GLUCOSE COATED NANOPARTICLES FOR MESENCHYMAL CANCER CELLS RECOGNITION

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Italian Abstract


Questo progetto di dottorato si è proposto di sviluppare una nuova metodica diagnostica in grado di distinguere le cellule mesenchimali del cancro da quelle epiteliali tramite lo studio del loro metabolismo e senza l’ausilio di anticorpi. Essendosi prefisso, infatti, come scopo finale quello di fornire un contributo nel campo della diagnosi preventiva e della prognosi a basso costo; una degli intenzioni principali del presente progetto era quello di ridurre al minimo l’utilizzo di anticorpi nel processo di identificazione dei due sottotipi tumorali. Questo progetto ha così sviluppato un sistema basato su nanoparticelle magnetiche (MNPs), in particolare di cobalto ferrite, con lo scopo di favorire l’ avanzamento nel campo delle attuali tecniche di isolamento magnetico.

Nello specifico le MNPs sono state sintetizzate e funzionalizzate con un analogo fluorescente del glucosio (il 2-2-(N-(7-Nitrobenz-2-oxa-1,3-diazol-4-yl)Amino)-2-Deoxyglucose, 2-NBDG) e caratterizzate con spettroscopia infrarossa e microscopia elettronica. Al fine di poterne monitorare in vitro la captazione da parte delle cellule tumorali, sono state utilizzate due ben note linee cellulari di tumore alla mammella, le MCF7 e le MDA-MB-231, definite rispettivamente: epithelial-like e mesenchymal-like. Ne è stata valutata la captazione e quindi l’internalizzazione da parte di queste due linee di cancro al seno, dimostrandola con 3 differenti metodiche (microscopia confocale, saggio di immuno-cito-chimica e analisi con microscopia elettronica accoppiata a milling ionic micro-guidato). In particolare è stato dimostrato come aumentando la concentrazione di glucosio nel mezzo (da 5.5 a 25 mM), le cellule epiteliali riducano drasticamente l’uptake di MNPs mentre esso si conservi nel
sottotipo mesenchimale. Tale differente comportamento è infatti basato sul differente metabolismo dei due sottotipi tumorali, il quale consente, senza l’utilizzo di anticorpi, di individuare il sottotipo mesenchimale, più metabolicamente attivo (e tipicamente più aggressivo), da quello epiteliale. Successivamente è stato ricercato il meccanismo molecolare responsabile di tale internalizzazione tramite l’inibizione del più espresso trasportatore di membrana del glucosio, Glut1, con un inibitore selettivo (STF-31). La specifica captazione da parte delle cellule mesenchymal-like è stata infine studiata come potenziale caratteristica da sfruttare per eventuali trattamenti di ipertermia, o termoterapia. Nello specifico è stato utilizzato un laser infrarosso che focalizzato in maniera accurata e precisa su uno degli aggregati di MNPs (dimensionalmente compatibile con la risoluzione della microscopia ottica), presenti all’interno delle cellule, ne ha permesso l’induzione selettiva di morte (necrosi o apoptosi in base tipicamente al tempo e all’intensità dell’esposizione). La possibilità di indurre una morte selettiva nelle cellule tramite la somministrazione di nanoparticelle magnetiche, è attualmente ben nota sia in ricerca che in terapia, ma esclusivamente tramite l’ausilio di campi magnetici oscillanti, onde radio o raggi infrarossi non focalizzati.

Infine per le applicazioni diagnostiche, che questo progetto si era prefissato, è stato studiato e sviluppato un dispositivo microfluidico utile all’isolamento di cellule tumorali tramite il principio di displacement magnetico. Tale dispositivo è stato studiato tramite simulazioni a computer ed in seguito prodotto per i successivi test. Ne sono stati valutati i parametri fondamentali ed infine studiato sperimentalmente con biglie magnetiche di dimensioni comparabili alle cellule, riportando infine i calcoli teorici per l’applicazione su campioni contenenti cellule tumorali.

Le dimostrazioni applicative presentate in questa tesi di Dottorato potranno portare, nel prossimo futuro, ad un avanzamento nel campo della diagnostica del cancro, consentendo di sviluppare sistemi a basso costo e quindi check-up più frequenti e mirati. Infine le prove di ipertermia eseguite, sfruttando le nanoparticelle magnetiche, forniscono interessanti spunti per possibili nuove terapie mirate.
Abstract

In cancer diagnosis the recognition of epithelial and mesenchymal cancer cells is one of the most difficult challenges. The former subtype have a well recognized method for their identification that uses an anti Epithelial Cell Adhesion Molecule (Ep-CAM) antibody. In contrast, the mesenchymal subtype lacks of a well spread and well expressed membrane marker lowering, consequently, the possibilities to develop a widely-usable assay. Currently, the proposed methods and techniques use several specific antibodies to recognize and isolate the mesenchymal cancer cells from whole blood. In this Doctorate thesis we exploit the possibility to use metabolism instead of membrane markers to recognize and isolate mesenchymal cancer cell from a complex environment like the one in the presence of cells with other subtype characteristics.

The metabolism of cancer cells is characterized by a higher rate of glycolysis respect to non-neoplastic cells. Mesenchymal cells, in particular, exhibit a hyper activated ATP production and an enhanced glucose uptake. Based on these fundamentals, we developed a new approach for mesenchymal cancer cells detection by means of magnetic CoFe$_2$O$_4$ nanoparticles (MNPs) coated by 2-2-(N-(7-Nitrobenz-2-oxa-1,3-diazol-4-yl)Amino)-2-Deoxyglucose (2-NBDG), a fluorescent glucose analogue and by D-glucose molecule, as control. MNPs were synthesized, functionalized and finally characterized by Fourier Transform Infra Red (FTIR) spectroscopy and Scanning Electron Microscopy (SEM). MNPs mean size was around 27 nm for every sample. Considering two types of BC cells: epithelial-like (MCF-7) and mesenchymal-like (MDA-MB-231), it has been demonstrated that increasing the glucose concentration in the medium from 1 g/L (low glucose) to 4.5 g/L (high glucose), results in a selectively MNPs internalization by the mesenchymal subtype. In particular we used a breast cancer (BC) cell lines co-culture and thus we demonstrated the mesenchymal predisposition to uptake glucose coated MNPs both in normal and in high glucose conditions. We observed that the mesenchymal-like cells (MDA-MB-231), respect to the luminal-epithelial ones (MCF7), internalized a statistically significant higher amount of glucose coated CoFe$_2$O$_4$ NPs in both glucose concentrations. Internalization was investigated using advanced techniques as immunofluorescence, immuno-cyto-chemical assay, confocal microscopy and Focused Ion Beam (FIB) - SEM. The internalization MNPs mechanism has been further investigated by the selective blockage of glucose transporter channels, via a specific inhibitor (STF-31), resulting in a proportional uptake decrease as a consequence of the treatment.
From a therapeutic point of view, the presence of MNPs inside the mesenchymal-like cells has been exploited for hyperthermia studies by exposing the cells to a localized Infra Red (IR) laser beam. Cells without MNPs were not affected by the IR laser while cells positive to MNPs have been warmed due to the IR absorbance characteristics of cobalt ferrite core, leading to cell damages and rearrangements.

We, moreover, investigated the ability of White Blood Cells (WBCs), obtained by healthy donor, to internalize the $\text{CoFe}_2\text{O}_4 - 2\text{-NBDG}$ NPs at both glucose medium concentrations, leading to a possible diagnostics applications in whole blood cells analysis. For this purpose, a microfluidics device has been developed for a possible isolation of MNPs-positive cells by applying an external magnetic field. The microfluidics chip was fabricated by the soft optical lithography method and the so obtained PDMS mold was bound to a glass slide by oxygen plasma treatment.

Finite element computer simulation has been carried out for better understanding the magnetic displacement principles in microfluidics devices. Finally, the set up has been fabricated and tested with micron-sized magnetic beads for the separation yield evaluation.

Concluding, thinking in terms of diagnostics we could infer that this method may lead to an alternative technique for mesenchymal cancer cells detection and isolation thanks to the magnetic properties of the MNPs used. Indeed, by applying an external magnetic field to a mixed sample of mesenchymal/epithelial cancer cells and WBCs it would be possible to isolate the mesenchymal-MNPs-positive ones. Moreover, our demonstration of localized heating could lead in the future to a possible therapeutics application in thermotherapy field.
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1. Introduction
**Chapter Summary:** in this chapter we will introduce Cancer from the metabolic point of view, in particular the differences between epithelial and mesenchymal subtype will be analyzed. Particular attention will be given to the Warburg Effect and the differences between epithelial and mesenchymal cancer phenotypes. We will moreover discuss the actual diagnostics methods that may provide effective isolation and enrichment of both cancer subtypes. This chapter will be concluded discussing about nanoparticles magnetic properties and their therapeutics applications with a critical analysis of particularly pertinent literature.
1.1 Cancer Metabolism: The Warburg Effect

In 1931, Otto Heinrich Warburg, a German scientist, received the Nobel Prize for discovering that the root cause of cancer is linked to a de-regulation of cells glucose uptake and lactic acid production\(^1\). In fact, more the oxygen lacks inside the cells, more an acidic environment is created. According to Warburg, "the veins contain more lactic acid than the arteries, hence in every case lactic acid is formed as the blood passes through the tumor". That is, if there is an excess of acidity, automatically there will be a shortage of oxygen in the body as well, if the oxygen is insufficient, the acidity will increase. Moreover, he demonstrated that if a healthy cell is deprived for almost 35% of its oxygen for 48 hours can be converted into cancerousness. Contrary to what happens for the healthy cells that have an absolute need of oxygen, cancer cells, which are anaerobic, encounter death in presence of high levels of oxygen, as Warburg discovered and reported in his works\(^1,2\), where he demonstrated that tumors are characterized by two conditions: blood acidosis and hypoxia. Besides the lack of oxygen, to promote tumor growth there is also the presence of glucose. In fact as Warburg stated “in order to kill tumor cells, through want of energy, it is necessary, as in experiments in vitro, to stop respiration as well as fermentation” ending with “tumor cells can be killed through want of oxygen, only when the sugar required for fermentation is lacking”.

Although the Warburg effect is not yet fully understood and not broader agreed upon, his hypothesis have been confirmed by several tumors with different genetic background\(^3\).

The cause of the favorable conditions for cancer growth is an initial accumulation of lactic acid, produced by tumor metabolism, and the subsequent selection of cells that, to cope with the acidity, express a proton extrusion pumps hyperfunction\(^4,5\). Over the last century, cancer therapy has been focused on understanding and targeting the genetic basis of tumor development and progression. This approach significantly improved personalized cancer therapies, generating progress on tumors targeting; however cancer cells survival and proliferation remain a stumbling block\(^6\). Therefore cancer genetic heterogeneity suggests the need for innovative and broader approaches able to target a wider range of epigenetic and genetic pathways currently known as “fuel for cancer growth”\(^6\).

Despite the fact that is still unclear if the Warburg effect is the cause or the consequence of genetic dysregulation\(^4\), cancer glucose metabolism has been exploited for several diagnostics applications\(^7,8\), such as in Positron Emission Tomography using \(^{18}\text{F}\)-Fluorodeoxyglucose\((^{18}\text{F}\text{FDG})\)^\(^9,10\). Recent studies by Alvarez and co-workers\(^11\) clearly demonstrated that
FDG uptake by channel Glut-1 is higher in aggressive Her2-positive mammary tumors. Indeed the possibility to specifically treat hyper activated cancer cells by targeting their metabolic pathways remains one of the best approaches for overcoming drugs resistance. Although glucose-channels-targeting drugs have been proposed, their clinical applications in cancer therapy have not yet materialized.

Normal cells generate Adenosine Tri Phosphate (ATP) primarily by glucose oxidation in the presence of oxygen via the highly efficient mitochondrial oxidative phosphorylation pathway. The normal metabolic pathway begin with the internalization of a glucose molecule by a specific passive transporter (mediated by Glut-1/4 transmembrane protein), and a subsequent phosphorylation by the hexokinase enzyme. This aerobic based pathway, that belongs to Krebs cycle, produces 36 ATP for each glucose molecule, clearly providing more chemical energy to the cell. In contrast, tumor cells have inefficient glycolytic pathway, for ATP generation, that provide them just 2 ATP molecules due to the lactic acid production mechanism, even in presence of oxygen (see Figure 1.1). This, subsequently, brings cancer cells to an increased glucose consumption rate and a maintained energy request. As tumour cells are able to deregulate the glycolytic pathway by entering the lactate pathway instead of the oxidative phosphorylation (pyruvate via), they also must evade the checkpoints control in the proliferation pathway, that normally block the proliferation in stressful metabolic conditions.

Moreover, recent studies have shown that both normal and tumoral cells contain efficient mitochondria. They produce, however, excessive amounts of lactate suggesting that, improved glycolytic pathway may confer a growth advantage. In this context, the importance of external environment and in particular tumor niches interactions in generation and/or maintenance of the uncontrolled proliferation and tumorigenic characteristics (tumor plasticity) should not be underestimated. The resulting lactate produced and excreted outside the cells by the tumor peculiar aerobic glycolysis provide the tumor proliferation, angiogenesis and invasion increased ability. Hence, several works are now focusing their interest in therapies targeting glycolytic metabolism, like specifically enzyme depletion by siRNA silencing, specific bio-mimetic compounds and in silico designed drugs.

One of the last and most appreciated work in this direction was developed by Chan and colleagues in 2011, where they provided a possible therapeutic approach, for renal cancer patients (and the von Hippel-Lindau Syndrome). In particular they identify a third generation compound, called STF-31 able to specifically inhibit Glut-1 transporters. By blocking the
Glut-1 transporters family they observed a drastic reduction of the renal cancer cells proliferation (due to the lower amount of glucose available in ATP production pathway). Moreover, another bio compound, glutamine, is strictly related to tumor proliferation maintenance and it is now becoming an elective target in cancer therapy due to its involvement in tumor metabolism\textsuperscript{22,23}. Indeed, it is well known that glutamine is involved in hyper activated metabolism\textsuperscript{24} and, in particular, in proliferation preservation\textsuperscript{25,26}. Glutamine is an essential aminoacid and, as glucose, is quite accepted to have a crucial role when its catabolism pathway is de-regulated. As well for glucose also glutamine it is nowadays exploited as novel compound for applications in cancer diagnosis and therapy by the metabolism-based targeted approaches\textsuperscript{27}.

