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c0060 Zinc Oxide Nanostructures in Biomedicine

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s0010 12.1 Introduction

p0010 Zinc oxide (ZnO) is a well-known metal oxide, studied from decades for its semiconducting, piezoelectric, pyroelectric, and optical absorption and emission properties [1]. From the biomedical and biological point of views, it is classified as a "GRAS" (generally recognized as safe) substance by the Food and Drug Administration (FDA) [2]. Actually, it can be easily found in many commercial formulations for healthcare, as in baby- and sun creams preparations [3]. Nevertheless, the materials considered GRAS are above the micrometer range. ZnO, when reduced to the nanoscale, as many other substances, can develop new structural, physicochemical, and optical properties, such as the increase of the surface area-to-volume ratio and a higher chemical reactivity, since a large percentage of atoms in nanosized materials are at the surface and due to quantum effects [4]. Therefore, unique biological and nano-biomedical applications could potentially arise from these new properties, as well as new possible mechanisms of toxicity not present in the bulk counterpart.

p0015 The possible interactions between ZnO nanostructures (NSs) and the biological systems are mainly ruled by different morphological parameters such as size, shape, aspect ratio, surface area, surface charge, and chemical reactivity [5–8].

p0020 Actually, the size of nanoparticles (NPs) is comparable to naturally occurring biological molecules, so their internalization into living cells enables them to affect the cellular behavior and viability.

p0025 The high surface reactivity of the NSs amplifies their capability to interact with other species yet to be loaded with active principles and to deliver these drugs to the target cells and tissues.

p0030 Both surface charge and chemical reactivity drastically affect the colloidal stability and the biological interaction of ZnO NPs with cells. Since cancer cells frequently have highly

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negative membrane potentials, showing a large amount of anionic phospholipids and negatively charged proteins and carbohydrates [9], interactions with positively charged ZnO NPs are expected to be promoted by electrostatic interactions. Thus, with respect to healthy eukaryotic cells decorated by neutrally charged zwitterionic phospholipids [4], cancer cells would selectively support cellular uptake of positively charged ZnO NPs and ultimately be affected by their potential cytotoxic behavior or drug release capability.

p0035 In particular, for in vivo experiments, the accurate design of ZnO NPs with appropriate size, surface charge, and chemistry is essential to avoid rapid circulation clearance, accumulation in end organs, and related toxicity [10], as well as to promote efficient cancer cell internalization and cytotoxicity.

p0040 In the present chapter, we describe the most recent applications of ZnO nanomaterials in the biomedical field. Starting from the synthesis of biocompatible ZnO NPs suitable for the contact with biological fluids, we then consider also the requirements for their hemocompatibility. We evaluate the potential cytotoxicity risks toward living cells once NPs are administered. We also focus on the multiple strategies developed for cancer diagnosis and therapies based on ZnO nanoconstructs and on their application in the tissue engineering (TE) and in the regenerative medicine contest.

s0015 12.2 Synthesis of Biocompatible Zinc Oxide Nanoparticles

p0045 As mentioned earlier, in order to build biofunctional materials, it is important to look at different aspects. First of all, it is fundamental to find an optimal size range to maximize the active area and to exploit different structural, physical, and chemical properties of the nanostructured material. The tuning of size is also important concerning cytotoxicity aspects (ZnO NPs in the size range of 5–30 nm have proven to show low toxicity levels) [11], and engineering the surface interaction with biomolecules can drive therapeutics approaches in a specific way.

p0050 One simple way, that allows controlling both the oxide particle size and functionalization, concerns the use of appropriate surfactants during the NP synthesis. In this way, the chemical reactivity and the colloidal stability in biological fluids of NPs also can be effectively controlled [8]. Recently, interesting examples of this approach were described in studies focused on ionic or nonionic polymers (such as poly(ethylene glycol), PEG; poly(*N*-vinyl-2-pyrrolidone), PVP; propylene glycol, PG; poly(vinyl alcohol), PVA [12,13]), or other specific surfactants (i.e., TritonX, Tween 20) in ZnO NP preparations, guaranteeing a good level of surface functionalization. Obviously, these surfactants must exhibit a very low toxicity and high solubility in the reaction media. The proposed synthetic approach of coating the ZnO NPs with polymers/surfactant results into more biocompatible surfaces, able to better interact with cells or, in general, with biological environments for both therapeutic and bioimaging applications. In addition, the electrostatic characteristics (i.e., *Z*-potential) of the surface are modified, avoiding the tendency of ZnO NPs to agglomerate due their high surface energy [14].

