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Some biomedical applications of ferrofluids*

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Abstract. Ferrofluids are colloidal solutions of iron oxide magnetic nanoparticles in either a polar or no polar liquid. We present here two biological applications using maghemite (γFe_2O_3) ferrofluids: magnetic cell sorting and magnetocytolysis. The first application employs magnetic particles binding a biological effector, which is capable to recognize the target cells specifically. These cells become magnetic and can be sorted in a gradient of magnetic field. We describe first the various steps of the synthesis of a biocompatible ferrofluid and the grafting an effector protein onto the particles. We then describe the use of particles carrying annexin V in the separation and quantification of damaged erythrocytes in blood samples. This very sensitive technique can be used to follow the erythrocytes ageing of normal blood samples during their storage under blood bank conditions or to detect the membrane modifications that are associated with some pathologies such as *malaria* or *Alzheimer's* disease. The dependence of the magnetic susceptibility *versus* the frequency is a way to transform magnetic energy into thermal energy. Magnetocytolysis is the destruction of cells, carrying magnetic particles, through the action of an alternating magnetic field (about 1 MHz). We present here preliminary experiments with macrophages, which demonstrate the method's feasibility and the formation of the non-specific interactions between the cells and the magnetic particles.

PACS. 75.50.Mm Magnetic liquids – 87.22.Pg Biothermics – 87.15.By Structure, bonding, conformation, configuration, and isomerism of biomolecules

1 Introduction

Over more than fifteen years, our team of chemists and of physicists devoted their research activity to the synthesis and the study of colloidal suspensions of magnetic nanoparticles of iron oxide (ferrofluids). Some of us are interested in the development of biomedical applications of aqueous ferrofluids as magnetic vectors, in association with biochemists of INSERM. These vectors are used in various areas:

- *in vitro*
  - magnetic cell sorting of biological molecules or cells labelled by magnetic vectors bearing specific effectors,
  - magnetocytolysis: destruction of magnetic vectors bearing cells in an alternating magnetic field;
- *in vivo*
  - medical imaging: either MRI (superparamagnetic compounds are used as contrast agents) or scintigraphy in a magnetic field (magnetic vectors associated with radioisotopes),
  - therapeutics: drugs or radioisotopes targeted by magnetic guidance.

Commercialy available magnetic vectors are destined mainly for the cell sorting or MRI. They contain frequently a core of magnetite, Fe_3O_4, or maghemite, γFe_2O_3.

The magnetic vectors belong to one of the main groups: magnetic nanoparticles, magnetic microspheres and magnetoliposomes. Magnetic nanoparticles are often coated with various polymers such as dextran [1–5], albumin [6–8], organosilanes or methacrylates [9–11]. The size distribution is quite broad and the mean diameter varies from several nm for the individual particles, to about a hundred nm for the aggregats. With such a small size, they can be used either *in vitro*, mainly for the cell sorting (MACS® from Miltenyi), or *in vivo*, mainly for MRI (Endorem® from Guerbet). Microspheres (such as Dynabeads® from Dynal) are monodisperse polymer beads (with a diameter of several μm) in wich magnetic iron oxide is dispersed. They are used for the cell sorting and to remove biological target molecules from heterogeneous suspensions. Magnetoliposomes [12,13] are phospholipid vesicles, filled with ferrofluid. These compounds, not yet marketed, are intended for the magnetic guidance of drugs.

An example of magnetic cell sorting and preliminary assays of magnetocytolysis are presented in this work. For the cell sorting, magnetic vectors must be labelled by biomolecule (the effector) able to recognise an accessible receptor of the target cell (*cf.* Fig. 1). We describe

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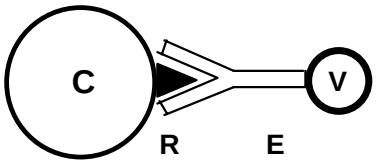


Fig. 1. Magnetic vector (V) linked with target cell (C) by the intermediary of a membrane receptor (R) and a specific effector (E).

