Iron oxide nanocubes in nanomedicine: theranostic approach for cancer treatment and diagnosis

Doctoral Thesis presented by

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To my grandparents

Francesco and Aurelia

... these droplets are not atoms, but they are sufficiently small and light to be not entirely insusceptible to the impact of one single molecule of those which hammer their surface in perpetual impacts. .... Their movements are determined by the thermic whims of the surrounding medium; they have no choice. If they had some locomotion of their own they might nevertheless succeed in on getting from one place to another, but with some difficulty, since the heat motion tosses them like a small boat in a rough sea.

(E. Schrödinger, 1941)

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Declaration

Herewith I declare that the present thesis is a presentation of my original doctoral work. Wherever contributions from others are involved, all of them are clearly marked with reference to the literature, licence and acknowledgement of collaborative research.

Genoa, 15th December 2017

Marco Cassani

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Thesis abstract

The present dissertation discloses the doctoral work carried out during the past three years at the Italian Institute of Technology (IIT) and the University of Genoa. The work was focused on the functionalization of iron oxide nanocubes (NCs) for different biological applications. Tuning the surface features of these magnetic nanocubes, as well as their assembly and intrinsic chemical and physical properties, resulted in the development of suitable nanotools for cancer theranostic, *i.e.* the combination of diagnosis and cancer therapy.

The first chapter deals with the synthesis of magnetic nanoclusters, referred as magnetic nanobeads (MNBs). Here, maghemite (γ-Fe_2O_3) nanocubes were tightly enwrapped into an amphiphilic polymer able to solubilize and stabilize them in water-based solutions. The synthetic route, reported in literature, was improved in order to obtain more stable polymeric shells that can be further functionalized with PEG-derivatized molecules. Due to the higher magnetic moment found for the MNBs, compared to that of single nanocubes, they were investigated for magnetic cell sorting. Therefore, a targeting feature was added to their surface by attaching a PEG molecule derivatized with folic acid (PEG-FA). This approach provides: 1) the binding of the MNBs to folate receptors overexpressed on the cell membrane of some cancer cells; 2) stability in complex biological media; 3) distance of the FA from the polymeric surface; 4) degree of freedom to the bioactive folic acid. A cancer cell line having high folate receptor expression profile (KB cell line) was chosen as a model for testing the sorting ability of the MNBs. The results obtained showed a significantly higher sorting efficiency for the MNBs functionalized with PEG-FA in comparison to the one observed for the MNBs functionalized with a non-biologically active PEG. This outcome reveals the potential of PEG-FA functionalized MNBs for the isolation of relevant folate receptor positive cancer cells, *e.g.* ovarian cancer cells, from biological tissues.

In the second chapter, the structural transformation of core-shell wuestite/maghemite (FeO/γ-Fe_2O_3) nanocubes into maghemite is reported. Here, an approach that is not often applied by material scientists was followed for the transformation under aqueous conditions. The non-interacting nature of core-shell nanocubes, due to their low magnetization, allowed for an easy and quantitative transfer in water, using a standard protocol for the coating with an amphiphilic polymer. Then, it was found that a mild oxidation process, carried out in water at 80 °C, promoted the conversion of the core-shell structure into fully maghemite nanocubes, enhancing their magnetic features, especially the specific absorption rate (SAR). Thus, the nanocubes were functionalized with PEG-FA and the annealing treatment was repeated.

Noteworthy, the oxidation strategy developed did not compromise the bio-functionality of the PEG-FA molecule. Unfortunately, the SAR values of the obtained one-phase nanocubes were found to be viscosity dependent, discouraging their use for magnetic hyperthermia in cellular environment. Instead, because annealing increased the magnetic moment of the nanocubes, they were efficiently used for the magnetic sorting of KB cells. The sorting efficiency found for these nanocubes was comparable to that of the MNBs reported in chapter 1, suggesting that a high amount of single nanocubes bound to the cell membrane increases the magnetic moment of the whole nanocubes-cell system. Thus, the methodology adopted for tuning the magnetic properties of core-shell iron oxide-based materials into one single phase NPs was proven appropriate for the preparation of nanocubes for magnetic driven cell sorting.

The third chapter discusses the use of maghemite (Fe_2O_3) nanocubes for developing multimodal nanotherapeutics to treat ovarian cancer. The intriguing high heat performance of the nanocubes was exploited to perform magnetic hyperthermia. At the same time, the high surface area available on the nanocubes has been used for drug delivery and specific antibody-mediated tumor targeting towards ovarian cancer cells. The NCs were functionalized with both an oxaliplatin-derivatized PEG (PEG-Pt) and a PEG-Bis(carboxymethyl)-lysine (a nitrilotriacetic derived molecule) complex for the binding of an his-tag antibody fragment (scFv) specific for folate receptor α (αFR). The functionalized nanocubes were able to recognize their target and to be efficiently internalized by the desired cells *via* endocytosis pathway. Once inside the cells, the nanocubes delivered the Pt compound, which induced toxicity by intercalating the DNA. Thanks to their crystal structure and size, these nanocubes exhibited a viscosity-independent behavior, keeping high SAR values even in highly viscous media. Indeed, once incubated with the cells, the nanocubes were able to heat the tumor mass up to 42 °C, promoting cell death. The contribution of the cytotoxicity from both Pt delivery and hyperthermia highlighted the use of these nanocubes for cancer multitherapy. Thus, combining targeting, drug delivery and magnetic hyperthermia, a suitable platform for a synergistic treatment of cancer has been developed.

Introduction. Nanoparticles in medicine

1. What is nanomedicine?

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Nanomedicine concerns the use of precisely engineered materials at the nano-scale size (**Figure 1.1**), generally with at least one dimension defined in the 1-100 nm range,[1-5] to develop therapeutic and diagnostic modalities.[1] It can be regarded as the application of nanotechnology (*i.e.* the understanding and control of matter in the nano-size scale) in medicine.

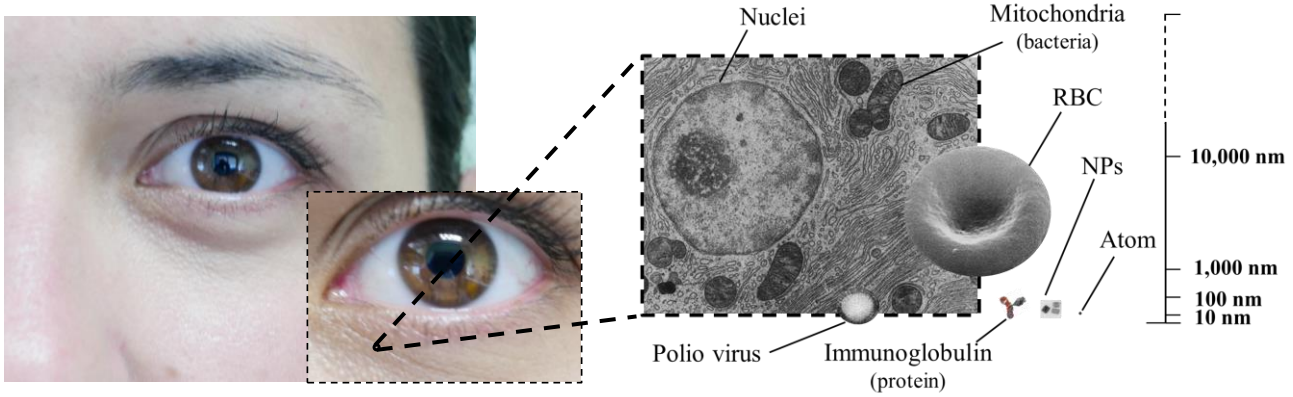

Figure 1.1. Size at the nanoscale. Nanomedicine faces with biological components in the micron/nanometer size, making interaction at the atomic scale. Scale on the right is logarithmic.

Indeed, nanomedicine is an interdisciplinary field, where nanoscience, nanoengineering and nanotechnology interact with the life science to achieve improved patients' outcome and quality of life.[3, 6] It also involves the design of colloidal materials and technologies for *in vivo* diagnosis and therapy, as well as new scaffolds and surfaces for regenerative medicine.[3] "*The right size is Nanobiotechnology*" was the statement by which Georges M. Whitesides in 2003 ventured that, by working together, physics and biology (from cellular to molecular biology, biochemistry and immunology) could have unlocked a fascinating way to lead the comprehension of the life mechanisms to an outstanding level of knowledge.[7] The enormous interest for understanding nanomaterials' interaction with the "bio-world" relies on the certainty that they have the potential to contribute to new modalities in molecular imaging, sensing and therapeutic intervention.[8, 9] Nanomaterials along with chemistry would play a main role in this process. Indeed, chemistry places a bridge between physics and biology, making real the theoretical physical laws and taking back biology and biologist to reality. However, physics, biology and chemistry occupy just a small portion of the whole nanomedicine subject. Mathematical modelling and bioinformatics,[10] molecular dynamics[11], biomedical engineering[12] and microbiology[13] contribute as well to face the many problems and challenges related to nanomedicine. All together, these subjects optimize research, saving time and money. It is, by now, clear that nanomedicine is a multi- and interdisciplinary field, in which only the strict collaboration and reciprocal consideration of the different disciplines will succeed in tuning its many aspects. The nanotools here exploited have unique size- and shape-dependent optical, electronic and magnetic properties, which are different from those of bulk materials of the same composition.[1, 3, 5] To date, several types of engineered nanomaterials have been developed, including both inorganic (*e.g.* iron oxide,[14] gold,[15] silver,[16] silica[17]), organic (*e.g.* lipid micelles,[18] polymeric nanostructures,[19] protein constructs,[20] layer-by-layer assemblies[21, 22]) or a

combination of them (**Figure 2.1**).[23] First, they can carry a large variety of drugs (*e.g.* doxorubicin[24-26]) or therapeutic biological molecules (*e.g.* siRNA[27-29]) and protect them from degradation.[30] Moreover, since most of the drugs commonly used in standard therapy are slightly or sparely soluble in water[31] (*e.g.* cisplatin and docetaxel), the use of smart nanocarriers can enhance their biodistribution and prolong their circulation time.[32] Nanomaterials can also act as multiple drug carriers for a more effective therapy,[33-37] overcoming the multidrug resistance (MDR) expressed by many cancer cells,[38, 39] due to the possibility to target different metabolic pathways at the same time. The second important feature of the nanoparticles relies on their high surface to volume ratio, which allows for the grafting of multiple functional ligands at high density. These ligands can serve as stabilizing agents (*e.g.* PEG-based molecules[40]), for increasing the nanoparticles solubility and bioavailability,[41] or as targeting ligands (*e.g.* peptides,[42, 43] folic acid[44] or antibodies[45]) for the selective guidance of the nanocarriers to the desired cells or tissues.[46, 47] The third critical point regards the release of the loaded cargo in a controlled manner.[9].[48] Indeed, conventional therapy has sometimes tremendous side effects, related to the intrinsic toxicity of the therapeutic agent involved in the pathologic treatment.[48] Then, to spare the healthy tissues in favor of a more localized therapy, the so called precision medicine, has acquired enormous importance in modern medicine. Several therapeutic strategies based on nanoparticles were developed, all focused on the selective induction of damage to only unhealthy cells, minimizing the side effects of the anticancer drugs while enhancing the efficacy of clinical treatments.[4]

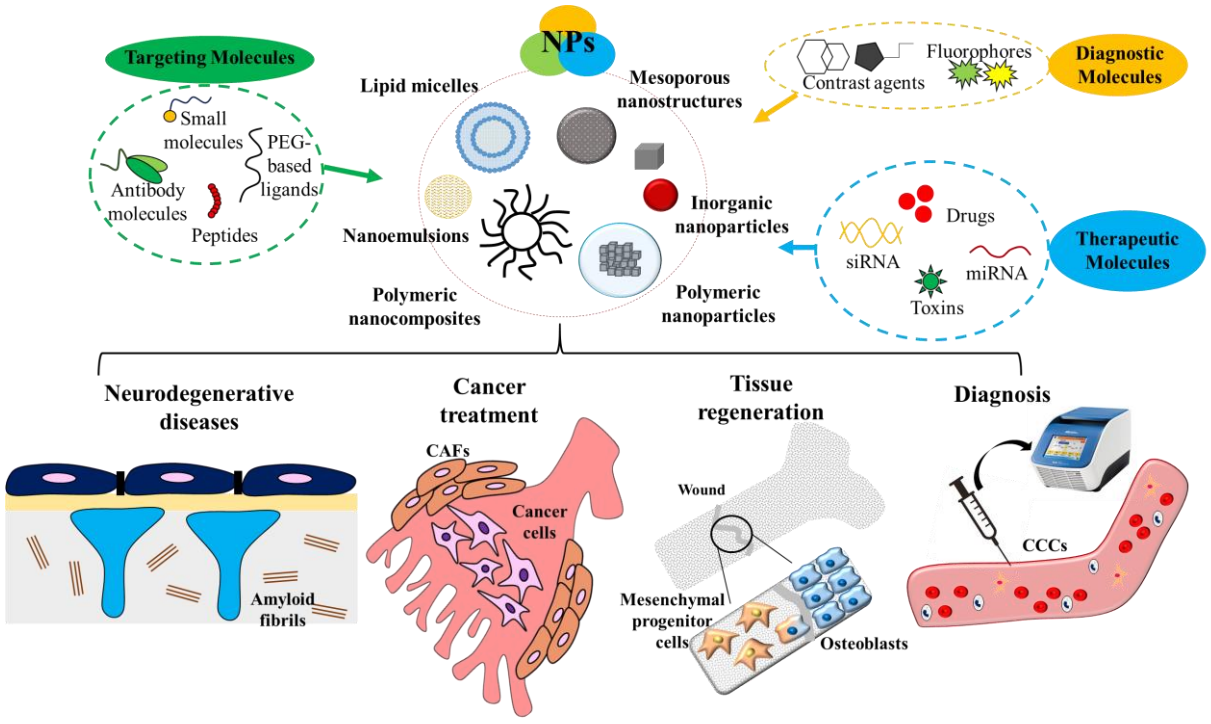

Figure 1.2. Toolbox of the applications of nanoparticles in medicine. Several kinds of functionalization, from the addition of targeting ligands or diagnostic agents, to the conjugation with therapeutics, can make nanoparticles, of different sizes and compositions, suitable for a wide range of uses in nanomedicine CAFs = Cancer Associated Fibroblasts; CCCs = Circulating Cancer Cells.

