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Antibody functionalized magnetic nanoparticles for circulating tumor cells detection and capture using magnetophoresis

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Abstract. Circulating tumor cells (CTCs) are cells present in the blood stream during the metastasis process. They can originate from primary or secondary tumors. Circulating tumor cells can be used for early diagnosis or they can be used for prognosis evaluation and even treatment efficiency evaluation. Circulating tumor cells can be captured based on specific antigens found on their surface that differ from those of normal blood cells, they can be captured using specific electrical signatures using dielectrophoresis and they can also be captured using induced magnetic properties and magnetophoresis. In this paper we describe a method for synthesizing and functionalizing superparamagnetic nanoparticles. The nanoparticles will be covered with polyethylenglicol (PEG) molecules to reduce agglomeration and non-specific cell adhesion or blood proteins fouling. The PEG covered magnetic nanoparticles will be functionalized with anti-EpCAM antibodies that are going to make the nanoparticles specifically bind to CTCs present in the blood sample. The samples will be further processed in a microfluidic device that will separate the targeted cells through magnetophoresis.

1. Introduction

In recent years nanotechnology has sky rocketed in the research and industrial fields with chemical, environmental and medical applications. Nanotechnology is the engineering and manufacturing of technologies or materials at nanoscale. One of the materials of great interest is represented by magnetic nanoparticles which gained a lot of attention for different medical applications. They are used for magnetofection, cell labelling, cell separation, cell targeting and controlled drug delivery and release, and localized magnetic hyperthermia for cancer treatment [1]. There are two types of superparamagnetic nanoparticles commonly used in medical applications: maghemite (γFe_2O_3) and magnetite (Fe_3O_4). Also

they can be further devised in superparamagnetic iron oxide particles (SPIOs) with a particle mean diameter >50 nm, ultrasmall superparamagnetic iron oxide (USPIOs) nanoparticles with a mean diameter between 10-50 nm and very small superparamagnetic iron oxide (VSPIOs) with a mean diameter <10 nm.[2] Their application is dictated by properties like the size of the iron oxide crystals, their coating, surface charge and the hydrodynamic size of the particles.[3]

Most of their applications are in magnetic resonance imaging (MRI), since they are being used as contrast agents [4-7]. Xie et al. used PEG, PEG/PEI and PEG/PEI/Tween80 modified superparamagnetic nanoparticles to perform *in vivo* mouse brain MRI. The used nanoparticles demonstrated vascular imaging effects in different lobes of the brain after a 24 hours intravenous injection.[4] Sanjai et al. created superparamagnetic iron oxide nanoparticles encapsulated in chitosan-triphosphate by using an ionotropic gelation method. They demonstrated that their chitosan covered nanoparticles can be used as tissue-specific MRI contrast agent. [8] Unterweger et al. exhibited that their developed dextran coated SPION nanoparticles (SPION^{Dex}) exceptionally safe and perfect candidates for further clinical trials [7].

Because superparamagnetic nanoparticles respond to magnetic fields they are suitable for targeted drug and gene delivery. If stimulated by high-frequency magnetic fields they can also be used for heat-triggered drug release and hyperthermia treatment.[9-12] Apart magnetic hyperthermia there are two other emerging methods for cancer treatment: photodynamic therapy (PDT) and photothermal therapy (PTT) [13, 14]. Magnetic particles have also been used for cell identification and separation techniques [15-20].

Based on their application, iron oxide nanoparticles are synthesized with controlled shape, size and composition. The manufacturing methods can be divided into top-down or bottom-up technologies. Top-down methods refer to obtaining nanoparticles by reducing the size from the bulk materials. Bottom-up technologies refer to creating magnetic nanoparticles from molecular reagents to the desired nano-size. The preferred method is the bottom-up approach, since it gives you a good control over their size and shape. The simplest and most efficient way of producing magnetic nanoparticles is by using the method of iron salts co-precipitation in aqueous solution. The mean diameter of the obtained particles is smaller than 50 nm. However, because of the broad size distribution of particles obtain this way, the nanoparticles require further size sorting to reduce the polydispersity index.[21] The size distribution can be better controlled in the presence of surfactants like alkanolamines,[22] (cetyl trimethylammonium bromide (CTAB), polyvinyl pyrrolidone (PVP) and sodium cholate [23]. Solvents like diethyleneglycol (DEG) can play the role of surfactants. It has chelating properties and it gives you the possibility to work at high temperatures [24].