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p0055 For example, in a simple one-step procedure at very mild conditions (precursor solution mixed for 24 h at room temperature), the surface functionalization with 3-mercaptopropionic acid occurred during the ZnO crystal formation. The functionalization acts as conjugation agent for a drug delivery system, allowing to improve both stability and solubility of curcumin molecule for therapeutic applications [15].

p0060 A similar approach was also reported [16] for studying the role of different capping agents (PEG and PVP) of functionalized ZnO NPs as antibacteria agents, with specific enzymatic inhibition activities against α-amylase enzyme.

p0065 Via a simple chemical route, it was possible the controlled co-precipitation of functionalized ZnO NPs from a water solution of precursors and of the two selected capping agents, without using complex procedures and postsynthesis treatments.

p0070 Another approach is to functionalize ZnO NPs after their synthesis, in a two-step process. This method results promising due to a discrete concentration of active chemical groups (i.e., hydroxyl groups or negatively charged oxygen atoms: $ZnOH^-$, ZnO^-) on the ZnO surface. These groups could easily react with specific molecules that will be covalently bound to the particle surface [17].

p0075 A typical methodology consists of the functionalization with amino [18] or carboxylic groups [19] using a condensation reaction, starting from a ZnO NP suspension in a solution of functional precursors, like 3-aminopropylphosphonic acid or 3-aminopropyltrymethoxysilane (3-APTES) for amino groups or citric acid for the carboxylic one. Also in this case, the synthetic processes result to be quite simple, refluxing the reactive suspension for several hours, followed by centrifugation and washing steps, in order to eliminate the unreacted precursor residuals onto ZnO NPs that could negatively influence any following procedures.

p0080 The interesting result consists of an NP surface that could better interact directly with biological environments, thanks to the combination of various weak interactions driven by the remaining –OH groups and by new functional groups, determining an overall more lipophilic NP behavior.

p0085 At this stage, this functionalization still encloses reactive groups opening to a wide range of further possible engineering phases driven by the final target application. For example, anticancer functions or antigen carrier for vaccine development should be accomplished by a second functionalization step of ZnO NPs, by combining a high-affinity peptide or specific antibody to the ZnO NPs [20] to directly activate a specific recognition toward target molecules. This principle should also be used in order to develop specific biosensors or predictive analytical bioassays.

p0090 An interesting alternative application of functionalized ZnO NSs in biological media could be a protein preconcentrator [21]. The study shows a hydrothermal synthesis of substrate-supported ZnO, followed by a preliminary functionalization using a silanization reaction with 3-APTES, providing sites for amide ($–CONH_2$) covalent bond formation on ZnO NPs. Therefore, the functionalized NS are biofunctionalized via drop casting with specific antibodies. In this way, the antibodies result immobilized on the surface and able to efficiently capture the target protein biomarker (in this case myoglobin) from a dilute and large volume sample.

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p0095 As demonstrated by the reported studies, the ease of introduction of different types of "biointeractive" species onto ZnO NSs or directly in ZnO synthesis may extend the molecular space in the search for new bioapplications (like antibacterial, drug delivery, or diagnostic agents) without complex chemical synthesis or sophisticated techniques.

p0100 The modification of ZnO NPs should take advantage of the possibility to rationalize the presence of reactive groups, for example, hydroxyl groups, driving condensation reactions, or hydrogen bond interactions, and being exploited to adapt the molecule surface at the required application.

s0020 12.3 Biostability and Biodegradation of Zinc Oxide in Biological Media

p0105 As mentioned earlier, size, shape, and surface chemistry are the key for the biological fate of NPs, since these parameters can regulate their action and biodistribution in living organisms [22] and their interaction with the immune system [23]. The control of charge and surface properties is of paramount importance also for the study of cellular response in vitro, since they affect the colloidal stability of NPs and their aggregation behavior in biological media [24]. In fact, the phenomenon of aggregation has been shown to strongly influence the cellular uptake and the toxicity profile of the NPs both directly (due to their increased size) and indirectly (due to their altered diffusion and sedimentation velocities) [25].

