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# Magnetite Nanoparticles for Cell Lysis Implanted Into Bone - Histological and TEM Study

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## 1. Introduction

Magnetite nanoparticles are frequently used to eliminate by heating in a high frequence oscillating magnetic field the tumor cells into which they are introduced in order to directly kill the cells or to make them more sensitive to radiotherapy (Ito, A., et al., 2005; Jordan, A., et al., 2001).

The appearance of bone metastases is a sign of a dissemination of primitive cancers. They rapidly become resistant to chemotherapy and radiotherapy and are often very painful necessitating local and/or alternative treatments in order to reduce the osteolysis triggered by the cancerous cells. The osteolysis is due to local activation of the osteoclasts and macrophages by factors synthesized by the tumor cells (Shimamura, T., et al., 2005). It is the osteolysis that is very often responsible for the pain.

We have developed a biomaterial containing magnetite nanoparticles which can be introduced into bone metastases in order to release naked nanoparticles in the contact with both the cancerous and the osteolytic cells. The material is made of a calcium sulphate paste containing a small percentage of nanoparticles which can be injected inside the metastasis (fig. 1). It sets within a few minutes *in situ*. The degradation of the calcium sulphate matrix within a few days releases the nanoparticles which are then available for cell internalisation. *In vitro*, these particles can be internalised in high amounts by metastatic cells from adenocarcinoma. The number of nanoparticles found inside the cells depends on the nanoparticle size, however the mass internalized seems to be almost independent of their size (Frayssinet, P., et al., 2005).

The nanoparticles did not show *in vitro* any signs of cell toxicity. This is consistent with previous reports which showed that cytotoxicity of magnetite nanoparticles could be due to several factors such as coating (Häfeli & Pauer, 1999). Furthermore, they are intented for use in very low doses (a few mgs). The degradation products of iron oxide are well known. They do not have a reported toxicity and are easily eliminated from the organism (Schoepf, U., et al. 1998, Okon, E.E., et al. 2000, Okon, F., et al. 1994).

Migration of the nanoparticles can however be a cause of concern due to the possible unwanted heating of other regions of the organism when submitted to a magnetic field.

When injected as a suspension in the blood, they were shown to be mostly taken up by the spleen and liver in the days following injection (Magin, R.L. et al., 1991). They can also migrate into the lymph nodes when directly injected into tissues.

