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Gold nanoparticles, radiations and the immune system: Current insights into the physical mechanisms and the biological interactions of this new alliance towards cancer therapy

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ABSTRACT

Considering both cancer's serious impact on public health and the side effects of cancer treatments, strategies towards targeted cancer therapy have lately gained considerable interest. Employment of gold nanoparticles (GNPs), in combination with ionizing and non-ionizing radiations, has been shown to improve the effect of radiation treatment significantly. GNPs, as high-Z particles, possess the ability to absorb ionizing radiation and enhance the deposited dose within the targeted tumors. Furthermore, they can convert non-ionizing radiation into heat, due to plasmon resonance, leading to hyperthermic damage to cancer cells. These observations, also supported by experimental evidence both *in vitro* and *in vivo* systems, reveal the capacity of GNPs to act as radiosensitizers for different types of radiation. In addition, they can be chemically modified to selectively target tumors, which renders them suitable for future cancer treatment therapies. Herein, a current review of the latest data on the physical properties of GNPs and their effects on GNP circulation time, biodistribution and clearance, as well as their interactions with plasma proteins and the immune system, is presented. Emphasis is also given with an in depth discussion on the underlying physical and biological mechanisms of radiosensitization. Furthermore, simulation data are provided on the use of GNPs in photothermal therapy upon non-ionizing laser irradiation treatment. Finally, the results obtained from the application of GNPs at clinical trials and pre-clinical experiments *in vivo* are reported.

ABBREVIATIONS

APCs: antigen-presenting cells; Au: gold; GNPs: gold nanoparticles; CTLs: cytotoxic T lymphocytes; DCs: dendritic cells; EPR: enhanced permeability and retention; EGFR: epidermal growth factor receptor; ECM: extracellular matrix; HATs: histone acetyltransferases; HMTs: histone methyltransferases; HSPs: heat shock proteins; IR: ionizing radiation; LEEs: Low energy electrons; LEM: local effect models; NPs: nanoparticles; NIR: non-ionizing radiation; SPR: surface plasmon resonance; LSPR: localized surface plasmon resonance; PEG: polyethylene glycol; PTT: photothermal therapy; RT: radiation therapy

1. THE INTRODUCTION OF NANOPARTICLES IN CANCER THERAPY

Cancer is currently one of the leading causes of death worldwide and a major public health concern, despite the advances that have been made towards its early diagnosis and treatment. In 2012, 14.1 million new cancer cases were estimated around the world; 7.4 million in men and 6.7 million in women (Ferlay, et al., 2012). More recent data published by the American Cancer Society further attest to its prevalence, with 1,685,210 new cancer cases and 595,690 cancer deaths projected to occur in the United States alone in 2016 (Siegel, Miller, & Jemal, 2016), while 23.6 million new cases of cancer are expected each year by 2030 (Stewart, 2014).

Ionizing radiation (IR) therapy, alongside chemotherapy, presents a major modality for cancer treatment following surgery applied to more than 50% of all cancer patients (Atun, et al., 2015). Despite the advancements made both in medical imaging and radiation sources with the development of new modalities such as intensity modulated radiotherapy (IMRT), stereotactic ablative radiotherapy (SABR), and image guided radiotherapy (IGRT) it is still a great challenge to restrict the curative dose of radiation on tumor tissue, sparing at the same time the adjacent normal tissues (Ngwa, et al., 2017). A great deal of work has been undertaken on IR therapy treatment modelling, planning and delivery either alone or in combination with chemotherapy; in all cases, however, the radiation-induced toxicities to adjacent non-tumor tissues represent the major dose-limiting factor (Zhao, Zhou, & Li, 2016).

A strategy to address the issue of radiotoxicity is to use radiosensitizers that confer additive and synergistic advantages to the tumoricidal effect of IR (Y. Mi, Shao, Z., Vang, J., Kaidar-Person, O., & Wang, A. Z. , 2016). In this way, lower radiation doses can be used to eradicate tumors with the same efficiency or even better, while causing minimal damage to surrounding normal tissues (James F. Hainfeld, Dilmanian, Slatkin, & Smilowitz, 2008). Up to date, a plethora of radiosensitizers have been developed and evaluated based on different attributes, such as dose enhancement, generation of radical oxygen species (ROS), and alteration of diverse biological responses to radiation. (Nikitaki, Hellweg, Georgakilas, & Ravanat, 2015).

Non-ionizing sources of radiation including microwaves, radiofrequency and ultrasound are also employed to treat cancer through the generation of heat (Sethi & Chakarvarti,

2015). Photothermal therapy (PTT) or microwave therapy can kill cancerous cells by targeted tissue hyperthermia induced by internalized therapeutic agents with a high photothermal conversion efficiency under external laser irradiation. PTT has recently attracted considerable attention owing to its controllable treatment process, high tumor eradication efficiency and minimal side effects on non-cancer cells (Q. Chen, et al., 2016). Numerous studies have shown that metallic nanoparticles (NPs), can act both as radiosensitizers for IR therapy as well as PTT agents due to their unique optical and electrical properties. In particular, gold nanoparticles have attracted considerable attention due to their high absorption coefficient, metallic properties and biocompatibility.

A number of reviews on GNPs and their applications in targeted cancer therapy and the enhancement of the effect of radiation (both IR and NIR) has been published up to date (Haume, 2016; Her, Jaffray, & Allen, 2015; Lim, Li, Ng, Yung, & Bay, 2011; Ngwa, et al., 2017; Swain, Sahu, Beg, & Babu, 2016). In this review, a simulation of the effects of hyperthermia induced by the ablation of GNPs localized at solid tumors upon exposure to NIR has been included; The latest experimental advances on the *in vivo* and/or *in vitro* administration of GNPs and their application as IR and PTT sensitizers are also presented. In addition, great emphasis has been given on the biological interactions of GNPs with blood components (both plasma proteins and cells of the immune system) and the effect of these interactions on radiation therapy (RT) and, hence, on cancer treatment.

2. THE 'BEHAVIOR' OF GNPS IN A PHYSIOLOGICAL ENVIRONMENT

NPs exhibit great diversity in their chemical composition. Typical inorganic or hard NPs include those derived from metals (e.g., gold, silver), semiconductors (e.g., quantum dots), carbon dots, carbon nanotubes, or oxides (e.g., iron oxide); organic or soft NPs include polymers, liposomes, micelles, cellulosic NPs, and DNA-linked NPs (Dennis, Delehanty, & Medintz, 2016). Irrespective of NP's composition, the biological identity of a NP largely depends on its synthetic identity (size, shape, architecture, surface chemistry and post-synthetical modifications), the physiological environment under which it is dispersed and the duration of its exposure to it (A. L. Chen, et al., 2016; Gunawan, 2014). Moreover,

parameters such as the physiological/biological medium's ionic strength, pH and temperature can further alter the interaction between the solid NP and the liquid medium as well as the forces generated by this interaction (Braun, DeBrosse, Hussain, & Comfort, 2016; Nel, 2009). It is becoming apparent, therefore, that NPs interacting with proteins, membranes, cells, DNA and organelles establish a series of NP-biomolecule interfaces that depend on colloidal forces, as well as dynamic physicochemical interactions (Dennis, et al., 2016). These interactions lead to the formation of protein coronas, particle wrapping, intracellular uptake and biocatalytic processes that could have either biocompatible or bioadverse outcomes (Nel, 2009). A great deal of these interactions is unanticipated, reflecting an extremely complex environment around the NP itself and the NP's interfaces with biological fluids and cells (Palchetti, et al., 2016).

In this review, emphasis is mainly given on GNPs and their interactions with proteins in biological fluids, as well as with cancerous cells, but most importantly with components of the immune system. This is because GNPs have a number of advantages in comparison to other NPs (discussed in detail in a subsequent section), including easy manufacturing, selective targeted delivery of chemotherapeutic drugs to tumors, and, most importantly, good biocompatibility (Haume, 2016).

2.1 Size of GNPs

Numerous studies have been published regarding the effect of nanoparticles' size on their bio-distribution and blood circulation lifetime as well as their cell uptake. The results of these studies are inconclusive, however, as there is no ideal size for a nanoparticle to be used as a radiosensitizer. The general consensus is that nanoparticles of intermediate sizes (20 -60 nm) exhibit maximum cell uptake (He, Hu, Yin, Tang, & Yin, 2010); they, however, have been proven to be problematic in terms of tumor penetration and even intratumoral distribution (Haume, 2016; Her, et al., 2015). Specifically, large GNPs tend to be captured by the liver, while GNPs with a diameter smaller than 5 nm tend to be excreted rapidly through the kidneys (Albanese, Tang, & Chan, 2012). As far as their cellular uptake is concerned, receptor-mediated endocytosis and diffusion have been proposed as likely mechanisms for the uptake of GNPs into endocytic vesicles. The size of these GNPs is similar to the size of viruses, that is, less than100 nm (Doherty & McMahon,

2009). The size of the formed vesicles, which depends both on the endocytotic mechanism involved and the cell type, is itself a determining factor for the nanoparticles to be internalized (Clift, et al., 2008; Geiser, et al., 2005). It has been experimentally verified that smaller GNPs (less than 50nm) do not necessarily get endocytosed more readily, since their docking on the plasma membrane does not produce enough free energy to completely envelop them, and small GNPs have to cluster together in order to get endocytosed (Chithrani & Chan, 2007). GNP aggregation is an often overlooked issue, which, nevertheless, can significantly differentiate experimental observations from theoretical models. For example, Albanese et al. showed that increased cellular uptake of GNPs is only apparent for large aggregates of smaller particles (larger than 50 nm) (Albanese & Chan, 2011). As already discussed, the cell type also influences the uptake of GNPs of different sizes. For example, embryonic fibroblasts preferentially internalize 25 nm GNPs rather than larger GNPs, whilst epithelial cells prefer 50 nm GNPs over 25 nm or 70 nm GNPs (Chithrani, Ghazani, & Chan, 2006). Most studies conclude that 50 nm is the optimum size of GNPs for cellular uptake, either bare GNPs or decorated GNPs (Lu, Wu, Hung, & Mou, 2009; S. H. Wang, Lee, Chiou, & Wei, 2010). However, Levy and coworkers demonstrated that the optimum size for a GNP expressed as the number of particles in a cell might differ when their mass is considered, which further attests to the complexity of GNP size and its effect on bioavailability and cellular uptake (Levy, Shaheen, Cesbron, & See, 2010).

