Chapter 4

Cytotoxic and Antiproliferative Effects of Nanomaterials on Cancer Cell Lines: A Review

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http://dx.doi.org/10.5772/intechopen.71685

Abstract

Cell models for the study of antiproliferative and/or cytotoxic properties of engineered nanoparticles are valuable tools in cancer research. Several techniques and methods are readily available for the study of nanoparticles' properties regarding selective toxicity and/or antiproliferative effects. Setting up of those techniques, however, needs to be carefully monitored. Harmonization of the wide range of methods available is necessary for assay comparison and replicability. Although individual or core laboratory capabilities play a role in selection and availability of techniques, data arising from cancer cell models are useful in guiding further research. The variety of cell lines available and the diversity of metabolic routes involved in cell responses make *in vitro* cell models suitable for the study of the biological effect of nanoparticles at the cell level and a valid approach for further *in vivo* and clinical studies. The present systematic review looks at the *in vitro* biological effects of different types of nanoparticles in cancer cell models.

Keywords: cancer, nanoparticles, organic, metallic, nanobiotechnology, cytotoxicity, antiproliferation

1. Introduction

Toxicity studies are needed for nanoparticles' (NPs) intended application on biomedical theranostics. Nanostructures are being designed and fabricated with a wide range of potentialities, including those in cancer therapeutics, medical imaging and diagnostics. Thus, research on cell models and *in vivo* toxicity is growing as the nanostructures that are being fabricated will find possible uses in biomedical, clinical medicine and health-related sectors. NPs have interesting physical-chemical properties that are of value when engineering drug delivery

systems, diagnostic platforms and nanotechnology-based imaging strategies. The high surface-to-volume ratio of NPs allows the use of different molecules, such as those intended for targeted drug delivery [1]. The above properties, however, might render NPs a toxic *in vitro* and *in vivo* profile. Since many NPs are entering the health market, it becomes increasingly necessary to perform toxicological investigation along with NP fabrication.

Earlier and recent toxicity studies on human cell lines have found a range of nanostructures that might be selectively toxic for particular cellular lines, including cancerous ones [2, 3]. This selective toxicity against specific types of cancer is a promising research field with potential implications in (pro)diagnosis and therapeutics [4, 5]. Human cell models are available for a variety of malignancies, serving as suitable platforms for exploring antiproliferative and cytotoxic effects of nanostructures [6]. Data from cancer cell models and NP exposure are valuable for guiding and designing *in vivo* testing and, potentially, for developing new anticancer theranostic strategies [7].

In this review, we compile and discuss the findings of several recent works using cancer cell models and exploring selective NP toxicity and/or antiproliferative effects for potential therapeutic applications in cancer. We looked for particularly interesting scientific papers from indexed journals published within 2015–2017. The focus of this review is on methodological aspects of NP treatment on human cell–based models, i.e. viability assessment techniques, experimental design for investigation of mechanisms of cellular damage, cell culture protocols and NP stability assessment, including in biological media. Results of this review are presented by nature of NPs. Studies exploring new cell culture techniques for assessment of NP toxicity on cancer cell lines were also included.

2. Physicochemical characteristics of nanomaterials and their influence on toxicity

The potential for biomedical applications of several NPs is enormous. There are, however, several shortcomings regarding interactions of engineered NPs with biological environments. Toxicity concerns for NPs intended for use in biomedicine have limited their translation into clinical settings. NP properties such as size, surface-to-volume ratio, shape, surface functionalization and stability on biological media, among others, have been demonstrated to influence the toxicological profile of the nanostructures and their biocompatibility in general [7, 8]. It has been also demonstrated that the level of toxicity varies depending upon cell type, which reflects on particular cell line biology and genetics [9].

Interactions of NPs inside a biological environment, e.g. eukaryotic cells, have been widely studied [10]. Proteins, lipids or any biomolecule may be absorbed by NPs, affecting not only the original synthetic structure but its biological effect. Assessing antitumor properties of NPs requires stability in investigation under *in vitro* cell culture conditions. The interactions between NPs and biomolecules present in the culture media, such as proteins and lipids, could change nanomaterial's characteristics [12]. For instance, research has demonstrated the formation of protein corona around NP surface due to interaction with cellular media, resulting in modifications of their physical properties and leading, for instance, to aggregation and

Nanoparticles		Anticancer properties	
		Advantages	**Disadvantages**
Metallic and nonmetallic nanoparticles	Naked [94, 95]	• High antitumor activity • Storage and release of energy to other molecules quite effectively • Improvement of sensitive single-molecule detection techniques • External stimuli responsive, e.g. light and magnetism modulate its activity • Tunable physical and chemical properties	• Conformational changes • Coalescence • Stabilizers do not function properly in different solvents • In a large extent, synthesized with toxic chemicals for health and/or environment
	Coated [13, 16, 96]	• Easy conjugation to drugs, proteins, and/or nucleotides • Attenuated cytotoxicity against normal cells due to surface functionalization • Specific site of action	• Biological effect varies among different coatings • Formation of a protein corona • Sedimentation and/or aggregation
Liposomes [96]		• High biocompatibility • Capability of conjugation with soluble and insoluble drugs • Targeted drug release • Low toxicity	• Colloidal stability and biodegradability • Complex and expensive synthesis

Table 1. Advantages and disadvantages of (non)metallic nanoparticles and liposomes application in cancer research.

sedimentation [13]. Thus, NP characterization during *in vitro* experiments is essential to understand the relationship between physical properties and mechanisms of *in vitro* toxicity.

In general, smaller NPs are more toxic than larger ones [14]. Several works have confirmed this relationship and some authors have identified NP sizes that correlate well with the level of toxicity observed on *in vitro* tests [15]. A range of toxic mechanisms leading to apoptosis, necrosis and genotoxicity is triggered by NPs of different range of dimensions. The net cytotoxic effect is usually cell and NP concentration dependent [9].