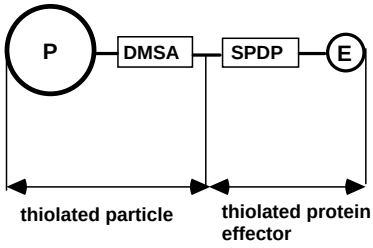


Fig. 2. Synthesis plan of effector-particle conjugate.

first, the synthesis of an aqueous ferrofluid of maghemite nanoparticles (precursor ferrofluid) and their ligation with proteins which serve as effector.

2 Synthesis of effector-particle conjugates

The linkage between the effector and the particle must respect three prerequisites: the linkage must be stable, the biological specificity of the effector must be preserved, and the effector-particle conjugate must be peptizable in biological liquids.

For these reasons we have chosen to thiolate the particles with dimercaptocuccinic acid (DMSA) and the protein with N-succinimidyl 3-(2-pyridyldithio) propionate (SPDP) and to link them by a disulfure bridge (*cf.* Fig. 2). The first step is the synthesis of maghemite nanoparticles and their treatment with DMSA to form a precursor ferrofluid. The second step is the grafting of the effector thiolated with SPDP, onto the particles.

2.1 Synthesis of precursor ferrofluid

2.1.1 Synthesis of acidic ferrofluid

By a process perfected in our laboratory [14,15] we obtain maghemite nanoparticles peptizable in acidic medium, without any surfactants.

The first step is the formation of magnetite particles by precipitation of a stoichiometric mixture of ferrous and ferric chloride, in an ammoniac medium. The second step is the oxidation of magnetite particles into maghemite using ferric nitrate in acidic medium. The diameter of the maghemite particle varies between 3 and 15 nm (the mean diameter is 7.5 nm). Each nanoparticle is a monodomain and the magnetic moment m_s is given by:

$$m_\mathrm{s} = M_\mathrm{s} V$$

where M_s is the bulk magnetisation and V the volume of the particle.

A cationic ferrofluid is obtained by a spontaneous dispersion of the particles in an dilute nitric acid solution. In aqueous media, amphoteric hydroxyl groups are linked with superficial iron (III) atoms (represented here by $\equiv$ Fe–OH). The particles are positively charged in acidic medium and negatively charged in an alkaline medium:

$$\equiv \mathrm{Fe\text{–}OH} + \mathrm{H_3O^+} \rightleftarrows \equiv \mathrm{Fe\text{–}OH_2^+} + \mathrm{H_2O},$$
$$\equiv \mathrm{Fe\text{–}OH} + \mathrm{OH^-} \rightleftarrows \equiv \mathrm{Fe\text{–}O^-} + \mathrm{H_2O}.$$

The isoelectric point (IEP) is at pH 7.5. The electrostatic repulsion between charged particles is responsible for the stability of the aqueous ferrofluids. Between pH 6 and pH 10, the superficial charge density is too low and the particles are flocculated.

2.1.2 Treatment of the particles with DMSA

Stable ferrofluid at pH 7 can be obtained by substitution of superficial hydroxyls with ligands such as tartaric acid [HOOC–CH(OH)–CH(OH)–COOH], represented as $\mathrm{LH_2}$ [16]:

$$\equiv \mathrm{Fe\text{–}OH} + \mathrm{LH_2} \rightleftarrows \equiv \mathrm{Fe\text{–}LH} + \mathrm{H_2O},$$
$$\equiv \mathrm{Fe\text{–}LH} + \mathrm{OH^-} \rightleftarrows \equiv \mathrm{Fe\text{–}L^-} + \mathrm{H_2O}.$$