![Figure 1.1](image)

**Summary of the molecular mechanism involved in the Warburg Effect.**

- **a**: fine regulation of the normal non-proliferating cells;
- **b**: deregulated pathways, in particular glycolysis, in proliferating tumor cells. Glucose uptake is related to functional Glut (1/4) transmembrane proteins by a semi-active, ATP dependent, way; it enters the glycolytic pathway producing pyruvate and lactate that is rapidly excreted, acidifying the external medium. Glycolysis is regulated by several proteins, few of them essentials for normal/tumoral state switch such as c-Myc, p53, mTOR and HIF that are widely considered as possible oncogenes. *Source: Macmillan Publishers Limited* \textsuperscript{17}
1.2 Circulating Tumor Cells and Epithelial to Mesenchymal Transition

Circulating Tumor Cells (CTCs) have been defined as cancer cells of solid tumor origin found in the peripheral blood. It is commonly accepted that these cells detach from original site for going into blood vessels. CTCs are very rare, with an estimated frequency of 1 per $10^8$ to 1 per $10^9$ blood cells. Patients with advanced solid tumors (e.g. prostate, breast, pancreatic, lung) have been pointed out as eligible for CTCs detection applications (from peripheral blood). In particular their presence may provide information about hematogenous phase of metastasis, since CTCs are not found until tumors generate neovascularization. Few peculiar phenotypic characteristics have been exploited consistently to identify CTCs in the peripheral blood. CTCs were initially characterized as non-WBCs, deriving from epithelium and disclosing significantly larger diameters and total membrane areas than normal blood cells. However, the morphological features of CTCs are now known to be less clearly defined, and may vary by disease, disease stage, or disease state (e.g., pre- vs. post-treatment setting). In addition, one of the first causes of miss-recognition is the epithelial antigens loss and the consequent replacing of the typical phenotypic markers in the, so called, Epithelial to Mesenchymal Transition (EMT). This is a phenotypic change that is, nowadays, thought to be an important feature in the metastatic process that could allows detaching of tumor cells form initiating site for traveling to the new proliferating one, through blood or lymphatic vessels, thus originating metastasis formation. An important and yet unidentified issue is how the mesenchymal cells transform themselves to get back to epithelial subtype. Even though the main hypothesis pertains a sort of mesenchymal to epithelial, intra-blood, transition it is still unclear how and whether it happens.

From a molecular point of view, EMT is a specific changing in the gene expression pattern that leads to the activation of several embryonal genes such as transcriptional factor, growth receptors and so on. This transformation may confer a competitive advantage as a higher proliferation rate, a diminished expression of normal-markers, an absence of cell-cell growth inhibition and an inherent ability for not requiring cell-surface adhesion (Figure 1.2). All these alterations furnish to these cells the ability to detach from the initiating site, typically a tissue, and to enter into blood circulation for reaching a new a proliferation site and thus originate metastasis formations. Actually It is not known if CTCs represent the most aggressive cells subtype, originated by tumor mass, however phenotypic assessments suggest that at least
some subset of CTCs could originate clonal metastatic lesions with an increased tumorigenic activity and achieved aggressiveness\textsuperscript{32,33}. Another important issue that seems to be correlated with the EMT is the stem-like property that could lead to genetics transformation with a consequent gain of function\textsuperscript{34}. Indeed the loss of cell-cell contacts and the cytoskeleton remodeling, followed by polarity losses, could lead to cancer progression and in particular to the mesenchymal phenotype\textsuperscript{35}.

![Schematic representation of Epithelial to Mesenchymal transition in cancer.](image)

**Figure 1.2 | Schematic representation of Epithelial to Mesenchymal transition in cancer.**

It is highlighted the typical markers expressed from epithelial (left) and mesenchymal (right) cancer cells. The typical disposition as joined-hexahedrons for the epithelial subtype is due to their behaviour to grow tight one to the other. In contrast the mesenchymal subtype is represented as single rounded cells as their tendency to grow isolated and with a not-conserved shape.

### 1.3 EMT and CTCs in Breast Cancer

BC (BC) and high grade BC in particular is one of the first cause of death that occur in women over the age of 50 with an Overall Survival (OS) of only 20\%\textsuperscript{36}. This poor prognosis is due to the low response to therapy (chemotherapy and radiotherapy) and often to the late diagnosis of the tumor mass. In particular this tumor is typically subdivided in 2 big groups, the HER2 positive and the HER2 negative. Human Epidermal growth factor Receptor 2 (HER2) is a tyrosine-protein kinase that drives the growth of the epithelial cells and its gene is
considered as oncogene because its recurring mutations in BC pathology.\textsuperscript{37} HER2 gene expression in cancer cells is a recognized biomarker for the BC classification and in particular its expression is associated with the high grade ones. Unlike the EMT in BC is associated with the gain of function of HER2 gene, thus increasing the invasion ability and the tumor aggressiveness. Indeed, being a proliferation transduction signal protein, HER2 gene is subjected to specific mutations that lead to a functional protein that, even in the absence of ligand-receptor interaction, communicates the maintenance of S/M cell cycle phases (bringing to DNA duplication and cell mitosis). For all these reasons HER2 (gene and protein) has become, in the recent years, the specific target for high grade BC therapy.\textsuperscript{38} Moreover, the presence of CD44-positive BC cells is associated with the poor prognosis, typically due to its mesenchymal characteristics. Studies regarding the tumor initiating ability of CD44\textsuperscript{+}/CD24\textsuperscript{-} cells on xenograft mouse models highlighted that the contemporary presence of these markers with the aldehyde dehydrogenase (ALDH) correspond to a higher proliferation rate and invasive behavior for the mesenchymal state displayed.\textsuperscript{39,40} EMT in BC (summary example given in Figure 1.3) is unfortunately associated with the poor prognosis even for the enhanced invasion ability and hematogenous metastasis formation, that CTCs can generate. Their number in the last decade, has started to be considered as a prognostic marker, for diagnosis and therapy control in BC. Recently an interesting microfluidic device has been proposed for the enumeration and isolation of HER2-positive CTCs that in the next future could lead to important advancements in better and faster BC diagnosis.\textsuperscript{41}

![Figure 1.3 | Breast Cancer EMT and CTCs.](source: scientificamerican.com)

The case of intra-tumoral EMT and consequent dissemination is reported. From the primary solid tumor, epithelial cells undergo the EMT acquiring the ability to enter into blood circulation and thus originating the hematogenous metastasis. Source: scientificamerican.com.
1.3.1 Epithelial- and Mesenchymal-like Breast Cancer Cell Lines

In this dissertation is presented work on two BC cell lines: MCF7 (epithelial-like) and MDA-MB-231 (mesenchymal-like). The former one has an Ep-CAM+/E-cad+/CD44- phenotype and is so classified as luminal and weakly metastatic. Despite its epithelial origin, MDA-MB-231 cancer cell line exhibit a CD44+/Ep-CAM/E-cad- and it is so classified as a mesenchymal-like phenotype. This BC cell line shows 85±5 % of CD44+/CD24- population, and it is positive to CD105 marker with a demonstrated tendency to metastasize. They have an unconserved morphology with a spindle-like shape predisposition and softness characteristics. Due to metastatic tendency they often grow isolated interacting with other cells by lamellipodia, filopodia and membrane prolongation. These two cell lines can be so recognized both molecularly and morphologically and an example of specific biomarker is given in Figure 1.4. These two cells have been chosen for their respective well-known characteristics and wide-accepted typing identification from literature.

![Image of two cell lines](image.png)

Figure 1.4 | Two examples of Epithelial-like and Mesenchymal-like BC Cells Lines.

**Left:** Ep-CAM+ (green) population of MCF7 epithelial-like cancer cells. **Right:** CD44+ (red) MDA-MB-231 mesenchymal-like cancer cells. In blue the nucleus. Scale bars: 20 µm.
1.4 CTCs Enrichment Techniques

Cell separation techniques are typically based on common biological or physical differences such as size, density, membrane antigens and stiffness/softness characteristics. A large number of these techniques bear laborious manual sample preparation steps (such as centrifugation, antibodies labeling and rinsing), that often generate inconsistent results with consequently high costs. Moreover these techniques need significant blood volumes (>7.5 mL) which influence and often limit their possible clinical applications. Nowadays, cell separation techniques based on membrane antigens recognition bear a massive antibodies usage and can be divided in two big categories: Fluorescence Activated Cell Sorting (FACS) and Magnetic Activated Cell Sorting (MACS). The former one is based, as the name says, on fluorescent antibodies and the antigens amount evaluation is based on fluorescence-expression levels (previously calibrated on dyes intensities). Usually, in semi-automated FACS analyzers, the marked cells are hydrodynamically focused for individual sorting thanks to a fluorescence activated sensor able to recognize and count positive cells. Even if this technique could in principle provide highly pure (about 95%) cell subtypes, it does require expensive instrumentation and consumables with the further limitation of a slow throughput (almost 100 cells per hour).

On contrary MACS method uses, typically, magnetic beads functionalized with specific Ab for the enrichment of desired target cells. In principle this technique might allow processing parallelization providing faster separation (almost one thousand cells per hour) and a high-purity isolated population. The common element between MACS and FACS is the time needed for the sample preparation that, typically, require almost 30-60 minutes of incubation for labels attaching (Ab-coated beads and fluorescent-Ab, respectively). Few affinity-based methods are known and several size-based separation techniques are nowadays largely studied and in the development stage. Size-based approaches are normally used in the case of unknown membrane biomarkers or well-known and sharp distributed dimensions. In these separation assays, cell strainers are largely used, where the cell suspension is separated according to size, where the smaller cells can pass through holes of a specific diameter and thus restraining bigger cells onto the sample holder.

As it is known from literature, cancer cells and in particular mesenchymal ones have soft characteristics that allow to be squeezed and so escape the restrainers network. Purity and throughput are two essential parameters for a consistent and reliable enrichment process, but
at the same time, difficult to be obtained together. Typically the results lead to a compromise about the ratio between false positive and false negative\textsuperscript{30}. All the size- and density-based techniques have both a limitation of purity and throughput. For instance FACS semi-automated systems show acceptable purity with a high throughput. In contrast MACS maintains both high purity and throughput, but demanding big and expensive instrumentations for the huge amount of sample volumes\textsuperscript{51}. These inherent advantages and disadvantages of MACS-based technique highlight the need for innovative diagnostic technique able to give high throughput maintaining high purity with the added value of low cost and the no need of antibodies labeling.

Several magnetic-based isolation methods (also called immunomagnetic methods) have been developed, so far, for tumoral cells isolation in particular for CTCs enrichment processes. These peculiar cells are deeply studied since last century as diagnostic and prognostic marker in the oncologic area and also as index for tumor aggressiveness and ongoing therapy control\textsuperscript{52}. Unfortunately, several cases are reported in literature about the extravasation ability of CTCs, with the unavoidable consequence to produce metastasis in large variety of body districts\textsuperscript{53}. Up so far, the unique instrument approved by the US Food and Drug Administration (FDA) for CTCs counting is the CellSearch\textsuperscript{®} (Veridex LLC, Johnson & Johnson Co.). The basic principle of this instrument is to enrich the CTCs population from a whole blood sample of 7.5 ml by means of magnetic beads coated by anti Epithelial Cell Adhesion Molecule (Ep-CAM) specific Ab. A second confirmation of the so isolated cells is then performed by three additional staining: two positive and one negative. The formers are Cytokeratin 8-18-19 and DAPI, the latter is a CD45 control. Cytokeratin (CK) is a term used for identify a complex matrix of intra-cytoplasmic keratin filament proteins useful for the assessment of the epithelial origin. There are several kind of these proteins: CK 4, 7, 8, 10, 13, 14, 18, 19 and 20.

CD45 is a specific white blood cells marker and in particular is a specific transmembrane glycoprotein exposed by mature leukocytes. On the contrary Ep-CAM is a transmembrane glycoprotein that allows homotypic cell-cell adhesion with a Ca\textsuperscript{2+}-independent way acting as negative control for cadherins-mediated adhesion\textsuperscript{54}. It is moreover known that can promote proliferation by influencing growth pathways\textsuperscript{54} and the MYC oncogene\textsuperscript{55}. Therefore, for all these reasons, Ep-CAM is a specific and widely used marker for epithelial origin neoplasm cells identification, isolation and targeted treatment approaches\textsuperscript{56}. While epithelial and few White Blood Cells (WBCs) express Ep-CAM molecule on their membrane, mesenchymal
cells do not, thus CTCs that have undergone the Epithelial to Mesenchymal Transition (EMT) are lost in the CellSearch®, first, enrichment step. This limitation determines, consequently, important lack of information about tumor characteristics and aggressiveness\textsuperscript{57,58}. Another important limitation of this CTCs enrichment instrumentation pertains the large amount of antibodies required and the low reliability of the results obtained\textsuperscript{59}. A further limitation regarding the CTCs fixation process is that does not allow any later molecular analysis on them, because the cells are left non-viable.

One of the most criticized question about the CellSearch® system is the system inability to recognize mesenchymal-type CTCs, with the consequent lack of knowledge about tumor characteristics.

The absence of a widely-expressed mesenchymal marker, lead to a more complex scenario that consequently opens a multi-parameters approach like the simultaneous usage of several antibodies\textsuperscript{60}. The membrane expression of mesenchymal cancer cells vary a lot in between tumours, maybe due to their highly undifferentiated phenotype and proliferation rate, and so the current proposed methods exploit the possibility to have a multiple positive staining with regards to CD15\textsuperscript{61}, HER-2\textsuperscript{30}, CD34\textsuperscript{62}, CD44\textsuperscript{62}, CD45\textsuperscript{63}, CD47\textsuperscript{63}, Plastin-3\textsuperscript{64} and Vimentin\textsuperscript{65}.

Actually, some investigators approach with negative selection, where only leukocytes are pulled out and tumoral cells enrichment does not depend on epithelial/mesenchymal specific markers. However, based on literature evidence, positive selection approaches is still currently preferable due to its isolation efficiency respect to the negative ones (56% vs 23% detection). In the last decades, in order to successfully reduce processed volumes, for cells identification and separation, the entire device dimensions have been subjected to an enormous decrease in particular with the microfluidics techniques.

