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New insights for the biological contribution of gold-based nanoparticles in radiosensitization effect.

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Acknowledgments

Before start talking about this very interesting topic, there are some people I would like to thank:

first, prof. ***MICHIELS Carine****, the person that allowed me to live this experience: she proposed me to study this topic and opened the doors of UNamur laboratories;*

dott. ***PENNINCKX Sébastien****, my internship supervisor and the person that answered all my question about this topic I never addressed;*

prof. ***LUCAS Stephane****, chief of the LARN laboratory, that allowed me to access the lab even with the COVID-19 pandemic spreading in the city.*

Lastly, prof. ***MORETTI Elisa*** *that coordinate my activities from Italy and was always available to answer my questions, either regarding the protocols I followed or the writing of this thesis.*

This work represents my first approach to the fight of cancer through nanotechnology, something I have never studied but I would like it to become the main topic of my research activity in the future.

Therefore, I would like to dedicate this manuscript to you all!

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Abstract

According to the World Health Organization (WHO) and the National Institute of Health (NIH), cancer is the second leading cause of death with 18.1 million new cases and 9.6 million deaths worldwide in 2018. Despite all the efforts put in research, an "universal" treatment to cure all 36 different kinds of cancer is still leading to the development of several treatment modalities.

Amongst them, we tried to increase the efficacy of radiotherapy by combining nanoscale materials with conventional radiation therapy to increase the energy deposited in tumor tissues sparing healthy ones: this concept is represented by a simple parameter called *therapeutic ratio* (defined as the differential radiation dose deposited in tumor cells versus the one deposited in healthy tissues). In fact, the main problem faced up with this treatment is the damage to healthy tissues surrounding the tumor: these damages cause side effects whose intensity can be considered directly proportional to the dose deposited in normal tissues. Therefore, maximization of the therapeutic ratio is one of the main goals to achieve in modern radiotherapy treatment. For this purpose, the use of charged particles instead of conventional X-rays (a new kind of radiotherapy called hadrontherapy) is growing worldwide since it ensures a more effective tumor targeting. At the same time, advances in nanotechnology are providing new nanoagents that offer the possibility to specifically target neoplastic cells and block their proliferation from the inside.

When gold nanoparticles (AuNPs) are irradiated with either X-rays or proton beam, the amount of cancer cells that undergoes apoptosis is higher than an identical irradiation without AuNPs; this enhanced cell death is known as *radiosensitization*. The mechanism behind this effect is not completely understood yet; however, according to the actual scientific literature, only a physico-chemical hypothesis is sufficient to explain this enhancement. Ionizing radiation (IR) interacts with cells, damaging the genetic material enclosed in the nucleus; at the same time, it may encounter AuNPs leading to the emission of low-energy electrons. These electrons interact with the surrounding medium, producing reactive oxygen species (ROS), which can damage biological targets. However, simulation works highlighted that the probability of this interaction is too low to explain this enhancement by itself and other contribution(s) must be involved. Therefore, a second hypothesis, based on a biological mechanism, has been proposed: AuNPs disrupt cell homeostasis, predisposing it to death after irradiation. More specifically, a time-dependent mitochondria membrane depolarization and inhibition of thioredoxin reductase (TrxR) are the main ideas behind this hypothesis.

This thesis was born with the aim of verifying or disproving the second model, in particular by studying how AuNPs of different size can affect TrxR inhibition. Unfortunately, because of the COVID-19 pandemic, the time spent in the laboratory was significantly limited and the great majority of experiments could not be performed because of limitations imposed by the authorities. However, this represents a preliminary study, the beginning of a new set of experiments.

Section 1: Introduction

Cancer is a large group of diseases that can originate in any part of the body and consists in an abnormal, uncontrolled cellular growth able to spread in surrounding tissues and invade other parts of the body. This disease can start almost anywhere in the human body, which is made up of trillions of cells. Normally, healthy cells grow and divide to form new cells as the body needs them; when these cells die, new cells take their place. Cancer cells can break this process, with damaged cells able to survive when they should die. These cells can divide uncontrollably, and this process may end in tumor formation.

The World Health Organization WHO estimated that 9.6 million people died because of cancer in 2018, accounting for 18% of total deaths around the world. The cancer death rate rose until 1991, then fell continuously through 2017, resulting in an overall decline of 29% that translates into an estimated 2.9 million fewer cancer deaths. This progress is driven by long-term declines in death rates for the 4 leading cancers (lung, colorectal, breast, prostate); however, over the past decade (2008-2017), reductions slowed for female breast and colorectal cancers, and halted for prostate cancer.

The most frequently diagnosed cancers (listed in descending order according to estimated new cases in 2018) are lung and female breast cancer (both representing the 11.6% of all cases), followed by colorectal cancer, stomach and liver cancer. It is possible to observe that the lifetime probability of being diagnosed with cancer is slightly higher for men (40.8%) than for woman (37.5%). However, more than one-third of the world population (39.5%) will be diagnosed with cancer at some point during their lifetime.

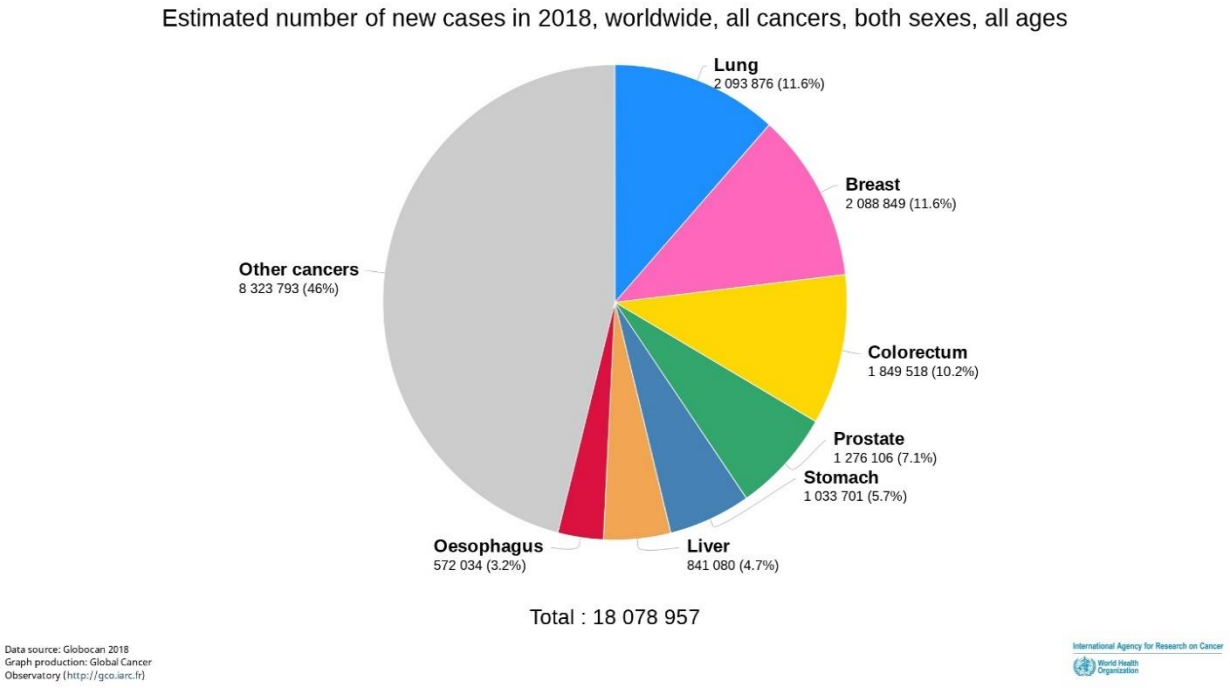

Figure 1.1: World Health Organization estimated number of new cancer cases in 2018, worldwide.

According to the Surveillance, Epidemiology, and End Results (SEER) program, cancer death rates decreased 1.8% per year (on average) among males and 1.4% per year on females. Although overall cancer death rates continued to decline among both males and females, incidence rates leveled off among males and increased slightly among females (***Figure 1.2***).

These trends reflect population changes in cancer risk factors, screening test use, diagnostic practices, and treatment advances. The decrease in incidence and mortality of lung, larynx, and bladder cancers are probably due to the decline in cigarette smoking. At the same time, these gains are being offset by increasing incidence trends for cancers related to excess body weight and physical inactivity. Finally, advances in screening tests allowed early detection of cancer and subsequent timely effective treatment, reducing mortality even more.

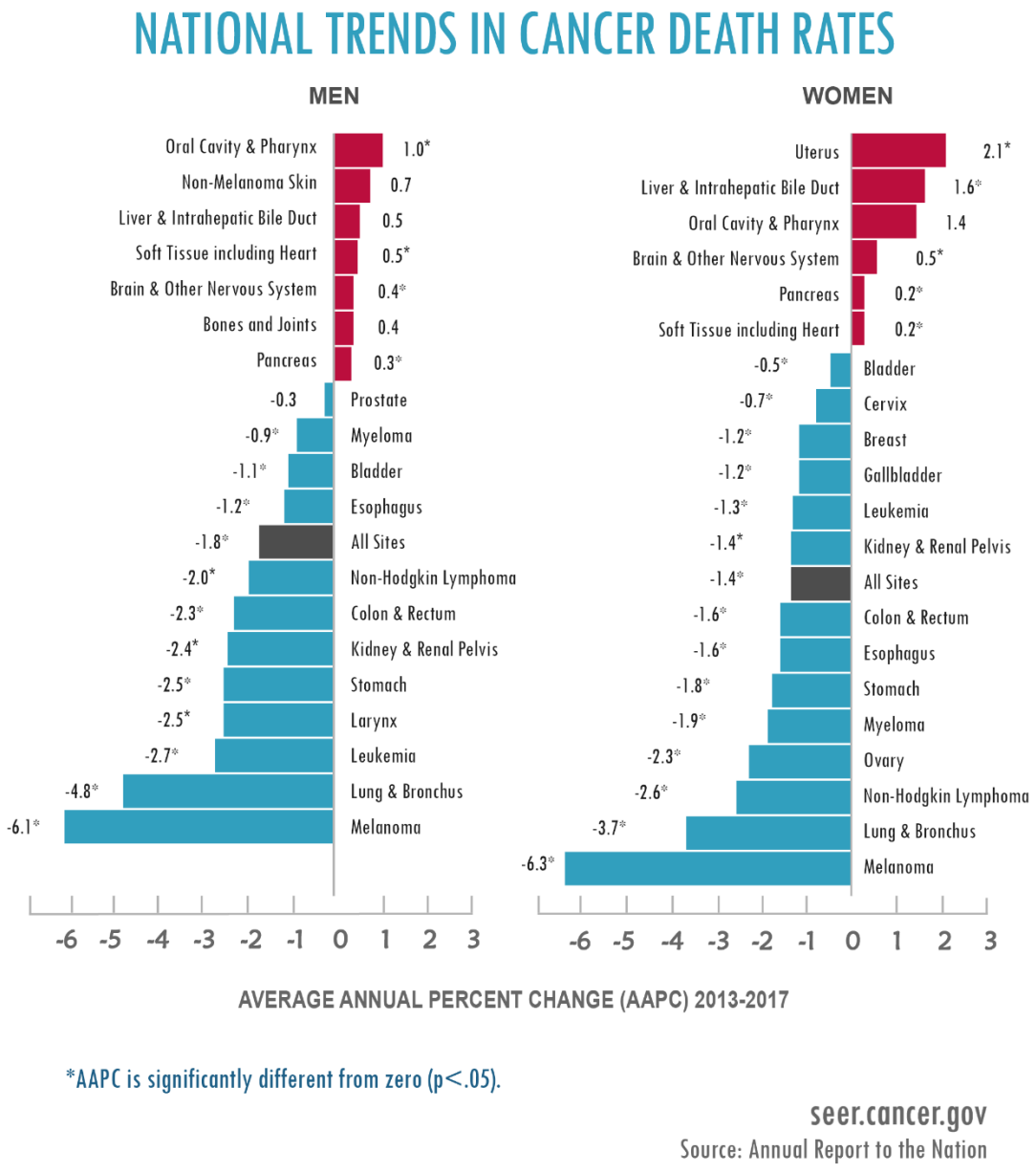

Figure 1.2: The average annual percent charge (AAPC) in age-standardized death rates for 2013 through 2017 are illustrated for all sites and for the 19 most common cancer deaths in men and the 20 most common cancer deaths in women. (adapted from [1])

As previously stated, many of these deaths (around 30-50%) could be prevented avoiding well-known risk factors like the use of tobacco, abuse of alcohol, reduction of exposure to UV radiation and, at the same time, implementing evidence-based prevention strategies such as maintenance of healthy weight, eat plenty of vegetables and fruits, practice exercise regularly and so on.

A conceptual organizing principle

All growth that are grouped together under the rubric of "cancer" exhibit mutated cell genomes, highly variable timelines of pathogenesis and progression to symptomatic and metastatic disease, with a plethora of pathological effects. Therefore, it was possible to propose a generic set of cancer hallmarks allowing researchers to rationalize this disease through these capabilities. These hallmarks can be seen as a set of acquired functional capabilities that act in combination to produce most forms of cancer, despite genetic and pathologic differences. Moreover, each of these capabilities could be acquired by developing cancers through several alternative means, representing different solutions to the common challenges facing all incipient neoplasias. This concept was first proposed by Hanahan and Weinberg in 2000[2] and then refined, by the same authors, in 2011[3]: this classification has proven to be a useful heuristic tool for distilling the underlying foundations of this disease.

In the current conceptualization, there are 8 hallmarks that are common to many forms of human cancer; each of these capabilities serve a distinct role in supporting the development, progression, and persistence of tumors and their constituent cells, as briefly explained below:

1. *Sustaining proliferative signaling*: the essence of this disease is a deregulated programme that instructs cancer cells to grow and divide, doing so at inappropriate times and places, chronically. Many of the mutations that convert normal genes into oncogenes serve to trigger cancer progression by stimulating cellular growth-and-division cycles. At the same time, other mutations alter regulatory circuits involving secreted growth-stimulatory proteins that bind their cognate cell-surface receptors. This process is followed by a cascade of protein-protein association and phosphorylation. The most prominent of these signaling channels being the growth-promoting signals leading to an unstoppable proliferation[3];
2. *Evading growth suppressor*: in addition to sustaining positively acting growth signals, cancer cells are also able to circumvent mechanism that negatively regulate cell proliferation. Cells proliferation is regulated by several mechanisms whose action is heavily dependent on the microenvironment where cells reside (to ensure that cells proliferation is not an entirely cell-autonomous process). Direct regulators of the cell division cycle (the retinoblastoma protein (pRb) and several cyclin-dependent kinase inhibitors) and the intracellular monitoring system, centered upon the p53 protein, are the most prominent brake to uncontrollable cells proliferation. In many human cancers, some components of these mechanisms may be prevented to efficiently stop cell proliferation because of epigenetic mechanisms, notably those involving DNA and histone methylation[3];

3. *Resisting cell death*: there are three distinct ways normal cells can face programmed death, and the most prominent of these programs is apoptosis (this process can be triggered by cell-intrinsic as well as non-cell-autonomous signals) This mechanism can be circumvent by overexpressing anti-apoptotic regulators or, on the contrary, downregulating pro-apoptotic factors. Necrosis is the second mechanism, with cells releasing their contents and leaving their carcasses as debris. The third program, called autophagy, is frequently seen when cells face nutrient deprivation; by degrading cellular organelles they are able to generate nutrients that cannot be found in their surroundings. All these mechanisms can be circumvent or attenuated by cancer cells in the great majority of human neoplasia[4-6];

4. *Inducing angiogenesis*: as all kind of tissues, cancer cells require continuous sustenance of nutrients and oxygen as well as evacuation of waste produced as consequence of metabolic activities. To satisfy all these requirements cancer cells stimulate the formation of new blood vessels (through vasculogenesis and angiogenesis) that grow in parallel with the tumor to facilitate its proliferation[3];

5. *Enabling replicative immortality*: cancer cells are able to replicate indefinitely, a behavior in marked contrast to the one observed in healthy cells that can pass through only a limited number of successive growth and division cycles before experiencing apoptosis or crisis. When telomere repeats – the repetitive nucleotide sequence at the end of each chromosome which avoid fusion between neighboring chromosomes - is reduced below a certain threshold, a tripwire is triggered, causing p53-dependent cell cycle arrest or apoptosis (the latter termed "crisis"). Most cancer cells circumvent this natural barrier by activating a mechanism of telomere maintenance by overexpressing the telomere-extending enzyme telomerase[3];

6. *Activating invasion and metastasis*: is the spread of a tumor from the primary site to a different (secondary) site within the host's body. A key role for this capability is played by E-cadherin, a protein involved in the formation of cell-formed sheets that maintain cells quiescence. Its overexpression is unfavorable to metastasis while its downregulation increases the likelihood for this process to occur[3];

7. *Deregulating cellular energetics and metabolism*: the concept that cancer cells alter their utilization of energy sources to support their proliferation was introduced almost 90 years ago by Otto Warburg[7]. He observed that certain cultured cancer cells exhibited enhance glucose uptake, which was then largely metabolized by glycolysis. Further studies highlighted that the "aerobic" glycolysis produces many of the building blocks for the cellular macromolecules that are required for cell growth and division[3,8];

8. *Avoiding immune destruction*: incipient neoplasias are able to circumvent active surveillance by the immune system that would otherwise eliminate aberrantly proliferating pre-malignant cells. The reason why immune system cannot detect cancer cells is still unknown. The phenomenon of immune tolerance may be part of the answer: because a normally functioning immune system develops a tolerance toward self-antigens, a tumor may pass under the radar and evade recognition and attack, as it expresses only these normal tissues antigens. However, rules of immune engagement remain unknown and ambiguities across the spectrum of human cancers are quite common. [3,8]

The process of tumor development reflects the need of evolving cancer cells to acquire the eight capabilities described above. How are these functional capabilities acquired then? Currently, there are two clearly established means by which these hallmarks are acquired: genome instability and the resulting mutation of hallmarks-enabling genes and inflammation by cells of the immune system help to provide such capabilities.

