Original paper

Characterization of human erythrocytes as carriers for iron nanoparticles

DANA ZDREMŢAN[1*], LARISA CALU[2], CIPRIAN VALENTIN MIHALI[3], IRIS MARIA TUŞA[2], FLORIAN DUMITRACHE[4,3], CORALIA ADINA COTORACI[1], DANIELA BRATOSIN[2,5]

[1]Vasile Goldiş Western University of Arad, Faculty of Medicine, Pharmacy and Dental Medicine, Department of Biochemistry, Arad, Romania
[2]National Institute for Biological Science Research & Development, Bucharest, Romania
[3]Vasile Goldis Western University of Arad, Institute of Life Sciences, Department of Experimental and Applied Biology, Arad, Romania
[4]National Institute for Laser, Plasma and Radiation Physics, Bucharest, Romania
[5]Vasile Goldis Western University of Arad, Faculty of Medicine, Pharmacy and Dental Medicine, Department of Biology, Arad, Romania

Abstract   Carrier red blood cells (RBCs) can be used for vascular delivery of encapsulated drugs, biologicals and nanoparticles of various compositions with multiple advantages including bioavailability, biocompatibility, large carrier volumes and vascular residence time. Iron-based magnetic nanoparticles (MNPs), with size less than 15 nm became superparamagnetic and they can be used for both, diagnostic (imagistic) and therapeutic purposes, including as controlled release drug delivery systems. For these reasons, the objective of this study was to employ human erythrocytes for the encapsulation of carbon shell iron-based core nanoparticles (obtained by continuous wave infrared laser pyrolysis of $Fe(CO)_5$ by a transient opening of cell membrane pores using different hypo-osmotic dialysis. In order to evaluate the encapsulation of nanoparticles into red blood cells (RBCs), normal, unloaded and loaded red blood cells (RBCs) were examined by Scanning/Transmission Electron Microscopy and Energy-dispersive X-ray spectroscopy analysis (EDX). Here, it is reported that these composite iron-based nanoparticles that were synthesized and tested could lead to their use in conjunction with the red blood cells (RBCs) as a promising agent in biomedical applications.

✉ *Corresponding author: DANA ZDREMŢAN, General Medicine Faculty, "Vasile Godiş"Western University, str. Henri Coanda, nr. 1, 310429, Arad, Romania;
Tel. +40745312502
E-mail: dana_zdremtan@yahoo.com

Introduction

The term carrier RBCs was first introduced in 1979 and today red blood cells (RBCs) can be used for vascular delivery of encapsulated or surface-bound drugs, biologicals and nanoparticles of various compositions (VILLA et al [1]).

As carriers, RBCs offer a multitude of advantages including bioavailability, biocompatibility, large carrier volumes and longevity in circulation (approx. 120 days in humans), which makes them a highly attractive vehicle for vascular delivery (ZIMMERMANN, [2], JAITELY et al [3]). At present, magnetic nanoparticles (MNPs), with less than 100 nm and especially those with superparamagnetic feature can be used for both, diagnostic (imagistic) and therapeutic purposes, including as controlled release drug delivery systems (YEZHELYEV et al [4], EMERICH & THANOS, [5], CUENCA et al [6], JAIN, [7]). In Magnetic Resonance Imaging (MRI) MNPs based on iron oxides represent a great potential (BONNEMAIN, [8]) providing early cancer detection and specific therapy, reducing invasive procedures and side effects for healthy surrounding tissues (MOGHIMI et al [9], PEER et al [10]), in a malignant lesion that can apply a controlled external magnetic field to direct the transport of particles to the therapeutic target.

Moreover, iron carbide nanoparticles can be used for magnetic-dependent diagnosis, due to their high magnetization and moderate coercivity (YU et al [11]) allowing them to be considered not only as promising MRI contrast agents but also as an active material in magnetic hyperthermia. (SZABO & VOLLATH, [12]). Also, studies have been carried out to show the potential of RBCs, loaded with magnetic ferrous nanoparticles being used as imaging tracer materials (GLEICH & WEIZENECKER, [13]). With the entire positive outcome, nanoparticles have a certain degree of toxicity for the cells (TUSA et al [14]) and the unprotected zero valent iron or Fe^{2+} nanophase induce oxidative stress (KENANN et al [15]). This is the reason why it was evaluated the possibility of encapsulating such iron-based nanoparticles in erythrocytes.