Several coating strategies have been tested for lowering the cytotoxic effects of many engineered NPs intended for medical applications. Metallic NPs have been extensively investigated and are excellent candidates as drug nanocarriers, for imaging strategies and for immunological platforms in biomedicine [11]. Toxicity concerns have, however, slowed their faster development and translation. Green chemistry or biologically mediated synthesis of coated metallic NPs is on the rise, and consequently their nanotoxicity evaluation on biological media has been pursued and published [15, 16].

Nanostructures such as semiconductor quantum dots (QDs) are also being investigated for biomedical purposes. Since the toxicity of these nanostructures is known, different coating procedures have been investigated in order to reduce their toxicity. For instance, zinc sulfide (ZnS) QDs functionalized with chitosan have shown no toxic effects on human leukocytes, contrary to the highly toxic cadmium sulfide (CdS) QDs that, even coated with biocompatible chitosan, showed to be toxic in a concentration and time-dependent manner [17]. A summary of the *pros* and *cons* of the use of NPs in cancer research is shown in **Table 1**.

3. The selective toxicity of nanomaterials on *in vitro* cancer cell models

Several mechanisms are involved in NP-mediated *in vitro* toxicity in normal (i.e. noncancerous) and cancerous cells. Cellular responses to NP exposure might include those at cell, organelle and gene level or a combination of them [18]. Direct cytotoxic effects might be apoptosis or necrosis (or both) mediated, with a number of mechanisms leading to cell death, changes in proliferation patterns and effects on cell differentiation. High levels of reactive oxygen species (ROS) production, downregulation of antioxidant enzyme coding genes, lipid peroxidation and genotoxic effects, among others, may be involved in the integrated cellular response to NPs [19, 20].

In spite of the number of studies providing useful information on nanotoxicological profiling, there remains particular information with regard to cell-NP specificity interactions. In addition, investigation on the toxicity of nanostructures and biointeractions rely on data from a wide variety of experiments with several different methods and techniques that are chosen on the basis of laboratory capabilities and researchers´ technical expertise [21, 22]. Then, there are, as today, no standard cell panels or defined protocols available for assessment of cancer cell responses to NPs; therefore, data arising from those studies are difficult to compile and integrate. Moreover, there is still the risk that the toxicological picture from a particular study on specific NPs and cell lines might not be "complete" enough and that toxic risks may be overlooked.

Apoptosis is a common response of cells to NP treatment. Azizi and colleagues found that albumin-coated silver NPs (AgNPs) LD50 were several times lower for breast cancer cells than for normal white blood cells. Apoptosis assays such as Annexin V and microscopy counts of apoptotic bodies demonstrated that albumin-coated AgNPs exert proapoptotic selective effects on breast cancer cells while normal blood cells remained viable at the tested concentrations and times of exposure [5].

In a recent work on several murine cancer cell lines, Namvar and colleagues investigated the antitumor properties of biosynthesized zinc oxide NPs (ZnONPs). They found that cancer cell proliferation was inhibited by NPs in a time- and concentration-dependent manner and that the mechanism of cell death was primarily apoptosis via procaspases activation and intrinsic mitochondrial pathway triggering [2].

NP exposure may cause cancer cell death by oxidative stress through varied mechanisms, including ROS production, inhibition of antioxidant enzymes, mitochondrial damage and lipid peroxidation [20]. For instance, Matulionyte, et al. demonstrated that photoluminescent gold

http://dx.doi.org/10.5772/intechopen.71685

nanoclusters have specific toxicity against MCF-7 breast cancer cells and were less toxic on MDA-MB 231 breast cancer cells, a highly drug-resistant cell line. The mechanism of cell death was apoptosis, necrosis and generation of ROS, effects that were more evident in MCF-7 cells [23].

Several other mechanisms are involved in the selective toxicity of NPs against different cancer cell lines. Endoplasmic reticulum (ER) autophagy is a well-known process related with NP exposure. A study by Wei, et al. found that silica NPs (SiNPs) induced ER autophagy in colon cancer cells. The authors showed a time-dependent effect of NP exposure, but interestingly, autophagy was present only at either low or high NP concentrations [24].

Due to the complexity of cell responses to NPs, it is important to evaluate the biological effect of NPs from different perspectives, from toxicology assessment to both *in vitro* and *in vivo* testing, to better understand NP-induced cellular responses and the mechanisms behind them (**Figure 1**).