Genome instability and the consequent mutation of hallmarks-enabling genes is the primary means of acquiring hallmarks capabilities. Cell genome is daily subject to damages inflicted by multiple sources, such as reactive species resulting from normal metabolism, environmental insults, and by errors in DNA replication during cell division. The resulting defects, if left unrepaired, can become cell-heritable mutations; according to the lesion's severity, many processes may be activated. If the genome is irreparably damaged, cells undergo the most dramatic process, apoptosis, that is orchestrated by the *p53* tumor-suppressor gene, which has therefore been named the "guardian of the genome" because of its function. [8]

Tumor-promoting immune inflammation is the other important means by which developing cancers can acquire hallmark capabilities. As previously described, cancer cells are able to avoid immune destruction by blocking infiltrating cytotoxic T cells. At the same time, other agents of the innate immune system fight against neoplastic cells (one of which is called infiltrating immune cells, or IICs). In principle, IICs-induced inflammation may be though as failed attempts to eradicate the disease. However, new evidences clearly show a quite different role: IICs help in the acquisition of multiple hallmark capabilities.[9] The identities of the recruiting signals that bring IICs into tumors are still incompletely understood.[8]

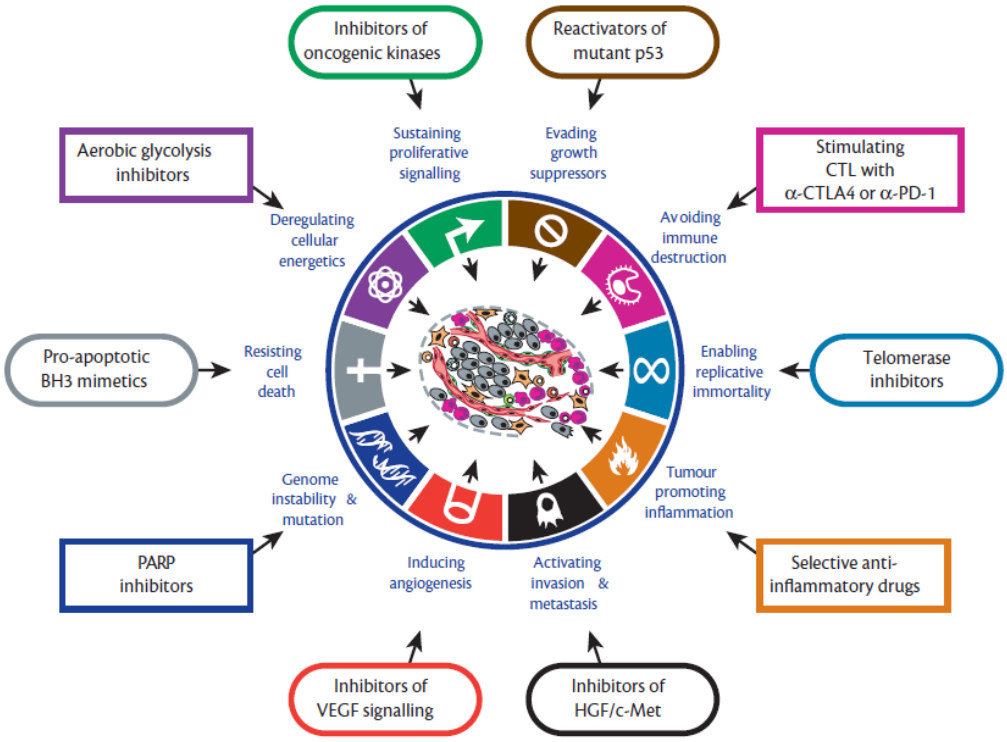

Figure 1.3: Therapeutic targeting of the hallmarks of cancer. Drugs have been developed for both the eight hallmark capabilities and both the enabling characteristics. Some of them are already approved for clinical use whereas other are in late-stage clinical trials. (reproduced from [8])

This conceptualization may be used for several purposes: the most obvious is to elucidate the molecular and cellular mechanisms by which particular forms of human cancer develop and progress to malignancy. At the same time, this classification may be used as guide for the development of novel mechanism-targeted therapies. From this point of view, there are either approved drugs or in late-stage clinical trials that target each of the eight hallmark capabilities and both of the enabling characteristics.

Unfortunately, such mechanism-based therapies have not proven to be very effective for the treatment of late-stage tumor, with adaptive resistance mechanisms appearing after a period of clinical response.[8]

The last strategy involves applying the concept of the hallmarks as independent (or quasi-independent) and necessary components of a malignant cancer: by simultaneously targeting multiple hallmarks, it may be more difficult for cancer cells to concurrently develop multiple resistance mechanisms, allowing improvements in both initial efficacy and duration of the clinical responses. As is always the case with multi-drug treatments, a major complication will arise from the toxicities that often accompany the application of such therapeutic protocols.

Section 2: Cancer treatment

Nowadays there are two main kinds of cancer treatment, systemic and local treatment: the first approach uses drugs that spread throughout the body to treat cancer cells wherever they are located[10], the latter focuses on a specific organ, or a limited area of the body, treating cancerous cells and sparing healthy ones.[11]

Chemotherapy is probably the most effective treatment to fight against cancer: it uses medications, called cytostatics, to stop cancer cells from keep dividing uncontrollably. These drugs are usually injected through infusion into a vein affecting consequently the whole organism.[12] Modern chemotherapy uses a set of drugs with different mechanisms of action: the most widely known is surely cisplatin that is able to crosslink with purine bases leading to DNA damages, interfering with DNA repairing machinery and therefore inducing apoptosis.[13]

Radiotherapy and hadrontherapy

Another treatment as famous as chemotherapy is radiotherapy; it belongs to the second family of treatments since a highly energetic radiation is sent precisely where cancer cells are located. This technique has high versatility since may be curative of several kind of cancer as stand-alone technique (if it is localized in a specific area of the organism) or can be used in combination with other treatments such as chemotherapy.

The principle under radiotherapy is quite simple: the radiation sent against cancer cells damages DNA leading to cellular death. This damage may be both direct or indirect: the first occurs when the beam damage one or multiple basis building the genetic chain while the latter is a result of water ionization (the most abundant component in cells) leading to the formation of free radicals that damage the DNA.

Almost immediately after the introduction of radiation therapy in 20th century, side effects that this treatment could generate on patients were known. One of the first advances was the fractionation of radiotherapy: the total dose delivered to the patient was divided in smaller amounts, enabling healthy tissues to recover between each irradiation session. Nowadays, this principle is still used, and its efficacy is coupled with improvement of imaging techniques which enable a better localization of the tumor.[11]

Another promising approach is hadrontherapy where charged particles are used instead of photons. With this technique it is possible to limit damages to healthy tissues because of the unique physical and radiobiological properties of these particles; they are able to penetrate tissues, with little diffusion, and deposit the maximum energy just before stopping. This allows the precise definition of the region to be irradiated.

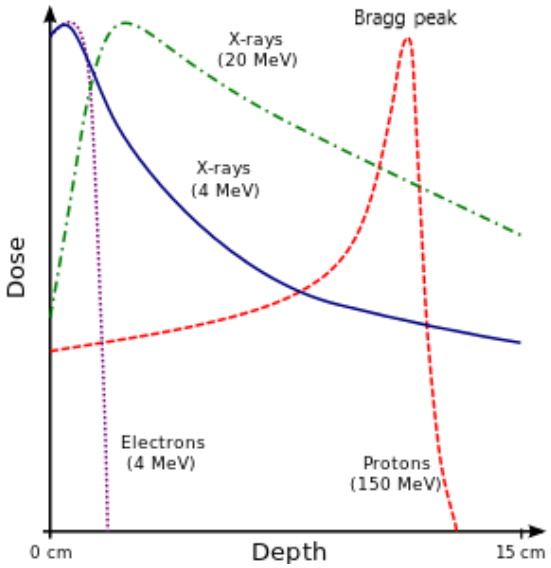

Figure 2.1: Depth-dose profile for electron (purple), X-ray (blue and green) and proton (red) beams. The maximum point for the proton beam is called Bragg peak and represent the depth where the maximum energy deposition occurs. (reproduced from [14])

The peaked shape of the hadron energy deposition is called *Bragg peak* and allows to minimize damages to healthy tissues in the surrounding of the tumor. By finely tuning the kinetic energy of these particles it is possible to precisely irradiate specific positions of the body located at different depths.

The idea to use protons for cancer treatment was first proposed in 1946 by Robert Wilson[15] but the application of this kind of treatment was limited to few parts of the body because accelerators were not powerful enough to penetrate deep in tissues. In the late 1970s improvements in accelerators technology and advances in medical imaging made proton therapy a viable medical option.[10] Nowadays, because of the high cost to obtain protons, hadrontherapy is used to treat cancer located near very sensible and fundamental organs such as brain stem, optic nerve or spinal cord.[11]

Spatial dose distribution

The most remarkable advantage of charged particles therapy over conventional one is the *spatial dose distribution*. X-rays transfer their energy to the tissues along their path with a deposition profile that shows an initial increase within a few centimeters. After that, the beam intensity decreases according to the well-known Lambert-Beer law irradiating tumor cells as well as healthy ones (see ***Figure 2.1***). This side effect may be detrimental to the patient, especially if sensible or fundamental organs are irradiated; it was also shown that conventional radiotherapy may also give rise to new cancerous sites because of the high energy deposited by the beam at the beginning of its path.[13]

To avoid these side effects, the use of charged particles therapy is growing worldwide: as discussed before, interaction of charged particles with the tissues of the body results in a decrease of its velocity. The specific location where the particle stops its path is the one where the highest energy is deposited; therefore, by irradiating the tumor with charged particles with very narrow kinetic energy, it is possible to target only cancer cells, reducing drastically the energy deposition in healthy tissues upstream the tumor and completely sparing tissues downstream it.

Water radiolysis

In a radiobiological context, the absorbing medium in a cell can be considered, in a first approximation, as water. As a photon interacts with a water molecule three main stages take place on different time scales:

- the so-called *physical stage*, which is achieved about 1 fs after the initial matter-ionizing radiation interaction, that consists in energy deposition followed by fast relaxation process. This process leads to the formation of ionized water molecules and sub-excitation electrons;
- the *physico-chemical stage* (from 10^{-15} to 10^{-12} seconds), where several processes take place such as ion-molecule reactions, dissociative relaxation etc;
- in the *chemical stage* (from 10^{-12} to 10^{-6} seconds) species react with each other and with surrounding molecules.

All these stages are summarized in the following image (***Figure 2.2***):

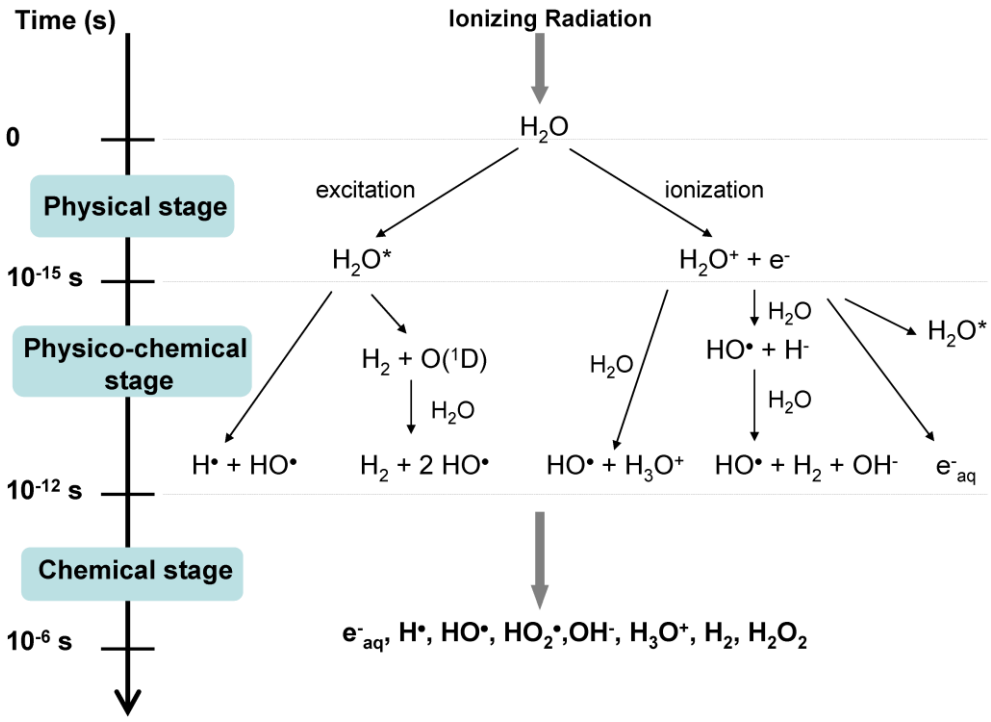

Figure 2.2: Main reactions occurring during the three stages of water radiolysis. (reproduced from [16])

Reactive Oxygen Species (ROS) are produced during the last stage; these species can diffuse in the surrounding and initiate other chemical reactions. Although ROS formation mechanism is not completely understood yet, many species have been identified including hydrogen peroxide (H_2O_2), hydroxyl radical ($OH^{\bullet}$) and dihydrogen (H_2).

Molecules of this family have a double-edged sword role acting as messengers for physiological phenomena but also inducing oxidative damages to the cell when the delicate balance between ROS production and elimination is perturbed.

In mammalian cells ROS are continuously produced as byproducts of various metabolic pathways, localized in different cellular compartments, that are scavenged by specific defense components.[17] Mitochondria are the prime source of endogenous ROS due to its role in ATP production, in which molecular oxygen is reduced to water in the electron transport chain. Moreover, organisms have developed efficient antioxidant machinery composed of enzymatic entities, such as superoxide dismutase (SOD), glutathione reductase (GR), and a non-enzymatic components that work together to scavenge ROS.[18] Furthermore, once these components exert their action reducing ROS to non-reactive molecules, their antioxidant capability is restored by specific enzymes[19] such as:

- *thioredoxin reductase* (TrxR): one of the main thiol-dependent electron donor system in the cell that plays a critical role in the regulation of the cellular redox environment.[20] Three isoforms of Trx have been identified in mammalian cells, each in a specific compartment of the cell with all containing the conserved active site: TrxR1 in the cytoplasm, TrxR2 in the mitochondria and the testis specific thioredoxin glutathione reductase TGR.[21] A more detailed description is presented in section 3;
- *glutathione reductase* (GR): part of an enzyme pool which serves to maintain glutathione in the reduced form. It can exert its action with either NADH or NADPH as hydrogen donor but was shown that only NADPH is effective *in vivo*. The dimeric form of two glutathione molecules (GSSG) is frequently observed (since glutathione is the most abundant thiol present in cells) when ROS donate an electron.[22]

DNA damages and repair mechanisms

DNA consists of two long polynucleotide chains composed of four types of nucleotide units that coil around to form a double helix. Each strand is composed of monomeric units, called nucleotides, that are made by a nitrogen-containing nucleobase (cytosine C, guanine G, thymine T and adenine A), a sugar molecule (2-deoxyribose) and a phosphate group. The 2 strands are joined by hydrogen bonds between the nitrogenous bases according to the well-known base pairing rules (A with T and G with C). The biological function of this structure is to carry genetic information in small DNA sequences called genes.

DNA damages, due to environmental factors and metabolic processes inside the cell, occurs at a rate of 10^4 to 10^6 lesions per day.[23] These can be divided in 2 categories according to the causing agent:

- *endogenous damages* due to the presence of reactive oxygen species resulting from the normal metabolic pathway of the cells;
- *exogenous damages* due to external agents like radiation (from UV to gamma rays of the electromagnetic spectrum), certain toxins or chemicals such as aromatic compounds (that can intercalate between different basis) or viruses.[24]

Once either endogenous or exogenous factors interact with the genetic material within the nucleus, 3 different types of damages can be observed:

- *base damages*, when the nitrogenous base's structure is somehow altered. ROS, that may be produced by radiation, are able to react with unsaturated bonds present in bases leading to new, highly reactive, radical intermediates. In some cases, these reactions can completely remove the nitrogenous base from the backbone, generating apurinic/apyrimidinic sites in DNA.[25]

- *single strand breaks* (SSBs) that are discontinuities in one strand of the double helix. This type of damage may directly arise from the interaction of the genetic material with ionizing radiation or through oxidation by ROS. It has been estimated that more than 10000 SSBs are generated per mammalian cell each day, representing the most common type of DNA lesion.[26]

- *double strand breaks* (DSBs) that occur when both strands of the double helix break within a distance of 10 base pairs. Contrarily to SSBs, where the healthy strand is used as a template to correct the lesions, in DSBs this repair approach is not possible; because of that, the repair mechanism can lead to mutations, loss of heterozygosity and chromosome rearrangements that result in cell death or cancer development. This type of lesion is present in all organisms and may arise as a result of exposure to ionizing radiation.[27]

Ionizing radiation can be considered as a "double-edged sword" since it damages healthy tissues but may also lead to loss of clonogenic survival of tumor cells. In everyday life we experience a lot of DNA lesions[28] due to ROS action that are generated by either endogenous processes and interaction with exogenous agents. To limit the impact these lesions have on their lifecycle, cells have evolved very efficient systems to find and repair these kind of damages and maintain their physiological functions.[29] Ionizing radiation is interesting because the wide range of lesions it can induce, including all the lesions previously described (base damage, single-strand breaks, double-strand breaks, all with a wide range of severity) plus DNA cross links.