p0110 According to DLVO (Derjaguin, Landau, Vervey, Overbeek) theory, the colloidal stability of NP suspension is favored by higher net surface charge, responsible of electrostatic repulsive forces [26]. In case of metal oxide NPs, such as ZnO, the charge behavior is regulated by oxydrile (–OH) groups present on their surfaces [27]. In high pH solutions, the chemisorbed protons (H^+) leave the surface, inducing a negatively charged surface with partially bonded oxygen atoms (ZnO^-). On the contrary, for low pH, protons from the medium are transferred to the particle surface, leading to positively charged $ZnOH_2{}^+$ groups. In particular, ZnO has an isoelectric point (IEP) around pH 9–10 that provides a strong positive charge under physiological conditions, such as blood or tissue fluid, which have neutral pH [28]. This characteristic is particularly interesting for biomedical applications because electrostatic interactions between positively charged nanomaterials and negatively charged cell membrane are believed to play an important part in cellular adhesion and uptake [29]. Specific studies, in fact, indicate that this characteristic might be the reason that ZnO nanoparticles display high cytotoxicity compared to many other oxides that have relatively lower IEPs [30].

p0115 The colloidal behavior of NPs in cell culture media or in physiological fluid is also strongly influenced by the fluid's composition, in terms of ionic content and strength, and in terms of presence of proteins and other macromolecules [25]. Hydrophobicity, size, radius of curvature, charge, and coatings are all factors that can influence the formation of protein corona around NPs [28] that define their biological identity and thus their

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biodistribution. Studies on ZnO and TiO_2 aggregation behavior in biological media show Q4 that a number of different plasma proteins (including immunoglobulins, lipoproteins, acute-phase proteins, and proteins involved in complement pathways and coagulation) effectively bound to these NPs, providing a first insight of in vivo biological response to metal oxide nanomaterials [31]. Concerning the interaction with inorganic components of media used in the biological tests, ZnO is particularly reactive toward phosphate ions, contained at different extent in all common cell culture media [32]. In particular, several studies [33] indicate that extracellular soluble zinc has the tendency to form poorly soluble zinc-phosphate precipitates that decrease the concentration of Zn^{2+} to insufficient level to elicit any appreciable cytotoxicity. These observations highlight the necessity of a detailed study on dissolution and speciation of ZnO NPs in biological media to avoid erroneous estimates of their toxicity.

s0025 12.4 Hemocompatibility of Zinc Oxide

p0120 NPs for in vitro and in vivo studies should be able to perform the required function avoiding any defective effects. Usually, NPs for drug delivery and imaging applications are intravenously administered and are suddenly exposed to the immune system that, in case of lack of biocompatibility, can recognize them as nonself, evidencing an immunemodulatory and/or cytotoxic activity [34]. Nanomaterials for biomedical application must fulfill all the possible biocompatibility needs and cytotoxicity studies that are usually performed together with hemocompatibility assays. NPs, micellar systems, and drug carriers should not induce coagulation activity or unveil thrombus formation, and in this contest only hemocompatibility testing is able to identify and quantify interactions of exogenous nanomaterials with blood, characterizing adverse and side effects.

p0125 The immune system is generally divided into two types of immunity, the innate and the acquired or adaptive one. The first one is guided by cells of both hematopoietic (natural killer cells, mast cells, macrophages, neutrophils, eosinophils, and dendritic cells) and nonhematopoietic (epithelial cells of respiratory, gastrointestinal, and genitourinary tracts) origin. It is focused on the early recognition of the conserved molecular patterns on pathogens receptors [35]. The high specificity of the acquired immunity provides memory and is centered on the activation of specific T cell types and on antibody production by B cells. The reaction of the immune system to NP exposure is crucial especially during in vivo applications. Escaping immune system recognition is one of the most important characteristics for NPs used for diagnosis, therapy, and early management of relapsing.

p0130 Many tests such as hemagglutination, hemolysis, platelet aggregation, and activation can be applied to check the hemocompatibility of NPs interacting with the different blood cells.

p0135 The interaction of NPs with erythrocytes may determine hemagglutination and/or hemolysis and simple colorimetric tests are able to quantify a possible toxic effect. Platelets instead have a key role in blood clotting and thrombogenicity. In such cases, NPs

