

Influence of magnetic iron oxide nanoparticles on red blood cells and Caco-2 cells

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ABSTRACT

The interactions of two types of cells (red blood cells, Caco-2 cells) with magnetic iron oxide nanoparticles (non-grafted, citrate-grafted, dendrimer-grafted) of 11 nm in size have been investigated. We focused on two important physiological parameters of the cells, the intracellular pH and the intracellular Ca²⁺ content. The results show that the nanoparticles do not have a significant influence on the pH and Ca²⁺ content of Caco-2 cells. The Ca²⁺ content of red blood cells is also not affected but the intracellular pH is slightly reduced.

Keywords: Red Blood Cells; Caco-2 Cells; Ca²⁺ Content; Intracellular PH; Magnetic Iron Oxide Nanoparticles

1. INTRODUCTION

One of the main topics in biotechnology relies on the development of various systems, ranging in the nano- and submicronic scales for diagnostics, imaging, drug delivery, or cancer therapy [1,2]. These systems comprise association between organic self-assembled vehicles (vesicles, emulsions, microbubbles, dendrimers) and inorganic nanoparticles. Designing and synthesizing hybrid materials combining at least two of these components are of the utmost importance since such materials can ensure a strong versatility and adaptability. This is the aim of project in which this study has been achieved [Nanomagdye FP7 EU Project grant agreement nr NMP3-SL-2008-214032]. However, before combining each component, the toxicity towards cells has to be studied.

In our studies, magnetic iron oxide nanoparticles non-coated or coated with citrate or dendrons have been

tested on cells. We first investigate the effect on physiological parameters of RBCs. As fundamental physiological parameters, the intracellular Ca²⁺ content as well as the intracellular pH (pH_i) have been chosen. These parameters can be measured using fluorescent dyes. For comparison and as an example of cancer cells, which are targets for nanoparticles in medical treatment, Caco-2 cells have been chosen. It should be mentioned that RBCs and Caco-2 cells differ in one main fundamental aspect – no endocytosis occurs in RBCs whereas Caco-2 cells have such a mechanism [3].

Many publications report experiments on the interaction of targeted tissues or cells and nanoparticles. However, it is sometimes unclear whether these particles show unspecific reactions with other tissues or cells they come in contact with [4]. For example, nanoparticles injected into the blood stream and therefore, coming in contact with red blood cells (RBCs), could adhere to the surface of the cells (and in this case not move to the target cells) or even enter the cells. Such an uptake of nanoparticles by RBCs has been reported by Geiser *et al.* [5] and Rothen-Rutishauser *et al.* [6]. In addition, in both cases nanoparticles could affect physiological parameters of the cells. For instance, it has been reported that iron oxide nanoparticles can increase the membrane permeability of human microvascular endothelial cells [7].

2. MATERIAL AND METHODS

The influence of bare iron oxide nanoparticles (11 nm diameter) and decorated with an organic shell (citrate or dendrimer) on the intracellular pH (pH_i) and the intracellular Ca²⁺ content of RBCs and Caco-2 cells has been investigated.

2.1. Nanoparticles

The bare, citrated or dendronized nanoparticles used were

synthesized at IPCMS, Strasbourg. The non grafted iron oxide nanoparticles were prepared according to the method of Daou *et al.* [8] by coprecipitation at 70°C from ferrous Fe²⁺ and ferric Fe³⁺ ions and a (N(CH₃)₄OH) solution. The nanoparticles were characterized by X-ray diffraction (XRD), magnetic measurements and microscopy techniques. They exhibit the magnetite spinel structure and a lattice parameter of 0.8379 ± 0.0004 nm corresponding to an intermediate phase between maghemite and magnetite written Fe_{3-x}O₄. They are 11 ± 2 nm in size deduced from TEM measurements and XRD characterizations. A generation zero dendritic molecule (C₄₁H₇₇O₂₂P, 953.02 g/mol) was grafted at their surface by mean of a phosphonate coupling agent following the method described by Daou *et al.* [9] and Basly *et al.* [10].

Citrate coated nanoparticles were obtained by introducing sodium citrate in a water suspension of nanoparticles and adjusting the pH at 4.0 with a buffer solution. Then the suspension was mechanically stirred for 24 h, washed with water and centrifuged several times. Water is finally added in order to obtain a stable suspension of citrated nanoparticles.