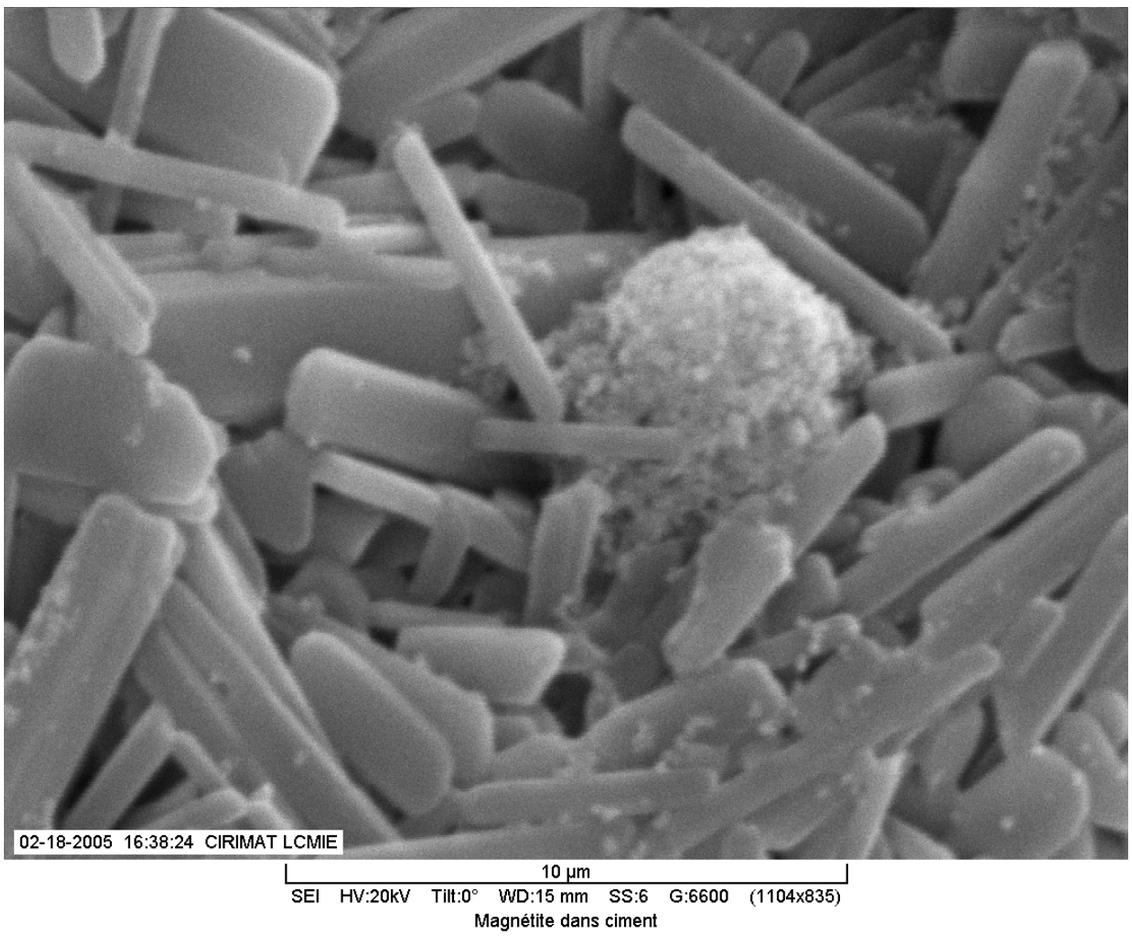


Fig. 1. SEM of calcium sulphate (flat crystals) matrix containing nanoparticles of magnetite. Isolated nanoparticles can be evidenced at the surface of the calcium sulphate crystals or as agregates between the crystals.

We have demonstrated that the nanoparticles used in this device penetrate adenocarcinoma cells by endocytosis *in vitro* (Frayssinet et al. 2004). The aim of this experiment was to check the uptake of these nanoparticles by the various types of bone tissue cells *in vivo,* their migration in the lymphatic tissue and the time *needed* for their *elimination*.
Thus, the nanoparticles were introduced through a bone defect drilled into the cancellous bone while the implantation zone and lymph nodes draining the region were examined by light and transmission electron microscopy.

## 2. Materials and methods

### 2.1 animal model

In order not to lose the nanoparticles, they were placed inside an open titanium alloy chamber implanted inside the cancellous bone of external condyles in the sheep. A titanium

alloy was used because it was demonstrated that there was no or a very limited foreign body reaction against this kind of alloy and device implanted inside the bone (Aspenberg et al., 1996). The chamber in which the particles were inserted was tunnel shaped and open at the ends communicating with the bone allowing tissue at all stages of bone regeneration to cross the tunnel and come in contact with the nanoparticles . Using this device it is straight forward to locate the nanoparticles for histological and TEM sections.

The device containing the chamber can be screwed into the cancellous bone to avoid any micromovement between the bone and the chamber which would shear the regenerating tissue. It can also be opened to facilitate the collection of the tissue to analyse (fig. 2).

Two sheep were used for each sample implanted for a 3 weeks. 0.1 mg of sterile nanoparticles were placed inside the chambers which were then implanted in the external condyle by a lateral approach.

After the three-week exposure the animals were killed by a Nembutal injection and the chambers the inguinal and aortic lymph nodes draining the implantation zone retrieved. The operation and the care of the animals followed the European commission guidelines concerning animal experimentation.