2. Cancer

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Cancer biology

Cancer is not simply composed by homogeneous malignant cells but is rather a complex ecosystems containing tumor cells, and also endothelial, hematopoietic and stromal cells that influence its function as a whole.[49] There is increasing awareness that intratumoral heterogeneity contributes to therapy failure and disease progression[49] Cancers originally develop from normal cells that gain the ability to proliferate aberrantly and eventually turn malignant. These cancerous cells grow clonally and have the potential to metastasize.[50] The clonal origin of tumors does not imply that the original progenitor cell, that gives rise to a tumor, has initially acquired all of the characteristics of a cancer cell. On the contrary, the development of cancer is a multistep process in which cells gradually become malignant through a progressive series of alterations.[51] The basis of cancer development is mainly genetic and involves the mutations and the selection for cells with progressively increasing capacity of proliferation, survival, invasion and metastasis.[51] The tumor initiation results in genetic aberrations and abnormal proliferation of a single cell. New cell clones originate, with the further increase of genetic alterations that promote the cells heterogeneity. As the tumor progresses, modifications of the cellular environment take place. Different hallmarks of the cancer, intended as distinctive and complementary capabilities that enable tumor growth and metastatic dissemination, provide helpful fundaments to better understand what tumorigenesis is (**Figure 2**).[52] The remodeling of the extracellular matrix (ECM) is one of the main events of carcinogenic processes.[53-55] Indeed, ECM is composed of molecules like collagens, laminins, fibronectin, glycoprotein and proteoglycans that have a structural and functional role. The deregulation of all these components has been associated with the development of malignancies.[55-57] Concomitantly to the induction of ECM remodelling processes, the cells of the tumor microenvironment secrete growth factors that promote angiogenesis, which is the formation of new blood vessels. Like normal tissue, tumors require nutrient and oxygen to sustain their growth. Angiogenesis, which occurs also during embryogenesis and wound healing in a transient manner, is deregulated in tumor and constantly activated.[52] Tumor-associated neo-vasculature rises in response to tumor-associated cells secreting growth factors that stimulate the proliferation of endothelial cells in the capillaries of the surrounding tissue, resulting in the outgrowth of new vessels.[57] Angiogenesis and tumor development may be associated with cancer cell migration form the original site. This event is promoted by the downregulation of adhesion molecules that ensure the contact between cell and ECM. The local invasion of the adjacent tissues is followed usually by the protrusion of those cells in the blood circulation, from which they can extravasate and start the growth of micrometastasis in distant tissues and organs. This colonization can lead to the establishment of a new tumor.[52] The chronic and often uncontrolled cell proliferation that represents the essence of neoplastic disease involves not only deregulated control of cell proliferation but also corresponding adjustments of energy metabolism in order to fuel cell growth and division.[52] As consequence, tumors sustain their growth in hypoxic environment, which produce a decrease of the pH of the surrounding vasculature and tissues. Another, still-unresolved issue surrounding tumor formation involves the role that the immune system plays in resisting or eradicating formation and progression of incipient neoplasias. The long-standing theory of immune surveillance proposes that cells and tissues are constantly monitored by immune system, responsible for recognizing and eliminating the majority of nascent cancer cells. According to this, solid tumors that do appear have managed to avoid detection by the

various components of the immune system or have been able to limit the extent of immunological killing, thereby evading eradication.[52]

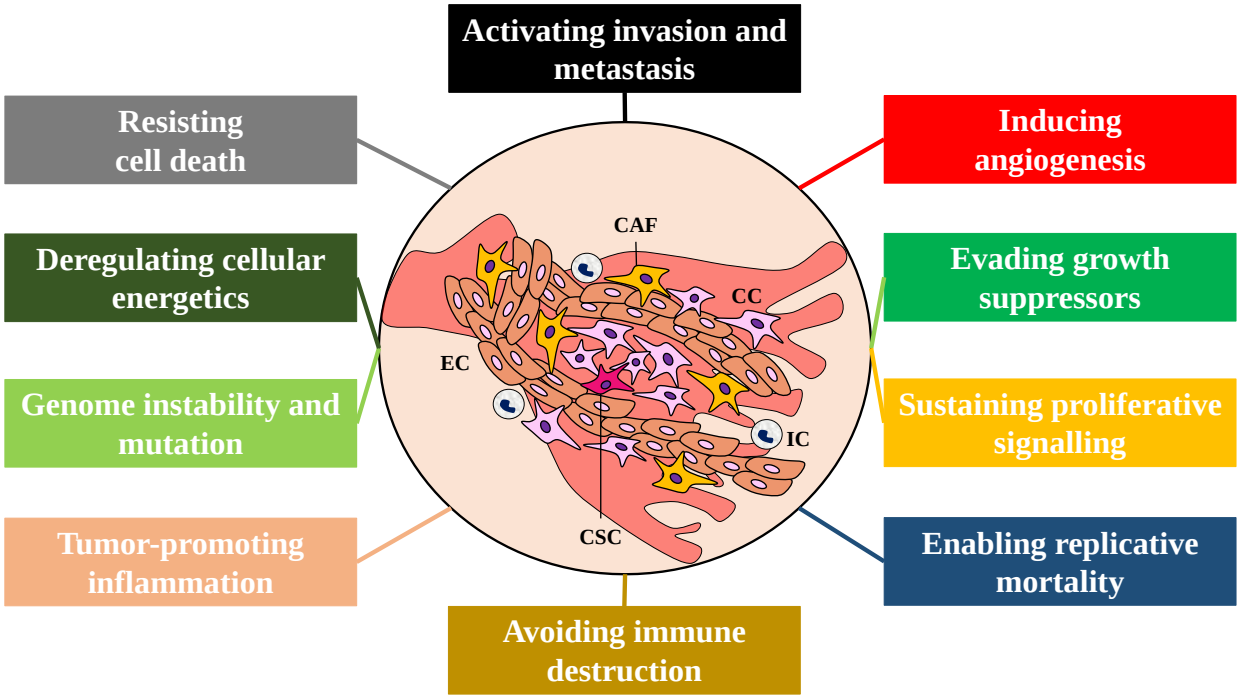

Figure 2.1. Cancer hallmarks. The distinctive features and properties of cancer development are highlighted. CAF = cancer associated fibroblast; CC = cancer cell; EC = endothelial cell; CSC = cancer stem cell; IC = immune inflammatory cell.[52] *Adapted from Hallmarks of cancer: the next generation. Cell, 2011. 144(5): p. 646-74., Hanahan, D. and R.A. Weinberg, 2011, with permission from Elsevier.*

Half century of challenges

Cancer is an abnormal growth of cells caused by multiple changes in gene expression leading to dysregulated balance of cell proliferation and cell death and ultimately evolving into a population of cells that can invade tissues and metastasize to distant sites, causing significant morbidity and, if untreated, death of the host.[58] This statement, proposed by *Weinberg* in "The biology of cancer" [58] masterfully resume the cancer concept. Cancers figure among the leading causes of death worldwide. Global demographic characteristics predict an increasing cancer incidence in the next decades, with 19.3 million new cancer cases annually expected by 2025. An overview on the most recent data, shown in **Figure 2.2**, indicates the most common cancers estimated to occur in men and women by 2016. Prostate, lung and bronchus, and colorectal cancers account for 44% of all cases in men, with prostate cancer alone accounting for 1 in 5 new diagnoses. For women, the 3 most commonly diagnosed cancers are breast, lung and bronchus, and colorectal, representing one-half of all cases; breast cancer alone was expected to account for 29% all new cancer diagnoses in women.[59]

There has been a steady rise in cancer death rates in the western countries during the past 50 years. The major reasons of this increment may be found in the higher life expectancy, which has progressively risen over the last decades,[58] and in the adoption of unhealthy lifestyles that became dramatically common with the achievement of economic welfare.[60] What has becoming clear is that cancer treatment was a more arduous challenge than what was initially expected. Despite the advancements of modern medicine in therapy and diagnosis, many patients still fail therapy, resulting in disease progression, recurrence, and reduced overall survival[49]. However, the continuously growing knowledge of molecular and tumor biology has notably changed cancer treatment paradigms

during the past 15 years.[61] Cancer therapy is moving gradually in the direction of more selective and safe treatments, aimed to reduce or avoid the destructive side effects of conventional therapies as surgery, radiotherapy and chemotherapy. New cancer treatment methods as genotype-directed precision oncology or targeted therapy, by means of antibody or other components of the immune system, are currently emerging and show a realistic possibility to be used in routine pharmacology.[61] However, in most of the cases conventional therapies are still cheaper and more efficient than the new arising technologies. Therefore, great efforts have to be made by researchers to develop the next generation of precision medicines with effective cost/benefit advantages.[61] Besides the treatment of the disease once it is already established, the early diagnosis has remarkable importance. Until the second half of 20th century cancer was diagnosed only when symptoms could have been clearly recognized. In many cases, cancer would have already spread, thus limiting the efficacy of the treatment.[62] The poor outcomes for cancers diagnosed at advanced stages of development have been the main rationale behind research into techniques able to detect the disease before symptoms are manifest.[62] Besides the therapeutic treatment, nanomedicine has moved in the direction of developing efficient tools for the screening of cancer biomarkers at the early stages of tumor development.[63]

Estimated New Cases

	Males			Females	
Prostate	180,890	21%	Breast	246,660	29%
Lung & bronchus	117,920	14%	Lung & bronchus	106,470	13%
Colon & rectum	70,820	8%	Colon & rectum	63,670	8%
Urinary bladder	58,950	7%	Uterine corpus	60,050	7%
Melanoma of the skin	46,870	6%	Thyroid	49,350	6%
Non-Hodgkin lymphoma	40,170	5%	Non-Hodgkin lymphoma	32,410	4%
Kidney & renal pelvis	39,650	5%	Melanoma of the skin	29,510	3%
Oral cavity & pharynx	34,780	4%	Leukemia	26,050	3%
Leukemia	34,090	4%	Pancreas	25,400	3%
Liver & intrahepatic bile duct	28,410	3%	Kidney & renal pelvis	23,050	3%
All Sites	**841,390**	**100%**	**All Sites**	**843,820**	**100%**

Estimated Deaths

	Males			Females	
Lung & bronchus	85,920	27%	Lung & bronchus	72,160	26%
Prostate	26,120	8%	Breast	40,450	14%
Colon & rectum	26,020	8%	Colon & rectum	23,170	8%
Pancreas	21,450	7%	Pancreas	20,330	7%
Liver & intrahepatic bile duct	18,280	6%	Ovary	14,240	5%
Leukemia	14,130	4%	Uterine corpus	10,470	4%
Esophagus	12,720	4%	Leukemia	10,270	4%
Urinary bladder	11,820	4%	Liver & intrahepatic bile duct	8,890	3%
Non-Hodgkin lymphoma	11,520	4%	Non-Hodgkin lymphoma	8,630	3%
Brain & other nervous system	9,440	3%	Brain & other nervous system	6,610	2%
All Sites	**314,290**	**100%**	**All Sites**	**281,400**	**100%**

Figure 2.2. Ten leading cancer types for the estimated new cancer cases and deaths by sex, in the United States by 2016. *Reprinted, by permission, from Rebecca L. Siegel, Kimberly D. Miller, Ahmedin Jemal, Cancer statistics, 2016 Jan 7, 2016 (24), Cancer Journal for Clinicians. Copyright © 1999 - 2017 John Wiley & Sons, Inc.*

3. Magnetic materials

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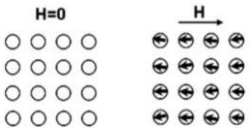
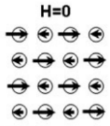
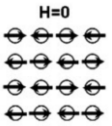
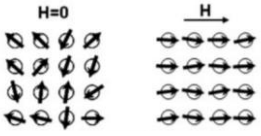
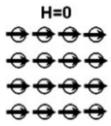
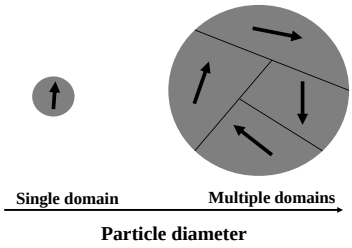
Introduction to magnetic nanoparticles

Magnetic nanoparticles (MNPs), made by a metal core like iron, cobalt, manganese or metal oxides have been differently used in the development of modern technology.[64] In particular, in the last decades, MNPs have attracted the attention not only of researchers but also that of chemical and pharmaceutical companies focusing on applications in the biomedical field. The properties of such nanoparticles can be finely tuned depending on the desired application, which might range from medicine for MRI imaging,[65] drug delivery[66] and magnetic hyperthermia,[67] to bioremediation, for the removal of contaminants from wastewater[68]. Materials in the nano-size scale have physical-chemical properties significantly different from those of the corresponding bulk materials, thanks to their high surface to volume ratio and tunable shape, composition and surface structure.[69] An important example of size-governed property change is related to magnetism. The attractive or repulsive forces between magnetic materials can be described in terms of magnetic dipoles, which can be considered as tiny bar magnets with opposite poles. Materials can thus be classified into diamagnetic, paramagnetic, ferromagnetic, ferrimagnetic, and antiferromagnetic according to the arrangement of their magnetic dipoles in the absence and presence of an external magnetic field.[70] **Table 3.1** summarizes the different types of magnetic materials.

Table 3.1. Features and behavior of the different types of magnetic materials. [70, 71]

Weak magnetic interactions		**Strong magnetic interaction**			
 **Diamagnetic**	1) Does not have magnetic dipoles in the absence of an external field and has weak induced dipoles in its presence; 2) magnetization responds in the opposite direction of the magnetic field	 **Ferrimagnetic**	1) Magnetic dipoles always exist in the absence and presence of an external field; 2) displays a permanent magnetic moment	 **Antiferromagnetic**	1) Adjacent dipoles are antiparallel in the absence of an external magnetic field and cancel each other
 **Paramagnetic**	1) Randomly oriented dipoles that can be aligned in an external field; 2) magnetization responds in the same direction as the external field	 **Ferromagnetic**	1) Magnetic dipoles always exist and are aligned antiparallel to the adjacent 2) strong dipoles in the absence of an external magnetic field	**a** 	

Each magnetic moment shown for the different materials can be considered inside the same multidomain structure. **Picture a** shows the size-dependent difference between single domain and multidomain. *Adapted, by permission, from U. Jeong,X. Teng,Y. Wang,H. Yang,Y. Xia, Superparamagnetic Colloids: Controlled Synthesis and Niche Applications, Dec 12, 2006 (28), Advanced Materials. Copyright © 1999 - 2017 John Wiley & Sons, Inc.*

In general, magnetic materials refer to those characterized by either ferro- or ferrimagnetic features.[70] Different factors such as saturation of magnetization (M_s), coercivity (H_c), blocking temperature (T_B) and relaxation time (τ_N and τ_B) contribute to control and optimize the key magnetic properties of MNPs.[71] M_s is the maximum magnetization possible and arises when all the magnetic dipoles are aligned in an external magnetic field. This parameter increases with nanoparticles size until it reaches the bulk value.[71] **Figure 3.1a** shows a typical magnetization curve for ferromagnetic or ferrimagnetic nanoparticles, showing the remanent magnetization (M_r, induced magnetization, which is conserved after an applied field is removed) and coercivity (H_c, the intensity of an external magnetic field needed to force the magnetization to go back to zero).[70] Magnetism, which arises from the collective interaction of atomic magnetic dipoles, is highly volume and temperature dependent. When the size of a ferro- or ferrimagnet decreases to a certain critical value r_c, the particles change from a state with multiple magnetic domains to a single domain.[71] In a single domain material, the magnetic moments of the atoms align accordingly to the reciprocal interactions below a critical temperature (T_B). Blocking temperature defines the transition from ferromagnetic to superparamegnetic regimes and can be considered as the thermal energy needed for spin reorientation.[70, 72] As shown in **Figure 3.1b**, if the size decreases to a value r_0, the thermal energy becomes comparable to that required for flipping the magnetic moment in the opposite directions, leading to the randomization of the magnetic dipoles in a short period of time.[70] Such small particles, known as superparamagnetic nanoparticles, do not have permanent magnetic moments in the absence of an external field and behave like a giant paramagnetic "molecule" in which the magnetic moment is able to rotate randomly. Nevertheless, they can respond fast to an external magnetic field.[70] As a consequence, the magnetization changes spontaneously above a critical temperature, which is significantly lower than in the case of single domain materials and approximate the room temperature.[64, 70, 73] Indeed, T_B increases with particle size.[71] The limiting size between monodomain and superparamagnetic MNPs (SPIONs) depends on their crystalline structures (in particular anisotropy constant K), composition and size.[64, 70] Figure 3.1c shows the maximum diameters for superparamagnetic (D_{sp}) and single-domain (D_{sd}) nanoparticles of different compositions [71, 72] and Figure 3.1d their magnetization curves. While for multidomain or single domain ferromagnetic nanoparticles a hysteresis loop is produced due to the presence of several magnetic moments that affect Mr and Hc (red line), for superparamagnetic nanoparticles, a sigmoidal curve with no hysteresis is originated (green line). In addition, the curves of diamagnetic (black line) and paramagnetic (blue line) nanoparticles are shown, with opposite or weak response to magnetic field, respectively.[71]The lack of remanence magnetization enables superparamagnetic nanoparticles to maintain their colloidal stability and avoid agglomeration, which is very important for biomedical purposes:[64] The main biomedical applications include magnetic hyperthermia (MH),[74] MRI imaging[75-77] and magnetic separation[44, 78] and will be discussed in paragraph 4.

