Chapter 3

# The Application of Nanodiamond in Biotechnology and Tissue Engineering

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**Abstract**

Diamond in the allotrope form consists of carbon atoms arranged in a cubic crystal structure covalently bonded in $sp^3$ hybridization. Diamond has emerged as a very promising material for various biomedical applications due to its excellent mechanical properties (hardness, low friction coefficient, good adhesiveness to the underlying substrate, good interlayer cohesion), optical properties (the ability to emit intrinsic luminescence), electrical properties (good insulator in the pristine state and semiconductor after doping), chemical resistance (low chemical reactivity and resistance to wet etching) and biocompatibility (little if any toxicity and immunogenicity). For advanced biomedical applications, diamond is promising particularly in its nanostructured forms, namely nanoparticles, nanostructured diamond films and composite scaffolds in which diamond nanoparticles are dispersed in a matrix (mainly nanodiamond-loaded nanofibrous scaffolds). This chapter summarizes both our long-term experience and that of other research groups in studies focusing on the interaction of cells (particularly bone-derived cells) with nanodiamonds as nanoparticles, thin films and composites with synthetic polymers. Their potential applications in bioimaging, biosensing, drug delivery, biomaterial coating and tissue engineering are also reviewed.

**Keywords:** diamond nanoparticles, nanocrystalline diamond films, nanodiamond-polymer composites, bioimaging, biosensing, drug delivery, nanodiamond cytotoxicity, biomaterial coating, tissue engineering

## 1. Introduction

The approaching nano- and biotechnological era expects the implementation of "smart" and "functional" materials, which will be used not only as a mechanically passive substrates, but also as active devices (i.e., electronic or optical devices and sensors, micro-electro-mechanical systems, smart drug delivery systems, bioactive substrates for the cell adhesion and growth, etc.).

Diamond represents a class of perspective multifunctional materials with morphological, chemical, optical and electronic properties tailorable on demand for specific needs, especially for life science, tissue engineering or regenerative medicine.

The bonds between the carbon atoms in the diamond lattice are covalent and predominantly of $sp^3$ hybridization [1, 2]. The diamond lattice of covalently bonded carbon atoms endows the diamond with extraordinary combination of intrinsic properties, particularly high hardness and thermal conductivity [3, 4]. The fracture toughness of the diamond has been measured to be 2 MPa $m^{1/2}$, which is a relatively high value compared to other gemstones or other optical materials [5]. Diamond toughness is also dependent on the crystallographic plane on which the fracture force is expressed. The lowest cleavage energies were measured for the <111> plane [6]. The combination of the highest Young's modulus, a high fracture toughness, a low friction coefficient and a low thermal expansion predetermines it as a material for protective layers and coatings. From optical point of view, diamond offers a unique combination of transparency in most of the ultraviolet, visible and infrared regions (from 227 nm to far IR). The refractive index of diamond is 2.41–2.44 (656–486 nm). As a semiconductor, diamond has an excellent combination of properties (except of electron mobility) [7, 8]. Other remarkable properties of diamond include a high wear resistance [9–11], pressure resistance [12], strong adhesion to the underlying substrate, i.e., when deposited in the form of films [10, 13, 14], a low friction coefficient [15] and high chemical stability (e.g., low reactivity and resistance to wet etching [2]). These properties can be further improved by various techniques of material engineering, e.g., by modulating the synthesis and structure of diamond and by various combinations of diamond with other elements and compounds. For example, hardness, fracture toughness, Young's modulus and wear resistance of diamond can be markedly enhanced by the preparation of diamond in the form of aggregated diamond nanorods [9], its hardness and thermal stability can be increased by nanotwinning with cubic boron nitride [4] and the adhesion of diamond films can be improved by laser treatment of the underlying substrate [14].

The optical properties of the diamond are also excellent. Pure diamond transmits visible light and appears as a clear colorless crystal. However, diamond is capable of high optical dispersion (i.e., the ability to disperse light of different colors). In addition, although the diamond lattice is very strong and rigid, it can contain defects or be contaminated with foreign atoms (N, B, H, Ni, Co, Cr, Si), e.g., during the growth of diamond lattice. These elements can also be introduced into this lattice by ion implantation. The lattice defects and the presence of foreign atoms are responsible for the various colors of diamond, e.g., yellow and orange (nitrogen), brown (nitrogen and lattice defects), blue (boron), green (trace amounts of nickel or radiation exposure), gray (the presence of boron or unsaturated forms of carbon), black (inclusions of

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graphite and iron) or also purple, pink and red (due to changes in the electron structure by plastic deformation during traveling of diamonds from the Earth's mantle to its surface *via* magma) [16]. The defects and the presence of foreign atoms in the diamond lattice also produce the intrinsic luminescence (fluorescence) of diamond [2, 17–19]. The electrical properties of diamond can be tuned by dopants. Pure diamonds can act as excellent electrical insulators but after chemical doping, e.g., the incorporation of boron, they are converted into a p-type semiconductor [20] or superconductor [21]. Boron is by far the most widely used dopant but other dopants are also possible [22].

For all these outstanding properties, diamond is an attractive material for technical and industrial application, e.g., for polishing, machining, cutting and drilling tools. These tools are also useful for biomedical applications, e.g., for polishing biomaterials with diamond paste or cutting them with a diamond saw [23, 24], for bone resection or craniotomy using a diamond threadwire saw [25, 26] or for dental drilling in stomatology [27]. Other important fields for diamond application are electronics, optics or the jewelry trade.

This chapter will concentrate on newly explored applications of diamond in biotechnologies and life sciences, such as bioimaging, biosensing, tissue engineering, controlled drug and gene delivery and for detecting and capturing various biomolecules and coating body implants. For some of these uses, diamond is attractive mainly in its nanosized or nanostructured form, namely free nanoparticles (1D), planar films from nanodiamonds (2D), and composites of diamond nanoparticles (DNPs) and other molecules, particularly polymers in the form of 3D scaffolds, as shown in **Figure 1**.