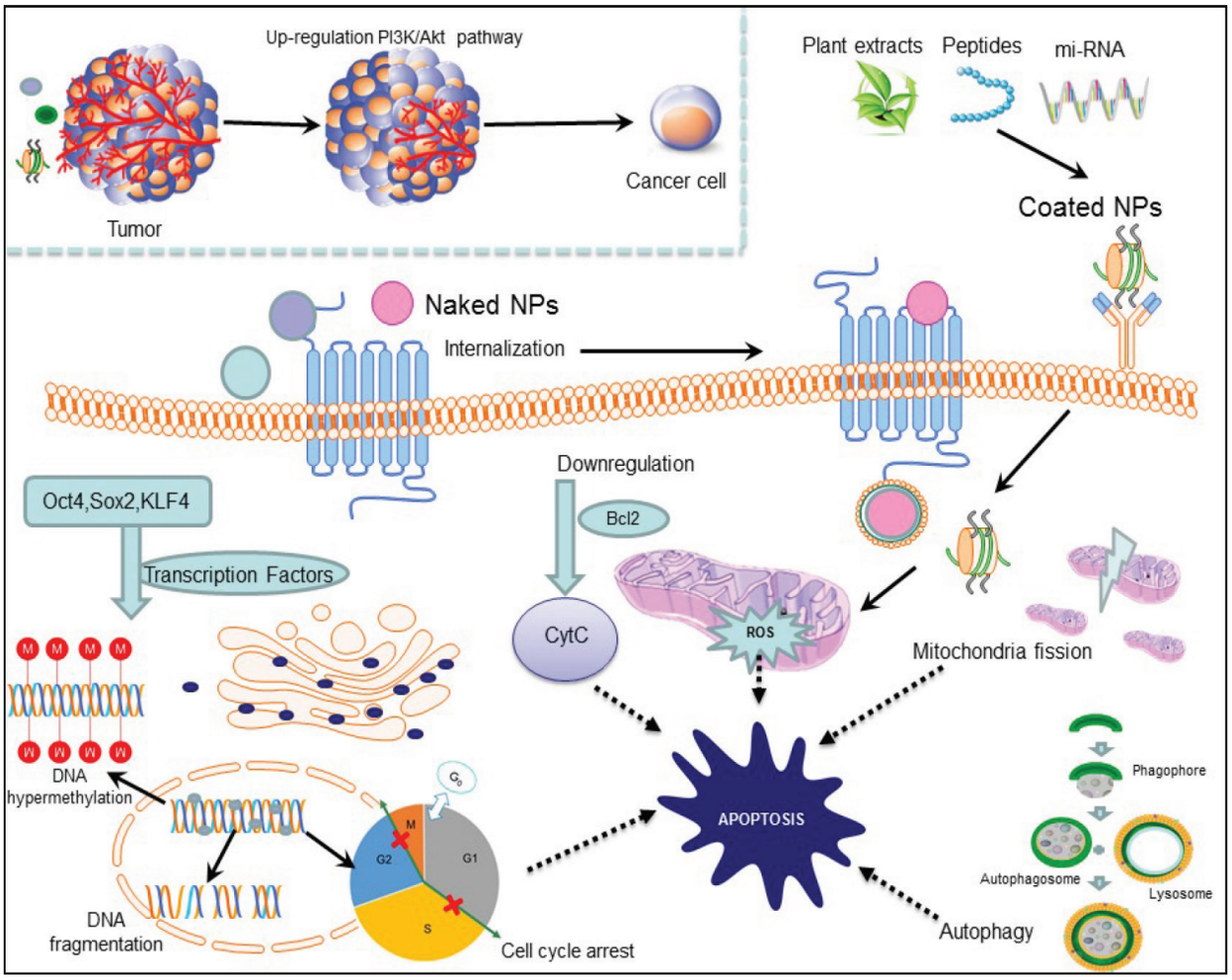

Figure 1. Schematic interpretation of nanoparticle (NP) cellular effects. NPs undergo internalization by nonspecific or specific endocytosis and remain in the cytoplasm or inside intracellular vesicles, either individually or in aggregates. NPs might release ions that enter the nucleus and cause DNA fragmentation/hypermethylation and/or cell cycle arrest in cancer cells. Furthermore, NPs' inhibitory effect on cellular viability is due to downregulation of antiapoptotic genes, e.g. Bcl2, generation of reactive oxygen species (ROS), mitochondria fission and autophagy and events that finally induce cell death through apoptosis. NPs could decrease the expression of transcription factors involved in stemness and thus inhibit angiogenesis.

In the following sections, we discuss the cytotoxic and antiproliferative *in vitro* properties of different types of NPs and their potential application in nanotherapeutics.

3.1. Metallic nanoparticles: noble metals and selective antitumor properties

Inorganic nanostructures exhibit interesting physical properties such as magnetism, fluorescence and localized surface plasmon resonance, which in combination with NPs' small dimensions make them suitable for biological applications. An advantage over other types of nanostructures is that inorganic NPs could respond to external stimulation with light or magnetic fields [1]. Among inorganic NPs, noble metals have been commonly used for the synthesis of nanomaterials. For instance, silver, gold and platinum NPs are of interest in cancer research as multifunctional anticancer agents due to their particular properties [25, 26]. In the subsequent sections, antitumor properties of noble metallic NPs are discussed focusing on their *in vitro* effects on several cancer cell lines.

3.1.1. Silver nanoparticles

Silver nanoparticles (AgNPs) possess particular physicochemical properties that determine their extent of cytotoxicity in biological systems [27]. It is well documented that AgNPs exert an antiproliferative effect on cancer cell lines [19, 28]. According to Choi, et al., AgNPs develop a potential cytotoxic effect on A2780 ovarian carcinoma cells and ovarian cancer stem cells (OvCSCs) at high concentrations. The inhibitory effect on cellular viability is caused by the upregulation of p53 and caspase-3 genes. In contrast, AgNPs might promote cell proliferation at low concentrations. The relevance of these findings is that OvCSCs present more sensitivity to the treatment with AgNPs, which is particularly interesting due to the fact that CSCs might increase the risk of acquired resistance to chemotherapy [19].