When only one of the two strands has a defect, the other strand can be used as a template to correct the mistake; to perform this action, cells have developed several mechanisms where the damaged nucleotide is removed and replaced, with an undamaged one, complementary to the "healthy" DNA strand (that is used as template).[30]

The most important mechanisms are:

- *base excision repair* (BER) that allow to correct small base lesions that do not significantly distort the DNA helix structure (such as deamination, oxidation or methylation). The process is initiated by a specific protein (DNA glycosylase) that recognize and remove the damaged base, leaving an abasic site that is further processed with the undamaged-strand complementary base to complete the BER;[31]

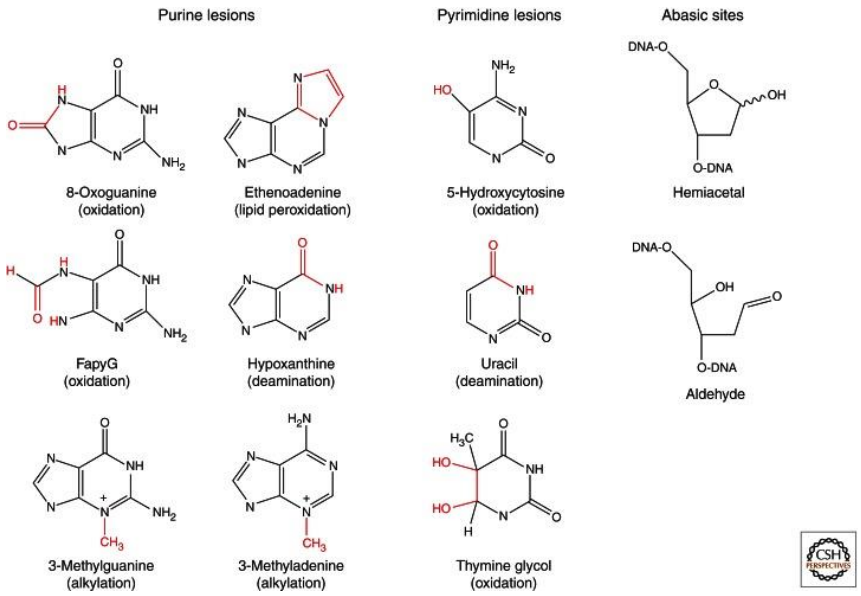

Figure 2.3: Most common lesions and abasic sites. (reproduced from [31])

- *nucleotide excision repair* (NER) is a multistep mechanism consisting in the recognition, removal and re-synthesis of an oligonucleotide sequence. The process starts with the recognition of the damage base and the removal of a 12-13 nucleotides long oligomer (in prokaryotes) and in a 24-32 nucleotides long oligomer in eukaryotes; the sequence is then resynthesized to fill the gap maintaining the complementarity with the undamaged strand and finally ligate to regenerate an intact molecule;[32]

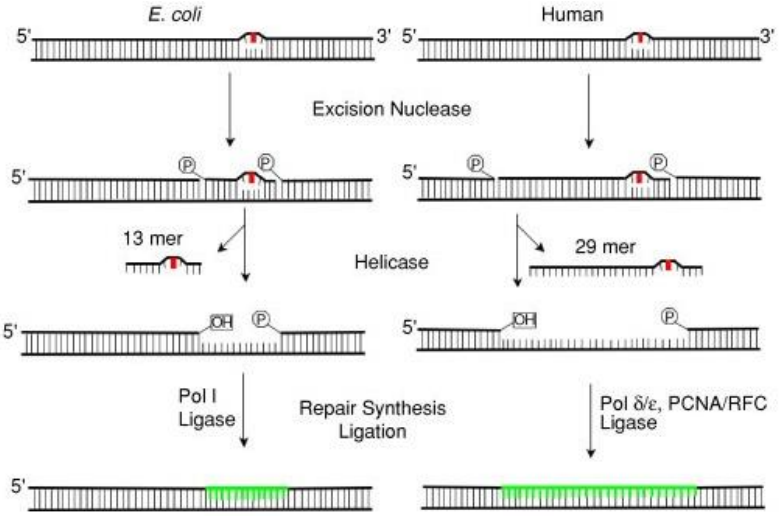

Figure 2.4: Schematic representation of NER in prokaryotic (left) and eukaryotic (right) cells. Is possible to notice that basic steps are conserved: damage recognition and DNA oligonucleotides excision followed by resynthesis and ligation to restore the original oligonucleotide polymeric structure. (reproduced from [32])

- *mismatch repair* (MMR) is a highly conserved mechanism present in prokaryotic and eukaryotic cells. This system allows to repair erroneous insertion of bases during DNA replication; in this process, one of the 2 strands is used as template and nucleotides are inserted according to well-known coupling rules (GC and AT). MMR machinery exerts its action with an array of different protein that are able to recognize the newly synthesized strand, because of the presence of nicks, and direct endonuclease protein toward the mismatch to repair it. [33]

ROS and cell death

ROS is an acronym used to describe a number of reactive molecules and free radicals derived from molecular oxygen. In physiological conditions ROS are indispensable for cell survival, apoptosis and differentiation while high levels of ROS contribute to carcinogenesis and other diseases related to oxidative damage.[34]

There are several sources of ROS coming from both exogenous and endogenous stimuli: as previously stated, the main source of intracellular ROS is the mitochondrial respiratory chain where O_2 is reduced to water in order to produce ATP. During this process, electrons released from the mitochondrial electron transport chain incompletely reduce O_2 to form superoxide such as H_2O_2 (it is estimated that the 1-2 % of the O_2 used is incompletely reduced leading to superoxide species).[35]

The wider plethora of exogenous factors able to induce ROS formation includes UV and ionizing radiation, quinone compounds, chemical found in tobacco smoke, environmental toxins and various pharmaceutical agents. These exogenous radicals are able to rapidly react with O_2 to generate superoxide free radicals or other ROS such as H_2O_2.[36]

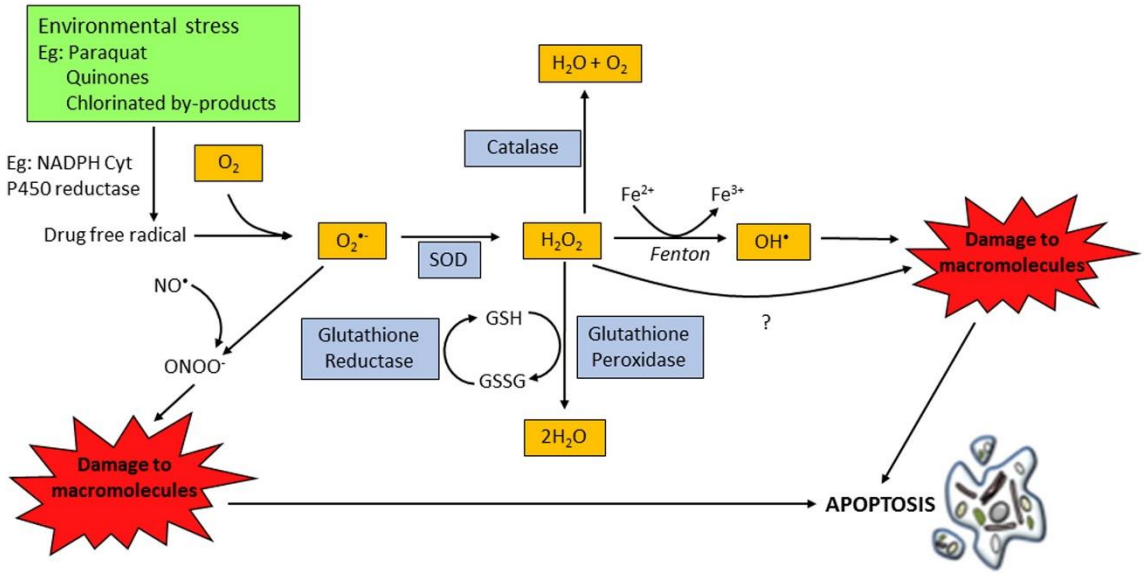

Figure 2.5: Environmental stress generates ROS that cause cellular damage and apoptosis. Toxic compounds (xenobiotics) are able to produce radicals whose rapid reaction with oxygen result in superoxide production; these can react with nitric oxide to produce peroxynitrite ($ONOO^-$) or undergo dismutation to generate H_2O_2, catalyzed by SOD. Hydrogen peroxide can be either detoxified by antioxidants or generate $OH^\bullet$ by the metal-catalysed Fenton reaction. Both $OH^\bullet$ and $ONOO^-$ cause damage cellular proteins, lipids and nucleic acids, which can lead to demise of the cell by apoptosis. (reproduced from [36])

To keep ROS at a low concentration, cells need to maintain an endogenous antioxidant capacity, which acts as a detoxification system, transforming ROS into unreactive molecules by metabolic conversion.[19] This function is performed by several molecules that can be classified into 2 categories: enzymatic (such as superoxide dismutase SOD, catalase CAT, glutathione peroxidase GTPx and so on) and non-enzymatic antioxidants (like vitamin A, C and E, β-Carotene and glutathione).[37]

If, for any reason, antioxidant detoxification systems fail to maintain tolerated levels of ROS, excess cellular levels of these species are observed and oxidative stress is triggered. This condition is characterized by a serious imbalance between ROS generation and antioxidant defenses in favor of the former, causing excessive oxidative damage to proteins, nucleic acids, lipids, membrane and organelles.[38] When this condition is reached, essential biological targets (like mitochondria) are damaged. This may end in mitochondria membrane depolarization that interferes with the electron-transport chain leading to cytoplasmic release of $O_2^{\bullet}$ radicals and dysfunction in ATP production. Furthermore, membrane permeability pores may be opened resulting in the release of pro-apoptotic molecules that may result in apoptosis. The reduced ATP production may also affect those processes that require energy to occur like the DNA repair system or enzymes involved in DNA replication.

Finally, ROS production seems to have a size-dependent behavior with smaller AuNPs (but with higher surface area exposed to the environment) the most efficient in inducing ROS formation.[39]

Section 3: Introduction to the Radiosensitization Effect

In this section, the radiosensitization effect and the agents responsible for it are analyzed. Among them, a particularly detailed description of the enzyme *thioredoxin reductase* (TrxR) and its inhibition is presented since it represents the main topic of this thesis. After a general description of the radiosensitizing agents known until now, a special effort is devoted to describing gold nanoparticles, spanning from their chemical synthesis to their application as radiosensitizers.

Tumor Control Probability TCP

One of the main goals in radiotherapy is to damage cancerous cells minimizing, as much as possible, damages to healthy tissues. At the same time the dose associated to the cure (i.e. eradication of the tumor) is not very different to the dose associated to the development of complications. Because of that, the dose delivered to any patient is carefully controlled and may be different case by case.

These aspects can be posed as an optimization problem where the *tumor control probability* (TCP) is used as parameter to measure the probability to achieve a specific goal – the elimination of all metastatic cells – while the *normal tissue control probability* (NTCP) is used to evaluate the damages induced by radiotherapy on healthy tissues.[40] It follows that the *probability of cure without complication* (PCWC) can be expressed as function of the TCP and NTCP by the formula:

$$PCWC = TCP \cdot (1 - NTCP)$$

All these probabilities are represented by sigmoid functions as shown in ***Figure 3.1***:

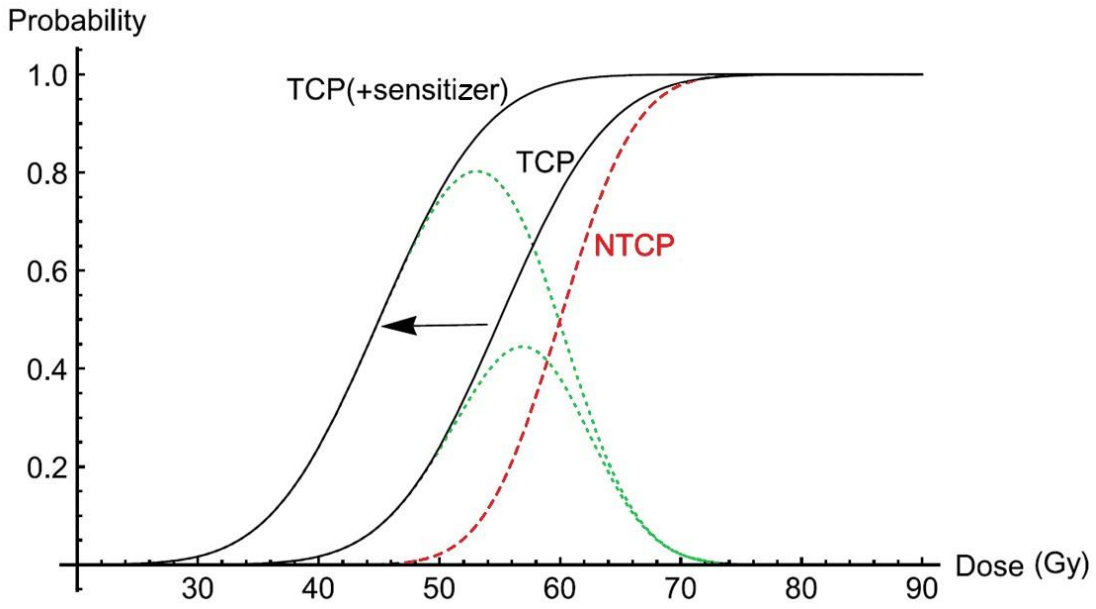

Figure 3.1: Schematic representation showing the TCP (black-solid line) and NTCP (red-dashed line) as a function of the radiation dose; 2 different scenarios are presented, one with TCD_{50} of 55 Gy and a second with TCD_{50} of 45 Gy to show the action of a radiosensitizer targeted to the tumor. In both cases, PCWC function is represented as green-dotted line. (adapted from [41])

The TCP is characterized by the radiation dose required to give a control probability of 50%, and is denoted by TCD_{50}, i.e. tumor control dose 50% (that may be considered as analogous to the lethal dose LD_{50} used to evaluate the effect of a drug on biological systems). When a radiosensitizer is linked to a tumor, its presence in the neoplastic site result in a modest decrease in the TCD_{50} (black-solid line) but in a much more intense increase in PCWC maximum and width (green-dotted line). On a practical point of view, this modification allows a bigger margin of errors during treatment planning.

Although these arguments are very simplistic and ignore many of the complications involved, they very clearly show the potential benefits of targeting radiosensitizers to the tumor. To give a wider view of the topic, the classification proposed by G.E. Adams[42] is now presented. In his pioneering work, he classified radiosensitizers in 6 categories:

- *inhibitors of endogenous radioprotective substances*: ionizing radiation exerts biological effects through ROS production. As previously described, cells exploit antioxidant systems (like the Trx system) to counteract ROS overproduction and keep them at physiological levels. However, molecules such as L-buthionine-SR-sulfoximine, a cysteine analogue, show the ability to interfere with antioxidant biosynthesis, reducing the cell capability to counteract ROS production;

- *molecules that exacerbate DNA damage*: in this category are present those molecules able to enhance DNA damages after irradiation, such as cisplatin or 5-fluorouracyl (5-FU). The former forms both inter- and intrastrand DNA adducts that produce SSBs when removed by DNA mismatch repair processes. Moreover, these breaks can be converted to lethal DSBs by irradiation. 5-FU is a pyrimidine analog that inhibits the biosynthesis of deoxyribonucleotides for DNA replication; its use as radiosensitizer is based on its ability to inhibit the repair of DSBs and enhance radiation-induced DNA damage through the production of reactive uracilyl radiacals and halide ions. However, 5-FU is particularly toxic to dividing tissues and its clinical use is limited by its severe side effects on normal cells.[43]

- *molecules able to inhibit repair processes*: in general, upregulation of DNA damage response (DDR) activity is frequently observed in many cancer cells due to their high replication rate. For this reason, the inhibition of these processes may be a powerful strategy in cancer treatment. Curiously, hyperthermia was the first mechanism investigated for clinical use because it was believed to indiscriminately denature DNA repair proteins.[44] Several drugs are also used with the same purpose such as gemcitabine, a nucleoside analogue, that interferes with nucleotide metabolism leading to DNA repair inhibition. Other molecules, like the novel P13K inhibitor HS-173, showed a marked increase in radiation sensitivity of different kind of cancers. The phosphatidylinositol-4,5-bisphosphate 3-kinase (PI3K) pathway plays a crucial role in cell growth, proliferation, survival and is known to be disrupted in many cancers. Targeting this family of enzymes led to an inhibition of repair response to DSBs and subsequent enhancement of radiation sensitivity.[45]

- *cell-cycle disturber*: to maintain cellular integrity after irradiation multiple pathways are activated, and cell cycle regulation is perhaps the most important. A common cellular response to DNA-damaging agent is the activation of cell cycle checkpoints where several sensor, transducer, and effector genes are involved. In addition, cell cycle phase determines a cell's relative radiosensitivity, with cells being most radiosensitive in the G_2-M phase, less sensitive in the G_1 phase, and least sensitive during the latter part of the S phase. Based on these considerations, multiple drugs were developed with the aim of somehow modify neoplastic cells cycle phase to make them more sensitive toward radiation. One example is paclitaxel, whose cytotoxicity is associated mainly with the binding to β-subunit of microtubulin, leading to block in the G_2-M phase.[46] As result, cells are synchronized in the most radiosensitive phase of the cell cycle.[47]

- *oxygen-mimetic sensitizers*: as tumor growth outstrips the ability of the surrounding vasculature to supply blood and nutrients to new cells, necrotic tissues are gradually formed, more frequently in the inner area, because of the very low oxygenation level. Due to this lack of oxygen, ROS production is limited in these cells and, therefore, the radiosensitization effect is much weaker. Based on this model, where oxygen delivery is compromised by limited diffusion inside tumors, early approaches to improve oxygen status of tumors involved administration of hyperbaric oxygen. Even though this treatment increased the oxygen diffusion distance, reducing chronic hypoxia, this modality did not address acute or anemic hypoxia.
  To solve this problem, oxygen mimetic molecules were developed. These molecules possess the same chemical properties of molecular oxygen with better diffusion properties into anoxic tissue. Moreover, unlike oxygen, which is rapidly consumed by respiring cells, these agents are less rapidly metabolized by tumors enabling better diffusion and penetration into hypoxic regions.[48] One of the most studied class of oxygen mimetics is that of the nitroimidazoles: these agents have no intrinsic activity but their effect becomes evident in the presence of ionizing radiation to "fix" or stabilize DNA radical lesions in an oxygen-deficient cell.[49]

- *Small Interfering RNA* (siRNA) technology: siRNA is a class of double-stranded non-coding RNA molecules, typically 20-27 base pairs long, able to bind and degrade complementary mRNA strands immediately after transcription. In this way, specific enzymes expression is downregulated by preventing translation. One example belonging to this class of agents is given by GLUT-1 inhibition. As previously described, GLUT-1 is expressed at high levels in many cancer types; because of that, Zhou *et al*. [50] inhibited this enzyme's expression by transfecting $CD133^+$HEp-2R cells with GLUT-1 siRNA, observing a irradiation dose-dependent reduction in proliferation, colony forming efficiency and invasive capability.

