersed and cluster forms (C and D); it is important to note that there is not any presence of iron oxide adherent on RBC surface. However, not all RBCs present encapsulated nanoparticles and the

Table 1: T1 and T2 of human Synomag®-D-PEG-OMe-RBCs, obtained dialyzing 500 μl of RBC 70 % Ht with 1.4, 2.8, 5.6 or 11.2 mg Fe (L1-, L2-, L3-, L4-Synomag®-D-PEG-OMe, respectively). Values of loaded-RBCs were compared to UL-RBCs. *p<0.05, **p<0.01 were obtained with Student t-test.

Samples	T1 (ms)	T2 (ms)	mM Fe (from r1)	MCV (fl)	MCH (pg)	MCHC (g/dl)	cell recovery (%)
UL-RBCs	2054±7	52.3±0.1	/	78±4.2	24.3±0.6	31.2±0.8	70±1.4
L1-Synomag®-D-PEG-OMe	1468±130	18.8±2.1*	0.37±0.11	75.5±3.5	23.3±0.8	30.9±0.5	69.5±3.5
L2-Synomag®-D-PEG-OMe	1238±139	11.6±1.1*	0.61±0.16	72±2.8	21.6±0.1	30±1.2	70.5±0.7
L3-Synomag®-D-PEG-OMe	741±54*	5.2±0.1**	1.59±0.17	66.5±2.1	17.1±0.8*	25.7±0.6	70.5±3.5
L4-Synomag®-D-PEG-OMe	822±79*	5.9±0.6**	1.36±0.21	63±2.8*	13.3±1.1*	21.2±0.8	73±8.5

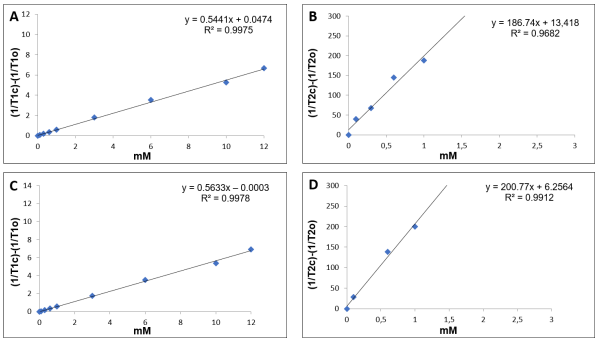

Figure 2: NMR dose-response curves generated by adding known amounts of Synomag®-D-PEG-OMe nanoparticles to human (A and B) and murine (C and D) RBCs at 44 % Ht.

results are different from those obtained with ferucarbotran that shows a remarkable efficiency of incorporation (E and F). The total preparation procedure resulted in a cell recovery of about 70 %, similar to that for unloaded cells.

Synomag®-D-PEG-OMe-loaded RBCs were slightly smaller on average than the untreated cells (MCV 63±2.8 fl for L4-RBCs vs. 78±4.2 fl of UL-RBCs) with less haemoglobin per cell, but this result is significant only when higher iron amount were used. Moreover, Synomag®-D-PEG-OMe-loaded RBCs present a near normal mean cellular hemoglobin concentration, Table 1. The final mM iron concentration encapsulated in the human RBCs was calculated using the calibration curve, obtained as described in Material and methods. The r1 and r2 relaxivities for human Synomag®-D-PEG-OMe-RBCs are 0.5441 and 186.74 sec-1 mM-1, respectively (Figure 2, A, B). Similarly, r1 and r2 relaxivities for murine

Table 2: T1 and T2 NMR measurements of RBCs loaded with Synomag®-D-PEG-OMe or Ferucarbotran obtained dialyzing 500 μl of RBC 70 % hematocrit in the presence of 5.6 mg Fe.