2.2 Surface charge of GNPs

Regarding the influence of GNP surface charge on their cellular uptake, GNPs that bear a positive charge exhibit increased chances of both binding to and being internalized by a cancer cell, as it was anticipated. This can be attributed to a number of factors, such as the primary negatively charged phospholipid bilayer, which in fact tends to be further negatively charged in cancer cells due to the increased glycoprotein content (Paszek, et al., 2014). Most of the published studies show that increase of positive surface charge or charge itself enhances particle intracellular uptake (Harush-Frenkel, Debotton, Benita, & Altschuler, 2007; Jin, Xu, Ji, & Shen, 2008; Rouhana, Jaber, & Schlenoff, 2007). Contradictory results reported by He *et al*. (He, et al., 2010) could be attributed to the

aggregates formed due to the low zeta potential of the nanoparticles examined and not to their negative surface charge. Zero surface charges, either due to neutral surface groups or zwitterionic (ions with both a positive and a negative charge) ligands, have been shown to invariably lead to low cellular uptake compared to charged particles. In addition to the surface charge of the nanoparticles, the cellular membrane potential has also been implicated in the intracellular uptake of GNPs, while it has been shown that by altering membrane potential, GNPs may modulate their own uptake (Arvizo, et al., 2010). Positively charged GNPS, for instance, induce rearrangements in the plasma membrane, allowing in this way their entry into cells through translocation or endocytosis (Beddoes, Case, & Briscoe, 2015).

2.3 Protein corona

A further issue that should be considered is that once a GNP enters biological fluids, a protein corona inevitably forms around it. GNPs must be treated, as mentioned previously, as biological entities rather than inorganic ones. However, until recently, only the chemical composition of a GNP and its physical properties were taken into consideration. Nevertheless, the numerous proteins and small molecules that are present at high or, surprisingly, low concentrations in biological fluids are known to be adsorbed to the NP surface, forming a cloud of aggregated proteins, known as a 'protein corona' (Kharazian, Hadipour, & Ejtehadi, 2016). These coronas constitute an effective interface between the nanoparticle and the surrounding biological medium and can also modify to a great extent nanoparticle' biological behavior (Soleimani, et al., 2016).

Protein coronas are further distinguished into hard and soft ones, depending on the strength of the interactions that develop between the proteins absorbed onto the NP; these interactions are far more complicated than mere electrostatic attractions or repulsions, encompassing everything from Van der Waals forces to H-bonding (Gunawan, 2014). As mentioned above, protein coronas evolve constantly, a phenomenon which can last up to several days (Grafe, et al., 2016; Walczyk, 2010). Furthermore, their composition does not necessarily reflect the relative abundance of proteins or small molecules in the medium that surrounds a given nanoparticle. For example, albumin, one of the major components of the blood plasma, is hardly ever found associated with nanoparticles, irrespectively of

the physical and chemical properties of the nanoparticle. On the other hand, apolipoproteins and opsonins, the blood serum concentrations of which are low, are the main constituents of the protein corona (Ho, Poinard, Yeo, & Kah, 2015). In fact, only a few of the proteins present in plasma are to be found associated with a NP. The 'adsorbome', a term coined by Walkey *et al*., consists of 125 different blood plasma proteins that have been observed to be adsorbed to at least one nanomaterial. The same group has shown that 2 to 6 proteins are adsorbed with high abundance and many more proteins adsorbed with low abundance, irrespectively of the nanoparticles's composition (Walkey, 2012).

The protein corona plays an important role in determining the biological fate of a nanoparticle, that is, the nanoparticle's subcellular organization and organ distribution, as well as its rate of clearance and cytotoxicity (Hamad-Schifferli, 2013; Zarschler, et al., 2016). Efforts have been made to predict the protein corona that would form around a nanoparticle by measuring the binding affinities of a panel of small molecules to its surface (Xia, Monteiro-Riviere, & Riviere, 2010). This approach, however, is not as straightforward as it appears, due, in part, to the observation that the same proteins could change their conformation and orientation depending on the particle's size, roughness and curvature (Walkey, 2012). Moreover, various techniques used to analyze the composition of the protein corona may produce erroneous or inaccurate results as well as different protein makeup profiles for the same nanoparticle. Even if the protein corona 'fingerprint' is identical between two particles of slightly different physical properties, it still cannot be predicted how many of these proteins would retain their tertiary structure (Ban & Paul, 2016; Hamad-Schifferli, 2015). Another important consideration is that the original protein corona at the point of the nanoparticle's entry (e.g., blood, lung or other) is not the one that determines the biodistribution and its effects *in vivo* but rather a corona modified during translocation (Monopoli, Aberg, Salvati, & Dawson, 2012). Therefore, the fate of the original corona, as it passes through membranes and barriers and interacts with the extracellular matrix, cannot be predicted. Several studies have focused on mapping the proteins found to be associated with nanoparticles (Hamad-Schifferli, 2015; Sund, Alenius, Vippola, Savolainen, & Puustinen, 2011), although more work is needed to ensure predictable biological and *in vivo* outcomes (Azhdarzadeh, et al., 2015). Thus far, much

attention has been given on blood plasma-induced corona on nanoparticles, while studies of the corona of NPs recovered from many other biologic fluids (e.g., urine, synovial fluid, cerebrospinal fluid and pleural effusion) are also emerging (Martel, 2011; Mirshafiee, Kim, Mahmoudi, & Kraft, 2016).

3. TARGETING TUMORS WITH GNPS

To overcome the inherent limitations of GNPs, such as nonspecific distribution, biocombatibility, rapid blood clearance and poor solubility in physiological environments (K. Cho, Wang, Nie, Chen, & Shin, 2008), various GNP coatings are used. These coatings do not only overcome the aforementioned limitations, but can also be exploited to deliver GNPs to target cancer cells either passively or actively (Akhter, 2012) (**Figure 1**).

3.1 Passive targeting

The passive method of targeting cancer cells is possible due to the enhanced permeability and retention (EPR) effect, which is based on the fact that GNPs leak into tumor tissue preferentially through permeable tumor vessels and are then retained in the tumor bed due to reduced lymphatic drainage (Ajorlou & Khosroushahi, 2016; Needham, et al., 2016). This effect could explain why macromolecules and nanoparticles are found at higher concentrations in tumorous tissues compared to the normal surrounding tissues (Ranganathan, et al., 2012; M. Wang & Thanou, 2010). Although the EPR effect has been extensively utilized to deliver GNPs and nanosize drugs to tumors, recent research studies suggest that this method is not as efficient as previously thought, since GNP-based drug delivery does not increase more than 2-fold compared to unassisted drug delivery. In addition, barriers such as the capillary wall's resistance further prevent the delivery of GNPs to tumors (Nakamura, 2016). Given that inter- and intratumoral variability can affect the architecture of the neovasculature and the tumor microenvironment, it becomes apparent that passive targeting of nanoparticles to tumors may be more complicated than originally assumed (Prabhakar, et al., 2013) and would depend on the size, surface charge and shape of the nanoparticle (Bertrand, Wu, Xu, Kamaly, & Farokhzad, 2014; Gmeiner & Ghosh, 2015). Efforts have been also made to improve/build on the EPR effect either by

remodeling the extracellular matrix (ECM) to increase the intratumoral mobility of colloids or by increasing the perfusing pressure (Bertrand, et al., 2014). In all cases, assessing the tumor microenvironment in individual patients and predicting patients' susceptibility to the EPR effect may eventually become the main determining factors when choosing the optimal therapeutic regimens (Carmeliet & Jain, 2011). On the other hand, it might be inefficient to rely upon the EPR effect or artificially augmenting it, as the behavior of drugs and their affinity for the intratumoral environment has to be taken into account when designing passively-targeted NPs. It would be pointless to deliver NPs to a tumor site if these NPs have no affinity for cancerous cells and would consequently diffuse back to the blood vessels (Dreher, et al., 2006; Ullal, et al., 2011).

3.2 Active targeting

The property of a ligand to bind preferentially to malignant relatively to nonmalignant cells, resulting in selective delivery of nanoparticles or drug activation when in proximity to malignant cells, can be exploited in order to actively and preferentially target malignant cells (Danhier, Feron, & Preat, 2010). One can therefore build upon the EPR effect and the passive accumulation of nanopartciles at a tumor by conjugating ligands to the surface of nanoparticles, thereby increasing the affinity of the latter for the cancer cells (Haume, 2016). A wide variety of such ligands has been used to date, ranging from antibodies to aptamers and even glucose molecules, which have been extensively reviewed in previous publications (Bertrand, et al., 2014; Geng, et al., 2014; Her, et al., 2015). Regarding GNPs, taking into consideration GNPs' capacity to act as radiosensitizers, the ligands of choice should not only facilitate discrimination between cancerous and non-cancerous cells, but also induce GNPs' cellular internalization (Kong, et al., 2008). For example, folate conjugation of GNPs has been shown to increase 6-fold GNPs' cellular uptake (Khoshgard, Hashemi, Arbabi, Rasaee, & Soleimani, 2014). Equally significant improvements have been demonstrated when transmembrane receptors, overexpressed in a large subset of cancers, such as the epidermal growth factor receptor (EGFR) (J. Liu, Liang, Liu, Li, & Yang, 2015) and HER-2 (Bhattacharyya, Gonzalez, Robertson, Bhattacharya, & Mukherjee, 2011), were targeted. A number of factors should be taken into consideration when selecting the ligand to target a tumor, including its molecular weight (MW), targeting

affinity, valency and biocompatibility (Gmeiner & Ghosh, 2015). It has been shown, for example, that a ligand might adversely affect the time a nanoparticle could remain in circulation (Singh & Erickson, 2009). Lastly, the potential effect of the formed protein corona on the ligand decoration of a GNP should also be taken into account and determined experimentally, as it has been shown to significantly alter the expected targeting efficiency of the nanoparticle (Salvati, 2013).