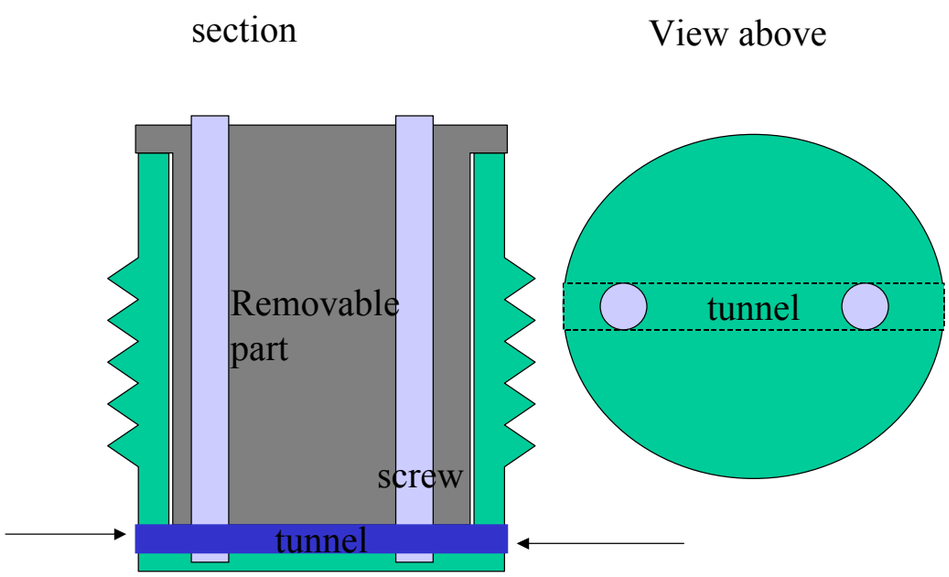


Fig. 2. The titanium device containing the tunnel in which the nanoparticles were introduced was screwed in the cancellous bone of the condyles. The healing tissue ingrews the tunnel according to the arrows. After the implantation period, the removable part was extracted to open the tunnel and the tissue containing the nanoparticles was available for histology.

### 2.2 Magnetite powder characteristics

The powders were constituted by $Fe_3O_4$ (99%). Their shape was polyhedral. Three different particle size groups were used: 70 nm, 150 nm, 500nm.

### 2.3/ Histological methods

The retrieved tissues were immediately immersed in isotonic phosphate buffer containing 2% glutaraldehyde for 2 days at 4°C. All the samples were then cut and either processed for light microscopy or TEM.

For light microscopy, the samples were dehydrated in increasingly concentrated ethanol solutions and embedded in hydroxyethyl methacrylate. 5 µm thick sections were cut and coloured with Perl's stain to reveal the nanoparticles in the sections. The tartrate resistant acid phosphatase activity (TRAP) of the cells was evidenced using a commercial leukocyte acid phosphatase kit (Sigma, St Louis, MO).

For TEM, the sections were dehydrated in increasingly concentrated ethanol solution and embedded in an epoxy polymer before ultrathin sections were performed and stained with uranyl acetate. Observations were made at 20 kV.

## 3. Results

### 3.1 Bone healing in the titanium tunnels

After the three-week experimental period, the tunnel was filled by loose connective tissue which did not show any signs of ossification.

Under light microscopy, the tissue in the tunnels showed that numerous cells contained nanoparticles. Some of the cells showed a black tattoo while in others the nanoparticles were not visible but were revealed by the blue-grey color of the cell after Perl's staining (fig. 3). The nanoparticles were either contained inside $TRAP^+$ or TRAP- cells. These $TRAP^+$ cells were uniformly dispersed inside the tunnel volume and were not aggregated around the foreign material as is usual for a foreign body reaction (fig. 4). The tissue appearance was the same for the three size of nanoparticles.

Under TEM, all tissues present in the tunnels contained cells loaded with the nanoparticles. It seemed that the smallest particles (70nm) penetrated the cells in larger numbers than the biggest ones (500 nm); at least their density was more uniform inside the cells. The 500 nm sized particles formed smaller aggregates in the cells than the 70 nm ones. These late particles could constitute large aggregates at the contact of which multinucleated cells were found. The cells dissociated or fragmented the aggregates before internalising the nanoparticules (fig. 5).

Inside the cells, the nanoparticles were found inside lysosomes, or phagolysosomes depending on the size of the particle aggregates. There were almost no particles alone inside cell vesicles (fig. 6). They occurred in groups of a few units to several dozen.

### 3.2 Lymph nodes

Using light microscopy, it appeared in most sections that nanoparticles had migrated to the lymph nodes after the three-week contact. Their number was very low, as only a few cells on each section were blue from Perl's stain. The nanoparticles were not found in the lymphoid follicles but in the capsule and subcapsular sinus. The migration was identical for the three types of nanoparticles.