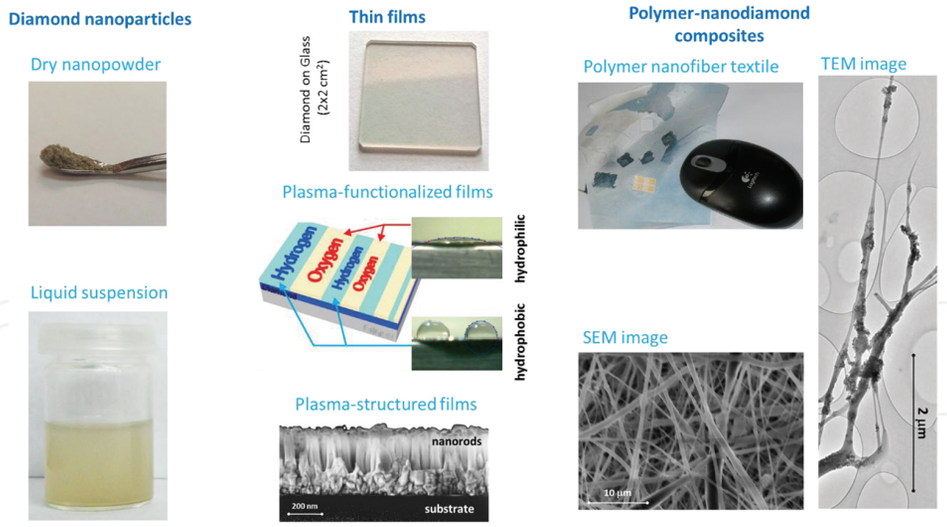


**Figure 1** Schematic view of three diamond material forms representing free nanoparticles (1D), planar nanodiamond films (2D) and composite 3D scaffolds.

Our earlier studies included in this chapter were focused mainly on nanocrystalline diamond (NCD) films as substrates for the adhesion, growth and osteogenic differentiation of human bone-derived cells in the form of commercially available cell lines, namely human osteoblast-like MG 63 cells [20, 28–33] or Saos-2 cells [34–41], primary osteoblasts [42] and bone marrow

mesenchymal stem cells (MSCs) [34, 42, 43]. The cell behavior on NCD films was further modulated by the size of the surface irregularities, measured by the root mean square (RMS) roughness parameter [34, 35], by the shape of these irregularities (nanocones and nanorods [36, 37], by termination of the NCD films with oxygen or hydrogen [38–42] and by doping of these films with boron [20, 32, 33]. NCD films micropatterned with H-terminated and O-terminated domains were also used for construction of biosensors [44]. Some of our studies were also dedicated to the construction of polymeric nanofibrous scaffolds reinforced with DNPs as potential scaffolds for bone tissue engineering. All the mentioned studies are summarized in **Table 1**.

| References | Topic | Cell model |
|---|---|---|
| Bacakova *et al*. [28] | Cell adhesion and proliferation on nanostructured and hierarchically organized submicron- and nanostructured NCD films | MG 63 |
| Grausova *et al*. [29] | Cell adhesion and proliferation on nanostructured and hierarchically organized submicron- and nanostructured NCD films | MG 63, endothelial CPAE cells |
| Grausova *et al*. [30] | Adhesion, proliferation, viability, mitochondrial activity and osteogenic differentiation of cells on nanostructured and hierarchically organized submicron- and nanostructured NCD films | MG 63 |
| Grausova *et al*. [31] | Adhesion, osteogenic cell differentiation and immune activation of cells on nanostructured and hierarchically organized submicron- and nanostructured NCD films | MG 63 |
| Broz *et al*. [34] | Regulation of the cell adhesion by the surface roughness of NCD films | Saos-2 MSC |
| Kalbacova *et al*. [35] | Regulation of the osteogenic cell differentiation by the surface roughness of NCD films | Saos-2 |
| Babchenko *et al*. [37] | Regulation of the cell adhesion by the shape of surface irregularities of the NCD films | Saos-2 |
| Kalbacova *et al*. [36] | Regulation of the cell adhesion, activity of focal adhesion kinase and osteogenic cell differentiation by the shape of surface irregularities of the NCD films | Saos-2 |
| Rezek *et al*. [38] | Adhesion and growth of cells on NCD films patterned with O-terminated and H-terminated microdomains | Saos-2 |
| Ukraintsev *et al*. [40] | Adsorption of fetal bovine serum proteins and cell adhesion on NCD films patterned with O-terminated and H-terminated microdomains | Saos-2 |
| Rezek *et al*. [39] | Adsorption of fibronectin and cell adhesion on NCD films patterned with O-terminated and H-terminated microdomains | Saos-2 |

| References | Topic | Cell model |
|---|---|---|
| Liskova *et al*. [41] | Adhesion, growth and osteogenic differentiation of cells on O- and H-terminated NCD films | Saos-2 |
| Liskova *et al*. [42] | Adhesion, growth and osteogenic differentiation of cells on O- and H-terminated NCD films | MSC, primary osteoblasts |
| Izak *et al*. [44] | Application of NCD films patterned with O- and H-terminated microdomains for construction of impedance-based sensor for real-time monitoring of cell growth | MG 63 |
| Kopecek *et al*. [33] | Adhesion, growth and osteogenic differentiation of cells on boron-doped NCD films | MG 63 |
| Kromka *et al*. [20] | Adhesion, growth and osteogenic differentiation of cells on boron-doped NCD films | MG 63 |
| Grausova *et al*. [32] | Adhesion, growth and osteogenic differentiation of cells on boron-doped NCD films | MG 63 |
| Parizek *et al*. [45] | Cell adhesion and growth on nanofibrous PLGA-nanodiamond scaffolds | MG 63 |
| Brady *et al*. [43] | Cell adhesion and growth on nanofibrous PLGA-nanodiamond scaffolds | MSC |
| Bacakova *et al*. [46] | Cell adhesion and growth on nanofibrous PLLA-nanodiamond scaffolds | MG 63, Saos-2 |

**Table 1.** Summarization of our earlier studies on cell performance on NCD films and polymeric nanofibrous scaffolds loaded with diamond nanoparticles.

## 2. Diamond nanoparticles

Individual DNPs can be advantageously used for drug and gene delivery, bioimaging technologies and biosensor construction. For these purposes, it is necessary to attach various atoms or molecules on the DNP surface, to achieve good dispersiveness of the DNPs in aqueous solutions, such as drug vehicles, buffers, physiological solution, cell culture media or body fluids and also to add chemical functionality to the DNPs in order to enable the uptake of DNPs by cells. DNPs have a high surface/volume ratio, i.e., they have a relatively large surface, on which not only atoms and small molecules, but also macromolecules (e.g., various drugs, nucleic acids and proteins) can be attached by physi- or chemisorption (for a review, see [47]). However, this attachment can be limited, for example, due to DNP hydrophobicity and tendency to aggregate in aqueous solutions. Thus, it is necessary to engineer the surface of DNPs for their specific applications [2]. The attachment of synthetic and biological molecules to the nanodiamond surface and the dispersion of DNPs in the aqueous media can be improved by various types of functionalization of the nanodiamond surface, e.g., by oxygen-containing chemical functional groups, such as –OH, –COOH [48–50], amine groups [48, 51] and by various biomolecules, such as lysine [52], biotin [53], thionine, trimethylamine [49], polygly-

cerol [54, 55] and RGD-containing oligopeptides, i.e., ligands for adhesion receptors on the cell surface [54, 56]. This functionalization can also improve the penetration of DNPs into cells (i.e., by crossing cytoplasmic and nuclear membranes) or by their internalization through clathrin-mediated endocytosis [57–59]. Functionalization of the DNPs with thiol groups (−SH) enables their conjugation with gold and silver nanoparticles [60, 61]. Such complexes are promising for photothermal therapy against tumors [60].