3.3 Further GNP coatings to improve plasma circulation time and intracellular uptake

In practice, it is common to use both methods of targeting to improve the nanoparticle's localization to the tumor. Irrespectively of the method selected, the coating applied on the GNPs can also improve both the time GNPs remain in plasma circulation and their cellular uptake (Alkilany & Murphy, 2010; Krpetic, Anguissola, Garry, Kelly, & Dawson, 2014). It has been widely reported that the nanoparticles have to be 'concealed' from the host's immune system in order to even have a chance to reach their target, avoiding in this way to be detected and destroyed by it (Grabbe, Landfester, Schuppan, Barz, & Zentel, 2016). Towards this end, small hydrocarbon chains, primarily PEG, are used for coating, in order to improve the biocompatibility of GNPs and, at the same time, prevent the formation of aggregates. PEGylation effectively alters the pharmacokinetics of a variety of drugs, including GNPs, and dramatically improves the drug efficacy by reducing drug leakage, cytotoxicity and immunogenicity, as well as increasing drug's plasma circulation time and tumor cell targeting potential (Mishra, 2016). The applied coating also allows to control the surface charge of GNPs, as this has been shown to influence their life time and cellular uptake dynamics (Saptarshi, Duschl, & Lopata, 2013). Care should be taken so that the applied coating does not interfere with the chosen ligand for active targeting; thus, shorter PEG chains that do not exceed ligand's length should be employed (Dai, Walkey, & Chan, 2014) and their concentration should also be carefully monitored (Shmeeda, Tzemach, Mak, & Gabizon, 2009). Another concern, especially in the case of GNPs intended to be used as radiosensitzising agents, is that the coating may absorb secondary electrons emitted from NPs' metal core, leading to a reduction in the number of the generated radicals (Gilles, 2014)

4. GNPs and radiation therapy

4.1 GNPs and IR therapy

It was first observed by Spiers *et al*. (1949) that high atomic number (Z) elements, such as iodine and barium, are not only useful as medical contrast agents, but also have much higher energy absorption coefficients compared to soft tissues. Therefore, their presence at a target zone, a tumor site for example, should increase the effective dose delivered to this area, thereby paving the way to use High-Z elements in radiotherapy. This field has attracted increasing interest in the last decade, with a particular focus on GNPs, which are excellent radiation absorbers, as already mentioned (James F. Hainfeld, et al., 2008). One of the first experiments to be conducted by Hainfeld *et al.* verified GNPs' potential as radiosensitizers by demonstrating natural tumor specificity and substantial improvements in tumor size control in mice receiving IR therapy minutes after the administration of GNPs. This study has prompted further theoretical and experimental work on the radiation sensitizing effects of GNPs, with promising results both *in vitro* and *in vivo* (James F. Hainfeld, et al., 2008). Radiosensitization has been demonstrated for various IR types, including keV photons and kilovoltage (kV) sources, as well megavoltage (MV) photons, megaelectron volt (MeV) electrons, and heavy charged particles (James F. Hainfeld, et al., 2008; Jain, et al., 2011; Schuemann, et al., 2016). Experimental studies which have employed GNPs of different size, shape and surface coatings, resulted in conflicting results as to the radiosensitizing effects of GNPs. These effects appear to be dependent on the animal model system being studied, with differing results in different cell lines *in vitro* (Butterworth, McMahon, Currell, & Prise, 2012; Schuemann, et al., 2016). The aforementioned concerns have likely hampered the development of GNP-based therapies and their application in clinical trials.

4.1.1 Radiosensitization mechanisms of GNPs in IR therapy

A series of mechanisms are activated upon exposure of biological systems to IR. These mechanisms can be broadly divided into physical, chemical and biological and differ in the time required for their effects to take hold. In the physical mechanism, IR interacts with

biomolecules causing ionization and excitation of the latter, as well as the generation of free radicals. By absorbing sufficient energy, the emitted electrons travel further and collide with subsequent atoms, eliciting a cascade of ionization events, with DNA being the ultimate target of the generated electrons and free radicals like OH·, H_2O_2, e_{aq} and others. In the chemical mechanism, the free radicals and the low energy electrons undergo several reactions to restore the cellular charge equilibrium. Lastly, in the biological mechanism, a series of cellular processes are activated to repair the radiation damage. What characterizes IR therapy is the formation of highly clustered DNA damage sites, especially in the case of particle radiation. Failure to repair damage in the DNA caused by IR, leads to cell apoptosis or genomic instability (Georgakilas, 2008; Georgakilas, O'Neill, & Stewart, 2013). It was initially thought that GNPs could solely be used for physical 'dose enhancement' by exploiting the enhanced photoelectric absorption of gold and the generation of a large number of localized electrons that cause damage in their vicinity. However, both the chemical and the biological mechanisms contribute to the radiosensitization as demonstrated by experimental data that suggest a role of GNPs in modulating all three mechanisms of interactions with radiation (Butterworth, et al., 2012).

4.1.2 The physical mechanisms

The principle idea behind the development of GNPs as radiosensitizers is based on the differences in energy absorbance between gold and the surrounding soft tissues, which enables a radiation dose enhancement in the presence of gold. This enhancement works better for keV photons, rather than MeV photons, as it was demonstrated for the first time *in vivo* by the intravenous injection of 1.9 nm GNPs into mice bearing subcutaneous mammary carcinoma (J. F. Hainfeld, Slatkin, & Smilowitz, 2004). Photons interact with matter in three main ways: 1) pair production, 2) Compton scattering, and 3) the photoelectric effect (Butterworth, et al., 2012; James F. Hainfeld, et al., 2008). Pair production occurs at high photon energies, about 1.22 MeV, where the incident photon energy exceeds twice the rest mass energy of the electron. For gold, the photoelectric advantage at those beam energies is lost (James F. Hainfeld, et al., 2008). For photons above 500 keV, Compton scattering and excitations are observed (Mesbahi, 2010). An incident photon is scattered upon collision with a weakly bound electron. In this process,

an amount of energy is transferred from the photon to the electron and the electron is emitted from the atom. The Compton scattering results in atom re-excitation and production of more Compton electrons, leading to the photoelectric effect (Butterworth, et al., 2012; James F. Hainfeld, et al., 2008; Mesbahi, 2010; Paunesku, Gutiontov, Brown, & Woloschak, 2015). In contrast to the Compton scattering, the photoelectric effect is the predominant mode of interaction for photons with energy between 10 and 500 keV (James F. Hainfeld, et al., 2008; Mesbahi, 2010). An incident photon is absorbed by a bound electron, leading to its emission from its electron shell. The vacancies created in a K, L, or M shell is swiftly filled with by outer-shell electrons moving into these cells. In this process, lower energy photons are produced (fluorescent) and a cascade of secondary electrons, such as Auger electrons, are released (Cooper, Bekah, & Nadeau, 2014; James F. Hainfeld, et al., 2008; Retif, et al., 2015). This low energy electrons have ranges of a few micrometers and are expected to cause highly localized ionization events (Rosa, Connolly, Schettino, Butterworth, & Prise, 2017). The X-ray cross section, which refers to the probability of a given material to interact with radiation, is dependent on its atomic number (Z); for the photoelectric effect, the X-ray cross section ranges approximately between Z^3 and Z^5 (Kaplan). The underlying physical mechanisms of GNPs-dependent enhancement of the biological effect have been investigated in studies with plasmid DNA (Shukla, et al., 2005), where radiation-induced DNA damage was assessed at the molecular level in the absence of biological responses to radiation. It has also been demonstrated that low energy electrons play a critical role in dose enhancement by GNPs (discussed in detail later in the manuscript). Experimental findings from these plasmid DNA studies suggest that secondary low energy electrons generated from GNPs are the result of the localized energy deposition in the vicinity of the nanoparticle, leading to dose enhancement and radiosensitization (Zheng, Hunting, Ayotte, & Sanche, 2008).

4.1.3 The chemical mechanisms

GNPs involved in the 'chemical mechanisms' of IR radiosensitization are activated through radical reactions or through induction of an 'open' chromatin structure, which allows access of damaging agents to DNA. Depending on the subcellular localization of GNPs, there are two main chemical mechanisms triggered upon IR exposure namely, the

radiochemical sensitization of DNA and the increasing catalytic surface activity and radical generation by GNPs' surface (Her, et al., 2015). While both mechanisms can lead to increased radiosensitization, the former mechanism requires the nuclear localization of GNPs; however, the majority of GNPs studied to-date are usually restricted to the cytoplasm (Her, et al., 2015). In spite of this, both processes provide critical information on the chemical radiosensitization through GNPs, which could be utilized towards the design of nanoparticles that could achieve the maximum possible dose enhancement. Electrons with energies below the ionization threshold (e.g., 10 eV), which are also being referred to as low energy electrons (LEEs), play an important role as secondary electrons in radiosensitization. In particular, Zheng *et al.* demonstrated that electrons in those energy levels fail to produce considerable secondary electrons through interactions with GNPs, but they can cause a great deal of DNA damage (Zheng, et al., 2008). This result was attributed to LEEs, which produce short-lived negative ions that weaken the hydrogen bonds in DNA. Yao *et al.* have also found that radiosensitization changes depend on the size and charge of GNPs (Yao, Huang, Chen, Yi, & Sanche, 2015). As a result, the tight binding of the small positively charged GNPs (5 nm) to the phosphate groups of DNA could lead to serious DNA damage. Larger, negatively charged GNPs (15 nm) showed a much weaker binding to DNA. Therefore, small GNPs can be localized easier to the nucleus and bind electrostatically to DNA, enabling in this way their full exploitation towards chemical enhancement.