Samples	T1 (ms)	T2 (ms)	mM Fe (from r1)	MCV (fl)	MCH (pg)	MCHC (g/dl)	cell recovery (%)
UL-RBCs	2054±7	52.3±0.1	/	78±4.2	24.3±0.6	31.2±0.8	70±1.4
L3-Synomag®-D-PEG-OMe	741±54*	5.2±0.1**	1.59±0.17	66.5±2.1	17.1±0.8*	25.7±0.6	70.5±3.5
L3-Ferucarbotran	110±25***	2.6±1***	6.81±1.43	68.7±2.3	19.9±1.4*	29.1±3.1	65.4±12.1

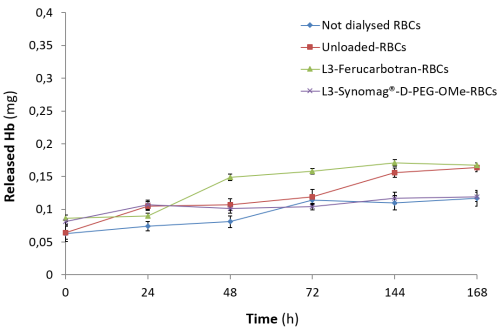

Figure 3: *In vitro* stability of human L3-Synomag®-D-PEG-OMe loaded RBCs was performed as reported in Material and methods. The release of Hb was compared to that of L3-ferucarbotran-loaded RBC sample and control cells (unloaded and not dialyzed RBCs).

Table 3: T1 and T2 NMR measurements of murine RBCs loaded with different amounts of Synomag®-D-PEG-OMe obtained dialyzing 500 μl of RBCs 70 % hematocrit with 1.4, 2.8 or 5.6 mg Fe (L1-, L2-, L3- Synomag®-D-PEG-OMe, respectively).

Samples	T1 (ms)	T2 (ms)	mM Fe (from rl)	MCV (fl)	MCH (pg)	MCHC (g/dl)	cell recovery (%)
UL-RBCs	2297±106	57.3±0.9	/	44.5±3.5	13.8±0.6	33.1±1.3	54.9±4.4
L1-Synomag®-D-PEG-OMe	1718±120	16.9±0.8	0.27±0.08	40±1.4	11.6±0.3	29±0.3	44.5±0.7
L2-Synomag®-D-PEG-OMe	1416±279	10.9±2.6	0.47±0.17	39.5±2.1	12±1.1	29.9±1.3	37±5.7
L3-Synomag®-D-PEG-OMe	884±55.3	5.3±0.4	1.1±0.1	40.5±0.7	10.9±0.7	26.9±1.6	27±8.6

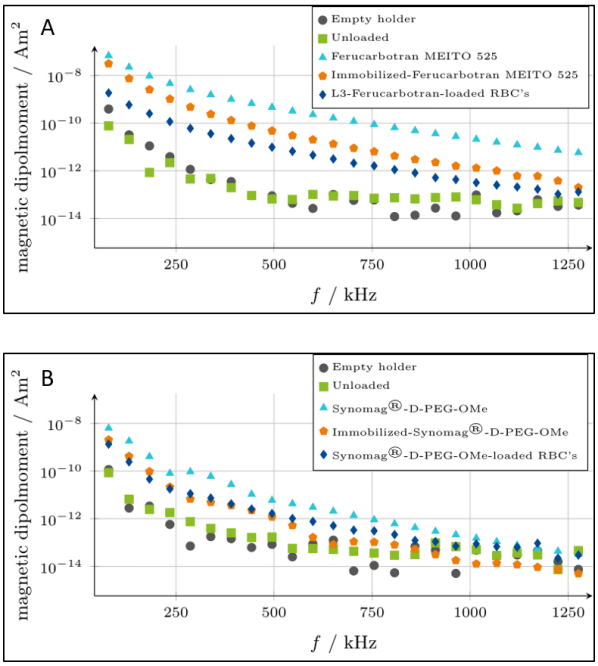

Figure 4: MPS spectra of representative L3-Ferucarbotran Meito-loaded RBC (A) and Synomag®-D-PEG-OMe-loaded RBC (B) samples in comparison with the respective immobilized and liquid nanoparticles.

Synomag®-D-PEG-OMe-RBCs are 0.5633 and 200.77 sec-1 mM-1, respectively (Figure 2 C, D).

