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Gold nanoparticles and cancer: Detection, diagnosis and therapy

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ABSTRACT

Gold nanoparticles (AuNPS) represent one of the most studied classes of nanomaterials for biomedical applications, especially in the field of cancer research. In fact, due to their unique properties and high versatility, they can be exploited under all aspects connected to cancer management, from early detection to diagnosis and treatment. AuNPs have thus been tested with amazing results as biosensors, contrast agents, therapeutics. Their importance as potent theranostics is undoubted, but the translation to clinical practice has been hampered by a series of aspects, such as the unclear toxicity in humans and the lack of thorough studies on reliable animal models. Still, their potential action is so appealing and the results so impressive that an outstanding number of papers is being published every year, with the consequence that any review on this topic becomes obsolete within a few months. Here we would like to report the latest findings on AuNPs research addressing all their functions as theranostic agents.

1. Introduction

Engineered nanomaterials massively emerged about two decades ago, at the beginning of the third millennium, and revolutionized most of the technological and scientific fields [1]. Human health was not an exception, and the term "nanomedicine" [2] was coined to describe the application of nanoscience and nanotechnology to the therapy, diagnosis and prevention of diseases, especially cancer, for which the superiority of nanomedicine over conventional treatments was immediately recognized. In fact, traditional anticancer therapies lack in selectivity and specificity, affecting healthy cells as much as sick ones. Nanomaterials, instead, not only can be designed for tumour targeting, drug delivery and enhancing conventional cancer immunotherapy, but also to create biosensors, increase imaging performances, repair damaged tissues: they can act both as therapeutic and diagnostic tools, for which another new term was coined: "theranostics" [3,4]. This would lead to the development of the so-called "personalized medicine", one of the most crucial challenges for the future of healthcare [5].

Despite the so many evident benefits brought to the biomedical field by nanomaterials, only a few of them made it to the clinical trials [6],

Abbreviations: A549, adenocarcinomic human alveolar basal epithelial cell line; acpcPNA, (2′R,4′R)-nucleobase-substituted proline and (1S,2S)-2-amino-cyclopentanecarboxylic acid; ADAM10, A disintegrin and metalloprotease domain 10; ASGPR, asialoglycoprotein receptor; AuNPs, gold nanoparticles; Bax, apoptosis regulator protein, or bcl-2-like protein 4; C-6, glioma cell line; C11Pc, a phthalocyanine derivative; CCD-841, epithelial-like cell line; CCND1, cyclin D1 protein; COS-7, African green monkey kidney fibroblast-like cell line; EGCG, epigallocatechin gallate; EGFR, epidermal growth factor receptor; EPR, permeability and retention; EW-CRDS, evanescent wave cavity ring-down spectroscopy; FITC, fluorescein isothiocyanate; GSH, glutathione; GM2AP, GM2 activator protein; H520, human lung cell line; HCT-116, colon carcinoma cell line; HEK 293, human embryonic normal kidney cell line; HeLa, cervical cancer cell line; HepG2, hepatocellular carcinoma cell line; HIV, human immunodeficiency virus; HPV, human papillomavirus; HT-29, human colorectal adenocarcinoma cell line; LSPR, localized surface plasmon resonance; MCF-7, breast adenocarcinoma cell line; MDA-MB-231, triple-negative breast cancer cell line; MICU1, mitochondrial calcium uniporter 1; MMP, mitochondrial membrane potential; MMP-2, matrix metalloproteinase-2; MRI, NMR imaging; NCF, normal neutrophil chemotactic factor cell line; NIR, near infra-red radiation; NPs, nanoparticles; PARP, poly (ADP-ribose) polymerase; PC3, prostate cancer cell line; PDPN Ab, podoplanin antibody; PEG, polyethilenglycol; PLK1, polo-like kinase 1; PrPC, prion protein; PSA, prostate specific antigen; PSMA, prostate specific membrane antigen; QCM, quartz crystal microbalance; ROR1, receptor tyrosine kinase-like orphan receptor 1; ROS, reactive oxygen species; SERS, surface-enhanced Raman scattering; SiNC, Si(IV)-naphthalocyanine; SpCas9, CRISPR associated protein 9; SW-948, human colorectal adenocarcinoma cell line; TAT, trans-activator of transcription protein; TRPV6, transient receptor potential vanilloid subfamily member 6 channel; WRL-68, hepatic cell line.

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and an even lower number were clinically approved and commercialised, such as liposomes and iron nanoparticles for NMR imaging (MRI) or photodynamic therapy [7,8]. The key aspect in the nearly systematic failure of nanomaterials in clinical translation, lies in the almost chronical lack of any assessment of the potential nanodrug under the pharmacological point of view, that is, almost no evaluation of its intrinsic toxicity, solubility, stability in physiological conditions, bioavailability, proneness to biotransformations etc., is carried out before its application to *in vitro* cell cultures or *in vivo* models. Nevertheless, the increasing incidence of cancer and other neoplasms demands that these new formulations could find real, safe and widespread application, thus their design should take in consideration also the abovementioned properties, including their potential toxicity, about which researchers are still debating, and the need of improving traditional testing on animal models [9–11].

Anyway, among the highly promising nanomaterials, gold nanoparticles are one of the most extensively studied, due to their outstanding and probably unique physical, chemical, optical and electronic properties, that make them suitable for the creation of highly multifunctional platforms for biochemical applications [12,13]. Moreover, their shape can be modelled into a variety of forms, such as nanocages, nanoboxes, nanorods, nanowires, hollow nanospheres, and nanoflowers (also called nanourchins or nanostars), each with its peculiar characteristics, behaviour and applications. They can be made out of pure gold, form composites (coated with polymers, such as polyethilenglycol (PEG), and other organic compounds) or be "doped" with other metals (Ag, Se, Mo, Mn, Pt, Fe_3O_4, for instance) to give new hybrid materials which can be further capped, functionalised or conjugated with drugs or other molecules for cell targeting and drug delivery. As theranostics, they show high direct anticancer activity, but are also active in the photothermal/photodynamic therapy, in biosensing and immunoassay, as contrast or imaging agents, or nanozymes [12].

Here we would like to give an account of the most recent findings and applications of gold nanoparticles in the field of cancer detection, diagnosis and treatment. In less than five years (2017-early 2021) more than 4000 articles and reviews have been published on this topic, nearly one thousand per year (sources: Scopus, Pubmed, Google Scholar), showing an impressive interest for these nanostructures and the remarkable efforts done to carry out research in the diagnosis and treatment of cancer with gold nanotechnology, resulting in a rapid "outdating" even of the most recent papers.