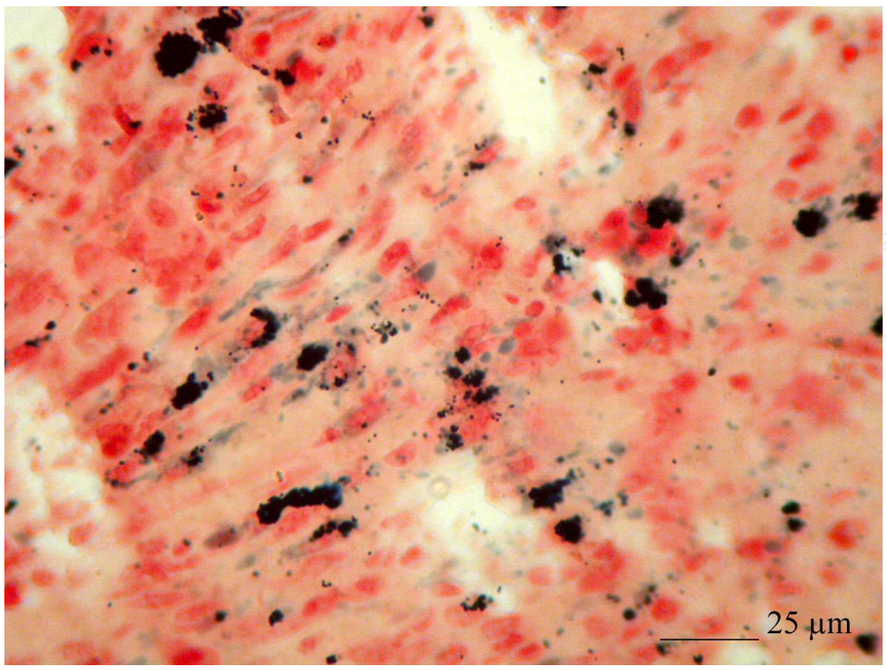


Fig. 3. The particles (70 nm) under light microscopy appear uniformly dispersed in the ingrown tissue in the titanium chambers. They can form aggregates inside the cells which are visible under the microscope or with Perl'staining a kind of blue grey tattoo

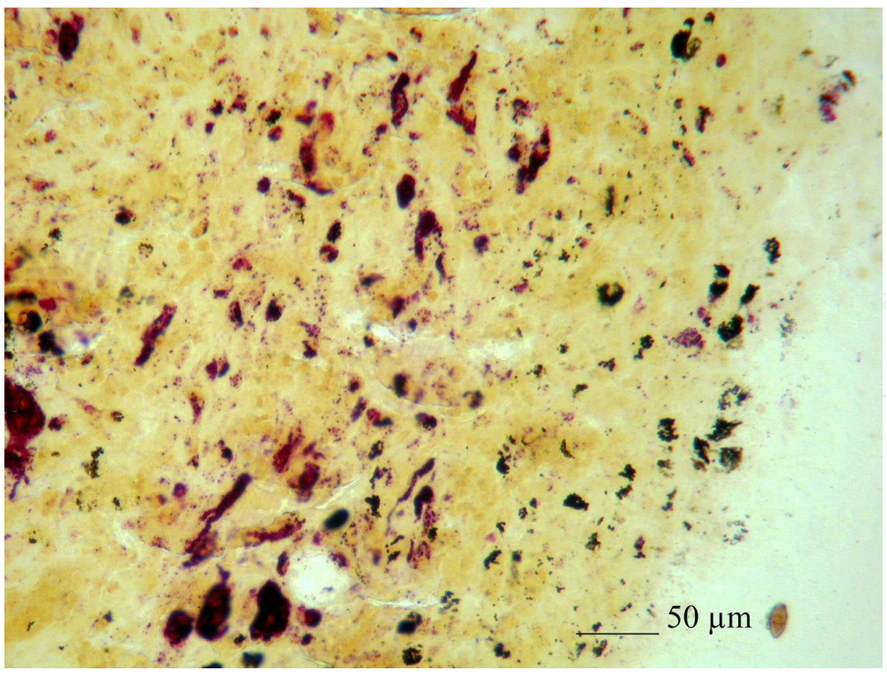


Fig. 4. Section of a Ti chamber containing 70 nm particles showing the TRAP+ cells in red. The particles are not necessarily internalised within the TRAP+ cells.