### 2.1. Controlled drug and gene delivery

Nanodiamond-based drug delivery has been developed particularly for advanced tumor therapies, e.g., the treatment of multidrug-chemoresistant leukemia. In experiments *in vitro*, daunorubicin conjugated with DNPs was efficient against a multidrug-resistant K562 human myelogenous leukemia cell line, which was able to overcome treatment with daunorubicin alone [62]. Other anticancer drugs, which were successfully conjugated with DNPs and delivered to cells, were 10-hydroxycamptothecin [63], polymyxin [64] or doxorubicin, an apoptosis-inducing drug widely used in chemotherapy [54, 65]; for a review, see [1, 66]. A nanodiamond-mediated doxorubicin delivery system was designed to inhibit the lung metastasis of breast cancer [67] and also to treat malignant brain gliomas [68]. The nanodiamond-doxorubicin complexes were more significantly efficient in killing glioma cells *in vitro* and also *in vivo* in rats than the uncomplexed drug [68].

Other therapeutic molecules which can be delivered into cells through DNPs include cell growth and differentiation factors, antibodies, vaccines and anti-inflammatory drugs. For example, basic fibroblast growth factor (bFGF) and bone morphogenetic protein-2 (BMP-2) were loaded onto DNPs by physisorption, forming a stable colloidal solution. This solution enabled the release of both factors in slightly acidic conditions, induced the proliferation and osteogenic differentiation of osteoblast progenitor cells and was injectable. Thus, this solution was an effective alternative to the currently used delivery of bFGF and BMP-2 to the surgical site by the implantation of bulky collagen sponges [69]. Carboxylated DNPs conjugated with growth hormone were tested for a specific targeting growth hormone receptor in cancer cells and potential anticancer therapy [70].

DNPs can also serve as a stable delivery platform for therapeutic antibodies, as revealed by studies on the transforming growth factor β (TGF-β) antibody. The complexes of this antibody with DNPs were stable in water, but under physiological conditions simulated in serum-supplemented cell culture, the release of the active antibody was detected [71]. An interesting newly developed application of DNPs is delivering vaccines through the skin for painless and efficient immunization. These nanoparticles could improve limited drug delivery through the stratum corneum, which has a very dense structure and only allows the penetration of small molecules with a molecular weight of below 500 Da (for a review, see [72]). Fluorescent nanodiamonds functionalized by hyperbranched polyglycerol also proved to be promising carriers for delivering aluminum oxyhydroxide, i.e., a compound widely used as an immunologic adjuvant of vaccines [73].

For localized drug delivery, DNPs can be incorporated into various materials, e.g., films for potential implant coating [74]. For example, aqueous dispersible detonation nanodiamonds

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were assembled into a closely packed multilayer nanofilm with positively charged poly-L-lysine *via* the layer-by-layer deposition technique, and integrated with drugs suppressing the release of inflammatory cytokines (tumor necrosis factor-alpha, interleukin-6) and nitric oxide synthase induced by lipopolysaccharides [65].

DNPs can also be effectively used for the proteolysis and digestion of glycopeptides. Trypsin and peptide-N-glycosidase F, immobilized on detonation nanodiamond, were more efficient than the free enzymes or commercially available beads immobilized with trypsin [75].

As for gene delivery, DNPs can act as excellent nonviral vectors for binding and transferring plasmid DNA and small interfering RNA (siRNA), particularly after functionalization, e.g., with –OH groups [49] or lysine [52]. DNPs, rendered cationic by coating with polyethyleneimine or by termination with hydrogen in plasma, efficiently delivered siRNA into Ewing sarcoma cells and blocked the expression of EWS/FLI-1 oncogene in these cells. In addition, the association of EWS/FLI-1 silencing by the siRNA/nanodiamond complex with a vincristine treatment potentiated the cytotoxic effect of this chemotherapeutic drug [59]. Similarly, DNPs conjugated with RGD oligopeptides and siRNA for vascular endothelial growth factor (VEGF) can be used in antiangiogenic gene therapy to inhibit tumor growth *via* the downregulation of the VEGF expression [56]. Last but not least, an array of DNPs in the form of nanoneedles facilitated the delivery of double-strand DNAs (dsDNA90) into cells to activate the pathway involving the stimulator of interferon genes (STING). In addition, this array was successfully utilized to isolate the transcriptional factor, NF-kB, from intracellular regions without damaging the cells, upon STING activation [76].

### 2.2. Bioimaging

Due to stable and controllable photoluminescence, DNPs are also promising for advanced bioimaging techniques. Like the various colors of diamonds, mentioned above, this photoluminescence is based on crystallographic defects and particularly on optical centers created by the incorporation of foreign atoms (N, Si or Cr) into the diamond lattice. DNPs with a nitrogen-vacancy (NV) color center were detectable in living cells *in vitro*, and also after application into chicken embryos *in vivo*, using a standard confocal microscope [19]. In addition, DNPs with NV color centers offer great potential for advanced imaging techniques, such as optical super resolution nanoscopy, magneto-optical (spin-assisted) subwavelength localization and imaging, single molecule spin imaging or nanoscale imaging of biomagnetic fields [17]. Photoluminescent nanodiamonds of size less than 50 nm were used to investigate the mechanism of their uptake by living cells. By selectively blocking different uptake processes, it was shown that DNPs enter cells mainly by clathrin-mediated endocytosis. Intracellularly, the largest nanoparticles and aggregates were localized in vesicles, while the smallest particles appeared free in the cytosol [58]. Fluorescent DNPs were also used for tracking the transplanted lung stem cells in mice with naphthalene-induced lung injury, and these stem cells were found at terminal bronchioles of the lungs for 7 days after intravenous transplantation [77].