Solutions are currently being sought for more efficient products, and their effectiveness is often compromised by their recognition and removal by the reticuloendothelial system (RES) before they reach the target tissue. Taking into account the large number of clinically approved iron oxide-based imaging agents, for this purpose, it is attempted the modelling of the nano-transporters according to the erythrocyte model in order to conceive the last generation cessation platforms (DOSHI & MITRAGOTRI, [16], MERKELA et al [17])

To merge nanoparticles and RBCs there are three strategies: non-specific binding of nanoparticles to RBCs, specific binding ligand-receptor mediated or binding with the help of chemical conjugation, and encapsulation in the RBCs of the nanoparticles. Commonly, particle entrapping in the RBCs is based on the similar principle to hypotonic swelling that leads to the opening of the pores in the cell membrane and allows nanoparticles to diffuse inside the cell. The RBCs are then returned to isotonic conditions in order to seal the pores (WU et al [18]). Through this method is necessary that the nanoparticles are small enough to pass by the pores. Other studies used erythrocytes-encapsulated nanoparticles obtained by magnetization and ultrasound stimulation while preserving the integrity of the cell during stimulation (WU et al [18]) for a therapeutic benefit. For example, RBCs were loaded with aspirin and with iron oxide nanoparticles, in the same time, for the treatment of arterial thrombosis and magnetic delivery of therapeutic molecules in dogs (OREKHOVA et al [19], ANTONELLI et al [20]). Certain studies have presented the preparation method of erythrocytes magnetically responsive for therapeutic drug targeting using the co-encapsulation of certain substances with certain ferrofluids like magnetite (VYAS et al [21]). Encapsulation of gold nanoparticles can possibly be used together with X-ray absorption for the tracking of erythrocytes' flow in blood vessels (AHN et al [22]).

Ferrous and ferric oxide are the main components of magnetic nanoparticles and, considering that they are attracted by a magnetic field, they can be used for processes of bio-separation including cell sorting and drug targeting (ITO et al [23]). The size of the particle varies a lot and influences their pharmacokinetic and physicochemical properties (ALLKEMPER et al [24]). Erythrocytes loaded with superparamagnetic iron oxide are artificially made products, effective as contrasting agents, mainly for imaging and for detecting the altered angiogenesis and occluded vessels in well-defined parts of the body and damaged vessels that may result in bleeding. The drug delivery based on magnetism is used as a replacement or as an augmenter of the chemotherapy/radiotherapy treatment (LUBBE et al [25], HILGER et al [26], ZHOU et al [27]). Contrast agents that incorporate nanoparticles of superparamagnetic iron oxide (SPIO) have shown potential as a way to visualize the labelled cells using MRI. Erythrocytes exposed to dialysis with a hypotonic buffer, firmly uptake nanoparticles of superparamagnetic iron oxide and can be used as MRI contrast agents. Such RBCs may also be used as vehicles for drug delivery.

The objective of this study was to utilize human erythrocytes for the encapsulation of Fe@C nanoparticles (obtained by continuous wave laser pyrolysis using the protocol described in reference article (DUMITRACHE et al [28]) by a transient opening of cell membrane pores using different hypo-osmotic dialysis. In order to evaluate the encapsulation of nanoparticles into RBCs the as-synthesized composite iron-based nanoparticles were examined by TEM (Transmission Electron Microscopy), XRD (X-ray diffraction) and EDX (Energy-dispersive X-ray) analyses. Human unloaded and loaded RBCs

were examined again using SEM (Scanning Electron Microscopy), TEM and EDX. Here, we report a procedure to incorporate these iron-based nanoparticle into RBCs for various medical applications.