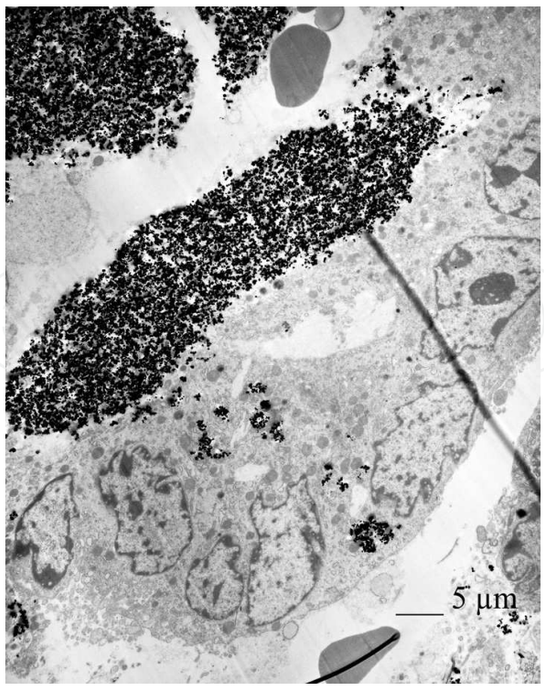


Fig. 5. When aggregates of 70 nm sized nanoparticles are formed, there can be multinucleated cells in contact with the material. The aggregate is dissociated by the cells which internalize the nanoparticles under the form of much smaller groups of particles.

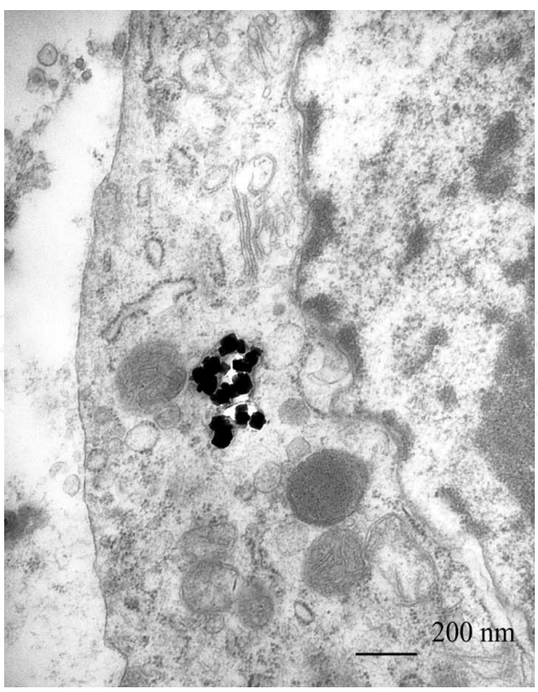


Fig. 6. Inside the cells the magnetite particles are found inside cell vesicles such as endosomes, lysosomes or phago- lysosomes.

By TEM, the nanoparticles were observed in the vesicles of macrophages and lymphocytes (fig. 7, 8,9, 10). All the particles seen in the lymph nodes showed one or several degradation signs. Nanoparticles of all three sizes were found in the lymph nodes, however in very low number.