The therapeutic effect of AgNPs in multidrug resistant (MDR)-cancer cells has also been investigated. Kovacs, et al. demonstrated that AgNPs induce apoptosis-mediated cell death in drug-sensitive (Colo 205) and drug-resistant (Colo 320) colon adenocarcinoma cell lines, in a dose-dependent manner [28]. The internalization of AgNPs was observed in both cell types; thus, they remained in the cytoplasm. In addition, AgNPs may act synergistically with anticancer drugs to enhance their tumor-killing effects in MDR cells due to their capability of modulating efflux activity [28]. It is important to highlight the risk of exposing normal cells to AgNPs. To illustrate, a hippocampal neuronal cell model (HT22) was treated with AgNPs, obtaining a decrease in cell viability, oxidative damage and hypermethylation in DNA due to the internalization of AgNPs. These effects in normal cells may be prolonged since harmful impacts remain after AgNP removal [29]. Similar reports were found by Gao, et al., demonstrating that AgNPs can potentially damage mouse embryonic stem cells [30]. A novel approach to reduce cytotoxicity against normal cells is the functionalization or modification of AgNP surface [16]. Extensive research has been conducted to validate the hypothesis that AgNPs could inhibit angiogenesis, a complex process that is involved in the formation of new blood vessels and tumor progression [31]. For instance, Gurunathan, et al. concluded that the treatment of bovine retinal endothelial cells (BRECs) with AgNPs might activate PI3K/Akt pathway resulting in the inhibition of capillary formation [32]. Based on

this evidence, AgNPs are potent antineoplastic agents with acute cytotoxic effects that modulate several metabolic pathways leading to decreased cell viability, independently or in combination with other anticancer drugs. This synergistic effect will be further discussed along this chapter.

3.1.2. Gold nanoparticles

Compatibility of gold with biosystems has been well demonstrated since metallic nanoscale materials were originally developed [33]. In recent years, synthesis and application of gold NPs (AuNPs) in the biomedical field have substantially increased due to their ductility physicochemical properties and biocompatibility. AuNPs can be synthesized in different shapes including spheres, rods, cubes, triangles, cones and shells [34]. Therefore, based on their size and shape, "naked" AuNPs possess several applications, e.g. as antitumor agents, drug nanocarriers, hyperthermia enhancers and radio sensitizers [1, 35].

AuNPs exert *in vitro* cytotoxicity on several human cancer cell lines including cervical (HeLa), prostate (PC-3), hepatocellular carcinoma (HepG2) and breast cancer (MDA-MB-231) [3, 36–38]. Wozniak, et al. proved that spherical and rod-shaped AuNPs are more efficient than other shapes in reducing cell proliferation of cancer cells *in vitro* [36]. Positively charged naked AuNPs interact with negatively charged cell membranes, increasing cellular uptake, preferably with smaller diameter particles rather than larger ones [37]. Also of interest, AuNPs can be used in combination with other anticancer molecules. For instance, Ke, et al. reported that AuNPs improved the responsiveness of Calu-1 epidermoid carcinoma cell line to tumor necrosis factor (TNF)-related apoptosis-inducing ligand (TRAIL) [39]. This combined approach induced DNA fragmentation, mitochondrial fission and a decrease in cell viability due to apoptosis. By contrast, the effect on cell viability was minimal in the BEAS-2B normal lung cell line [39].

In addition, AuNPs could act as enhancers of hyperthermia-targeted therapy because they efficiently absorb laser light and convert it into thermal energy [40]. The synergistic effect of AuNPs and laser-induced thermotherapy renders thermally exposed cancer cells susceptible to be ablated with minimal exposure times and lower laser intensities [33]. Rau, et al. showed that AuNPs could cause severe damage in the cytoskeleton of MG63 osteosarcoma cells in combination with laser treatment, increasing the calcium content inside the cells and leading to mineralization [41]. Another technique to induce hyperthermia in tumors is directed ultrasound. Kosheleva, et al. discovered that the combined treatment of ultrasound and AuNPs exerted a more acute cytotoxic effect on A549 lung cancer cells compared to BEAS-2B normal lung cells when cultivated separately and in coculture [42]. These findings suggested that AuNP-assisted thermotherapy could cause targeted cancer cell ablation, while avoiding damage to surrounding noncancerous cells.

AuNPs can be uptaken by cancer cells via endocytosis and trigger apoptotic events [43]. As a consequence, an improvement in radiation therapy has been observed when cancer cells are previously exposed to AuNPs [43]. Likewise, high atomic number in AuNPs increases radiation absorption from the target tumor [43]. Literature suggests that AuNPs act as radiosensitizers in several cancer cell lines, such as U251 glioblastoma, which in clinical practice could

increase radiotherapy efficacy and prevent the development of drug-resistant tumors [44]. Another approach thoroughly studied by Rezaee, et al. showed that electroporation enhances radiosensitizing effect of AuNPs in HT-29 colon adenocarcinoma cells as a result of increasing cell membrane permeability. In this study, AuNPs' radiosensitizing effect was more prominent in cancer cells than in normal counterparts [43].

3.1.3. Platinum nanoparticles

Several investigations have addressed the antiproliferative effect of platinum nanoparticles (PtNPs) in cell models [45–48]. Bendale, et al. concluded that the harmful effect of PtNPs on cancer cell viability depends on the cell type. At the same PtNP concentration, an acute cytotoxic effect was observed in lung (A549), ovary (PA-1) and pancreatic (Mia-Pa-Ca-2) cancer cells [45] . In this study, no significative effect on cell viability was observed in breast, renal, colon and leukemia cancer cell lines. Interestingly, peripheral blood mononuclear cells (PBMCs) were not affected either, suggesting that PtNPs could preferably target tumor cells [45]. According to Kutwin, et al., PtNPs severely affect the proliferation rate and morphology of U118 and U87 human malignant glioma cell lines, and as a consequence, cells suffer from membrane disruption, reduced density and decreased migration [46]. Gehrke, et al. did not find any adverse effect on cellular viability when HT29 colon carcinoma cells were treated with PtNPs. It was observed, however, that smaller PtNPs enter the cells and remain in the cytoplasm or inside intracellular vesicles, either individually or in aggregates. Additionally, PtNPs released Pt ions that may bind to DNA leading to strand cleavage damage [49]. Another important feature is the synergistic antitumor activity between platinum and gold NPs. Ahamed, et al. reported that platinum-coated gold nanorods (AuNRs-Pt) affected cell viability on MCF7 breast cancer cells at relatively low doses. The mechanism of action of AuNRs-Pt involved impairment of normal morphology resulting in rounded cells, cell cycle detention at SubG1 phase, increased expression levels of proapoptotic genes caspase-3 and caspase-9 and generation of ROS [47]. Manikandan, et al. demonstrated that PtNPs could improve photothermal treatment in cancer cells. Neuro-2a brain neuroblastoma cells were exposed to the combined scheme of laser irradiation and PtNPs, which resulted in induction of apoptosis [48]. There was no significative effect on cellular viability when PtNPs and laser treatment were applied separately [48].

