TOPICAL REVIEW

Biomedical applications of nanodiamond (Review)

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Topical Review

Biomedical applications of nanodiamond (Review)

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Abstract

The interest in nanodiamond applications in biology and medicine is on the rise over recent years. This is due to the unique combination of properties that nanodiamond provides. Small size (∼5 nm), low cost, scalable production, negligible toxicity, chemical inertness of diamond core and rich chemistry of nanodiamond surface, as well as bright and robust fluorescence resistant to photobleaching are the distinct parameters that render nanodiamond superior to any other nanomaterial when it comes to biomedical applications. The most exciting recent results have been related to the use of nanodiamonds for drug delivery and diagnostics—two components of a quickly growing area of biomedical research dubbed theranostics. However, nanodiamond offers much more in addition: it can be used to produce biodegradable bone surgery devices, tissue engineering scaffolds, kill drug resistant microbes, help us to fight viruses, and deliver genetic material into cell nucleus. All these exciting opportunities require an in-depth understanding of nanodiamond. This review covers the recent progress as well as general trends in biomedical applications of nanodiamond, and underlines the importance of purification, characterization, and rational modification of this nanomaterial when designing nanodiamond based theranostic platforms.

Keywords: Nanodiamond, Theranostics, Drug delivery, Biomedical imaging, Carbon nanomaterials

(Some figures may appear in colour only in the online journal)

1. Introduction

The need for better quality and more affordable healthcare is one of the greatest challenges faced by our society. In developed countries the aging population leads to an increasing proportion of the so-called ‘aging population diseases’, including cancer, cardiovascular, musculoskeletal, and central neural system disorders. In poor countries, infections and other pathogens continue to take a heavy toll on the young and working-age population. All around the world these factors steadily increase the burden on healthcare systems. Although our successes in treating many diseases are truly impressive, recent cases of emergence of antibiotic-resistant superbugs in the USA [1], the deadly Ebola virus outbreak [2], unexpected Zika virus infection [3], as well as old unsolved problems such as cancer (called ‘the emperor of all maladies’), continuously challenge our progress with threat detection, diagnosis, treatment strategies, and development of new drugs.

Nanomedicine, personalized medicine, targeted therapy are some of our recent responses to these challenging societal problems, where nanomaterials play a very important role combining therapeutic and diagnostic modalities in one robust theranostic platform. Through tuning chemical, optical,
electric, and magnetic properties of materials, nanotechnology offers medicine a strong progressive treatment for patients [4]. For example, it may help in reducing severe adverse effects of chemotherapy through the targeted delivery of therapeutics to the malfunctioning site. Carbon nanomaterials are especially attractive for biomedical applications because carbon is the main constituent of all living organisms on Earth, including the human body. Since the advent of the era of nanotechnology in the 1990s, carbon based nanomaterials such as fullerenes, carbon nanotubes, graphene, and nanodiamond have been in the focus of researchers developing theranostic platforms. Among different carbon nanomaterials, nanodiamond particles (ND) are outstanding due to their biocompatibility and low toxicity, chemical inertness of diamond core with highly tailorable and fully accessible surface, exposing a number of functional groups that can be used to tailor their affinity to different environments, non-covalent or covalent attachment of drugs or biomolecules, as well as incorporation into composites and hybrid materials for biomedical applications. Some of these NDs bear fluorescent centers in their cores providing opportunities for in vitro and in vivo imaging. Others have a very small size (5 nm or less in diameter) being potentially able to penetrate the smallest pores in the body, for example, in the nuclear membrane (nucleolemma), or kidney filtration system. With all these unique properties combined in one particle, NDs outperform other nanoparticles that provide some of these properties but not all. These advantages of NDs unmatched by any other carbon or non-carbon nanomaterial are already important enough to render ND the superior nanomaterial for theranostics (figure 1). And yet, ND offers more benefits: it can be relatively easily and inexpensively produced by detonation on a large industrial scale and is already available commercially for quite an affordable price. All these factors contribute to growing interest in using the hardest material (diamond) in its nanoscale form to fight some of the hardest problems faced by human society.

The fundamental properties of NDs have been reviewed before [5–7]. Some of their applications, for example in composites, have also been covered recently [8, 9]. Here we provide a review of recent and most important, in the authors’ opinion, results related to biomedical applications of these little gems.