2.2. Preparation of RBCs

The blood used for experiments was given by healthy human donors, stored at 4°C not longer than 24 h, and washed three times by centrifugation at 2000 g for 5 min at room temperature in a physiological solution containing 145 mM NaCl, 7.5 mM KCl, 10 mM glucose, 10 mM HEPES, pH 7.4.

2.3. Preparation of CaCo-2 Cells

The Caco-2 cells were cultured in Dulbeccos modified Eagle medium. For detaching and separation the cells were trypsinated and washed three times by centrifugation at 2000 g for 5 min at room temperature in physiological solution.

2.4. Loading with Fluorescent Dyes

For intracellular Ca²⁺ and the intracellular pH measurements the fluorescent dye fluo-4 AM and BCECF AM were used, respectively. Stock solutions of the fluorescent dyes (1 mM) in Pluronic F-127 20% in DMSO (Molecular Probes, USA) were prepared. The dyes were added to the washed and re-suspended cells (0.2% v/v) at a concentration of 2 nM. After incubation for 30 min at 37°C the cells were washed three times (2000 g, 5 min) to remove free dye in the medium surrounding the cells.

2.5. Treatment of Cells with Nanoparticles

The cells loaded with the dye (fluo-4 AM or BCECF AM) were incubated in a physiological solution containing 5 -

50 µg/ml of nanoparticles for 30 min at 37°C. The exposure time of 30 min has been selected to be able to measure changes of fast as well as slow processes of cellular reactions.

2.6. Fluorescence Measurements

After the 30 min incubation the cells were transferred on a cover slip and after 5 min (necessary time to let the cells settle down) the fluorescence intensity was measured with a fluorescence microscope (Eclipse TE2000-E, Nikon, Japan) for single cell investigations. Flow cytometry (FACScalibur, Becton Dickinson Bioscience, USA) measurements were carried out to investigate an higher amount of cells. In this case the cell suspension was immediately measured after the 30 min incubation.

2.7. Calibration for pH_i Determination

A calibration curve of the fluorescence intensity depending on pH was created, using the K⁺/H⁺ ionophore nigericin. For this reason 5 µM nigericin was added to RBCs suspended in the calibration buffer solutions containing 135 mM KCl, 10 mM NaCl, 10 mM glucose, 10 mM HEPES/NaOH [11,12]. The calibration curve was linear in the pH range 6.5-8.0.

2.8. Reagents and Dyes

The chemicals used were purchased from VWR (Germany) and Sigma Aldrich (USA). Fluorescent dyes from Molecular Probes (USA) were used for analysing the physiological parameters.

2.9. Statistical Treatment of Results

The data are presented as mean values ± SD of independent experiments. The significance of differences was tested by paired or unpaired Student's *t*-test. Differences were considered significant if *p* < 0.05. Each experiment was repeated at least 3 times on blood from different donors. For Ca²⁺ and pH_i determinations using the fluorescence microscope at least 3 measurements (with blood from 3 different donors and at least 10 single cells from each blood, i.e. 30 single cells) were carried out for each experimental condition. For the FACS experiments 3 measurements (with blood from 3 different donors and 30.000 cells from each blood) were carried out and analysed under each experimental condition.

3. RESULTS AND DISCUSSION

The effect of non grafted, citrate grafted or dendronized nanoparticles on the intracellular Ca²⁺ content as well as on the pH_i of RBCs and Caco-2 cells is shown in **Figure 1**. These two cellular parameters are largely independent from each other and play an important role for many relevant physiological processes. For example, they in-

fluence the activity of cellular and membrane proteins, membrane ion transport as well as other membrane-bound processes (e.g., the interaction of the cytoskeleton with the cell membrane). In addition, changes in the pH_i of RBCs are expected to happen in a relatively short time (some seconds), whereas changes in the intracellular Ca²⁺ content of RBCs are expected to occur in some

minutes only [13,14].