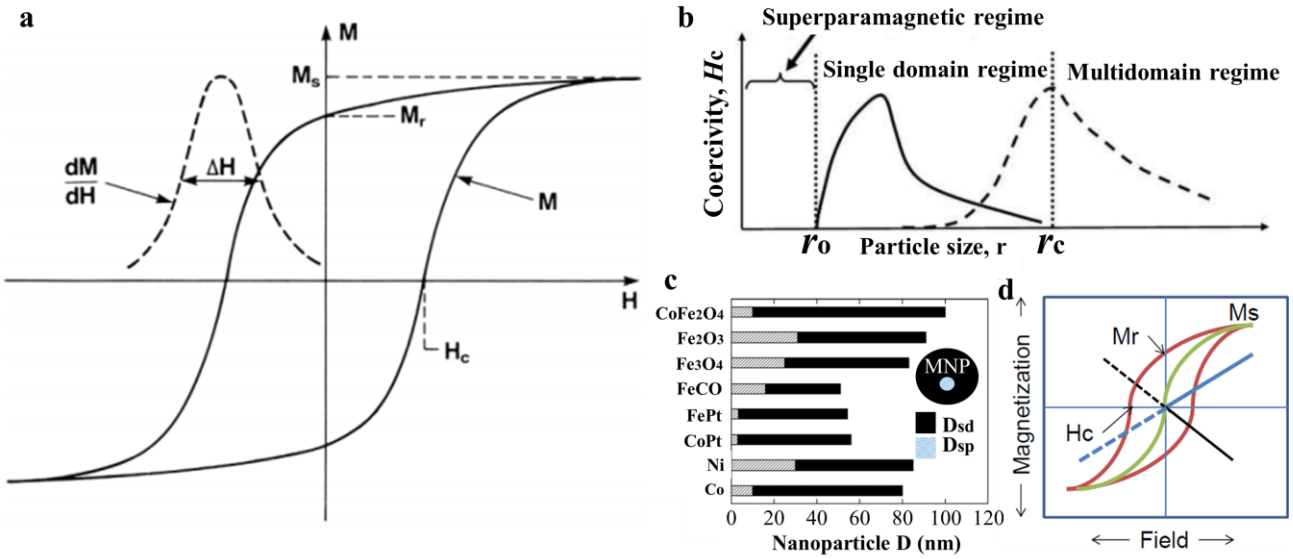


Figure 3.1. a) The typical magnetization curve of a ferro- or ferrimagnetic material, from which one can identify the saturation magnetization M_s, the maximum value of M; the remanence magnetization Mr, the residual magnetization at zero field strength; and the external field required to bring magnetization back to zero known ascoercivity H_c,.[70] **b)** Schematic illustration of the dependence of magnetic coercivity on particle size. In the single-domain regime, the coercivity can follow either the solid curve for non-interacting particles or the dashed line for particles that have coupling between them. The coercivity falls to zero for superparamagnetic colloidal particles.[70] **c)**, Different magnetic materials have different transition size from single domain (D_{sd}, diameter single domain) to superparamagnetism (D_{sp}, diameter superparamagnetism). For diameters, $D < D_{sp}$, they exhibit superparamagnetic behaviour; for $D > D_{sd}$, they split into multiple domains to minimize their overall energy and in between, $D_{sp} < D < D_{sd}$, they are ferromagnetic and single domain.[72] **d)** Magnetic behavior, under the influence of an applied field, for different magnetic materials. The X-axis is the applied field (Oe), and the Y-axis is the magnetization of the sample as a function of field exposure (emu/g). Typical behavior of multidomain ferro(i)magnetic materials shows hysteresis loop (red line); single domain materials/superparamagnetic nanoparticles do not show hysteresis and have coercivity close to 0 (green line). Paramagnetic materials slightly follow the external magnetic field (blue line), while diamagnetic materials respond in the opposite direction of the applied field (black line). *(a-b) Reprinted, by permission, from U. Jeong,X. Teng,Y. Wang,H. Yang,Y. Xia, Superparamagnetic Colloids: Controlled Synthesis and Niche Applications, Dec 12, 2006 (28), Advanced Materials. Copyright © 1999 - 2017 John Wiley & Sons, Inc. (c-d) Reprinted, with permission, from Krishnan, K.M., Biomedical Nanomagnetics: A Spin Through Possibilities in Imaging, Diagnostics, and Therapy, IEEE Transactions on Magnetics, 2010. 46(7): p. 2523-2558.*

MNPs: iron oxide nanoparticles above all

Among the different kinds of magnetic nanoparticles developed so far, iron oxide-based nanoparticles (IONPs) are the most used for biological applications. Indeed, IONPs express the best compromise between good magnetic properties (such as M_s) and stability under oxidizing conditions.[64] The most investigated iron oxides are FeO (wuestite), γ-Fe_2O_3 (maghemite) and Fe_3O_4 (magnetite), which have remarkably different magnetic properties.[70] Wuestite, being paramagnetic at room temperature, is less attractive than maghemite and magnetite. Nevertheless, new opportunities begin to emerge for nanoparticles with a mixed composition of wuestite and ferrimagnetic oxides, as it will be discussed in chapter 6. γ-Fe_2O_3 and Fe_3O_4 are instead ferrimagnetic[79] Both have spinel structure, with the oxygen ions forming a close-packed cubic lattice and iron ions located at the interstices.[64, 80] The saturation of magnetization (M_s) for bulk material, at low temperature (ca. 5 K), is higher for magnetite (98 emu/g) than for maghemite (83.5-87.4 emu/g). The highest value reached so far for magnetite IONPs was 92 emu/g, while for maghemite it was 77 emu/g.[81] Despite the better magnetic

properties, magnetite is poorly stable under ambient conditions.[73] Indeed, Fe_3O_4 nanoparticles tend to oxidize to γ-Fe_2O_3, which instead are more stable in aqueous solution, ensuring durable magnetic performances.[64, 73] Several methods for the synthesis of IONPs were developed and are now available. **Table 3.2** reports the procedures used for the synthesis of iron oxide nanoparticles.[73, 80, 82, 83]

Table 2.2. Synthetic routes of iron oxide nanoparticles with tunable size and shape.[73, 80, 83, 84]

Synthetic method	Reaction T (°C)	Reaction time	Solvent	Capping ligands	Size distribution	Yield
Co-precipitation	20-90	minutes	Water, base	Oleic acid (OA)	< 50 nm	High/scalable
Thermal decomposition	100-320	hours	Organic, *e.g.* octadecene (ODE)	OA, hexadecylamine (HDA), decanoic acid	2-40 nm[85, 86]	High
Microemulsion	20-50	hours	Water-organic, *e.g.* heptane,[87] octane[88]	Dioctyl sulfosuccinate (AOT)[87], cetyltrimethyl-ammonium bromide (CTAB)[88]	4-15 nm	Low
Hydrothermal	65	hours	water - ethanol	Silica coating	10-30 nm[89, 90]	Medium
Magnetobacteria[91]	220	days	Growth medium, water based	Lipids and proteins	~45-55 nm	Low (compared to chemical methods)

In addition to the methods shown in Table 3.2, other synthetic routes can be identified: sol-gel and polyol method, microwave-assisted synthesis, sonolysis or sonochemical methods, electrochemical methods, aerosol/vapor methods, electron beam lithography, gas-phase deposition, oxidation method, flow injection method and supercritical fluid method.[73]

Among all the listed synthetic approaches, one of the most studied for the synthesis of IONPs is thermal decomposition of iron-oleate, developed by *Hyeon et al.*[92, 93] due to its scalability and versatility. However, a variety of parameters (*e.g.* atmosphere, the amount and type of reducing agent, surfactant, precursor, solvent) make this type of synthesis quite critical. Monitoring the effect of those factors on the magnetic behavior, as well as on the size and shape distributions, is of great relevance for developing suitable IONPs for biomedical applications.[81] During the synthesis, the IONPs are covered by capping ligands, which contribute to separate the magnetic domain of each single nanoparticle and to hold the attractive forces apart. For both the syntheses preformed in aqueous or organic solvents the binding of the capping ligands to the IONPs surface is mediated by coordination complex between the electron donor groups (*e.g.* carboxylic,[86] hydroxyl,[94] phosphonate[95] or amine groups[96, 97]) and Fe^{2+} and Fe^{3+} species (**Figure 3.2a**).[98] Because iron is extremely reactive towards oxidizing agents and in the presence of water, the protection of MNPs is of prime importance for obtaining physically and chemically stable colloidal systems. Such protection can be achieved by: 1) surface coating of the MNPs (**Figure 3.2b**), using polymeric stabilizers/surfactants, like dextran,[99]

carboxydextran,[100] poly(vinyl alcohol) (PVA)[101] or poly(ethylene glycol) (PEG);[102] 2) by deposition of layers of metals (*e.g.* gold)[103] or oxides(*e.g.* SiO_2);[104] ,3) by the formation of lipid-like coatings (liposomes/ lipid NPs)[105, 106] around the magnetic core.[80] All the three listed cases can lead to the formation either of single-coated nanoparticles or clusters. Since many of the synthetic routes produce nanoparticles soluble in organic solvents, surface functionalization of IONPs with water-soluble ligands or polymers is crucial for their bio-applications. The surface coating strategy can involve: 1) the replacement of the capping ligands with more strongly binding ligands, usually referred as ligand exchange, **Figure 3.2c,** which increases the nanoparticles solubility in aqueous media;[107] 2) the addition of amphiphilic molecules, which express affinity for both the hydrophobic component of the nanoparticle surfactants and for the aqueous surrounding environment known as polymer coating, **Figure 3.2c**[107, 108]. Besides improving the colloidal and physical stability of the particles, the surface coating may provide the scaffold for further conjugation of bioactive molecules or targeting ligands in order to obtain multifunctional MNPs (**Figure 3.2d**).[80]

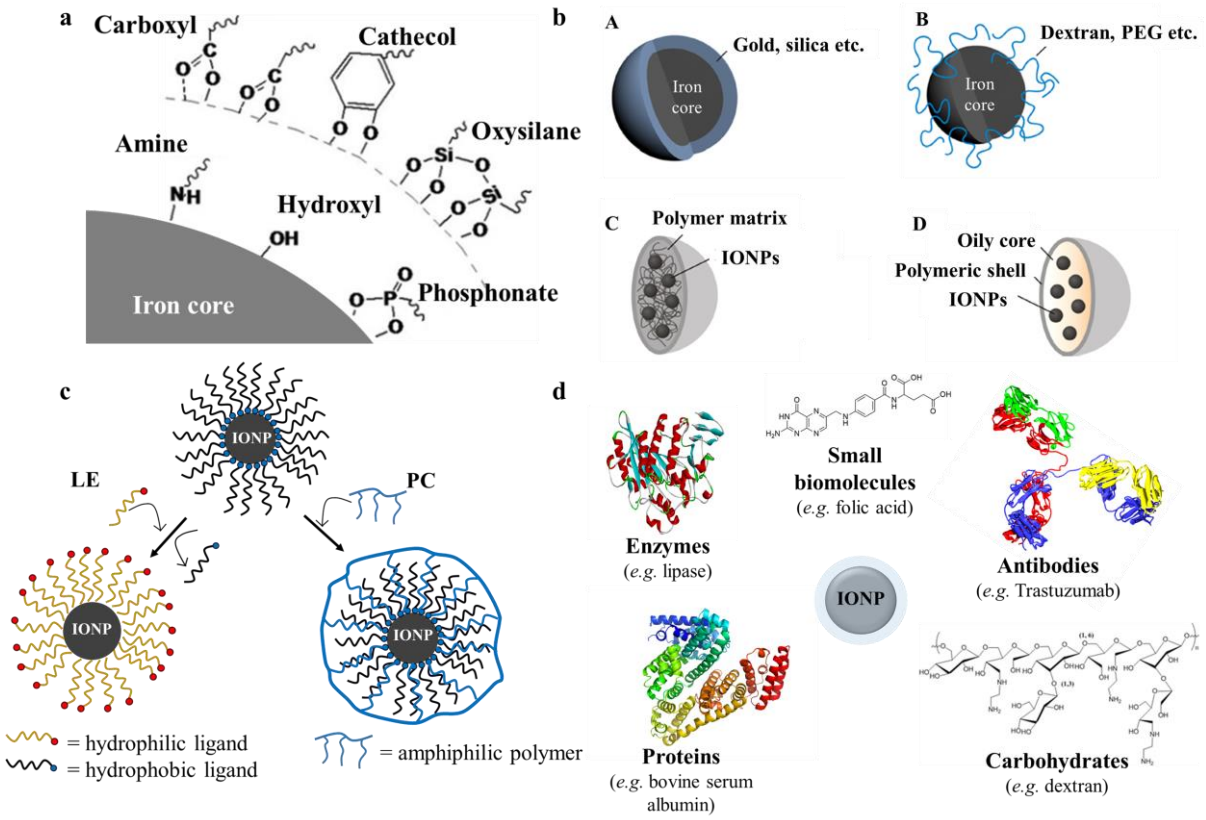


Figure 3.2. Surface coating provides stability and functionalization to IONPs. a) Nucleation process with different surfactants. **b)** Just after the crystal growth or in a second step of synthesis, IONPs can be coated with several molecules able to prevent the degradation of the inorganic iron core. **c)** Ligand exchange (LE) or polymer coating (PC) are the two main techniques used for stabilizing the nanoparticles in aqueous media. **d)** The functionalization with biomolecules such as enzymes, proteins, antibodies, small bioactive molecules (*e.g.* folic acid or biotin) or carbohydrates makes IONPs able to interact with the biological environment. *(a) Adapted from (Ref 97) with permission of the Royal Society of Chemistry. (b) Adapted with permission from L. Harivardhan Reddy, José L. Arias, Julien Nicolas, and Patrick Couvreur, Magnetic nanoparticles: design and characterization, toxicity and biocompatibility, pharmaceutical and biomedical applications. Chem Rev, 2012. 112(11): p. 5818-78. Copyright © 2012 American Chemical Society.*