This classification was based on the mechanism of DNA damage and repair; with the development of nanotechnology, nanomaterials possessing good radiosensitizing effects and metabolic properties are appearing. Among them, any high Z nanoparticles represent a very good candidate for this approach to improve cancer therapy; more specifically, gold (Z = 79) received the great majority of attention because of its high biocompatibility and easy functionalization.[41]

A focus on thioredoxin reductase TrxR

In order to maintain the endogenous antioxidant power, several enzymes are assigned to regenerate the antioxidant pool once it exerts its action. One of these enzymes, called thioredoxin reductase TrxR, is the main topic of this thesis.

The mammalian thioredoxin reductase (TrxR) is a selenium-containing pyridine nucleotide-disulphide oxidoreductase with a conserved -Cys-Val-Asn-Val-Gly-Cys- active site. TrxR is the only enzyme able to catalyze the NADPH-dependent reduction of the redox protein thioredoxin (Trx) and, therefore, it is involved in all those pathways in which thioredoxin acts as reducing substrate.[51] Thioredoxin reductase, together with thioredoxin and NADPH, is part of a bigger system called thioredoxin system. Beside Trx, TrxR can directly reduce other substrates, such as peroxides (including lipid hydroperoxides), hydrogen peroxides, and protein disulfide isomerases, which participate in the posttranslational folding and processing of cellular proteins.[52] Moreover, it participates in the regeneration of some antioxidant molecules with antioxidant activity such as dehydroascorbate[53], lipoic acid[54], and ubiquinone[55].

From the structural point of view, 3 isoforms of TrxR can be found in mammalian cells: TrxR1 and TrxR2, with the former located in the cytosol and nucleus, whereas the latter is mainly localized within mitochondria. The third isomer, named TrxR3 (or Thioredoxin Glutathione Reductase TGR), is produced only by specialized tissues (such as testis).[56] TrxR1 and TrxR2 have similar overall structure and share the same catalytic mechanism catalyzing the NADPH-dependent reduction of oxidized Trx.[57]

All isoforms contain selenium in the form of selenocysteine, the naturally occurring selenium analogue of cysteine. The residue is located at the C-terminus, within a tetrapeptide motif (-Gly-Cys-SEC-Gly-) conserved between species.[58]

The most important functions of thioredoxin reductase are summarized in the following picture:

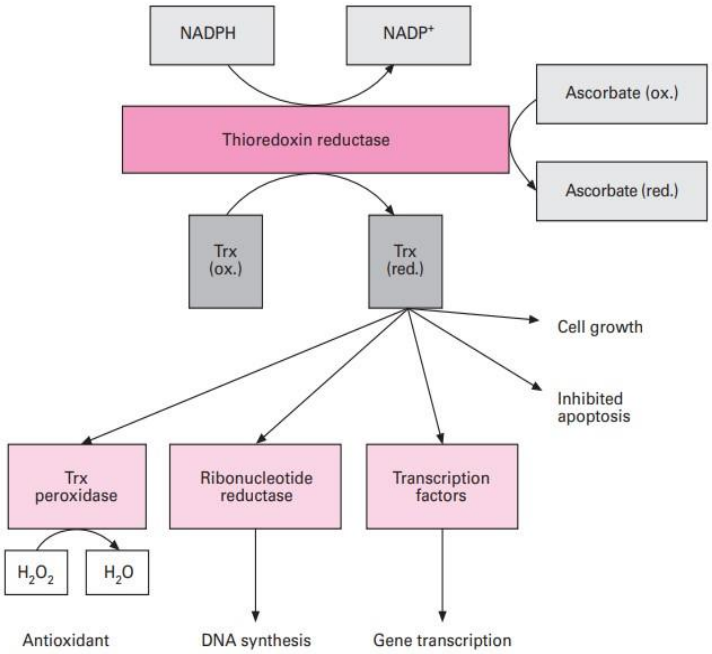

Figure 3.2: Reactions and functions of TrxR in the cell (from [51]).

More interestingly, elevated TrxR levels were found in human tumor cell and induced murine tumors.[53,59] If, in normal cell lines, TrxR is fundamental to maintain redox homeostasis and protect against oxidative stress, in malignant cells it supports tumor growth and progression. In a study published in 2006, Hatfield *et al.* [60] exploited RNA interference technology to confirm the involvement of TrxR in murine tumor growth: the TrxR was downregulated resulting in a drastic decrease of tumor progression. This was the first *in vivo* investigation where the tumorigenic activity of TrxR was shown; it also confirmed this enzyme to be an excellent target for cancer therapy.

Additionally, cancer cells usually display higher ROS level, compared to healthy cells, because of their uncontrolled proliferation and high metabolism rate. To maintain the redox balance, cancer cells upregulate the antioxidant system to counteract the increase of ROS levels (that is essential for maintaining tumor phenotypes, but also renders cancer cells vulnerable to oxidative stress). The modification of the redox environment may be thought as the "Achilles' heel" of cancer cells and could be exploited as target for cancer therapy. Therefore, there is an increasing interest in modulating cellular redox signaling, especially the Trx system, as a potential strategy for cancer treatment.

Since Trx system inhibition was shown to improve therapeutic potency of many anticancer treatment where high levels of ROS are generated (such as chemotherapy and radiotherapy), agents able to decrease TrxR efficiency seem very promising.[61]

To achieve this goal, the first strategy is to focus on the selenocysteine residue located on the flexible C-terminal arm of this enzyme. To date, several drugs have been tested as Trx system inhibitors; one of these is auranofin, an anti-arthritis drug approved by FDA in 1985, that is being investigated for potential therapeutic application in other diseases such as cancer and neurodegenerative disorders. In his work, Wang *et al*. [62] showed a dose-dependent TrxR inhibition, ROS overproduction and mitochondrial damage associated with this drug. Although its ability to counter cancer, its use is associated to cytotoxicity (IC_{50} value of 19 and 11μM for 4T1 and EMT6 cell lines respectively).

Therefore, less toxic alternatives are required and this is why AuNPs are tested for this application.

Thioredoxin reductase inhibitors: state of art

Over the last years an increasing number of research groups became engaged in the discovery and development of compounds acting on thioredoxin reductase. Several drugs were proven to inhibit TrxR functions, such as gold-based, silver-based and platinum-based compounds.[63] What they have in common is the inhibition mechanism by binding to the catalytic selenocysteine residue.[19]

In her work, Becker *et al.* [64] observed a direct link between TrxR gene expression and the onset of prostate cancer, showing the main role of this enzyme in tumor proliferation. Therefore, a lot of effort is put to find new drugs able to target this enzyme in order to slow down tumor proliferation.

Interestingly, this is not the only reason to choose TrxR as molecular target: it was observed that elevated TrxR levels are associated with chemotherapy resistance, hence TrxR-targeting may contribute to prevent or reverse this resistance mechanism.

By considering the fundamental role exerted by TrxR in tumor proliferation, a brief overview of the most recent data obtained about thioredoxin inhibition is presented.

Gold-containing drugs have been validated as potential TrxR inhibitors *in vitro* in the nanomolar range. Gold is known for its high affinity toward thiols, rendering the nucleophilic selenolate of reduced TrxR the prime target site or modification by this metal. As previously reported, auranofin - (PEt_3)Au(I)thioglucose - was used to treat leukemia on mice because of its ability to induce ROS overproduction and damages to mitochondria. Au(I) is also able to inhibit glutathione reductase's function, even though less efficiently that TrxR. This may be due to the high affinity of gold for the selenocysteine residue present in the active site of TrxR, but lacking in GR.

In addition to gold (I) other gold compounds, with different oxidation state, have recently been evaluated and confirmed to be able to inhibit TrxR. In such sense, gold (III) compounds are emerging as a new class of metal complexes with outstanding cytotoxic properties and are being evaluated as antitumor agents.[65] Gold (III) di-thio-carbamate derivates were shown to be able to inhibit DNA and RNA synthesis and were active against cisplatin-resistant leukemia cell lines. Other studies proved their ability to inhibit TrxR action inducing better antiproliferative effect and higher apoptosis rates.[66]

Platinum compounds belong to a group of metal complexes with promising TrxR-inhibition properties. These compounds are suggested to exert their function by intercalating between DNA bases. Cis-di-ammine-di-chloro-platinum (CDDP) complexes of nitrofuran have been shown to possess promising *in vitro* antitumor and anti-TrxR activity.[67] These Pt compounds are also able to inhibit glutathione reductase in parallel, although less efficiently.

Pt (II) compound, such as terpyridineplatinum(II) complexes, are able to strongly bind nucleophiles like protein thiols like those present in TrxR's active site.[68]

At the same time, Pt (IV) complexes are being tested for oral anticancer therapy as they combine a rapid reaction with their target with a greater inertness than Pt (II) complexes leading to reduced toxicity. Several studies also showed their ability to induce ROS overproduction resulting in necrotic tumor cell death. [69]

Additional TrxR inhibitors are tellurides compounds as they possess a growth inhibition capacity in the submicromolar range. A similar action was observed with organotellurium and organoselenium compounds that provided interesting properties related to TrxR-driven apoptosis in tumor cells. However, the mechanism(s) behind of TrxR inhibition is not yet elucidated although multiple hypothesis have been proposed.[70]

Nitroaromatic compounds, such as 2,4-di-nitro-chloro-benzene (DNCB), are able to derivatize the C-terminal selenocysteine preventing TrxR functions. A series of other nitroaromatic compounds can be found within this group, all of them with a striking activity against mammalian TrxR and apoptosis-provoking effects in human cancer cells most likely due to inactivation of cellular TrxR.[71]

The last group of inhibitors is composed by natural occurring and widely consumed compounds like polyphenols that have recently been identified to be efficient oxidative stress-inducing with potential antitumor activity in multiple cancer cell lines.[72]

Nanoparticles: from chemical synthesis to biomedical applications

Nanoparticles (NPs) in both crystalline and amorphous forms received many attentions worldwide for their uses in many commercial applications. In comparison to either small molecules or bulk materials, nanoscale structures possess particular, intrinsic reactivity as a result of increased surface area to volume ratio. Among the different types of nanomaterials, metal NPs, especially AuNPs, have attracted huge interests from different fields of science, due to their peculiar features: high X-ray absorption coefficient, ease of synthetic manipulation, enabling precise control over the particle's physico-chemical properties[73], strong binding affinity to thiols, unique tunable optical and distinct electronic properties.[74]

Based on dimension, nanoparticles can be classified in:

- *one-dimensional NPs*, like nanorods, nanowires, nanotubes and nanobelts;
- *two-dimensional NPs*, with the family of nanoplates such as stars, pentagons, rectangles, hexagons etc;
- *three-dimensional NPs*, like nanospheres and nanopods.

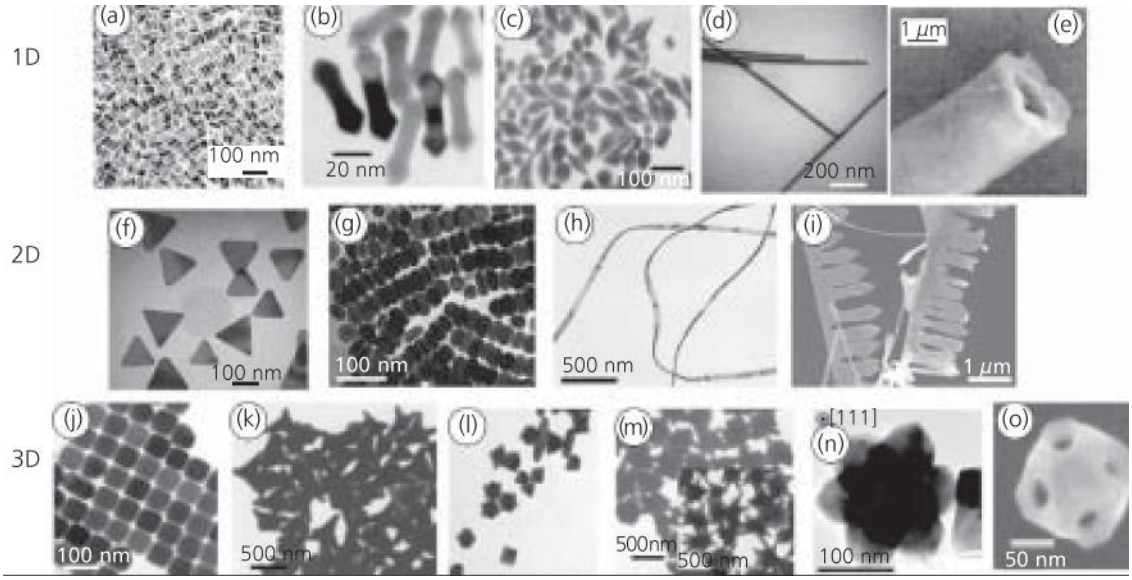

Figure 3.3: TEM and SEM images of one-, two- and three-dimensional noble metal nanoparticles: (a) nanorods; (b) nanoshuttles; (c) nanobipyramids; (d) nanowires; (e) nanotubule; (f) triangular nanoplates; (g) nanodiscs; (h) nanoribbons; (i) nanobelts; (j) nanocubes; (k) nanotetrapods; (l) and (m) star-shaped nanoparticles; (n) nanohexagon; (o) nanocage. (reproduced from [75])

Regardless this classification, AuNPs are becoming a milestone in biotechnology because they display shape-dependent optoelectronic properties, large surface-to-volume ratio, excellent biocompatibility and low toxicity. Their most known feature, the surface plasmon resonance SPR, and all their physical properties depend on their morphology and physiology.

In the biomedical field AuNPs are exploited for multiple applications like photodynamic therapy (PDT) (where apoptosis or necrosis is induced in tumor cells by highly-active free radicals) or photothermal therapy (with AuNPs generating heat, used to kill malignant tumor cells, after absorption of light in the visible or near IR).

Another very promising application consists in using AuNPs as nanocarrier in drug delivery systems (DDSs) because of their ability to transfer various drugs (such as peptides[76], proteins[77], etc.) throughout the whole organism.[78] PEGylation of the NPs still remains a fundamental step to avoid recognition of the immune system and to provide a better pharmacokinetic profile. This occurs because the PEG layer forms a hydrophilic barrier on the surface of NPs and blocks receptor interaction via steric hindrance.[79]

When nanoparticles, or more generally nanotechnologies, are applied in health science, is more appropriate to talk about nanomedicine. In this field, nanoobjects are employed for multiple applications because of their high surface-to-volume ratio, physico-chemical characteristics and the ability to modulate or improve the pharmacokinetics and pharmacodynamics profile of drugs. Moreover, nanomedicine compounds can alter the biodistribution of drugs by allowing them to accumulate preferably at the tumor site, the so-called enhanced permeation and retention (EPR) effect, that will be treated more specifically at the end of this paragraph. In addition to EPR, many other key points promote the use of nanotechnologies as nanomedical agents.

First, nanoparticles may help to overcome solubility problems and chemical stability of anti-cancer drugs. Indeed, poor water solubility limits the bioavailability of a compound and may hamper the development of anti-cancer agents identified during early drug screens.[80] One example is wortmannin, a P13K and radiosensitizer, whose development was stopped because of poor solubility and chemical instability; trough a lipid-based nanocarrier system, its solubility was increased from $4\,\frac{mg}{L}$ to $20\,\frac{g}{L}$ while increasing its *in-vivo* stability.[81]

Second, nanocarrier can protect anti-cancer drugs from biodegradation influencing the pharmacokinetics profile of a compound. For example, enzymatically-cleaved drugs (like siRNA by RNAses in the plasma or proteins by pepsin/trypsin in the stomach) can be prevented from being degraded by enzymes. One possibility to achieve this goal is the encapsulation of these drugs within liposomes.

Third, nanotechnology can improve distribution and targeting of anti-cancer medication. Distribution of anti-cancer drugs is defined by their physico-chemical properties and is limited by drug penetration into tumor tissue; however, nanomedicine compounds can be constructed with the aim to improve drug penetration and to redirect chemotherapy or targeted compounds selectively to tumor cells or cells of the stromal compartment.

Fourth, nanocarriers can be designed to release their payload upon a trigger resulting in stimuli-sensitive nanomedicine therapeutics. For example, drugs whose delivery is not primarily pH-dependent, such as doxorubicin, can be conjugated with a pH-sensitive NPs to increase cellular drug uptake and intracellular drug release.[82]

Finally, targeted nanomedicine therapeutics may decrease resistance of tumors against anti-cancer drugs; indeed, these may enhance the circulation time of a compound and mediate stimuli-responsive drug release as well as endocytic drug uptake, leading to a reduced resistance of tumor cells against targeted nanocarriers.

Anyway, the greatest breakthrough leading to a more general targeted anti-tumor therapy was the discovered of the EPR effect, firstly reported by Matsumura and Maeda in 1986[83] and subsequently described in greater detail by Maeda *et al.*[84-86] Their investigation showed that most solid tumors have blood vessels with defective structure and usually produce extensive amounts of various vascular permeability factors. The great majority of tumors exhibit enhanced vascular permeability, which ensure a sufficient supply of nutrients and oxygen to tumor tissues rapid growth. As result, macromolecules larger than 40 kDa selectively leak out from tumor vessels and accumulate in tumor tissues.

This effect becomes extremely relevant when considered with another characteristic of tumor tissues, the lack of effective lymphatic drainage.[83] In normal tissues, the lymphatic system's role is the recovery of macromolecules and lipid particles from the interstitial space. In cancerous tissues this recovery occurs much slower, enabling these molecules to stay there for longer time.[84]

The great majority of clinically available nanocarrier-based cancer therapeutics are passively targeted first-generation drugs. By passive targeting is meant the nanocarriers ability to travel down a tumor's vasculature system and, because of the EPR effect, accumulate in its surrounding. As more carriers undergo this faith, large amounts of drug accumulate at the tumor site. The first two generations of nanomedicine drugs exploit this mechanism to exert their biological function.