Regarding the second mechanism of chemical radiosensitization, an increasing number of studies have reported that GNPs are capable of catalyzing chemical reactions due to their electronically active surface (Ionita, Conte, Gilbert, & Chechik, 2007). Particularly, small GNPs can exhibit great catalytic activity and electron transfer from surface-bound donor groups to O_2, thus generating free radicals. As a result, the alteration in the electronic configuration of surface atoms enables radical production at the reactive surface of GNPs (Her, et al., 2015). The catalytic surface activity has been demonstrated *in vivo* by Ito *et al.*, where 15 nm GNPs conjugated with citrate enhanced the cytotoxic effects, and combined with photodynamic therapy raised the production of ROS (S. Ito, et al., 2009). The enhanced ROS production has also been demonstrated *in vitro* in the absence of radiation (Chompoosor, et al., 2010; Mateo, Morales, Avalos, & Haza, 2014; Pan, et al.,

2009). ROS production enhancement was initially associated with secondary electron and photon emission from GNPs responsible for the secondary radiolysis of water. A variety of free radicals is produced by water radiolysis, causing indirect damage to DNA, proteins and lipid membranes through oxidation which could lead to the initiation of apoptotic cellular death and/or senescence (Pateras, et al., 2015). Increased levels of ROS has been reported for GNPs of various sizes, shapes and surface functionalization *in vitro* (Chompoosor, et al., 2010; Mateo, et al., 2014). It has been demonstrated that the increase of the time-dependent level of ROS leads to the cell death of GNPs of diameter 1.4 nm but not for 15 nm particles with the same chemical properties (Pan, et al., 2009). The GNP's size was also shown to play a key role in cytotoxicity, where increased levels of apoptosis for 4.8 nm PEG-coated GNPs were observed compared to larger PEG-coated GNPs (Zhang, et al., 2012). Related oxidative effects have also been demonstrated for iron-core nanoparticles coated with gold, leading to the suggestion that gold plays a key role in the oxidative response (Y. N. Wu, et al., 2011). The chemical interaction of the GNPs themselves with macromolecules could also provide an explanation for the mechanism through which they induce oxidative stress. Despite the large amount of evidence suggesting the induction of oxidative stress as a central mechanism to GNPs' radiosensitization, a small number of reports suggest that GNPs can act as anti-oxidants, depending on their surface functionalization, thereby further highlighting the complexity of the chemical mechanisms involved in the GNP-mediated radiosensitization (Tournebize, et al., 2012).

4.1.4 The biological mechanisms

There are a few biological models based primarily on *in vitro* studies where the radiation dose enhancement ratio is examined (Butterworth, et al., 2012; Haume, 2016; Schuemann, et al., 2016). The dose enhancement effect (DEF) for cancer cell killing usually ranges between 1.1-2.0 for low-LET radiatons (X-, γ-rays), clinically relevant doses (<2 Gy) and GNP sizes above 2 nm and various coatings as recently reviewed in Rosa (Rosa, et al., 2017). Sensitization in local effect models (LEM) depends greatly on the cellular location of GNPs. In general, cell survival decreases with increased cellular internalization of

GNPs; moreover, the closer GNPs are located to the nucleus of target cells, the larger the effect (James F. Hainfeld, et al., 2008; J. F. Hainfeld, et al., 2004; J. F. Hainfeld, Smilowitz, O'Connor, Dilmanian, & Slatkin, 2013). Of particular note, all these calculations are extremely sensitive to dose distributions on the nanometer scale, while the dose enhancement effect is expected mainly for low-LET radiations (X-, γ-rays) rather than high-LET particles, as in the case of protons, therefore raising questions regarding the applicability of GNPs in proton therapy (Y. Lin, McMahon, Paganetti, & Schuemann, 2015). Considering the large number of papers that have been published in the past decade, only a few studies provide detailed evidence on the biological interactions of GNPs combined with IR and present viable biological models. Recently, a model considering the dosimetric characteristics of GNPs and the LEM has simulated very nicely experimental survival curves for X-rays (McMahon, et al., 2011) and therapeutic beam protons (Y. Lin, et al., 2015). In all cases, a perinuclear distribution of GNPs is assumed and usually evidenced, but recent technological advancements in the field led to the use of solid gold nanospheres, with a cancer cell penetrating/pro-apoptotic peptide and a nuclear localization sequence (NLS) peptide (Mackey, Saira, Mahmoud, & El-Sayed, 2013). These nuclear-targeted 30 nm GNPs were found to enhance 5-Fluorouracil drug efficacy in human oral squamous cell carcinoma (HSC-3) cells *via* regulation of the cell cycle, a chemosensitization technique that could potentially be expanded to different cancer cell lines and different therapies including radiation-based ones. Nevertheless, very few *in vivo* experimental evidence exists. It is expected that tumor control and survival will be enhanced via the use of GNPs based on preliminary animal work using murine models. For example, glioblastoma (GBM) cells implanted into nude female athymic mice exhibited radiosensitivity following selective targeting of brain tumors with PEG-GNPs (12.5 nm) (Joh, et al., 2013). In another study, mice irradiated using a combined protocol of 250 kVp X-rays and 1.9 nm GNPs showed an improved (by a factor of 4) 1-year survival when compared to X-ray therapy alone (J. F. Hainfeld, et al., 2004).

4.2 GTPs and NIR therapy

Metallic NPs strongly absorb and scatter light close to their localized surface plasmon resonance (LSPR), and that is why they can be used as heat 'nanosources' in various

applications (Rodrigo & Alieva, 2016). Most importantly, the increased localized heating offers the possibility to selectively target tumors without inflicting harm to the surrounding healthy tissues. Plasmon resonance is a nanoscale electronic effect that causes metallic nanoparticles to absorb and scatter electromagnetic radiation of wavelengths considerably larger than the particles themselves (Pissuwan, Valenzuela, & Cortie, 2006). Plasmon resonance originates from the free electrons of the nanoparticle itself. As a result, a plasmon resonant wave is generated in the interface of the metal and the dielectric medium due to the highly delocalized state of the electrons (plasmon state). The plasmon resonant wave is related to an evanescent electromagnetic field (de Mol & Fischer, 2010). As a result, resonant NPs convert the electromagnetic energy into heat, thereby delivering hyperthermic damage to cancer cells (A. L. Chen, et al., 2016). Photothermal therapy (PTT), therefore, causes hyperthermia that leads to cell death either by cell apoptosis or cell necrosis. Cell apoptosis takes place when the heating temperature ranges from 41°C to 47°C. Proteins like Caspase-8 and Caspase-9 induce a cascade of events that leads to cell death. On the other hand, excessive cell necrosis by heat shock occurs at temperatures above 50°C. Necrosis leads to rapid cell death, as opposed to apoptosis, and is based on protein denaturation (Cherukuri, Glazer, & Curley, 2010).

PTT presents a potential tool for the treatment of solid tumors, especially due to the low thermotolerance of cancerous tissue relatively to noncancerous tissue, which is caused by the poor blood supply of the former. Nevertheless, the development of 'thermotolerance' by tumors must also be taken into account in order to target tumors effectively with NIR-induced hyperthermia, since it could limit the effects of thermal treatment (Cherukuri, et al., 2010). For example, two heating treatments performed at intervals of 12-48 hours caused an increased blood flow in the target tumor and increased tumor thermotolerance, as opposed to thermal treatments administered all at once and which can likely decrease tumor perfusion (Griffin, Dings, Jamshidi-Parsian, & Song, 2010).

GNPs provide desirable optical properties for PTT. Gold, when fabricated on the nanoscale, exhibits enhanced surface plasmon resonance (SPR). The SPR frequency strongly depends on the GNP geometry, and can be tuned within a broad range of the electromagnetic spectrum, from visible to NIR (Pattani & Tunnell, 2012). Several experimental studies have shown that gold nanorods have a higher temperature increase

per mass gold (von Maltzahn, et al., 2009) than gold nanoshells and the nanorod absorption cross-section is twice as high as that of gold nanoshells (Hu, et al., 2006). A recently published study showed that gold nanoshells of the same diameter with spherical GNPs actually generated more heat, per nanoparticle, than nanorods, primarily due to their overall larger geometric cross-section (Pattani & Tunnell, 2012). Recently published studies have highlighted another role for the heat shock proteins (HSPs)-induced thermotolerance in antitumor immunity. In particular, Ito and coworkers applied hyperthermia to tumors through magnetite cationic liposome and recorded increased levels of intracellular HSP-peptide complexes, enhanced processing of endogenous peptides and increased density of MHC class I-peptide complexes at the cell surface (A. Ito, Honda, & Kobayashi, 2006). The authors suggested the following sequence of events: 1) MHC class I-restricted cytotoxic T lymphocytes (CTLs) recognize the tumor cells, 2) the tumor cells that die due to CTLs or hyperthermia release the intracellular HSP-peptide complexes and other peptides, 3) the released HSPs and/or antigenic peptides activate neighboring monocytes to produce proinflammatory cytokines and recruit antigen-presenting cells (APCs), 4) the HSP-peptide complexes are taken up by dendritic cells (DCs) and are in turn presented to T cells via MHC class I and/or II antigens (A. Ito, et al., 2006).