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may start blood clot formation also in the absence of mechanical injury, and it can result in the formation of thrombi and in the worst case in stroke or heart attack [36]. The effect of NPs on platelet activation may be evaluated through blood clotting tests considering the release of clotting factors; by separating red cells from whole blood, platelet-rich plasma is obtained, and the hemocompatibility is usually tested by means of the measurement of factors related to thrombus formation such as platelet factor 4 (PF4) and β-thromboglobuin [37]. In addition, flow cytometry analysis allows establishing the platelet activation grade, monitoring the upregulation of the platelet receptors glycoprotein (GP) IIb/IIIa (CD 41) and of the P-selectin (CD 62P). The effect of NPs on different coagulation parameters such as fibrinogen, thrombin time, prothrombin time (PT), and activated partial thromboplastin time (APTT) can be manually or automated monitored [38].

p0140 The ever-increasing number of NP applications in the biomedical field [39] is nowadays leading to the optimization of more and more reliable biocompatibility and hemocompatibility tests. ZnO NP hemocompatibility investigation states to the direct and side effects of the interaction between the materials and each component of the blood. Different kinds of tests can be used in this context including the study of nanomaterials on blood coagulation, measuring the PT and APTT, adsorption of plasma proteins, and erythrocyte hemolysis. ZnO NPs are characterized by a positive surface charge and, when dispersed in Q5 the blood, they interact with many proteins present in the plasma leading to potential unfavorable distribution and toxicity. The assessment of the hemocompatibility of ZnO NPs is considered a main aspect for their in vivo applications and it is strictly related to the physicochemical characteristics, design, composition, charge, and functionalization of their outer layer. More in detail, often an enhancement of hemocompatibility is referred to the protein coating over the ZnO NP surface.

p0145 The hemolysis assay of ZnO NSs functionalized with bovine α-lactalbumin (BLA, the whole NS was here called FZnONSBLA) demonstrated that such ZnO, at a concentration of 300 μg/ml and 600 μg/ml, showed 7.7% and 75.3% hemolysis, respectively, however, with minimal hemolysis below 300 μg/ml (<5%). FZnONSBLA showed negligible hemolysis up to a concentration of 600 μg/ml (<3%). The results clearly demonstrated that FZnONSBLA was hemocompatible till 600 μg/ml [40].

p0150 In another case, lipid-coated ZnO NPs (LZnO NPs), loaded with the drug 6-mercaptopurine (6-MP), were developed for the treatment of lymphatic metastatic tumors [41]. The results of hemolysis and blood aggregation tests (Fig. 12.1) evidenced that no hemolysis occurred in the ZnO NP- and LZnO NPs-treated groups, although red blood cells were evidently aggregated in the ZnO NPs-treated group, most likely for their high positive *Z*-potential value.

p0155 Despite the diffuse use of ZnO NPs, more information regarding their biological effects is still necessary. Although hemolysis, lymphocyte activation, coagulation, and the activation of complement system are today systematically investigated, it will be necessary a Q6 deeper characterization of NPs, plasma protein coronas, their interaction with blood, and with its single cellular and proteic components.

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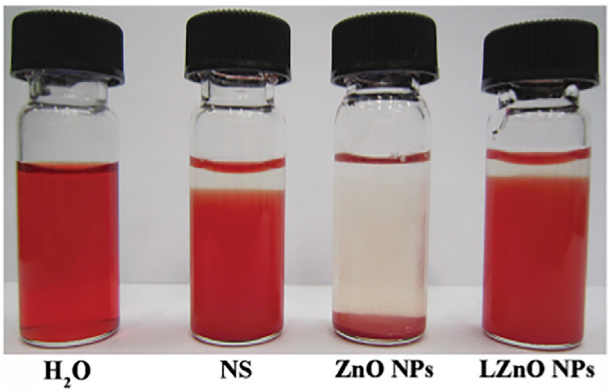

f0010 **FIG. 12.1** Photographs of red blood cell hemolysis and aggregation assay with ZnO NPs and LZnO NPs, using water (H_2O) and normal physiological saline (NS) solutions as positive and negative controls, respectively. *(Reproduced with permission from Ref. [41]; copyright (2015) The Royal Society of Chemistry.)*

s0030 12.5 Cytotoxicity of Zinc Oxide Nanoparticles

p0160 ZnO bulk materials have been considered as GRAS by the FDA [2]. However, in recent years, several in vitro studies have demonstrated that ZnO NPs have a strong cytotoxic effect on human [42] and animal [43] cells, bacteria [44], fungi [45], and protozoa [46]. in vivo toxicity was also evaluated [47]. Furthermore, many studies evidenced that cancer cells are more affected by ZnO NPs than their healthy counterparts even though the mechanisms underlining these effects were not clarified yet [48].