Consequently, all these cited problems left opened a large space where numerous innovative techniques and approaches for rare-cells identification are used, like microfluidic devices, cell sorting columns and chromatography restrainers.
1.5 Microfluidics for Cells Separation

Microfluidic devices have recently emerged as efficient and widely used method for biomedical miniaturization process\(^\text{66}\). Additionally, microfluidic devices are portable, low cost and easily to be integrated with analytical techniques (biochemistry and biotechnology such as PCR and electrophoresis). Microfluidic devices are traditionally produced by the so called, soft optical lithography technique that is useful in fabrication of micron-sized structures via a mold obtained with silicon pieces. A silicon wafer is used as basis for the pouring of a photolithographic resin that is then exposed to UV lights and developed for master creation; once obtained it a liquid elastomeric silicon-based material, typically poly(dimethylsiloxane) (PDMS), is slowly poured on the master structure, finally implemented with a baking time (15-20 minutes)\(^\text{67,68}\).

The soft lithography process allows the fabrication of micron sized pattern and then, by simply peeling off, removing the impressed PDMS for bonding it on clean plasma treated glass surfaces. This fabrication process allows a wide variety of microfluidic devices, ranging from simple parallel channels to highly complex systems\(^\text{69}\).

One of the first and well-known microfluidics device proposed in CTCs isolation was developed by Mehmet Toner and colleagues at Harvard Medical School (Boston, MA) in 2007\(^\text{70}\) (see figure 1.5).

An example of a microfluidics chip developed by Toner group at the Harvard Medical School. A cancer patient whole blood sample is fluxed inside for the identification and counting of CTCs number.
This CTCs chip is a microfluidics device that exploits the interaction between cells and Ep-CAM-coated pillars for the specific recognition of epithelial subtype of circulating tumor cells. The efficiency of cell capture was directly related to the contact persistent time length between the single CTC and the device contour. The same group developed, in 2010, an Herringbone-like chip, which provided an enhanced capture capability, respect to the previous one, maintaining the Ep-CAM approach. In 2013, they presented a new microfluidic chip that, by maintaining the usage of an anti-Ep-CAM, magnetic, antibody, was able to reach a processing velocity of 8 ml/hour, a really remarkable rate, with the added value to isolate the CTCs without any fixation process, so allowing molecular and cytological analysis. Other Ep-CAM based microfluidic devices have been developed in the last 8 years following the same concept of the CellSearch way. Few of them proposed multi markers approaches, as described previously, as for example in pancreatic tumor by the concomitant recognition of positive markers as Ep-CAM and MUC1/MUC4 and the negative depletion of CD45-positive cells.

1.5.1 Magnetic Based Sorting

Several magnetic micro- and nano-particles applications have been studied, so far, in microfluidic cell separation techniques. Actually, MACS approaches can be classified into two categories: (a) to capture cells into magnetic traps followed by release, and (b) to enrich cells by displacement from streamlines within a flow. In the former one, the discrimination capability is a particles-size independent way that may depend to target expression. Usually such kind of techniques demonstrate the inability to capture large amount of cells, because of the steric hindrance and the volume occupied into the restrainers. Depending on channel geometry and constraints, large particles-aggregates can clog the channels damaging the capture efficiency. Another shortcoming that may arise is non-specific binding of the cells to the magnetic pillars or traps causing, consequently, a reduction of cell release.

In the flux-based enrichment category (b), isolation can be achieved both by transversal magnetic gradient and by magnetic field guide. The basic principle of these techniques is to incubate cell suspensions with functionalized particles for a variable time and then flux them into microfluidics channels. Indeed, in this approach, cells are continuously flowed and exposed to an external magnetic field. This applied field results in streamline displacements
of the magnetoically-labeled target cells across the channel with a relative velocity, that is proportional to few parameters according to the following equation:

$$\vec{F}_m = V_m \cdot M_m \cdot \nabla \vec{B}_x$$

**Equation 1** | The equation represents the magnetic force ($F_m$) exerted, in one direction, on a certain mass of magnetic material ($M_m$) by a magnetic field ($B$).

where $V_m$ is the magnetic-particles total volume, $M_m$ is the magnetization mass value, and $\nabla \vec{B}_x$ is the gradient of $B$ (the magnetic field) in the $x$ direction (the one perpendicular to the streamlines).

So far, in MACS techniques, there is no evidence of CTCs discrimination possibilities, from normal-blood cells, by the application of metabolism, instead of classical membrane markers recognition.

In 2006, Pamme and Wilhelm\textsuperscript{73} proposed a permanent magnet (NdFeB) positioned alongside the microfluidic channel for the magnetic guided displacement of the magnetic-sensitive cells. In particular they administered carboxyl terminated ferrite nanoparticles ($\gamma$Fe$_2$O$_3$) to macrophages and HeLa, cultured cells, for their really high uptake ability and then put a NdFeB permanent magnet for the lateral displacement of cells from the normal streamline. This group was able to effectively displace two different cell types by tagging them with beads with different magnetic susceptibilities. However, this technique lacked precise tunability due to the absence of on/off capabilities. Furthermore, the permanent magnet size is orders of magnitude larger than the nanoparticles, resulting in low field gradient respect to the nano-magnetic structures (implemented in order to increase local magnetic field and applied field gradient) so decreasing the magnetic force exerted on target cells\textsuperscript{61}.

In this thesis a magnetic-nanoparticle-based method is presented that could, in the future, lead to a new interesting MACS system. In particular we developed a way to discriminate mesenchymal cancer cells by exploiting their metabolism, by the usage of glucose coated MNPs. In particular CoFe$_2$O$_4$ nanoparticles have been used for the functionalization with normal glucose and with a fluorescent glucose analogue, the $2\text{-}2\text{-}(\text{N}\text{-}(7\text{-}\text{Nitrobenz}-2\text{-}\text{oxa}-1,3$-
diazol-4-yl)Amino)-2-Deoxyglucose (2-NBDG), for the targeted recognition of the metabolic hyper activated cancer cells.

The use of metabolism to identify cancer cells and count CTCs has been recently introduced, for the first time, in our laboratory as a consequence of promising results (Patent pending, PCT/IB2014/067057).

This strong tropism of glucose coated MNPs for the mesenchymal cancer cells should be taken as an advantage for MACS diagnostics system development. Indeed the possibility to reduce the costs by avoiding the use of antibodies and in this way, to increase the number of check-ups and therapy monitoring to an almost daily rate should be the best goal to achieve. Furthermore, coupling the present type of analysis to the simple counting, of the full variety of CTCs, should deliver precious information on the role of mesenchymal cells in the spreading of metastatic cancer, that seems to be more complex than what previously thought (Daniela Cesselli et al., private communication).

1.5.2 Fluorescence Based Sorting

FACS is the other main technique used in cells targeting and isolation diagnostics systems. The method provides to label the cell sample by fluorescent dyes and subsequently to flux them inside fluidic channels for the excitation and recording. The labels are excited by sharp unique-wavelength lasers and the fluorescence so released is collected for quantification analysis. The selection can be: positive or negative. In the former case the cells that have to be recognized and/or sorted express specific targetable markers, in the latter it is known, only, that certain markers have not to be recognized. The classical FACS system are, basically, flow cytometers used in clinical diagnostics, for the high throughput and yield that they can reach in a really short time. These devices use mostly the hydrodynamic focusing principle in order to record one cell (called also event) at a time and so let the machine count every “event” that, usually, should correspond to a positive cell. In this way not only an univocal recognition is possible but also a subpopulation collection can be achieved. The main disadvantage of this technique is the high number of cells needed and the proportionally high errors, with the consequent impossibility to use it in CTCs counting assays. Fluorescence-based sorting can also be achieved by the infection of the desired cells by an adenovirus, like in the work of
Shigeyasu and coworkers\textsuperscript{74}, where a GFP-bearing virus has been used to transfect CTCs for their subsequent recognition by emission of fluorescence.

Another interesting approach provide Ep-CAM positive CTCs enrichment and analysis by the concurrence usage of FACS and MACS techniques\textsuperscript{75}.

A novel and promising approach that foresee to enrich CTCs by the means of fluorescent dyes is the so called CACTUS technique (extra ERC funding received on the 22\textsuperscript{nd} of September 2014). Developed by a collaboration between Professor Giacinto Scoles (at the University of Udine) and Professor Wilhelm Huck (at the University of Nijmegen) and their co-workers. The main goal of this approach is the development of a CTCs detection system that focuses its attention on the cells’ metabolism. This technique, which avoids the classical bulk usage of antibodies, is able to produce several pico-liter droplets enclosing each cell prepared by a water-in-oil emulsion. After each cell has been so separated from the rest, the droplets are interrogated for extra acidity that is taken for a sign of the presence of a cancer cell.
1.6 Magnetic Nanoparticles in Biomedicine: Diagnostic and Therapeutic Applications

MNPs are a cheap class of nanomaterials with the unique characteristic to behave differently from the bulk counterpart. MNPs, typically, are in the size range of few to tens of nanometers, placing them at comparable dimensions to those of biological entities (such as proteins: 5-50 nm). As the magnetic particle size decreases from microns to tens of nanometers size range, depending upon specific composition, the magnetic configuration changes from the multi-domain state, to high-coercivity single-domain regime to the superparamagnetic regime\(^7\) (see Figures 1.6 and 1.7).

![Figure 1.6](image)

Figure 1.6 | Type of magnetic behavior in presence and absence of an external field.

Magnetic properties for each magnetic material in the presence and absence of an external magnetic field. Materials are classified as: Diamagnetic, Paramagnetic, Ferromagnetic, Ferrimagnetic and Antiferromagnetic, based on their response of single or dipole alignments\(^7\).

![Figure 1.7](image)

Figure 1.7 | Change of magnetic regime in relation to dimension parameters

(a): transition from Superparamagnetic to Single-Domain and finally Multi-Domain regimes for correspondent increasing in particle size. (b): maximum diameters for Superparamagnetic and Single-Domain NPs for different chemical composition\(^7\).
One of the most important properties of superparamagnetic NPs is that they exhibit a large magnetic moment and a high susceptibility also in the presence of small fields; after the removal of the intensity field they ideally release all residual magnetization. In this condition the particles exhibit zero coercivity which makes them highly applicable for several uses. The characteristics of superparamagnetic NPs to exhibit a completely reversible magnetization in response to an external applied magnetic field allowed them to be exploited for varied purposes, including biomedicine: live Magnetic Resonance Imaging (MRI)\textsuperscript{77,78} and theranostics\textsuperscript{79,80} (with specific concerns about their safety in human treatments\textsuperscript{81}). MNPs have been studied for several applications in the biomedical field, as for example in MRI where they are used especially for their high response to the applied external magnetic field, giving the possibility to, clearly, reduce its intensity. The MRI is a low-invasive medical imaging technique used to investigate specific soft-tissues both in healthy and diseased patients, since 1983. This instrument, using radio electromagnetic waves, excite the water-electrons spin momentum thus allowing to create a 3D map of the body inner parts. The magnetic field intensity vary from 0.2 to 3 Tesla (T) and side effects have never been encountered. Due to the low risks provided, the 3D pattern supplied and the avoidance of ionizing radiation, MRI has substituted X-ray diagnostics in the several medical applications.

The other magnetic nanoparticles application in the medical field pertain to therapeutics. NPs in general and MNPs in particular have generated a huge amount of works focused on the possibilities to cure diseases, cancer as essential target, with the aim of reducing the concentration of administered compounds by targeting, almost exclusively the tumor cells. NPs stimulated drug delivery is becoming one of the main driving forces behind personalized medicine. Advancement in condensed matter physics, biomedical engineering, and molecular biology techniques will help in the understanding of nano-composite materials and their relevance in medical therapy\textsuperscript{82}. Nanoparticles in particular have been developed with different synthetic ways, such as made of chemical\textsuperscript{83} and biological\textsuperscript{84} materials. The first problem that the administrable NPs has to respect before being used on a human body is to have good ADMET parameters (Administration Distribution Metabolism Excretion Toxicology) so demonstrate that they can be fast metabolized without displaying toxicity. Metal based nanoparticles, in particular ferrite (Fe\textsubscript{2}O\textsubscript{3}) and its derivatives (such as CoFe\textsubscript{2}O\textsubscript{4}) have several advantages, explained and the beginning, but also disadvantages for their low excretion rate and low bio-compatibility. To overcome this limitations and increase the body-compatibility several biological functionalization have been proposed so far\textsuperscript{85,86}. By the implementation of
cover shells with bio-compatible compounds it is possible to avoid first the classical accumulation in filtering organs (liver and kidney) and second the inflammatory effects due to the interaction between the immune system and these metals. Another approach for drug delivery and live bio-medical imaging is provided by the biodegradable materials. In this case it is not, clearly, possible to have magnetic materials (or at least with current knowledge) but, in principle, this approach could drastically reduce the administered amount of drug, consequently reducing side effects.