4.3 Combined ways of treatment

Various studies have demonstrated, that PTT combined with IR therapy enhances both chemotherapy and radiotherapy (Griffin, et al., 2010; J. F. Hainfeld, et al., 2014) by increasing blood flow locally and, hence, allowing higher concentrations of chemotherapeutic agents to reach the cancerous tissue. Furthermore, hyperthermia could make chemotherapy even more effective since many drugs are activated by heat (van der Zee, 2002). Due to the limited tissue penetration of drugs, this combination would be more efficient in certain types of cancer. For example, chemotherapeutic drugs in conjunction with hyperthermia have been successful in melanoma patients (Cherukuri, et al., 2010). Despite this, RT continues to be the mainstay of cancer therapy due to its proven ability to kill cells and shrink tumors. Unfortunately, the radiation dose has to be reduced below the curative level in order to spare healthy surrounding tissue. PTT therapy presents a promising approach to kill radioresistant cancer cells and one of the most effective

adjuvants to RT. In addition to its direct toxic effect on tumors, hyperthermia can also eradicate radioresistant tumor cells, such as tumor cells in hypoxic, low pH regions and cells in S-phase, which are mainly responsible for tumor recurrence and deadly metastasis after radiotherapy (P. Li, et al., 2015). Clinical results have shown that the combination of hyperthermia and IR therapy can successfully decrease the tumor size more than 10% (Cherukuri, et al., 2010). In animal tumors, heating to 43.5 °C for two hours along with X-ray irradiation produced a thermal enhancement ratio of 8:1 (i.e., the ratio of the radiation dose alone divided by the radiation dose combined with heat for the same therapeutic effect), leading to the suggestion that hyperthermia plays a significant role in tumor radiosensitization (Horsman & Overgaard, 2007). The difficulty of delivering uniform heating to deep tumors presents a major problem which prevents the clinical utility of hyperthermia. There is also a certain time frame where combined hyperthermia and RT work best, such as simultaneous application of both or each alone within a 1–2 hour time frame (Horsman & Overgaard, 2007).

The aforementioned obstacles can be overcome with the application of GNPs in thermo-radiotherapy. As mentioned previously, spherical GNPs mainly absorb light at the visible spectrum, between 300 and 800nm. For instance, GNPs of 40 nm with a peak absorption at 530 nm have been irradiated with a 514 nm laser beam to kill cells *in vitro* (J. F. Hainfeld, et al., 2014). Spherical GNPs absorb only ultraviolet (UV) and visible light, and therefore the number of tissues targeted for heating is rather limited, since the penetration depth of these wavelengths in living tissue is restricted. The optimal wavelength for penetrating tissue efficiently is near infrared at 800 nm. Gold nanoshells were found to absorb in this range and were used to treat efficiently tumors in mice by administering an intravenous injection followed by 800 nm laser irradiation (J. F. Hainfeld, et al., 2014; P. Li, et al., 2015; M. Zhou, et al., 2015). The use of smaller spherical GNPs, instead of nanorods and nanoshells, would provide an advantage in tumor penetration, as well as a lower background absorption in non-tumor cells and blood, where the GNP concentration must be lower. Another advantage of using GNPs in tumors is that their capacity of absorbing high-energy X-rays also enables them to absorb radiation efficiently. Depending on the X-ray energy spectrum and the concentration of GNPs in the tumor, the effective radiation dose in the tumor can be increased by a factor of two or more (Cooper, et al., 2014; James

F. Hainfeld, et al., 2008; Retif, et al., 2015). Zhou *et al* found that PEG-modified GNPs conjugated with ^{64}Cu and combined with NIR laser and IR could inhibit the growth of cancer ATC cells by 83.14%, as opposed to GNPs and IR therapy or GNPs and PTT where the reduction was 74.96% and 50.87%, respectively (M. Zhou, et al., 2015). In a recent study, it was shown that 15 nm PEG-decorated GNPs combined with IR and NIR could increase the survival rate in 71% of mice, in contrary to other combinations (J. F. Hainfeld, et al., 2014). Furthermore, Pink *et al*. has established that arginine-glycine-aspartate peptides-conjugated gold nanorods which target melanoma cancer cells are rapidly heated up, after their exposure to 808 nm NIR. **Table 1** provides a list of recent publications on different forms of GNPs that have been used with IR or NIR therapy to treat tumors/cancer cells both *in vitro* and *in vivo*.

5. INTERACTIONS OF GNPS WITH THE IMMUNE SYSTEM

The interactions between GNPs, or even the protein corona-decorated entities, with components of the blood plasma as well as with white blood cells is an issue which is often overlooked. These interactions can either stimulate desirable immune responses or induce adverse effects such as inflammation or toxicity (Dobrovolskaia, Shurin, & Shvedova, 2016; Zolnik, Gonzalez-Fernandez, Sadrieh, & Dobrovolskaia, 2010). Furthermore, they can lead to increased susceptibility to infectious diseases, autoimmune diseases or cancer (Petrarca, et al., 2015; Zolnik, et al., 2010). Taking into account the widely held notion that successful treatments against cancer should recruit host's immune system, the interactions that develop between the GNPs and the immune system should be extensively studied and exploited in conjuction with IR and NIR-based therapies. The effects of the interactions of GNPs with both the innate and the adaptive immune system have been grouped as immunoactivatory or immunosupressory in the following paragraphs. Irrespectively of their immunomodulatory effect, increasing experimental evidence suggests that cells of the immune system interact with NPs through Toll-like receptors (TLRs) which have evolved for the recognition of pathogens (Yang, et al., 2015). As far as the cells of the adaptive immune system are concerned, nanoparticles interact most frequently with antigen presenting cells in the blood circulation, including B cells, macrophages, and dendritic cells (Gregory, Titball, & Williamson, 2013). Larger nanoparticles (>100 nm) are internalized

and transported by macrophages and dendritic cells of the innate immune system, while smaller nanoparticles can easily travel to and accumulate in lymph nodes and affect the B and T cells of the adaptive immune system.

In this study, we have performed a thorough systematic literature review on the interactions of GNPs with the immune system. To this end, the biomedical literature was searched through PubMed {Lu, 2011 #1012} using relevant keywords in order to retrieve gene/gene products that are involved in immunomodulation and inflammation/toxicity; the official HGNC (Gray, Yates, Seal, Wright, & Bruford, 2015) gene symbols were used (**Table S1**). The retrieved data were further analyzed using bioinformatics approaches. A Venn diagram displaying the genes/gene products shared by "immunomodulation" and "inflammation/toxicity" (**Table S1**) was created (**Figure 2**) using BioVenn (Hulsen, de Vlieg, & Alkema, 2008). The molecules implicated in both processes are associated with the immune system as expected. Statistically significant over-represented WikiPathways (Kutmon, et al., 2016) (the threshold for the FDR-corrected p-value was set at 10^{-4}) across the genes presented in **Table S1** were identified (see **Table S2**) by employing the WebGestalt (WEB-based GEne SeT AnaLysis Toolkit) (J. Wang, Duncan, Shi, & Zhang, 2013) toolkit. As expected, many of these pathways are related to the immune system (e.g., cytokines and inflammatory response, IL-1/IL-2/IL-3/IL-4/IL-5/IL-6/IL-9/IL12/IL17 signaling pathway and Th1-Th2). Moreover, the Toll-like receptor signaling pathway is over-represented. Of particular note, several enriched WikiPathways are associated with DNA damage repair (**Table S2**), including DNA damage response, homologous recombination, mismatch repair, G1 to S cell cycle control and apoptosis, thereby outlining the importance of these pathways in immunomodulation and inflammation/toxicity.

5.1 Immunoactivation

As far as the activation of the immune system upon interaction with nanoparticles is concerned, both the innate and the adaptive immune systems have been shown to be stimulated. Regarding the former, inflammatory responses and secretion of pro-inflammatory signaling molecules, such as cytokines and chemokines, have been widely reported. The observed stimulation is highly dependent upon the physicochemical properties of the NPs. For example, positively charged NPs usually possess a higher

inflammatory potential compared to negatively charged or neutral NPs (Siegel, et al., 2016). In particular, Yen *et al*. have shown *in vitro* that GNPs, irrespectively of their size, up-regulate the expression of the proinflammatory genes interleukin-1 (IL-1), interleukin-6 (IL-6), and tumor necrosis factor (TNF-alpha) (**Table S1**); it has been also postulated that the protein corona that forms around GNPs allows them to enter cells via the more complicated endocytotic pathway, thus increasing cytotoxicity and triggering inflammation (Yen, Hsu, & Tsai, 2009). *In vivo* experiments in rabbits have shown that colloidal gold increases the leukocyte content and a considerable increase in polynuclear forms (Dykman & Khlebtsov, 2011). Cho *et al*. have found gene expression patterns typical of apoptosis and inflammation in liver of mice that were administered GNPs (W. S. Cho, Kim, Han, Son, & Jeong, 2009). Interestingly, GNPs were also found trapped in neutrophil extracellular traps (NETs) which could lead to their clearance from the body by the immune system (Bartneck, et al., 2010). As previously mentioned, the physicochemical properties of the nanoparticle have been shown to affect the degree of immune activation, although there are publications opposing this notion (Yaswen, et al., 2015). The interactions between the nanoparticles and the components of the adaptive immune system are even more complicated. Nanoparticles most probably act as haptens that become immunogenic only upon attachment to a larger carrier molecule (Kononenko, Narat, & Drobne, 2015), although direct attachment to antibodies has also been recorded (Dobrovolskaia, Aggarwal, Hall, & McNeil, 2008). Their immunomodulatory effects on cells of the immune system and their role in cancer therapy has received considerable attention. GNPs used as adjuvants in vaccines were shown to stimulate the phagocytic activity of macrophages and affect the functioning of lymphocytes (Dykman & Khlebtsov, 2011). Lin et al. reported that GNP-mediated delivery of modified CpG can stimulate macrophages and inhibits tumor growth for immunotherapy (A. Y. Lin, et al., 2013), while Ahn et al. recently demonstrated that GNPs enable efficient tumor-associated self-antigen delivery to dendritic cells and induce antigen-specific cytotoxic T cell responses for effective cancer therapy (Ahn, et al., 2014) (**Table S1**).