The optical activity of the luminescent color centers in DNPs depends on their proximity to the nanoparticle surface and its surface termination [2]. The photoluminescence can also be

modulated by changes in the environment surrounding the DNPs. For example, coating the DNPs with a polymer film resulted in a reduction of the lifetime of NV centers and an average enhancement in their emission rate [78]. For photoacoustic imaging in biological tissues, nanodiamonds with very high absorption in the near-infrared range can be created by irradiation with the $He^+$ ion beam [79]. The fluorescence of DNPs can also be induced by surface passivation of the DNPs with bis(3-aminopropyl) terminated poly(ethylene glycol) [60]. A nanodiamond-polyglycerol-gadolinium(II) conjugate was designed and prepared as a novel nanodiamond-based magnetic resonance contrast agent dispersible in physiological media [55]. For combined bioimaging and drug delivery, photoluminescent DNPs were coated with a porous $SiO_2$ shell [80].

### 2.3. Biosensing

DNPs with an NV color center are also emerging tools for nanoscale sensing, e.g., sensing molecular fluctuations and temperatures in live cellular environments, detecting and measuring magnetic and electric fields, and measuring ion concentrations and cell membrane potentials [17, 18]. DNPs immobilized onto a gold electrode by direct adsorption were used as a biosensor for determining the concentration of glucose and lactate [81]. Arrays of DNPs in the form of nanoneedles were applied for intracellular sensing, e.g., of intracellular signaling through NF-kB, translocated from the cell cytoplasm to the nucleus region [76]. Other sensoric applications of nanodiamonds require the creation of continuous diamond films, and they are discussed in Section 3 (Nanostructured diamond films).

### 2.4. The potential cytotoxicity of diamond nanoparticles

For all the applications mentioned above, it is necessary to use nontoxic nanoparticles. DNPs are generally considered as materials with little, if any cytotoxicity. These nanoparticles dispersed in the cell culture media did not affect the adhesion, growth, viability and differentiation of various cell types (for a review, see [82, 83]). For example, the addition of DNPs in the culture medium did not affect the adhesion, growth and adipogenic or osteogenic differentiation of human adipose tissue-derived stem cells [84]. Also the labeling of lung stem cells with fluorescent DNPs *in vivo* in mice did not affect the ability of these cells to grow and to differentiate into type I and type II pneumocytes [77].

In spite of these encouraging findings, the number of reports on nanodiamond cytotoxicity is increasing. The first report demonstrated the damage and destruction of human erythrocytes and leucocytes by DNPs [85, 86]. For low and moderate concentrations, DNPs did not negatively influence the viability of isolated inflammatory neutrophils and even enhanced their response to bacterial agents. However, the viability and antibacterial reaction of these cells was suppressed at high concentrations of DNPs (≥1 g/l; [87]). Similar reactions of neutrophils were also observed *in vivo* after intravenous and intraperitoneal administration of DNPs into rats [88]. DNPs evoked significant activation of human platelets, and when administered intravenously in mice, they induced widespread pulmonary thromboembolism [89]. DNPs had antiangiogenic effects, exerted by the downregulation of the gene and protein

expression of the proangiogenic bFGF [90]. DNPs also induced apoptosis and necrosis of vascular endothelial cells [91, 92] and caused DNA damage of mouse embryonic stem cells [93] and human glioblastoma U87 cells [94]. Moreover, DNPs decreased the proliferation and viability of various cell types, e.g., human cervical carcinoma HeLa cells [95], human osteoblast-like MG 63 cells and primary rat MSCs [96].

The adverse effects of DNPs have usually been attributed to the oxidative stress generated by these particles [92, 95, 97, 98]. DNPs created by detonation synthesis affected the cellular content of glutathione and the activities of the main antioxidant enzymes (superoxide dismutase, catalase, glutathione peroxidase, glutathione reductase and glutathione S-transferase) in human umbilical vein endothelial cells *in vitro* [98]. Furthermore, after intravenous and intraperitoneal administration into rats *in vivo*, DNPs increased the activity of superoxide dismutase and decreased the activity of glutathione reductase and glutathione peroxidase within erythrocytes [88]. In addition, DNPs increased the adsorption and delivery of a large amount of sodium ions into cells, which induced osmotic stresses, cell swelling, increase in the intracellular levels of calcium and severe cellular damage [97].

The cytotoxicity of DNPs depends on their origin, size, surface functionalization, tendency to form aggregates and the presence of impurities. In studies reporting the adverse effects of DNPs, these nanoparticles were often prepared by detonation [85, 86, 90, 93, 96, 97], while in studies indicating the nontoxic effects of DNPs on cell behavior, the DNPs were prepared by non-detonation methods, e.g., milling diamond microcrystals [73, 99]. The detonation DNPs, also called ultrananocrystalline detonation diamond (UDD), are generally of smaller size (in nanometers) than non-detonation DNPs (tens of nanometers), and this small size has been considered as the main determinant of nanoparticle cytotoxicity [91]. The detonation diamonds often contain impurities, such as other carbon allotropes [53], iridium [100], carbon soot and oxides and carbides, including those of iron, chromium, silicon, calcium, copper, potassium, titanium and sulfur (for a review, see [1]). In a study by Keremidarska et al. [96], the increased cytotoxicity of DNPs was associated with their smaller size and presence of impurities, particularly other carbon allotropes. Thus, for biomedical studies, detonation nanodiamonds need to be extensively purified. For the removal of nondiamond carbon, the following chemicals can be used: ozone-enriched air, liquid oxidants such as $HNO_3$, $HClO_4$ or different acid mixture under pressure; metal impurities can be removed by treatment with HCl, $H_2SO_4$ and its mixtures with $HNO_3$ or potassium dichromate (for a review, see [1, 96]).

The functionalization influence of detonation DNPs on their biocompatibility is controversial. Detonation nanodiamonds covered by cobalt ions showed a protective effect against contact dermatitis in mini-pigs, induced by local exposure of the animal skin to these ions [101]. Detonation nanodiamonds with hyperbranched polyglycerol coating showed good cytocompatibility with A549 cancer cells and U937 macrophages [54]. Detonation nanodiamonds functionalized with thiol groups and conjugated with gold nanoparticles did not show significant cytotoxicity to a human lung carcinoma cell line [61]. On the other hand, detonation DNPs terminated with oxygen showed higher cytotoxicity to mouse embryonic stem cells than pristine unmodified DNPs, although this cytotoxicity was lower than in multiwalled carbon nanotubes [93].

In our studies, we compared the potential cytotoxicity of DNPs from three different origins. DNPs fabricated in the Nano Carbon Research Institute (Japan) under the product name NanoAmando were prepared by a detonation method and were hydrogen-terminated. A part of these DNPs was oxygen-terminated by annealing at 450°C in air. The size of both unmodified and O-terminated detonation DNPs was only about 5 nm. The DNPs, which were acquired from Microdiamant AG (Switzerland) named MSY (Monocrystalline SYnthetic), were prepared using a high pressure high temperature (HPHT) method and were oxygen-terminated due to postproduction acid treatment by the producer. These DNPs were not further modified and were provided in several sizes ranging from 18 to 210 nm. The DNPs acquired from Adámas Nanotechnologies, Inc. (Raleigh, U.S.A.) were prepared using the HPHT method and were already provided in a water colloid with a concentration of 1000 mg/l. The size of these nanoparticles was 40 nm. The Adámas DNPs had intrinsic red fluorescence based on NV defects in their diamond lattice, which was further activated by annealing in a vacuum at 800°C.