3.1.4. Other metal-based nanomaterials

Titanium dioxide (TiO_2), zinc (Zn), copper (Cu) and iron (Fe) are used in several industrial applications such as cosmetics, paint chemicals, food additives, pharmacological coatings, drug delivery systems, biosensor technologies and body implants. These nanomaterials have been also tested in cancer research and development of new therapeutics [22, 50].

Xia and coworkers reported the cytotoxic effect of cuprous oxide nanoparticles (CONPs) on HeLa, SiHa and MS751 human cervical cancer cell lines. Results demonstrated that CONPs are uptaken by cells and internalized into the cytoplasm, mitochondria and lysosomes; as a result, cell morphology alterations and decreased cellular viability were observed. Cell cycle arrest in the G1/G0 phase, induction of apoptosis and autophagy were also reported [51].

The antineoplastic effect of CONPs in PC-3, LNCaP FGC and DU145 human prostate carcinoma cells was investigated by Wang, et al. The results of this study suggest that CONPs might induce cytotoxicity selectively on cancerous cells without affecting normal prostate epithelial cells (RWPE-1). Moreover, a significant decrease in the expression of Oct4, Sox2 and KLF4 transcription factors related with stem-cell proliferation capability was observed [52].

Superparamagnetic iron oxide nanoparticles (SPIONs) are also included in a large extent in nanomedical products [1]. SPIONs develop magnetic properties within a magnetic field; therefore, they are able to act in specific target sites [1]. Several studies demonstrated that SPIONS can be approached as hyperthermia enhancers, contrast agents in magnetic resonance imaging, drug nanocarriers and anticancer candidates [1]. For instance, Du, et al. studied the combined effect of SPIONs and spinning magnetic field (SMF) on the survival rate of U-2 OS and Saos-2 osteosarcoma cell lines. This combined treatment exerts a more effective cytotoxic response triggering the intracellular ROS generation, autophagic cell death and apoptosis, than SPION treatment alone [53].

3.2. Nonmetallic and organically coated metallic nanomaterials: antiproliferative and cytotoxic properties

3.2.1. Green synthesis–based nanomaterials

Production of materials at the nanometric scale (1–100 nm) has been performed using several approaches [54]. The most common synthesis method involves the use of three elements: capping agent, reducing agent and solvent [54]. However, most of these elements are toxic, flammable, corrosive and even dangerous for the natural environment and living organisms. For this reason, a new green chemistry tendency emerged in the nanotechnology area to modify chemical processes and reduce or minimize the use of hazardous reagents [55]. The green-synthesis approach has been focused on finding nontoxic elements to develop a more eco-friendly design with improved efficiency [54]. Some of these new techniques require the use of solvents such as water, supercritical CO_2 or ionic liquids [56, 57]. For example, silver and gold nanoscale structures, due to their chemical and biological properties, have been widely used in green synthesis in combination with medicinal plants (photosynthesis) or bacterial/fungi/viral proteins (microbial-synthesis) [58]. This section provides further interesting examples of green-synthetized nanomaterials.

3.2.1.1. Photosynthesis

The importance of developing an alternative nanosynthesis protocol is not only for an environmental footprint reduction, but contributes also for the simplification of industrial production with the lack of expensive organic solvents and toxic chemicals [59]. The use of innocuous plant extracts with solvents such as water facilitates the production and further evaluation of green nanomaterials, which are fundamental for biological applications in critical areas e.g. drug production [59]. There are several nanoscale structures coated with plant extracts and their effect on living systems has been extensively studied [60]. For instance, Krishnaraj, et al. reported that Ag/Au biosynthesized NPs with *Acalypha indica* extract exerted a cytotoxic effect

in MDA-MB-231 human breast cancer cells. These NPs exhibited a proapoptotic effect through caspase-3 activation [58]. Another example of naturally coated AgNPs includes the effect of the *Erythrina indica* extract causing a dose-dependent reduction of viability in MCF7 breast cancer cells and HepG2 hepatocellular carcinoma [27]. The authors also demonstrated high antimicrobial activity for AgNPs against *Staphylococcus aureus*, *Micrococcus luteus*, *Escherichia coli*, *Bacillus subtilis*, *Salmonella typhi* and *Salmonella paratyphi* [27]. Moreover, AgNPs were synthesized using *Albizia adianthifolia* leaf extract; the AgNP analysis determined the presence of saponins and glycosides as stabilizing agents [61]. Toxicity analysis was performed on A549 lung cancer cells and normal peripheral lymphocytes [61]. The results showed a reduction in A549 cellular viability to 21% at 10 g/mL and 73% at 50 g/mL after 6 h of exposure [61]. In comparison, proliferation rates for normal cell lines were not altered [61]. Other applications of these nanostructured particles for disease treatment include antidiabetic effects, described with *Cassia fistula* AuNPs that reduce glucose levels in rats with streptozotocin-induced diabetes [62], and antimosquito larvicidal activity of *Nelumbo nucifera* AuNPs [63].