An important feature of this particular type of MNPs, *i.e.* IONPs, is their significantly lower toxicity and good biodegradability, which show outstanding compatibility towards biological systems, both *in vitro* and *in vivo*.[109] As result, the US Food and Drug Administration (FDA) and the European

Medicines Agency (EMA) have already approved the medical use of several magnetic iron oxides nanoparticles (**Table 3.3**).[110]

Table 3.3. Approved and available pharmaceuticals IONPs-based.[80, 110]

Compound/trademark	Company	Material	Application	Target
Nanotherm®	MagForce	Aminosilane-coated IONPs, 15 nm	Hyperthermia	Glioblastoma
Ferumoxytol/ Faraheme®	AMAG pharmaceuticals	SPIONs coated with polyglucose sorbitol carboxymethylether, 17-31 nm	Iron replacement therapy	Deficiency anemia in adult patient with CDK
Ferumoxide/ Feridex® - Endorem®	AMAG pharmaceuticals/ Guerbet	SPIONs with low molecular weight dextran coating, 120-180 nm[111]	Contrast agent in MRI imaging	Liver lesions associated with RES alteration
Ferumoxsil/ Gastromark® - Lumirem®	AMAG pharmaceuticals/ Guerbet	Siloxane-coated SPIONs, 300 nm[112, 113]	Contrast agent in MRI imaging	Lower intestinal system

SPION = superparamagnetic iron oxide nanoparticle; CDK = chronic kidney disease; FDA = Food and Drug Administration (USA); EMA = European Medicines Agency; PMDA = Pharmaceuticals and Medical Devices Agency (Japan); RES = Reticuloendothelial system.

many other compounds, initially approved for clinics, were subsequently withdrawn from the market due to emerging adverse effects or reduced market potential. Examples of these pharmaceuticals are: Abdoscan®, 300 nm IONPs used as gastrointestinal contrast agent; Clariscan®,[114] oxidized starch-coated SPIONs replaced on the market by a gadolinium-based contrast agent; Sirenem® (Combidex®)[115] dextran-coated SPIONs used as contrast agent to detect metastatic diseases in lymph nodes; Resovist®, carboxydestran-coated SPIONs contrast agent for liver imaging.[116] Remarkably, almost the all the listed IONPs-based systems are explored for MRI or iron deficiency therapy. Thus, despite promising results in humans, the marketing of iron oxide nanoparticles is currently at a standstill.[115]

4. IONPs applications

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Magnetic separation

Magnetic separation **(Figure 4.1)** can be used as a quick and simple method for the efficient and reliable capture of proteins, biomolecules (DNA and RNA), mammalian cells, bacteria and virus.[73] The switchable magnetization properties of nanoparticles, particularly those from superparamagnetic nanoparticles, enable the magnetically-driven transport of biological molecules.[70] Biological entities exploit specific interactions with high affinity between molecular pairs to achieve reciprocal recognition and trigger signaling processes.[64] Thus, the surface of the nanoparticles is modified with biocompatible and/or targeting molecules. The first ones refer to PEGs, carbohydrates or phospholipids, which provide good colloidal stability, while the second is obtained using antibodies[117] or small molecules like folic acid or biotin,[118] which confer different degrees of specificity and affinity towards the desired biological specie.[70] Several applications for bio-functionalize MNPs have been found so far, including protein and DNA separation, molecular biosensing and pathogen detection/sequestration.[64] The separation and enrichment of biologically relevant molecules/cells allow a fast and accurate identification of determinant markers inside a complex biological fluid, increasing the sensitivity of the commonly used analytical devices like flow cytometer[119, 120].

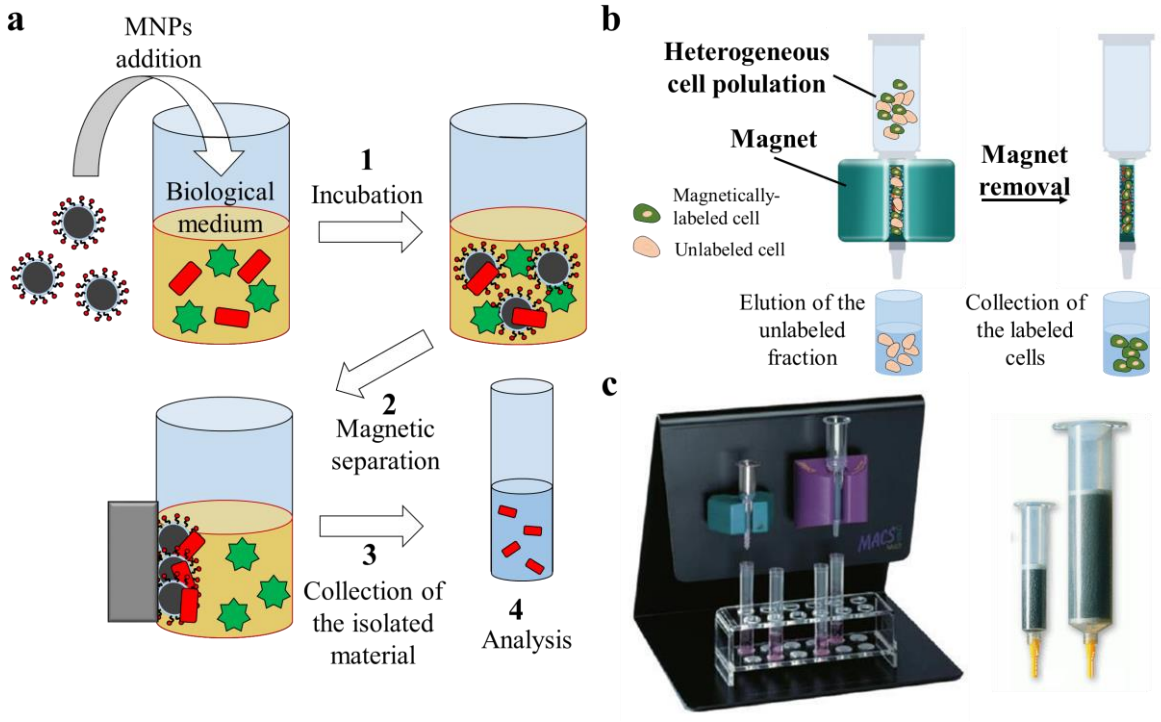


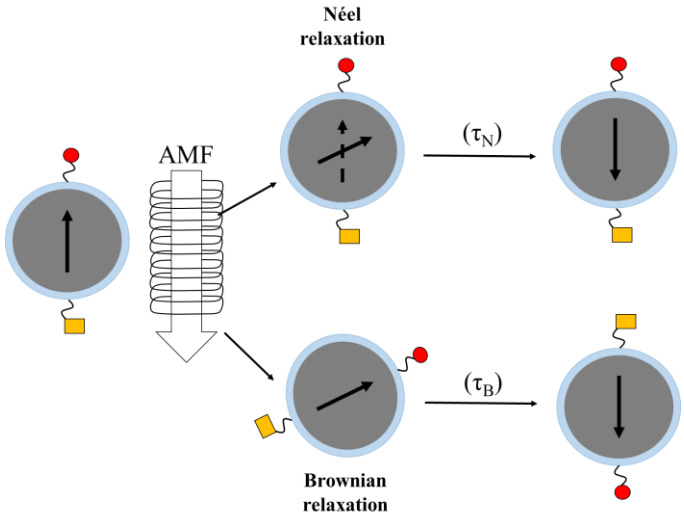
Figure 4.1. Magnetic separation. a) MNPs, functionalized accordingly to the desired research purpose, are incubated with the biological tissue of interest **(1)**. Here, nanoparticles are able to recognize specifically their target. Subsequently, they can be collected by applying an external magnetic field **(2)**. The magnetically isolated and enriched biomolecules (cells, proteins, antigens etc.) can be separated from the MNPs and washed, in order to remove debris **(3)**. Once re-dissolved in the desired medium, the sample can be analyzed using the commonly used analytical techniques, like Fluorescence Activating Cell Sorting (FACS) **(4)**. **b)** MACS® columns contain a matrix composed of ferromagnetic spheres covered with a cell-friendly coating that, when

placed on a magnetic separator, amplify the magnetic field and are able to hold cells magnetically labeled with, for example, MNPs. The unlabeled fraction is quickly eluted, while the desired labeled population of cells is collected just after the removal of the column from a compatible magnet. **c)** Stand for supporting the magnetic separator that fixes the magnetic columns of different sizes.

Nowadays, several magnetic tools based on magnetic micro/nano particles have been commercialized.[121] Among them, Dynabeads® (Invitrogen)[122] and MACS® microbeads (Miltenyi Biotech) can be considered the most popular.[123] Dynabeads® are uniform polystyrene beads with a magnetic core and dimeter of 4.5 µm, while MACS® (**Figure 4.1b**) microbeads are beads of 20-100 nm made by superparamagnetic iron oxide nanoparticles covered by a dextran coating. Opportunely functionalized with biological active molecules, they found important applications in the isolation of immune system cells and cells from bulk tissues. [124-127]

Magnetic Hyperthermia

Magnetic hyperthermia (MH) is a novel non-invasive treatment, now undergoing clinical trials on patients affected by glioblastoma multiforme, prostate cancer and pancreatic cancer.[64, 128] It exploits the heat generated by magnetic nanoparticles (MNPs) when exposed to an alternating magnetic field (AMF) (**Figure 4.3**).[8, 72, 129] The continuously switching rotation of the magnetic moment of the nanoparticles under AMF generates energy, which is dissipated in the form of heat. In small monodomain MNPs, the reorientation of the magnetic moments can occur due to Néel or Brownian relaxation. Néel relaxation (with time constant τ_N) occurs when the inner magnetic spin flips from the easy axis of the crystal structure to the opposite direction, thus overcoming the energy barrier related to magneto-crystalline anisotropy constant (K) and particle magnetic volume (V_m). In addition, it is insensitive to the viscosity (η) of the environment. Brownian relaxation involves the mechanical rotation of the whole particles, constituted by the magnetic core and the organic coating. Its time constant (τ_B) strictly depends on the viscosity (η) of the medium and the hydrodynamic size (d_h) of the particle.[64, 71] **Figure 4.2** shows the relaxation mechanisms involved in the dissipation of heat from the nanoparticles.



$$\Delta E = KV_m \quad (1)$$

$$\tau_B = \frac{3\eta V_H}{K_B T} \quad (2)$$

$$\tau_N = \tau_0 \exp\left(\frac{KV}{K_B T}\right) \quad (3)$$

$$\tau = \frac{\tau_B \tau_N}{\tau_B + \tau_N} \quad (4)$$

Figure 4.2. Relaxation mechanisms for nanoparticles. Néel (top) and Brownian (bottom) rotation of the nanoparticles. Red dot and orange square refers to biomolecules grafted on the NPs coating. The equations show the magnetization energy barrier (1), the Brownian (2) and Néel (3) relaxation times and the overall effective relaxation time of the particles (4). ΔE = thermal energy barrier; K = anisotropy constant; V_m =

magnetic volume; η = viscosity; V_H = hydrodynamic volume of the particle; K_B = Boltzmann constant; T = temperature; $\tau_0 = 10^{-9}$ s.[130]

The heating efficiency of the magnetic nanoparticles is expressed by their specific absorption rate (SAR)/specific loss power (SLP). [8, 64, 71, 130] The SAR value defines the temperature increase with time (ΔT/Δt) at a given MNP mass concentration (m), and is calculated by the following equation:

$$SAR = \left(\frac{W}{g}\right) = \frac{C}{m}\ x\ \frac{dT}{dt} \qquad (5)$$

where C is the specific heat capacity of the solvent. The concentration m of magnetic material is expressed as gL^{-1}. [86, 131] SAR depends on various factors, among them: 1) the applied magnetic field characteristics (frequency and amplitude); 2) the intrinsic magnetic properties (*i.e.* saturation magnetization, anisotropy); 3) the properties of the dispersing medium (*i.e.* viscosity, concentration, heat capacity).[128] From a biological point of view, hyperthermia is defined as the use of heat for killing tumors. [8] When biological tissues are exposed to temperatures higher than 41°C, damages are induced in cancer cells rather than healthy cells, due to the disorganized and compact vascular structure of the tumor mass, which hinders heat dissipation in comparison to healthy tissues.[8, 64, 130] Some medical constraints are imposed to the physical parameters of the AMF to ensure a safe application of hyperthermia to patients. Indeed, the product of the frequency by the magnetic field amplitude (H*f*) cannot exceed an established threshold of 5×10^9 $Am^{-1}s^{-1}$.[130, 132]

Gilchrist et al. were the first ones to use magnetic particles for the thermal treatment of tumors, back in 1957. In this pioneer work, they successfully used micrometer-size particles for heating lymph nodes in dogs.[133] The successful results obtained from MNP-based hyperthermia treatment of cancer in animal models, in the past three decades,[134-138] led to the establishment of the technique in clinical and industrial development.[64] Clinical studies, using combined hyperthermia and radiation therapy or chemotherapy, have shown that more than 80% of patients had tumor regression, of which 37% had a complete tumor ablation while 25% exhibited a tumor reduction above 50%.[64, 139] The efforts made in this field culminated with the approval of the therapy by FDA for use in the treatment of established non-surgical cancer additive treatments. [64, 139]