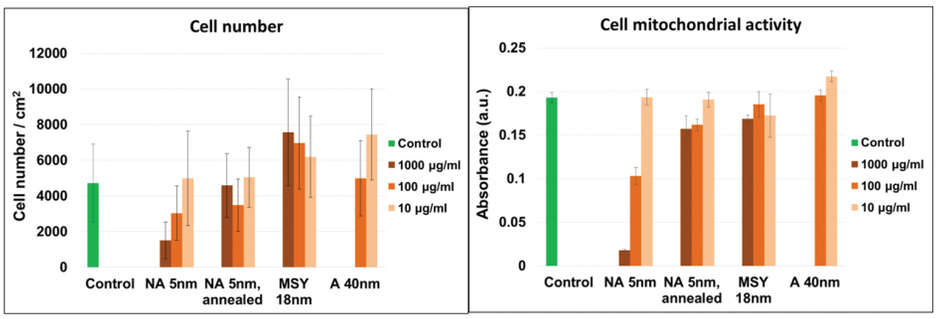


**Figure 2.** The number and mitochondrial activity (measured by MTS test) of human osteoblast-like Saos-2 cells in 3-day-old cultures exposed to NanoAmando (NA), MSY and Adámas (A) diamond nanoparticles suspended in cell culture medium (concentrations from 10 to 1000 µg/ml) for 48 hours of cultivation. Control cells were cultivated in pure medium without diamond nanoparticles. Mean ± S.D. (standard deviation).

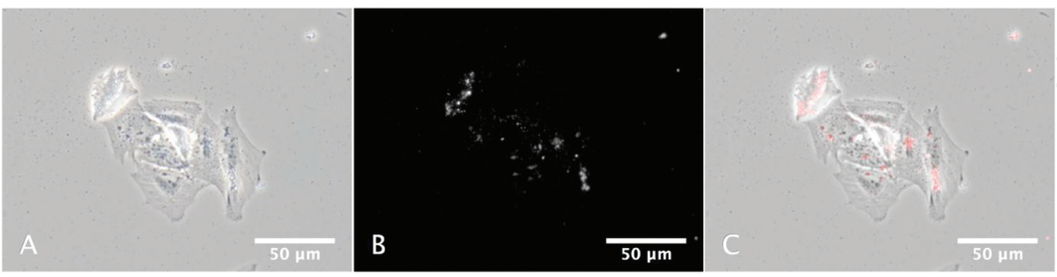


**Figure 3.** Micrographs of cells cultivated for 48 hours with Adámas DNPs Olympus IX 71, obj. 40×. Phase contrast image (A), red fluorescence of Adámas DNPs in grayscale (B), and merged images A and B (C).

The DNPs were dispersed by ultrasonication in water to obtain a colloid with a DNP concentration of 10,000 µg/ml, and added to 24-hour-old cultures of human osteoblast-like Saos-2 cells in concentrations of 10, 100 or 1000 µg/ml of the culture medium. After 48 hours, the cell number was evaluated by cell counting on recorded microphotographs using Image J software

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and correlated with the activity of mitochondrial enzymes measured by a commercially available MTS test (Promega). The lowest cell numbers and mitochondrial activity were obtained in cultures with unmodified NanoAmando DNPs, and these values decreased with increasing nanoparticle concentration (**Figure 2**). The cell numbers slightly improved in cultures with annealed NanoAmando DNPs, but they did not reach the average values obtained in MSY and Adámas DNPs. Only the mitochondrial activity of cells cultured with annealed NanoAmando DNPs reached similar values to the cells exposed to MSY and Adámas DNPs. The annealing of DNPs at a high temperature probably had a similar effect to the chemical purification of DNPs in oxidizing agents. The fluorescence of Adámas DNPs was detected inside the fixed cells after 2 days of cultivation using the standard epifluorescence microscope Olympus IX 71 (**Figure 3**).

## 3. Nanostructured diamond films

### 3.1. Diamond films as substrates for cell adhesion and growth

Our earlier studies and studies by other authors showed that NCD films provided excellent substrates for the adhesion, growth and phenotypic maturation of various cell types, such as neuronal and glial cells [102–104], epithelial cells [105], vascular endothelial cells [29], fibroblasts [106] and particularly bone-derived cells (for a review, see [82, 83, 107]). The latter cells included commercially available lines of osteoblast-like cells (MG 63 [20, 28–33], Saos-2 [30, 31, 35, 41, 108]), primary human osteoblasts [42] and human bone marrow MSCs [42, 108, 109].

The positive effects of NCD films on cell performance can be explained by their nanostructure, i.e., the presence of irregularities at the nanoscale level on their surface (e.g., an average roughness $R_a$ parameter equal or less than 100 nm). On these surfaces, the cell adhesion-mediating proteins present in biological fluids (e.g., fibronectin and vitronectin in the serum supplement of the culture media) are adsorbed in a favorable geometrical conformation enabling the exposure of specific adhesion sites in these proteins (e.g., RGD motifs) to the cell adhesion receptors, i.e., integrins. In addition, it is believed that the nanostructured surfaces preferentially adsorb vitronectin (due to its relatively small and linear molecule), which is favorably recognized by osteoblasts through its KRSR amino acid sequence [110, 111] (for a review, see [107, 112]). Thus, the NCD films are promising particularly for the surface coating of bone and dental implants. In accordance with this concept, submicron crystalline diamond films (grain sizes 200–1000 nm) and particularly microcrystalline diamond films (grain size up to 2 μm) supported the adhesion and spreading of osteoblasts to a lesser extent than NCD films [113, 114]. Also in our earlier study, the number of human osteoblast-like Saos-2 cells adhering to NCD films deposited on silicone substrates of various roughness (RMS of 20, 270 and 500 nm) decreased with the increasing substrate roughness [34]. In addition, the NCD films of RMS of 20 nm and 270 nm supported better the osteogenic differentiation of Saos-2 cells than the surfaces of a RMS of 500 nm, as manifested by a higher activity of alkaline phosphatase and a higher cellular content of calcium and phosphates [35]. Similarly, on NCD films with hierarchically-organized submicron and nanoscale surface roughness (RMS of 301.0 nm and 7.6

nm, respectively), the MG 63 cells adhered in lower initial numbers than on nanorough only surfaces (RMS 8.2 nm), although the subsequent cell proliferation was higher on the hierarchically-organized surfaces [28].