p0165 One of the mechanisms proposed for ZnO NP cytotoxicity is related to dissolution and release of Zn^{2+} ions. Zinc ions are in themselves nontoxic for the cell yet essential for life and involved in many cellular processes. In particular, their availability in specific cellular sites is hemostatically regulated by several pathways. Their cytoplasmatic concentration is strictly kept at low levels thanks to the action of zinc-transporting and zinc-sequestering proteins, called methallothioneine [49]. In this context, an uncontrolled increase of intracellular level of Zn^{2+} due to dissolution of ZnO NPs seems to be one of the major determinants in ZnO NP cytotoxicity [47]. In the literature it is reported that the accumulation of Zn^{2+} inside the cells disrupts zinc homeostasis and leads to a protein activity disequilibrium, affecting a wide range of crucial cellular processes [28]. In addition, unexpected elevated levels of zinc ions in the cytoplasmatic compartment induce a massive mitochondrial zinc sequestration and a consequent toxic effect for these organelles [50].

p0170 At present it is still to be ascertained whether the toxicity of zinc ions is due to their intracellular or extracellular release. In many works, toxicity of ZnO NPs is directly related to the release of toxic Zn^{2+} in the cell culture medium, and the cytotoxic effects are the result of both intracellular and extracellular dissolutions [51].

p0175 In contrast, many articles prove that extracellular dissolution is insufficient to cause cytotoxic effects [7]. This lack of toxicity is associated with the too high pH of the cell culture environment, the prevented dissolution by serum proteins, and especially the

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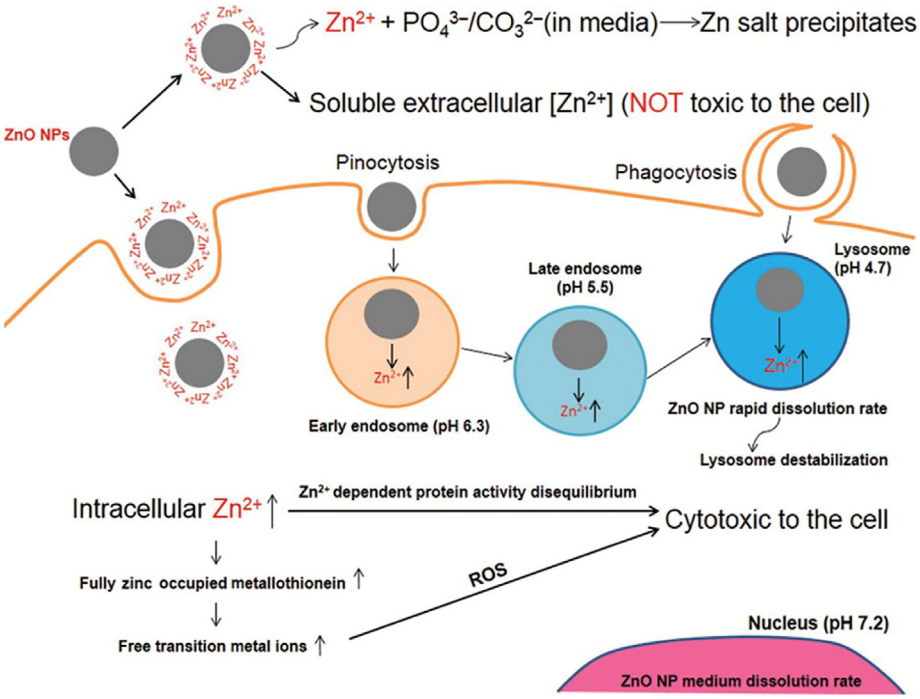

f0015 **FIG. 12.2** A possible mechanism of cytotoxicity of ZnO NPs. *(Reproduced with permission from Ref. [28]; copyright (2015) InTech Open.)*

formation of $ZnCO_3$ or Zn_2PO_4 precipitates [52]. The toxic effect is therefore due to the intracellular release of ions, which is enhanced by the low pH of the lysosome compartment [53] (Fig. 12.2).