2. Recent progress in characterization, purification, and deaggregation of ND

With greatly expanding development of nanomaterials for biomedical applications, their toxicology becomes of ever increasing importance. In addition to traditional factors determining the toxicological profile of bulk materials, moving from micro- down to the nanoscale adds more dimensions: besides elemental composition, potential toxicity of nanomaterials may depend on their size, shape, and dispersion state. Traditional purification techniques designed to produce highly pure large crystals or particles of bulk materials often fail for nanomaterials. In addition, when the surface to volume ratio becomes larger, the contribution of any contaminants that usually reside on the surface of particles...
becomes more pronounced. Therefore, control of purity and dispersion are no less important than synthetic efforts aimed at developing the nanomedicine platforms and bringing them to applications.

The majority of impurities in detonation nanodiamond (DND) originate from the material of the blast chamber, charge suspension device, and initiator (usually Pb, Cu or Ag azide). Carried out in a steel blast chamber, the explosion produces ND-containing detonation soot and introduces metallic contaminations along with non-diamond carbon. Therefore, for most of its applications, in particular, in biology and medicine, DNDs must be purified post-synthesis.

Plenty of techniques have been proposed to purify NDs. Some of them can be used for NDs of different origins, whether it is detonation DND or high-temperature high-pressure ND (HPHTND). A liquid phase oxidative treatment (with mineral acids, e.g. sulfuric, nitric, and perchloric acids or mixtures thereof) removes almost all graphic and metal impurities [12, 13]. However, some non-carbon functional groups, e.g., sulfonic, are introduced as a result of liquid oxidation [14]. In contrast, gas phase oxidation techniques produce only oxygen-rich carbon functional groups on the ND surface, although these methods require additional treatment in dilute acids to remove metal impurities [15, 16]. Microwave-assisted liquid oxidation requires a lower temperature compared to traditional liquid oxidation, although the most complete removal of metals is achieved when EDTA complexes are used [17, 18].

The NDs show no inherent toxicity, but may show toxicity that depends on the tailorable surface properties of the material, emphasizing the need for testing all surface modified NDs for their toxicity/biocompatibility (table 2). Thus, it is critical to analyze the impurities content in NDs developed for biomedical applications, although many researchers tacitly rely upon almost complete removal of metal and non-diamond carbon contaminations after air and liquid oxidation or liquid oxidation alone. The techniques for characterizing the ND purity and content of contaminants include inductively-coupled plasma mass-spectrometry and elemental analysis [17, 19–21], SEM/TEM [13, 22], XRD [23], XPS [24], and Raman spectroscopy [25–28]. In most cases, one method is not sufficient (for example, many manufacturers provide the C:N:O content but no phase composition of carbon, which is necessary to evaluate the content of diamond and non-diamond phases in the material), and complete characterization requires a combination of several techniques.

The notoriously strong aggregation of DNDs severely limits their potential in many applications, including polymer- and metal-matrix composites [8, 29–31], as well as biomedical applications [32]. Since the 1960s all traditional deaggregation techniques known in colloidal science/materials have consistently failed to yield single-digit NDs from DND aggregates [33, 34]. The problem was summarized by E Osawa: ‘The aqueous slurry of micron nanodiamond aggregates can be disintegrated by means of powerful 400 W ultrasound to 60 nm aggregates but never beyond’ ([32], table 1). Severe aggregation is generally explained by rich surface chemistry and small size of DNDs [5]. The presence of diverse functional groups on the ND surface, such as carboxyl, hydroxyl, lactone, etc, may result in formation of multiple hydrogen and even covalent bonds between the adjacent DND particles, making it difficult to separate them. DND primary particles are ∼5 nm in diameter, thus, various biological studies conducted with DND aggregates of 100–200 nm can hardly reflect the performance of single-digit ND particles. The shape of nanomaterials also has a great impact on their application as a therapeutic platform [35]. For example, the use of graphene oxide of spherical shape is more beneficial for photothermal ablation of tumors than the needle-like graphene oxide [36]. And in this respect too, nearly spherical primary DND particles are advantageous compared to the elongated or thin and sharp shapes of other nanocarbons.

The problem of ND deaggregation into single-digit particles was solved only in 2005 via bead-assisted ball milling ([34, 37, 38], table 1) and its successor bead-assisted sonic disintegration (BASD), ([34, 37–40], table 1). A few years later, salt-assisted dry attrition milling ([41], table 1) became available. Most recently, the salt-assisted ultrasonic deaggregation has been added to the arsenal, opening avenues to easily produced, inexpensive, ultra-pure single-digit ND colloids for a multitude of applications ([42], table 1). Below, we analyze these deaggregation methods from the standpoint of ND for biomedical needs.