For evident reasons, it will not be possible to include all these works in the present review, that will be necessarily limited to the last 2–3 years. Therefore, the authors want to apologize for those that will be inevitably excluded.

2. Gold nanomaterials in cancer therapy

Thousands of AuNPs, together with other inorganic nanoparticles, have been prepared for anticancer applications during the past decades, with different fortunes. In fact, a nanomaterial should possess precise chemical-physical properties to exert a therapeutic activity, such as adequate composition, size, shape, surface charge, coating, inner structure, lipophilicity/hydrophilicity, etc. (see for instance [14]). Small particles (<10 nm) are cleared by the kidneys, big ones (> 200 nm) are eliminated by liver and spleen; too small, they tend to be more reactive and can sometimes cause haemolysis; too big, they are not able to interact with cells and thus are ineffective [15]. The best dimensions to increase circulation times and effective delivery at the target site seem to fall in the range 10–100 nm, the optimal size being around 20 nm, to ensure the longest permanence in the blood stream, the most efficient tumour cell internalization and the highest concentration and retention inside the target cell. Yet, water-dispersible ultrasmall (< 2 nM) AuNPs, can be more biocompatible, exert minimal toxicity, and can be eliminated through renal clearance.

The shape of a nanoparticle, as well, can influence its permanence in the blood circulation and accumulation at the desired site, with non-spherical particles having a longer circulation time [15]. The surface charge, on the other hand, is responsible for the nanoparticle interaction with the cell and the internalization pathways, with neutral and anionic charged particles having higher cell permeability. The main mechanism through which this happens is endocytosis, a process leading to the engulfment of NPs in membrane invaginations, followed by formation of endocytic vesicles, which are then transported to specialized intracellular compartments. Endocytosis of nanoparticles can be reconducted to five main types: phagocytosis, clathrin-mediated endocytosis, caveolin-mediated endocytosis, clathrin/caveolae-independent endocytosis, and micropinocytosis, depending on the kind of cells involved and proteins, lipids, and other molecules taking part in the process [16]. It must also be reminded that most of the nanoparticles do not undergo degradation *in vivo*, and that AuNPs, although remarkably inert, biocompatible and safe, will remain inside the cell for a relatively long time. All these aspects should be carefully considered in the design of successful AuNPs for anticancer applications, to have optimal internalization of the nanoparticles while avoiding exceeding accumulation and toxicity.

2.1. Direct anticancer action

Bearing these considerations in mind, the availability of new AuNPs with anticancer activity during the past few years has considerably increased to offer a remarkable variety of shapes, morphologies, sizes, surface ligands, with the opportunity to tune their action against tumour cells. Coating with proper polymers can also be beneficial in improving the performances of these nanoparticles; for instance, PEG is useful in increasing their solubility, reducing aggregation and nonspecific binding, and thus can enhance their biocompatibility and circulation half-life. Moreover, the introduction of the so-called "green synthesis" for this kind of nanomaterials, in alternative to the "classical" reduction by e.g. hydrazine or sodium borohydride, improved not only their sustainability, but also their stability and biocompatibility (read for instance [17–19]). Biological fluids derived from various organic sources, going from plant extracts to bacterial cultures, are able to reduce gold salts into the nanometallic forms in one-pot, low temperature, eco-friendly processes, where the biomolecules present in the fluid not only carry out the reduction reactions, but are also deposited on the nanoparticle surface, forming an organic layer, or coating, able to stabilize the particles in small structures that tend to aggregate much less than the "classical" counterparts, and show a lower cytotoxicity towards the healthy cells while maintaining their activity against the cancer ones. This is a characteristic often found in biogenic AuNPs (*vide infra*). Moreover, the antineoplastic action of known biomolecules is enhanced when they are used as the reducing/capping agents in the synthesis of "green" AuNPs [20].

To quote just a few examples among the hundreds reported to date, the case of AuNPs prepared from flaxseeds (*Linum usitatissimum*) that were tested against different cancer cell lines (i.e. hepatocellular carcinoma HepG2, breast adenocarcinoma MCF-7, and colon carcinoma HCT-116), but were remarkably active and potent especially towards breast cancer cells [21]; gold nanoparticles synthesized from the extracts of a marine bacterium (*Vibrio alginolyticus*) were particularly effective against colon carcinoma through an apoptotic mediated cell death. They evidenced a dose dependent inhibitory effect on the growth of cancer cells with an IC_{50} of 15 μg/mL, and the maximum inhibition of the cell death (>75 %) recorded with a concentration of 25 μg/mL [22,23]; *Thymus vulgaris* leaf extracts produced "green" AuNPs that were found to be more active than doxorubicin in a myeloid leukemic rat model [24]; dragon fruit (*Hylocereus undatus*) extracts allowed the preparation of gold nanoparticles that displayed a significant growth inhibition on MCF-7 breast cancer cells, while no substantial toxicity was observed on mortal cells, thus indicating biocompatibility [25].

Not only plant extracts have been used to produce active AuNPs, but

also plant-derived reductants owing therapeutic properties *per se*, and the resulting nanomaterial had increased action once tested against cancer cells compared to the biomolecule alone. This has been seen, for instance, in the case of curcumin, a compound present in *Curcuma longa* (turmeric) to which the anti-inflammatory, anti-oxidant and anticancer activities of the tuber are ascribed [26], that has been extensively applied to the preparation of effective gold nanoparticles. AuNPs biosynthesized using curcumin as the reductant can assume a spheroidal shape and be highly stable to aggregation, up to six months. They were active against colon (MCF-7) and breast (HCT-116) cancer cell lines, evidencing increased antiproliferative and apoptotic action compared to free curcumin [27], or against C-6 glioma cells [28], and showed to release curcumin only under physiological conditions. With an analogously "green" synthesis, a gold nanocluster conjugated with curcumin was prepared and tested *in vitro* against mortal (COS-7) and cervical cancer (HeLa) cell lines, showing a much higher toxicity in the latter case respect to the former [29]. The fluorescence of this gold nanocluster also suggested a potential use in imaging applications. A mixture of curcumin-AuNPs together with other gold nanoparticles obtained from gold reduction with turmeric, quercetin, and paclitaxel indicated a synergistic action of the nanoparticles and was significantly efficient in inhibiting cell proliferation, angiogenesis, and colony formation in different breast cancer cell lines (MCF-7 and MDA-MB 231). All these nanoconjugates, alone or in association, were not cytotoxic towards human embryonic normal kidney cell line (HEK 293) [30]. Moreover, curcumin-AuNPs anchored on graphene oxide tested against human colon cancer cell lines (HT-29 and SW-948) showed concentration- and time-dependent cytotoxicity with relatively low IC_{50} values (~100 μg/mL), being at the same time non-toxic towards normal human colon (CCD-841) and liver cells (WRL-68), with high selectivity [31].