p0180 A second proposed cytotoxicity mechanism involves reactive oxygen species (ROS) formation induced by ZnO NPs. It was largely demonstrated that ZnO NPs, thanks to their semiconductor properties, are able to directly induce ROS production. Indeed, in the presence of radiation with an energy of more than 3.3 eV [44], the excitation of electrons from the valence (VB) to conduction band (CB) could generate positive holes and induce formation of hydroxyl radicals. In addition, the free electrons in the CB can reduce oxygen [44] and generate superoxides [54]. It was also reported in the literature that the presence of crystal defects increases the electron-hole pairs in ZnO NPs even without an additional stimulus [55].

p0185 Zn^{2+} moreover can induce a mitochondrial cristae remodeling that causes a decrease of mitochondrial membrane potential [56], the release of cytochrome C, and thus an increase of ROS production [57].

p0190 An overamount of ROS formation leads the cells to an oxidative stress situation [58], altering cell cycle [47] and promoting cell death through apoptosis [54] or autophagy [59]. ROS indeed are able to induce lipid peroxidation, associated with impairment of cell membrane structure [60], protein denaturation, and different types of DNA damage [61].

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p0195 Literature also reported that ZnO NPs can promote the activation of different signaling pathways, related to the oxidative stress [47]: in particular, the activation of the transcriptional factor NF-κB, resulting in the production of proinflammatory mediators, such as IL-1b, TNF-α, IFN-γ, and IL-12, and inducing inflammatory responses [62].

p0200 Finally, yet importantly, several articles have demonstrated that the cytotoxicity of ZnO NPs is strongly influenced by parameters like particle shape, size, and surface charge. For instance, Baek et al. showed that smaller and more positive particles exhibit higher cytotoxic effects compared to larger ones [63], and Zhou et al. assessed that surface functionalization is more closely related to the fate and effects of ZnO NPs than the core composition alone [64]. As a consequence, recent articles explored the possibility of tailoring the cytotoxic effect, modifying physicochemical properties of ZnO NPs [30] and covering them with appropriate polymer coating, as described in Section 12.2.

s0035 12.6 Therapeutic Activity of Zinc Oxide

p0205 As discussed in Section 12.5, several studies have proved that nanostructured ZnO can cause severe toxicity in different cell lines. This intrinsic property of ZnO can be exploited as an innovative nanomedical tool. Hanley et al. [65] compared the response of healthy human cells and cancerous cells to ZnO NP treatment. They found out that ZnO NPs induce toxicity in a cell-specific and proliferation-dependent manner, with rapidly dividing cancerous cells being the most susceptible to the treatment. Reddy et al. [66] studied the selective toxicity of ZnO NPs to prokaryotic and eukaryotic systems. They had minimal effect on primary human T cell viability even at concentrations highly toxic to both *Escherichia coli* and *Staphylococcus aureus* bacteria. Together, these findings suggest that ZnO NPs may potentially prove useful as selective nano-therapeutic agents for bacterial infections and cancer therapy.

p0210 Moreover, to achieve a remotely activated cytotoxic effect, the catalytic properties of semiconducting ZnO can be exploited. Nanosized ZnO has a band gap of 3.37 eV. Thus, irradiation of ZnO NPs with light having a wavelength of less than 400 nm can excite an electron (e^-) from the VB to the CB, leaving behind a positive hole (h^+). The "electron-hole" pair exhibits strong redox properties and is highly unstable. In aqueous environment, the photo-generated negative electrons can reduce oxygen molecules thus forming superoxide radical anion (O_2^-), while the positive holes can oxidize water molecules and hydroxide ions, generating hydroxyl radicals and hydrogen peroxide (H_2O_2) molecules [67]. The recombination of the electron-hole pair can generate emission of a photon (radiative recombination), that in turn can excite ground state oxygen generating singlet oxygen. These ROS can exert high cytotoxic effects if generated intracellularly, as reported earlier, and can be useful for killing tumor cells.

p0215 Using the electron paramagnetic resonance technique, Lipovsky et al. [68] revealed that ZnO NPs in aqueous suspensions generate OH·radicals and singlet oxygen under UV irradiation and visible light. Thus, the remotely activated photo generation of ROS at the surface of ZnO NPs can be exploited as effective therapeutic strategy, and it is called