NMR measurements evidenced that T1 and T2 values of Synomag®-D-PEG-OMe RBC sample remarkably decrease in comparison to values of control sample or unloaded RBCs (for L3-RBC sample; T1=741±54 ms versus 2054±7 ms; T2=5.2±0.1 ms versus 52.3±0.1 ms).

The data shows that the efficiency of iron encapsulation increases with the increased iron amounts used to perform loading procedure reaching about 1.6 mM Fe incorporated into cells when 11.2 mg Fe/ml RBCs are used. Higher Synomag®-D-PEG-OMe amounts do not appear to lead to an improvement of iron encapsulation, Table 1. This result is significantly different from that obtained with ferucarbotran contrast agent (~7 mM Fe) when loaded RBC are prepared at the same condition, Table 2.

The *in vitro* stability assay of human Synomag®-D-PEG-OMe-loaded RBCs evidenced that they are stable over time; the release of Hb amounts from these RBC-constructs is not significantly higher than ferucarbotran-loaded RBC sample and control cells (not dialysed and unloaded RBCs) during all the experimental time points, Figure 3.

In order to evaluate the suitability of Synomag®-D-PEG-OMe with murine RBCs, several loading experiments, similar to those with human RBCs, were performed and results were reported in Table 3.

As evidenced for human loaded-RBCs, Synomag®-D-PEG-OMe-loaded RBC samples showed a decrease in T1 and T2 values, compared to unloaded RBCs. In particular, this is evident for L3-Synomag®-D-PEG-OMe sample (T1=884±55.3 ms versus 2297±106 ms; T2=5.3±0.4 ms versus 57.3±0.9 ms). Furthermore, the highest iron concentration loaded into murine RBCs at the end of the loading procedure is 1.1±0.1 mM for L3-Synomag®-D-PEG-OMe sample, Table 3. However, cell recovery of murine Synomag®-D-PEG-OMe RBCs strongly decreases when the amounts of used nanoparticle increase (44.5±0.7 % for L1-, 37±5.7 % for L2- and 27±8.6 % for L3-Synomag®-D-PEG-OMe-loaded RBCs respect to 54.9±4.4 % for unloaded control sample). MPS analyses were performed on human L4- Synomag®-D-PEG-OMe-loaded RBC sample in order to verify their performance as MPI tracers. The MPS signal of the immobilized and liquid Synomag®-D-PEG-OMe nanoparticles was scaled down to the same mM Fe concentration obtained by NMR for L4-Synomag®-D-PEG-OMe-RBCs sample (1.36±0.21 mM). It is evident that the MPS signal is comparable to that provided from immobilized Synomag®-D-PEG-OMe, Figure 4. These results will permit the *in vivo* use of such constructs as convenient both in MPI and MRI without limitations.

IV. Conclusions

It was evidenced that Synomag®-D-PEG-OMe nanoparticles can be encapsulated in the human and murine RBCs and the efficiency of encapsulation is similar between human and murine RBCs leading to 1.1-1.6 mM Fe into cells. Moreover, PEG-OMe-functionalization of Synomag®-D nanoparticles improves the efficiency of Synomag®-D plain encapsulation into human RBCs already described [10, 12, 13]. Although, human Synomag®-D-PEG-OMe RBCs do not produce a stronger MPS signal with respect to ferucarbotran-loaded RBCs [10], the MPS signal strength is good and comparable to immobilized nanomaterial at the same iron concentration. This means that the signal quality does not decrease due to the loading process, but the new Synomag®-D-PEG-OMe-loaded RBC constructs do not appear to be optimal MPI tracers from these *in vitro* data. However, they could be evaluated through further *in vivo* studies to understand if they could be used as long circulating MPI tracers.

Acknowledgments

We thank Prof. Ichiro Kato for providing ferucarbotran gold standard (Meito Sangyo, Co., Ltd.). Research funding: EuroNanoMed III, (grant number: 13XP5060B, “MAGneTISe”) and ARS01_00876, “BIO-D”-CUP B32F20000270005-RNA-COR 3200158.

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