Zirconia microbead-assisted ball milling and BASD are now most used to produce single-digit DNDs for research, in particular, for adsorption and delivery of insoluble anti-cancer therapeutics [7, 32, 34, 37, 39, 43–45]. Both techniques require the use of ∼30 μm ZrO2 microbeads. In BASD, for example, the dense ZrO2 microbeads, propelled by the energy of cavitation, collide and crush ND aggregates trapped in-between (figure 2). BASD yields stable single-digit ND colloids of up to 10 wt% concentration with up to 80% yield relative to the initial ND mass [40]. However, BASD, as well as ZrO2 microbead-assisted ball milling have certain disadvantages, such as a high cost (ZrO2 microbeads are expensive, special mills have to be designed for the process, separation of microbeads from NDs is also costly) and difficult to remove ZrO2 debris (harsh acid or base treatment is needed to dissolve ZrO2, which negatively impacts production safety and contributes to the cost of the purified ND) [19–21, 38]. On the other hand, if ZrO2 is not removed completely, then the presence of this contaminant in uncontrolled quantities may negatively impact the prospects of clinical approval for ND enabled theranostic platforms. Thus, ZrO2 and similar ceramic contaminates may pose a serious obstacle on the way to low-cost and safe ND therapeutics.

On the contrary, water-soluble dry media-assisted attritor milling and SAUD [41, 42, 46] utilize inexpensive, non-toxic, and non-contaminating crystalline milling media such as sodium chloride or sucrose. Upon completion of the deaggregation process, the milling media can be easily washed out with water, providing a remarkable advantage over a process involving insoluble ceramic beads. However, during the dry media-assisted attritor milling, parts of the mill containate
Table 1. ND deaggregation techniques.

<table>
<thead>
<tr>
<th>References</th>
<th>Technique</th>
<th>Milling aid</th>
<th>Ultra-sound power, W</th>
<th>Post-treatment</th>
<th>Final ND diameter, nm</th>
<th>Comments</th>
</tr>
</thead>
<tbody>
<tr>
<td>[32]</td>
<td>Ultrasound</td>
<td>—</td>
<td>400</td>
<td>—</td>
<td>60</td>
<td>Does not produce single-digit NDs</td>
</tr>
<tr>
<td>[34, 37, 38]</td>
<td>Bead-assisted ball milling</td>
<td>Zirconia micro-beads</td>
<td>—</td>
<td>Dissolution of ZrO₂ contaminants in strong base or acid</td>
<td>&lt;10</td>
<td>A special mill is required. Difficult-to-remove contaminations originating from ceramic microbeads</td>
</tr>
<tr>
<td>[34, 37–40]</td>
<td>Bead-assisted sonic deintegration (BASD)</td>
<td>ZrO₂ or other ceramic micro-beads</td>
<td>400–450</td>
<td>Dissolution of ZrO₂ contaminants in strong base or acid</td>
<td>4–5</td>
<td>Difficult-to-remove contaminations originating from ceramic microbeads</td>
</tr>
<tr>
<td>[41]</td>
<td>Dry media-assisted attrition milling</td>
<td>Sodium chloride, sucrose</td>
<td>—</td>
<td>Acid treatment to remove Fe and other metals followed by pH adjustment to 11</td>
<td>&lt;10</td>
<td>An Attritor mill is required. Fe contaminations as result of the wearing of steel balls and parts of the mill</td>
</tr>
<tr>
<td>[42]</td>
<td>Salt-assisted ultrasound deaggregation (SAUD)</td>
<td>Sodium chloride, potassium chloride, sodium acetate, etc</td>
<td>150 or higher</td>
<td>Washing/centrifugation steps (x2)</td>
<td>5–10</td>
<td>Simple. Inexpensive. Leaves no contamination. Works with different commercial NDs</td>
</tr>
</tbody>
</table>
ND with Fe, Ni, and other components of steel, thus requiring an extra purification step. Furthermore, while significantly reducing the aggregate size from micrometer scale down to 50–30 nm, dry media-assisted attritor milling does not yield truly single-digit NDs unless the dispersion pH is adjusted to ~11 upon completion of milling [41].