Materials and Methods

1. Chemicals

In the present study iron-based nanoparticles Fe@C were synthesized by CW CO2 laser pyrolysis using Fe(CO)5 and ethylene as Fe and C donors, the experimental set-up and the more important nanoparticle synthesis parameters were shown in detail elsewhere (DUMITRACHE et al, [28], ALEXANDRESCU et al [29], MORJAN et al [30]; MORJAN et al [31]). The resulted nano-powder has the following elemental composition C 62%, Fe 31%, O 7% (from EDX). The nanocomposite core–shell structure and their quasi spherical shape of the as-synthesized particles are highlighted in the TEM image presented in Figure 1.
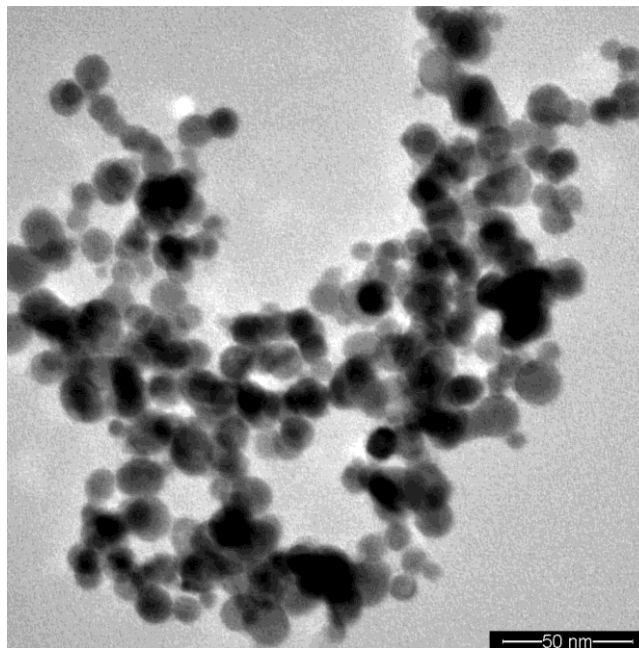

Figure 1. TEM image of the core–shell nanoparticles

The diameter of the iron composite nanoparticles is ranging from 6 to 17 nm with log-normal distribution centered at 11.3 nm. A well stabilized dispersion was prepared via a ultrasonic horn procedure, 15 min. into an ice cooled bottle (LIN et al [32]).

The X-ray diffraction (XRD) diagram for the as-synthesized nanoparticles is displayed in Figure 2. The different colored lines indicate the standard XRD peaks corresponding to α-Fe (green), Fe3C (red) and Fe7C3 (blue), the most probable identified crystalline phases. The only evidence of iron oxide phases is the very small peak centred at $2\theta=35.6°$ corresponding to the most intense peak of magnetite/maghemite phase. This fact revealed a good protective feature of C shell from the environmental oxidation. The Raman spectroscopy, not presented here, shows the characteristic D and G band of amorphous carbon.

2. Blood preservation for the study

Human blood type II Rh+, collected in heparin was centrifuged at 2,000 g; 4°C; 5 min. Plasma, platelets and leukocytes were removed by aspiration and the red blood cells were resuspended (10^7 cells per ml) for the experiment in isotonic phosphate-buffered saline (PBS) solution pH 7.4 (Na_2HPO_4 8.1 mM, KH_2PO_4 1.5 mM, NaCl 140 mM and KCl 2.7 mM). 1µl of RBCs sediment was diluted with 1 ml NaCl solution and the cell suspension obtained was again diluted, 100 µl cell suspension in 900 µl saline solution, to get a final concentration of 10^5 cells/ml. Erythrocytes were subsequently used for the incorporation of nanoparticles.

3. Entrapment of nanoparticles into human erythrocytes

The encapsulation of nanoparticles into RBCs is made possible by transient opening on the cell membrane of pores that are large enough (50-200 nm) to be crossed by the nanoparticles.