With DMSA [HOOC–CH(SH)–CH(SH)–COOH], this type of substitution is observed but in addition, the adsorption of DMSA is accompanied by an oxidation of thiol groups of DMSA by the surface Fe(III) atoms and a limited dissolution of the particles [17] according to the reaction:

$$4\mathrm{DMSA} \rightleftarrows (\mathrm{DMSA_{ox}})_4 + 6\mathrm{H^+} + 6e^-$$
$$\mathrm{Fe_2O_3} + 6\mathrm{H^+} + 2e^- \rightleftarrows 2\mathrm{Fe^{2+}} + 3\mathrm{H_2O}$$

with the balance:

$$4\mathrm{DMSA} + 3\mathrm{Fe_2O_3} + 12\mathrm{H^+} \rightleftarrows (\mathrm{DMSA_{ox}})_4 + 6\mathrm{Fe^{2+}} + 9\mathrm{H_2O}.$$

The adsorption of the anionic polydisulfides [designated by $(\mathrm{DMSA_{ox}})_4$] in acidic medium, results in a flocculation of the particles. A stable precursor ferrofluid is obtained after dissolution of the flocculate at pH 9 followed by neutralization. For this ferrofluid at pH 7, the polydisulfide molecules adsorbed onto the particle are organized as represented Figure 3.

The resulting precursor ferrofluid is stable in aqueous medium between pH 3 and pH 10 and in usual buffers at ionic strength up to 0.45 mol/l NaCl; it is heatresistant up to boiling and can be stored many months at 4 °C when protected from air.

The mean hydrodynamic diameter of the particles measured by photocorrelation spectroscopy is 50 ± 5 nm and the mean diameter of the solid core measured by electron microscopy is about 7.5 nm.

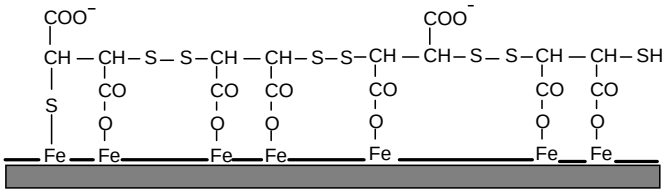
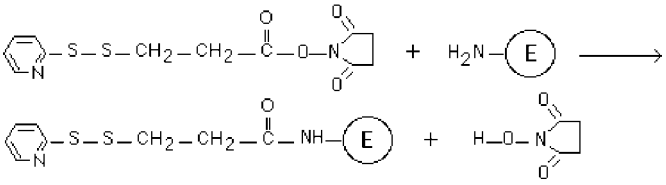


Fig. 3. schematic representation of polydisulfide molecule ($DMSA_{ox}$) adsorbed onto the maghemite nanoparticle surface.

2.2 Synthesis of thiolated protein effector

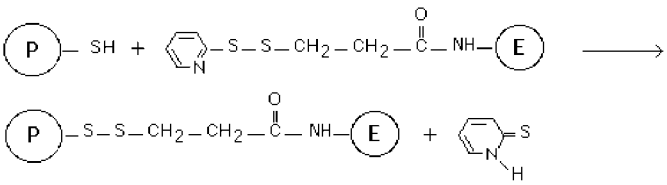
Most of the biological effectors grafted onto magnetic carriers are proteins. As can be seen in Figure 3, some SH thiol on the surface of magnetic particles remain non-complexed and can be used to bind the effector.The ratio [SH]/[Fe] lies in the range of 0.05% to 0.3%. Grafting of proteins to the particles was achieved through the use of an heterobifunctional agent such as N-succinimidyl-3-(2-pyridyldithio)propionate (SPDP).

SPDP is first coupled with the effector (E-NH2) through an amide bridge:



2.3 Synthesis of particle – effector conjugate

The thiolated effector reacts with the residual -SH groups on the magnetic particle (P-SH) to form a disulfide bridge according to the reaction:



Under our experimental conditions, each particle is bound on the average to one effector molecule.