5.2 Immunosupression

Since inhibition of the immune system may decrease host resistance to infections and cancer, as well as lead to thymic suppression and myelosuppression, identification of

undesirable immunosuppressive properties of engineered nanomaterials is an important component of establishing their safety profile. (Ngobili & Daniele, 2016). While several mechanisms attributing certain structural properties of nanoparticles to their proinflammatory immunostimulatory effects have been described, it is a rather grey area when it comes to their immunosuppressive properties. As Iliknskaya et al. have pointed out, the effect a nanoparticle has depends on the model studied and the end points (e.g. cytokine secretion, cell adhesion, cell viability) evaluated. The same nanoparticles may be beneficial in one model and/or using one end point, and adverse when using another model or end point (Ilinskaya & Dobrovolskaia, 2014). For example, administration of organo-gold compounds has been utilized for nearly a century to treat inflammation but it was only until recently that the anti-inflammatory activity was attributed to the inhibition of cellular responses induced by interleukin 1 beta (IL-1β) (**Table S1**), involved both in the innate and the adaptive immunity (James, et al., 2015), which contradicts the GNPs' pro-inflammatory responses described in the previous section. Dependency both on NP size and surface charge was demonstrated, where GNPs with zwitterionic charges were shown to completely block the IL-1β pathway (Fernandez, et al., 2015), while only GNPs of specific sizes and at specific concentrations have been shown to interfere with TLR9 trafficking and, therefore, the activation of the innate immune system. Citrate-stabilized GNPs have been shown to prevent the development of pro-inflammatory responses initiated by IL-1β in THP-1 cells (Sumbayev, et al., 2013) and to attenuate TNF-α induction, triggered by CpG oligodeoxynucleotides(Ivanov, et al., 2007). Villiers *et al*. have shown that accumulation of GNPs in dendritic cells lowers IL-12p70 levels, which is directly involved in T lymphocyte activation and thus in the regulation of the antigen specific immune response (Villiers, Freitas, Couderc, Villiers, & Marche, 2010). In conclusion, despite a number of reviews and experimental studies that have been published outlining the effects of GNP-induced immunosuppression (Ilinskaya & Dobrovolskaia, 2014; Ngobili & Daniele, 2016), there are still a lot to be deciphered.

6. EPIGENETICS AND CANCER: THE ROLE OF GNPS

The term "epigenetics" defines the study of heritable modifications in gene expression patterns, which are not related to changes in primary DNA sequence (Probst, Dunleavy, &

Almouzni, 2009). Epigenetics play a critical role in establishing specific cell-type expression patterns and thus contribute in the normal development of an adult organism. The most important and well characterized epigenetic mechanisms regulating gene expression are DNA methylation, histone modifications, chromatin remodeling (Golbabapour, Abdulla, & Hajrezaei, 2011) and non-coding RNAs (Bonasio, Tu, & Reinberg, 2010). In general, it is suggested that diverse epigenetic mechanisms regulate gene function in a coordinated and complex manner resulting in a chromatin state which enables and/or prevents access of transcription factors into gene promoter sites (Golbabapour, et al., 2011). Furthermore, the epigenetic machinery has been implicated in a number of physiological processes [e.g. (i) genomic imprinting (E. Li, Beard, & Jaenisch, 1993; Reik & Walter, 2001), (ii) X chromosome inactivation (Avner & Heard, 2001; Panning & Jaenisch, 1998), (iii) development of an embryo and placenta (Hamad-Schifferli, Schwartz, Santos, Zhang, & Jacobson, 2002; Hemberger, 2007; Maltepe, Bakardjiev, & Fisher, 2010)] and disease processes including carcinogenesis (Al-Haddad, et al., 2016; K. C. Chen, et al., 2011) (Lehmann, et al., 2008; Toyota, et al., 2008). In general, it is considered that pathologic conditions may arise due to a deregulation of the epigenetic machinery (Egger, Liang, Aparicio, & Jones, 2004) by means of changes in DNA methylation patterns which in combination with alterations in histone proteins cause changes in gene expression levels (Ziech, et al., 2010). More specifically, in carcinogenesis, these epigenetic changes are considered as a non-genotoxic mechanism of tumor formation by repressing tumor suppressor genes (hyper-methylation) and/or activating oncogenes (hypo-methylation) among other mechanisms (Ziech, Franco, Pappa, & Panayiotidis, 2011).

GNPs have been shown to induce desirable or adverse posttranslational modifications in gene expression patterns when used either alone or as therapeutic nanocarriers. A number of limited studies have already shown the successful delivery of therapeutic agents into cells by using GNPs-based vehicles. To this end, studies have demonstrated the utilization of GNPs as an effective delivery system in cancer treatment. More specifically, miRNA-145 (found to be down-regulated in prostate and breast cancer cells) was chemically immobilized onto the surface of GNPs and efficiently delivered in these cells (Ekin, Karatas, Culha, & Ozen, 2014). In line with these observations, a further study has shown

the successful transfer of siRNA into HeLa cells, using an GNP-siRNA-PBAE [poly(-amino esters)] platform thus enhancing the overall efficiency of the delivery (Lee, et al., 2009). Finally, another study has demonstrated the use of GNPs when coated with Charge-Reversal Polymer in order to enhance the delivery, release and function of siRNA into HeLa cells (S. Guo, et al., 2010). Thus, GNPs have been proven to be an important tool for various biomedical applications and therapeutic strategies.

When not used as therapeutic agents, it has been demonstrated in a number of *in vitro* and *in vivo* studies, that GNPs can affect global DNA methylation, patterns and/or mediate histone modifications (Jennifer, 2013). In this context, Ng and colleagues have shown possible toxic effects when human fetal lung fibroblasts were exposed to GNPs. Indeed, they were shown that GNPs were able to induce epigenetic changes through chromatin remodeling leading to alterations in gene expression patterns and specifically in the up-regulation of miR-155 which is found to be implicated in various pathological conditions including cancer ((Ng, et al., 2011). In another *in vivo* study, when pregnant mice were injected intraperitoneally with various sizes of GNPs (40 and 100nm), during gestation, modifications on miRNAs expression levels in both fetal lung and liver tissues were noted with GNPs of a 100nm but not with those of a 40nm size (Balansky, et al., 2013). These data suggest a link between GNPs' size and induction of cytotoxicity which needs to be considered before further use in a clinical setting.

7. PREDICTING THE EFFECT OF LASER-INDUCED GNP HYPERTHERMIA WITH SIMULATIONS

In this section, we present some general examples of the response of GNPs upon laser radiation localized in a tumorous tissue. More specifically, the main aspects of the simulation are the following: i) determination of the optimal thermal effect of GNPs depending on radiation wavelength, NP size, impurities on Au etc, ii) optimization of the diffusion time GNPs in the tumorous tissue and iii) distribution study of the thermal effect of the GNPs inside the tumorous tissue. We performed some simulations for the hyperthermic effect and GNP diffusion within the tumor. For more details about these simulations please refer to the *Supplementary information* (**Table S3**, **Figures S1-2**).

In the first study, we simulated the electromagnetic scattering in a 10 nm spherical silica-core GNP coated with gold (Qian, Zhou, Too, & Chow, 2011). The density power of the incoming laser beam was set at 20 W/cm^2. The beam is considered CW and the incoming wave is plane. In **Figure 3a**, the plasmon peak is found at about 0.58 μm (580 nm). In this region, the heat losses are maximized and the gold nanoparticle absorbs 2.3x10^{-10} Watts of heat. This simulation, although trivial, provides crucial information necessary to select the laser type and power.

We then simulated the diffusion of GNPs inside the tumorous tissue of human cutaneous squamous carcinoma for a 24-hour period. The geometric characteristics of the carcinoma was based on a similar previous simulation carried out by Sankar and Zhang (Sankar & Zhang, 2015). The tumorous tissue is spherical with a radius of approximately 3 mm. The concentration of NPs injected into the tumor is about 40 μg/ml (Qian, et al., 2011). The volume of the injected solution is about 14 mm^3. The solution is injected into the center of the tumor and the NPs diffuse radially, following a Gaussian form, outwards into both tumorous (r ≤ 3 mm) and surrounding healthy tissue (r>3 mm), thus forming a concentration gradient (**Figure 3b**).

The final case study presents the thermal effect of GNPs in tumorous and healthy tissue. In this study, it is assumed that, all nanoparticles have the same size and the interparticle distances are large enough to prevent thermal interactions. The final results show that the generated heat is diffused through the tumor and the surrounding tissue, giving the temperature profile shown in **Figures 3c-d** after 10 minutes of radiation treatment. The intratumoral temperature surpasses the threshold for cell damage, while the temperature of the surrounding healthy tissue remains at a safe level. These results could be utilized in future applications for precisely targeting of tumor cells.

7.1 Comparison of the simulation data with experimental studies

The experimental data used for the simulations were obtained from Qian *et al* (Qian, et al., 2011). Some of the nanoparticles that Qian *et al*. used had silica core and Au in the outer layer. The optimal concentration for Au is 40 μg/ml (Qian, et al., 2011). Maximum absorption was found at approximately 550nm, which is very close to the wavelength found in the simulation performed herein. Qian *et al*. concluded that heating the tissue with 20

W/cm^2 can produce enough heat to cause cell death {Qian, 2011 #553}, while the simulations have shown that GNPs can produce sufficient heat to damage the cancerous tissue in about 15 minutes (**Figure S2**).