Hypotonic dilution was the first method investigated for the encapsulation of chemicals into erythrocytes and this method is the simplest and fastest. A volume of packed erythrocytes is diluted with 2-20 volumes of an aqueous solution of a drug or nanoparticles. The solution is then restored by adding a hypertonic buffer. The resultant mixture is then centrifuged and the pellet is washed with isotonic buffer solution (TAJERZADEH & HAMIDI, [33]). Saline solutions of decreasing concentrations were prepared in a series of 19 hemolysis tubes, in which the sodium chloride concentration gradually decreased,

starting from tube number 1, with an isotonic solution, to the 19th tube, where there was only distilled water. RBCs were dialyzed in the presence of carbon-coated iron nanoparticles in saline solutions of decreasing concentrations *under sterile conditions* by incubating for 4 hours at room temperature on a shaking loose. The loaded RBCs were recovered by centrifugation at 400 g and washed 3 times with an isotonic solution to remove unentrapped particles. Following the same procedure, unloaded erythrocytes were prepared, with the exception that they were dialyzed in the absence of iron material.
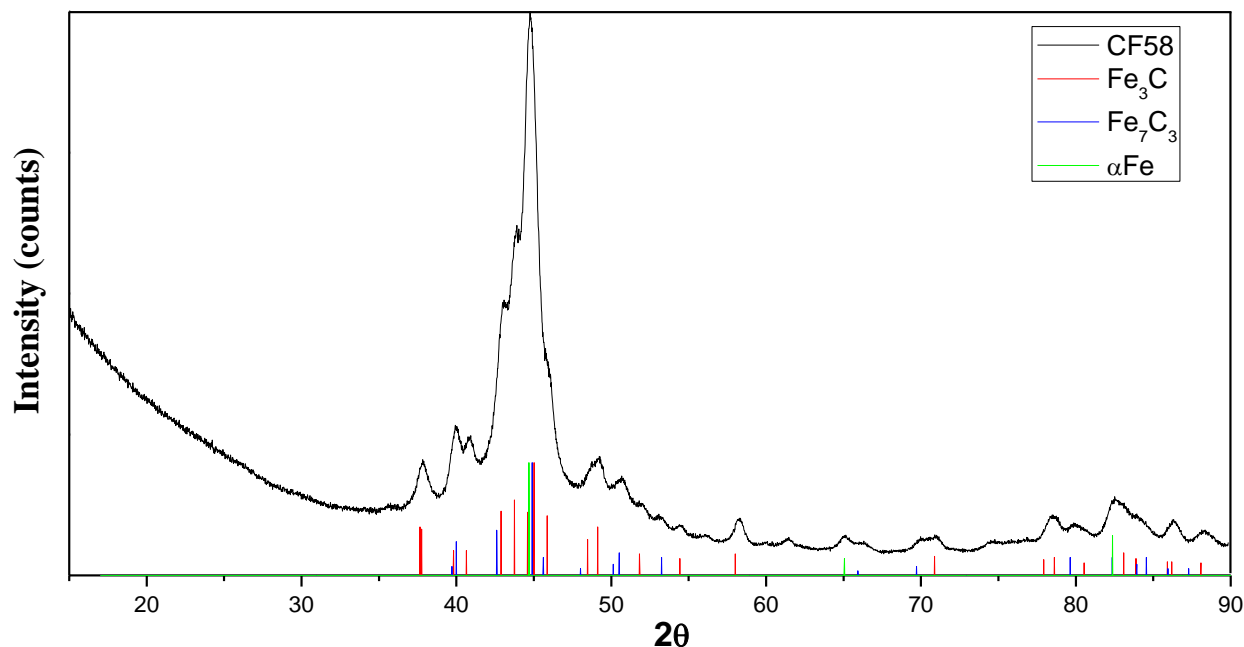

Figure 2. X-ray diffractogram of the as synthesized nanopowder and the theoretical peaks of the major crystalline phases identified.

4. Microscopic analysis

Scanning Electron Microscopy (SEM) analysis. Fresh erythrocytes were fixed for 4 hours in a 1.25% glutaraldehyde in cacodylate buffer (0.1 M, pH of 7.2), washed 3 times with distilled water, filtered through on 0.2 µm Anodisc filters and analyzed at a HITACHI SU1510.