A probable mechanism behind the anticancer activity of these nanoparticles can be found in the biotransformations they undergo after administration. In fact, as soon as the curcumin-AuNPs are in the biological environment, they bind proteins onto their surface [32], which help their internalization. Once inside the cancer cell (e.g. MCF-7), they are able to induce apoptosis. The process involved seems to be connected with the generation of ROS that, by disrupting the mitochondrial membrane potential (MMP), triggers the release of Bax, a pro-apoptotic protein, which in turn starts PARP cleavage and DNA fragmentation, thus causing cell death [33]. The anticancer properties of both curcumin and AuNPs, and the recognition of their synergistic action, led to the synthesis of new nanomaterials or nanocomposites in which curcumin was loaded onto the nanoparticles for cancer detection, drug delivery, selective release at target sites (e.g., in pH-responsive formulations) [34–36], or a combination of them [37], and related therapies [38].

The analysis of the literature on this topic revealed that in most of the cases the mechanisms through which AuNPs exert their anticancer actions are linked to apoptosis caused by ROS generation. For instance, the sequence of biological events connected with cell death in the treatment of ovarian cancer with AuNPs has been thoroughly investigated [39] and showed that formation of ROS caused a state of oxidative stress in the cell that could not be compensated, with subsequent damage of the genetic material and biological membranes, and finally initiation of apoptosis. Analogously, oxidative stress and mitochondrial dysfunctions were evidenced in the case of breast cancer cells treated with AuNPs [40], and a caspase-dependant, mitochondrial-mediated apoptosis in cervical cancer (HeLa) cells [41].

2.2. Tumour targeting and drug delivery

Nanoparticles in general can be exploited to carry drugs or other molecules to tumour tissues for multiple purposes (therapy, imaging, sensing, etc.); this strategy allows a faster delivery of the pharmaceutical species to the site of action, longer permanence in the tissues with reduced clearance or deactivation, and consequently lower doses and lower toxicity respect to the drug alone (Fig. 1) [42]. The carrier function is thus the delivery of various cargoes to targeted cells, and to do so it should be able to enter those cells and then release its cargo to perform the intended functions, generally via pH responsive stimuli, redox changes, etc.

Drug delivery can be made selective by appending onto the NPs surface a "trojan horse" able to interact with cancer cells that will recognize the target site thanks to a specific surface receptor; the targeting moiety often recognises a receptor that is overexpressed by tumour cells. This is referred to as the active transport and is typically achieved through the conjugation of the nanoparticles with antibodies, proteins, peptides, aptamers, carbohydrates, hormones, and low molecular weight molecules like folic acid. Furthermore, active transport can be obtained by triggering physical stimuli, such as pH and temperature of the environment [42].

NPs can also exploit passive transport to increase uptake, that is mainly due to the enhanced vascular permeability and retention (EPR) effect [43], caused by the "leaky" walls of blood vessels in tumours, that allow permeation also to the relatively big (compared to usual drugs)

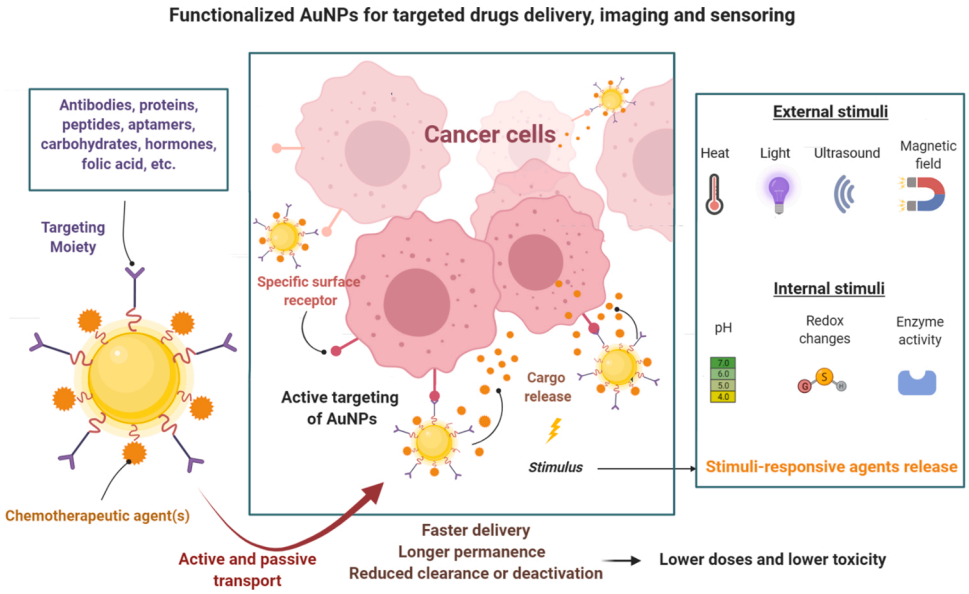

Fig. 1. Multifunctional AuNPs theranostic: targeted drug delivery, imaging and sensing in cancer cells.

nanoparticles. Moreover, NPs can accumulate inside the cancer tissues also because they show poor lymphatic drainage respect to healthy ones. Thus, nanoparticles have a facilitated uptake by cancer cells due to EPR effect, but they are not cleared away as efficiently, and their concentration selectively builds up inside the sick tissues compared to normal ones [44]. The two mechanisms, active and passive transport, are obviously synergistic since they can work together to enhance the efficacy of a nanodrug. And in fact, a relevant number of nanoformulations proposed for cancer therapy include conjugation with both a drug and a targeting agent for optimal performances.

AuNPs are particularly suitable for tumour targeting and drug delivery [45] due to their biological and chemical-physical properties, especially in terms of low cytotoxicity, ease of preparation and functionalization, stability and biocompatibility. Moreover, they add their intrinsic anticancer activity to that of the drugs they carry, further enhancing their effects [46]. Since AuNPs are generally not porous and rarely hollow, the best way to carry the active molecules is to append them onto their surface, and this can be done after proper functionalization and derivatization. Functionalization is normally carried out by reducing gold salts in the presence of thiols [47] or thiol-derivatized molecules; in this way, strong, non-labile, covalent Au-S bonds spontaneously form through thiol surface adsorption, which are very stable even respect to pH changes [48]. This process is also a key aspect for AuNPs toxicity because, if not properly functionalised to allow filtration by the reticuloendothelial system, they can accumulate in lymph nodes, spleen and liver [44].