3.2.1.2. Microbial synthesis

Bacterial survival in the presence of heavy metals is caused by a transformation (reduction/precipitation) of metal ions into insoluble nontoxic metal nanoclusters. These detoxification reactions are mediated by intracellular accumulation or a physicochemical process–denominated extracellular biosorption, which facilitates the concentration of contaminants, e.g. heavy metals, and binds them in their cellular structure, with variable levels of dispersity [64]. Based on these bacterial properties, Klaus, et al. described AgNP production in *Pseudomonas stutzeri*. This bacterium reduces silver ion to generate Ag^0 and AgS_2 NPs of different shapes and sizes located around the cellular poles [65]. Another interesting example is *B. subtilis* that reduces Au^{3+} to a neutral nanocompound (Au^0) [66]. Moreover, production of lipopolysaccharides and phospholipids in some bacteria mediates bioreduction, e.g. transformation of chloroauric acid ($HAuCl_4$) to AuNPs in *E. coli* DH5α [64]. Nonetheless, assembly of microbial NPs is also performed in several fungi species such as *Penicillium chrysogenum*, which has showed to be an AuNP producer in $HAuCl_4$ solution [67]. Another remarkable study optimized nanowire production with M13 virus as biotemplate for development of lithium batteries [68]. Based on this information, affordable and massive industrial production should be feasible with the use of biological nanofactories such as the above-mentioned examples. However, the lack of complete understanding of the molecular reaction mechanism is a major disadvantage of this methodology.

3.2.2. Organically coated metallic and nonmetallic nanomaterials

Nanobiotechnology as a mature biomedical field emerged in the last years [69]; for example, from gene-delivery systems to targeted drug delivery, it has several applications in cancer treatment, diagnosis (biomarkers), molecular biology and genetic/cell engineering [70, 71]. A nanomedicine-based therapeutic approach might be built on nanocarriers, e.g. liposomes and NPs that improve chemotherapeutic biodistribution [72] and have been useful for treating diseases such as cancer [73] and microbial infections [74]. In 1989, Matsumura and Maeda described the enhanced permeation and retention (EPR) effect, a controversial concept based

on the passive accumulation of macromolecular drugs in tumors due to the presence of a high number of abnormal blood vessels (angiogenesis), which lack lymphatic drainage, affecting in turn as a drug delivery system [75]. Despite the fact that this effect has been extensively studied but has failed in clinical trials [76], EPR is still one of the most used concepts in nanobiodistribution [76]. With this information in mind, this section discusses relevant aspects of metallic and nonmetallic coated nanomaterials, including liposomes as novel therapeutic agents for cancer.

3.2.2.1. Organically coated nanomaterials

Organic coating is used to stabilize NPs and maintain a balance between electrostatic and electrosteric repulsion forces [73]. NPs of different shapes might be covered with diverse capping agents such as citric acid, polysaccharides, surfactants, proteins, polymers and nucleic acids [77, 78]. However, despite the fact that they have the same core material, coated-NPs exert different biological responses. For instance, viability, genotoxicity and mutagenicity evaluation of AgNPs coated with anionic (citrate, SDS), neutral (disperbyk, tween) or cationic (byk and chitosan) compounds were performed by Kun, et al. using lymphoblast TK6 cell line and Chinese hamster lung fibroblasts. The methodology used for testing involved trypan blue exclusion assay, relative growth activity, cell morphology, HPRT mutation and comet assay. The results determined that AgNPs_byk and AgNPs_chitosan were the most cytotoxic, affecting cell morphology, inhibiting proliferation and inducing cell death through apoptosis or necrosis. Furthermore, AgNPs_byk showed significant mutagenic effects by inducing DNA strand breaks and oxidation. It is important to note that AgNPs_byk formed the smallest agglomerates in medium solution in comparison to other coated AgNPs, suggesting that size is an important factor in toxicity. To sum up, coated NPs display various biological *in vitro* effects depending mainly on their surface charge [12].

One of the well-known NP biointeractions is that with bovine serum albumin (BSA), which relies on principle that a protein corona is dynamically formed around NPs when they enter a biological environment [79]. A recent investigation performed by Zhou, et al. determined that ZnONPs bound to BSA elicited interleukin-6 (IL-6) production-mediated anti-inflammatory responses in HepG2 liver cancer cell line. Additionally, synthesized NPs induced mitochondria and lysosomal damage by increasing intracellular Zn ions production [79]. However, the analysis of the biological effect of ZnONPs bound to α-linolenic acid (LNA) did not show the same response [79]. Another interesting example is the evaluation of the response of SPIONs conjugated to the antitumor peptide ATWLPPR (A7R) on HUVEC human umbilical vein endothelial cells and MDA-MB-231 human breast adenocarcinoma [11]. These NPs might be adjusted carriers for targeted drug delivery systems using their magnetic properties. Furthermore, the presence of cell receptors for the A7R peptide facilitates the uptake of the nanocarriers. This is particularly important because the role of the receptor is to repress the vascular endothelial growth factor A (VEGF A). Consequently, NPs affect angiogenic events and impair cell proliferation [11].

The study of commercial anticancer drug formulations in nanoform has also been evaluated, with positive results in many cases. For instance, tamoxifen, an anticancer agent used

in estrogen receptor-positive (ER+) breast cancer, has been commonly used before surgery to reduce tumor volume [80]. However, tamoxifen resistance has become a significant problem in cancer treatment [80]. Devulapally, et al. synthesized biodegradable polymer NPs loaded with the active compound of tamoxifen (4-hydroxytamoxifen-4OHT) and the noncoding RNA (anti-miR-21). NPs showed antiproliferative and proapoptotic effects in human breast (MCF7, ZR-751, BT-474) and mouse mammary (4 T1) carcinoma cells [81].