EDX/SEM analysis (Energy-dispersive X-ray spectroscopy with scanning electron microscope). For this analysis, the samples were prepared the same as for the SEM analysis. EDX analysis became a common practice analysis being an essential part of a scanning electron microscope. Using this method, it is possible to determine the content of any sample. The information generated through EDX analysis is given as spectra with multiple peaks that correspond to all the diverse elements present in the analyzed sample. Each element has a characteristic peak of unique energy. Also, EDX is useful for both qualitative and quantitative analysis. Most of the scanning electron microscopes dedicated software provide auto-identification of peaks and calculate the atomic percentage of each detected element. Another advantage of this method is the non-destructive technique used for characterization, requiring little sample preparation. The device EDX was produced by OXFORD INSTRUMENTS and is equipped with AZTEC software.

Transmission Electron Microscopy (TEM) analysis. Unloaded and loaded erythrocytes were analysed by TEM with a FEI TECNAI G2 SPIRIT TWIN / BIOTWIN electron microscope. Erythrocytes were primarily fixed in 2.5% glutaraldehyde in phosphate buffer 100 mM, at pH 7.0 for 2 hours and then washed with phosphate buffer 200 mM. The sample was fixed in osmium tetraoxide 1% solution in 100 mM phosphate buffer solution, 2 hours at 4°C. Cells were washed 5 times with distilled water and blocking with uranyl acetate 2% aqueous solution, followed by acetone series dehydration. The sample was transferred in the Epon mix resin, put into matrices and polymerized.

Results and Discussions

The analyses made on as-synthesized powder confirm the laser pyrolysis as a promising technique to synthesize nanoparticle with controller dimension, structure and stability. The mean particle size (around 11 nm) is near the superparamagnetic-border at room temperature for zero valent iron structure and such feature might be optimal

for a bio-medical application (MRI or hyperthermia). For this reason, studying the possibility of encapsulating such nanoparticles into a biocompatible media, may lead to the development of a promising agent in biomedical research.

Some biological features of the erythrocytes obtained at the end of the iron nanoparticle encapsulation procedure were analyzed and compared with those of the native cells by Scanning/Transmission Electron Microscopy and EDX analysis.
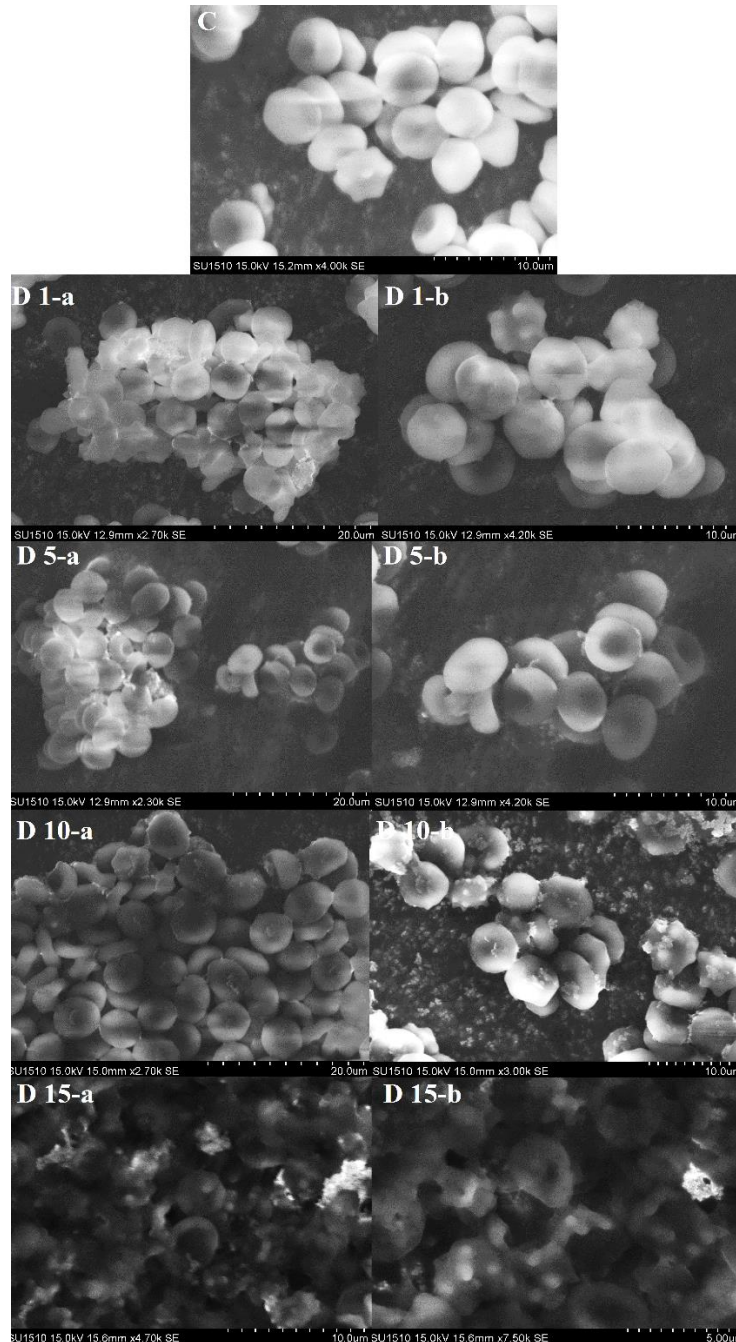
1. Scanning Electron Microscopy (SEM) analysis