A number of therapeutic agents have been appended onto the surface of AuNPs to be tested in drug delivery for cancer therapy. For instance, taxol (paclitaxel) [49] and its derivatives (e.g. docetaxel) [50], doxorubicin [51–53], methotrexate [54–57], 5-fluorouracile [58–60], cisplatin [61], etc.

Paclitaxel (taxol) is used to treat a number of tumours, including ovarian, breast, esophageal, lung, cervical, and pancreatic cancers, and Kaposi's sarcoma. It is poorly soluble in biological fluids and this limits its performances. For this reason, the use of taxol in the preparation of AuNPs conjugates (alone or in association with other active molecules) can enhance its bioavailability and cytotoxicity [49]. An example is given by a nanocomposite material used to delivery paclitaxel to mitochondria, based on epigallocatechin gallate (EGCG)-capped AuNPs, which were functionalized with poly-D-lysine grafted polyethylene glycol and triphenylphosphonium cation [62]. The latter is specifically used for mitochondrial targeting, as mitochondria became one of the latest objectives in anticancer therapy, since their impairment leads to the death of the whole cell. In this case, the nanocomposite was particularly active against human cervical carcinoma (HeLa) cells, preferentially localizing in the mitochondria as expected, and causing cell death via a caspase-dependent apoptosis mechanism. Moreover, it showed an enhanced activity compared to both the untargeted nanoparticles and the free drugs.

Docetaxel is a taxol derivative used to treat breast, head and neck, stomach, prostate and non-small-cell lung cancers. Its limits are again those connected to low solubility and high toxicity. To circumvent these drawbacks, docetaxel has been tested in nanoformulations directed to several lines of tumours. For instance, it was encapsulated by the non-covalent linkage method within AuNPs specifically designed for drug delivery to prostate cancer (PC3) cell lines. For this task, they were PEGylated with thiol-PEG-amine (SH-PEG-NH_2), and then conjugated with folic acid for cancer cells targeting. The results were encouraging, since they obtained a 40 % reduction of cell viability in a dose-dependent way [63]. In another case, the drug was inserted into AuNPs by reduction with sodium borohydride of the corresponding Au (III) complex containing also a dicarboxylic acid-terminated PEG. Upon reduction, hybrid nanoparticles were produced, where docetaxel was protected in the gold core embedded within the polymer chains. Finally, the nanomaterial was combined with the human anti-EGFR polyclonal antibody (which recognizes the hERG1 channel overexpressed on the membrane of human lung cancer cells) for therapeutic targeting [64]. Another nanocomposite for targeted drug delivery towards lung cancer (this time H520 cell lines) was produced by conjugating docetaxel and folic acid to AuNPs, the former via non-covalent interactions, while the latter was covalently bond [50]. This nanoformulation exhibited excellent binding specificity, good cytotoxicity, and biocompatibility.

Doxorubicin is a chemotherapeutic used to treat breast and bladder cancers, Kaposi's sarcoma, lymphoma, and acute lymphocytic leukemia. It has been employed to prepare polyvinylpyrrolidone-stabilized AuNP for drug delivery. The conjugated nanoformulation effectively released doxorubicin inside lung cancer cells, which were then brought to death via a ROS-mediated apoptotic pathway [65]. Moreover, the new nanomaterial was also able to disrupt mitochondrial membrane potential, inducing both early and late apoptosis, and upregulate the expression of tumour suppressor genes. Furthermore, pectin-capped gold nanoparticles were conjugated with doxorubicin for active transport to hepatocellular carcinoma (HepG2) cells overexpressing asialoglycoprotein receptor (ASGPR) [66]. Pectin had a triple role: reducing, stabilizing and targeting agent. These nanoparticles evidenced excellent stability to pH changes and modifications of the electrolytic conditions, and higher potency in killing cancer cells as compared to the free drug. Moreover, it was demonstrated that the nanoparticles were uptaken by HepG2 cells via a clathrin-dependent receptor-mediated endocytosis by asialoglycoprotein surface receptor. A different receptor was exploited for the targeting of colorectal cancer: the cellular prion protein PrPC, a cell surface glycoprotein overexpressed in this kind of cancer. So, the relative PrPC aptamer was conjugated to gold nanoparticles via the terminal thiol moiety, followed by hybridization of its complementary DNA for drug loading. Finally, doxorubicin was loaded onto the functionalized nanoparticles and tested for its anticancer properties [67]. The gold nanocomposite was able to generate ROS by decreasing catalase and superoxide dismutase activities. It was also observed that this nanomaterial inhibited mitochondrial functions by knocking down a series of mechanisms, from expression of peroxisome proliferator-activated receptor gamma coactivator 1-alpha, to complex 4 activity, and oxygen consumption rates.

In a different study, PEGylated AuNPs were conjugated with doxorubicin in association with another anticancer drug, bleomycin [68] and tested against HeLa cells. The nanohybrid benefited from the active targeting of the ovarian cancer cells and enhanced cytotoxicity due to the synergistic action of the two drugs, so that the effective drug concentration was reduced of a 50 %, with promising applications in therapy. Other different cases of co-delivery of multiple anticancer agents in association with doxorubicin have been reported in the literature. One is that of 5-fluorodeoxyuridine, where the two drugs were both loaded onto an affibody. The relative gold nanohybrid was assessed against HER2 positive breast cancer, inducing cell apoptosis, with a higher inhibition in HER2 overexpressing breast cancer cells respect to a simple mixture of the two drugs [69]. A second case is the co-delivery of doxorubicin and an aptamer against Forkhead box M1 obtained with a nanocomposite of chitosan-gold nanoparticles coated with a nucleolin aptamer. A549 and 4T1 cells, nucleolin+, were effectively able to internalize the nanoparticles, while Chinese hamster ovary cell and non-targeted cells were not, and also cell mortality followed the same trend [70].