The nanoparticles uptake by cells, and in particular cancer cells, is a controversial field due, mainly, to the existence of conflicting experimental results. Nanoparticles uptake is, indeed, an ATP-independent process because its mediated by electrostatic interactions among particles and membrane phospholipids. In other works it was shown how membrane transporters can influence the specific uptake by target cells. What it is absolutely sure is the possibility to induce a localized heating into cancer cells by the electromagnetic stimulation of MNPs-treated cells in the so called theranostics approach. Typically, to achieve a heating effect inside specific target cells after MNPs administration an oscillating magnetic field with a frequency rate of 100 kHz - 1 MHz and a typical intensity in the range of 10 to 50 mT (see Figure 1.8). Thermotherapy and hyperthermia are considered equivalent terms in the literature, except for some works where thermotherapy is associated with the usage of external chemical or biological material, whereas the second term means the usage of electromagnetic waves alone (infrared, microwaves and radio range spectra). In a recent, and really interesting, work carried out by Alphandery and co-workers a hyperthermia treatment of a heterotopic xenograft tumor mass, obtained with MDA-MB-231 BC cells was achieved by the administration of magnetosome chains extracted from bacteria. In this work are compared the response to thermotherapy, induced by an oscillating magnetic field at 198kHz with a 20 mT power, of a direct injection into tumor mass of MNPs or magnetosome chains. The interesting results are that the repetition of treatment for three times in 20 minutes led to a complete disappearance of the tumor mass after 30 days, only in the case of magnetosome treatment. In this specific case the MNPs were simply Fe₃O₃ coordinated by citrate or by PolyEthylene Glycol (PEG) with a diameter less than 20 nm. A reasonable motivation why MNPs does not exhibit tumor disappearance ability in this specific work could be associated to the absence of aggregates that do not allow a heating process due to their oscillation, or even to an intrinsic inability of MNPs to be internalized by cancer cells. Since the magnetosome chains have a non-symmetric shape, this allow an heavy response to the
oscillating magnet frequency, with a consequent increasing of local temperature. Moreover, the concentration of administered chains of magnetosome was ranging from 0.125 to 1 mg/mL, a cytotoxic amount\textsuperscript{92}, that is really difficult to be reached in the tumor mass in clinical applications, for the whole-body distribution and accumulation side effects. Even if the presence of tumor were localized to an accessible site in patients, it would be better to recur to a surgery resection that in principle have less collateral consequences respect to an injection of a so concentrated amount of magnetic-metal materials. In spite of these limitation, this work represent an important contribution to magnetic thermotherapy with consequent possible further explorative investigation about cells uptake properties and magnetosome functionalization processes.

Other works proposed the possibility to reach a whole body hyperthermia besides electromagnetic field, also by an optic fiber able to direct the light bundle on the tumor mass or even by applying ultrasounds\textsuperscript{90}. In the last case several MNPs have been developed and proposed to specifically recognize cancer cells for tumor mass targeting with thermotherapy techniques\textsuperscript{93,94} as is the case in the most known paper by Lukianova-Hleb and co-workers\textsuperscript{92}.

![Diagram](image)

**Figure 1.8** | **Example of Theranostics Approach Achieved with Gold Nanoparticles**\textsuperscript{92}.

**A**: gold NPs are collected by target cells \textit{via} endocytosis. **B**: laser pulse excitation exerts its influence especially on big clusters. **C**: optical scattering allows to provide real-time imaging of single-targeted cell.\textsuperscript{92}
2. Materials and Methods
**Chapter Summary:** in this chapter we report all the methods developed or used, pointing out, in particular, the technique and the instrumentations. Magnetic Nanoparticles have been characterized via FTIR spectroscopy and SEM. The cancer cells specific uptake has been evaluated by confocal microscopy, Perls’ iron staining, and via precise FIB-SEM milling. The mechanism that drives the MNPs internalization process has been analyzed by a selective inhibition of glucose transporter 1, achieved by STF-31 drug administration.

A Microfluidics device has been developed with soft optical lithography (SU-8 photoresist plus PDMS molding) and then functionalized and bonded onto a glass surface.
2.1 CoFe$_2$O$_4$ Nano Particles: Synthesis and Functionalization with 2-NBDG

CoFe$_2$O$_4$ NPs was synthesized accordingly to Massart’s method\textsuperscript{95} modifying the protocol as previously described by Nappini and colleagues\textsuperscript{96}, in order to have a stable solution suitable for cellular environment. Concentrated nitric acid (2 mL) was added to an aqueous solution of 1 M FeCl$_3$ (64 mL) and Co(NO$_3$)$_2$ (32 mL) and heated until reaching the boiling point. Afterward the mixture was, promptly, mixed under vigorous stirring with a 1 M NaOH solution (400 mL) and warmed up again until boiling. The stirring were maintained for 90 minutes as the boiling conditions. The so obtained particles were isolated by magnetic decantation, washed with bi-distilled water and added to a 2 M solution of HNO$_3$ (40 mL). The precipitate was separated as previously done, dispersed in a boiling solution of 0.5 M FeCl$_3$ (56 mL) and 0.5 M Co(NO$_3$)$_2$ (28 mL) and kept under vigorous stirring for 30 minutes. The precipitate of this reaction was isolated and washed with bi-distilled water. For obtaining larger nanoparticles the same synthetic procedure can be used, except that the Co(II), Fe(III), except for the NaOH mixture that has to be kept at the boiling temperature for 120 minutes instead of 90. Once obtained the particles, the citrate coordination has been carried out as follows: the precipitate was isolated, water-washed and dispersed in 250 mM TMAOH (tetramethylammonium hydroxide) and then added to a 100 mM citric acid solution (30 mL) and slightly stirred for 1 hour at room temperature. The MNPs were collected \textit{via} a permanent-magnet-dependent decantation, then dispersed in 20 mM trisodium citrate (30 mL) and further kept under stirring for 45 minutes. The obtained particles were separated by magnetic decantation and washed several times with water and acetone in order to remove any excess of citric acid. The citrate coated MNPs were dried under a gently nitrogen gas flux, dispersed in a buffer solution (10 mM HEPES, 107 mM NaCl, 5.3 mM NaOH, pH 7.4) and finally kept under, slight, stirring for 24 hours. The MNPs solution was washed 3 times with bi-distilled water and then placed in the previous buffer solution. The citrate coordination of the metal atoms on the CoFe$_2$O$_4$ NP surface was verified by ATR-FTIR spectroscopy.

The citrate coordination of MNPs was used to achieve the esterification reaction between the more reactive hydroxyl group of glucose molecule in 2-2-(N-(7-Nitrobenz-2-oxa-1,3-diazol-4-yl)Amino)-2-Deoxyglucose (2-NBDG, Life Technologies) and the free carboxylic group of citrate (not involved in NPs chemical coordination), in presence of HCl as catalyzing agent.
The reaction was carried out by mixing 15 mg/mL of CoFe$_2$O$_4$ NPs solution in a bi-distilled water 1:30 solution by weight, in presence of 1:30 HCl (1M) and 1:3000 of 14 mM 2-NBDG. Afterward, NPs were pelleted by magnetic decantation, washed 3 times with the buffer solution and stored in bi-distilled water at 4°C.

As control, the same reaction has been performed for the covalent functionalization of citrate coordinated CoFe$_2$O$_4$ NPs with the normal glucose molecule.

![Figure 2.1](image.png)

Figure 2.1 | Schematic Representation of CoFe$_2$O$_4$ NPs Functionalization Reactions

A schematic representation of the magnetic nanoparticles chemical functionalization is plotted. The chemical bonds are not in scale.
2.2 CoFe$_2$O$_4$ Nano Particles Characterization

For the experimental evaluation and validation of the 2-NBDG functionalized CoFe$_2$O$_4$ NPs an ATR – FTIR spectroscopy experiment has been carried out. Measurements were done in collaboration with SISSI Beamline at Elettra Synchrotron (Trieste, Italy) with a Vertex 70 spectrometer (Bruker® Co.), purged with nitrogen, using a DTGS (Deuterated Tri Glycine Sulfate) detector. MIRacle Single Reflection ATR box (PIKE Technologies) equipped with a diamond IRE (Internal Reflection Element) was used for experimental purposes. A 5µL drop of each water-dispersed sample was put onto the crystal and the measurements repeated until the complete disappearance of the combination band of bending and vibrational modes of liquid water (centered at ~ 2150 cm$^{-1}$). The background was collected on the clean IRE. Spectra were acquired averaging 128 scans with a spectral resolution of 4 cm$^{-1}$.

For the purpose of average dimension distribution a Scanning Electron Microscope (SEM; Supra 450, Zeiss, Germany) analysis has been chosen. The SEM principle is based on the possibility to observe nanometer-scale objects depending on the charging properties of the constituent material. MNPs sample were prepared by drying under nitrogen flux a drop of 1 µM concentrated CoFe$_2$O$_4$ – 2-NBDG on silica wafer. Sample was after washed twice with drops of milliQ water (electric resistance: 18mΩ) and dried again under a gently nitrogen flux. The SEM images were obtained by applying a 10 kV electron beam gun, and then analyzed with the “Analyze Particles” tool of ImageJ software$^{97}$ for the NPs dimension evaluation after setting up their brightness/contrast pattern. The so obtained diameter values were summed as integer and every count per diameter value was then plotted.

2.3 Breast Cancer Cell Lines Cultures and Co-cultures

Two BC cell lines representative, as described in the introduction, for the epithelial-like and the mesenchymal-like subtype were MCF7 and MDA-MB-231, respectively. Both cell lines were purchased from ATCC database, and cultured in Dulbecco’s Modified Eagle Medium (DMEM) with 10% of Fetal Bovine Serum (FBS) and supplemented with Penicillin/Streptomycin in a fully humidified atmosphere of 5% CO$_2$ at 37°C$^{36}$. The DMEM purchased were the one with 1 g/L of glucose (Low Glucose DMEM, Life Technologies) and the same with a 4.5 g/L of glucose (High Glucose DMEM, Life Technologies) to better discern the MNPs uptake behavior in these two different medium conditions. Both cell lines
were detached from flasks using Trypsin/EDTA (Life Technologies), subsequently fresh medium was added to stop trypsin/EDTA effect and centrifuged at 400 rcf.

For experimental setup: cells were seeded at densities of $4 \times 10^4$ cells/well onto 12 mm glass round coverslips (120 µm thick), previously put into a 24-wells plate, and incubated at 37°C for 24 h. Cell co-culture was obtained by mixing MDA-MB-231 and MCF7 suspensions at a 1:1 ratio before seeding onto glass coverslips. Examples of BC cell lines co-culture is showed in Figure 2.1.

Figure 2.2 | Images of MCF7 / MDA-MB-231 BC Cell Lines Co-culture

A: SEM image of MCF7 and MDA-MB-231 cell lines co-culture after fixation (with PFA 4%) and dehydration process. B: live phase contrast image of the same co-culture in A. Dashed Lines: MCF7 cells. Complete Lines: MDA-MB-231 cells. Morphology differences can be appreciated: MDA-MB-231 have a spindle-like shape predisposition; MCF7 show the typical epithelial shape with a flattened morphology. Scale bars: 20 µm.

2.3.1 Cell Cultures: CoFe$_2$O$_4$ – 2-NBDG NPs Treatment and Toxicity Evaluation

In order to evaluate any possible toxicity effect of CoFe$_2$O$_4$ – 2-NBDG NPs, the two BC cell lines, selected during exponential phase, where seeded 8.000 per well in a 96-wells plate in presence of low glucose DMEM. Cells were subsequently treated with CoFe$_2$O$_4$ – 2-NBDG NPs at different concentrations: 0.1, 0.5, 1, 2.5, 5, 10 and 25 µg/mL. Control cells were
treated with an equal volume of PBS. Every treatment was carried through 6 wells. Cells vitality was analyzed after 72 hours by MTT Assay (Life Technologies). 0.5 mg/mL of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide were added 3 hours before reading the absorbance at 570nm with a Tecan Infinite 200 PRO (Tecan Trading AG, Switzerland).

For the evaluation of MNPs uptake by BC cell lines, and to permit the optical/electronic microscope investigation, the treatments have been carried out as follows: cells were growth both in low (1 g/L) and in high (4.5 g/L) glucose DMEM (purchased from Life Technologies). After an incubation period of 24 hours at 37°C with 5% CO₂, cancer cells were treated for 2 hours with 2.5 µg/mL of CoFe₂O₄ – 2-NBDG NPs, dispersed in water, and then fixed in 4% ParaFormAldehyde (PFA) solution in PBS, for 15 minutes at room temperature.

### 2.3.2 Antibodies Staining for Experimental Cells Typing

Fixed MCF7, MDA-MB-231 and co-cultured samples were washed with PBS and incubated for 10 minutes with TritonX-100 diluted at 0.1% for cell membranes permeabilization. MDA-MB-231 and MCF7 samples were labeled with 1:400 phalloidin (Rhodamine conjugated, λex= 555 nm, Life Technologies) for highlight actin filaments and with DAPI for nuclei evidence. MCF7 were labeled with an anti Ep-CAM Ab (Ep-CAM-APC, Mylteni Biotec) at 1:100 for 30 minutes (See Figure 1.3). MDA-MB-231 cells were marked with a 1:100 primary anti-CD44 Ab (mouse, DAKO) and then a 1:600 anti-mouse Ab conjugated with a CY7 dye. Cells were, moreover, stained with specific antibodies for internalization process evaluation; in particular we focused our attention on LAMP1, a lysosomal marker and EEA1, an endosomal marker. Both Ab were used at 1:100 ratio and left in incubation overnight at 4°C (mouse Santa Cruz and rabbit Abcam, respectively).

Co-culture samples were stained with 1:100 anti E-cadherin Ab (rabbit, Dako), overnight at 4°C, for specifically marking membrane of epithelial cells. After incubation, samples were stained with a secondary anti-rabbit Ab, Cy-7 conjugated (goat, Abcam). Nuclei were marked with DAPI as previous.
2.3.3 Confocal Microscope Imaging and Uptake Evaluation

Fluorescence evaluation of CoFe$_2$O$_4$ – 2-NBDG NPs uptake by both MDA-MB-231 and MCF7 has been analyzed with a confocal microscope (DMRE, Leica, Germany). In order to cover all the cell thickness, a range of about 4 µm, for each sample, has been captured with a z-stacking of about 250 nm. The images representing the average level of fluorescence – based on all the captured images mediated on the number of z-level stacking – have been taken into account for the quantitative analysis with the ImageJ “ROI manager” tool after a single-cell contour selection by the “freehand” tool. The method used is described in the following. A contour of the cell area is drawn, then the fluorescence is calculated by the software as a sum of all the included pixels. The same contour is then moved to a background region (in the same image) and the value of fluorescence is measured in the same way. The total amount of cell fluorescence is calculated summing all the cell values. The same procedure was used for the control samples and the two resulting numbers finally divided and plotted as relative fluorescence. A ratio of 1 means no difference between the fluorescence value from the treated cells and that from the controls. The fluorescence for each sample was evaluated on about 200 positive-cells.