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photodynamic therapy (PDT). This therapy promises better selectivity and fewer side effects compared to traditional chemo- and radiotherapies. These benefits arise because the ZnO NPs can accumulate specifically within the region of interest so that when the light is directly focused only in that region the therapeutic effect is highly localized. PDT using ZnO NSs has recently shown great potential for cancer therapy. ZnO NPs irradiated with UVA-1 for 15 min induced in vitro cell death exclusively of human squamous cell carcinoma cells, while leaving oral mucosa cells unaffected [69]. Comparing the therapeutic effects of ZnO and TiO_2 NPs as photosensitizers in PDT, both nanomaterials were able to generate intracellular ROS and no differences were observed in terms of resulting in vitro cytotoxic effects in human hepatocarcinoma cells [70]. On the same cell line, PDT effects were size dependent in the range from 20 to 100 nm [71], where the smaller the NP size, the higher the cytotoxicity. UV irradiation of ZnO was also used to inactivate bacteria in vitro.

p0220 Recently, researchers have described the capability of ZnO NSs conjugated with porphyrin to synergistically induce cytotoxic effects in ovarian cancer cells on exposure to UV-A light, whereas little cytotoxicity was detected under dark conditions or with UV exposure in the absence of NPs [72]. However, the main limitation of PDT is the limited tissue penetration depth of UV light (<1 mm) used to excite the NPs, making PDT not suitable for deep-seated tumors. Further efforts are thus needed to increase the effectiveness of nanostructured ZnO as a photosensitizer agent.

p0225 Beside the use of bare ZnO NPs to induce selective cytotoxicity in cancer therapy, there is a growing interest in drug delivery systems based on nanostructured ZnO as carriers. Studies have demonstrated that coadministration of ZnO NPs loaded with chemotherapeutic drugs (i.e., daunorubicin, DNR) resulted in synergistic cytotoxic effects on cancer cells, which were enhanced further by UV irradiation [73].

p0230 Zhang et al. [74], using a simple one-step solid-state reaction, fabricated ZnO nanorods (NRs) as drug carriers of DNR. They demonstrated that NRs could enhance the intracellular concentration of DNR and increase its in vitro antitumor efficiency. Furthermore, they reported that PDT on ZnO NRs loaded by a chemotherapeutic agent could convey distinguished improvement in anticancer activity.

p0235 To enhance the low stability of ZnO quantum dots (QDs) loaded with doxorubicin (DOX), resulting from hydrophilicity and cationic charge characteristics, the NSs were capped with chitosan [75]. The use of chitosan-coated ZnO NSs proved to be an effective drug delivery system characterized by an initial burst of drug release followed by a more controlled release rate.

p0240 Similarly, biodegradable ZnO/polymer core-shell QDs were synthesized, and they were found to be sensible to pH with high water solubility and biocompatibility [76]. Poly(acrylamide) was employed as a protective coating for the ZnO QDs and DOX was selected as an anticancer drug to study the release process in human glioblastoma cells. Dicarboxyl-terminated PEG was also used to coat aminated ZnO QDs in order to stabilize the NSs in physiological fluids [77]. DOX molecules were successfully linked via formation of covalent metal-DOX complexes. After cellular uptake, the pH-sensitive ZnO QDs

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dissolved to Zn^{2+} in acidic endosome/lysosome, triggering the dissociation of the metal-drug complex and leading to a controlled DOX release.

s0040 12.7 Zinc Oxide in Tissue Engineering

p0245 ZnO was also explored for TE applications; NSs like nanoflowers (NFs), NRs, and nanowires (NWs) were investigated for promoting the adhesion, growth, and differentiation of different cell lines.

p0250 Colon et al. [78] successfully demonstrated for the first time the decreased *Staphylococcus epidermidis* adhesion and the increased osteoblast adhesion, alkaline phosphatase (ALP) activity, and calcium mineral deposition on nanostructured ZnO surfaces with respect to microstructured ones, mainly due to the use of surfaces having nanometer-size features. The topographic effect of NFs and flat ZnO films was observed on osteoblast growth and osteointegration [79]. After four days, ZnO NFs were completely covered by osteoblasts. Lamellipodia formation was observed both on ZnO films and on NFs, but NFs resulted in more active filopodia formation. This was ascribed to enhanced fibronectin adsorption, integrin binding, and expression of cytoskeletal proteins, finally promoting filopodia formation. The DNA content (total number of cells) and ALP activity (bioactivity of cells, a marker for bone cell differentiation) were also significantly higher for ZnO NFs than for flat films. After in vivo implantation of both NSs for four weeks, bone regeneration was successfully observed for NFs.