3.2.2.2. Nonmetallic nanomaterials

Despite the fact that most widely used nanomaterials have metal cores, a number of industry-relevant nonmetallic nanoscale particles such as SiO_2 and carbon NPs have been engineered. For instance, silica NPs have been extensively used in food additives [82], toothpaste and skin care products [83]. However, their use requires toxicology screening to determine their innocuity. According to Wittig, et al., commercially available nanosilica (Ø 12 nm) increases the growth of GXF251L human gastric carcinoma cells. The results showed an important proliferative effect through the activation of cellular epidermal growth factor receptor (EGFR) and mitogen-activated protein kinase (MAPK) signaling pathways [84].

On the contrary, research on antiproliferative properties have found that cerium oxide nanocrystals (nanoceria: CeO_2-NCs) can act as an anticancer drug [85]. The investigation conducted by Khan, et al. found that fluorescence microscopy assessments of nanoceria displayed a marked *in vitro* cytotoxic effect and reduced cellular viability on HT-29 human colorectal adenocarcinoma. The results showed downregulation of Bcl2 and BclxL protein expression suggesting proapoptotic effects. Additionally, this study confirmed previous reports [86] where cerium oxide exhibited a cytotoxic effect toward cancer cells with minimum toxicity to normal cells [86]. Another interesting example involved the evaluation of cytotoxic effects of smart-releasing NPs synthesized using cytochrome C (Cyt C) and hyaluronic acid (HA) [87]. This study, by Figueroa, et al., showed that A549 human lung adenocarcinoma cellular viability was reduced to 20% (0.16 mg/mL [Cyt C], 6 h of exposure), while OS-7 African green monkey kidney fibroblasts were not affected. Confocal microscopy imaging confirmed the release of Cyt C to the cytoplasm upon reaching the target. In this study, EPR effect is used to develop a new potential stimuli-driven nanoparticle for cancer treatment [87].

Liposomes were firstly described in the middle 1960s as spherical vesicles constituted with phospholipid bilayers [88]. These lipid-based nanoparticles have been used in several fields from biophysics to biology for many years [88]. With the advances of nanotechnology, liposomes have evolved in order to assure controlled delivery of active molecules to a specific site of action. For instance, a radiation therapy scheme in use for more than 50 years is boron neutron capture (BNCT), which is based on the specific delivery of the isotope (boron-10) undergoing a nuclear reaction to form boron-11, through exposure to a laser beam (neutron source [89]). This reaction causes a release of an α-particle that has a high linear energy transfer (LET) and kills the equivalent of one cell diameter [89]. In the above research, conducted by Maitz, et al., the effect of unilamellar liposomes (composed of cholesterol, 1, 2-distearoyl-sn-glycero-3-phosphocholine, K [nido-7-CH_3 (CH_2)15-7, 8-$C_2B_9H_{11}$] and core Na_3 [1-(2′-$B_{10}H_9$)-2-$NH_3B_{10}H_8$]) on mice bearing tumors (breast carcinoma EMT6 and colon carcinoma CT26) was studied.

Type	Nanoparticles	Coating material	Cell type	Biological effect	Ref.
Metallic	AgNPs	Naked	A2780 ovarian carcinoma	Cytotoxic	[19]
			OvCSCs ovarian cancer stem cells		
			Colo 205 colon adenocarcinoma	Proapoptotic, synergic with anticancer drugs	[28]
			Colo 320 drug-resistant colon adenocarcinoma		
			HT22 hippocampal neuronal model	Antiproliferative, DNA hypermethylation and oxidative stress damage	[29]
			Mouse embryonic stem cells	Transcriptomic alterations	[30]
			Bovine retinal endothelia	Angiogenesis inhibition	[32]
		Erythrina indica extract	MCF7 breast cancer	Cytotoxic	[27]
			HepG2 hepatocellular carcinoma		
		Albizia adianthifolia extract	A549 lung cancer		[61]
		Acalypha indica extract	MDA-MB-231 human breast adenocarcinoma	Cytotoxic, proapoptotic	[58]
	AuNPs	Naked	HeLa cervical carcinoma	Cytotoxic	[3, 36–38]
			PC-3 prostate cancer		
			HepG2 hepatocellular carcinoma		
			MDA-MB-231 human breast adenocarcinoma		
			Calu-1 epidermoid carcinoma	Proapoptotic, synergistic effect with anticancer molecules, DNA fragmentation and mitochondrial fission	[39]
			MG63 osteosarcoma	Cytoskeleton damage in combination with laser treatment	[41]
			A549 lung cancer	Cytoskeleton damage in combination with ultrasound	[42]
			U251 glioblastoma	Proapoptotic, radiosensitizer	[43, 44]
			HT-29 colon adenocarcinoma		
		Platinum coated	MCF7 breast cancer	Proapoptotic, ROS production and cell cycle arrest	[47]
		Acalypha indica extract	MDA-MB-231 human breast adenocarcinoma	Cytotoxic, proapoptotic	[58]
	PtNPs	Naked	A549 lung cancer	Cytotoxic	[45]
			PA-1 human ovarian teratocarcinoma		
			Mia-Pa-Ca-2 human pancreas carcinoma		
			U118/U87 human malignant glioma	Antiproliferative	[46]
			Neuro-2a brain neuroblastoma	Proapoptotic in combination with laser treatment	[48]