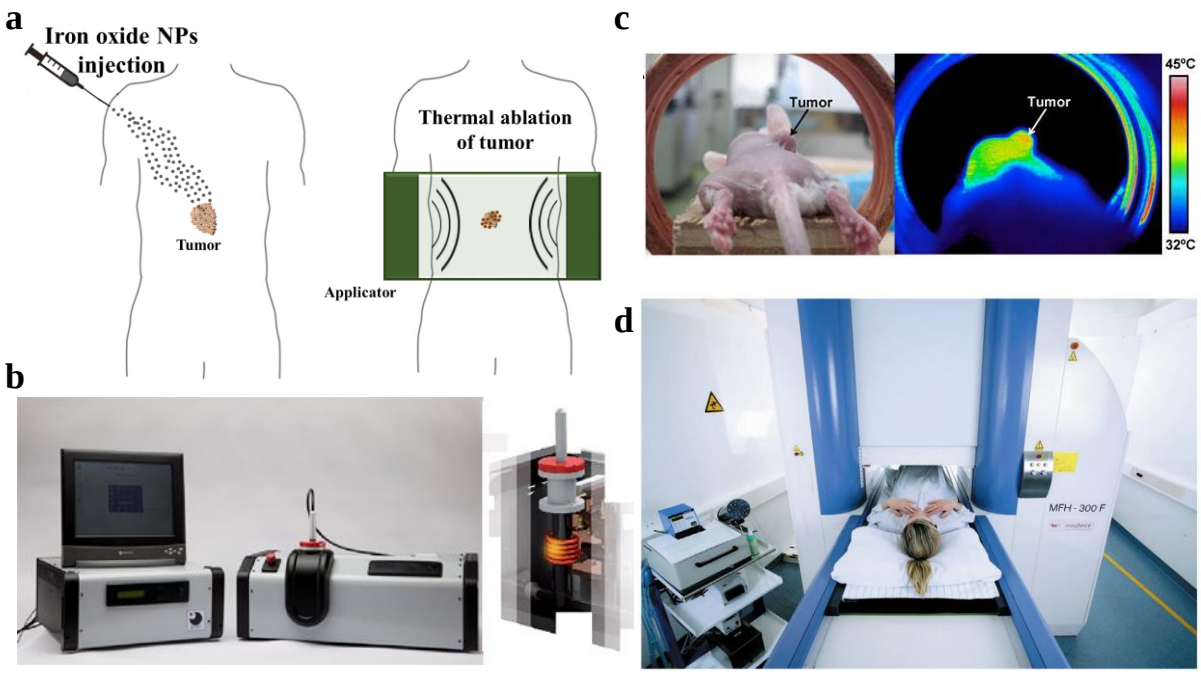


Figure 4.3. Magnetic hyperthermia application. a) Hyperthermia carried out using iron oxide nanoparticles relies on the accumulation of IONPs at the tumor site. During the exposition to an AMF, the nanoparticles

release energy in the form of heat, rising the surrounding tissue temperature up to 42-45 °C, thus promoting the disease ablation. **b)** Picture of the device used for measuring the heating performance of the nanoparticles *in vitro* (DM applicator series from nanoScale Biomagnetics Corp.). The probe, submerged in a solution of IONPs, is inserted inside the equipment coils able to generate AMF. The increase of temperature over time is recorded by the analyzer and the SAR values calculated according to the slope ($\Delta T/\Delta t$) obtained. **c)** Hyperthermia experiment on a mouse using similar instrumentation. Usually, the tumor xenografted into the mouse is locally injected with the nanoparticles and then exposed to cycles of AFM.[140] **d)** Thermotherapy treatment of the pelvic region in humans, after intratumoral injection of magnetic nanoparticles, using the alternating magnetic field applicator MFH 300F (MagForce Nanotechnologies AG, Berlin, Germany).[141] *(c) Reprinted, by permission, from Hayashi K, Nakamura M, Sakamoto W, Yogo T, Miki H, Ozaki S, Abe M, Matsumoto T, Ishimura K. Superparamagnetic Nanoparticle Clusters for Cancer Theranostics Combining Magnetic Resonance Imaging and Hyperthermia Treatment. Theranostics 2013; 3(6):366-376. Creative Commons License. (d) Reprinted, by permission, from Thiesen, B. and A. Jordan, Clinical applications of magnetic nanoparticles for hyperthermia, International Journal of Hyperthermia, 2008. 24(6): p. 467-474. Copyright © Taylor & Francis.*

Although different kinds of MNPs can be efficiently used for hyperthermia purposes,[64, 70, 130] ferrite nanomaterials like $CoFe_2O_4$[142, 143] and $MnFe_2O_4$[131], IONPs remain the main candidates, due to their higher heat performance. However, the intrinsic toxicity of Co^{2+} and Mn^{2+} [144, 145] can instill skepticism in the use of these materials for therapy, especially when it requires multiple exposures and prolonged circulation in the body of the nanoparticles. Contrarily, the superior biodegradability and biocompatibility of IONPs make these nanoparticles absolutely promising.[146] Nowadays, there are two main companies developing devices for MNP-based hyperthermia for clinical application: Sirex Medical and MagForce. Sirtex Medical designed a combined hyperthermia-radioteherapy system, using small magnetic micro-spheres (Thermosphere®, under development in collaboration with the Australian National University) in combination with resin-based microparticles impregnated with radioisotope yttrium-90, for the treatment of liver cancer.[64] Under magnetic induction conditions of 53 kHz and 30 kAm^{-1}, an intratumoral temperature of 48 °C can be reached in 5 minutes.[147] MagForce exploits iron oxide nanoparticles, with a diameter of 15 nm, coated with aminosilane for the treatment of cancer such as glioblastoma, prostate and pancreatic cancer.[64, 141, 148, 149] Using an applicator MFH®300F generating frequency of 100 kHz and fields between 0-18 kAm^{-1}, intratumoral treatment reaches temperatures ranging from 43 °C to 47 °C, at an injected dose of nanoparticles between 40-120 mg mL^{-1} (**Figure 4.3d**).[141, 150, 151] Despite these encouraging results, concerns have been raised regarding the toxicity for cancer-directed therapy, due to the possible thermal damaged to the adjacent healthy tissues. In order to minimize the potential side effects arising during the clinical treatments, the quantity of nanoparticles administered needs to be as low as possible.[152] Currently, this represents the main limitation of MH, *i.e.* is the poor heating efficiency of most of the used magnetic nanoparticles when administered at low doses.[152] Another drawback which comes from the high concentration of IONPs required for therapy is the impairment to monitor tumor progression by MRI, since the substantial dose of IONPs is incompatible with MRI imaging.[153] Although several research studies have aimed to design optimal heat mediators that would allow reduction of the IONPs dose, while maintaining the required heating performance, low heating efficiency remains among the current limitations for IONPs used in clinical trial.[154] Therefore, major efforts were made to optimize the NP heating efficiency by tuning key magnetic parameters such as size, shape and composition.

Nanoparticle's shape matters for hyperthermia: the importance of using nanocubes

Anisotropic-shaped magnetic nanoparticles have attracted enormous attention in the last few years owing to their superior magnetic properties.[128] The particles heat dissipation strongly depends on their physico-chemical features. Thanks to the advancements in thermal decomposition synthesis, in the latest decades a significant progress in size and shape control has made possible to obtain a large variety of spherical,[93] cubic,[155] star-,[156] flower-,[157] rod-[158] and octapod-shaped[159] iron oxide nanoparticles.[128] Recently, it was reported that anisotropic cubic-shaped particles reveal a superior heating performance in comparison to spherical ones..[152, 160] In particular, for 19 nm nanocubes (NCs) higher SAR values and higher saturation of magnetization values (M_s) were determined than for spherical IONPs of near size (25 nm).[161] Interestingly, the SAR values displayed by iron oxide nanocubes (with effective anisotropy constant K_{eff} = 9.1 x 10^4 J/m^3, and Ms = 1.7 x 10^6 Am^{-1}) are among the highest reported in the literature so far for IONPs. In detail, the SAR value found was about 3000 W/g at $\mu_0 H_{max}$ = 73 mT (58 kAm^{-1}) and f = 274 kHz.[152] Nevertheless, the synthesis of monodisperse NCs remains a great challenge and it is hardly attainable by any other method rather than high temperature colloidal synthesis.[128] The synthesis based on metal-organic precursors, using iron acetylacetonate,[81, 132, 153, 160] has proven to be successful for the preparation of homogeneous NCs, with tunable sizes (**Figure 4.4a**). However, among the different NCs sizes obtainable, *Guardia et al.*[162] demonstrated that nanocubes of 19 nm have the best heating performances in the biological threshold of frequency and field applied. Compared to bigger nanocubes of 25, 35 and 43 nm, 19 nm nanocubes show no hysteresis, with magnetic coercivity close to 0 Oe.[161, 162] Despite they appear to be the best candidate for designing a IONPs-based strategy for hyperthermia treatment, another parameter affecting the heating performance of the nanocubes has to be considered. As demonstrated by *Fortin et al.*,[163] viscosity (η) is a physical parameter that affect the nanoparticles heating efficiency. In addition, *Cabrera et al.* confirmed a strong decrease of the heat dissipation dependent on the increase of the medium viscosity when considering iron oxide nanocubes (**Figure 4.4b**).[164] In addition to the viscosity dependent SAR behavior due to the interaction of magnetic nanoparticles with biological samples, it has been shown that the magnetic heating efficiency is significantly reduce when MNPs are located inside cells or tissues.[67, 165] this might be explained by the increase of viscosity and/or nanoparticles aggregation imposed by the host biological matrices.[165] Hence, the need for nanoparticles able to retain their heating efficiency inside the biological environments, in order to obtain the maximum therapeutic effect from the hyperthermia treatment, has become a clear goal. Noteworthy, the viscosity dependent heating efficiency was also proved for spherical IONPs, which present lower SAR values in high viscosity media compared to nanocubes, within a size below 30 nm,.[161] Indeed, while for spherical IONPs the SAR values strongly decrease when increasing the viscosity of the medium, for 14 nm nanocubes SAR is constant among the different viscous media tested.[165] Even if 14 nm ones have lower heating performances, compared to the 19 nm nanocubes,[162] their viscosity independent behavior suggests their use in hyperthermia application on biological samples.[165].

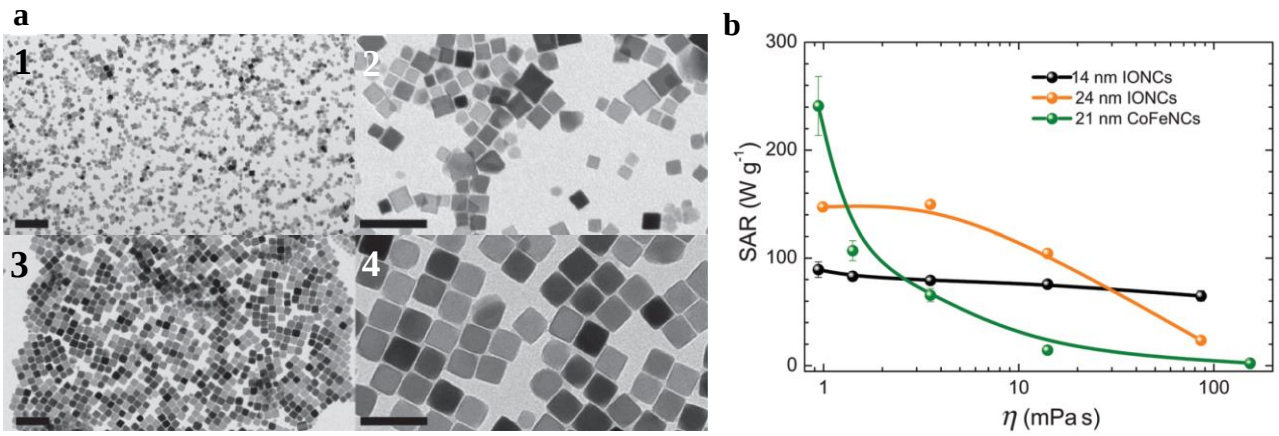


Figure 4.4. Iron oxide nanocubes. a) Representative transmission electron microscopy (TEM) images at low (left panel) and higher (right panel) magnification of iron oxide nanocubes for nanocubes edge with lengths of (1-2) 12 nm and (3-4) 19 nm. Scale bar 100 nm (left) and 50 nm (right). **b)** Viscosity dependence of SAR values for 14 nm (black dots) and 24 nm (orange dots) IONCs and 21 nm CoFeNCs (green dots). a) *Reprinted with permission from Pablo Guardia, Riccardo Di Corato, Lenaic Lartigue, Claire Wilhelm, Ana Espinosa, Mar Garcia-Hernandez, Florence Gazeau, Liberato Manna, and Teresa Pellegrino, Water-Soluble Iron Oxide Nanocubes with High Values of Specific Absorption Rate for Cancer Cell Hyperthermia Treatment.* ACS Nano, ***2012**, 6 (4), pp 3080–3091. Copyright © 2012 American Chemical Society. b) Reproduced from (Ref 164) with permission of the Royal Society of Chemistry*

5. Iron oxide nanoparticles for multimodal cancer therapy

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Cancer treatment: a multimodal approach

Considering the promising features of using IONPs for MH cancer treatments, new multimodal strategies for these materials might be envisaged. One of the most prominent is the addition of binding biomolecules to the nanoparticles surface to provide specificity and selectivity and/or the combination of MH with drug delivery. Thus, it might be possible to improve the accumulation of the IONPs at the tumor site and confine the thermal treatment exclusively to the malignant tissue, sparing the healthy ones. In addition to the target units, the surface might be also coated with polymers able to encapsulate drug molecules and enhance the effectiveness of cancer therapy. The drug release might be driven by a thermic stimulus generated by the magnetic nanoparticles under magnetic hyperthermia. When administered in the body, nanoparticles should circulate in the blood as long as possible to have a higher chance to find their cell target. To do so, their stability and the protection of both targeting molecules and loaded cargo must not be compromised. Once the IONPs will recognize the tumor cells, they are expected to be internalized and to release their therapeutic cargo enhanced by MH. To direct nanoparticles towards tumor cells, implies overcoming several biological barriers, as shown in **Figure 5.1**.