2.3.3.1 Statistical Analysis

A two-tailed t-test inter- and intra- BC cell lines, at both glucose conditions, was used for the evaluation of statistical significance of CoFe$_2$O$_4$ – 2-NBDG NPs uptake. In particular mean, standard deviation and the number of total cells per sample was put as input for the statistical validation.
2.3.4 Perls’ Iron Staining

The Perls’ method is an old (first discovery obtained in 1867) but widely used assay for the visualization of internalized nonheme iron inside cells and tissues\textsuperscript{99}. This method belongs to the formation of insoluble Prussian blue crystals onto the prepared samples by the acid (HCl) ferrocyanide reaction, in presence of reduced iron into sample tissues. This assay is, typically, used for osteocytes and hepatocytes iron accumulation diagnosis as histopathological analysis\textsuperscript{100}. The reaction has been carried out by a semi-automated instrument: the Artysan\textsuperscript{TM} Link Pro (Dako). This assay has been performed on three different treatment in order to investigate the MNPs uptake ability by the BC cell subtypes, in particular: with the CoFe$_2$O$_4$ NPs coated by 2-NBDG, coated by the normal glucose and citrate coordinated for both glucose medium conditions. Due to absence of tissues and presence of entire cells we simply modified the Perls’ protocol by introducing a 12 minutes incubation with a solution of TritonX-100, 1:100 diluted, in order to make the cells membrane permeable for the following reactions.

Figure 2.3 | Picture of the Instrumentation Used for the Perls’ Iron Staining
2.3.5 Glut1 Inhibition Studies

Glut1 transporter is the protein translated (and previously transcribed) from the gene called SLC2A1, and its function has been further elucidated in the introduction paragraph. As it is known from literature this fundamental glucose transporter is one of the most targeted and studied for cancer treatment and in particular a third generation inhibitor has been developed recently: the STF-31.

![STF-31 Chemical Structure](image)

Figure 2.4 | STF-31 Chemical Structure

In order to better investigate the role of the Glut1 glucose transporter in the glucose-coated NPs uptake, the STF-31 selective inhibitor has been used on both BC cell lines. In particular from literature was well known that MDA-MB-231 cells are Glut1 overexpressing, maybe as cause of the Warburg effect, and consequently by blocking this transporter family a decrease in proliferation (and in vitality too) should be observable. MCF7 too has a hyper expressed level of Glut1 mRNA even if less than mesenchymal-like counterpart.

The MTT test has been chosen for the toxicity evaluation of STF-31 on both cell lines as follows: the STF-31 has been administered at 1, 5, 10, 20, 50 µM (diluted in DMSO) for 16, 24, 32 and 72 hours. The experiments pertaining the Glut1-dependency of MNPs internalization were performed on both cell lines as follows: cells were prepared and seeded on glass rounded cover slips as described at the beginning of the paragraph 2.3 and then treated with 5, 10, 20 µM concentrated STF-31 for 2, 4, 8, 16, 24, 32 hours and then treated with 2.5 µg/mL concentrated MNPs (glucose, 2-NBDG and citrate) for 2 hours. The STF-31 has been added in serum-free DMEM, and then after 4 hours FBS was added at 10 %, in order to let the drug exerts its effect and so avoid possible interaction between STF-31 and serum proteins. After the incubation periods the cells were fixed with 4% PFA solution as described previously. The experiments were repeated three times (3 glass cover slips for each sample)
for 2 times. A toxicological in vitro analysis has been, further, performed on both cell lines via the MTT assay. The STF-31 concentration tested were 1, 5, 10, 20, 50 µM at both glucose medium conditions.

2.4 CoFe$_2$O$_4$ – 2-NBDG NPs Test on Healthy White Blood Cells

A sample of healthy White Blood Cells (WBCs) was isolated from the whole blood population using the Fraction Collector Omnicoll®. The blood sample was provided by “Istituto di Anatomia Patologica, Azienda Ospedaliero Universitaria Santa Maria della Misericordia, Udine” Italy). 1.5 mL of entire blood was gently added in a FACS tube containing 1.5mL of Omnicoll. Sample was subsequently centrifuged at 500 rcf for 20 minutes. WBCs population was removed and put on gently rotation in a 1.5 ml tubes ferris-wheel with 2.5 µg/mL of CoFe$_2$O$_4$ – 2-NBDG NPs for 2 hours in DMEM low glucose (Life Technologies) at 37°C with a 5% CO$_2$ in humidified atmosphere. Then an anti CD45-PE-Texas Red antibody was added. CD45 is a well known transmembrane protein expressed by leukocytes, as described in the introduction paragraph. Anti CD45 antibodies are, indeed, widely accepted by literature for WBCs identification. After a 15 minutes staining, the sample was gently washed and centrifuged at 300 rcf for 3 minutes and the supernatant removed. A a 5 µL mixture of 5 µg/mL DAPI solution was added to the sample. Pellet was resuspended in PBS 1X solution and a drop of each of them was put on a cover slip glass slide for microscope investigation.

2.5 MDA-MB-231 / MCF7 SEM Imaging and Focused Ion Beam Cutting

MDA-MB-231/MCF7 co-culture has been treated with 2.5 µg/mL of CoFe$_2$O$_4$ – 2-NBDG NPs and then fixed with 4%PFA for 15 minutes and dehydrated by increasing concentration of Et-OH (from 30% to 99%). Samples were dried by gently N$_2$ blow and coated with 20 nm thick Chromium film by sputtering to allow electric charges flowing. The SEM – Focused Ion Beam (FIB) analysis have been realized with a Carl Zeiss Cross Beam Microscope (Zeiss
International, Germany) for Gallium FIB precise milling and SEM inspection. The FIB technique on the focalization of an accelerated ion beam, Gallium in our case, that can be precisely directed on a sample portion. The possibility to physically determine, by the command software, an area and an intensity for the precise milling allows to properly erase portion of target materials. This technique is indeed used in material science and in biological science for its numerous applications. The coupled SEM finally permit to obtain the reconstruction of our biological samples, allowing to finely appreciate the presence of eventual conductive materials (MNPs) inside or outside cells. In our cases, the biological samples have been investigated with a SEM voltage in the range of 3 to 5 kV (kilo Volt).

2.6 Optical Microscope Setup for Hyperthermia Studies

A continuous wave infrared (IR) user-regulated laser beam was focused by the microscope lenses onto the target cell containing MNPs. The microscope was a Nikon (Eclipse TE2000-E, Nikon, Japan) and the camera a Hamamatsu (ORCA-Flash 4.0). The wavelength of the laser was 1064 nm, for which the cell adsorption is minimum. Moreover, the power at the sample was finely regulated from 1.0 to 15.0 mW. Therefore, the laser beam absorbed by the MNPs, internalized by the cancer cells, induced a local heating on the target.

Figure 2.5 | Optical Microscope Setup Used for Laser Therapy on BC Cells

The optical microscope, the high-resolution camera, the piezo-electric control for z-stacking and the laser controller can be appreciated.
2.7 Microfluidics Device: Design and Fabrication

A Finite Element Analysis (FEA) has been carried out with COMSOL MultiPhysics software for the microfluidic device simulation and evaluation with the specific “Microfluidic” module. A “fine” level was used for the Mesh resolution and the “Particle Trajectories” tool used in order to evaluate the percentage of diluted particles (could be considered as a cell sample) distribution inside the flow depending on the design (Figure 3.12).

An AutoCAD-based software has been used for the lithographic mask design and boundaries definition. Once obtained the mask from an external facility (Delta Mask, The Netherlands), the fabrication of the microfluidic devices has been achieved by the soft optical lithography technique\textsuperscript{102}. Several of these are well-known and widely-used in order to fabricate structures at the micro- and nano-level suitable for biological or medical applications\textsuperscript{103}.

The soft optical lithography in particular is a lithographic-based technique that allows to obtain structures that ranges from hundreds of nanometers to hundreds of microns. The structures were obtained on a silicon pieces previously cleaned with acetone and isopropyl alcohol (IPA) and dried with nitrogen flux. Afterward the silicon was heated up until reach 250°C for a complete dehydration of the material and then put onto a spin coating instrument. Before spinning the SU8-2050 photoresist on the silicon, a hundreds nanometers thin layer of OmniCoat\textsuperscript{TM} (Microchem Corp, MA, USA) has been deposited. SU8-2050 was deposited on a 4 inches rounded silicon wafer and spun at 3500 rpm for 2 minutes. A soft bake at 60°C for 3 minutes and at 95°C for 10 minutes has been carried out in order to obtain a 40 microns height structure, as described by the data sheet. After the heating procedure a self-reduction of temperature, until reaching 20°C, has been left for avoiding thermal stress effects inside the structures (cracks). The exposure with a, mercury lamp, UV light at 160 mJ/cm\textsuperscript{2} was left for 30 seconds and then the wafer has been put on the thermal plate, for the post exposure bake process, for 6 minutes at 95°C and then left, as previous, to cool down until room temperature. The development of the exposed structures has been achieved by immersing the wafer in the SU8 Developer until the complete disappearance of the unexposed photoresist portion. After the development the structures (fixed on the top of the wafer) has been washed vigorously with the IPA and then put on the thermal plate for 1 hour at 150°C for reduce the structures imperfections.
The microfluidic channel obtained by the soft optical lithography technique and covered by a 20 nm chromium layer is reported. The microstructure was made of SU8 photoresist and the dimensions are pointed out in green.

The replica of the channels has been fabricated by pouring the liquid polydimethylsiloxane (PDMS) on top of the SU8 microstructures. The PDMS was previously mixed, at 10:1 ratio, with the curing agent, and degassed in vacuum for almost 1 hour or until the complete disappearance of the bubbles. The PDMS has been poured onto the silicon wafer and then baked for 30 minutes at 115°C and finally put inside a plasma cleaner for an oxygen (30 ppm) plasma process for 50 seconds at 45 W for the surface activation and consequently allowing the binding on an activated glass surface (plasma treated as previous).

The microstructure can be appreciated thanks to the black background. The bottom part is a 1 mm thickness glass slide and on top a PDMS slice with the microstructures dug in is covalently bound on the glass via an oxygen plasma surface treatment. The holes from the top to the bottom allow the sample to flow in and out. Scale bar: 1 cm.
2.8 Microfluidics Device: Functionalization

Mainly based onto the applications of our microfluidics channels the devices were functionalized with an octadecylsilane (Sigma-Aldrich). A 10 µM solution diluted in tetradecafluorohexane (TDFH, Sigma-Aldrich) has been fluxed into the microfluidic channels in a parallel way (see Figure 2.8) by using a single automated syringe pump for 1 hour. At the end of this process the syringe were replaced with another one filled with acetone in order to remove TDFH residues. It was then fluxed for 1 hour and then finally washed with an IPA solution to remove the traces of acetone too. This process allowed to bind octadecylsilane on the glass hydroxyl groups and so reducing the hydrophilicity of the glass surface. Indeed this process is well-known and applied in order to avoid the protein/cells interaction with glass part of the microfluidic devices.

Figure 2.8 | Functionalization Process of Microfluidic Channels with Silane

Three microfluidic devices are functionalized in parallel with an automated pump. For the functionalization a 10 µM concentrated octadecylsilane has been diluted in tetradecafluorohexane and fluxed at 1ml/hr for 1 hr inside the devices.
For the evaluation of the hydrophobic functionalization of the glass a free piece of it has been treated as the PDMS/glass device and the contact angle measurements has been carried out. To achieve that a 1 μL drop of water has been put on the glass and a high definition camera provided with a specific software allowing the calculation of the contact angles was used. The experiments were repeated three times.

![Contact Angle Measurement Setup](image)

**Figure 2.9 | Contact Angle Measurement Setup**

The contact angle setup is provided with a laptop, a desk and a high resolution camera. All the instrumentation is placed in a class 10 Clean Room at IOM-CNR in the Elettra Synchrotron Light Laboratory.
2.9 Microfluidics Device Testing with Micron-Sized Beads

Ferrite magnetic beads of 10 µm in diameter were purchased from Sigma-Aldrich in order to test the microfluidic devices and so better understanding the flux streamlines. In order to better simulate the real context where cancer cells are diluted with WBCs in a ratio of $1 \times 10^4$-$1 \times 10^5$, the magnetic beads were diluted in physiological solution with latex-non-magnetic beads, of 2 µm in size, in a ratio of 1 to $10^3$. Moreover the magnetophoresis evaluation have been carried out by applying an external static magnetic field via a NdFeB permanent magnet put just in contact with the PDMS structure. The sample containing beads were fluxed at 1 mL/hr with an automated syringe pump.

Figure 2.10 | Microfluidics Device Put Under Microscope Setup.

Top left: the two syringe pump can be seen during operations of the microfluidics study. Top right: the PDMS microfluidic device can be appreciated with the tubes and the connections for performing the experiments. Bottom: an optical microscope image of the microfluidic device. Scale bar: 200 µm.
3. Results and Discussion
Chapter Summary: the main results obtained during the Doctorate program are highlighted in this chapter. A particular attention has been given to the MNPs uptake process, demonstrated by advanced techniques. The first demonstration of the MNPs uptake has been achieved by a confocal imaging analysis via the labeling of a cytoskeleton protein (actin filaments), subsequently the glucose dependency has been further proved by the comparison with simple citrate coated MNPs. These analysis have been performed by a cyto-chemical assay able to highlight the iron accumulated inside cells, the Perls’ iron staining.