In this paper we describe a method for synthesizing and functionalizing superparamagnetic nanoparticles for the detection and separation of circulating tumor cells (CTCs). The nanoparticles can be used in a microfluidic device that will separate the targeted cells through magnetophoresis. The nanoparticles will be covered with polyethylenglicol (PEG) molecules to reduce agglomeration and non-specific cell adhesion or blood proteins fouling. In order to make the superparamagnetic nanoparticles attach to the targeted cells, the PEG covered nanoparticles will be further functionalized with anti-EpCAM antibodies that will bind only to the cells with epithelial origin.

2. Experimental

2.1. Materials used for magnetic nanoparticles synthetisation
We synthesized the maghemite superparamagnetic nanoparticles by using the following chemical reagents: $FeCl_3 \cdot 6H_2O$ (236489 Sigma-Aldrich), $FeSO_4 \cdot 7H_2O$ (215422 Sigma-Aldrich), NH_4OH-28% v/v (320145, Sigma-Aldrich), HCl (320331, Sigma-Aldrich), poly(ethylene glycol) diamine (Mn6000) (752444, Sigma-Aldrich) and EDC (39391, Sigma-Aldrich). We functionalized the nanoparticles with anti-EpCAM mouse monoclonal antibodies (BSH-7402-100, Nordic BioSite).

2.2. Superparamagnetic nanoparticles synthesis method

The γ-Fe_2O_3 nanoparticles were synthesized by dissolving $FeCl_3 \cdot 6H_2O$ and $FeSO_4 \cdot 7H_2O$ (Sigma-Aldrich) into DI water (2:1 molar ratio). While stirring the solution, small droplets of NH_4OH were added with a pipette. The precipitate is collected with the help of a magnet and rinsed in DI water.

2.3. PEG covered superparamagnetic nanoparticles synthesis and antibody functionalization method
In order to increase biocompatibility and to reduce agglomeration and non-specific cell adhesion or blood proteins fouling and to facilitate directed antibody functionalization, the superparamagnetic nanoparticles were covered in poly(ethylene glycol) diamine. We did that by mixing 0.675g $FeCl_3 \cdot 6H_2O$, 0.350g $FeSO_4 \cdot 7H_2O$ and 6.0g PEG-diamine in 4 ml (DI water). The reaction was alkalinized with 800µL NH_4OH and then brought to acid pH (4.0) by using HCl (6M). The formed precipitate was separated with a magnet and washed in DI water. Antibodies were crosslinked to the amino groups of the PEG-diamine by using carbodiimides (EDC) crosslinking chemistry.

2.4. Sample characterisation
The bond between PEG molecules and maghemite nanoparticles was analysed by Fourier Transform Infrared (FTIR) spectrometry at room temperature using a Bruker Tensor 27 spectrometer, in the wavenumber 4000-400 cm^{-1} by averaging 64 scans and with a resolution of 4 cm^{-1} and KBr pellet technique. The magnetic characterization of our maghemite nanoparticles and functionalized maghemite nanoparticles with PEG-diamine was made by using the VSM unit from 7T Mini Cryogen Free Measurement System.

3. Results and discussion

3.1. Sample phase analysis
Infrared molecular absorption spectroscopy is an analytical technique used for the qualitative analysis of materials based on absorption spectra produced by vibrating atoms present in different molecular groups. Chemical processes and different chemical structures of chemical compounds can be studied with the help of Fourier Transform Infrared (FTIR) spectrometry. Thus, we obtained FTIR spectra of PEG, maghemite and PEG coated maghemite (Fig.1). PEG spectrum is characterized by spectral bands which can be attributed mainly to the stretching and deformation vibration modes of C-H bonds (in the spectral region 3000-2700 cm^{-1}, 1400-1250 cm^{-1} and 950-840 cm^{-1}). It can also be characterized by the vibration modes of the C-O bonds (1200-950 cm^{-1}) of the ether group. Maghemite spectrum shows spectral bands at 584 cm^{-1} and 444 cm^{-1} that can be attributed to the stretching vibration mode of the Fe-O bond. The spectral bands that are showing from the 4000-800 cm^{-1} range, are attributed to the solvent used during the washing process. It can be seen that the maghemite covered in PEG spectrum is composed of spectral band belonging to organic molecules and inorganic molecules of the sample. A shift can be observed in the spectral bands for both organic and inorganic components. The shift in the spectral band the 1100-1000 cm^{-1} domain and in the 630-400 cm^{-1} domain clearly indicates that PEG-diamine has bonded to the maghemite nanoparticles through Fe-O-C bonds. We can also observe a change in the 1400-1200 cm^{-1} region of the C-H bond vibration mode that shifted to 2950-2700 cm^{-1} region after PEG binding.