Figure 3. Electronic Scanning Microscopy (SEM) analysis for the hipotonic dilution 1 (D-1a and D-1b), dilution 5 (D-5a and D-5b), 10(D-10a and D-10b), and 15(D-15a and D-15b) with carbon-coated iron nanoparticles compared with natives RBCs (C).

The first analysis by scanning microscopy of the RBCs incubated with a suspension of carbon-coated iron nanoparticles in different hypotonic medium dilutions, shows that loaded erythrocytes have normal cell morphology and no significant differences with respect to control cells; the majority of the loaded erythrocytes appeared to have

a biconcave discoid shape with occasionally stomatocytes (hypotonic dilution 1 to 5). It is noted that starting with dilution 7 appears a slight morphological change which at dilution 15 is manifested even by the marked the stomatocytes and echinocytes forms, a phenomenon that characterizes the onset of red cell senescence (Figure 3).

2. EDX analysis with a scanning electron microscope (SEM)

EDX (energy dispersive X-rays) analysis allows the determination of elemental composition at the microscopic scale during the inelastic interactions between the electronic incident beam and matter to be analyzed in SEM (scanning electron microscope) set up.

The EDX analysis of native erythrocytes compared to erythrocytes dialyzed in presence of ferrous nanoparticles in various hypotonic saline dilutions solutions allowed us to evaluate the best dilutions of their inclusion.

It is noted that the native red blood cells, taken as a control sample spectrum, do not contain traces of Fe. The analysis was performed on 10 different samples.