Not only the size but also the shape of the surface irregularities on the diamond films is an important factor regulating the cell behavior. For example, in our earlier studies, two different types of nanoscale features, namely nanocones and nanorods, were prepared by plasma etching of nanodiamond films using Au and Ni masks, respectively. As revealed by immunofluorescence staining of vinculin, Saos-2 cells cultivated on relatively short and broad nanocones (height 5–100 nm, diameter up to 80 nm) formed less numerous but large focal adhesions, while the cells cultivated on relatively high and thin nanorods (height 120–200 nm, diameter 20–40 nm) formed more numerous, but very thin and fine focal adhesions [37]. The large focal adhesions on nanocones were associated with stronger cell adhesion, increased activation of focal adhesion kinase (FAK), and were better predestined for osteogenic cell differentiation [36].

In contrast to free DNPs, to the best of our knowledge, no study reported the cytotoxicity of NCD films. This may be due to the fact that NCD films are compact, chemically and mechanically stable and their surface is much better defined/controlled than the surface of DNPs. Moreover, planar NCD films do not interfere and/or penetrate into the cells. The surface termination of NanoAmando DNPs controlled by high-temperature treatment was shown as a crucial parameter for their cytotoxicity (see Section 3.4). NCD films were not cytotoxic even if NanoAmando DNPs were used for the substrate seeding prior to NCD deposition.

NCD films have usually been synthesized from methane or fullerene $C_{60}$ precursors using the microwave plasma-enhanced chemical vapor deposition (MW PECVD) method. NCD films have been deposited on various substrates such as the silicon, glass or metals currently used in orthopedics and stomatology, e.g., Ti [115] and Ti-6Al-4V [116] (for a review, see [82, 83]). On these substrates, the NCD films behave as well-adhering, highly cohesive and mechanically and chemically resistant coatings. Not only were their potential adverse effects suppressed, but no particles were released from the compact films, which in addition behaved as a passivation barrier for particles and ions from the underlying materials. For example, silicon wafers, which are currently being used as experimental substrates for NCD deposition and behaved as cytotoxic for human osteoblast-like MG 63 cells and vascular endothelial CPAE cells, were rendered highly compatible with these cells by their continuous and hermetic encapsulation with NCD films [29].

Cell performance on NCD films can be further modulated by their functionalization with various atoms, chemical functional groups or biomolecules. For example, the adhesion, growth and osteogenic differentiation of bone-derived cells were better on O-terminated than on H-terminated NCD films. Human osteoblast-like Saos-2 cells, primary human osteoblasts and bone marrow MSCs in cultures on O-terminated NCD films showed better-developed focal adhesion plaques (**Figure 4**), reached higher cell population densities and produced more collagen I and alkaline phosphatase (i.e., an early and middle marker of osteogenic cell differentiation, respectively). The cells on O-terminated surfaces also showed a higher activity of alkaline phosphatase, i.e., an enzyme participating in bone matrix mineralization, and

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accordingly with it, these cells produced more calcium in their extracellular matrix (ECM) [41, 42]. Furthermore, human dental stem cells cultured on O-terminated diamond films deposited more ECM than on H-terminated films, and this matrix contained higher levels of calcium, oxygen and phosphorus [117]. Similar behavior has also been observed in human renal epithelial HK-2 cells cultured on borosilicate glass with NCD films. On the films terminated with O, these cells adhered and grew better than on H-terminated NCD films and uncoated borosilicate glass [105]. When NCD films were constructed as micropatterned films, i.e., with domains terminated with O or H, e.g., in the form of stripes of 30–200 μm in width, the cells adhered and grew preferentially on the O-terminated domains [38, 117].

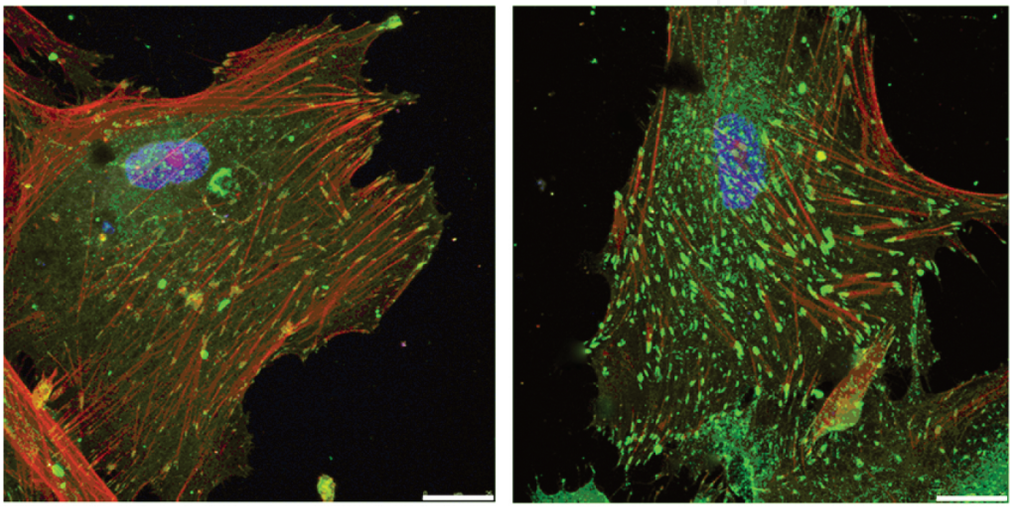


**Figure 4.** The immunofluorescence of paxillin (green), a protein of focal adhesion plaques, in primary human osteoblasts on day 7 after seeding on H-terminated (Diam. H) and O-terminated (Diam. O) NCD films. Actin cytoskeleton is stained with Phalloidin-Texas Red, cell nuclei with Hoechst #33342 (blue). Bar = 25 μm.

The positive influence of O-terminated diamond films on cell behavior has been explained by the higher polar component of the free surface energy and higher wettability of these films [118] (for a review, see [41]). It is known that wettable surfaces (similar to the nanostructured surfaces mentioned above) promote the adsorption of cell adhesion-mediating molecules in a bioactive and flexible geometrical conformation, and their effective recognition and binding by the cell adhesion receptors (for a review, see [112]). Our earlier study showed that the fetal bovine serum (FBS), i.e., an important supplement of standard cell culture media and an important source of cell adhesion-mediating proteins (e.g., vitronectin, fibronectin), formed more compact and better-adherent films on O-terminated NCD surfaces than on H-terminated NCD, where the FBS layer tended to peel from the material surface [40]. The following experiments with preadsorption of NCD films with O-terminated and H-terminated microdomains with FBS proteins revealed that the preferential growth of Saos-2 cells on O-terminated domains is mediated by fibronectin [39].