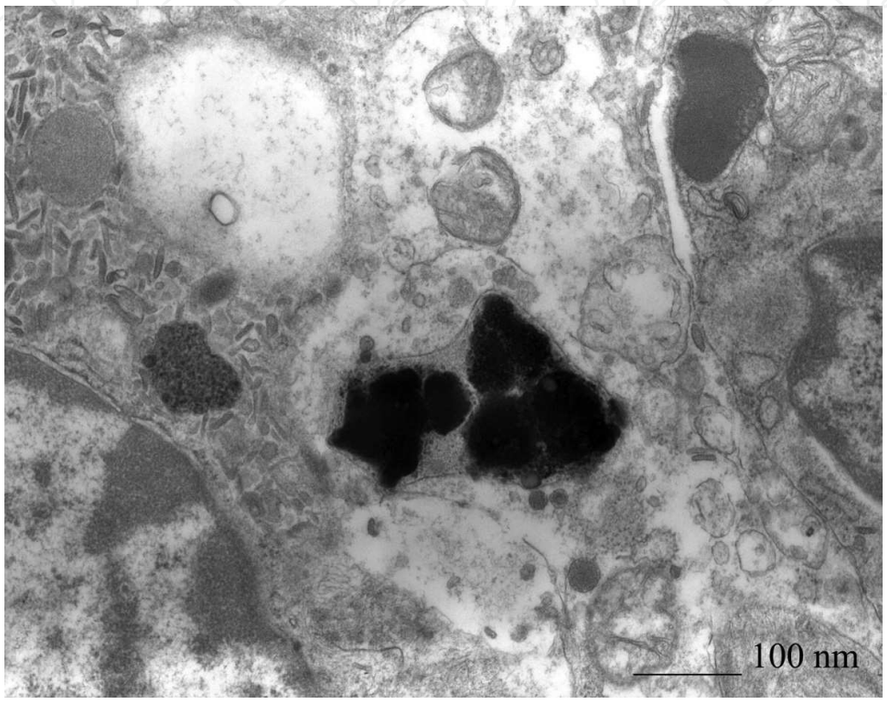


Fig. 7. TEM of 70 nm particles observed inside the lysosome of a lymph node macrophage. The particles show fuzzy edges, a modified shape and some have merged.

### 3.3 Degradation

Signs of nanoparticle degradation were evidenced both in the bone tissue and the lymph nodes (fig. 7, 8, 9, 10). The degradation took place in the cell vesicles. The big particles were sometimes fragmented. The small ones became fuzzy and lost their shape suggesting that material had dissolved. Very fine and needle like particles were sometimes observed in the periphery of the nanoparticles suggesting precipitation. Most of the particles, whatever their size, showed a modification of the shape and some particles fused together.

## 4. Discussion

Direct implantation of magnetite nanoparticles into the tumor instead of injection into bloodstream avoids their concentration inside liver, lung or spleen. It allows the use of lower doses of magnetite limiting its toxicity, if any, and side effects such as heating of the reticulo endothelial cells in these organs.

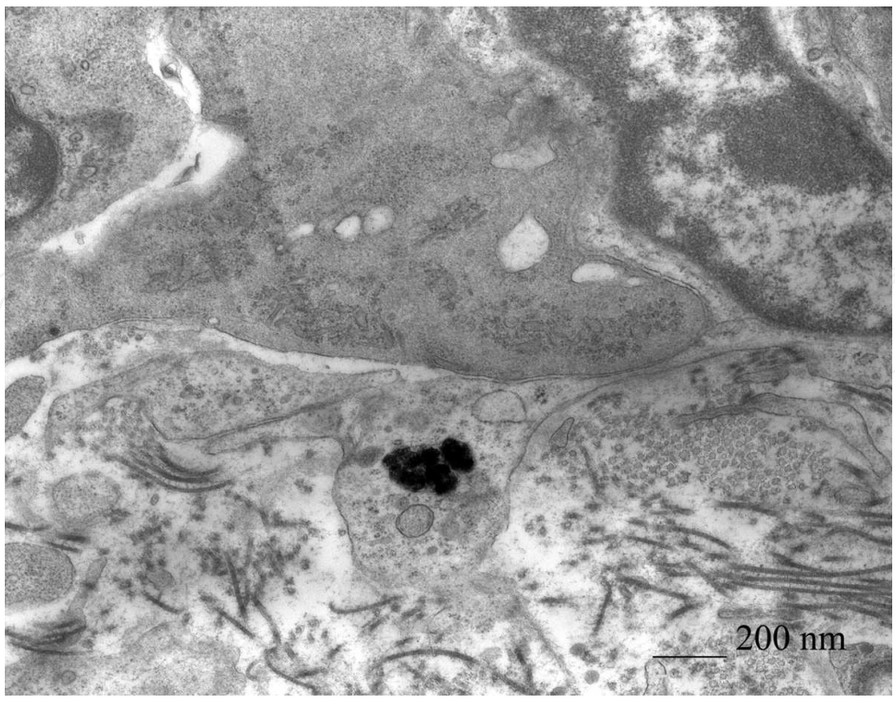


Fig. 8. TEM of 70 nm nanoparticles showing a precipitate formed in the lysosome around the nanoparticles.