Within the topic of AuNPs-mediated delivery, gene therapy finds considerable room. It consists in the use of exogenous DNA or RNA to treat a number of pathologies, among which, of course, cancer [71]. A considerable number of papers appeared in the literature during the past years to report about the preparation and results obtained with AuNPs designed for gene delivery [72], especially in consideration of the fact that genetic material is prone to fragmentation during its journey towards the target, but conjugation with AuNPs seems to protect nucleic acids, preventing their degradation by plasma and tissue nucleases [73]. Short interfering RNA (or silencing RNA, siRNA), is a class of double-stranded, non-coding RNA oligonucleotides, generally 20–27

base pairs long, that interfere with the expression of specific genes with complementary nucleotide sequences by degrading mRNA after transcription, thus hampering translation. A number of siRNA were reported to be involved in several cell processes, such as metabolism, metastasis, cell death, angiogenesis, and immunosuppression in cancer. Different types of nanovehicles have been used to deliver siRNA, such as lipid-based (e.g. liposomes) and non-lipid based (e.g. chitosan, cyclodextrins, dendrimers) nanovectors [74]. However, gold nanoparticles seem to be highly suited for this task, due to their chemical-physical and biological properties, and the possibility of multifunctionalization that allows different but synergistic anticancer properties [75]. The number of siRNA-based AuNPs for gene silencing has thus constantly increased during the past few years [76–81]. The nanovehicle based on an AS1411 aptamer modified, dsDNA and MMP-2 cleavable peptide-fabricated gold nanocage was loaded with both siRNA and doxorubicin, for a synergistic, triple attack to lung cancer: tumour-responsive genetic therapy, chemotherapy, and photothermal treatment [82]. This nanocomposite was administered to mice every three days for one month, after which the eradication of long-lived tumours was observed, with tumour inhibition higher than 90 % and a survival rate of approximately 67 % compared to the control group (passive genetic treatment) that recorded a 30 % tumour inhibition and 0 % survival rate. Analogously, co-delivery of doxorubicin and polo-like kinase 1 (PLK1) siRNA was made possible by using gold nanoparticles coated with polyethyleneimine to facilitate assembly of PLK1 on their surface. Doxorubicin was loaded on these AuNPs through a pH-sensitive linker carrying a thiol group at one terminal end for controlled release. Again, a synergistic effect of combined drug and gene delivery was demonstrated [83].

In another study, ADAM10 siRNA was loaded onto a hybrid nanomaterial, where a core of magnetic Fe_3O_4 was coated with a shell of gold nanoparticles, which in turn was capped with polyethylenimine. An outer layer of hyaluronic acid was added to improve biocompatibility. ADAM10 siRNA was then charged onto its surface, and tested against prostate cancer (PC3) cells, suppressing their growth *in vitro* [84]. Knockdown of HER2 gene in MCF-7 cells was attempted with multi-layered AuNPs obtained with 11-mercaptoundecanoic acid, calcium chloride and polyethyleneimine deposited in alternate charge successive layers which were finally coated with HER2 siRNA [85]. The new nanocomposite was tested against breast cancer (MCF-7) cells, showing an IC_{50} of 45.35 nM. The real time PCR based delta-delta Ct analysis evidenced a significant decrease (around 19 times) in the expression of HER2 gene as compared to untreated cells.

MICU1 is a novel glycolytic switch in ovarian cancer, and it was targeted by MICU1 siRNA delivered via an AuNPs-doped liposomal formulation [86]. The presence of gold nanoparticles prevented liposome degradation within lysosomes. Low-dose siRNA via transfection or conventional liposomes was not effective in MICU1 silencing, while the same dose carried by the auroliposome nanoformulation gave >85 % gene silencing. Both *in vitro* growth assays on ovarian cancer cells and *in vivo* tumour growth in human ovarian cell line and patient-derived xenograft models showed a high efficacy of this new siRNA vector.

ROR1 is an onco-embryonic gene overexpressed in many malignancies, thus its suppression by siRNA can represent a good strategy against cancer. ROR1 siRNA delivery was studied with AuNPs capped by HIV-1 TAT peptide and tested against breast cancer [87]. The positively charged nanoparticles were used in low concentrations because they showed high cellular uptake, leading to high gene transfection and negligible cytotoxicity. Subsequent downregulation of ROR1 and its targeted gene, CCND1, induced apoptosis in cancer cells.

Analogously to RNA interference (RNAi), examined above, where siRNA can be used to inhibit the expression of a specific mRNA for gene silencing, DNA interference (DNAi) represents a novel approach to inhibit transcription [88]. DNAis are single stranded sequences, 20–34 bases long, designed to bind to upstream DNA sequences of transcription initiation sites. Hybridization of the DNAi oligonucleotides to their target region leads to gene expression modulation at mRNA and protein levels. This approach was exploited to target Bcl-2 oncogene in breast cancer (MCF-7) cells by DNAi via the conjugation to AuNPs. Cellular uptake of these nanoparticles is size-dependent, with internalization of AuNPs around 42 nm being more efficient respect to smaller sizes (~14 and ~26 nm). Moreover, DNAi-conjugated gold nanoparticles were effective in silencing the targeted Bcl-2 gene, as expected, thus inducing apoptosis [89].

Not only siRNA and DNAi oligonucleotides have been loaded onto AuNPs for gene therapy, but also the CRISPR/Cas gene editing system was tested in this perspective. Gold nanoclusters (AuNCs) with SpCas9 protein self-assembled under physiological conditions were efficient in the delivery of SpCas9 into the cell nucleus. It was also demonstrated that the assembly process is highly pH-dependent and that a lower pH was able to cause SpCas9-AuNCs disassembly. This assembly-disassembly mechanism favours the delivery of SpCas9 into cells and cell nucleus, where it exerts its cleavage function. The SpCas9-AuNCs were effective in the knockout of the E6 oncogene, re-establishing the correct function of tumour-suppressive protein p53. They also induced apoptosis in cervical cancer cells with little effect on normal human cells [90]. Another kind of gold nanocluster was used to transport Cas9-sgRNA plasmids into cells and release them to achieve efficient genome editing. The AuNCs were capped with protamine and quickly assembled with Cas9-sgRNA plasmids to produce efficient nanocarriers for cellular delivery, while cationic protamine helps the release of Cas9-sgRNA plasmids into the cellular nucleus. These gold nanocomplexes are able to carry out successful gene editing in cells, but they can also knock out oncogenic genes for cancer therapy. In addition, the AuNCs are highly photoluminescent, so this new multifunctional nanoplatform can also be exploited for bioimaging [91].

These are just a few examples of the enormous number of research papers appeared in the literature during these very few years, indicating that tumour targeting in association with effective drug delivery carried out by properly designed gold nanoparticles is a very active field, with remarkable results that encourage further research with the aim of finding the optimal combination of drugs, coatings and carriers for gold nanoparticle-based precision therapy.