As one can see from **Figures 1(a)** and **1(b)** there is no significant effect of any type of nanoparticles on the Ca²⁺ content of both cell types. However, it must be noted that the error bars (S.D.) are relatively high for RBCs (**Figure 1(a)**). The arbitrary units of the fluorescence intensity of single cells in the presence of the dif-

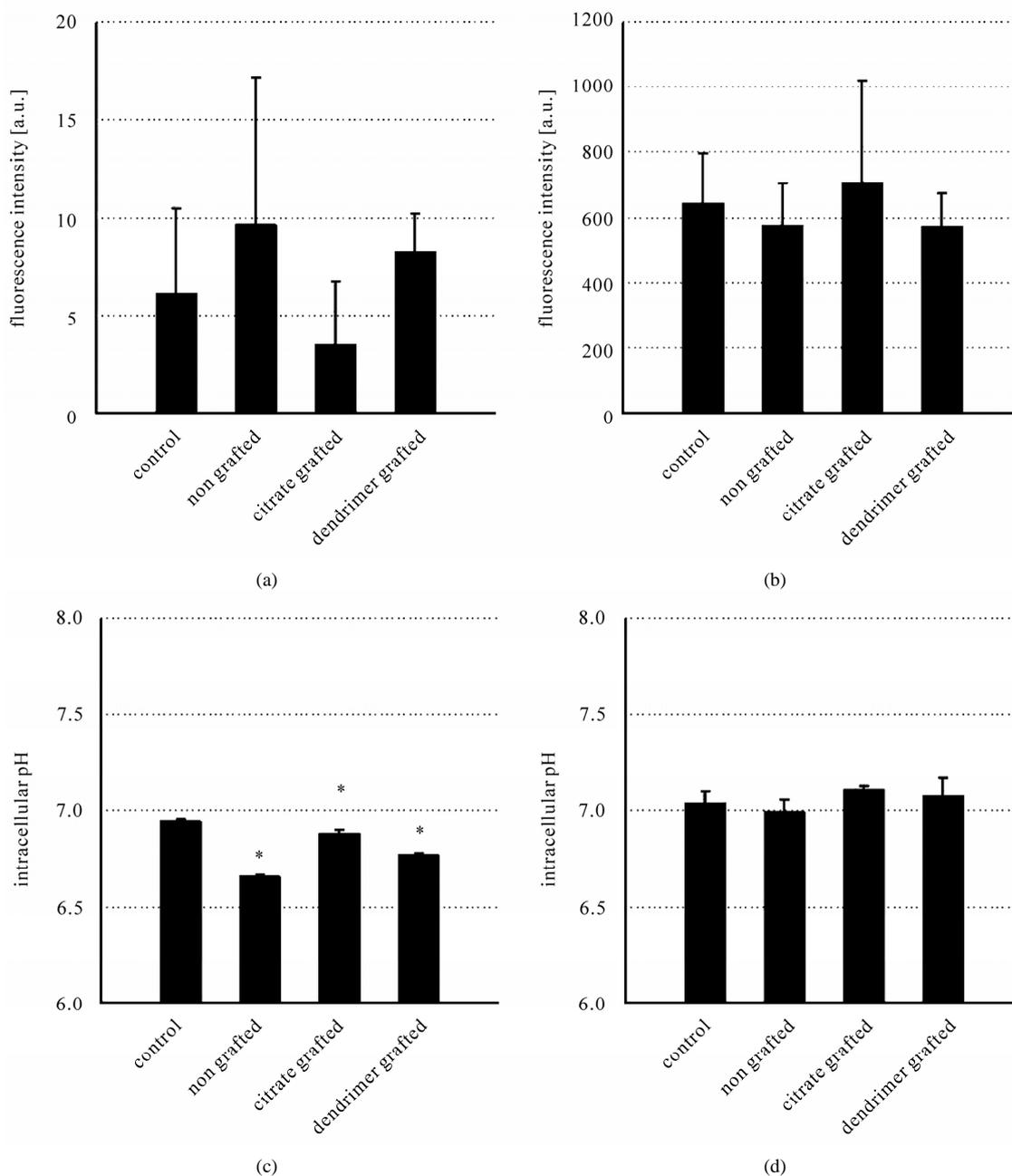


Figure 1. Fluorescence intensity of fluo-4 labelled RBCs (a) and Caco-2 cells (b) as well as intracellular pH (measured with BCECF) of RBCs (c) and Caco-2 cells (d). Measurements have been carried out with fluorescence microscope after 30 min of incubation of the cells with the nanoparticles. Each type of nanoparticles has been investigated with 10-20 cells of at least 3 different bloods. The results are shown as mean values \pm S.D. * indicate values which are statistical significant different from control ($p < 0.01$).

ferent nanoparticles vary only between 0 and 20. The reason is that the intracellular Ca^{2+} content of these cells is very low and close to the detection minimum using fluo-4 as the Ca^{2+} -sensitive fluorescent dye. It is well known that the intracellular Ca^{2+} content of RBCs is very low [15,16] and it has been previously reported by our group that the ratiometric Ca^{2+} -sensitive fluorescent dye fura-2 is not applicable for RBCs and that only fluo-4 is useful to monitor reasonable qualitative changes of the intracellular Ca^{2+} content (see [15] for more details).