http://dx.doi.org/10.5772/intechopen.71685

Type	Nanoparticles	Coating material	Cell type	Biological effect	Ref.
Metallic	Cuprous oxide CONPs	Naked	SiHa cervical squamous carcinoma	Cytotoxic, autophagy and proapoptotic	[51]
			HeLa cervical carcinoma		
			MS751 cervical cancer		
			LNCaP FGC human prostate carcinoma	Cytotoxic, reduction in transcription factors for proliferation	[52]
			PC-3 prostate cancer		
			DU145 prostate carcinoma		
	Iron oxide, SPIONs	Naked	U-2 OS osteosarcoma	Cytotoxic, autophagy and proapoptotic	[53]
			Saos-2 osteosarcoma		
		antiangiogenic peptide ATWLPPR (A7R)	MDA-MB-231 human breast adenocarcinoma	Reduce angiogenesis and proliferation	[11]
	ZnO	Bovine serum albumin	HepG2 hepatocellular carcinoma	Anti-inflammatory and mitochondria-lysosome damage inducer	[79]
	Biopolymer	4-hydroxytamoxifen and noncoding RNA (anti-miR-21)	MCF7, ZR-751, BT-474 breast cancer	Antiproliferative and proapoptotic	[81]
			4 T1 mouse mammary carcinoma		
Nonmetallic	SiO_2	Naked Ø 12 nm	GXF251L gastric carcinoma	Increase proliferation	[84]
	Cerium oxide CeO	Naked	HT29 human colorectal adenocarcinoma	Cytotoxic, antiproliferative and proapoptotic	[85]
	Cytochrome C (Cyt C) and hyaluronic acid (HA)	Naked	A549 lung cancer	Antiproliferative	[87]
Liposomes	Core: Na3 [1-(2′-B10H9)-2-NH3B10H8]	Cholesterol, 1, 2-distearoyl-sn-glycero-3-phosphocholine, K [nido-7-CH_3 (CH_2)15–7, 8-$C_2B_9H_{11}$]	EMT6 breast carcinoma	Increase radiosensitivity of tumors	[89]
			CT26 colon carcinoma		
	Lipid-core	Poly(ε-caprolactone), capric/caprylic triglyceride, sorbitan monostearate and polysorbate 80	SK-Mel-28 human melanoma	Cytotoxic, proapoptotic and cell cycle arrest	[92]

Table 2. Biological effects of different types of nanoparticles (NPs) on cancer cells.

The results showed a 50% tumor reduction after 45 min of radiation, despite lower boron concentrations inside EMT6 tumor, in comparison to CT26. The average time for tumor growth, set as three times the pretreatment volume, was 38 days for BNCT-treated mice in comparison to 4 days for untreated controls. In conclusion, the authors found that liposomes were useful elements for increasing inherent radiosensitivity in selected tumors [89].

The use of liposomes has also been found useful for drug delivery systems [90]. Sadhu, et al. evaluated the cytotoxicity and antiproliferative effects of liposomes designed to increase the intracellular glutathione disulfide (GSSG) on B16 murine metastatic melanoma tumor cells (B16F10), human metastatic lung carcinoma cells (NCI-H226) and *in vivo* on C57BL/6 mice. Glutathione (GSH) is fundamental in the antioxidant defense against ROS [91]. Oxidation of GSH is mediated by a sulfhydryl residue from oxidative species and results in GSSG [91]. Analysis of GSSG has been a challenge since it is not inducible and neither cell membrane permeant. The results showed an important effect in the apoptotic pathway affecting cell migration, invasion and adhesion. Dacarbazine (the treatment option for melanoma) and GSSG liposomes showed a significant *in vivo* reduction of tumor proliferation (90% and 85%, respectively) [91].

Drewes, et al. demonstrated that lipid-core nanocapsules containing poly(ε-caprolactone), capric/caprylic triglyceride, sorbitan monostearate and polysorbate 80 affected cell proliferation and triggered cell cycle arrest on SK-Mel-28 human melanoma cells. Furthermore, nanocapsules induced apoptosis and necrosis on a murine model B16F10 (H2b) bearing B16 melanoma cell line [92]. To sum up, GSSG liposomes and lipid-core nanocapsules are potentially useful for antimetastatic treatment and as drug delivery systems for melanoma treatment, respectively [91]. Organically coated nanostructures, including liposomes, might exert antiproliferative cytotoxic properties against cancer cells/tumors but may also induce cell proliferation depending on the type of tumor and nanostructure used.

The wide spectrum of known cancer cellular responses to nanomaterials is summarized in **Table 2**.

4. Conclusion

In vitro cellular models for the study of antiproliferative and/or cytotoxic properties of engineered nanomaterials are valuable tools in cancer research. Cancer cell lines represent very easy-to-use models where different codelivery treatments might be tested. For instance, including chemotherapeutic drugs, siRNAs and antibodies in the same NPs should help lower drug concentrations and side effects as well as improve the therapeutic effect. Taking advantage of this type of approach in cancer cell lines might be of value when testing NPs in personalized medicine applications, when tumor cells from the patients are collected and either cultured or injected into *in vivo* vertebrate models. Recently, Rita, et al. reported the use of zebrafish xenotransplants [93] using colon cancer cell lines, SW480, SW620, and HT29, HCT116 and Hke3. Larvae were injected with cancer cells to develop mono-/polyclonal tumors, which were treated with different antiproliferation drugs. Results displayed differential

drug sensitivities and support the potential application of this assay in personalized medicine and diagnostics. Such approaches should also decrease multidrug resistance rates.

Several techniques and methods are readily available for investigation of nanostructured particle properties regarding their selective cytotoxicity and/or antiproliferative effects. Setting up of those techniques, however, needs to be carefully monitored. Harmonization of the wide range of methods available is necessary for assay comparison and replicability.

To sum up, extended cell-based testing (*in vivo*) is necessary to obtain a complete understanding of the *in vitro* results. Although individual or core laboratory capabilities play a role in selection and availability of techniques, data arising from cancer cell models have demonstrated usefulness in guiding further research.

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