The main result obtained was the demonstration of the significant higher MNPs uptake by the mesenchymal-like cells respect to the epithelial-like ones both in low (5.5 mM) and high glucose (25 mM) medium concentration. In particular at 25 mM concentrated glucose in the culture medium the epithelial-like breast cancer cells internalized a quasi-null amount of glucose coated MNPs. The molecular process guiding the specific internalization of those has been evaluated and demonstrated as to be Glut1-dependent by the usage of a specific glucose channel inhibitor (the STF-31). The mesenchymal-like BC cells have been treated by an IR laser beam focused on MNPs aggregates in order to cause a localized heating and consequently observe the hyperthermia effects. Computer simulations about microfluidic channels have been carried out and then the devices were fabricated and used. To assess the microfluidic devices, a model experiments has been carried out using a magnetic beads sample of 10 µm in diameter and finally fluxed inside the devices for displacement studies. The conclusion of this chapter is dedicated to the displacement studies of the MNPs-positive cells for diagnostics applications.
3.1 **Glucose Coated CoFe$_2$O$_4$ Nano Particles Characterization**

ATR-FTIR allows to confirm the esterification reaction between the 2-NBDG and the citrate molecules adsorbed on the cobalt ferrite NPs. To evaluate the IR absorbance of the MNPs samples, three spectra have been reported in Figure 3.1: 1)citrate stabilized MNPs (in black), 2) CoFe$_2$O$_4$ NPs functionalized by the 2-NBDG (red line) and finally 3) the spectrum of glucose coated ones (in blue). The FTIR spectrum of citrate coated CoFe$_2$O$_4$ NPs is characterized by few and broad bands, the most intense of which centered at 1396 cm$^{-1}$ (COO$^-$ symmetric stretching) and 1580 cm$^{-1}$ (asymmetric stretching of COO$^-$). Free carboxylate moieties are revealed by this vibrational pattern, while the shifts of the carbonyl group, of carboxylic moieties, from 1710 cm$^{-1}$ in free solution (data not showed) to 1680 cm$^{-1}$ demonstrate the partial single bond character of the C=O group and consequently the chemisorptions of carboxylate ions onto the NPs surface$^{104,105}$. The carboxylate groups of citrate anion adsorbed onto the NPs express a partial single bond character that results in the weakening of the carbonyl bond and determines the shift to lower. It is, furthermore, possible to highlight that citric acid forms complexes with the metal cations of CoFe$_2$O$_4$ by chemisorption of one or two of the three COO$^-$ groups, depending on steric hindrance onto the surface, whereas the remaining carboxylate groups do not interact with the surface$^{106,107}$. This coordination scheme is also supported by the pronounced asymmetry of the symmetric stretching of COO$^-$, that envisages the peculiarity of at least two types of $\nu_{\text{sym}}$(COO$^-$). In accordance to this, at least one carboxylate group results exposed to the solvent, and this group could be, reasonably, the one involved in ester bonding formation$^{108}$. The coordination band is further shifted as a consequence of the functionalization with the glucose derivative, while the carboxylate spectral features are greatly suppressed due to esterification reaction involving the –OH group of the 2-NBDG. The spectroscopic demonstration of the covalent ester bonding formation is given by the absorbance peak at 1737 cm$^{-1}$ in MNPs – 2NBDG and MNPs-Glucose samples. The absorbance peaks in the spectral region between 1200 and 900 cm$^{-1}$ pertain the glucose and, in particular, 2-NBDG specific vibrational characteristics. CoFe$_2$O$_4$ NPs were further analyzed by SEM for the dimension evaluation. An average diameter of 27 nm by the weighted mean calculus with a standard deviation of 3 nm has been recorded for almost each sample. The data used for this evaluation are reported in Figure3.2.
Figure 3.1 | ATR-FTIR absorbance spectra of CoFe$_2$O$_4$ NPs.

The blue line pertains the FTIR absorbance data of CoFe$_2$O$_4$ NPs citrate coordinated. The black line is the 2-NBDG coated CoFe$_2$O$_4$ NPs and in red the glucose coated ones. The covalent functionalization of glucose (and 2-NBDG) is confirmed by the peak at 1737 cm$^{-1}$ corresponding to the ester bonding formation.

Figure 3.2 | CoFe$_2$O$_4$ NPs SEM image and Diameter Distribution Histogram.

The CoFe$_2$O$_4$ NPs samples have been prepared on silicon pieces and investigated with SEM. An example image is provided on the left. Scale bar: 200 nm. All the images collected have been analyzed with ImageJ software as described in text. On the right is reported the distribution histogram of diameters found and the Gaussian curve fitting.
3.2 Breast Cancer Cells: Evaluation of CoFe$_2$O$_4$ NPs Uptake

3.2.1 Dose-Effect and Toxicity Study of CoFe$_2$O$_4$ – 2NBDG NPs

Before proceeding with cell culture treatment the MNPs cytotoxicity were previously evaluated by the means of the MTT assay. Below 5 µg/mL, concentrated MNPs, no significant decrease in cell viability was observed for both cell lines. A 8±2 % and a 5±1 % of cell death has been encountered at 5 µg/mL for MDA-MB-231 and MCF7, respectively. MCF7 resulted, in each concentration tested, significantly less affected by the MNPs administration respect to the mesenchymal-like counterpart. They showed a significant cytotoxicity effect (higher than 5 %) at the 10 µg/mL and 25 µg/mL concentrations. Co-culture revealed, as expected, an average behaviour between the two cell lines (as can be seen in Figure 3.3). One of the main, possible, reason why the mesenchymal-like subtype exhibited a less tolerability, at the same concentration respect to the MCF7, will be better explained in the next paragraph.

![Figure 3.3](image)

**Figure 3.3 | Toxicity Evaluation of CoFe$_2$O$_4$ – 2-NBDG NPs on BC Cells.**

The CoFe$_2$O$_4$ – 2-NBDG NPs have been tested on BC cell lines (MCF7 and MDA-MB-231) for further evaluation of cytotoxicity at different concentration of administration in cell cultures. In blue are reported the data for the mesenchymal-like cancer cells, in red for the epithelial-like and in white for the co-culture environment.
3.2.2 Confocal Microscopy Study for CoFe$_2$O$_4$ – 2NBDG NPs Uptake

Due to the low toxicity observed on both BC cell lines after the administration of the MNPs showed on both cell lines, we choose the 2.5 µg/mL concentration for the CoFe$_2$O$_4$ – 2-NBDG NPs uptake studies. Therefore, this concentration corresponds to a total amount of about 4.6 x10$^{10}$ nanoparticles, that perfectly fit literature data$^{92}$. The specific uptake capability was accurately observed, with a confocal microscope in presence of two different glucose medium concentration: 1 g/L (5.5 mM) and 4.5 g/L (25 mM). At the low glucose concentration, both cell lines (and the co-culture too) revealed an evaluable amount of NPs internalized via the relative fluorescence analysis. Hence, the epithelial-like subtype exhibited about 1.5 times more fluorescence than the control cells, whereas the mesenchymal-like one showed a level of more than 2.2 times respect to the control. On the opposite, at the high glucose medium condition (4.5 g/L), the MDA-MB-231 cells exhibited a comparable uptake to the low one (See Figure 3.4 and 3.6), whereas MCF7 cells show a quasi-null or even completely absent amount of NPs internalized measured as fluorescence ratio between the MNPs-treated samples and the control ones (see Figure 3.5 and 3.7). The calculated relative fluorescence gave a comparable amount for the MDA-MB-231 in between glucose medium concentrations, whereas for the epithelial-like one a statistically significant p-value of less than 0.0001 was found. The same results were given by the comparison of the two BC cell lines. Details are reported in Figure 3.7.

The cytotoxicity effect of the MNPs, observed by the MTT assay, can now be justified with the significant uptake difference, between cell lines, registered by the fluorescence amount evaluation.
Figure 3.4 | CoFe$_2$O$_4$ – 2-NBDG NPs Uptake by MDA-MB-231 Cells in Low and High Glucose Medium.

An average confocal fluorescence image of a representative example for the MDA-MB-231 sample is provided. The confocal microscope z-stacking was around 250 nm and the total height evaluated, for each sample, was about 4 µm. **Left:** CoFe$_2$O$_4$ – 2-NBDG NPs channel in red for both glucose medium conditions. CoFe$_2$O$_4$ – 2-NBDG NPs have been administered at 2.5 µg/mL for 2 hours before fixing the cells with a 4% PFA solution. **Right:** the merged images of all fluorescence channels. In cyan are highlighted the actin filaments by the phalloidin staining, in red the nanoparticles and in blue the nucleus. Scale bars: 50 µm.
Figure 3.5 | CoFe$_2$O$_4$ – 2-NBDG NPs Uptake by MCF7 Cells in Low and High Glucose Medium.

An average confocal fluorescence image of a representative example for the MCF7 sample is provided. The confocal microscope z-stacking was around 250 nm and the total height evaluated, for each sample, was about 4 µm. **Left:** CoFe$_2$O$_4$ – 2-NBDG NPs channel in red for both glucose medium conditions. CoFe$_2$O$_4$ – 2-NBDG NPs have been administered at 2.5 µg/mL for 2 hours before fixing the cells with a 4% PFA solution. At the **Right:** the merged images of all fluorescence channels. IN cyan are evidenced the actin filaments by the phalloidin staining, in red the nanoparticles and in blue the nucleus. Scale bars: 50 µm.

Moreover, the co-culture samples reflected the behaviour of the single cultures by showing an intermediate effect both as fluorescence level (Figure 3.7) and as MNPs-positive cells (Figure 3.8). In this case, as it can be appreciated in Figure 3.6, the co-culture samples were incubated with an anti E-cadherin Ab that finely allow to discriminate the epithelial-like cells to the mesenchymal-like ones. Indeed, using this univocal-epithelial marker, it was possible to quantify the fluorescence emitted by the E-cad positive cells (MCF7) and by the E-cad negative ones (MDA-MB-231). MCF7 cells thus confirmed their uptake of CoFe$_2$O$_4$ – 2-NBDG NPs also in the co-culture environment showing a statistically significant uptake in the
1 g/L medium compared with 4.5 g/L one, as in the normal single culture. On the other hand, MDA-MB-231 showed a CoFe$_2$O$_4$ – 2-NBDG NPs uptake comparable in both medium conditions. The absence of MNPs internalization in the E-cad positive cancer cells and, at the opposite, the presence inside the E-cad negative ones evidenced and also confirmed the previous results.

Figure 3.6 | CoFe$_2$O$_4$ – 2-NBDG NPs Uptake by BC Cell Lines Co-Culture.

An average confocal fluorescence image of a representative example for the co-culture sample is provided. The confocal microscope z-stacking was around 250 nm and the total height evaluated, for each sample, was about 4 µm. Co-culture of MCF7 and MDA-MB-231 BC cells have been treated with 2.5 µg/mL of CoFe$_2$O$_4$ – 2-NBDG NPs for 2 hours, both in low and high glucose medium conditions. In red the channel of CoFe$_2$O$_4$ – 2-NBDG NPs, in cyan the E-cadherin and in blue the nucleus. Are evidenced how the epithelial E-cadherin protein-expressing cells do not internalize MNPs in the high glucose medium conditions. Scale bars: 50 µm.
The 3D reconstruction of the confocal z-stack images provided the possibility to associate the presence of the CoFe$_2$O$_4$ – 2-NBDG NPs inside the BC cells, thanks to the actin filament staining that allow to follow the NPs presence at the same level of the cytoplasm.

In addition to the relative fluorescent data also the MNPs-positive cells quantification is provided with the relative statistical p-value in between samples (Figure 3.8). MDA-MB-231 resulted in a higher uptake capability with a statistical significant p-value less than 0.0001 in comparison with MCF7 in both glucose conditions. A lower p-value (less than 0.05) was found for the comparison between MDA-MB-231 and the co-culture behaviour in the low glucose medium conditions as well for the comparison between co-culture and MCF7.

The results obtained clearly demonstrated the possibility to finely target the mesenchymal, hyper-activated cancer cells by the exploitation of their metabolism. In particular the interesting result pertains the possibility to decide whether recognize both cell lines or not by increasing the glucose medium concentration (in vitro) from a low level to a higher one.

Figure 3.7 | CoFe$_2$O$_4$ – 2-NBDG NPs Uptake: Fluorescence Evaluation Summary.

Measurements of the fluorescence ratio between the MNPs treated sample and the control ones is reported. Example images of the data used are reported in Figure 3.4, 3.5 and 3.6. The averaged confocal images of fluorescence have been used for the calculations. As can be observed, the MDA-MB-231 internalized a statistically significant higher amount of fluorescence particles in both glucose medium conditions respect to the epithelial-like cells. p-value < 0.0001 (co-culture row). The MCF7 as well displayed a statistical significant uptake itself by the comparison of the 2 glucose medium environment.
**3.2.3 SEM – FIB Investigation of CoFe$_2$O$_4$ – 2-NBDG NPs Internalization**

The registered different behaviour, in between BC cell lines, has been further and deeply explored by SEM-FIB inspection. The Gallium gun allowed to finely milling the tumoral cells in order to identify the metal part, and so link the “metal object” presence to the MNPs internalization. In both cell lines the FIB has been used and example images are provided in Figure 3.9. The Figure 3.9 highlights two examples of milled part of BC cells: in the MDA-MB-231 case a big CoFe$_2$O$_4$ – 2-NBDG NPs aggregate (around 600 nm), internalized by the cell, is visible; in MCF7 case a portion of a milled cell portion is zoomed where several single MNPs are evidenced as white “dots”. The white color is due to the electron conductance ability exerted by the metal objects, whereas the grey parts mean a less capacity in electron charges transfer, as it is for the cytoplasm and for the glass surface. The dimensions of the white particles in Figure 3.9 have a mean dimension of about 30 nm resulting in accordance with the averaged diameter of MNPs (previously calculated by SEM imaging and reported in Figure 3.2). In MCF7 as well for the MDA-MB-231 cells, MNPs aggregates were found (at the 5.5 mM glucose) both on top of the cytoplasm and on the nuclei. By the investigation and cutting of more than 50 cells per samples, a demonstration of the MNPs presence inside the nucleus has never been found, leading to the hypothesis of a specific recognition of the nano
objects by the cancer cells. High glucose samples of the epithelial-like, MCF7, cell line have been cut and investigated, as described for the low one, but no internalized NPs or kinds of aggregates were observed inside or outside (outer membrane) the cell body (see Figure 3.10). The explanation of that could be the dehydration and washing steps that the cell samples followed in order to be suitable for SEM imaging. Effectively these epithelial-like cells, due to the fact that they did not internalize MNPs at high glucose medium concentration, did not strongly interact with the MNPs resulting in a complete wash of the membrane and of the eventual MNPs on it. This occurrence was not registered in the low glucose medium samples.

Figure 3.9 | SEM images of FIB-Cut BC Cells in Low Glucose Conditions.