   The magnetic characterization of our maghemite nanoparticles and functionalized maghemite nanoparticles with Polyethylene glycol PEG-diamine was made by using the VSM unit from 7T Mini Cryogen Free Measurement System. The blocking temperature is determined by Zero Field Cooling-Field Cooling (ZFC-FC) magnetization measurements. From ZFC-FC measurements, Fig. 2(a), made on our maghemite nanoparticles, 10 nm in diameter, we obtained T_B=252 K. For temperatures larger than T_B a superparamagnetic behaviour is expected that is desirable for biomedical applications.

   The room temperature magnetization curve for our maghemite MNPs, Fig. 2(b), is well fitted by the Langevin function (1) indicating the superparamagnetic behavior of the nanoparticles.[25]
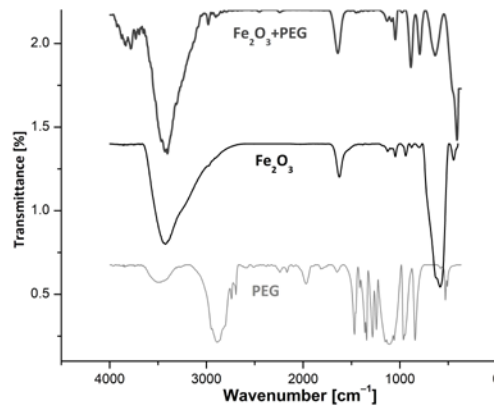
Figure 1. FTIR spectra of PEG, maghemite and maghemite + PEG

$$M(H) \approx M_S\left(1/\tanh(\alpha) - 1/\alpha\right), \; \alpha = \frac{\mu_0 H \cdot m_S}{K_B T}$$

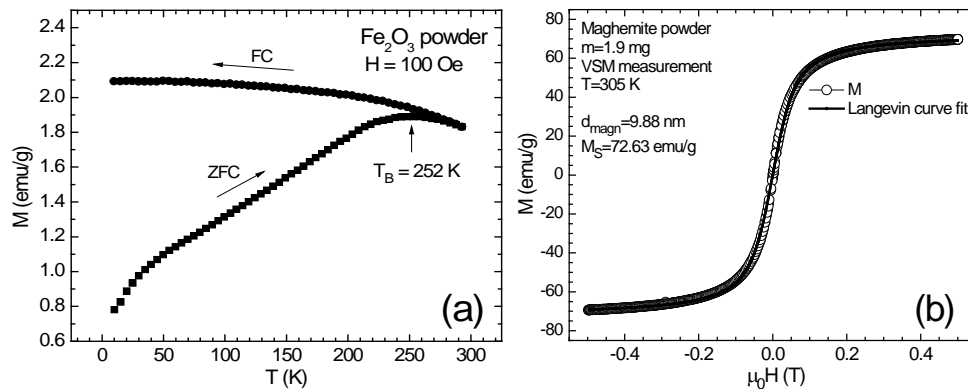where $m_s = \pi d^3 M_S/6$ and $M_S = 480$ emu/cm^3=480 kA/m (for maghemite).

Figure 2. (a) The ZFC-FC magnetization curves and (b) the magnetization curves obtained by VSM for maghemite nanoparticles; the particle diameter is determined from the fitting curve.

From the fitting curve we found an average value of the particle diameter, d_{magn}=9.88 nm. In many applications, like detection of magnetically labelled molecules of interest, the functionalised MNPs are found in aqueous solutions. Fig. 3(a) shows the magnetization curve at room temperature for 30 µl of aqueous solution containing maghemite nanoparticles functionalised with PEG-diamine. By comparing the magnetization curves presented in Figs. 2(b) and 3(a), we can see how large is the diamagnetic contribution of water and PEG molecules at fields higher than 0.1 T. Fig. 3(b) presents the magnetisation curves for the same solution when diamagnetic correction is applied. This behaviour shows that diamagnetic contribution of water and PEG molecules can lower the field produced by the functionalized MNPs when large magnetic fields are used for magnetophoresis and detection experiments. To lower the diamagnetic contribution, the maximum value of the magnetizing field must be carefully tuned. From Fig. 3(a) it comes that optimal value of the applied field in order to get a maximum response from the functionalized MNPs is 0.1 T.

