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Characterization of the Synomag®-D-PEG-OMe nanoparticles for the encapsulation in human and murine red blood cells

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Abstract

It was shown that the encapsulation of SPIO-based contrast agents in the red blood cells (RBCs) increases the circulation time in blood of these nanomaterials. Not all iron oxide particles are eligible for the entrapment into RBCs, depending on several factors and synthesis protocol. We have recently identified some type of nanoparticles that can be loaded with our method into RBCs to produce biocompatible SPIO-RBCs carriers that could be used as new intravascular tracers for biomedical applications, such as Magnetic Particle Imaging (MPI). Here, we report the first *in vitro* results obtained by using the Synomag®-D-PEG-OMe nanoparticles with both human and murine RBCs. MPS analysis showed that human Synomag®-D-PEG-OMe-loaded RBCs produced a signal that is weaker respect to the remarkable signal obtained with 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

Encapsulation of superparamagnetic iron oxide (SPIO) nanoparticles into red blood cells (RBCs) has been suggested to increase the blood circulation time of iron oxide 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. Recently, the suitability of different SPIO nanoparticles 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) [2] and Magnetic Particle Imaging (MPI) [3,4]. The use of RBCs as a diagnostic tool is related to urgent identification of biocompatible and long circulating nanomaterials potentially suitable for a cardiac vitality evaluation, tissue perfusion, detection of bleeding sources and brain diseases, such as stroke

[3]. 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 particles. It was shown that this nanomaterial 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 [5] but not for murine RBCs (data not shown); in fact, the use of Perimag®-COOH nanoparticles leads to the lysis of murine cells. In fact, native properties and cell integrity of human and murine RBCs can be irreversibly damaged by the use of some type of nanoparticles during the loading procedure. This result discriminates the suitability of an iron oxide nanomaterial for cell encapsulation without affecting the cell viability. 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.

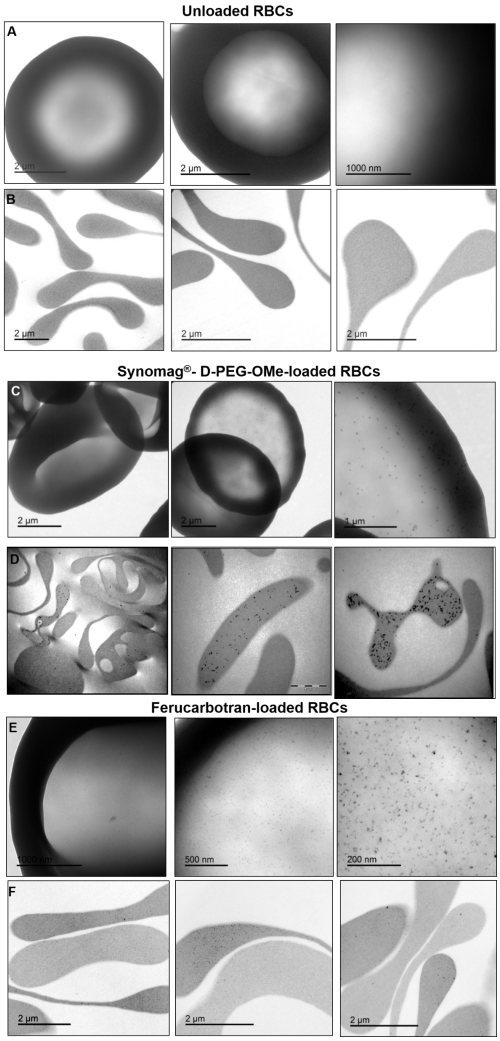

Figure 1: TEM images of whole RBCs (A,C,E) and sectioned-RBCs (B,D,F) of human UL-RBCs (A, B), Synomag®-D-PEG-OMe- (C, D) and ferucarbotran-loaded RBC (E, F) samples prepared by using 11.2mg Fe/ml RBC 70%.

II. Material 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 size). Ferucarbotran nanoparticles (iron concentration of 28 mg Fe/ml, 54 nm particle size) was provided by Meito Sangyo, Co. Ldt., Nagoya, Japan. To characterize the efficiency of Synomag®-D-PEG-OMe encapsulation into human or murine RBCs, three independent loading experiments were performed essentially as previously reported [2,4]. Some biological properties, such as mean corpuscular volume (MCV), mean hemoglobin concentration (MCH) and mean corpuscular hemoglobin concentration (MCHC) of Synomag®-D-PEG-OMe-loaded RBC samples were evaluated as well as the cell recovery at the end of loading procedure [2,4]. 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 [6]. The Fe concentration in human and murine Synomag®-D-PEG-OMe-loaded RBCs was determined by NMR relaxation measurements as reported previously [4,5]. T1 and T2 relaxation times of these samples at 44% of hematocrit (Ht) 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 both the ferucarbotran-loaded RBC sample, prepared at the same condition (L3-RBCs), and to control cells (unloaded and not dialysed RBCs) after their incubation at 37°C at 0.5% Ht. 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 12000g before spectrometric measurements at 540 nm. The amounts (mg) of hemoglobin (Hb) 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 Ref. 5.