8. GNPs' CLINICAL APPLICABILITY

Despite the big strides that have been made in many successful proof-of-principle studies with GNPs and their applications in cancer therapy, imaging and drug delivery as well as in their preclinical evaluation in both *in vitro* and *in vivo* models, clinical translation of GNPs has been hampered by a number of reasons. Issues such as their toxicity, efficacy, dosage and administration route as well as their clearance are still unclear and require focused research and attention. *In vivo* targeted cancer imaging using nanoparticles has rarely been achieved and even fewer nanoperticles exhibited tumor targeting efficacy that is sufficient for potential molecular imaging or molecular therapy applications in the clinical setting (Cai, Gao, Hong, & Sun, 2008). Libutti *et al*. conducted a Phase I clinical trial with PEGylated colloidal gold for the delivery of recombinant human tumor necrosis factor (Libutti, et al., 2010) and, although no toxicity was reported, the results were rather disappointing. This formula, marketed as Aurimmune by Cytimmune Sciences, has now entered Phase II clinical trials for the treatment of head and neck cancer (Shao, et al., 2013). A completed Phase I/II trial with silica-gold nanoparticles for the plasmonic PTT treatment of coronary atherosclerosis, on the other hand, proved very efficient (Kharlamov, et al., 2015). Finally, AuroLase® Therapy, marketed by Nanospectra Biosciences, is an investigational photothermal therapy based on the administration of GNPs which is available only through FDA-approved clinical study sites (Stern, et al., 2016). In the following paragraphs, studies that have been conducted both *in vitro* as well *in vivo* to decipher the toxicity as well as the biodistribution and renal clearance of GNPs are being discussed. In general, one possible concern with the use of GNPs can be the toxicity and the efficiency of GNP elimination via the liver. An earlier study reported a 9% decrease in the content of gold in the liver after 6 months, following the intravenous injection of 40-nm colloidal GNPs (Sadauskas, et al., 2009). However, these hypothetical GNP-side effects can be disregarded for example in patients with poor cancer prognosis. To this direction, several GNP formulas have already entered clinical trials for cancer treatment,

including CYT-6091 (27 nm citrate-coated GNPs bound with thiolated PEG and TNFalpha) (Libutti, et al., 2010) and AuroShell® particles (~150 nm, silica core with a gold shell, clinicaltrials.gov identifier # NCT00848042 assessed 1/3/2017). Currently, very few ongoing clinical trials incorporate the use of GNPs and only one registered trial towards cancer treatment, that is, a spherical nucleic acid (SNA) gold nanoparticle NU-0129 targeting BCL2L12 in recurrent Glioblastoma multiforme or gliosarcoma patients (clinicaltrials.gov identifier # NCT03020017, assessed 1/3/2017).

8.1 GNPs and cytotoxicity

The issue of GNP cytotoxicity has been extensively studied and reviewed by a number of research groups (Alkilany & Murphy, 2010; Khlebtsov & Dykman, 2011) with results that are more often than not contradictory. GNPs have been found to be "nontoxic" according to many reports (Connor, Mwamuka, Gole, Murphy, & Wyatt, 2005; Shukla, et al., 2005), while other groups reporting on the 'toxic' nature of GNPs have shown that cytotoxicity is influenced by a number of factors. For example, Pan *et al*. found that smaller 1.4 nm particles triggered necrosis, mitochondrial damage, and induced an oxidative stress on all examined cell lines, whereas larger particles were relatively nontoxic (Pan, et al., 2009), a conclusion that was further supported by the findings of Tsoli *et al* (Tsoli, Kuhn, Brandau, Esche, & Schmid, 2005). A clear dependence on the surface charge of GNPs has also been established, where Goodman *et al*. found that cationic GNPs (2 nm in diameter) are toxic, while the same nanoparticles with a negatively charged surface were nontoxic (Goodman, McCusker, Yilmaz, & Rotello, 2004). These conflicting results could arise not only from variations in the GNPs' chemical/physical properties, but also on the cell lines and the dosage used. Patra *et al*, have shown that citrate-capped gold nanoparticles were found to be toxic to a human carcinoma lung cell line but not to human liver carcinoma cell line (Patra, Banerjee, Chaudhuri, Lahiri, & Dasgupta, 2007), while Pernodet *et al*, demonstrated that high concentrations of GNPs could penetrate the membranes and accumulate in vacuoles (Pernodet, et al., 2006). Vacuolar damage was also proposed to be the causative reason for cytotoxicity in another study; however, it was the large GNPs (45nm) that caused it rather than the small ones (13nm) (Mironava, Hadjiargyrou, Simon, Jurukovski, &

Rafailovich, 2010). Contrary to the findings of Patra *et al*., Villiers showed that GNPs are nontoxic in dendritic cells even at high concentrations {Villiers, 2010 #967}. As a matter of fact, the majority of the published studies negates the toxicity of GNPs especially if they are functionalized and not bare gold (Gannon, Patra, Bhattacharya, Mukherjee, & Curley, 2008; Murawala, Phadnis, Bhonde, & Prasad, 2009; S. Wang, et al., 2008). The toxicities of GNPs tested on various cell lines with information on their physicochemical properties and coatings, as well as their plasma circulation time are presented in **Table 2**.

A number of hypotheses have been proposed to explain the toxicity of GNPs observed by some researchers, as such phenomena were not observed upon administration of other colloidal metals. The conversion of Au(0) to Au(I) under the effect of certain sulfur-containing amino acids was proposed as one possible explanation (Khlebtsov & Dykman, 2011). Furthermore, the simplest GNP solution contains the core material (gold) and surface-bound stabilizing ligands, and, potential leftover chemicals from its synthesis. The observed toxicity from a GNP solution, therefore, could arise from any of these components. Therefore, evaluating the contribution of each component is essential to understand the origin of toxicity (Alkilany & Murphy, 2010). For example, Uboldi *et al*. suggest that the residues of sodium citrate adsorbed to the GNP surface are responsible for causing toxicity (Uboldi, et al., 2009){Uboldi, 2009 #1254}, while in a recently published *in vivo* study it was shown that the coatings usually applied to GNPs prior to administration might be degraded by proteolytic enzymes (Kreyling, et al., 2015). However, in an excellent review on GNP cytotoxicity by Khlebtsov *et al.*, it was suggested that the replacement of sodium citrate with other biocompatible modifiers such as PEG reduces the cytotoxicity. Furthermore, Khlebtsov *et al*. suggest that if the upper particle concentration limit does not exceed the threshold of 10^{12} particles/ml, particles even with small diameters (3-5 nm) are not cytotoxic and it is only really small GNPs that can induce cellular damage and apoptosis through their association with the DNA in the nucleus (Khlebtsov & Dykman, 2011).

In vivo cytotoxicity studies are quite limited compared to the available *in vitro* data. Stefan et al (Stefan, et al., 2013) examined the effects of chitosan-coated GNPs of two different sizes (12nm and 22 nm) in rats, and found that there could be brain damage caused especially in the case of the larger nanoparticles. Intravenously administered PEG-coated

GNPs to mice were found to induce acute inflammation and apoptosis in the liver (W. S. Cho, Cho, et al., 2009), while GNPs administered to both rats and mice by Pocheptsov *et al*. were found to accumulate to liver Kupffer cells and the spleen, respectively (Khlebtsov & Dykman, 2011). Similar findings in mice were published by Chen *et al*. (Y. S. Chen, Hung, Liau, & Huang, 2009). Further work by Cho *et al*. on the possible genotoxic effects of GNPs revealed differential expression of those genes associated with the cell cycle, response to stress, signal transduction, and the metabolism (W. S. Cho, Kim, et al., 2009), which was further supported by a more recent study by Balasubramanian *et al*. (Balasubramanian, et al., 2010). As in the case of *in vitro* studies, the general consensus is that GNPs are not cytotoxic *in vivo* unless administered at high doses (Khlebtsov & Dykman, 2011).

8.2 GNP biodistribution and clearance

Bioavailability studies conducted in mice showed that GNPs of small diameter (up to 10 nm) cross the gastrointestinal tract more readily than larger ones (i.e., 30-60nm) and can be subsequently located in blood, brain, lungs, heart, kidneys, spleen, liver, small intestine and stomach (Hillyer & Albrecht, 2001), while a study conducted by Zhang *et al*. demonstrated the presence of 13.5 nm GNPs even in the bone marrow (Zhang, et al., 2010). Interestingly, it was shown that intermediate GNPs (18nm) are more abundant and hence are taken up by the gastrointestinal tract more readily than smaller ones, which might be attributed to the protein corona that forms around these particles, a factor that should also be taken into account and which is further discussed below (Schleh, et al., 2012). The main pathways suggested for GNP uptake are the transcellular and the paracellular, across tight junctions for smaller size particles, while the larger ones rely exclusively on the transcellular pathway (Schleh, et al., 2012). Furthermore, it is worth mentioning that administered GNPs can be degraded and taken up as ions and then reform as nanoparticles in the tissues, which further complicates the effect of the initial size of GNPs and their bioavailability (Das, Debnath, Mitra, Datta, & Goswami, 2012). Intravenous administration of unmodified GNPs to rats revealed that the smallest nanoparticles studied (10nm) had the most widespread distribution, whereas the 50, 100 and 250 nm particles were only significantly deposited in the liver, spleen and blood (De Jong, et al.,

2008).Moreover, PVA coated GNPs administered to mice were found to accumulate in the liver (Wojnicki, et al., 2013). Similar experiments conducted in rats and rabbits with PEGylated GNPs (15nm and 50nm) were in agreement with the findings reported in the previously cited studies (Terentyuk, et al., 2009). Blanco *et al*. recently published a review to summarize the latest data on the design parameters of a nanoparticle for enhanced circulation, distribution, and uptake (Blanco, Shen, & Ferrari, 2015).