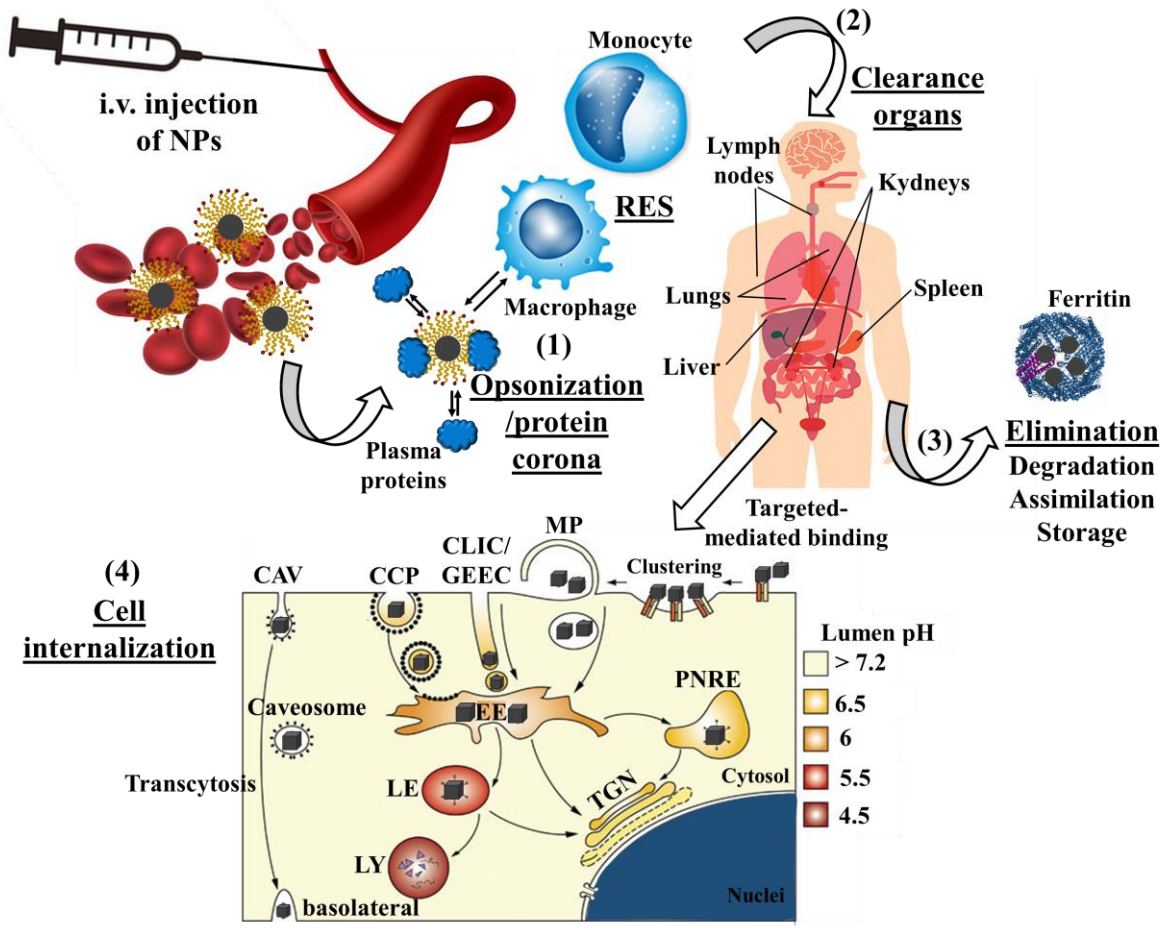

Figure 5.1. NPs in the body. Overcoming the biological barriers. Despite their potential for targeting and drug delivery purposes, the nanoparticles face a complex series of biological barriers that severely limit site-

specific bioavailability, preventing achievement of proper therapeutic outcomes. These obstacles include opsonization and subsequent sequestration by the mononuclear phagocyte system (MPS) and reticuloendothelial system components (RES) **(1)**, nonspecific distribution **(2)**, degradation and elimination from the body by the clearance organs and tissues **(3)**, cellular internalization and escape from endosomal and lysosomal compartments **(4)**. MP = macropinosome; CLIC/GEEC = clathrin-independent carrier/ Glycosylphosphotidylinositol - anchored protein (GPI-AP) enriched compartment; CCP = clathrin-coated pit; CAV = caveolin; EE = early endosome; LE = late endosome; LY = lysosome; TGN = trans Golgi network; PNRE = perinuclear recycling endosome.

Bioactivity and biodistribution of nanoparticles

For iron oxide nanoparticles, as well as for most of MNPs, different physical-chemical parameters of the material affect the biodistribution of the nanoparticles in human body. Nanoparticles size for instance is one of the main features to be considered when designing a therapeutic strategy against cancer. The adsorption of plasma proteins (protein corona)[166, 167] onto the NPs was shown to be lower for smaller nanoparticles (6% of plasma proteins adsorbed onto 80 nm NPs) than for bigger ones, with an trend increase trend dependent on the nanoparticles size (23% and 34% of plasma proteins adsorbed onto 170 nm and 240 nm particles).[80] This has important consequences for the clearance of the nanoparticles promoted by the cells of the reticuloendothelial systems (RES) and, in particular, by monocytes circulating in the blood (mononuclear phagocyte system, MPS), macrophages located in different organs (like liver, spleen, lymph nodes, bone marrow, lung an brain) and Kupffer cells in the liver.[168-170] Thus, the systemic clearance of the smaller nanoparticles was slower than that of the bigger NPs.[80] Moreover, the nanoparticles within a size above 200 nm undergo filtration in the spleen, whereas particles with sizes less than 100 nm have higher probability of getting trapped into the hepatic parenchyma.[80] Very small particles, below 8 nm of diameter, are instead filtered by the glomerular capillary membrane of the renal tubules and cleared by the kidneys through the urine.[169]

Another key parameter, which affects the biodistribution of nanoparticles, is the surface charge. Even if it is reported that neutral nanoparticles have low level of opsonization thus showing an extended circulation time, [80, 169] the absence of charge and consequently electrostatic repulsions might promote the aggregation of IONPs *in vitro*.[171] This, in turn, might compromise the nanoparticles circulation in the blood system, the effectiveness of drug delivery as well as the hyperthermia, since aggregation drastically compromises the heating performance of the IONPs. [171] Therefore, designing charged IONPs is preferable. Usually, IONPs with surface charge below -20/-30 mV or above +20/+30 mV present good colloidal stability. Negatively charged IONPs shows hepatic and splenic uptake but generally a higher blood circulation compared to positively charged nanoparticles that tend to be rapidly cleared from the systemic circulation.[80, 169] Another interesting behavior for differently charged nanoparticles was reported by Rotello's group.[172] Using an *in vitro* system simulating the complex tumor environment, they showed that 6 nm spherical gold nanoparticles, with negative or positive charges of +30 mV and -36 mV, respectively, were able to rapidly penetrate the cell mass. Interestingly, while positive nanoparticles interacted fast with the surrounding cells, negative particles were able to go deeper in the tumor mass.[172]

Along with size and surface charge, shape plays also a major role for the biodistribution of NPs in the body. It has been shown that rod-shaped and non-spherical nanoparticles exhibit a longer blood circulation time compared to spherical ones, in rat model studies.[173] In particular, short-rod mesoporous silica nanoparticles, which have roughly a spherical profile, were found to accumulate in the liver, whereas long-rod-shaped particles accumulated into the spleen. Moreover, short-rod mesoporous silica nanoparticles showed a faster clearance rate via urine and feces compared with

long-rod mesoporous silica nanoparticles.[174] This behavior is likely to be explained based on the different interactions that rod-shaped and spherical nanoparticles have with macrophages.[175] In addition, the surface organic coating of the particles can dramatically change the composition of the protein corona.[169, 170] Noteworthy, the coating should be non-toxic and biodegradable in order to ensure safety of the IONPs in clinics. Besides using several polymeric coatings,[176, 177] which should provide biological activity while reducing the clearance and the non-specific proteins adsorption, recently, new strategies were developed for increasing the biodistribution and the "blood-compatibility" of the nanoparticles. For instance, the development of biomimetic coatings consisting of cell membrane portions isolate from leukocytes[178] and red blood cells[179] demonstrated a decreased uptake rate by the MPS, RES and Kupffer cells and so a prolonged circulation time in the blood.[178, 179] Other approaches focus on the beneficial effects of the protein corona formation for modulating nanoparticles stability and drug delivery.[180] Hamad-Schifferli and co-workers demonstrated the efficient formation of controlled protein corona around gold nanoparticles of different shape.[181, 182] The protein scaffold was used for payloading cargo molecules such as DNA or doxorubicin and their passive releasing.[181, 182] However, since protein corona is a very dynamic process relying on the presence of different proteins and their relative concentration in the serum, the effectiveness of this approach has to be demonstrated *in vivo* before being accepted as an alternative method.[183] Given all the parameters discussed, one can conclude that the NPs clearance mechanism is a very complex and hardly predictable phenomenon, which must consider the combination of several factors such as size, charge, shape and coating.[170] It is of utmost importance that particles remain stable while in circulation in order to prevent non-required activity in undesired organs and to maximize bioavailability at the targeted site.[184] However, once they have accomplished their therapeutic purpose, IONPs should leave the organism in a safe way without affecting the body homeostasis. Indeed, one of the main concerns is related to the problems that can arise from a long-time persistence of the nanomaterials in the body: they can trigger chronic inflammation upon the interaction of the partially degraded NPs with the immune system or the generation of toxic by-products.[146] Specific methodologies for monitoring the fate of iron oxide nanoparticles in complex organisms are the following: electron paramagnetic resonance (EPR), which allows to distinguish at room temperature IONPs from endogenous paramagnetic iron species, MRI, magnetic manipulation of the nanoparticles inside organs or tissues, transmission electron microscopy (TEM) and elemental analysis.[146] As general rule, the fraction of IONPs that do not reach the liver, spleen or kidneys, accumulates inside the endosomal/lysosomal organelles of the cells where they undergo degradation processes (**Figure 5.2a** and **d**). During iron oxide dissolution, IONPs become surrounded by ferritin proteins, which are deputed to the storage and transportation of endogenous iron in the body (**Figure 5.2d**). Since ferritin regulates the metabolism of iron species, which can be deleterious if not opportunely complexed, the colocalization of the protein with the IONPs suggests remediation and recycling processes, ensuring the innocuousness of the nanoparticles.[146] In addition, it was shown that the IONPs aggregation state influences the degradation rate of the iron oxide, with single and isolated nanoparticles that erode faster than aggregated ones.[146] In mouse model studies, *Kolosnjaj-Tabi et al.* could follow the degradation of IONPs by following the reduction of MRI contrast of nanocubes in spleen and liver over time, which returned to normality after four months post-intravenously (i.v.) injection (**Figure 5.2b**).[132] In this work the sequestration of the iron by ferritin proteins, after nanoparticles degradation, was also proven.[132] Considering the presented data, IONPs can be assumed safe from a therapeutic point of view and their application for disease treatment is suitable for being largely extended. Of course, considerable attention has to be paid to the coating of the nanoparticles, and case-by-case study is needed for understanding the safeness of a IONPs-based formulation.

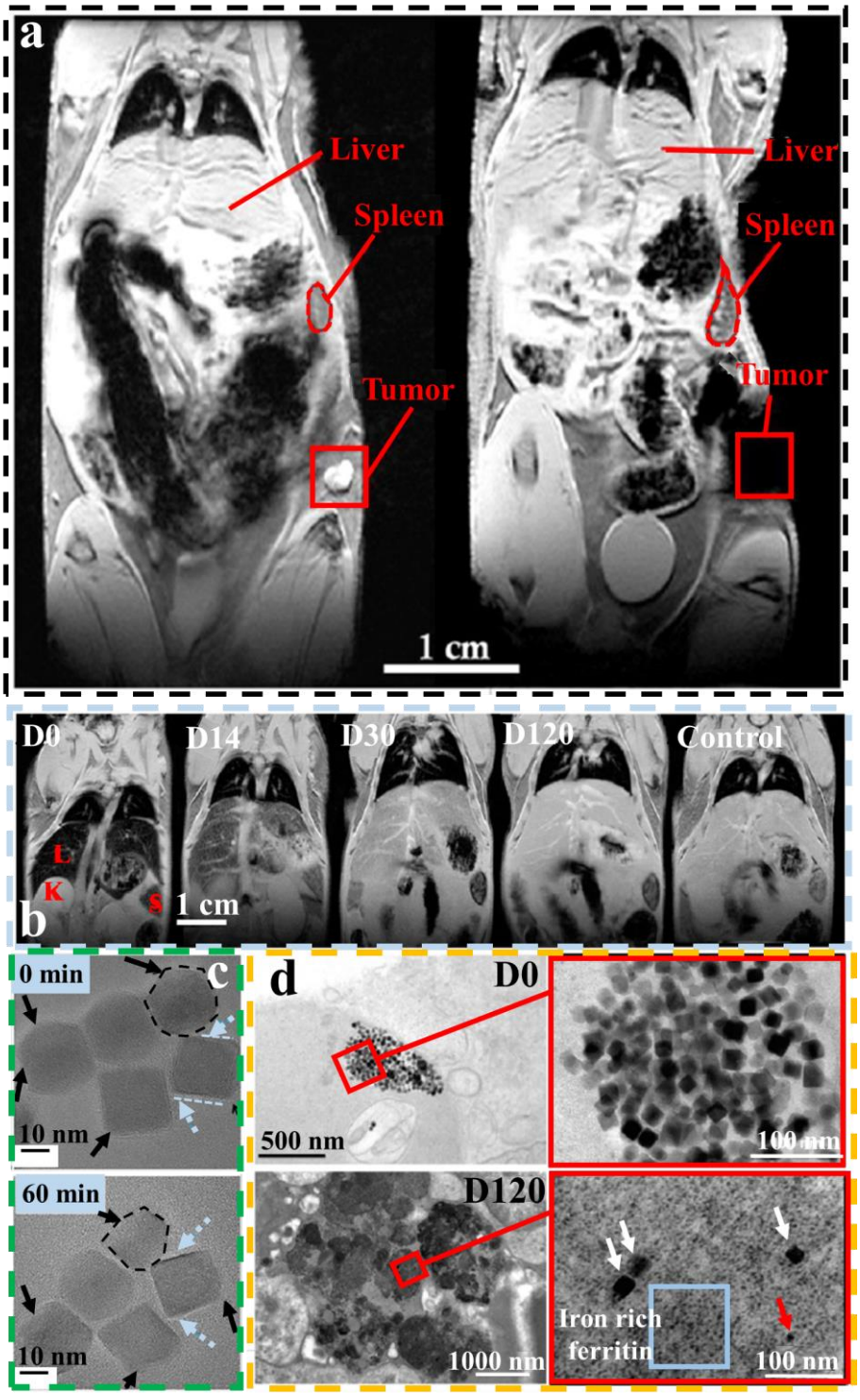

Figure 5.2. Biotransformation of IONPs. a) Black dashed box. Comparison of MRI scans of a non-injected mouse (left) and a treated mouse intratumorally injected with nanocubes (right) taken in vivo at day 6 post-injection. A large hypointense signal, mainly localized in the tumor region denoted by a red square, is observed in the injected mouse. **b)** Light blue dashed box. Comparison of MRI scans of a control mouse and intravenously injected mice taken at D0, D14, D30, and D120 post-injection, showing a pronounced hypointense signal, mainly localized in the liver and spleen (letters L, S, and K denote the liver, spleen, and kidney, respectively) which is attenuating over time. **c)** Green dashed box. Degradation over time of nanocubes in lysosome-like medium. After 60 minute, the shape and morphology of the nanocrystal dramatically change (black dashed box and black arrows), indicating its partial degradation. The organic coating appear completely degraded after 60 minutes (light blue dashed line and arrows). **d)** Orange dashed box. TEM micrographs

showing a representative intracellular endosome loaded with nanocubes (left) and its magnified view (right), found in the spleen on the day of cube injection (D0, top). Bottom micrographs show spleen sections harvested 4 months after injection: characteristic electron-dense lysosomes (right, and left for the magnified view) reveal the coexistence of scattered intact nanocubes (white arrows), a cube leftover (red arrow), and numerous monodisperse iron-rich ferritin proteins (blue square). *(a-b) Adapted with permission from (Ref 131). Copyright (2014) American Chemical Society. (c-d) Adapted from Biotransformations of magnetic nanoparticles in the body, Jelena Kolosnjaj-Tabi, Lénaic Lartigue, Yasir Javed, Nathalie Luciani, Teresa Pellegrino, Claire Wilhelm, Damien Alloyeau, Florence Gazeau, Volume 11, Issue 3, June 2016, Pages 280-284, with permission from Elsevier.*