The so-called first generation of nanomedicine drugs mainly rely on controlling the pharmacokinetics and biodistribution of a compound by modulating its physico-chemical properties.[87] Because of the EPR effect, accumulation of nanomedicine therapeutics in tumor's location occurs without any specific ligand attached to the surface of the nanocarrier. However, many problems were associated with this approach: passive targeting, based exclusively on the EPR, is not sufficient to control the side effects of cytotoxic drugs and fully exploit the benefits of targeted delivery. The heterogeneity of the tumor and its stroma, such as a hypoxic gradient, can severely impact on the efficacy of drugs delivered and passive targeting may result in a reduced transportation of the compound in the tumor.[88] Similarly, increased interstitial pressure and the extracellular matrix of certain malignancies may limit nanomedicine access to the tumor.[89,90] Finally, passive targeting may result in nanodrug accumulation in other organs with fenestrated endothelium such as the liver and spleen.[91] One example of first-generation nanomedicine drug is PEGylated liposomal Doxil®, the first FDA-approved nanodrug. Because of EPR effect, Doxil is passively targeted to tumor and its doxorubicin is released and becomes available to tumor cells, even if the mechanism behind it still remains unknown.[92]

The following drugs generation, the second-generation nanomedicine compounds, are based on drug-delivery technology with an active targeting vector or smart nanocarriers with stimuli-responsive properties. The principle behind these nanocarriers is the active targeting, that enables an extremely high affinity between a ligand, attached to nanocarriers surface, and its cognate receptor on the targeted cells.[93] Several drugs belonging to this generation have been developed at a preclinical level, and only a few have entered early clinical testing, even though none of them has been approved for commercial use yet. One example of second-generation nanodrug is CT-2103, a novel conjugate of paclitaxel, used to treat recurrent ovarian, fallopian and peritoneal cancer.[93]

The last step is represented by the nanocarriers of the third-generation: they can be described as multifunctional systems able to offer new degrees of particle sophistication and an improved probability to localize therapeutic payloads at the disease site. These systems perform simultaneously multiple functions, such as bio-recognition, protection from degradation, avoidance of toxicity and overcoming bio-barriers. This ability is given by the employment of multiple nanoscale-based products that synergistically provide distinct functionalities.[94,95] As example, Ferrari *et al.* [96] have recently developed a multi-stage technology platform able to deliver drug-loaded nanoparticles to the disease's site resolving many of the previously described problems. The multi-stage drug-delivery system is made by silicon microparticles specifically designed to exhibit improved margination and adhesion properties to the tumor site.

The aforementioned concepts are summarized in ***Figure 3.4***, where the classification of nanocarriers into the three generations is based on their purpose and intended functions.

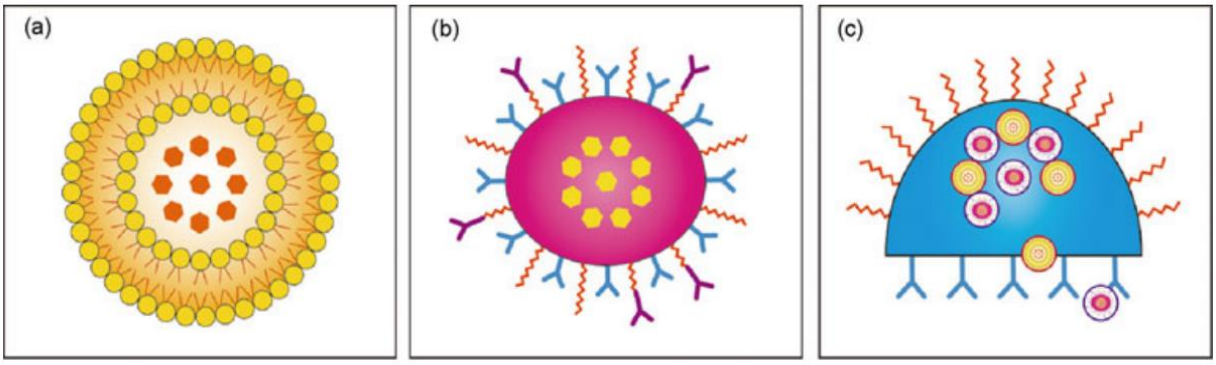

Figure 3.4: Hierarchy of nanocarriers. (a) first-generation nanocarriers (such as liposomes) whose primary role is to enclose therapeutic or diagnostic agents and then localize in tumor by the EPR effect. (b) second-generation nanocarriers improved by incorporating further modification allowing for specific targeting via antibodies or other recognition biomolecules or "stealthing" from MPS sequestration. (c) third-generation nanocarriers advanced the field by creating platforms capable of incorporating and preforming multiple complex functions due to their nanoscale features. (adapted from [97])

High Z NPs as radiosensitizer

Radiotherapy is one of the most applied treatment strategies for effectively controlling and eradicating unresectable parts of tumor in current clinics and is frequently combined with chemotherapy and surgical therapy. Whit respect to killing cancer cells, it is much unquestionable to need a high-energy dose of ionizing radiation that causes severe damage to adjacent healthy tissues. At the same time, reducing the radiation dose or increasing the radiation time may result in the emergence of radiation resistance of cancer cells; this leads irreversibly to a decrease in radiotherapeutic efficiency and, ultimately, to the failure of radiotherapy.[97] To overcome these limitations, radiosensitizers are nowadays frequently used in clinical treatment. Physical toxicity and severe side effects generated by these drugs cannot be tolerated by patients. Therefore, radiosensitizers with (I) good biocompatibility, (II) enhanced tumor accumulation and retention and (III) rapid renal clearance need to be developed as soon as possible.[98]

By definition, radiosensitizers are classified as those agents that increase cytotoxicity of ionizing radiation. They may be further classified as *true* – if meet the strict criterion of being non-toxic by themselves but acting only as potentiators of radiation toxicity – or *apparent* – when their action is due to a combination of potentiators of radiation toxicity and toxicity carried by the agent itself.[99] The mechanisms lying behind radiosensitization are not completely understood yet: however, it is possible to state that the increase in radiotherapy efficiency is due to the cooperative action of multiple contributions, all occurring simultaneously. In this field, high atomic number (Z) nanoparticles have drawn the attention of many scientist because of their ability to enhance radiotherapy efficacy and ease of synthesis.

Ionizing radiation is known to generate ROS (such as $HO^{\bullet}$, $O_2^{\bullet}$ and H_2O_2) through water radiolysis; *Geng et al.* [100] showed that glucose bound AuNPs Glu-AuNPs enhance the production of intracellular ROS when irradiated with 90 kVp or 6 MV X-rays in SKOV-3 human ovarian cancer cells. Xu *et al.*[101] found that irradiation of A375 melanoma cells, tagged with AuNPs, induced a range of cell line-specific responses, including decreased clonogenic survival and increased apoptosis.

The physical reason behind this phenomenon must be searched in the different number of electrons belonging to the species involved in the irradiation. If no sensitizers are used, water is the radiation recipient and the Compton effect is the most probable mechanism by which this interaction can occur, with the incident photon transferring part of its energy to a water electron. Moreover, the direction of the photon is deviated from its original path and the electron may be ejected from the atom if enough energy is absorbed. When high Z nanoparticles are introduced, the total absorption cross-section is larger (due to the higher number of electrons per atom) and this leads to a higher energy absorption per unit mass when radiation pass through high Z-NPs than water. Ultimately, an increase in local dose is observed even when small amount of metal NPs are introduced.

Similarly, this very same treatment can be extended to charged particles used in hadrontherapy. In this case, interaction between protons and high Z materials leads to a higher stopping power than with water, translating into a higher energy deposition per unit length.

In both cases, the increase in local dose deposition enables electron emission from NPs which subsequently deposit their energy in the surrounding medium leading to extra H_2O ionization and ROS overproduction (that is known to increase DNA damages and subsequently inducing cell death). This cascade of events highlights that high Z NPs can play the role of radiosensitizer through a physical enhancement mechanism.

To somehow quantify the radiosensitization effect carried by all kinds of radiosensitizers an arbitrary unit – the dose enhancement units (DEU) – was introduced; an enhancement of 1 DEU means that dose delivered double when these are used. Focusing on AuNPs, several studies highlighted that this enhancement was significant with low-energy beams (20 – 30 keV)[102] while negligible with high-energy beams (>500 keV).[103,104]

However, a lot of studies agrees to state that the physical enhancement is only a part of the entire radiosensitization effect: Roa *et al.* [105] investigated the radiosensitization effect of AuNPs in prostate cancer cells, predicting a physical enhancement of 0.07 DEU based on their uptake data after irradiation with a 2 MeV X-rays. Surprisingly, they reported an experimental enhancement between 0.5 and 1.0 DEU, meaning that the physical contribution cannot be the only one behind the whole effect.

The same considerations can be extended to hadrontherapy, where charged particles are used: Heuskin *et al.* [106] demonstrated that the interaction probability of AuNPs with the incident beam is very low when protons are considered. They estimated that only a small fraction (between the 0.0001% and 0.001%) of the total NPs content interacts with the radiation.

Therefore, new mechanisms need to be proposed to explain the AuNPs-induced radiosensitization process; in this sense, the involvement of thioredoxin reductase TrxR seems to be the most likely.

AuNPs in cancer treatment

One of the most employed high Z NPs are gold nanoparticles (AuNPs) because of their very interesting properties such as good biocompatibility, very narrow size distribution for a wide range of diameters between 1 – 400 nm and ease in surface functionalization. Their versatility lead to applications in several fields such as drug carrier[107] or radiosensitizer in cancer treatment, colorimetric sensor[108] in food industry or as electric and electrochemical sensor[109] to detect contaminants in water and as catalyst for many chemical reactions. ***Figure 3.5*** try to summarize the great versatility of these nanoparticles, which can be synthetized with very different shapes and dimensions; moreover, the easy functionalization of their surface and the possibility to modify their coating enable their utilization for a wide plethora of applications.

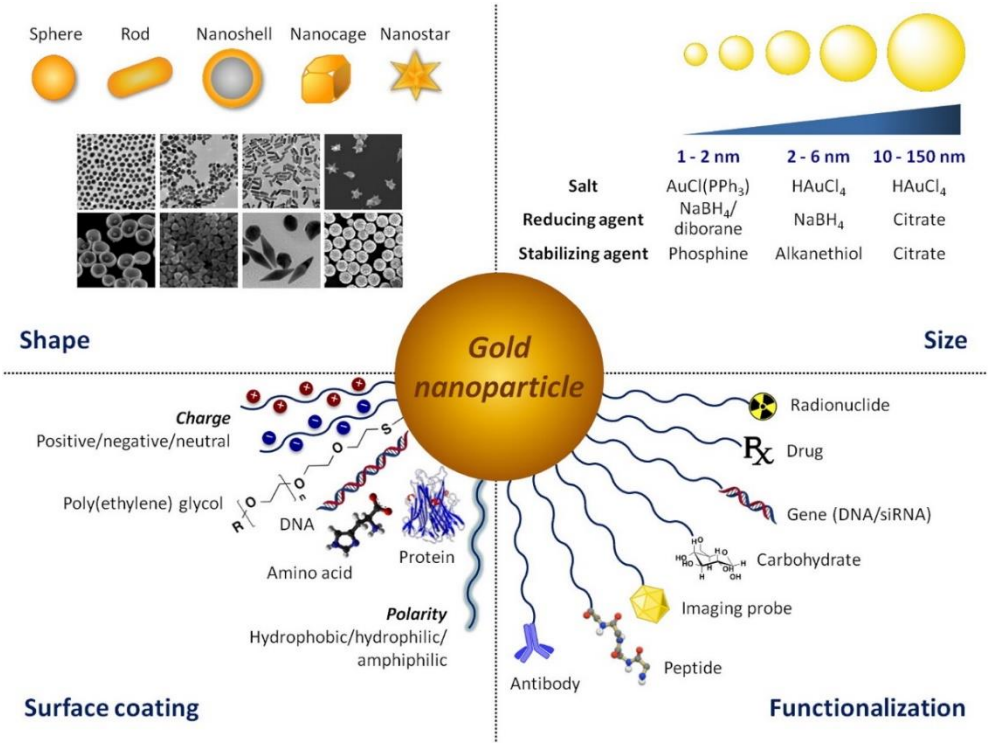

Figure 3.5: The synthetic versatility of AuNPs. AuNPs offer a unique platform for straightforward manipulation of particle size, shape, surface coating and functionalization, enabling fine-tuning of particle properties.[110]

Several groups documented this radiosensitization effect: Penninckx *et al.*[111] irradiated different cancer cell lines using 225 kVp X-rays with and without incubation with AuNPs for 24 hours. What resulted was a cell type-dependent resistance against radiation but with all the lines sharing a decrease in survival fraction when pre-incubated with AuNPs.

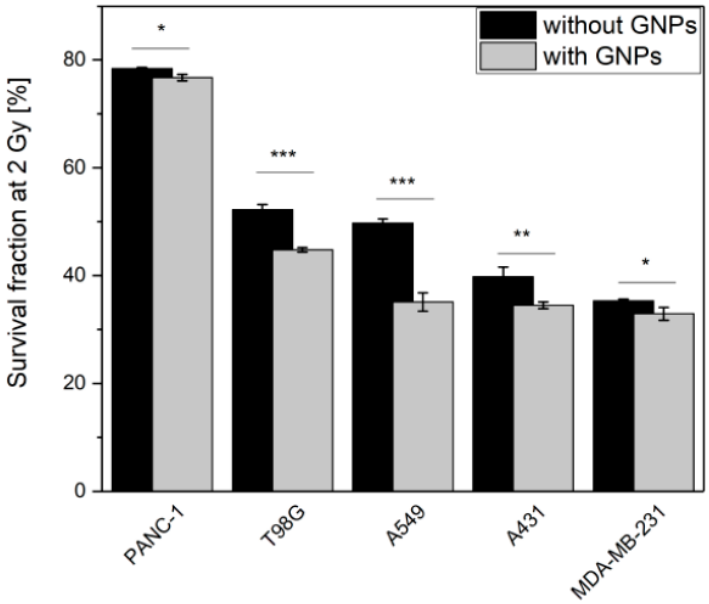

Figure 3.6: Survival fraction of different cell lines with and without incubation with AuNPs after irradiation with 225kVp X-rays. (reproduced from [111])

In a study conducted by Misawa *et al.* [112] the radiosensitizing effect of AuNPs (with a size between 5 – 250 nm) was investigated under X-ray irradiation. The result revealed that the ROS could be induced and enhance by AuNPs with smaller sizes under this condition. In another study, performed by Liu *et al.* [113] survival rates of EMT-6 (mammary carcinoma cell line) and CT26 (colorectal carcinoma cell line) were investigated in the presence of PEG-coated 6 nm AuNPs under exposure doses up to 10 Gy. Cell survival rates were found to be lessened as a function of the radiation dose and the concentration of NPs. Devika *et al.* [114] studied the *in-vivo* radiosensitization effect of AuNPs ranging in size from 14 to 74 nm on HeLa cells. Radiosensitization was largely dependent on the number of internalized AuNPs into the cells and their size. Moreover, AuNPs of 50 nm displayed the highest cellular uptake and induced the greatest radiosensitization factor compared with the smallest or bigger counterparts.

Local Surface Plasmon Resonance LSPR

When matter is reduced from bulk to nanometric scale new properties emerge, including optical, magnetic, electronic, and structural properties, making nanosized nanoparticles very promising for a wide range of biomedical applications. Plasmonic (noble metal) nanoparticles distinguish themselves from other NPs (semiconductor quantum dots, magnetic and polymeric nanoparticles) by their unique and peculiar localized surface plasmon resonance, LSPR.[115] This phenomenon occurs when conduction electrons on the metal surface undergo a collective oscillation when are excited by light at specific wavelengths.

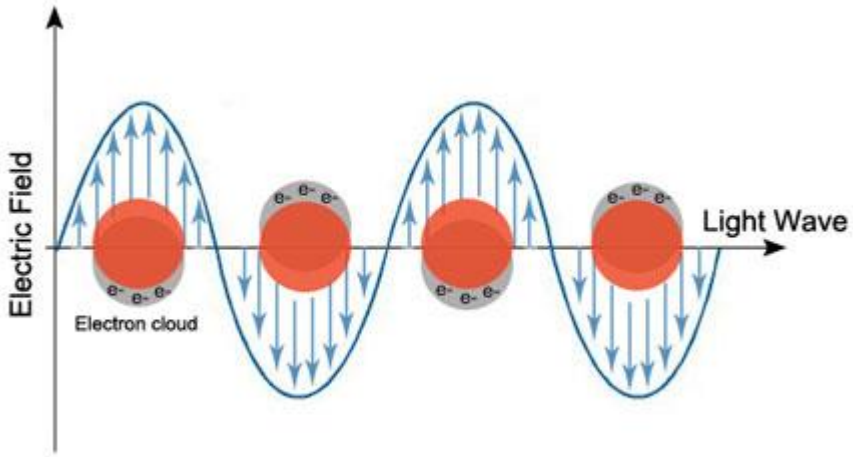

Figure 3.7: Schematic representation of the collective oscillation of valence electrons once surface atoms of a noble metal NPs are excited with an appropriate radiation.

The amplitude of the oscillation reaches maximum at a specific frequency, called surface plasmon resonance, causes a strong absorption of the incident light that is frequently detected by many analytical techniques. This because band intensity and position depend on several factors affecting the electron charge density on the particle surface such as metal type, particle size, shape, structure, refractive index on the particle surface, dielectric constant of the material. Gold nanoparticles of 10 nm size typically show a SPR band centered at 520 nm, in the visible region.[116] But this optical phenomenon is strongly affected by the particle size as shown by ***Figure 3.8*** where a red shift is observed for bigger NPs.