Clearance of GNPs takes place primarily through the kidneys. Filtration of particles through the glomerular capillary wall is highly dependent on molecule size. Molecules smaller than 6 nm are typically filtered, while those larger than 8 nm are not typically capable of glomerular filtration (Longmire, Choyke, & Kobayashi, 2008). The charge also plays a significant role in determining which particles are going to be filtered through, as it contributes to the formation of a protein corona which increases the overall diameter of the particle and at the same time determines its interactions with the fixed negatively charged renal capillary walls (Deen, Lazzara, & Myers, 2001). To account for enhanced blood circulation time, accumulation and retention at a tumor and rapid clearance through the kidneys, a number of recent *in vivo* studies propose the replacement of the extensively used PEG-coating with glutathione with very promising results (J. Liu, et al., 2013) (Simpson, Salleng, Cliffel, & Feldheim, 2013) (C. Zhou, Long, Qin, Sun, & Zheng, 2011).

9. CONCLUSION AND FUTURE PERSPECTIVES

In this review, we have presented the latest advances in cancer treatment based on the combination of GNPs with different types of radiation. As it has become apparent, GNPs present an indispensable part of the future armory against cancer due to a number of indisputable advantages over traditional treatment regimes, such as the ability to selectively target tumors and act as radiosensitizers for both IR and NIR. Despite their benefits, very few nanoparticle-based therapies have been approved to enter clinical trials and even fewer have been commercialized, like Doxil or Abraxane (Y. Mi, Shao, Vang, Kaidar-Person, & Wang, 2016). Most of these efforts have failed primarily due to the decreased efficacy observed in humans compared to animal models. Passive tumor targeting due to the EPR effect is challenged by more and more clinical data. For this reason, a deeper understanding of the tumor microenvironment is required, as the moderate increase of therapeutic GNP-

delivery by the EPR effect is not sufficient to cure cancer (Y. Mi, Shao, Z., Vang, J., Kaidar-Person, O., & Wang, A. Z. , 2016). There is also a growing consensus that delivery of nanoparticles within the tumor subvolume may be necessary but not sufficient to enhance therapy, and that subcellular targeting may be crucial in maximizing therapeutic efficacy (Ngwa, et al., 2017). Future efforts, therefore, should be directed towards deciphering the correlations among the *in vitro*, *in vivo* and patient results in order to gain a deeper understanding of the biological mechanisms behind the observed phenomena instead of the design of increasingly sophisticated nanoplatforms.

At the same time, failure of GNPs to accumulate at a tumor site and subsequent retention might lead to the prolonged circulation time of nanoparticles which eventually increases systemic toxicity. To address this issue, further characterization of the protein corona and the development of new techniques and protocols to standardize its composition across laboratories could be of help (Hamad-Schifferli, 2013). Most importantly, the protein corona could be exploited in order to achieve better bio-distribution and cell targeting, longer circulation times, reduced cytotoxicity and even increased drug loading and release. Moreover, the interactions of GNPs with components of the immune system should also be exploited in combinatorial treatment strategies of radiotherapy with immunotherapy and chemotherapy. In this review, the delivery of chemotherapeutic agents with the use of GNPs was not discussed, however the advantages of such platforms have been well documented elsewhere (Fontana, Liu, Hirvonen, & Santos, 2017). Tao *et al*., for example, developed gold nanorods co-loaded with doxurubin and an adjuvant CpG oligonucleotide to combine chemo- with immune-therapy (Tao, et al., 2014). These nanorods could also be used for PTT. Guo *et al*, combined PTT with immunotherapy using copper-sulfide NPs along with encapsulated CpG (L. Guo, et al., 2014). Gene delivery based on nanoparticle systems has also been proposed for cancer gene therapy (K. Wang, Kievit, & Zhang, 2016). The aforementioned examples further support the applicability of GNPs in cancer treatment either alone or combined with different traditional treatments. Most importantly, though, they underline the necessity to further develop techniques and protocols to standardize their use and progress from animal models to clinical evaluation. Modelling of the effect of GNPs on the targeted tumor cells, the surrounding healthy tissue and the immune system will be required, which should be based on detailed simulations like the one we have

included in this review. Finally, the complexity of biological systems and the interactions between their different components should be further elucidated in order to accurately predict GNP's toxicity, distribution and clearance.

FIGURE LEGENDS

Figure 1. Key aspects of optimized GNPs-based cancer therapy. a) Passive targeting of cancer cells by GNPs due to the EPR effect and active targeting achieved by the conjugation of ligands to the surface of GNPs. b) Dose enhancement due ionizing radiation (IR) and c) Localized hyperthermia due to non-ionizing radiation (NIR).

Figure 2: Venn diagram comparing the genes/proteins of "immunomodulation" and "inflammation/toxicity".

Figure 3. Examples of simulating GNPs' hyperthermic effect after laser (non-ionizing radiation; NIR) exposure. a) Heat losses vs wavelength. Plasmon peak is found at ~590 nm. b) Distribution of nanoparticle solution inside the tumorous tissue. c) Thermal distribution after 10 minute of continuous laser irradiation. d) Snapshots of thermal distribution during radiation.

TABLES

Cell line	AuNP-type	[AuNP]	Uptake time	Therapy type	Shape	size (nm)	Reference
MCF-7	AET-GNPs	3.85 nM	24h	X-radiation	sphere	10.8	(Kong, et al., 2008)
MCF-7	Glu-GNPs	15 nM	24h	X-radiation	sphere	10.9	(Kong, et al., 2008)
MDA-MB-231	Thiol -GNP	12 µM	24h	X-radiation	sphere	1.9	(Jain, et al., 2011)
HeLa	Citrate-GNP	1 nM	24h	X-radiation	sphere	14 50 74	(Butterworth, et al., 2010)
EMT-6	PEG-GNP	500 µM	48h	X-radiation	sphere	6.1	(C. J. Liu, et al., 2010)
HeLa	folic Acid-GNP	255 µM	6, 12, 24, 48h	X-radiation	_	50	(Khoshgard, et al., 2014)
MCF-7	Glu-GNP	100 µM	2h	X-radiation	sphere	16	(Rostami, Toossi, Sazgarnia, & Soleymanifard, 2016)

SK-OV-3	thio-glu-GNP	5 nM	1-24h	X-radiation	sphere	14.37	(Geng, et al., 2011)
HSC 313	Anti-EGFR-GNP	0.2 nM	40 min	514 nm laser	sphere	40	(El-Sayed, Huang, & El-Sayed, 2006)
HSC 3	Anti-EGFR-GNP	4 nM	40 min	540 nm laser	sphere	30	(Huang, Qian, El-Sayed, & El-Sayed, 2007)
MCF-7	aptamer−Ag-Au	_	30min	808 nm laser	brances	80	(P. Wu, Gao, Zhang, & Cai, 2012)
SK-OV-3	Anti-HER2 GNPs	5 μg/mL	_	628 nm laser	brances	60.4	(Van de Broek, et al., 2011)
MDA-MB-361	anti-HER2--PEG-GNP	4.8 mg/(g tumor)	48h	X-radiation	sphere	30	(Chattopadhyay, et al., 2013)
EMT-6	GNPs	2.7 g/(kg body weight)	-	X-radiation	sphere	1.9	(J. F. Hainfeld, et al., 2004)
Prostate cancer cells	goserelin -PEG-GNRs	0.1-10μg/(g body weight)	15min - 72 h	X-radiation	nanorods	_	(Wolfe, et al., 2015)

Tested *in vivo* using Foxn1 mice							
Tu-2449 Tested *in vivo* using GFAP-v-src transgenic mice	GNPs	4 g/kg	Injected for 15h	X-radiation	nanoprobes	11nm	(J. F. Hainfeld, et al., 2013)
MCF-7 Tested *in vivo* female BALB/c mice	PEG-GSNs-Transferrin	1mg/mL	6h	808 nm laser	nanoshells	30	(H. Liu, et al., 2012)
B16-F10 Tested *in vivo* in nude mice	Doxorubicin-GNPs	1μM	24	660 nm laser	sphere	20	(Nam, et al., 2013)

Table 2. GNPs' physical properties and their relative toxicity.

Chemical composition		Size (nm)	Shape	Concentration	Exposure time	Tissue Cells	Cytotoxicity
Water-soluble GNPs (Fan, Hung, Li, & Yeh, 2009)		5 10 30	various	31.6 µg/mL 71.1 µg/mL 71.1 µg/mL	5 days 3 days 3 days	hBMSCs HuH-7	cytotoxic non-cytotoxic non-cytotoxic
Aqueous GNPs –Au(0), Au(I), Au(III) (Shukla, et al., 2005)		3-8	various	10-100 µM	72 h		non-cytotoxic cytotoxic cytotoxic
Isolated GNPs (purified) (Karthikeyan, et al., 2010)		50	various	>300 nM <300 nM	24 h 24 h	BRPE	cytotoxic non-cytotoxic
GNPs citrate stabilized (Coradeghini, et al., 2013)		5 15	various	>50 µM	72 h	Balb/3T3 mouse fibroblasts	cytotoxic non-cytotoxic
Thioglucose-conjugated GNPs (Geng, et al., 2011)		14	sphere	5nM	8-96h	Ovarian cancer	non-cytotoxic
PEGylated GNPs conjugated to Herceptin 0 (Chattopadhyay, et al., 2013)		30nm	sphere	2.4 mg/mL	24h	Breast cancer (in vivo and in vitro)	subtoxic
GNPs conjugated to folate		50nm	Nano rods	12.5 µM		Human gastric carcinoma cells	non-cytotoxic

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