Smart drug delivery: NPs get into action

The need for drug nanocarriers that efficiently target diseased areas in the body arises because drug efficacy is often altered by nonspecific cell and tissue biodistribution, and because some drugs are rapidly metabolized or excreted from the body.[185] Several ways for exploiting a controlled release of drugs from NPs were developed so far, aiming to take advantage of the metabolic and environmental differences occurring between healthy and pathological tissues, or to trigger a synergistic response with the nanoparticles' intrinsic features like magnetism or heating performance.[186] The strategies studied so far include redox release,[187] enzymatic degradation,[188] thermo-responsive and pH-responsive release.[189] Redox-sensitive nanosystems rely on the cleavage of disulphide bonds by glutathione (GSH), which is abundant in the intracellular environment (~2–10 mM) but relatively low concentrated in the extracellular environment of healthy tissues.[185] Moreover, due to the high metabolic activity, some tumors express elevated glutathione concentration in comparison to non-diseased tissues.[190] Reducible polymers, GSH-sensitive crosslinking agents or thiol-cleavable bonds were used for grafting on the nanoparticles surface drugs for the treatment of cancer cells.[185] Enzyme-sensitive systems take advantage from the altered expression profile of specific enzymes (such as proteases, phospoholipases or glycosidases) observed in pathological conditions, such as cancer or inflammation, for triggering enzyme-mediated drug release with accumulation of drugs at the desired biological target. Most of the systems devoted to enzyme-mediated drug delivery exploited the presence of enzymes in the extracellular environment, like matrix metalloproteinases (MMPs).[185] Thermo-responsive (TH) polymers, which can undergo reversible changes in physical properties in response to changes in the solution temperature, have become very popular. The possibility to rise the local temperature, not only of the pathological environment but also of the NPs coating via hyperthermia, suggested intriguing application of IONPs for the synergistic treatment of cancer. Indeed, while treating cancer cells by heat, it is possible to trigger the release of drugs from thermo-responsive polymeric shells, in which the therapeutic cargo is encapsulated.[191-193] This combination of treatments increases the effectiveness of the therapy, promoting an efficient eradication of the malignancy. Another strategy exploits the local changes in pH that occur naturally at different organ, tissue and cellular levels, *e.g.* the stomach versus the intestine environment, the intracellular endosomal-lysosomal compartments versus the cellular cytoplasm. This pH difference occurs also in some pathological conditions, such as the inflammatory or tumor microenvironment. pH-responsive polymers show changes in stability, solubility and volume in response to environmental pH changes. These features have been merged with NPs to produce new probes for drug delivery systems, tracking and imaging.[185] pH-responsive polymers are usually synthesized by using amines or derivatives, which confer to the nanoparticles shell a positive charge.[185] Positively charged nanoparticles have been reported to promote the endosomal escape of the nanoparticles after cell uptake.[194] This phenomenon, referred as "proton sponge effect"[195] acquired relevance for enhancing the cytosolic release of cargo molecules, avoiding their degradation inside the lysosomes.[196] Despite promising results were obtained for the delivery of drugs and genetic materials,[197, 198] it was shown that highly positively charged nanoparticles may induce acute generic cytotoxicity.[199, 200] Thus, the rational design of the polymeric shell and/or the linkers that bound a chosen drug to the NPs surface is critical for obtaining the desired therapeutic effect and strictly influences the disease outcome.

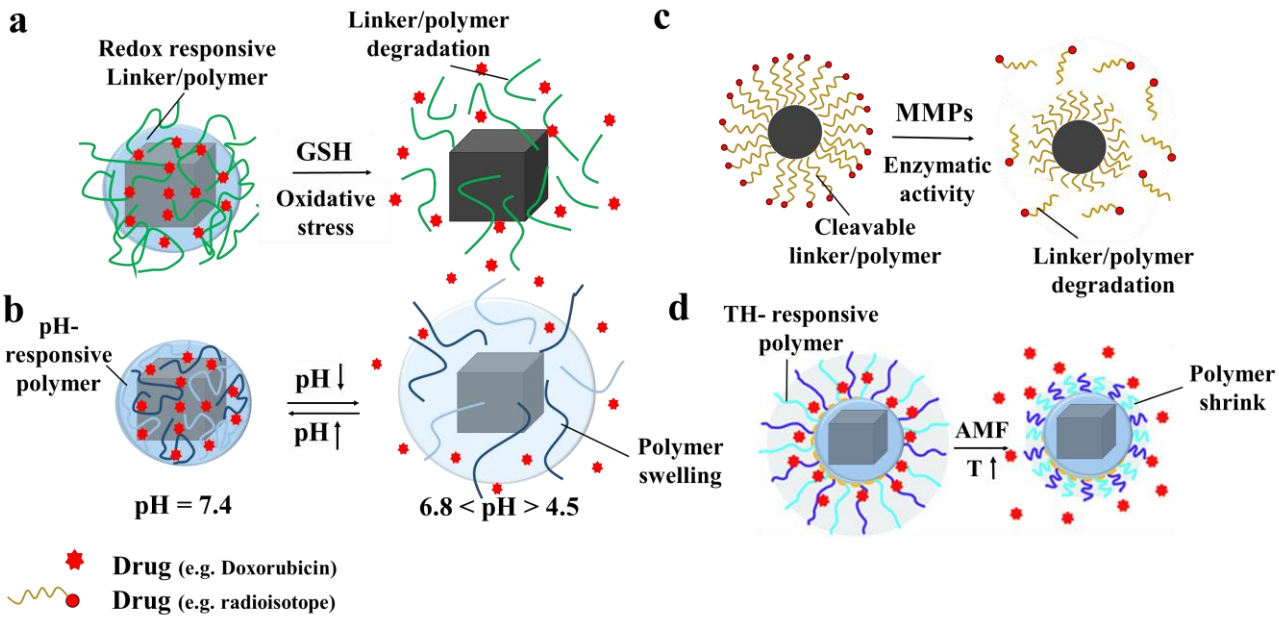

Figure 5.3. Stimuli-responsive drug release. a) Redox-stimuli systems exploit the oxidative reduction of reducible linkers or polymers which bind or encapsulate drugs cargoes. **b)** Upon the proteolytic activity of enzymes specifically active at the tumor environment, therapeutic molecules are released from the enzyme-sensitive nanosystems. **c)** The local decrease of pH at the tumor site or the low endosomal/lysosomal pH that the nanoparticles experience after the endocytosis process induce the release of the NP-bound/encapsulated drug following the polymer swelling. **d)** The local increase of temperature generated by magnetic hyperthermia may promote the shrinking of thermo-responsive polymer grafted on the IONPs surface. The drugs encapsulated inside are then release in the surrounding environment. *(d) Adapted from (Ref 192) with permission of the Royal Society of Chemistry.*

Targeting, *i.e.* how to localize IONPs at the desired site

To direct the nanoparticles towards the intended site, two different routes are usually followed: passive or active targeting (**Figure 5.4**). The first relies on the passive targeting of IONPs, based on the enhanced permeation effect (EPR) typical of some tumors. The presence of irregular blood vessels that present a discontinuous epithelium and lack the basal membrane of normal vascular structures were considered for a long time as a good opportunity for the passage and accumulation of tiny IONPs (30-100 nm in size) at the tumor site.[201, 202] However, several concerns have been raised, recently, about the reliability of the EPR as general hallmark of cancer.[203, 204] Indeed, different groups reported that the blood vasculature architecture can greatly change from one tumor to another and that, while for highly permeable tumors the EPR remains an affordable factor for nanoparticles targeting, in more compact and dense tumor masses only small, long circulating NPs may slowly extravasate.[205] Therefore, linking to the IONPs surface biomolecules able to actively recognize its target may help to localize the therapeutic drug at the desired site. This methodology, involving an active or ligand-mediated targeting, relies on the affinity of immobilized ligands on the nanoparticle's surface towards specific molecular targets present on the tumor cells.[206]

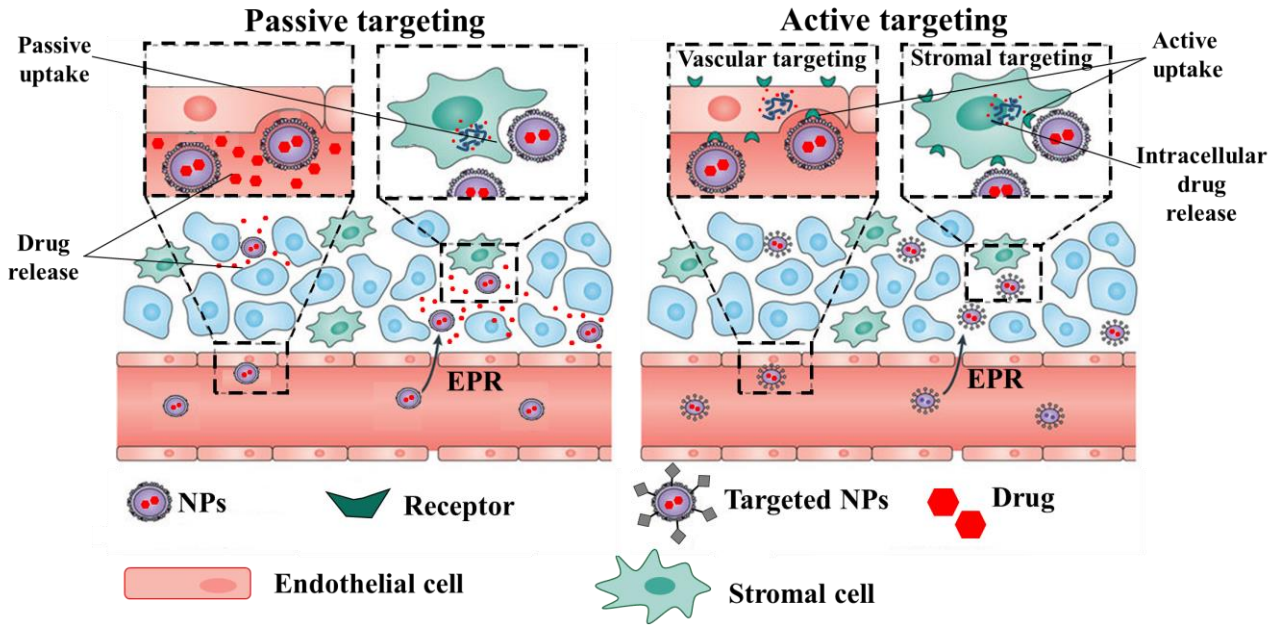

Figure 5.4. Passive vs active targeting. Passive targeting exploits the EPR effect for accumulating the nanoparticles, with the relative therapeutic cargo, at the tumor site. The fenestrations that are present in the disorganized and fast growing tumor vasculature promote the extravasation of the NPs and their interaction with the cells of the unhealthy environment. The drug is released in the tumor ECM or intracellularly upon the passive uptake of the nanocarriers. Active targeting instead relies on the ligand-mediated interaction of the biomolecules bound to the NP surface with specific markers overexpressed or preferentially expressed on the cancer cells membrane. The targeting may be directed to the cells of the tumor mass or to the endothelial cells of the tumor vasculature. Active targeting may also take advantage of the leaky tumor vasculature and increase the NPs uptake at the tumor site. *Adapted by permission from Macmillan Publishers Ltd: Nature Reviews Cancer (reference 9), copyright t (2017).*

Active targeting

Nanoparticles have been successfully functionalized with several biological active molecules in order to obtain specific targeting. Small molecules, peptides and antibodies are the most frequently used for that purpose.[207] Small molecules or peptides allow for a high density coating on the nanoparticles surface, increasing the avidity for the target. Moreover, a homogeneous coating can also provide better stability to the nanoparticles in solution. [43, 208] Compared to small molecules or peptides, monoclonal antibodies (mAb) express higher specificity and affinity for their target. However, they are usually very expensive, poorly stable, immunogenic and difficult to handle and produce. Lately, small antibody fragments, differently composed of the variable heavy (V_H) and light (V_L) regions of an entire antibody and connected through natural or synthetic loop, have acquired increasing interest in the field of bioconjugation (**Figure 5.5**).[45] Among them, single-chain fragment variable recombinant antibodies (scFv) have shown a great potential.[45, 209, 210] Due to their small size (typically in the range of 20-30 kDa) they are more stable and do not present a high immunogenic profile.[211] Even if they express a reduced affinity for their target compared to their parent mAb, the binding of multiple fragments on the surface of the nanoparticles produces an increment in the avidity for the target, compensating the reduction of affinity.[211]

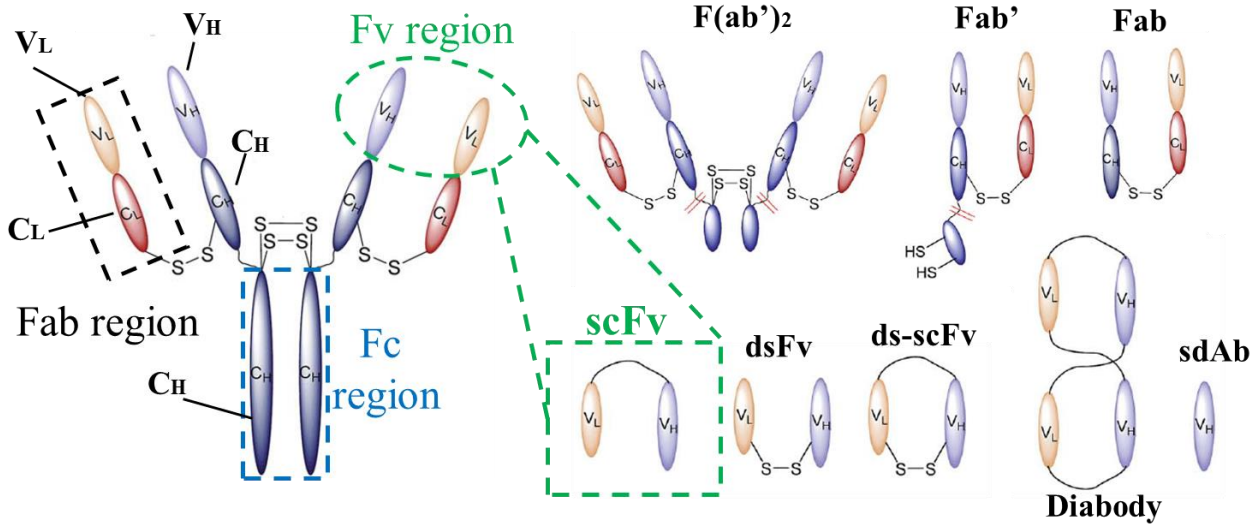

Figure 5.5. Antibody and antibodies fragments for NP-mediated targeting. Monoclonal antibodies (mAbs) consist of four protein chains; two identical ca. 25 kDa light chains (*i.e.* L-subscript) and two identical ca. 50 kDa heavy (*i.e.* H-subscript) chains. These chains contain multiple domains which are characterised by their degree of sequence variability. The N-termini of the chains constitute the variable domain (V), which form the antigen-binding region. Further from the terminus, the structure becomes more conserved and is called constant region (C). The heavy and light chains are held together by several inter-chain disulfide bonds (ds) to form a Y-shaped structure. The overall structure can be divided into two distinct segments; the fragment antigen-binding (Fab) region and the fragment crystallisable (Fc) section. Fabs can be further divided into variable (Fv, $V_{H/L}$) and constant ($C_{H/L}$) regions.[45] Fab, Fab', F(ab')$_2$, were isolated and used alone or in combination with nanoparticles. Additionally, other classes of antibody fragments emerged as targeting molecules for NPs, such as the ScFv (green dashed box), ds-Fv, ds-ccFv, single domain antibodies (sdAb), and diabodies. *Adapted from (Ref 45) with permission of the Royal Society of Chemistry.*