3 Biomedical applications

We present here, two aspects of biomedical applications of magnetic nanoparticles:

– the first one is the cell sorting of biological cells: magnetic particles linked to the membrane of a cell permit to obtain magnetic cells which can be acted by a gradient of magnetic field. The magnetic force $F_{\rm m}$ is given by:

$$F_{\rm m} = \mu_0 N m_{\rm s} \nabla H$$

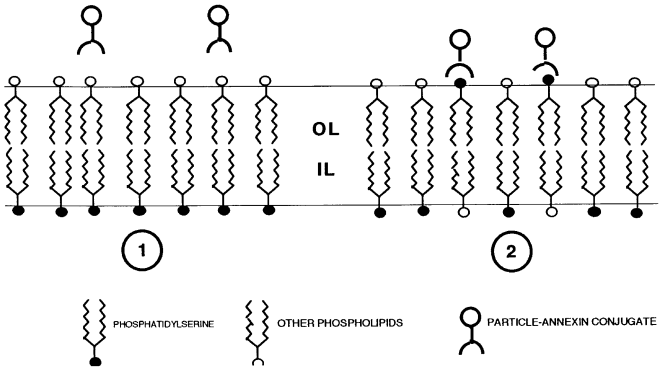


Fig. 4. Labeling of damaged erythrocytes with magnetic particle. Annexin conjugate (1): normal. Erythrocyte membrane (2): damaged erythrocyte membrane. OL: outer leaflet. IL: inner leaflet.

where N is the number of nanoparticles linked to the cell and $m_{\rm s}$ the magnetic moment of one particle;

– the second one is to use the behavior of the magnetic susceptibility $\chi(\omega)$ *versus* the frequency ω:

$$\chi(\omega) = \chi_0 / \left(1 + i\omega\tau_{\rm N}\right).$$

For superparamagnetic particles, which are the case of maghemite, the magnetic moment is governed by the Neel time $\tau_{\rm N}$:

$$\tau_{\rm N} = \tau_0 \exp(+E_{\rm a}/KT) \quad \text{with} \quad \tau_0 \cong 10^{-9}\ \text{s}.$$

$E_{\rm a}$ is the anisotropic energy and KT the thermal energy.

For a frequence ω, such as $\omega\tau_{\rm N} \cong 1$, magnetic energy is transformed in thermal one; this effect increases the temperature of the particles and then, the temperature of the cell (magnetocytolysis).

3.1 Numeration of damaged erythrocytes

Normally, phospholipids are organised asymetrically in cell membranes: phosphatidylcholine and sphingomyelin are found on the outer leaflet of the plasma membrane and phosphatidylserine (PS) only on the inner one, facing the cytoplasm. This asymetric distribution is disrupted in erythrocytes either in certain pathologies or in the course of their aging, notably during blood conservation *in vitro*, resulting in a randomization of PS distribution in cell membranes. The same phenomenon is also observed during apoptosis and in activated platelets. The presence of PS on the outer leaflet can be detected by annexin V. Annexins belong to a family of ubiquitous intracellular proteins having in common the ability to bind specifically to phosphatidylserine and other anionic phospholipids, in presence of Ca^{2+}.

The use of annexin V as an effector grafted onto magnetic nanoparticles allows the targeting (Fig. 4), the magnetic separation and the numeration of damaged RBC.

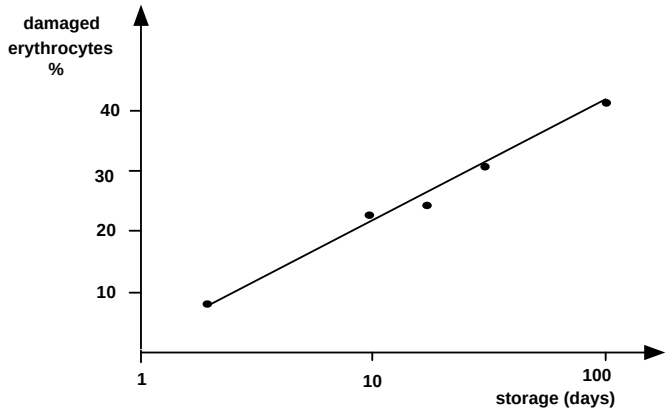


Fig. 5. Numeration of damaged erythrocytes *versus* storage duration at 4 °C.