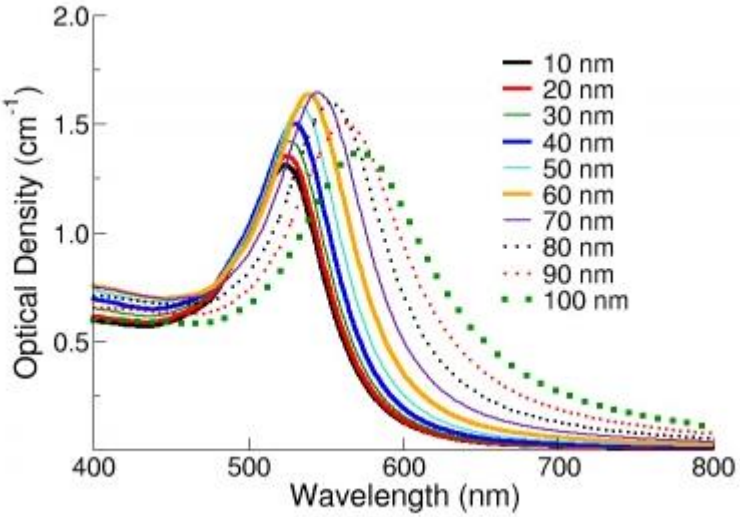

Figure 3.8: Dependence of surface plasmon resonance on spherical nanoparticle size.

The optical properties of gold nanoparticles change when particles aggregate: when this occurs, SPR shifts to lower energies, causing the absorption and scattering peaks to red shift to longer wavelengths.

The shape of the nanoparticle is another key parameter affecting surface plasmon resonance. By considering rods instead of spheres, the SPR band is split in two bands: a strong band in NIR region corresponding to electron oscillation along the long axis, the longitudinal band, and a weaker one, centered in the visible region, usually called reverse band.

While the latter is insensitive to the size changes, the longitudinal band is red shifted from the visible to NIR region with increasing the so-called aspect ratio (defined as the ratio between length and width of the nanoparticle) that can be precisely controlled by changing the experimental parameters of the synthesis reaction (***Figure 3.9***).

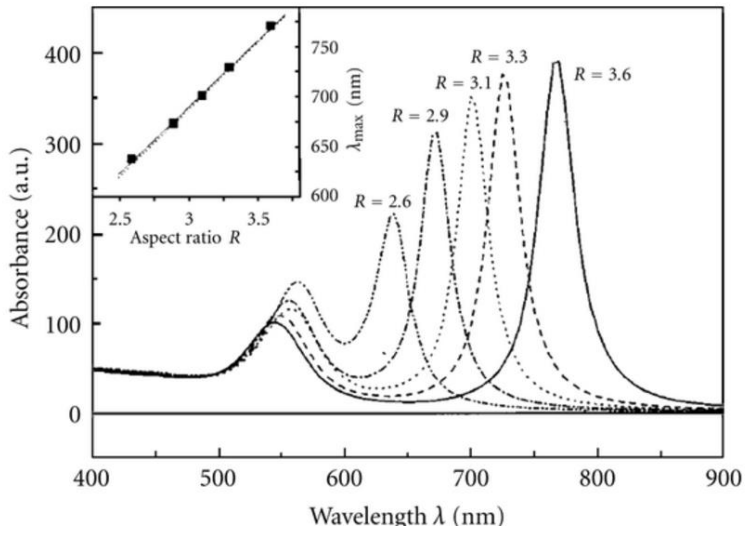

Figure 3.9: Dependence of SPR on the aspect ratio for gold nanorods. (reproduced from [117])

Finally, modification of the nanoparticle structure can lead to similar variations as seen in nanoshells and nanocages.[116]

The former can be composed by a silica core and a thin shell of gold about few nanometers; in this case the SPR wavelength can be easily controlled by changing the shell thickness.

Gold nanocages are a type of hallow and porous nanostructure formed by a galvanic replacement between silver nanocubes and auric acid in aqueous solution. In this case by controlling the amount of auric acid solution is possible to move the SPR maximum wavelength at lower or higher wavelengths.[116]

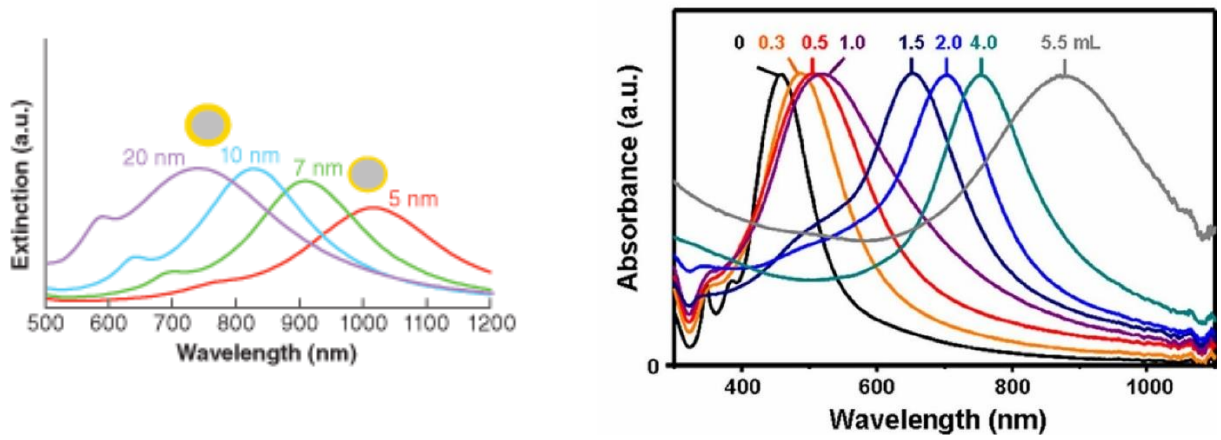

Figure 3.10: Dependence of SPR on (left) shell thickness for gold nanoshells and (right) the amount of auric acid for gold nanocages. (reproduced from [116])

Cellular uptake

In order to exert their radiosensitization effect, AuNPs need to be internalized within cells. Therefore, a description of the processes leading to AuNPs internalization performed by cells is required.

In general, all types of cells in the body exploit endocytosis or exocytosis to communicate each other, and with the environment, allowing them to internalize and externalize ions, nutrients and signaling molecules. When NPs are administered for biomedical purposes the endocytosis of such NPs depend on several parameters such as size, shape and surface chemistry of the nanoobjects but also on the cell-type.

Once NPs are injected into the body the most obvious obstacle they have to face is the extracellular membrane: this is a selectively permeable membrane that allows the passage of any kind of agents (which size may reach hundreds of nm) through its pores.[118]

In their work Lin *et al.*[119] showed the shape-dependent behavior of AuNPs with comparable size (≈ 50 nm); gold nanostars (GNSs), gold nanorods (GNRs), gold nanotriangles (GNTs) were PEGylated (to exclude interference of external factors) and incubated with the RAW264.7 cell line, which are mouse leukemic monocyte macrophage. After a 24 hours incubation, a markedly different internalization was observed, with 0.38%, 2.04% and 3.33% of gold uptake for GNSs, GNRs and GNTs, respectively. Anyway, the uptake of spherical NPs was shown to be 500% more than that of GNRs of similar size.[120]

Dimension is the second key parameter in determining the efficiency of cellular uptake.[121] Several studies have indicated that for cellular uptake of NPs, there is an optimum size of 50 nm at which NPs are internalized more efficiently and with higher uptake ratio; smaller (between 15 – 30 nm) or bigger (between 70 – 240 nm) nanoparticles shown a decrease efficiency of this process.[120]

Surface charge is another parameter that may greatly affect the cellular uptake of NPs. It is well known that nanoparticles may be easily functionalized (in particular, AuNPs) with many functional groups leading to a positively or negatively charged surface. In general, positively charged NPs are better internalized than neutral and negatively charged ones because of the interaction with the negatively charged cell membrane.[122] Once internalized, NPs may induce a charge-dependent effect on the cell: for example, positively charged NPs may disrupt the integrity of the membrane and lead to an increase in toxicity, inducing to cell death.[123] For what concerns the cell membrane, it was shown that internalization of positively charged NPs increases its fluidity while internalization of negatively charged NPs leads to its gelation.[124]

Section 4: Mechanistic Investigation

Although the influence of several parameters on this radiosensitization effect were investigated, the mechanism(s) responsible for it still remains unclear. The great majority of the papers published in scientific literature attribute the origin of the measured enhancement to an increased adsorption of ionizing radiation by nanomaterials. But recent studies are trying to shift the nature of this phenomenon to the biological point of view focusing on the action of nanomaterials once internalized within the cell.

In general, after exposure to ionizing radiation, biological systems undergo a series of processes that can be divided into three phases: physical, chemical and biological enhancement that differ in terms of time scale (***Figure 4.1***).

Radiosensitization by AuNPs was initially believed to stem solely from physical enhancement, exploiting the elevated photoelectric absorption of Au. However, an increasing number of studies are shifting the attention to biological and chemical enhancement, suggesting roles for AuNPs in all three steps resulting from the interaction of cells with ionizing radiation. While the exact mechanisms behind AuNPs-induced radiosensitization effect remain to be fully elucidated, several hypotheses have been proposed.

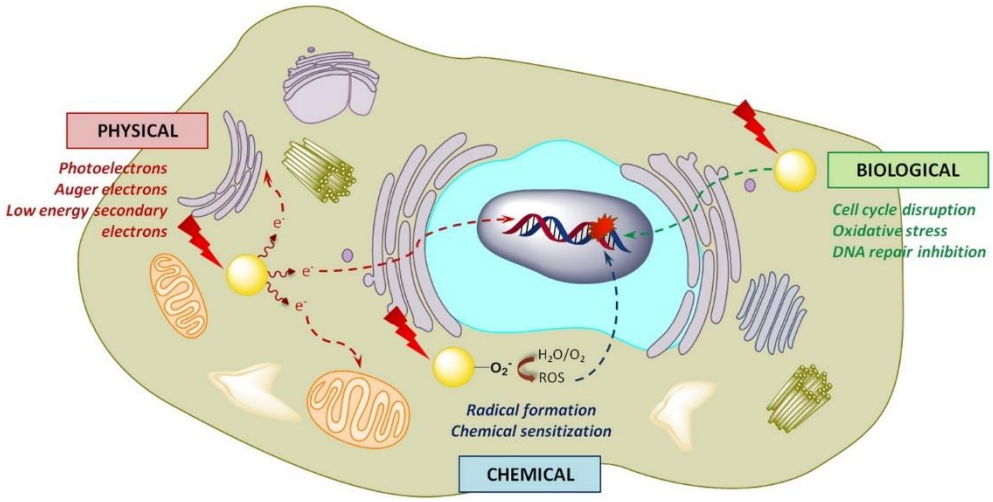

Figure 4.1: Mechanisms of AuNPs radiosensitization. The ability of these nanoobjects to enhance radiation effects is the combination of a physical, chemical and biological effect.
In the first phase, AuNPs exert cellular damage by increasing the production of photoelectrons, Auger electrons and low energy secondary electrons. In the second phase, the electronically active surface of AuNPs catalyzes the formation of radicals such as ROS. Finally, in the last phase, AuNPs enhance the effects of IR via oxidative stress, cell cycle disruption and DNA repair inhibition. (from [110])

What follows is a more detailed discussion about the three subsequent phases occurring after irradiation with ionizing radiation.

Physico enhancement

The contribution to the physico-chemical part is mainly given by the difference in dose absorption between metal NPs and the surrounding that enables a dose enhancement in NPs internalizing cells. Many groups have theoretically depicted ROS mechanism formation as a 3-steps process: X-ray absorption by the nanomaterial, the emission of an electron and lastly the interaction of this electron with its surrounding and cellular components.

In the kV energies, photons interact with matter mainly though Compton or photoelectric effect. In the former, the incident photon is scattered after the collision with a weakly bound electron. Because of the interaction, part of the energy is transferred from the photon to the electron, that may leave the atom if its energy content is high enough. In the photoelectric effect, the incident photon is absorbed by an atom-bound electron reaching an excited state that may result in the ejection of an inner-shell electron. An outer-shell electron falls to fill the vacancy releasing, at the same time, photons (fluorescence) and/or secondary electrons (Auger electrons) to get rid of the energy excess.

With low energy ionizing radiation photoelectric effect is the most probable to take place resulting in the absorption of the incident photon followed by the ejection of an electron. This effect is prevailing until the photon energy reaches a threshold energy (dependent on the irradiated element) with a cross-section in the range Z^4 to Z^5.[125] For higher energies Compton effect become the most likely phenomena: in both cases, the low-energy secondary electrons emitted are believed to be responsible to produce ROS and to cause the majority of cells damages.[126]

By exploiting the atomic number difference between Au (Z = 79) and soft tissues, which are mainly composed by organic material with low atomic number, AuNPs can be used to deliver a significantly greater amount of energy per unit mass, increasing the local dose of radiation deposited at the target site.

As seen in section 3, irradiation with high energy photons failed to produce dose enhancement; this observation was explained by considering the decreased contribution of the photoelectric absorption by Au at higher energies, providing support for physical dose enhancement.

Several studies were published, using AuNPs in combination to radiotherapy, to have a clearer view about the radiosensitization effect: most of these agree to the critical role played by low-energy electrons LEEs in dose enhancement. Zheng *et al*.[127] reported a 2.5-fold increase in doble strand breaks DSBs and single strand breaks SSBs when DNA-AuNP complexes were exposed to 60 keV radiation compared to bare DNA.

Chemical enhancement

In comparison to the physical and biological enhancement, chemical enhancement is the less investigated contribution to the radiosensitization effect. The role of AuNPs in the chemical enhancement is through radical formation or by weakening DNA bonds, making it more susceptible to radiation-induced damages. According to AuNPs localization inside the cell, two different mechanisms of chemical enhancement have been proposed: (I) chemical sensitization of DNA to ionizing radiation and (II) increased radicals formation via AuNPs surface. What differs between them is the localization of AuNPs: the first mechanism requires AuNPs located within the nucleus in order to bind DNA while the latter does not.

For what concern internalized AuNPs localization, experimental data suggest a size-dependent behavior with smaller NPs able to get closer to the nucleus than bigger NPs. For example, Penninckx *et al.* [128] incubated A431 cells with a non-toxic concentration of well-dispersed 5 and 10nm AuNPs for 24 hours. By taking scanning confocal microscopy images they confirmed that internalization of both 5 and 10nm AuNPs occurred; moreover, smaller NPs were able to reach the nuclear envelope while bigger NPs did not. In another study, Sanche *et al.* [129] synthetized 2 different types of AuNPs with different size and surface charge: a positively-charged 5nm AuNPs and a negatively-charged 15nm AuNPs. They observed that smaller NPs, with positive charge, were able to bind the negative charge carried by the phosphate groups of the DNA backbone, leading to significant damages and an enhancement factor of 4.5. On the other hand, bigger NPs bound randomly DNA, resulting in a substantially lower degree of radiosensitization.

Although AuNPs are chemically inert, their surface seems to be electronically active and able to catalyze chemical reactions.[130] Moreover, small AuNPs (< 5 nm) possess higher surface area, resulting in superior catalytic activity and ease in superoxide radicals formation.[131] Similar results were found by Ito *et al.* [132] where 15 nm citrate-capped AuNPs enhanced ROS production. More specifically, these NPs catalyzed the formation of superoxide and hydroxyl radicals in a two-step process. In addition to this, several other studies have demonstrated enhanced ROS production *in vitro* without exposure with IR. They all confirm that catalysis by AuNPs occurs predominantly through surface interaction with molecular oxygen, which facilitate electron transfer to generate ROS.

To investigate the influence of AuNPs on ROS production, Penninckx *et al.* [133] incubated A549 cells with AuNPs for defined incubation time (ranging from 0 to 24 hours) and ROS levels were measured at the end of each of these. What they observed is an increase in ROS production within the first 6 hours (with 1.4-fold increase in the maximum) followed by a gradual and constant reduction until the end of the experiment.

Taken together, these studies prove that AuNPs can catalyze radical reactions and increase ROS production that can damage irreversibly cellular fundamental structures leading to cell death.

Biological enhancement

The first doubts that radiosensitization cannot be explained solely considering physical enhancement are quite practical: although kV radiation is used to treat patient with superficial tumors, it suffers from short penetration depth. Therefore, to treat patient with deeper tumors, radiation with higher energy must be used; indeed, MV IR is used when cancer is localized in brain, prostate, pancreas etc. But, as previously stated, radiosensitization in this condition is expected to be negligible if only photoelectric effect is considered. Therefore, a new model, freed of any radiation-dependence, able to explain the observed radiosensitization effect is needed.

In addition to the physico-chemical approach, a biological one was proposed by Carine *et al.*[111] The first one require a direct interaction between the incident beam and the internalized AuNPs. However, an increasing amount of simulations evidenced that the number of hits in a cell containing AuNPs is very low, especially in case of charged particles.[106,134] Consequently, the calculated physical enhancement effect due to AuNPs is very low compared to the radiosensitization observed in *in-vitro* studies.

Moreover, several studies reported significant radiosensitization effect with megavoltage X-rays where little or no increase in overall dose deposition would be expected according to the theory. [135] This suggests that other mechanisms have to take place and bring a greater contribution than this "ballistic" approach to the radiosensitization effect.

In their work, Carine and colleagues demonstrated the involvement of the Trx system, suggesting a biological mechanism. They performed an invalidation of the TrxR expression in A549 cell line using siRNA technology leading to a residual 15% TrxR protein level. These invalidated cells were then irradiated without AuNPs, evidencing a significant radiosensitization effect.[133] This allowed them to hypothesize a new (biological) mechanism: following cell uptake through a receptor-mediated endocytosis, AuNPs-containing endosomes fuse with lysosomes. By decreasing the pH inside the vesicle, lysosomes trigger AuNPs degradation leading to the release of Au^{+} ions, well-known TrxR inhibitors. This inhibition induces various dysfunction of pathways leading to a cytoplasmic ROS accumulation, a decrease in ATP production and DNA damage repair alterations. This mechanism is represented in ***Figure 4.2*** where is possible to observe the TrxR inhibition by Au^{+} ions emitted as consequence of AuNPs degradation.