2.3. Photothermal and photodynamic therapy

One of the most characteristic aspects of AuNPs are their outstanding optical properties, as they are able to absorb and scatter light with remarkable efficiency. The interaction of metal nanoparticles with light at specific wavelengths causes the collective oscillation of the conduction electrons on their surface. This oscillation, known as a localized surface plasmon resonance, determines the absorption and scattering intensities of AuNPs, which are consistently higher than identically sized non-plasmonic nanoparticles [92]. Thus, AuNPs absorb visible light with extinction coefficients several orders of magnitude higher than those of many highly absorbing organic dyes. The frequency of the absorption band is strongly correlated with both size and shape of the AuNPs [93]; this is an important aspect, since the absorption of gold nanoparticles can be controlled by tuning these two determining factors. The presence of surface plasmon resonance in AuNPs is critical for their clinical applications, because the strong absorption of light makes the nanoparticles useful as therapeutic agents in the so-called photothermal and photodynamic treatments [94].

When AuNPs are irradiated with light of the same wavelength of their surface plasmon absorption band, their surface electrons are excited and resonate intensely, so that light is rapidly (about 1 ps) converted into heat. In this way cancer cells can be heated up to 41–47 °C for tens of minutes and are destroyed by the induced hyperthermia in a process of photothermal ablation, known as photothermal therapy (read for instance [95,96]). This technique with AuNPs has several advantages: their resonance wavelength allows for high absorption in the near infrared (NIR) region, since in this range (between 800 and 1200

nm) the body tissues are moderately transparent to NIR light, that can thus penetrate more deeply in cancer tissues, for a more efficient treatment; photothermal therapy with AuNPs can be combined with targeted drug delivery or enhanced tumour detection, thus increasing their efficacy [97–99]. The gamut of such gold-based nanomaterials is wide and has reached high levels of technology and complexity. This is the case, among the others, of stimuli responsive nanodevices based on the DNA structure. pH changes have often been exploited with this aim, like in the case of i-motif DNA that could be folded into four-stranded (C-quadruplex) structure when the pH of the environment varies from 7.4 to 5.0, which is important since extracellular pH in cancer tissues is often slightly more acidic than physiological values. This pH responsive i-motif DNA was conjugated to AuNPs, together with MUC1 aptamer as the targeting moiety to enhance specific cellular uptake. Finally, doxorubicin was loaded onto this nanodevice by intercalation in the CG base pairs of the DNA [100]. The nanovehicle had an excellent photothermal conversion efficacy under 808 nm near-infrared (NIR) irradiation due to the presence of AuNPs, and anticancer activity supplied by doxorubicin, with a synergistic effect of the chemo- and photothermal therapies.

Photothermal therapy was also performed with NIR photoactivated fluorouracil-gold nanoparticle complexes, that produced a mild hyperthermia for the intraperitoneal treatment of colon cancer metastasis, together with a chemotherapeutical action [101]. In another study, small-sized gold nanoparticles (~3 nm) coated with glutathione (GSH) were employed. These AuNPs were pH-sensitive, responding to changes in pH conditions, especially between normal (~7.4) and cancer tissues (6–6.5), that help differentiation in their toxic action. GSH-AuNPs accumulated in cancer cells for both green and NIR laser irradiation, which promotes photothermal effects into deeper cancer tissues [102]. A double action (chemo- and photothermal therapy) was exerted by a multifunctional nanoplatform for drug delivery based on polyethylene glycol-stabilized gold nanoparticles conjugated with doxorubicin and podoplanin antibody (PDPN Ab). This system exhibited low toxicity, high drug loading capacity and cellular uptake efficiency; upon laser irradiation, its anticancer effects where greatly enhanced, showing synergistic effects of the two functions both *in vitro* and *in vivo* [53].

Photodynamic therapy is funded on different concepts respect to the photothermal technique based on plasmon resonance. It employs incident radiation, a photosensitizer and (indispensably) the oxygen molecule present in the tissues. In fact, photosensitizers used in photodynamic therapy are mainly of the Type II kind: they are excited by a light source into a triplet state; the excited photosensitizer then reacts with a ground state, triplet oxygen molecule, exciting it into the singlet state. In this way, O_2 becomes a reactive oxygen species, and is able to produce other ROS, such as hydroxyl radicals ($^{\bullet}OH$) and superoxide (O_2^{-}) ions [103]. Photosensitizers may be of different nature: organic molecules (containing highly conjugated systems), noble metal organic complexes (e.g. ruthenium, iridium and rhodium coordination compounds), and nanomaterials (e.g. nanorods, quantum dots, etc.). The photosensitizer has to be injected in the tissue, then light of an appropriate wavelength is applied to excite the photosensitizer, in this way radicals and/or ROS are generated, that induce apoptosis [97]. Analogously to photothermal therapy, also in this case AuNPs have been used for their well-known properties, and also because the application of nanoparticles (in general) in this field has benefits in terms of bioavailability, solubility and degradation [104]. The possibility of creating multifunctional nanoplatforms, again, enhances the effects of photodynamic therapy by combining other powerful tools against neoplastic cells, like imaging for a more localized administration, targeting for selective delivery to tumour tissues, and anticancer action. This is the case of a multifunctional gold nanocage, based on Pluronic-polyethylenimine assembled into micelles to encapsulate paclitaxel and at the meantime providing reduction sites for gold cage formation via a “green” synthesis [105]. The gold cage had a surface plasmon resonance peak at near-infrared region in a broad window for optimal photodynamic/photothermal applications. The composite nanomaterial was tested against androgen-resistant prostate cancer with high control of drug release, and caused blocking of the TRPV6 cation channel, enhanced cell cycle arrest, increased temperature and produced ROS leading to apoptosis. Again, gold nanocages were used to incapsulate an infrared photosensitizer, SiNC (Si(IV)-naphthalocyanine), and then coated with glycol chitosan further functionalised with an enzyme-cleavable peptide linkage to prevent the premature release of the photosensitizer and improve the biocompatibility of the nanostructure [106]. The nanocage was highly phototoxic and showed remarkable tumour suppression efficacy in a glioblastoma model.

Bifunctional nanoprobes based on gold AuNPs bearing Chlorin e6 and Epidermal Growth Factor attached onto their surface were effectively internalized by triple negative breast cancer with an average rate of 63 nanoparticles per minute, and a concentration of 0.2 μg/mL was found to be strongly cytotoxic for cancer cells (86 %) while being unharmful for healthy ones [107]. Photodynamic treatment in cancer cells also generated ROS in 9-fold higher amounts respect to normal cells.

In another study, AuNPs were PEGylated and covalently functionalised with the phthalocyanine C11Pc as the photosensitiser, and used a short peptide for the targeting of epidermal growth factor receptor overexpressing cancers [108]. Selective phototoxicity was observed already at nanomolar concentrations with minimal dark toxicity.