Analysis of the 20 samples of red blood cells by embedding nanoparticles upon dilution 1, showed a composition of (%) in the Fe that varied between a minimum of 10.24% and a maximum of 43.00%, with an average of 19.57%, which denotes an encapsulation of ferric nanoparticles in the blood. For the samples of RBCs from dilution 5 embedding nanoparticles, the percentage composition (%) for Fe ranged from a minimum of 8.14% and a maximum of 69.25%, with an average of 38.58%, which shows a superior encapsulation of ferric nanoparticles in the blood, consistent with the results obtained by the rest of the assays. In the analysis of erythrocytes from the hypotonic saline dilution 10 in presence of ferrous nanoparticles, the percentage composition (%) in the Fe ranges from a minimum of 6.31% and a maximum of 41.94%, with an average of 16.35%, which shows a lower encapsulation of the previous one. RBCs from dilution 15 embedding nanoparticles, the percentage composition (%) for Fe ranged from a minimum of 7.15% and a maximum of 26.62%, with an average of 18.58%, which shows an encapsulation similar to that obtained at dilution 10.
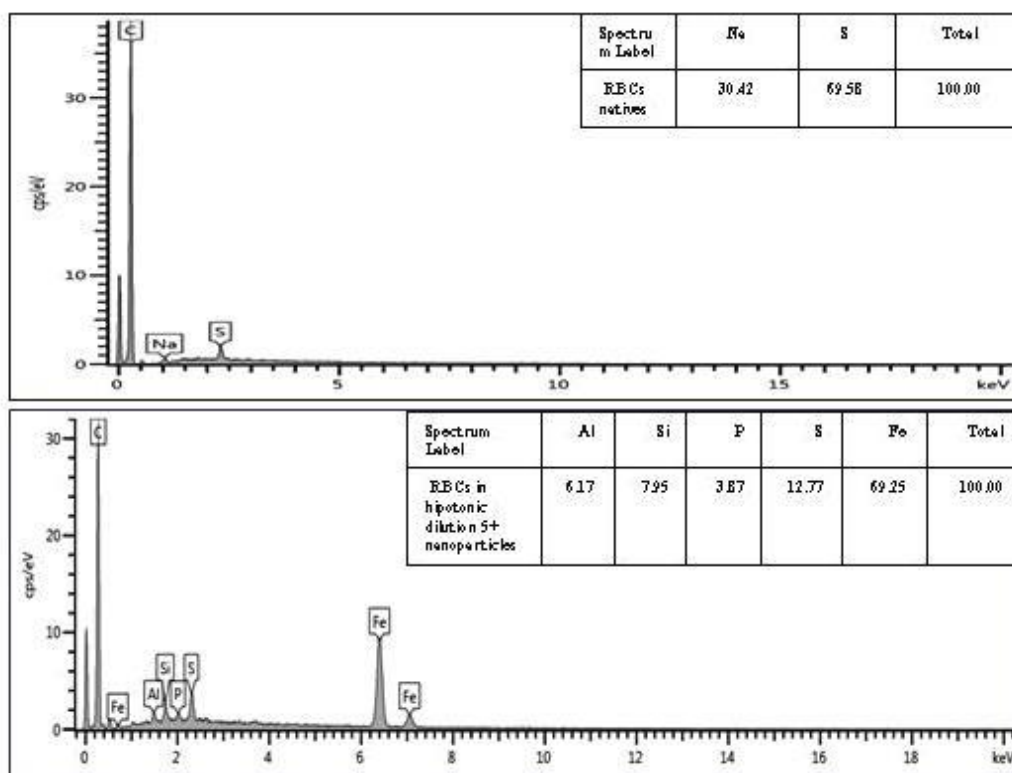

Spectrum Label	Na	S	Total
RBCs natives	30.42	69.58	100.00

Spectrum Label	Al	Si	P	S	Fe	Total
RBCs in hipotonic dilution 5+ nanoparticles	6.17	7.95	3.87	12.77	69.25	100.00

Figure 4. Example of EDX analysis with SEM for entrapment of nanoparticles into erythrocytes at a dilution of 0.7% NaCl (hypotonic dilution 5) compared with native RBCs.