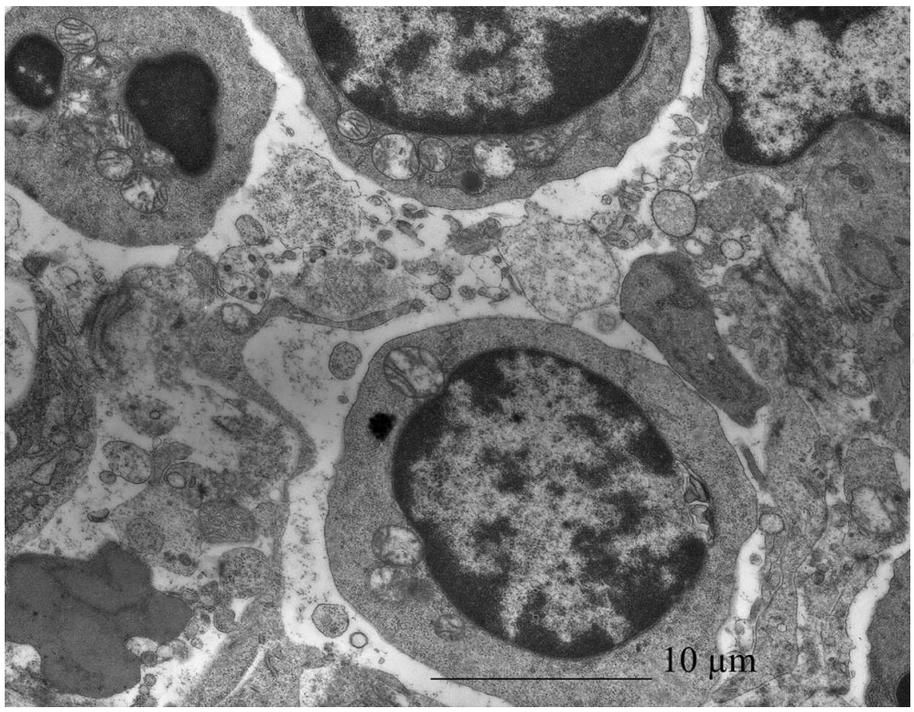


Fig. 9. 70 nm nanoparticles showing degradation signs in vesicles located inside a lymphocyte from a lymph node.

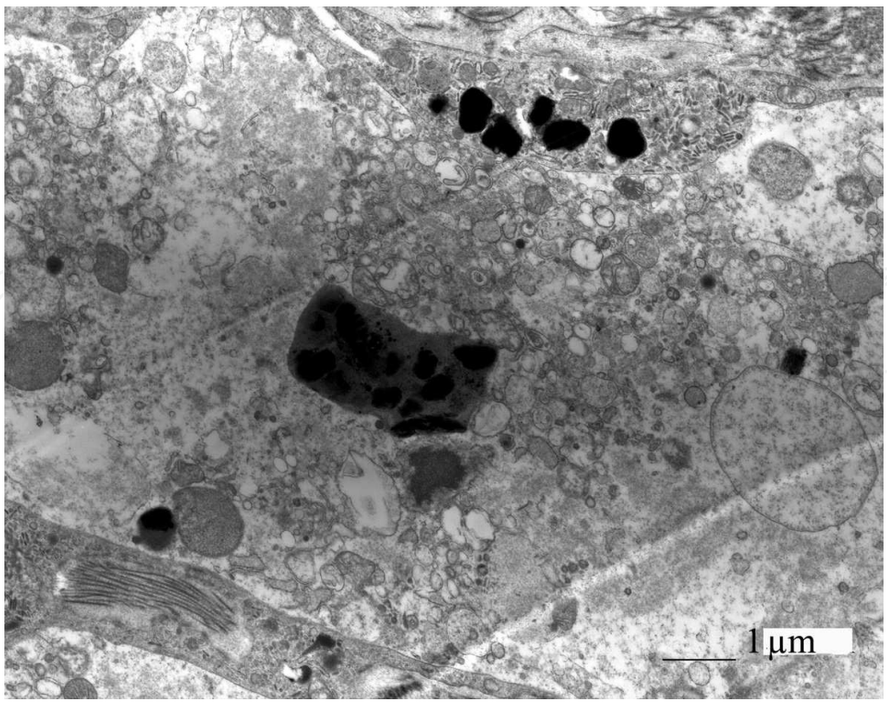


Fig. 10. 500 nm sized particles inside the vesicles of a cell going into apoptosis inside a lymph node. The nanoparticles have lost their shape and show a core surrounded by a fuzzy zone.