To clarify the big variation of the fluorescence intensities of fluo-4, i.e. the Ca^{2+} content of the RBCs shown in Fig. 1a, comparable flow cytometry (FACS) investigations have been carried out. This method allows considering 30.000 cells in one single experiment, i.e. for one blood sample. **Figure 2** shows a characteristic example of such flow cytometry measurement (one of three) for 2 types of nanoparticles in comparison to control. It can be clearly seen that the fluorescence peaks are identical, supporting the finding that there is no significant increase of the intracellular Ca^{2+} content when the RBCs are exposed to the nanoparticles. It should be mentioned that one big advantage of fluorescence microscopy is that single cells can be monitored over a certain time period. This allows seeing differences in cellular reactions and morphological changes and possible destructions of individual cells. In addition, some physiological parameters of RBCs are affected by shear forces [17,18], which might be also the case during the process of cell flow through the capillary in a flow cytometer.

Figure 1(c) shows that the pH_i of RBCs is slightly but significantly lowered after the cells are in contact with the different nanoparticles (compared to the control value). However, such effect in the range of 0.1 to 0.3 pH units seems not of fundamental physiological relevance. It has to be stated that the pH_i value for the control is already about 0.1 to 0.2 units below the expected (literature) value [13]. The small effect of the nanoparticles on the pH_i of RBCs could be due to the fact that RBCs can regulate their pH very efficiently and rapidly. Each RBC contains 10^6 copies of a protein (band 3) responsible for the gas ($\text{HCO}_3^-/\text{Cl}^-$) exchange and some other proteins for pH regulation like the Na^+/H^+ exchanger [14,19]. The band 3 protein occupying a substantial area of the outer membrane surface [20] could be easily affected by the nanoparticles. Although HCO_3^- was not explicitly added to the solutions, there is enough bicarbonate present (from the air-containing CO_2 dissolving in the solutions) that the $\text{HCO}_3^-/\text{Cl}^-$ exchanger is fully functioning. Furthermore, this exchanger also operates as a Cl^-/Cl^- exchanger and therefore normally no

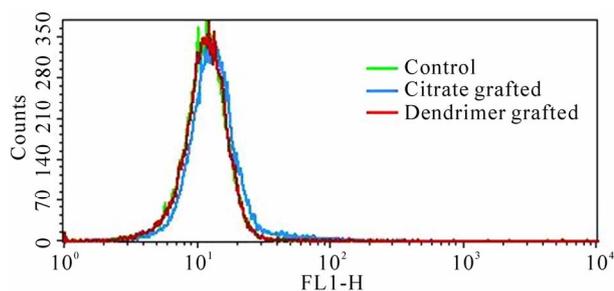


Figure 2. Fluorescence intensity of fluo-4 labelled RBCs. Measurements have been carried out with flow cytometry after 30 min of incubation of the cells without (control) and with nanoparticles (citrate grafted, dendrimer grafted). For each measurement 30.000 cells were analysed. The figure shows one single experiment, representative for 3 different blood samples.

HCO_3^- has to be added when investigating RBC membrane transport [14].

Figure 1(d) shows that there is no significant effect of the various nanoparticles on pH_i of Caco-2 cells.

In the described experiments investigating the effect of nanoparticles on RBCs (Figs. 1a and 1c) no haemolysis was observed. On the contrary, a haemolytic effect of magnetic nanoparticles (magnetite colloidal nanoparticles stabilised with citric acid) on animal RBCs was reported by Creanga *et al.* [21]. However, the authors mentioned that further investigations must be done to explain how nanoparticles can induce haemolysis of RBCs.

4. CONCLUSION

Nanoparticles can influence physiological parameters of cells. In our investigations we found a small but significant effect of nanoparticles on the intracellular pH (pH_i) of RBCs. The pH_i of Caco-2 cells was not affected by the nanoparticles. The intracellular Ca^{2+} content of RBCs as well as Caco-2 cells did not significantly change when the cells were exposed to nanoparticles. Therefore, nanoparticles have to be tested not only with respect to their effect on target cells but also in respect of interactions with cells they can come in contact with, especially RBCs.

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