A nanobioconjugate prepared using AuNPs, a photosensitizer (AlPcS4Cl) and a specific antibody was employed against cancer stem cells, since they are behind tumour recurrence through inhibition of drug-induced cell death that reduces the effects of chemotherapy and photodynamic therapy. The nanoparticles were tested against lung cancer stem cells, where they localized in integral organelles involved in cell homeostasis, and caused significant cell toxicity and cell death. Moreover, when applied to photodynamic therapy they obtained enhanced effects with cell destruction to the point of eradication [109].

A different concept in making nanomaterials for photodynamic applications can be found in the case of nanometre-thick gold nanosheets. These are 2D nanomaterials, meaning that two of the three dimensions of these particles exceed the nanoscale. The Au nanosheets of this study [110] were synthesized with a uniform thickness (2–8.5 nm) and their outstanding efficacy in photodynamic therapy, with an extraordinary photon conversion, was most probably due to their uniform 2D morphology. In fact, these Au nanosheets had low cytotoxicity, but were able to kill cancer cells under NIR irradiation after 5 min with little heat generation, a result that could be obtained with 10 times mass loading of “conventional” AuNPs.

The literature on this subject is obviously much more extended than the few examples that have found room in this chapter, nevertheless they already give a hint of the huge possibilities behind the applications of gold nanoparticles to the field of light-induced therapies in cancer treatment.

3. Diagnostics

Another important advantage of AuNPs in the treatment of cancer is that they are, rather often, also very versatile tools for *in vitro* and *in vivo* diagnosis [111], since their optical and electronic properties make them remarkable agents in imaging [112], sensing and detection [113]. Their efficiency as theranostics, performing at the same time accurate diagnosis and precision imaging-guided therapies, has thus been variously exploited in the search for optimal and complete treatment of malignancies.

Gold nanoparticles represent, among all nanomaterials, powerful candidates as contrast agents for instrumental diagnosis, and their applications span from computed tomography (CT) and nuclear imaging to all the other “I” techniques: magnetic resonance (MRI), positron emission tomography (PETI), fluorescence (FI), photoacoustic (PAI) and X-ray fluorescence (XRFI) imaging. Moreover, being able to perform two

or more of the abovementioned diagnostic methods at the same time, they can function as multimodal agents. This is highly desirable since none of these techniques alone can offer the optimum combination of properties (e.g. sensitivity, resolution, cost, availability), while in combination they can afford a more reliable and complete diagnosis.

Research carried out on this topic has been freshly reviewed [112–114]. The most recent novelties in this field of application include a glutathione-AuNPs complex with lactoferrin that was successfully used as a contrast agent in angiography carried out via X-ray CT. Once administered to mice, the retention time of these nanoparticles was 1–3 h, considerably longer than standard contrast agents for angiography (e.g. iopamidol), and allowed imaging of morphological changes in identical tumour vessels for several days [115]. Imaging-guided CT was carried out also on osteosarcoma cells through engineered macrophages with dendrimer-entrapped gold nanoparticles [116]. The dual mode CT/MR imaging of a breast cancer model *in vivo* was obtained with multifunctional core-shell tecto dendrimers incorporated with gold nanoparticles [117].

PET images of HeLa cells were obtained in cancer xenograft mouse models using radiolabelled AuNPs carrying doxorubicin [118]. Multimodal (computed tomography, photoacoustic and photothermal) imaging was performed to guide photothermal synergistic chemotherapy with a porous gold nanoshell incorporating mayransine as the targeting agent and co-decorated with methoxy polyethylene glycol (mPEG) and trastuzumab towards breast cancer cells [119].

A multifunctional nanoprobe was prepared with AuNPs nanostars linked to Fe_3O_4-NPs using polydopamine and combined with antibody-FITC or antibody-quantum dots for *in vitro* or *in vivo* fluorescence labelling (FL) and magnetic resonance imaging (MRI). The latter showed that this new nanocomposite is able to increase the signal intensity respect to pure Fe_3O_4 nanoparticles of 65 %. Under the precise guidance of multimodal imaging, the nanoprobe was able to carry out homogeneous photothermal ablation of bulky solid tumours (~ 400 mm^3) in a xenograft mouse model [120]. Another formulation based on a core-shell combination of these two materials was prepared by coating Fe_3O_4 nanoparticles with gold, and it was used as a dual function theranostic in magnetic resonance imaging and photothermal therapy. The Fe_3O_4 inner core is responsible for the remarkable performance in MRI, while the outer gold shell allows NIR photothermal activity with high efficacy [121].

Radiolabelled AuNPs have been extensively applied to cancer imaging and therapy, as recently reviewed [122]. One of the latest examples is given by ^{89}Zr-labeled gold nanoparticle–antibody conjugates, which were used for the immunoPET imaging of pancreatic tumours [123] while ^{99m}Tc-labeled ultrafine gold nanobioconjugates were employed for targeted imaging of folate receptor positive cancers [124].

AuNPs have been used as bio- and chemical sensors and detectors of RNA, DNA [125], biomarkers [126], antigens, proteins, and cells, providing useful information for the diagnosis of cancers [113]. Gold nanoparticles have a double function: they work as a signal amplifier for the detection of ultralow amounts of the analyte, and support the conjugation of specific molecules to target and interact with the analyte itself.

Detection of prostate cancer, among others, is a significant example of how AuNPs biosensing can be differently addressed by the use of properly functionalised gold nanoparticles directed towards PSA (prostate specific antigen) (see for instance [127]), PSMA (prostate specific membrane antigen), and miRNA. PSMA expression is significantly higher in malign hyperplasia, so it can be effectively exploited to discriminate other benign prostatic conditions. For this reason, AuNPs conjugated with Concanavalin A, a carbohydrate-binding protein of the lectin family able to interact with PSMA (a glycoprotein), were employed on an aluminium interdigitated electrode. The technique exhibited a linear detection range from 10 pM to 100 nM with detection limit and sensitivity of 10 pM and 1.65 nF/pM respectively [128]. Similarly, PSA was detected with an interdigitated mini-electrode on which gold nanoparticles conjugated with a PSA specific aptamer were deposited [129]. In this way PSA was determined at a 45 aM concentration with a sensitivity of 30 aM. In another study, PSA was detected using a DNA-AuNPs conjugated and measured via evanescent wave cavity ring-down spectroscopy (EW-CRDS), reaching an impressive lower detection limit of 0.54 fM [130]. A different approach for PSA detection exploited localized surface plasmon resonance (LSPR) biosensing of gold nanoparticles conjugated with anti-PSA monoclonal antibody 8G8F5, reaching a detection limit of 0.2 ng/mL [131]. Similarly, a quartz crystal microbalance (QCM) biosensor was designed as a simple device for point-of-care diagnosis based on PSA quantification in body fluids. The QCM sensors were covered with antibody against PSA (anti-PSA monoclonal antibody mouse type) and gold nanoparticles modified with specific antibody against PSA (anti-PSA polyclonal antibody rabbit type). The PSA biosensor reached a limit of detection of 0.054 μg/l and limit of quantification of 0.18 μg/l [132]. Prostate cancer can be also revealed by different biomarkers. This is the case of a graphite sensor system modified with an AuNPs-peptide nanotubes composite for the impedimetric quantification of a possible biomarker, circulating miRNA 410, released into peripheral blood system by prostate cancer cells. The modified sensor showed high sensitivity for the recognition of miRNA 410 based on the hybridization process, and a detection limit as low as 3.90 fM with an ample linear range from 10 fM to 300 pM [133].