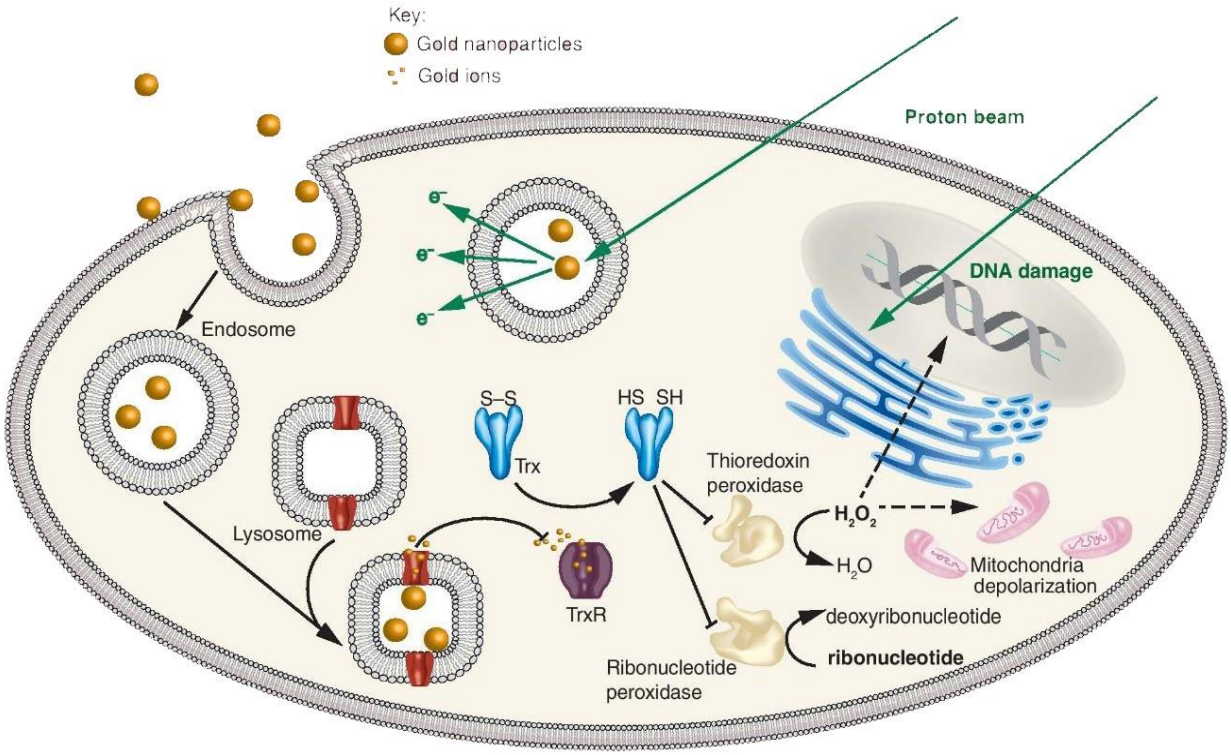

Figure 4.2: Schematic representation of the biological mechanism proposed by Carine *et al.* Once internalized into the cell, endosomes containing AuNPs fuse with lysosomes. The decrease in pH triggers *in situ* degradation of the nanoparticles resulting in gold ions emission; these are well-known to establish strong Au-S covalent bonds with selenocysteine residue in TrxR active site, preventing its function. As consequence, the decrease in the amount of reduced Trx leads to the accumulation of ROS triggering the oxidative stress condition. These radicals can further interact with mitochondria leading to membrane depolarization and consequent drop in ATP production. (reproduced from [133])

To investigate the dependence of ion release with time, Pompa *et al.* [136] dispersed metallic (Au and Ag), magnetic (Fe_3O_4) and semiconductor (CdSe/ZnS) nanoparticles in acidic solutions to mimic lysosomal environment (37°C, pH 4.5) as well as in neutral ones to mimic the cytoplasmic environment (37°C, pH 7). For all the NPs they observed a significant ion release in the acidic environment and no measurable release in neutral conditions; moreover, these NPs undergo degradation with consequent loss of morphology and weakening of the magnetic properties.

They also studied TrxR inhibition in these conditions, observing a 35% of inhibition due to the great amount of ions release in the acidic environment while the negligible amount of ions release in the control did not significantly affect the enzyme activity. The same experiment was performed *in vivo* by measuring TrxR activity after 48 hours of incubation; even in this case they observed a clear reduction of the enzyme activity. These observations indicate that ions release by AuNPs are active against this enzyme.[136]

Section 5: Objectives

Radiotherapy is one of the reference treatments to cure cancer that can be used as stand-alone technique or in combination with other approaches (like chemotherapy). When ionizing radiation is sent toward cancer site, healthy cells in its surrounding are damaged as well. This inevitable interaction give rise to side effects whose intensity can range from modest to very severe. Therefore, new approaches that allow the maximization of the therapeutic ratio (like the use of radiosensitizers or the switch from conventional radiotherapy to hadrontherapy) are under study.

The advent of nanotechnology offered the opportunity to take advantage of nanoscale materials for medical applications such as radiosensitizers in oncology. In this field, many studies highlighted the ability of high-Z nanoparticles to increase the local dose deposited by the beam. Unfortunately, even though many papers have been published, the mechanisms behind the radiosensitization effect is not completely understood yet.

To date this effect is mainly attributed to a physical-chemical mechanism: DNA damages induced by ionizing radiation and an increased ROS content result in apoptosis. In addition to these, a biological mechanism, centered on thioredoxin reductase inhibition and mitochondria membrane depolarization, have been recently proposed.

The goal of this thesis is to validate or disprove this novel mechanism, in particular by studying how AuNPs affect TrxR inhibition. More specifically, the aim was to investigate the relation between Au^{+} ions release and AuNPs dimension when these are placed in a lysosome-mimicking buffer; indeed, these ions are well-known TrxR inhibitors by binding its active site and preventing its biological function.

Data shown in this thesis may reinforce this novel biological enhancement, helping to clarify an effect whose mechanism is not completely understood yet.

Section 6: Experimental Part and Results

Materials and methods

Table 5.1: chemicals used for the synthesis.

Reagent	Molecular formula	Assay	Molecular weight $\frac{g}{mol}$
Tetra-cloro-auric acid tri-hydrate	$HAuCl_4 \cdot 3H_2O$	99%	393.83
Sodium borohydride	$NaBH_4$	99%	37.84
Milli-Q water (18.2MΩ)	H_2O		18.01
	$TA\text{-}PEG_{550}\text{-}OCH_3$	95%	550
	$TA\text{-}PEG_{400}\text{-}NH_2$	95%	400
Citric acid	$C_6H_8O_7$	99%	192.19

As previously described, gold nanoparticles were used in the experimental part. More specifically, AuNPs of 4 different sizes were synthesized: 4 nm, 6 nm, 7.5 nm and 18 nm. To achieve such a diversity in dimension, 2 different protocols were followed.

A revisited Turkevich method[137] was followed for the synthesis of the smaller NPs (from 4 to 7.5 nm) where a passivation step was performed immediately after AuNPs were obtained.

First, tetracloroauric(III) acid ($HAuCl_4$) was mixed in milliQ water with the ligand $TA\text{-}PEG_{550}\text{-}OCH_3$ to promote the formation of Au-ligand precursor under vigorous stirring. The progression of this reaction could be checked by naked eyes since the solution color gradually turned from pale yellow to colorless. Then, addition of the reducing agent $NaBH_4$ triggered gold ions reduction and the subsequent formation of gold nanocrystals. Even this time the progression of the reaction could be check visually since the color of the solution gradually turned from light to intense, dark red as the reducing agent was added.

Passivation step followed immediately after; nanoparticles surface was functionalized through the reduction of the thioctic moiety of the amino-functionalized PEG by $NaBH_4$.

In the following picture (***Figure 5.1***) both steps are presented:

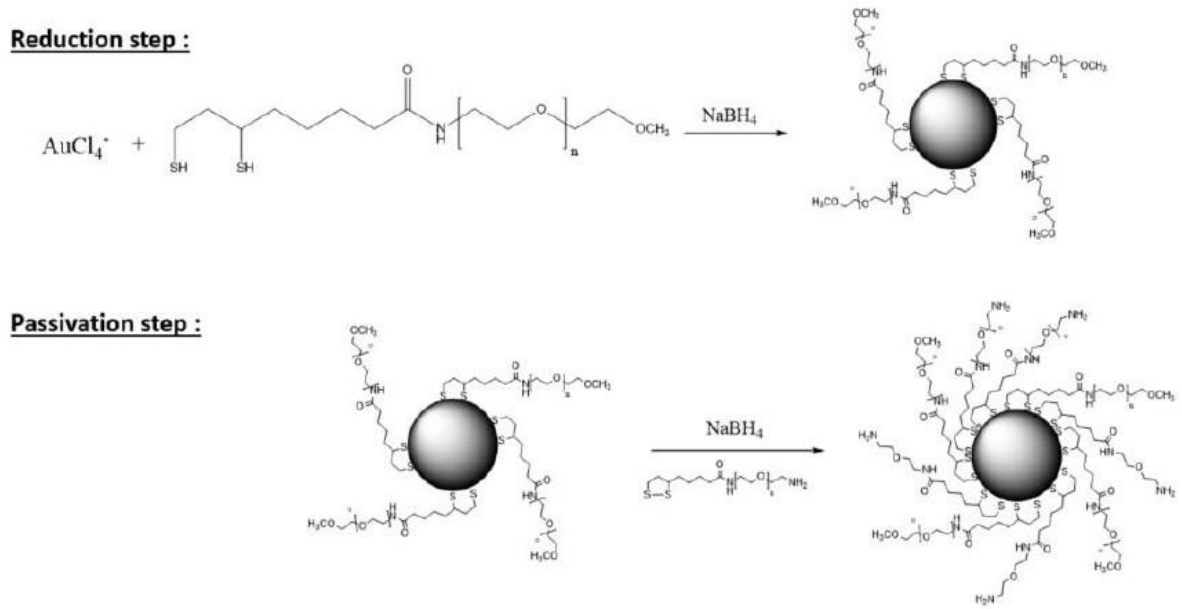

Figure 5.1: Schematic representation of the smaller AuNPs synthesis method.

The most interesting feature of this protocol is the possibility to synthesize AuNPs of different size by simply tuning the molar ratio between gold and the ligand added in the first step.

A remarkable aspect that should be highlighted is the different role of the PEG ligands used in these steps: the ligand used in the reduction step enable to obtain a specific nanoparticles dimension while the second enable the functionalization of their surface.

For the synthesis of the bigger nanoparticles (18 nm) a seed-growth method[138] was followed. For this procedure, citric acid was added to milliQ water and the mixture was heated and vigorously stirred. To avoid excessive evaporation, a cooling column was used. Once the solution reached its boiling point, $HAuCl_4$ and the ligand TA-PEG_{550}-OCH_3 were simultaneously added to it. The color of the solution turned grey in a first moment to become light purple few seconds after. Two further additions of $HAuCl_4$ were made, each separated from the previous one of 15 minutes. At the end the heating mantle is removed, and the solution is naturally cooled down to room temperature.

For the passivation step, a defined amount of TA-PEG_{400}-NH_2 (in order to achieve a molar ratio Au/PEG = 1) was added to the solution, that was stirred for other 3 hours.

Finally, the mixture is purified to remove all the contaminants.

NPs characterization

UV absorption spectra, obtained with *GENESYS 40 Vis/50 UV-Vis Spectrophotometer*, are presented in ***Figure 5.2***: as shown, all plasmon bands appear in the range between 520 – 525 nm suggesting AuNPs with a diameter in the range 5 – 20 nm. The narrow peaks, in particular for the bigger NPs, suggest a narrow size distribution. Finally, is possible to notice a red-shift of the absorption maximum as the NPs size increases.

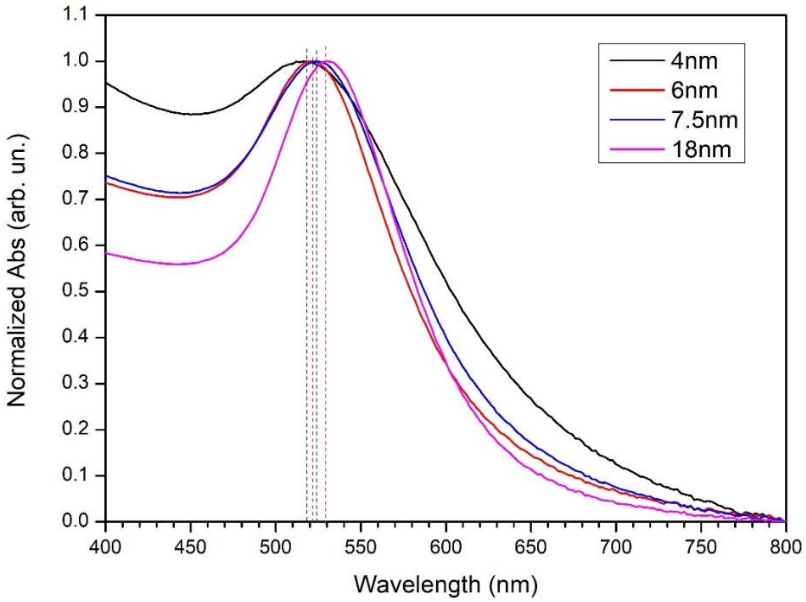

Figure 5.2: Normalized UV-Vis absorption spectra of the synthesized AuNPs colloidal solutions.

To better evaluate NPs dimension, TEM pictures were taken. A statistical analysis was performed by measuring the diameter of approximately a thousand NPs per dimension with ImageJ; this software also allowed to evaluate the average nanoparticles diameter and size distribution.

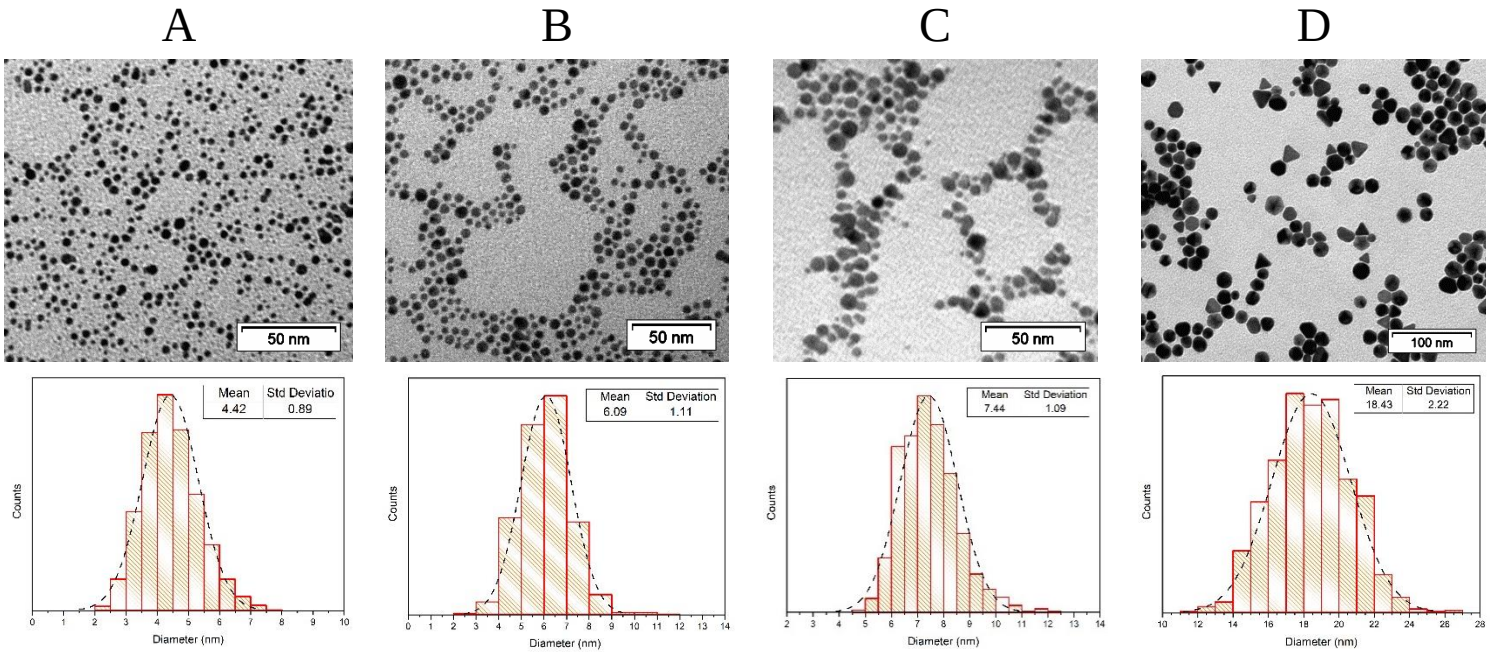

Figure 5.3: SEM images and particles size distribution of the (A) 4 nm, (B) 6 nm, (C) 7.5 nm and (D) 18 nm AuNPs.

As seen by these micrographs, all syntheses have led to the formation of spherical nanoparticles with very narrow size distributions. The only exception can be observed for the 18 nm AuNPs where triangular-shaped nanoparticles are present; the formation of these nanoobjects is attributed to the followed seed-growth method according to several authors.[139,140]

In order to assess the stability of all the AuNPs, Zeta potential analysis was performed.