Positive effects on surface polarity, wettability and cell adhesion were also observed in NCD films terminated with amine groups, which upregulated the adhesion of human femur

osteoblasts [115, 118], or supported the adhesion of dorsal root ganglion neurons and Schwann cells isolated from Wistar rats and NG108-15 neuroblastoma-glioma hybrid cells [102].

However, in the case of the neural stem cells derived from mouse embryos, both the O-terminated and H-terminated NCD surfaces effectively supported well the cell proliferation, and this proliferation was even slightly better on H-terminated surfaces. Moreover, H-terminated NCD films were able to spontaneously induce (i.e., without the presence of differentiation factors in the culture medium) differentiation of the stem cells into neurons, while on O-terminated NCD films, the cells preferentially differentiated into oligodendrocytes [103]. The neuronal cell differentiation on H-terminated NCD films was explained by the high accumulation of fibronectin on these films, which then activated a signaling pathway involving $\beta_1$-integrin adhesion receptors, FAK and mitogen-activated protein kinase/extracellular signaling-regulated kinase1/2 (MAPK/Erk1/2) [104]. Thus, the H-terminated surfaces seemed to have a greater potential to promote the regeneration of neurons in a damaged central nervous system (which is considered as impossible in human patients) than O-terminated surfaces.

Thus, the results of cell performance on H-terminated and O-terminated NCD films, obtained by various authors, are controversial. This disproportion can be attributed to different NCD qualities (the presence of non-diamond carbon phases, different grain size, etc.), the different reactivity of various cell types (neural, epithelial, osteogenic) or different cultivation conditions. For example, the studies by Chen et al. [103, 104] were performed using a medium with a low concentration of serum (only 2% of FBS), while the experiments with osteogenic cells were generally carried out in standard cell culture media supplemented with 10–15% of FBS. When osteogenic cells were cultured in a serum-free medium, no differences in cell adhesion on O- and H-terminated NCD domains were observed [38]. Another explanation could be the relatively high hydrophilicity of O-terminated surfaces (water drop contact angle ~20° *vs*. ~78° for H-terminated surfaces) in studies by Chen et al. [103, 104]. It is generally known that cell adhesion and growth is optimal on moderately hydrophilic surfaces (for a review, see [112]). On highly hydrophilic surfaces, the adsorption of cell adhesion-mediating proteins is weak and unstable, which hamper or disable cell adhesion. For example, extremely hydrophilic O-terminated nanostructured diamond surfaces (contact angle ~2°) almost completely resisted the adhesion of human bone marrow MSCs, whereas less hydrophilic H-terminated nanodiamond surfaces (contact angle ~86°) gave good support for the adhesion and growth of these cells [109]. Also in our studies, human osteoblast-like MG 63 cells on O-terminated NCD films (contact angle about 20–35°) showed a higher tendency for spontaneous detachment at later culture intervals, when the surfaces had to hold a large number of cells [29–31] than the cells on H-terminated NCD films (contact angle about 85–90° [33], (for a review, see [112].

Another important modification of NCD films influencing cell behavior is the boron doping of these films. Lower concentrations of boron (approximately 100–1000 ppm of B) supported the proliferation and early osteogenic differentiation of human osteoblast-like MG 63 cells, which was manifested by an increased cell number and increased concentration of collagen I and alkaline phosphatase in the cells. Higher concentrations of boron (6700 ppm of B) enhanced cell adhesion and osteogenic cell differentiation, manifested by increased concentrations of

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vinculin, a focal adhesion protein, and osteocalcin, a calcium-binding ECM glycoprotein. These effects were probably due to the increased electrical conductivity of NCD films after boron doping [32]. It is known that electroactive materials are able to enhance the adhesion, growth and phenotypic maturation on various cell types, even without active stimulation with an electrical current (for a review, see [83, 112]).

Also, composite apatite-nanodiamond coatings were shown to improve the performance of bone cells. When electrodeposited on stainless steel, these coatings markedly enhanced the attachment, spreading and formation of focal adhesion plaques in osteoblast-like MG 63 cells, compared to the pure stainless steel and apatite coatings without nanodiamond, which was mediated by an increased adsorption of fibronectin on the composite coatings and its deposition by the cells [119].

### 3.2. Diamond films as substrates for biosensing and localized drug delivery

Nanocrystalline and polycrystalline diamond films are also important for constructing biosensors. For example, NCD films patterned with O- and H-terminated microdomains were used for the construction of an impedance-based sensor for real-time monitoring of cell growth, and successfully applied in cultures of human osteoblast-like MG 63 cells [44]. An aptasensor for platelet-derived growth factor (PDGF) was designed on a NCD surface functionalized with carboxylic and amine groups, which served as units for immobilizing PDGF-binding aptamers [120]. Arrays based on polycrystalline diamond surfaces grafted with odorant binding proteins (i.e., small soluble proteins present in olfactory systems) were fabricated for potential artificial olfaction applications [121]. Electrodes coated with boron-doped diamond (BDD) films are also excellent materials for the construction of biosensors, especially after further modifications with other nanoparticles and molecules [122]. For example, a BDD electrode modified with silver nanoparticles was developed as a cholesterol sensor [123]. A biosensor for the detection of L-serine was fabricated using a BDD electrode modified with polycrystalline nickel and nickel(II) oxide (Ni-NiO) half nanotubes [124]. A BDD electrode modified with platinum nanoparticles (serving as a highly conductive catalytic transducer, and coupled with a competitive magneto-enzyme immunoassay), was used for the detection and degradation of the pesticide atrazine [125]. An electrochemical sensor for glutamate, an important neurotransmitter in the mammalian central nervous system, was fabricated on doped chemical vapor deposition diamond electrodes and graphene nanoplatelet structures [126]. Diamond coatings also enhanced the sensitivity and operation range of optical fiber sensors [127].

As for the drug delivery, oxidized ultrananocrystalline diamond thin films were functionalized with type I collagen and an anti-inflammatory drug dexamethasone, in order to fabricate a hybrid therapeutically active substrate for localized drug delivery [128].