SEM images of MCF7 and MDA-MB-231 cells after the CoFe₂O₄ – 2-NBDG NPs administration are reported. Both cell lines underwent the FIB precise milling to better explore the inner part of the treated cells. The SEM technique allowed to point out the presence of metal-origin parts inside the cells cytoplasm, as white objects due to their conductive capability. An internal view of the cells is provided thanks to the precise focused Gallium ion beam milling. The grey part on bottom is the glass coverslip (indicated by the sign) where cells were grown. In MCF7 case several white objects
(particles) inside the cytoplasm, with average size in agreement with the one of the MNPs, can be interpreted as to be CoFe$_2$O$_4$–2-NBDG NPs internalized by the cell. MDA-MB-231 example image points out a big aggregate zoomed in, where also few single separated nanoparticles are visible. The cytoplasm is grey due to the lack of metal part inside it, instead as it is for MNPs and membrane (Chromium coated) with an increased electron contrast. Upper scale bars are 2 µm. Middle scale bar is 600 nm. Bottom scale bars are 200 nm.

![Figure 3.10](image)

**Figure 3.10 | MCF7 Cells Treated with CoFe$_2$O$_4$–2-NBDG NPs in High Glucose Medium**

A representative SEM image of a MCF7 sample after the administration of CoFe$_2$O$_4$–2-NBDG NPs at 2.5 µg/mL at high glucose medium concentration (4.5 g/L). The main result highlighted by the figure is the absence of MNPs on the cell membranes. The explanation of that is why at high glucose medium concentration the MCF7 cells did not internalize glucose coated MNPs. Indeed the epithelial-like cells reach a sort of threshold of glucose uptake that somehow inhibit further internalization. Scale bar: 30 µm.

### 3.2.4 Specific vs. Unspecific NPs Uptake: Perls’ Iron Staining

A further confirmation of MNPs uptake has been obtained, on the two single cultures, by the Perls’ iron staining (see paragraph 2.3.4). This method has been chosen for the MNPs evidence demonstration by the presence of the Prussian blue spots in correspondence to the cells cytoplasm (observed with an optical microscope). The results obtained allow to clearly identify big differences between samples; D-glucose- and 2-NBDG- coated MNPs displayed a
time-dependent internalization with a maximum reached after 2 hours of incubation. Citrate coated MNPS, on the other hand, revealed an unspecific and almost constant internalization by the BC cells treated. While MDA-MB-231 cells did internalize a quite constant amount of MNPs, despite the glucose medium concentration, confirming the data obtained in the confocal analysis (with fluorescence), MCF7 cells exhibited a clear opposite uptake ability between low and high glucose environment. After 2 hours of incubation, the MDA-MB-231 cell line showed a percentage of MNPs-positive cells higher than 80% in both glucose conditions, instead MCF7 cells showed an amount of positive cells of about 60% at the 1 g/L environment, and a quite null uptake at the higher one. Regarding the citrate coated NPs uptake, the results obtained with this technique showed a quasi time-dependent behaviour with a percentage of positive cells variable between 5 and 15 % in every cases. The remarkable results pertain the MCF7 cells uptake of citrate coated MNPS in the high glucose conditions. In this specific case, a quite comparable amount, ranging from 5 to 15 %, has been registered regardless the coating type (Figure 3.11).

BC cell lines have been investigated also after 3 and 4 hours of incubation, but any substantial difference with the 2 hours samples were observed.

For every treatment a time-dependent uptake can be appreciated. The MCF7 exhibit a strange
behaviour at the high glucose concentrated medium where they internalize quite the same amount of MNPs for every NPs type.

The percentage of positive cells after 2 hours of incubation with the different MNPs used are reported in table 3.1.

<table>
<thead>
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<th>MDA-MB-231_1g/L</th>
<th>MDA-MB-231_4.5g/L</th>
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<tr>
<td></td>
<td>Mean[ %]</td>
</tr>
<tr>
<td>CoFe2O4 Glucose</td>
<td>82.5</td>
</tr>
<tr>
<td>CoFe2O4 2-NBDG</td>
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</tr>
<tr>
<td>CoFe2O4 Citrate</td>
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<table>
<thead>
<tr>
<th>MCF7_1g/L</th>
<th>MCF7_4.5g/L</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Mean[ %]</td>
</tr>
<tr>
<td>CoFe2O4 Glucose</td>
<td>59.3</td>
</tr>
<tr>
<td>CoFe2O4 2-NBDG</td>
<td>54.8</td>
</tr>
<tr>
<td>CoFe2O4 Citrate</td>
<td>21.4</td>
</tr>
</tbody>
</table>

Table 3.1 | Mean Percentage of the MNPs-positive Cells after 2 hours of incubation.

The average percentage of the treatment with every MNPs for each cells and medium condition after 2 hours of incubation are reported. The same data with the time dependent results are plotted in figure 3.11. Mean is expressed as percentage of MNPs-positive cells, SD is the standard deviation and N is the total number of positive-cells counted per sample.
The Perls’ technique moreover allowed to claim the internalization of MNPs instead of a simple interaction. Indeed in previous experiments we did not add the TritonX-100 incubation and only few blue dots on cancer cells were highlighted, whereas after the “soap” addition several blue dots were pointed out, clearly confirming the cytoplasmic internalization of the MNPs. Details are reported in Figure 3.12.

The difference in MNPs amount identified, as a consequence of Prussian blue deposition, is reported for both cell lines. In particular the Perls’ assay have been simply modified by adding a TritonX-100 incubation in order to improve the membrane permeability and so the assay reaction yield. The clear results can be appreciated in both cases as numbers of Prussian blue spots. Scale bars: 50 µm.

The possibility to understand the glucose functionalization (both as single and as fluorescence analogue) dependency on MNPs uptake gave interesting suggestions about the driving molecular mechanism. These results, indeed, together with the previous one on the MNPs presence outside the nuclei, furnished the basis for the following step pertaining the internalization process. The fact that the glucose medium concentration act somehow as a threshold for the epithelial-like cells, influencing the uptake process too, led to the hypothesis of glucose transporters mediated mechanism.
3.3 The Molecular Mechanism Driving The Internalization Process

MDA-MB-231 are widely-recognized, by literature, as to be Glut1 overexpressing cells, whereas MCF7 cells own a normal expression. SLC2A1 is the gene responsible for the GLUT1 encoding and, as it is described in the introduction, is one of the glucose-specific channels family involved in the de-regulated metabolism involved in the Warburg effect, by enabling the uptake of the glycolysis substrate. The experiments performed on BC cell lines with the purpose to better understand the driving mechanism standing at the basis of the glucose (and 2-NBDG) coated MNPs uptake, have been investigated at the molecular level. In particular the glucose specific Glut1 channels were molecularly blocked by the usage of a selective inhibitor, the STF-31. This drug was firstly tested for cytotoxicity evaluation at different administration time length and concentration. MDA-MB-231 exhibited a comparable effect among different glucose medium conditions at 16, 24 and 32 hours after the drug administration. MCF7 cells, as well, exhibited comparable results but a higher sensitivity to the compound respect to the mesenchymal-like cells. This behaviour can be explained by the relative lower percentage of Glut1 transporter on their membrane, thus reducing the necessary dose to reach the same inhibition effect. A higher effect of cytotoxicity were evaluated after 72 hours of the STF-31 administration, in both cell lines. The details are reported in Figure 3.13.

Based on toxicity results the following evaluation of STF-31 effect on MNPs internalization were performed at different concentration for the two cell lines. The experiments performed on luminal-epithelial and mesenchymal-like BC cell lines furnished interesting results about the molecular mechanism for the channel-mediated internalization of CoFe$_2$O$_4$ NPs. On MCF7 cells the decrease of 2-NBDG-NPs internalization was observed at 5 µM concentrated STF-31 beginning from 4 hours of treatment with a peak at 8 hours, where a few MNPs inside were found (both as fluorescence and as Prussian blue spots). MDA-MB-231, on the other hand, did not exhibit a significant uptake decrease after 8 hours at 5 µM, consequently the drug concentration as well as the incubation time have been increased. 32 hours after the drug administration, the MNPs amount internalized significantly decreased until reaching a 4% of positive cells. In order to quantify the STF-31 affection on glucose coated MNPs two type of quantification were used. First the fluorescence were quantified as before with the averaged images respect to the control, whereas for the number of MNPs positive cells the Perls’ Iron
staining were performed (Figure 3.17). The fluorescence analysis results for the luminal-
epithelial subtype are reported in Figure 3.14, whereas for the mesenchymal-like one is
plotted in Figure 3.15. The p-value obtained from the quantification of the fluorescence
analysis are reported on the graphs, where three asterisks means a p-value less than 0.0001
and two asterisks less than 0.01.

Figure 3.13 | **STF-31 Cytotoxicity on BC Cell Lines.**

Different concentration and different incubation time are reported for the toxicity evaluation after the
Glut1 inhibition by STF-31 in both glucose medium conditions. In Y axes are reported the ratio of
alive cells (1 means a viability of 100 %) and in X axes the incubation time. The data were obtained
via MTT test.
Figure 3.14 | MCF7: Glut1 Inhibition Effect on CoFe₂O₄ – 2-NBDG NPs Uptake.

Four fluorescence microscope images, for different incubation time at 5 μM concentrated STF-31, with the quantification plot, are reported. In cyan is highlighted the actin filament, in red the CoFe₂O₄ – 2-NBDG NPs and in blue the nucleus. It is appreciable the reduction of the MNPs internalized by the sequential decreasing of red amount inside cells beginning from 2 hours and reaching the maximum after 8 hours. A representative image for each treatment has been chosen and provided. The p-value are reported as asterisks among samples. Scale bars: 50 μm.

The data obtained with the fluorescence evaluation were confirmed also by Perls’ iron staining assay, where analogous results have been achieved (see Figure 3.16 and 3.17). These results confirmed the hypothesis that the CoFe₂O₄ – 2-NBDG NPs (or glucose coated) are internalized via a Glut1 dependent mechanism, due to the reduction of internalization observed after its fine molecular blocking. This pathway means, probably, an interaction between the NPs and the glucose transporters that consequently stimulate an active endocytosis (clathrin or non-clathrin mediated) that finally lead to a MNPs-bearing vesicles internalization.
Figure 3.15 | MDA-MB-231: Glut1 Inhibition Effect on CoFe$_2$O$_4$-2-NBDG NPs Uptake.

Four fluorescence microscope images, for different incubation time at 10 µM concentrated STF-31 with the quantification plot, are reported. In cyan is highlighted the actin filament, in red the CoFe$_2$O$_4$-2-NBDG NPs and in blue the nucleus. It is appreciable the reduction of the MNPs internalized by the sequential decreasing of red amount inside cells. A representative image for each treatment has been chosen and provided. The p-value are reported as asterisks among samples. Scale bars: 50 µm.
Figure 3.16 | Perls Iron Staining after STF-31 Administration on BC Cell Lines.

**Left:** MDA-MB-231 cells after 8 hours of treatment with a 10 µM concentrated STF-31 drug. In blue are pointed out the 2-NBDG coated MNPs, and around 50% of cells resulted as positive. Scale bar: 50 µm. **Right:** MCF7 cells after 6 hours of treatment with a 5 µM concentrated STF-31 drug. In blue are pointed out the 2-NBDG coated MNPs, and around 20% of cells resulted as positive. Scale bar: 30 µm.

Figure 3.17 | 2-NBDG coated MNPs internalization after Glut1 inhibition.

Evaluation of *in vitro* reduction of MNPs internalization after the treatment with the Glut1-specific inhibitor, STF-31. The drug has been administered at different concentration and for same incubation time points. In Y axes are
plotted the number of MNPs positive cells (calculated by the Perls’ iron staining) respect to the control. MCF7 cell line was not further treated at high glucose medium due to the previous discovered low MNPs uptake at that concentration.

For a further investigation of the molecular basis driving the internalization processes and the consequent management of MNPs by cancer cells we also tried to identify the cytoplasmic pathway. In order to better understand it, we marked the cells with a lysosomal marker (anti LAMP2 Ab) and with an endosomal one (anti EEA1 Ab), but a lacking of any co-localization of fluorescence between the MNPs spots and the vesicles ones have been found. Future, deeper analysis have to be carried out for a whole comprehensive molecular pathway reconstruction, maybe by a live imaging system.

### 3.4 White Blood Cells Staining with CoFe$_2$O$_4$ – 2-NBDG NPs

A sample of WBCs from a healthy patient was collected and processed as described in the 2.4 paragraph. No evidence of CoFe$_2$O$_4$ – 2-NBDG NPs uptake has been pointed out in WBCs sample (see Figure 3.18). These results gave interesting insights for future applications in cancer diagnosis and in particular in such a complex scenario, like the whole blood analysis for CTCs enrichment. Due to the lack of internalization by healthy WBCs at the concentration tested, a future implementation of the present methods could be now provided.

![Healthy WBCs treated with 2.5 µg/mL CoFe$_2$O$_4$ – 2-NBDG NPs.](image)

The WBCs did not internalized a fluorescent-evaluable amount of MNPs after the 2 hours incubation. The CD45 positive cells are highlighted in red. In blue are the nuclei and in green the MNPs.
3.5 Infrared Laser Hyperthermia on Breast Cancer Cells

The glucose coated CoFe$_2$O$_4$ NPs and in particular the 2-NBDG functionalized ones have been investigated for the hyperthermia (or thermotherapy) treatment. The specific uptake of mesenchymal cancer cells has been, indeed, exploited for a better comprehension of the possible therapeutic applications. The IR laser beam used has been focused precisely on the MNPs aggregates that the cancer cells internalized and this was confirmed by two controls: first the light scattered from the metal particles and second the fluorescence emitted by the 2-NBDG functionalized particles. CoFe$_2$O$_4$ NPs have a relatively low IR absorbance so they also exhibit a reflective part that was used, by checking the scattered light, for the MNPs aggregates identification. Each treated cell has been recorded during laser focusing for about 5 minutes in order to observe all the possible morphological modifications induced and finally checked after 30 and 60 minutes for death confirmation (by apoptosis or necrosis). The power of the laser onto the samples ranged from 0 mW to 15 mW with steps of 1 mW, in order to observe the power increasing effects. The main reactions noticed were morphological rearrangements, of the mesenchymal-like subtype, like bubbles formation and contours shrinking. These modification were generated by the local heating provoked by the MNPs that increasing the temperature in the surrounding region determined all the responses cited. Indeed, while cancer cells were not affected by the IR laser, at those power, the nanoparticles specific absorbance allowed the hyperthermia induced effects (see Figure 3.19). Around 10 MDA-MB-231 cells have been irradiated by the laser and each one encountered, in the next 60 minutes, the cell death. To exclude possible IR laser side effects a sample of MDA-MB-231 cells has been subjected to a 15 mW laser focusing for about 5 minutes, without any previous MNPs administration, and no effects have been noticed both earlier (during the 5 minutes irradiation) and later (after 1 hour). MCF7 epithelial cells subjected to the administration of CoFe$_2$O$_4$ – 2-NBDG NPs have been irradiated by the IR laser for 5 minutes at 15 mW of power (see Figure 3.19). No morphological effects or apoptosis/necrosis have been achieved, during and after irradiation time, confirming the low amount of MNPs internalized by the epithelial subtype (Figure 3.19E and 3.19F).