p0255 The adhesion, proliferation, and differentiation of two mammalian cell lines (PC12, as a model of neuronal cells, and H9c2, as a model of muscle cells) over ZnO NWs were also investigated [80]. Independently from the cell line, NWs supported high cell viability (>95% after 72 h), also represented by lots of cellular protrusions and conformal contacts between cells and NWs. However, contrasting behaviors were obtained after seven days from differentiation induction. PC12 cells exhibited a well-developed neurite network, with neurite approaching 100 μm in length. In contrast, H9c2 cells displayed a disordered arrangement, without showing the typical tubular shape of H9c2 myotubes. This was due to the mechanical stiffness of the NW substrate, suitable for neuronal cell adhesion and differentiation but inadequate for differentiation of muscle cells into myotubes.

p0260 Several studies also proved the promising use of ZnO NSs as adhesion-resistant biomaterials. Compared to flat ZnO substrates, ZnO NRs strongly reduced the adhesion and viability of fibroblasts and endothelial cells [81]. A deeper investigation revealed the lack of focal adhesion assembly in the cells due to the excessive spacing among NRs. This limited cell spreading and the formation of lamellipodia, thus inducing apoptosis. Although cell Q7 death was mainly ascribed to nanotopography effects, the toxic effect due to cellular uptake of ZnO NRs was also hypothesized.

p0265 The combination of the effect of nanotopography, cytotoxicity of ZnO NRs, and sputtered films on macrophages cell culture growth was further explored [82], showing that the number of adherent macrophages was reduced in both cases, if compared to control cells seeded on glass.

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p0270 Prevention of macrophage adhesion and viability was observed also by Wang et al. [83], ruling out the pore density-dependent cytotoxicity of porous ZnO films on the metabolism and spread of fibroblasts. By decreasing the pore density, the spread and proliferation of cells were inhibited accordingly, as suggested by their round shape coupled to low metabolic activity. Petrochenko et al. [84] also investigated the cytotoxic effects of nanostructured ZnO thin films and their leachates against macrophages. A lower toxicity was observed in cells grown directly on the ZnO thin film with respect to those exposed only to leachates. However, cytotoxicity was dependent on the leachate concentration of the extract solution. Cytotoxicity of the ZnO surface and the considerable ROS generation for the undiluted leachate solution also prevented adhesion of macrophage cells.

p0275 As a whole, these findings suggest that nanotopography could modulate cell adhesion but also pointed out the role of cytotoxicity due to Zn^{2+} ions release and ROS, irrespective of the morphology. However, a pioneering study [85] reported the proangiogenic properties of ROS generated by ZnO NFs, inducing the formation of matured blood vessels. In this case, a higher proliferation of human umbilical vein endothelial cells (HUVECs) was obtained when treated in vitro with 5 μg/ml of ZnO NFs. Moreover, ZnO NFs significantly induced angiogenesis and vascular sprouting during in vivo chick embryo angiogenesis assay. NFs also promoted endothelial cell migration, showing the ability to close a scratch in a wound healing assay. In all the cases, green fluorescence emission signal due to H_2O_2 (a signaling molecule for angiogenesis) was detected only for HUVECs treated with ZnO NFs. Therefore, the authors claimed that ROS generation, like H_2O_2, was the plausible mechanism for the observed angiogenic properties.

s0045 12.8 Conclusions

p0280 ZnO nanomaterials have a tremendous potential in the biomedical field, ranging from anticancer therapeutics, to antibacterial agents, and to TE scaffolds.

p0285 In this chapter we have overviewed the properties of ZnO at the nanoscale and considered the synthetic approaches to obtain biocompatible, biostable, and hemocompatible ZnO NPs. A careful evaluation about the mechanisms underlying the intrinsic cytotoxicity of ZnO nanomaterials was also offered. However, the pioneering exploitation of these cytotoxic mechanisms enables to obtain efficient ZnO-based therapeutics, antimicrobial agents, and innovative TE scaffolds.

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