## 4. Polymer-nanodiamond composites

Another promising group of materials for biomedical applications, particularly for bone tissue engineering, is polymer-nanodiamond composites. Polymers in general are too soft and elastic

for bone tissue engineering, and thus they require reinforcements with harder and stronger materials, e.g., ceramic, metallic or carbon nanoparticles, including DNPs. Polymers reinforced with DNPs have been used in the form of thin films, e.g., poly(L-lactide) (PLLA) films [129, 130], or as electrospun nanofibrous scaffolds made of poly(lactide-co-glycolide) (PLGA) [43, 45]. Both types of composites showed improved mechanical properties and effectively supported the adhesion and growth of osteoblast-like MG 63 cells and human bone marrow MSCs (**Figure 5**). In the case of PLLA films, fluorescent DNPs were used in order to localize these materials in tissues after implantation and to investigate their behavior *in vivo* [129, 130]. In the case of nanofibrous PLGA scaffolds, the DNP-loaded scaffolds supported the growth of MG 63 cells to a similar degree as the pure scaffolds, but accelerated the growth of MSCs [43, 45]. This might be due to the fact that the primary or low-passaged cells, such as MSCs, were more sensitive to the physical and chemical properties of their growth substrate than the cell lines, which were better adapted to the *in vitro* conditions.

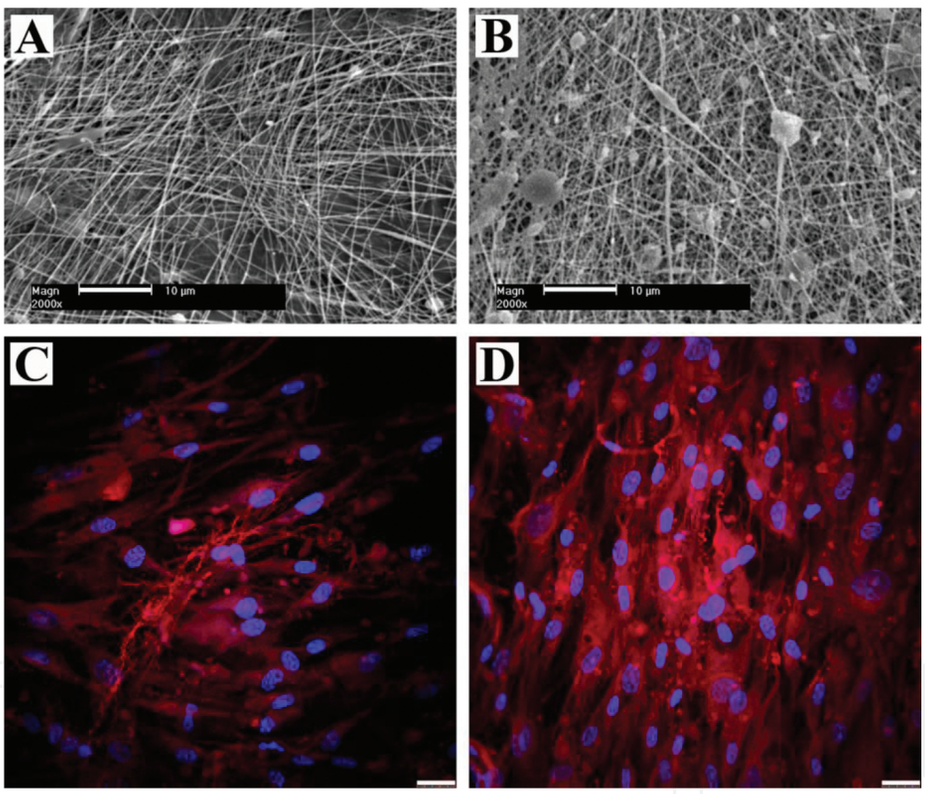


**Figure 5.** The morphology of pure PLGA nanofibrous scaffolds (A), PLGA scaffolds loaded with ~23 wt.% of diamond nanoparticles (B) and human bone marrow mesenchymal stem cells on day 9 after seeding on the PLGA (C) or PLGA-nanodiamond (D) scaffolds. A, B: scanning electron microscope, bar = 10 μm. C, D: cell membrane and cytoplasm stained with Texas Red $C_2$-Maleimide; cell nuclei counterstained with Hoechst #33342. Leica SPE confocal microscope, bar = 20 μm.

However, nanofibrous PLLA-nanodiamond scaffolds exerted rather adverse effects on their colonization with bone cells. The number, mitochondrial activity and expression of some markers of osteogenic differentiation at the mRNA and protein level in the human osteoblast-like MG 63 and Saos-2 cells cultured on these scaffolds decreased with the increasing concen-

tration of DNPs in these scaffolds (from about 0.4 to 12 wt.%; [46]). At the same time, the concentration of DNPs in nanofibrous PLGA-nanodiamond scaffolds was considerably higher, i.e., ~23 wt.% [43, 45]. The different cell behavior on the PLGA- and PLLA-based scaffolds was probably due to the different origin and properties of the DNPs used for loading these scaffolds. For PLGA scaffolds, the DNPs were prepared using a radio frequency plasma activated chemical vapor deposition (RF PACVD) method [131], while for the PLLA scaffolds, pristine NanoAmando DNPs (see Section 2.4) were used. These DNPs were much smaller, hydrophobic, and induced cytotoxic effects in Saos-2 cells when added into the cell culture medium. They were probably released from the scaffolds, and may have also increased the hydrophobicity of the scaffolds, which could negatively affect cell functions.

DNPs have been also added in materials other than polymers. For example, these nanoparticles were used to increase the corrosion resistance of Mg, i.e., a material promising to construct biodegradable bone implants. Specifically, the corrosion rate of Mg was reduced by 4.5 times, when 5 wt.% of DNPs were uniformly dispersed in the Mg matrix. At the same time, L-929 fibroblasts cultured on the Mg-nanodiamond composites maintained high cell viability and a healthy morphology [132].

## 5. Conclusion

It can be concluded that diamond in the form of nanoparticles, nanostructured films and composite scaffolds, particularly nanofibrous scaffolds loaded with DNPs, is an exceptionally promising material for a wide range of biomedical applications. DNPs are suitable for drug and gene delivery, bioimaging, biosensing and also as additives to polymeric scaffolds for bone tissue engineering, particularly those nanofibrous, in order to improve their mechanical properties and increase their bioactivity. Nanodiamond films can be applied for localized drug delivery, construction of biosensors and particularly for bone implant coating due to their favorable effect on cell adhesion, growth and osteogenic differentiation. The cell performance on the nanodiamond films can be modulated by their roughness and topography, e.g., the size and shape of the surface irregularities, by termination with various atoms (e.g., O and H), chemical functional groups and biomolecules and by boron doping. However, free DNPs and DNPs dispersed in a material matrix should be applied with caution because of their higher reactivity with the surrounding environment and potential penetration into cells, which may finally result in their cytotoxicity.

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