The exploitation via a laser beam irradiation not only allowed us to appreciate the localized hyperthermia effect at the single cell level but also to ensure the MNPs heating production capability. The application of this technique for in vivo purposes is not currently feasible or even a possible way to follow. The main reason why we chose this
procedure was due to the necessarily demonstration of the detailed possibility to cause hyperthermia on mesenchymal-like BC cells via the exploitation of the glucose coated MNPs. The foremost controversial aspect of the MNPs administration in vivo regarding the tissue-specific cytotoxicity with particular attention to the liver and kidney accumulation problem. Hence by coupling the possibility to develop new biological-safe magnetic material with the well-known oscillating-magnet-field therapy, future approaches could be translated to clinics.
The hyperthermia effect can be appreciated by the morphological modification events achieved by focusing an infrared laser beam directly onto the MNPs internalized by the mesenchymal-like cancer cells (picture A, B, C,D). In A and B are reported an MDA-MB-231 cell, respectively, before and after the laser beam focusing procedure on MNPs and a bubble formation is pointed out by the red arrow. In C and D another MDA-MB-231 cell is reported with the same protocol as A and B and a huge morphological modification is visible (retraction of a cell prolongation). In E and F a MCF7 cell is illustrated before and after, respectively, the same laser treatment and as can be seen, no morphological effect can be appreciated.
3.6 Microfluidics Device

3.6.1 Device Geometry Characterization

The computer simulations for channel geometry optimization have been carried out with COMSOL™ Multiphysics software. The geometry was selected for the purpose of rare cancer cell sorting as explained in previous paragraphs. The first problem encountered was to achieve acceptable velocity, in order to be compatible with blood sample volume processing. For this reason a low dynamic or even static channel was not usable, and moreover no branching with angle above 30 degrees could be inserted, otherwise the yield of the cells collected at the outlet decrease exponentially. With these limitations a microfluidic channel was previously produced and published, like the one from Hou et al. in 2010\textsuperscript{109}. Despite this paper, where the channel geometry was chosen for lateral displacement of malaria infected red blood cells, mainly driven by visco-elastic properties; in our case the geometry was a function of the velocity to output ratio. In Figure 3.20 it is reported a simulation carried out for better understanding the percentage of cells that, without any external agent (like magnetic forces), go into each microfluidics branch. As it can be seen, at 1 mL/hr, the percentage of cells that goes inside the “lateral” branches is less than 30% each. In this way after the MNPs-positive cancer cells displacement, achieved by a magnetic field, only the 29.3% of the total amount of initial cells will be collected at the same exit. This should be implemented with an in-series device or directly with a finer method that can choose the single positive cells (MNPs internalized) and consequently sort them in a diluted environment, like the DEPArray™ System\textsuperscript{110}.

![Particle Statistics for the Microfluidics Geometry Chosen at 1 mL/hr](image)

The final part of the microfluidics channel is detailed and zoomed in. The relative percentages, of flux amount for each branch, are pointed out. As it can be seen, less than 30% of the total amount of particles goes into the two lateral branches, at the simulated velocity of 1 mL/hr.
3.6.2 Hydrophobic Functionalization Measurements

The microfluidic device obtained by the soft optical lithography has been used for the first tests and hypothesis validation. In order to avoid clogs formation inside the micron-sized channels, the glass surface has been treated with octadecylsilane as described in paragraph 2.8. Biological samples and in particular cells are indeed able to strongly bind to the glass if any functionalization process has been previously followed. For a better comprehension of what kind of surface tension energy was determined by the just mentioned protocol a contact angle measurement was performed as described in the paragraph 2.8. The results obtained with the contact angle measurements are reported in the Table 3.2 and as it can be appreciated, also from the Figure 3.21, the difference in between the untreated and the silane-treated glass portion is huge. From the typical initial value of 40 degrees, for the hydrophilic surface, the glass layer gone to hydrophobic values after the silane treatment, in particular in our case reached 104 degrees.

![Contact Angle Measurements](image)

Figure 3.21 | Contact Angle Measurements

Hydrophobic evaluation of a glass surface with two drop of water deposited on it. Left drop is lain down onto the glass portion treated with 10 µM octadecylsilane for 1 hour. Right drop is laid down onto the glass portion not treated. The surface forces differences are hence confirmed by the contact angles measurements carried out by coupled camera-software analysis.
Table 3.2 | Contact Angle Measurements Data

The contact angle degree measurements data pertain the drops reported in Figure 3.21. The software calculated the angle between the glass layer (assumed as to be linear) and the point of contact with the water drop lay down on it.

An example of the microfluidics channel wetability is given in the figure 3.22.

![Figure 3.22 | Microfluidic Device Wetting After Silanization](image)

An optical microscope image of the microfluidics device after silane treatment for hydrophobic functionalization. The water cohesion forces can be appreciated by the meniscus formed in the interface with air, inside the microfluidic channel. Scale bar: 100 µm.
3.6.3 Microfluidics Magnetic Displacement of Micron-sized Beads

To observe and trace the flux streamlines inside the microfluidics channel a first test with a fluorescent latex beads sample (Ø = 2 µm) has been executed. The better results with the chosen channels structure was achieved at 1 mL/hr, where, indeed, no clogs formation was observed and a confirmation of the simulation previously carried out has been reproduced. The streamlines were found to be in accordance with the theoretical ones, calculated by software simulations, as can be observed in Figure 3.23.

![Figure 3.23](image)

**Figure 3.23 | Computer Simulations vs. Microfluidics Test**

**Left**: an image of velocity field, in the range of 0.01 m/s to 0.1 m/s (represented by arrows thickness), carried on with COMSOL™ Multiphysics software. **Right**: an image of the microfluidic channel obtained, by an exposure time of 10 seconds, with a high resolution camera is reported; the test was achieved by using a latex fluorescent beads solution (2 µm in size). Scale bar: 100 µm.

The interesting results were obtained by mixing together latex non-magnetic (Ø = 2 µm) and ferrite-magnetic beads (Ø = 10 µm), where a specific displacement, under a permanent magnetic field, has been observed. In Figure 3.24 is showed the beads positioning without any applied magnetic field, and as can be appreciated, latex and ferrite beads occupy different position. In Figure 3.25 is reported a magnetic bead displacement, with a streamline maintenance predisposition, due to the external magnetic field applied (gradient goes from top to bottom part in the image provided).
Figure 3.24 | **Microfluidics Test With Magnetic Beads Diluted in Latex Beads.**

In figure can be appreciated three magnetic beads (the ones indicated by arrows) occupying different streamlines. The smallest beads are latex ones of 2 µm in size. Scale bar: 100 µm.

Figure 3.25 | **Microfluidics Magnetic Displacement of 10 µm-sized Magnetic Beads.**

Two consecutive frames showing the magnetic displacement exerted on a bead, by a permanent magnet of about 200 mT placed at 1 cm from the channel, is provided. Arrows indicate the magnetic bead displaced attracted by the magnetic force applied consequently following the border. The magnetic beads have been diluted 1:1000 in a solution of non-magnetic latex beads. The latex non-magnetic beads (Ø = 2 µm) can be seen as little black dots (in motion due to the flux velocity). Scale bars: 100 µm.
3.6.4 Beads vs. Cancer Cells Displacement: Theoretical Measurements

Theoretical calculations for cancer cell displacement have been performed for better understanding the required magnetic field after the results reached with the magnetic beads and the permanent magnet. In the case just discussed for magnetic beads, of 10 µm in diameter, the percentage of ferrite inside was about 50% and the outside shell material was polystyrene (purchased from Sigma Aldrich Co. LLC). In this case the density of each single bead was an average between the one of polystyrene (1.05·10³ kg/m³) and the one of ferrite (7.80·10³ kg/m³), so it was assumed around 3.9·10³ kg/m³. In the case of CoFe₂O₄ NPs the density was calculated to be 5.3·10³ kg/m³. The ferrite mass magnetization (Mₘₐₜₜ) was known, from the literature, to be in the range of about 160 to 200 A·m²/Kg, for both normal ferrite (FeO) and cobalt-ferrite particles¹¹¹,¹¹². In order to displace magnetic material (beads or cells) in addition to the magnetic force (Equation 1) acting on it there is another force (that by acting in the opposite direction respect to the motion exert a limitation on the particles displacement), i.e. the Stokes’ drag force.

\[ \vec{F}_m = V_m \cdot M_m \cdot \nabla B_x \]

Equation 1 | Magnetic Force.

The equation represents the magnetic force (Fₘₐₜₜ) exerted, in one direction, on a certain mass of magnetic material (Mₘₜₜ) by an applied magnetic field (B).

\[ \vec{F}_S = -6 \pi \cdot \mu \cdot r \cdot v \]

Equation 2 | Stokes’ Force Acting on a Particle.

π is the Greek pi, µ is the dynamic viscosity of the fluid (medium), r is the particle radius and v is the relative velocity between the particle and the fluid.

All the parameters cited, so far, have the same order of magnitude, so the only one big difference, between magnetic beads and MNPs-positive cells, is the magnetization volume. Indeed magnetic beads had around 50 % of their volume occupied by a magnetic material,
while the cells have a completely different ratio. The quantification carried out by fluorescence confocal microscope images analysis gave a volume ratio between cell volume \( V_{\text{cell}} \) and internalized-MNPs total volume \( V_{\text{MNPs}} \) equal to 1/50, as average (Equation 3).

\[
\frac{V_{\text{Cell}}}{V_{\text{MNPs}}} = \frac{1}{50}
\]

Equation 3 | Mean Cell to Internalized-MNPs Volume Ratio.

The ratio between mean cell volume and total amount of MNPs internalized (per cell) was calculated by confocal microscope images analysis.

Taking into consideration that for the magnetic beads displacement a permanent magnet of NdFeB was used and the magnetic gradient exerted was about 0.5 T/m, in the case of MNPs positive cell displacement a 25 times higher magnet should be used. This can be right if the nano-sized material and the large one behave in the same way, but as it is known from literature the single NPs reduce their magnetization properties of almost a half (from 200 to 100 A·m\(^2\)/Kg). This introduces another variable that has to be considered in the theoretical displacement calculation increasing the needed magnetic gradient of 50 times with respect to the one used in our experiments, meaning 25 T/m. Moreover the ratio value of 1/50 calculated does not take into consideration the distribution tails for the uptake were certain mesenchymal-like cells internalized significantly less MNPs, down to a 1/500 ratio, leaving the necessity to further increase the magnetic gradient of 1 order of magnitude since the whole cell-population is needed. In this case the magnetic field gradient might need to be 250 T/m that is a huge and not macro-scale reachable value. For all these reasons a micro-fabricated system is necessary, as several known from literature, were up to \( 10^4 \) T/m gradient have been achieved and reported\(^{61}\). We conclude from these calculations that the separation between mesenchymal and other type of cells is feasible in a system like ours.
4. Conclusions and a Perspective on the Future
One of the most currently debated issues in cancer diagnostics, in any separation method that does not kill the cells pertains to the possibility of identifying and investigating CTCs molecular characteristics. Tumor invasion and metastatization is, indeed, linked to the presence of tumoral cells in the blood stream circulation. The number of Ep-CAM positive CTCs has a clinical prognostic value even if it is not yet clear why epithelial cells can be found into the circulating blood. Considering that epithelial cells detach from initiating tumor mass only after the EMT they should be found as mesenchymal subtype and not as Ep-CAM positive. For these reasons an intra-blood mesenchymal to epithelial transition (MET) has been proposed in recent papers. Since it is clear that CTCs are both of mesenchymal and epithelial subtype we have tried to give our contribution by the development of cancer cells specific recognition method. Actually, the possibility to identify both subtypes could add a significant improvement to the cancer comprehension and treatment.

Even if the mesenchymal cancer cell metabolism characteristics are well known, they are not yet explored for diagnostics or prognostic applications. The concept of glucose covalent functionalization to therapeutic compounds is not new but nanoparticles functionalization with glucose has not yet been investigated. The innovative solution, of this work, regards the possibility to correlate the glucose massive uptake of cancer cells with the development of new low cost diagnostic methods. The low cost is an important issue in order to allow a larger access to diagnostics check-ups.

Concluding, the results we obtained can be summarized as follows:

1. The covalent glucose functionalization were exploited for a targeted delivery of magnetic nanoparticles inside cancer cells, with a particular attention to mesenchymal-like cells by increasing the glucose concentration within the medium. Due to the hyper-activated metabolism exerted by the more aggressive cancer cells we, indeed, were able to avoid the MNPs internalization by the epithelial, and less invasive-prone, cell population by tuning the medium characteristics.

2. The MNPs uptake inside cancer cell cytoplasm has been demonstrated with advanced technique such as focused ion beam and scanning electron microscope. The Perls’ method, an histo-chemical assay, has been slightly modified, for the purposes we required, as a further MNPs uptake evidence.
3. The Glut1-dependent internalization mechanism, for glucose coated compounds, has never been directly demonstrated before this work. The specific inhibition of the glucose transporter channel 1 allowed to, definitely, correlate it to the glucose functionalization. This stringent dependency has moreover been evaluated for avoiding eventual unspecific uptake by means of citrate coated MNPs, finding statistically significant differences between treatments.

4. A microfluidic devices has been proposed and developed, as a proof of concept, with the scope to use it in cancer cells displacement studies.

The results that we obtained on just two breast cancer cell lines suggest a new diagnostics and therapeutics possibilities that need to be demonstrated on bigger data sets such as other cell lines and cancer patients cell samples. However our consistent results can lead to a new interest in exploitation of cancer metabolism for a demanding personalized and targeted therapy.
5. References


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