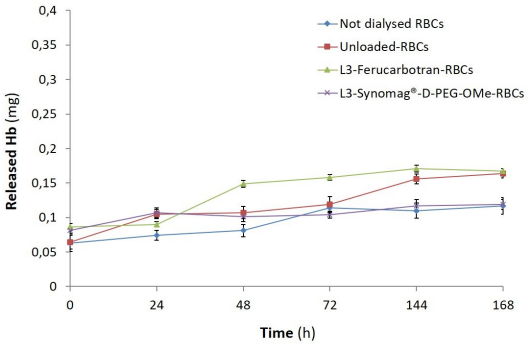

Figure 2: *In vitro* stability of human L3-Synomag®-D-PEG-OMe loaded RBCs was performed as reported in Material and methods. The release of Hb was compared to that of L3-ferucarbotran-loaded RBC sample and control cells (unloaded and not dialyzed RBCs).

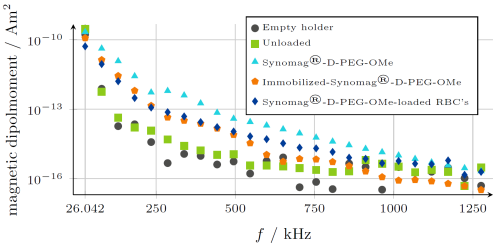

Figure 3: MPS spectra of a representative L4-Synomag®-D-PEG-OMe-RBC sample in comparison to the immobilized and liquid Synomag®-D-PEG-OMe.

III. Results and discussion

The data evidenced that Synomag®-D-PEG-OMe iron oxide nanoparticles are suitable for encapsulation into human and murine RBCs (Table 1 and 2). Figure 1 shows the entrapment of Synomag®-D-PEG-OMe nanoparticles into human RBCs in comparison to control cells (unloaded RBCs, Figure 1 A and B) and 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 results are different from those obtained with ferucarbotran that shows a remarkable 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 hemoglobin per cell, but this result is significant only when higher iron amounts were used. Moreover, Synomag®-D-PEG-OMe-loaded RBCs present a near normal mean cellular hemoglobin concentration,

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 (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

Table 2: T1 and T2 of murine Synomag®-D-PEG-OMe-RBCs obtained dialyzing 500 µl of RBCs 70% Ht with 1.4, 2.8 or 5.6 mg Fe (L1-, L2-, L3- Synomag®-D-PEG-OMe, respectively).

Samples	T1 (ms)	T2 (ms)	mM (from r1)	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

Table 1. The final mM iron concentrations encapsulated in the human RBCs were calculated with r1 and r2 relaxivities for Synomag®-D-PEG-OMe that resulted to be 0.5441 and 186.74 sec-1 mM-1, respectively. T1 and T2 values of Synomag®-D-PEG-OMe RBC sample remarkably decrease in comparison to values of control sample (for L3-RBC sample; T1=741±54 ms and T2=5.2±0.1 ms versus 2054±7 ms and 52.3±0.1 ms, respectively of UL-RBCs). 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 oxide encapsulation, Table 1. This result is significantly different from that obtained with ferucarbotran contrast agent (8 mM Fe) when loaded RBC are prepared at the same condition [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 2.

Results obtained with murine Synomag®-D-PEG-OMe-RBCs were reported in Table 2. As evidenced for human loaded-RBCs, Synomag®-D-PEG-OMe-loaded RBC samples showed a decrease in T1 and T2 values, compared to UL-RBCs.

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). About 1mM encapsulated Fe was obtained. Cell recovery of murine Synomag®-D-PEG-OMe RBCs strongly decreases when the amounts of used nanoparticle increase, Table 2.

MPS analyses were performed on human L4-Synomag®-D-PEG-OMe-loaded RBCs 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 3. 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 described in Ref. 7. Although, human Synomag®-D-PEG-OMe RBCs do not produce a stronger MPS signal with respect to ferucarbotran-loaded RBCs [4], 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.

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Author’s statement

Authors state no conflict of interest. Informed consent has been obtained from all individuals included in this study. Ethical approval: The research related to human use complies with all the relevant national regulations, institutional policies and was performed in accordance with the tenets of the Helsinki Declaration, and has been approved by the authors’ institutional review board or equivalent committee.

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