3. Analysis by Transmission Electron Microscopy (TEM) for entrapment of nanoparticles into erythrocytes

TEM images of Figure 5 show the intracellular presence of electron dense nanoparticles in the RBC samples loaded with carbon-coated iron nanoparticles, dispersed into the cell cytoplasm, differ to unloaded cells. As can be seen, in the case of sample of RBCs incubated with nanoparticles in 0.7% NaCl solution is observed the diffusion of nanoparticles into erythrocyte.
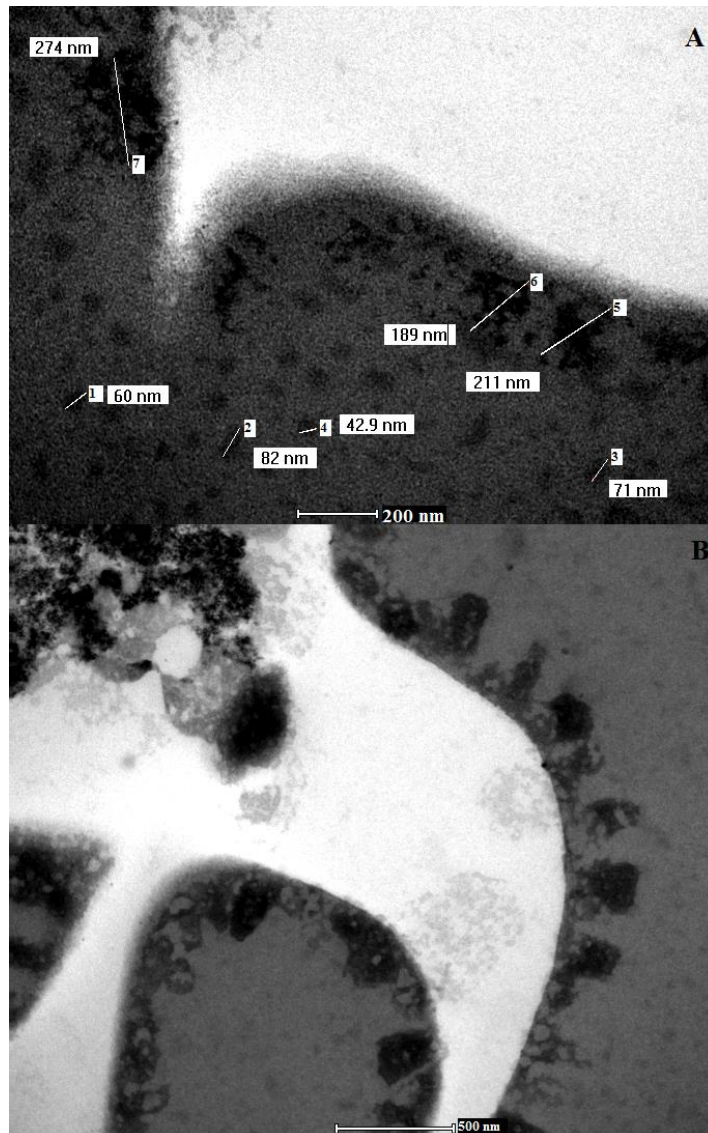
Figure 5. Analysis by Transmission Electron Microscopy (TEM) for entrapment of nanoparticles into erythrocytes at a dilution of 0.7% NaCl (hypotonic dilution 5) and diffusion of nanoparticles through the cell membrane. A - 43 kx magnification, B – 26500x magnification

On the basis of these observations, we have concluded that the best incorporation in erythrocytes being at 0.7% NaCl solution and for this hypotonic dilution, the results were confirmed by analysis of RBCs by flow cytometry in FL1(data not shown). TEM analyses reveal that these carbon-coated iron nanoparticles are not bound on the external erythrocyte membranes, which is an important result because the modification of the cell surface activates the elimination of the loaded cells by the immune system.

Conclusion

In our investigation, we have focused our attention on the possibility to encapsulate new magnetic core-shell iron-based nanoparticles with different structural and morphological features that are preferentially internalized into human erythrocytes through the open membrane pores without affecting the cells.

From the analysis of the obtained results, it is observed that the entrapment of the Fe@C nanoparticles through the action of a hypotonic medium is possible, the optimal saline concentration being around the dilution 5, *i.e.* a concentration of 0.7 g NaCl. At this concentration, SEM analysis showed and confirmed the maintenance of the morphological characteristics of the nanoparticle-bearing erythrocytes and by EDX analysis it has been proven at this dilution embedding carbon-coated iron nanoparticles with the highest yield.

The efficiency of the encapsulation procedure has also been proven by TEM images that clearly demonstrate the intracellular presence of iron nanoparticles, confirming the efficiency of the encapsulation procedure and a uniform

distribution of iron-containing nanoparticles throughout the cell cytoplasm without any accumulation in the RBC membrane. Results of our loading procedure are different from those described by other authors (BRÄHLER et al [34]) who have encapsulated iron oxide nanoparticles. In their RBCs, the iron nanoparticles are also strongly attached to the external membrane that is a restriction for the application of these loaded erythrocytes *in vivo* because their survival in the bloodstream could be compromised by spleen macrophages uptake.

In conclusion, we are confident that these new Fe based nanoparticles that we synthesized and tested could lead to their use in conjunction with the RBCs as promising intravascular imaging contrast agents in biomedical applications.

Acknowledgments

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