An astonishing amount of binding chemistries are available for the functionalization of the nanoparticles surface,[212] either exploiting the natural affinity of the chemical groups of the nanoparticle surface and biomolecule or relying on the modification of one or both components. [212] Parameters such as the ratio of biomolecule per NP, the orientation of the biomolecules onto the nanoparticle, as well as its distance form NP surface, control over the attachment affinity and finally, yet importantly, the cheapness and replicability of the chosen strategy have to be strictly considered (**Figure 5.6**). Instinctively, one can think that higher is the number of molecules bound on the NP surface, better would be the recognition of their target and consequently more efficient and specific the treatment. However, despite the possibility to exploit the high surface to volume ratio of the particles with the grafting of small molecules at high density, the impact of the steric hindrance of the biomolecules and their reciprocal interactions onto the NP surface should not be underestimated, especially for big biomolecules. Indeed, *Colombo et al.* reported that increasing the number of antibodies bound on the surface of gold nanoparticles reduced their effective specificity towards the desired cells. Their work showed that the binding of one antibody resulted in an efficient recognition of the target, while the nanoparticles functionalized with two antibodies were inefficient.[213] Intuitively, also the orientation of the biomolecules plays a crucial role for an efficient bioconjugation strategy. The activity of proteins, enzymes, antibodies but also small molecules like folic acid or biotin depends on the availability/accessibility of their active site to interact with the target molecule. A rational plan of the distance and orientation parameters of the biomolecules grafted on the NP surface is essential to ensure a precise bioconjugation construct. Indeed, non-specific chemistry or electrostatic interactions can result in heterogeneous attachment and impair the activity of the final conjugate.[212]

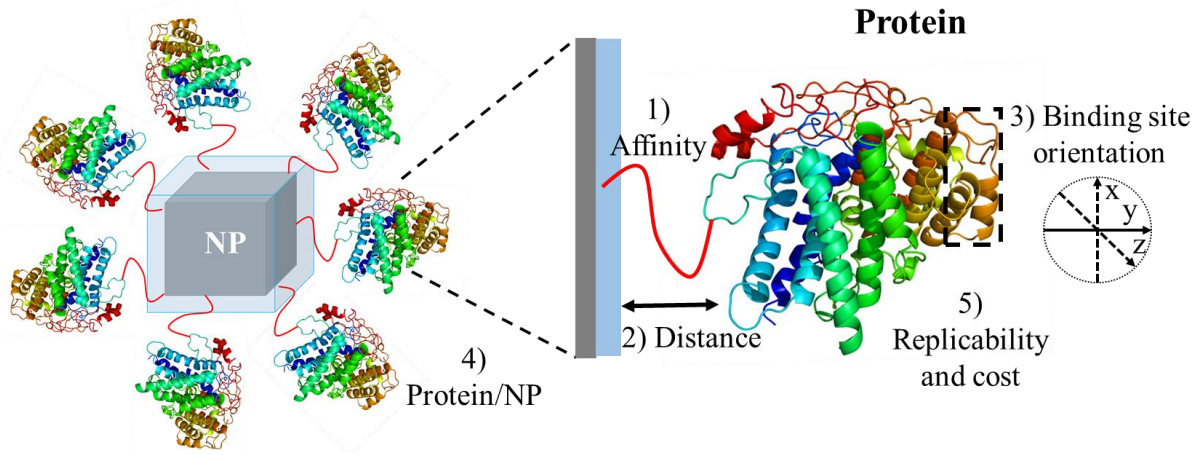

Figure 5.6. Rational binding of the biomolecules to the NP surface. Taking into consideration a toolset of parameters would allow controlling the attachment of any protein or biomolecule to any NP. These criteria include 1) control over attachment affinity; 2) control over relative separation distance from the NP; 3) control over the orientation and exposition of the active site on the NP; 4) control over the valence of biomolecule per NP. A further parameter that should be considered is the replicability of the binding strategy, *i.e.* the possibility to extend it to other biomolecules. Moreover, the intrinsic cost of the procedure chosen may facilitate the clinical/market translation of the nanosystems developed. *Adapted with permission from (Ref 211). Copyright (2013) American Chemical Society.*

In addition, the stability of the bioconjugate should be assessed for its final utility. A permanent linkage would be preferable for providing long-term stability to the nanoconjugate, while a reversible binding could be more desirable in case of NP-mediated drug delivery.[212] The later can be the case of antibodies labeled with radionuclides used for both targeting and for killing cancer cells. pH-cleavable or matrix metalloproteinase (MMPs)-cleavable linkers can be used for spreading the nanoconjugate activity at the tumor site, once that the active drug or compound is released from the nanocarrier.[214] When designing an active targeting strategy for cancer therapy, the protein corona formation around the nanoparticles must be carefully considered. Indeed, the opsonization of plasma proteins on the NP surface can mask the active site of the immobilized protein, thus preventing the interaction with its target. As presented by *Salvati et al.*, silica NPs conjugated with transferrin lost their ability to recognize transferrin receptor on cells in presence of high concentration of serum proteins.[215] This work spreads serious criticism on the thousands of published papers that carried out targeting studies at serum protein concentration of 10% (amount usually used for cell culture), which compared to pure plasma does not resemble the *in vivo* conditions. This result questions the relevance of the *in vitro* tests for NP-ligand/receptor recognition.[215] Furthermore, some doubts were raised on the real efficiency of an active targeting strategy compared to the non-active one. Indeed, designing a ligand-mediated targeting strategy can be onerous in terms of cost and time, but if it does not lead to an improved outcome in patients it may remain interesting only scientifically and not clinically.[6] Noteworthy, in animal models, it was observed that the tumor accumulation for a wide range of nanomaterials with targeting was modest compared to nanoparticles without targeting and not always higher.[216] However, the presence of targeting molecules can facilitate the uptake of the nanoparticles and promote their internalization through specific pathways,[217] like caveolin-mediated endocytosis, that facilitate the activity of a cargo drug avoiding its degradation inside the lysosomes.[184] In conclusion, due to the many challenges and factors that influence the efficiency of a ligand-mediated targeting strategy, the development of a suitable nanoplatform has to be carefully planned and evaluated for all the single aspects composing it: 1) size and shape of the magnetic support; 2) coating material and charge; 3) size and composition of attached biomolecules ; 4) carrier target specificity and escape to clearance.

6. How far we are?

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To better understand the potentiality of nanomedicine and fix the milestones for future research, is opportune to ask ourselves "How far can we go?". Several questions were moved to the nanomedicine in the early 2000's.[218] Most of these questions still have to receive suitable answers. Nanoscientists have to face that even if great improvements were made in the direction of a more clear comprehension of the behavior of nanoparticles in biological environment, still major challenges remain. Bioavailability, accumulation at the desired site and efficient release of the therapeutic cargo are just a small part of the tasks that a nanodrug has to accomplish inside the biostructures of a complex organism, like a human being. Poor understanding of the biological barriers, misinterpretation of drug delivery concepts, cost-effectiveness, manufacturing and scaling up, and regulatory issues, have also affected the clinical translation of nanomedicines.[219] Another major limitation is related to the investigation approaches hitherto used. Although cell culture studies and small animal models continue to be essential for the investigation of fundamental bio-nano interactions, it remains challenging to use these types of models to predict clinical performances.[6] *In vitro* experimentation, the use of 2D culture, which has proven to be a valuable method for cell-based studies and basic molecular interactions, presents intrinsic limitations. Indeed, this model does not adequately take into account the natural 3D environment of cells *in vivo*, in which they are surrounded by other cells and extracellular matrix[220, 221] Currently, in drug discovery, the standard procedure for screening compounds starts with the 2D cell culture-based tests, followed by animal experimentation. The most commonly used model for these studies is the mouse, often immunodeficient, which may poorly represent the behavior of a human body.[6] Three main issues can be pointed out from the use of these mice: 1) the impossibility to assess the role played by the immune system in cancer and how its interaction with nanomedicine does affect the therapy;[219] 2) the enhanced permeability and retention (EPR) effect which is not accurate for such models; 3) the different size of the xenograft tumors implanted in mice compared to a human tumor. The relative size of a tumor (2 – 10 g) in a patient (70 kg) is in the range of 0.003 – 0.01%. Unlikely, the relative mass of a tumor (0.1 - 4 g) xenografted into and growing in a nude mouse (15 – 30 g) is in the range of 0.3 – 30%.[6] A tumor of this size in a mouse would correspond to the size of a basketball in a human.[6] As consequence, one soon realizes that it is rather easier for nanoparticles to encounter the tumor in mouse model, while in human this cannot be easily assumed. Thus, the differences between human and current animal models (tumor microenvironment, dosing regimens, bioavailability, pharmacokinetics, pharmacodynamics, as well as a lack of standardization in the conducting and reporting of preclinical studies) may originate discrepancy between what is expected from preclinical mouse results and what is actually observed in clinics. So far, it seems that cancer nanomedicine has dramatically focused on treatment of mice rather than humans. Indeed, the EPR-driven xenograft tumor models are ideal for demonstrating enhanced therapeutic efficacy of nanomedicine formulations compared to free drug but are useless from a translational point of view.[219] However, having cleared the complexities and challenges that are emerging with nanomedicine, several advances in the development of new *in vitro* and *in vivo* assays are being proposed (**Figure 6.1**). For example *in vitro*, 3D tumor models and spheroids,[220-222] microfluidic-based assays[223] and culturing of tumor explants and organoids *ex vivo*[224, 225] are being pursued and have shown promising results.[6] Considering *in vivo* alternatives, it is possible to count the use of immunocompetent animal models in which tumors develop spontaneously and comparative oncology, which examines both cancer risk and tumor development across species. However, thinking of being "on the right way" could turn out

to be a simplistic illusion. Instead, it is reasonable to imagine that what is until now firmly established will have to be revised in the future. Nanoscientists have the duty to lead the nanomedicine to the development of better devices, drugs and technologies for early diagnosis or treatment of a wide range of diseases with high specificity, efficacy and personalization, thus overcoming the nowadays limitations for a precise theranostic approach (combination of diagnosis and therapy).[3, 226]. It is possible that "magic bullet" concept is definitively outdate.[227] Identify the weakness of NPs is the best way to find what will.

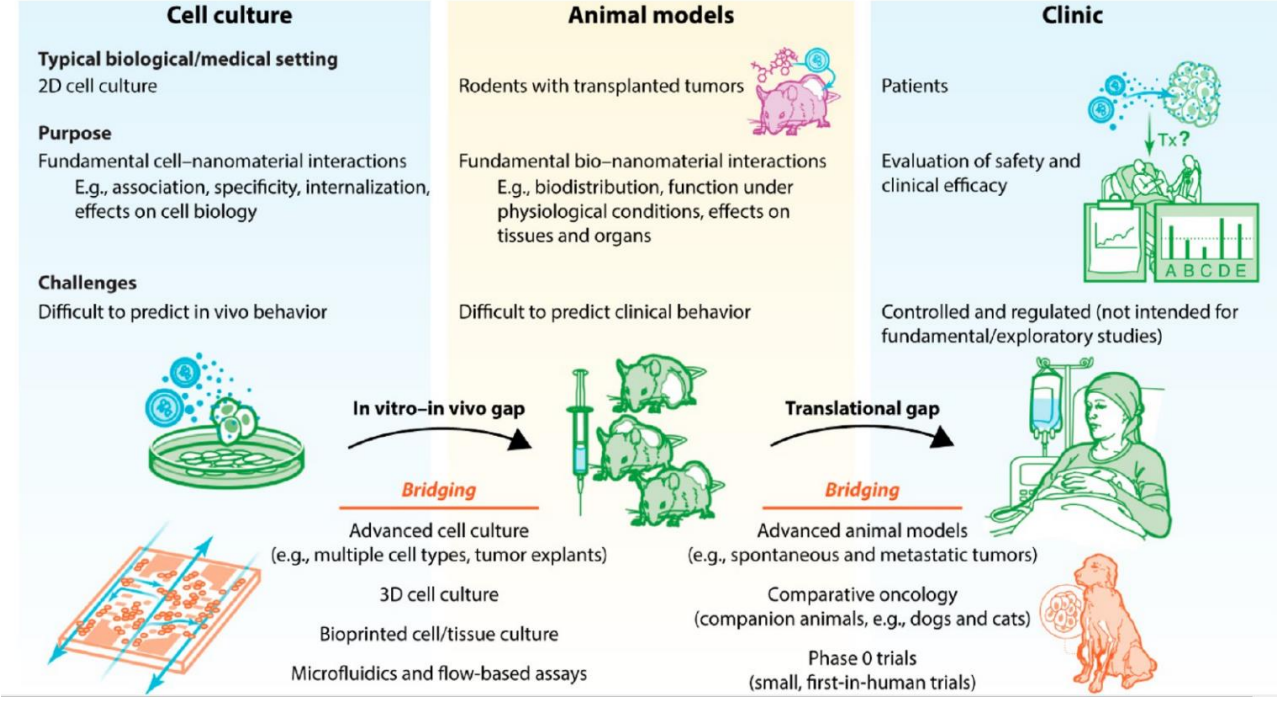

Figure 6.1. Overview of the approaches for developing nanomaterials for cancer treatment. The "*in vitro–in vivo* gap" is that *in vitro* results cannot be easily translated to *in vivo* settings. The "translational gap" is that strategies developed with the help of animal models can be difficultly translated to human patients. New approaches are emerging, which can help to overcome these challenges, for example 3D cell cultures and comparative oncology.[6] *Reprinted with permission from Mattias Björnmalm, Kristofer J. Thurecht, Michael Michael, Andrew M. Scott, and Frank Caruso, Bridging Bio-Nano Science and Cancer Nanomedicine.* ACS Nano, *2017, 11 (10), pp 9594–9613. Copyright © 2017 American Chemical Society.*

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