Fig. 4(a) presents the high field magnetisation curve, as measured, for 1.27 mg powder of functionalized maghemite nanoparticles. The high field magnetization curve, allows us to estimate the

diamagnetic contribution of the PEG-diamine which has a linear dependence with the applied field as seen in Fig. 4(a). To obtain 1.27 mg of powder, we used 107.7 mg of aqueous solution which was dried at 56 °C. A higher temperature would destroy the PEG molecule. After applying the diamagnetic correction and fitting the magnetization data from Fig. 4(b) with the Langevin function, we obtained the "magnetic diameter" of the functionalized maghemite nanoparticles as d_{magn}=11.48 nm.
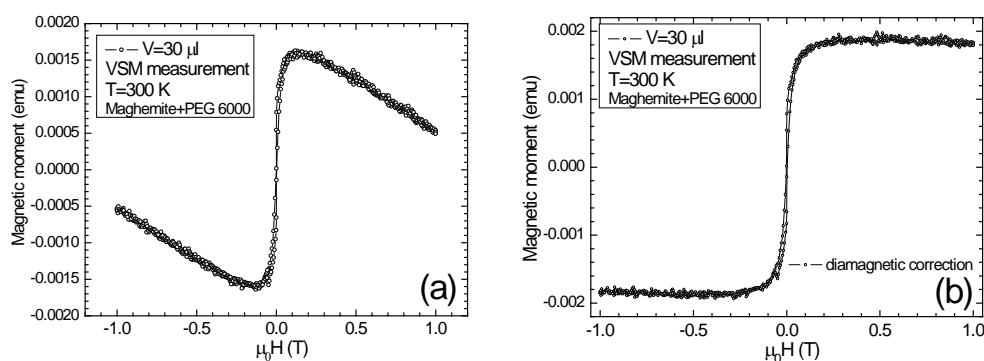

Figure 3. (a) The magnetisation curve obtained by VSM at room temperature for 30 µl of aqueous solution containing maghemite nanoparticles functionalised with PEG-diamine and (b) the same curve when diamagnetic correction is applied.

The PEG-diamine covered nanoparticles have been functionalized with anit-EpCAM antibodies using carbodiimides (EDC) crosslinking chemistry, in order to make the nanoparticles attach to CTCs of epithelial origin. To enhance cell attachment, the antibodies have to be linked with the crystallisable fragment (Fc) to the nanoparticles so that the antigen binding region (Fab), especially the paratope will be facing outwards, so that the availability of antigen binding sites will be maximized. EDC will react with the carboxylic acid (-COOH) that exists at the end of the Fc region of the antibody activating it, thus linking to the NH_2 molecules present on the magnetic nanoparticles. The quantity of used PEG-diamine, EDC, antibodies and the antibody functionalization process needs further optimization.
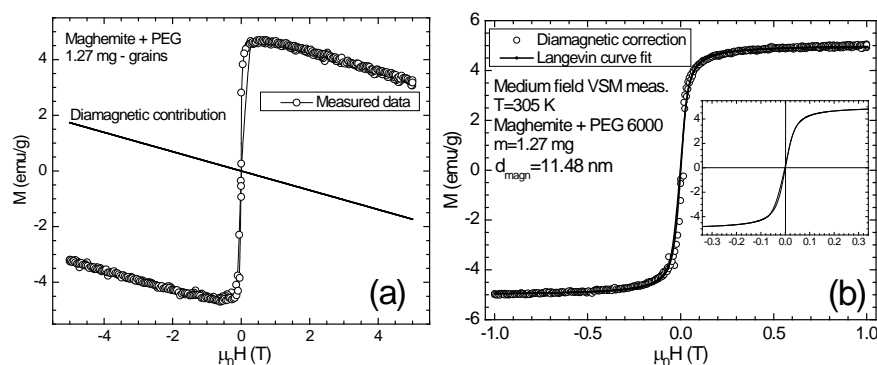

Figure 4. (a) Magnetization curve obtained by VSM for maghemite nanoparticles functionalised with PEG-diamine as powder and (b) the extracted curve after diamagnetic correction; the inset shows the low field magnetization curve.

4. Conclusions

The γ-Fe_2O_3 superparamagnetic nanoparticles coated with PEG-diamine have been synthesized and characterized by FTIR spectrometry and magnetically characterized by using the VSM unit from 7T Mini Cryogen Free Measurement System. Phase analysis was performed by FTIR spectrometry, showing spectral bands at 584 cm^{-1} and 444 cm^{-1} which can be attributed to the Fe-O bond. After PEG-diamine bonding, FTIR spectra demonstrated the formation of Fe-O-C bonds. Magnetic characterization

showed that the diameter of the nanoparticles was d_{magn}=9.88 nm. The diamagnetic contribution of water and PEG molecules can lower the field produced by the functionalized MNPs when large magnetic fields are used for magnetophoresis and detection experiments. The "magnetic diameter" obtained with the Langevin function of the PEG-diamine coated superparamagnetic nanoparticles was d_{magn}=11.48 nm. Future work is focused on optimizing the antibody functionalization process, which will improve CTCs detection.

Acknowledgements

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