This experimentation shows that when implanted into bones the magnetite nanoparticles can migrate within the cells having internalised them. At this time however, the nanoparticles did not seem to be present in sufficient amounts to induce secondary heating as only a few particles appeared in each section of the lymph nodes.

The particles were found inside lysosomes or phagolysosomes. The physico-chemical environment in these vesicles is very aggressive with a low pH and many hydrolytic enzymes (Dell'Angelica et al., 2001). These conditions could explain the degradation of the material which was seen to occur in some of them. It must be noted that magnetite degradation at a low pH has already been demonstrated (Florindo et al., 2003, Gruendle et al., 2002). The rate depends on the pH and the type of acid present.

It is not clear how the endocytosis takes place. There is no indication of specific endocytosis. The amount of nanoparticles endocytosed by non- transformed cells is lower than the amount endocytosed by cancer cells. It is suggested that fast dividing cells show a large particle uptake (Jordan et al., 1999). This means that, in this case, the cells of the regenerating tissue would internalise fewer nanoparticles than bone metastasis cells.

From the TEM pictures it seemed that, when the nanoparticles formed aggregates in the implantation zone, isolated nanoparticles or small groups were able to penetrate inside the cells in contact with the aggregates. This suggests that the cells are able to separate the nanoparticles which are internalized from the aggregates.

There was no true foreign body reaction against this material even when large aggregates formed as TRAP+ cells were uniformly dispersed in the tunnel volume and not aggregated in contact with the material. Furthermore, the internalisation of the particles was not limited

to the TRAP+ cells which are known to be among professional macrophages. This suggests that in bone metastasis these particles can potentially penetrate both the cancer cells and the cells of the monocyte lineage involved in osteolysis.

After three weeks of implantation, the healing tissue was similar to that occurring when there are no particles (Frayssinet, unpublished results). This result must be compared to *in vitro* experiments showing that when grown with a primary line of monocytes the small particles do not trigger the synthesis of cytokines or TGF and so do not activate these cells (Frayssinet, unpublished results) suggesting that the cells do not recognize the magnetite nanoparticle having this range of size as a foreign body.

Breakdown of iron oxides in the organism can form several kinds of degradation products: fragments of the material; salts such as iron chloride or hydroxide; complexes of the salts with organic molecules (Michel, A., Bénard, J., 1964). It was demonstrated that the iron released from magnetite can be incorporated into haemoglobin, erythrocytes or feritin (Weissleder et al., 1989). It is important to know the degradation rate of these nanoparticles and the location of the degradation because the degradation products show altered magnetic properties. Thus, knowing the migration and degradation rates of the material will help avoid heating unwanted zones.

All three types of particles tested migrated from the bone into the lymph nodes, however, the proportion of particles found in the lymph nodes was very low and the percentage of the lymph node cells containing nanoparticles was also very low.

It must also be pointed out that the nanoparticles were not found only in the macrophages of the lymph nodes but also in the lymphocytes.

This has direct consequences on the functionality of the biomaterials using iron oxide as seen to heat tissues under oscillating magnetic fields. The heating ability (SAR) of the nanoparticles decreases within weeks after implantation. Thus, the heating protocol must be performed in a window of time to be specified.

It is also probable that the material does not persist in the organism for a very long time. However, as long as a magnetic core persists in the particles it is possible to follow the migration of the cells containing the particles by MRI; It could be very useful to follow the migration of metastatic cells before they can form a visible tumor by MRI.

## 5. Conclusions

Magnetite nanoparticles can easily penetrate the cells with which they are in contact whether or not they are professional phagocytes. This property is particularly useful and is the essential rationale for injecting a medical device releasing iron oxide nanoparticles inside a bone metastasis. The aggregation of the magnetic nanoparticles outside the cells does not seem to impair their endo or phagocytosis inside the cells as isolated nanoparticles.

Magnetite nanoparticles able to be used for heating bone tumors can migrate with the cells in which they are internalised. They are degradable in the cell vesicles indicating that they will lose their magnetic properties in a few weeks to months depending on the structural modifications they undergo during their migration through the different cell compartments and the different cells of the site. During this time, their heating properties will decrease but as long as they have some magnetic properties they will be able to serve as a probe to follow the cells migrating from the tumor.

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