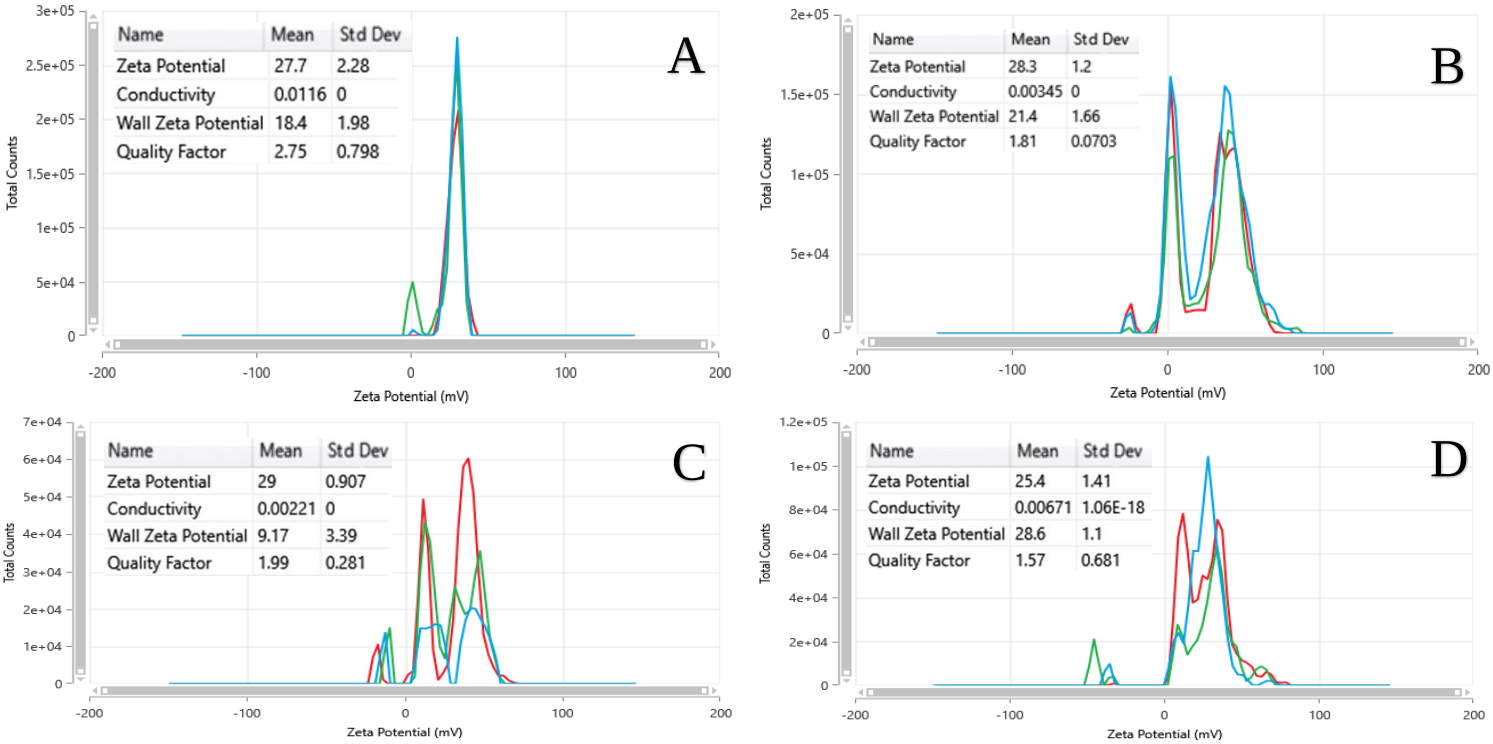

Figure 5.4: Z potential graphs of (A) 4 nm, (B) 6 nm, (C) 7.5 nm and (D) 18 nm AuNPs resuspended in deionized water.

All the NPs exhibit a zeta-value around +25 mV when suspended in deionized water; in general, colloidal solutions displaying a zeta-value around ± 30 mV are very stable while lower zeta-values, between + 5 mV and – 5 mV, indicate low stability and, therefore, fast aggregation.

Ion release

As previously mentioned, TrxR is inhibited by gold ions that may bind the protein in 2 different locations: between the Cys residues on the *si*-face of FAD cofactor and between other Cys residues (at positions 520 and 574).[19]

Thiol groups constituting the active site of TrxR are classified as soft bases according to Hard-Soft Acid Base theory (*HSAB*); this theory states that soft acids react faster and form stronger bonds with soft bases, whereas hard acids react faster and form stronger bonds with hard bases, keeping constant all other parameters. This means that thiol groups in TrxR active sites are more likely to react with those ions classified as "soft" in *HSAB* theory such as Cu^{+}, Ag^{+}, Au^{+}, Cd^{2+}, Pt^{2+}, Hg^{2+}.[141] Therefore, AuNPs were used to perform this experiment because gold is frequently used in radiosensitization experiment and AuNPs may be produced in different sizes and functionalized quite easily.

What was interesting to evaluate is if AuNPs can release Au^+ ions and investigate if there is some kind of relation between Au^+ ions release and AuNPs size. To answer this question an ion release experiment was performed.

First, two different buffers were prepared: a *citric acid/sodium citrate* buffer at pH 4 and a *PBS* buffer at pH 7 (the former to mimic the environment NPs would encounter once internalized in lysosomes, the latter to have a negative control). Both buffers were 150 mM to simulate the ionic strength found within lysosomes. 24 centrifuge filters were used, half filled with the first medium and the remaining with the other. AuNPs were added to these buffers and then incubated for 24, 48 or 72 hours (37°C, 0% CO_2); the final concentration of each mixture was 100 $\frac{\mu g\ Au}{mL}$.

After each incubation time, centrifugation was performed in order to remove the NPs from each solution (since these could not pass through filter pores) and analyze the supernatant by ICP-AES.

The results are reported in ***Figure 5.5***:

Inc. time (h)	4 nm	6 nm	7,5 nm	18 nm
24	0,00%	0,06%	0,11%	0,09%
48	0,08%	0,08%	0,14%	0,14%
72	0,14%	0,17%	0,22%	0,17%

Inc. time (h)	4 nm	6 nm	7,5 nm	18 nm
24	0,01%	0,01%	0,01%	0,003%
48	0,01%	0,00%	0,002%	0,001%
72	0,01%	0,01%	0,010%	0,001%

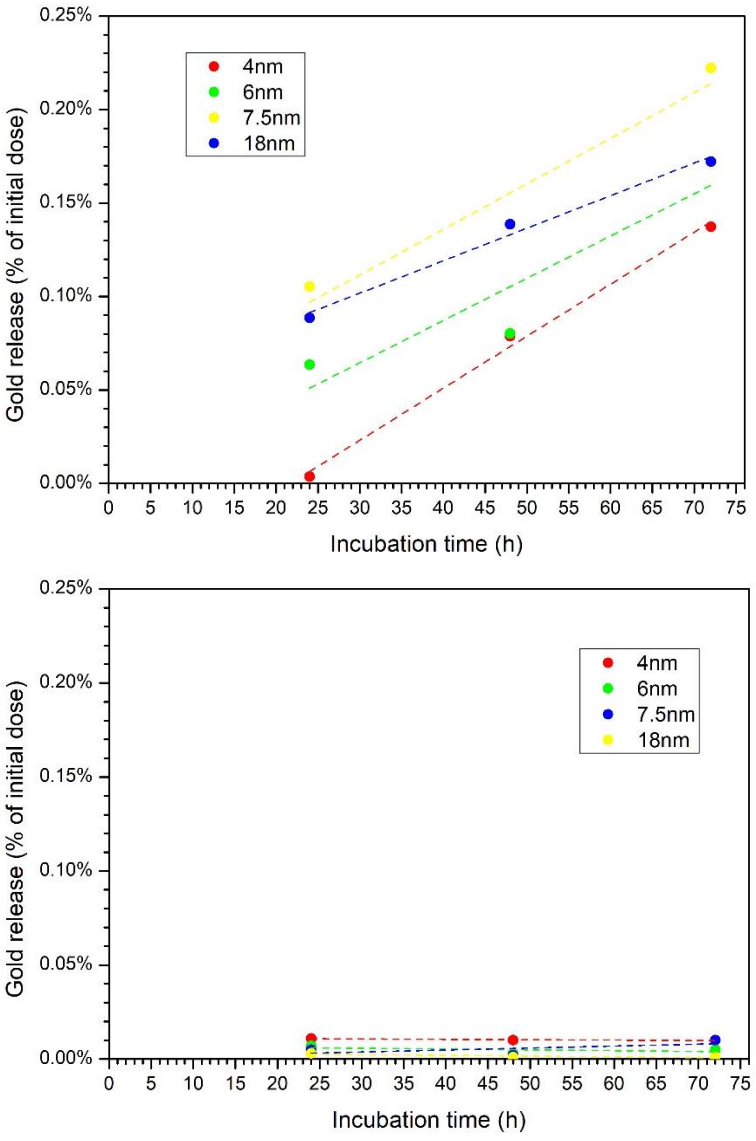

Figure 5.5: ICP-AAS data of the ion release experiment for the pH 4 (above) and pH 7 (below) buffers.

The trend displayed by these graphs confirms that Au^+ concentration increases with time for the acidic condition whereas it remains almost constant for the control. At the same time, a surface-dependent behavior is highlighted, with the 7.5 nm AuNPs releasing the highest amount of Au^+ ions.

To understand what happens to the NPs in these buffers, UV-Vis spectra of both conditions were taken after each incubation time (***Figure 5.6***).

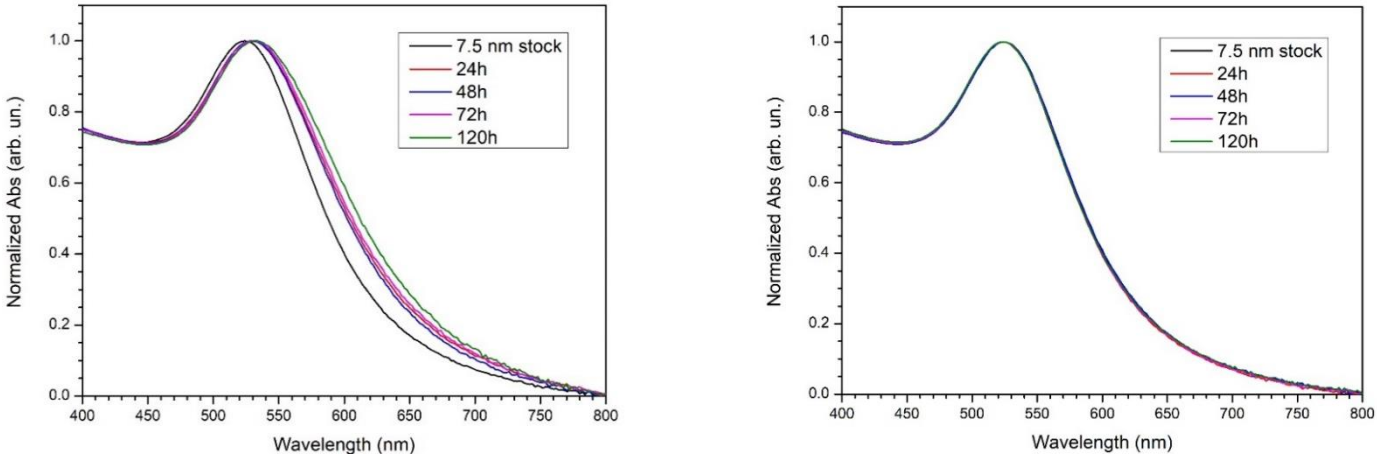

Figure 5.6: Normalized UV-Vis spectra of the 7.5 nm AuNPs after different incubation times in acidic (left) and physiological (right) buffer.

From the graphs obtained analyzing the acidic buffer is evident that an aggregation process is occurring as the peak's tail is increasing with the incubation time. Moreover, the peaks wavelength is shifting toward higher values (red-shift) meaning that NPs aggregates are forming in the solution. This is probably due to the degradation of the amino-PEG group that act as stabilizing agent; without its action, NPs are not stabilized and agglomeration into larger aggregates may occur. As expected, this process affects also the LSPR effect with a red-shift of the band maximum.

This hypothesis is confirmed by the CPS curves of the NPs incubated in the pH 4 buffer:

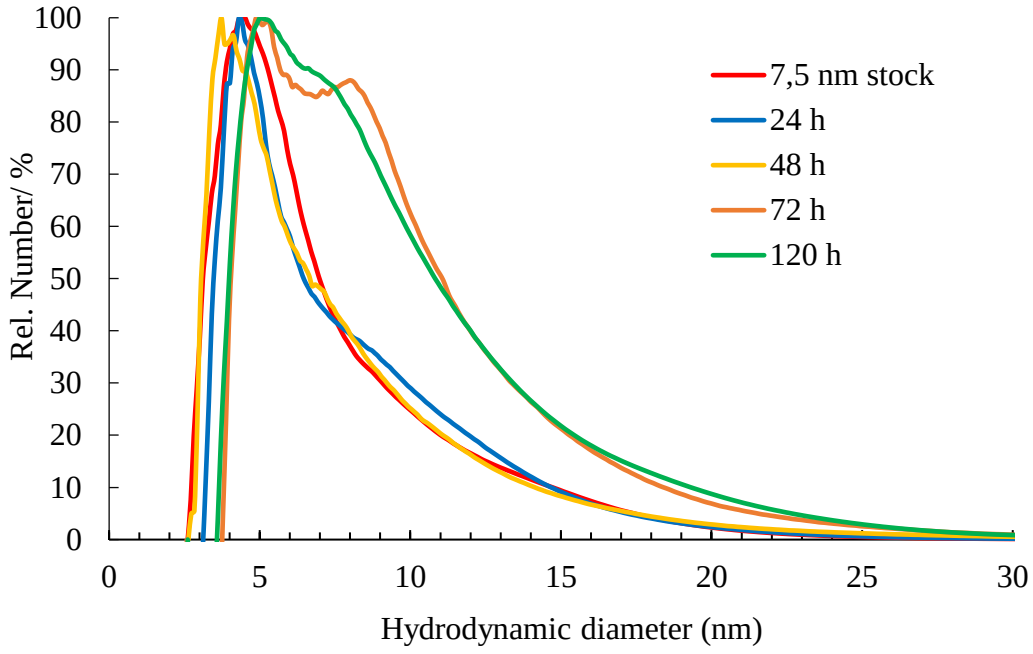

Figure 5.7: CPS profiles of the 7.5 nm AuNPs in acidic buffer after different incubation times.

An initial decrease of the hydrodynamic diameter maximum, followed by an increase, can be observed by studying the peak of each curve. This trend may be explained as the initial degradation of the stabilizing amino-PEG group, decreasing the time required by the NPs to reach the laser beam and, therefore, a smaller hydrodynamic diameter detected by the technique. Then, because of the weakened stabilization, NPs start to aggregate forming bigger agglomerates that travel across the liquid gradient with higher resistance (and that is why an increase in the hydrodynamic diameter is observed). At the same time, no aggregation process is taking place in the other solution since the peak seems to be fixed at the same wavelength and all the graphs are essentially superimposable (data not shown).

Finally, by considering the concentration of the colloidal solutions and the density of gold it was possible to estimate the number of NPs added to each buffer and the total surface that these NPs expose to the buffer. By plotting the ion release rate versus the total NPs surface (***Figure 5.8***) is possible to observe that the higher is the surface exposed to the buffer and the higher the amount of ions released.

Diameter NPs	**4 nm**	**6 nm**	**7.5 nm**	**18 nm**
Surface	$3.513 \cdot 10^{22}$	$2.579 \cdot 10^{22}$	$2.087 \cdot 10^{22}$	$8.425 \cdot 10^{21}$

pH 4				
Slope	0.0028%	0.0023%	0.0024%	0.0017%

pH 7				
Slope	0.000%	0.000%	0.000%	0.000%

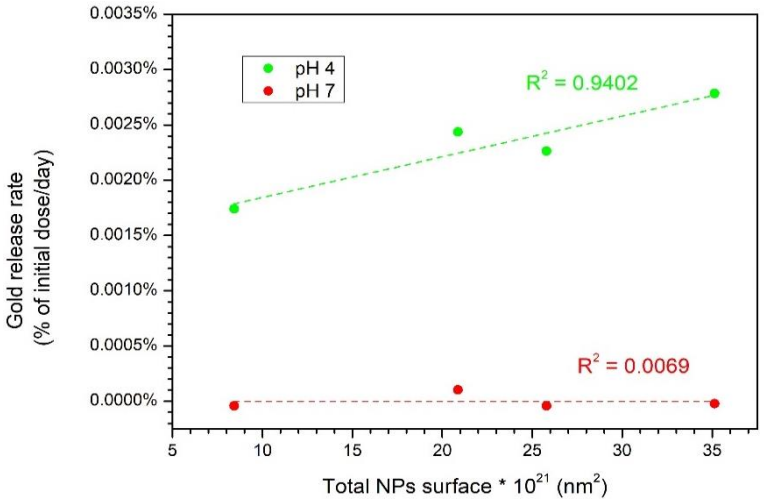

Figure 5.8: Evaluation of the ion emission rate from AuNPs of different size.

This suggests that, if the intracellular environment is considered, TrxR inhibition is more pronounced when smaller AuNPs are internalized (due to the higher amount of Au^{+} ions they can release).

Section 7: Conclusions and Prospects

Over the past decade, the sensitization effect carried by AuNPs has undergone a growing interest for radiation therapy improvement driven by their unique physico-chemical properties. Excellent clinical outcomes on the use of AuNP-based radiosensitization have further facilitated the production of multimodal AuNPs functionalized with homing ligands to bind cancer cells. Although the radiosensitization effect was mainly attributed to a physical contribution, many clues are suggesting that a biological contribution is also present, and that it may be more relevant than the physical one.

Once internalized within lysosomes, it was shown that AuNPs emit Au^{+} ions in a size-dependent way and these ions are thought to bind thioredoxin reductase TrxR in its catalytic site, preventing the action of the whole detoxification system.

Results presented in this study are meant to be preliminary and give a tiny insight on the radiosensitization effect, whose causes are not yet well known.

Other experiments need to be performed. First, AuNPs should be incubated with different tumor cell lines to evaluate the amount of internalized nanoagents; many authors pointed out that NPs parameters (in particular, their size and shape) greatly affect cellular capability to internalize such nanoobjects. Once internalized, gold content should be quantified by inductively coupled plasma mass spectroscopy (ICP-MS).

To investigate the influence of internalized AuNPs on TrxR activity, atomic absorption spectroscopy (AAS) should be performed in order to evaluate the amount of gold ions Au^{+} emitted by AuNPs within lysosomes. Then, TrxR activity should be measured (there are many commercially available kits) to propose a relation between Au^{+} ions emission and TrxR inhibition.

At the end, irradiation of tumor cells, pre-incubated with AuNPs, should be performed in order to evaluate the enhanced cell death.

The same experiments could also be performed considering nanoparticles of different shapes (for example by employing gold nanorods), but also with other high-Z elements, such as silver (Z = 47) or platinum (Z = 78).

Considering the enormous impact that cancer has on our society, the need to find new or more effective treatments is enormous. In such sense, even though its mechanism has to be further investigated and clarified, radiosensitization seems to be a great opportunity to increase radiotherapy efficiency.

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