The high sensibility of this method allows its use to detect damaged RBC in various cases:

- quality test of human blood during storage *in vitro* at 4 °C under blood bank conditions [20] (Fig. 5);
- evolution of erythrocyte populations during parasitic pathologies such as *malaria* [21] or degenerative such as *Alzheimer's* disease [22];
- detection of membrane modifications involved in complex biological process such as apoptosis which is an extensively studied phenomenon aspecially in cancer research.

This method is patented [18] and the marketing of magnetic particle-annexin conjugate like is being evaluated.

3.2 Magnetocytolysis: preliminary experiments

Magnetocytolysis (or thermocytolysis) is the destruction, of ferrofluid-bearing cells in an alternating magnetic field.

Our first experiments [23,24] have been carried out on human or mouse macrophages with two different kinds of ferrofluids designated: FF-DMSA and FF-DTT.

- FF-DMSA is the precursor ferrofluid, where particles have only few strong non specific bonds with cells ($[SH]/[Fe] \leq 0.3\%$).
- FF-DTT is a precursor ferrofluid treated with dithiotreitol (DTT) to break the disulfur-bridges of the ligands adsorbed onto FF-DMSA particles. The ratio [SH]/[Fe] is then about ten times higher. In this case, particles can bind with cells by the intermediary of –S–S– bridges.

Without magnetic field or in a constant magnetic field no cytolysis or toxic effect is observed with FF-DMSA or FF-DTT. Under an alternating magnetic field of 100 Oe at a frequency of 1 MHz, the temperature of a concentrated ferrofluid ($[Fe_{total}] = 1$ mol/l) increases from 37 °C to 80 °C in about 2 minutes. However, for magnetocytolysis experiments, ferrofluids are used at so low concentrations ($[Fe_{total}] = 5 \times 10^{-6}$ mol/l) that no increase of temperature can be detect in the bulk solution.

The cells are incubated 1 hour at 37 °C with ferrofluid. The alternating field is then applied for 5 minutes. The surviving cells are counted at various times. In these conditions, it has been observed that 40 to 50% of the mouse or human macrophages are killed but only 3 to 6 hours after the magnetic field application. These results show that the binding of the particles onto the cell membrane and perhaps their phagocytosis are essential for the cytolysis.

These trials show that cytolysis under an alternating magnetic field can be realised but many questions are still unanswered:

- is it possible to realize the magnetocytolysis of other cells, recognized by specific effectors?
- where (intra or extracellular) and how (individual or aggregated) are magnetic particles active?
- is there, on the cellular level, an increase of temperature and if there is what is its range?
- why cytolysis is observed only after several hours?

In an attempt to answer to these questions, we are using other techniques such as electron microscopy and ferromagnetic resonance [25].

4 Perspectives: in vivo application

Interesting biomedical applications of magnetic nanoparticles are in specific imaging, concentration of drugs and radioelements to a given tissue and magnetocytolysis. They all depend on a successful targeting of cells and tissues.

For *in vivo* applications of ferrofluid, the study of the biocompatibility and biodistribution is a prerequisite. Most of the intravenously injected magnetic vectors are recognised by the body as being foreign with the ensuing immun reactions. They must be able to reach target cells before their hepatic sequestration. To this end, the particles must be free of non specific interactions with blood components and hepatic macrophages. It has been shown that the biodistribution of the particles depends upon their size and their surface state [26]. A final treatment of the particles can be useful to mask the excess of reactive chemical groups and suppress non-specific interactions between particles and plasma proteins. Unlike the case of nanoparticles coated with macromolecules where effector is fixed to these macromolecules, the treatment of our particles can be easily adjusted for the intended use without change of effector nor of its binding to particles.

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