Prostate cancer is surely one of the most frequently studied cases for the application of AuNPs biosensing, but an impressive number of papers has been published in the last few years in a broad range of research areas, so it will possible to mention here just a very few of them. For instance, an electrochemical immunosensor for highly sensitive detection of GM2 activator protein (GM2AP), a new lung cancer biomarker, was prepared modifying a screen-printed carbon redox electrode by covering it with phosphomolybdic acid and polyethyleneimine-coated AuNPs. A decrease in the current response of phosphomolybdic acid redox probes was at the basis of GM2AP detection. The new sensor reached a limit of detection of 0.51 pg/mL [134].

Carcinoembryonic antigen and cancer antigen 15–3 are two relevant breast cancer biomarkers, which were detected with biosensing probes made of an AuNPs three–dimensional graphene hydrogel nanocomposite bearing the aptamers of the two biomarkers [135]. The detection limits were found to be 11.2 pg/mL and 11.2 × 10^{-2} U/mL for the two biomarkers, respectively.

Fucose is a cancer biomarker, and the determination of its presence in urine can be critical for the preliminary screening of neoplasms. Detection with amperometric biosensing methods is fast, simple, and precise but suffers from interferences with other analytes, such as ascorbic acid, uric acid and dopamine. An amperometric method devoid of interferences was then developed with a L-fucose biosensor that employs direct electron transfer type bioelectrocatalysis of pyrroloquinoline quinone-dependent pyranose dehydrogenase from *Coprinopsis cinerea*. A suitable domain of this enzyme was isolated and immobilized on AuNP-modified electrodes. The detection limit for L-fucose was 13.6 μM, and the method does not require pre-treatment of the urine sample [136].

AuNPs coated with two analogues of Prussian blue (containing Cu^{2+} and Pb^{2+} instead of Fe^{2+} ions, respectively) showed two different surface-enhanced Raman scattering (SERS) emissions. Once they were modified with aptamers of epithelial cell adhesion molecule and epidermal growth factor receptor, which are both expressed in breast cancer MCF-7 and MDA-MB-231 cell lines but in different levels, the SERS nanoprobes were able to simultaneously identify the expression of these biomarkers on the cell surface, since each marker corresponds to a single SERS emission, thus differentiating between the two cell lines [137].

Cytosensors are simple cell-based electrochemical biosensor having a vital role in early stage cancer diagnosis, thus they are highly demanded in order to improve survival rate in cancer patients. Cytosensing based

on fiber optic localized surface plasmon resonance (LSPR) has been used for the efficient detection of different types of cancer cell lines, such as HepG2, Hepa 1–6, MCF-7, A549, and normal cell lines (NCF and LO2) by using graphene oxide decorated with AuNPs and copper oxide nanoflowers to increase sensitivity [138]. Such device was impressively sensitive, since it was able to detect almost all the cell lines with a limit of detection of 2–4 cells/mL, being slightly less sensitive towards normal NCF strain (10 cells/mL). Analogously, AuNPs derivatised with MUC1 aptamer, were used in a cytosensor based on Ω-shaped fiber optic localized surface plasmon resonance for detection of breast cancer MCF-7 cells. The sensor displayed a low limit of detection of 12 cells/mL with excellent selectivity and no inference when the measurements were carried out in fetal bovine serum. The analysis execution was rapid, being completed in just 37.5 min [139].

Finally, an interesting application was reported about the detection of human papillomavirus using a sensor integrated with a smartphone. The paper-based DNA sensor was able to reveal the presence of papillomavirus with a change of colour and had a low detection limit of 1 nM. Moreover, under optimal conditions, linearity lied in the range from 1 to 1000 nM. The biosensor is based on dextrin-stabilized AuNPs, which are the colorimetric reagent. The colour change is caused by aggregation of the functionalized gold nanoparticles and is induced by the positively charged acpcPNA, (2′R,4′R)-nucleobase-substituted proline and (1S,2S)-2-aminocyclopentanecarboxylic acid. acpcPNA can form antiparallel hybrids with complementary DNA with high affinity and sequence specificity. So, it reacts with the HPV DNA target to give duplex formation, and the residual probe can cause AuNPs aggregation to various extent, resulting in a colour modification, that can be quantified by analysing its intensity through a smartphone application [140].

4. Conclusions

Gold nanoparticles and gold-based nanomaterials represent one of the most investigated topics of research related to cancer diagnosis and treatment, since they possess properties that can also be found in other metals, but rarely all reunited in the same material, and this determines the uniqueness of gold. Stability, biocompatibility, low toxicity, ease of preparation, functionalization and derivatization, size and shape control, aqueous dispersibility, together with remarkable optical and electronic properties make AuNPs fundamental for the preparation of multifunctional nanoplatform to be employed either in the diagnosis and/or treatment of cancer via a wide series of mechanisms. This variety allows extensive applications to nearly all aspects of cancer management. In spite of these outstanding properties and achievements, translation of AuNPs into clinical practice is poor, fundamentally due to the two aspects previously reminded: lack of assessment of the potential nanodrug under the pharmacological point of view, and need of improving traditional testing on animal models. Nanomaterials appeared on the stage almost two decades ago, and still, there is no agreement in the scientific community about their safety, with controversial results obtained on this topic. This issue should be resolved once for all in order to see AuNPs finally enter clinical trials.

Meanwhile, investigation on this fascinating topic will go on at an impressive rate. The few (respect to the extraordinary mole of published papers) examples here reported can give just a hint of the active research carried out in the field of AuNPs for anticancer applications, underlining its high content of innovation that causes rapid “obsolescence” of the result obtained.

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Declaration of Competing Interest

The authors report no declarations of interest.

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