SAUD uses ultrasonic power delivered by a standard lab horn sonicator into suspensions of different water-soluble crystalline media (e.g., sodium chloride, potassium chloride, sodium acetate, etc) to yield single-digit ND colloids without any pH adjustments (figure 2). Since no ZrO2 is used, SAUD completely eliminates zirconia or any other difficult-to-remove impurities in ND. The mechanical action of salt crystals in SAUD is combined with formation of a corresponding salt of Na+, K+, etc with COO− groups of ND, thus enhancing the single-digit ND colloid stability.

In another approach, hydrogen annealing of NDs at 800 °C–850 °C gives rise to deaggregated hydrogen and −OH terminated NDs [47]. These hydrogenated NDs show high colloidal stability in water due to their high positive zeta potential. More information on hydrogenated nanodiamonds is provided in section 3 of this review.

3. Modification of ND surface chemistry for biomedical applications

With the advent of the era of theranostics, the interest in nanoparticles combining drug delivery, imaging, and diagnostic modalities in a single robust platform has been growing. For a theranostic nanoparticle, well-defined surface chemistry is a key requisite, determining its biodistribution and efficacy, strongly influencing its toxicity, and impacting its clinical approval prospects. Surface groups to a large extent determine ND behavior in biological environments and dictate strategies for its chemical modification. In many cases surface homogeneity helps to suppress inter-particle interactions [6, 48], which may lead to undesirable aggregation. DND exposes a vast array of functional groups on its surface, such as carboxyl, hydroxyl, epoxide, lactone, anhydride, and alkenes [5, 6, 48]. This chemical diversity enables the use of rich and well-known organic chemistry transformations to tailor ND surface chemistry for different environments. In addition to modification of surface functional groups, researchers cover ND particles with protective biocompatible silica or polymers, producing core–shell structures for prolonged in vivo stability. The shell post-modification further contributes to the diversity of these core–shell structures [49].

Graphitization of sp2 carbon of DND starts in vacuum at 700 °C–750 °C proceeding from the surface inward and gives rise to hydrophobic NDs with no surface functional groups but with a layer(s) of sp2 carbon [50] (figure 3(A)). When done in a controlled way, it results in a core–shell ND-graphitic carbon structure [51], which is anticipated to have interesting optical and photothermal properties. Additionally, the sp2 carbon shell around the diamond core is amenable to various C–C bond formation reactions, for example, arylation by arylidiazonium salts [50, 52–54], Diels–Alder reaction [52, 55], Prato reaction [54], and Bingel–Hirsch reaction [56]. Hydrogenated ND (ND-H) can also be used for C–C bond generation [57], e.g., arylation with diazonium salts [58]. Fully hydrogenated ND can be obtained either by annealing in a hydrogen atmosphere at 800 °C–850 °C [59] or plasma-assisted hydrogenation [60] (figure 3(B)). For presently unclear reasons, ND-H has a highly positive ζ-potential in aqueous colloids [47]. Hydrogen terminated diamond is known to have negative electron affinity [61], thus ND-H is anticipated to have negative electron affinity too. These distinct differences of ND-H from NDs terminated with oxygen containing functional groups have been exploited to generate oxygen species and solvated electrons via photoirradiation of ND-H [57]. The synthesis, properties, and biological applications of ND-H were recently reviewed [62].
Air oxidation of ND at 425 °C for several hours removes non-diamond carbon and when done in a controlled way [63, 64], it may be used to change the size of the primary ND particles, while transforming surface functional groups into carboxyls (ND-COOH) or their corresponding dehydrated forms—anhydrides [25, 48]. Anhydrides can be converted to COOH by hydrolysis for further functionalization. In those cases, when hydrolysis is not performed, differences in stoichiometry and reactivity of carboxyl and anhydride groups must be taken into consideration. For example, the reaction of carboxyl group (converted into acyl chloride, see below) with amine yields amide, while the anhydride directly forms amide and an extra COOH that may be involved in further reactions or used for ND stabilization (figure 3(C)). Prior to any chemical modification of ND, the presence and, if possible, the quantity of anhydrides, carboxyl groups, lactones, esters, imides, etc, must be established by FTIR, XPS, or titration [48].

To suppress Brønsted acidity of carboxyl groups in certain reactions, they need to be converted into the corresponding acyl chlorides. For example, octadecylamine [65], octylamine [66], amino-polyethylene glycol [67, 68], lysine [69], hydroxyl-polyethylene glycol [70], or ethylenediamine [71] react with ND-acyl chloride forming the corresponding amide or ester bonds. Reaction between ND-acyl chloride and diamines results in amino-terminated ND (ND-NH₂) [71] (figure 3(D)), whereas ND-COOH would give corresponding salts. The modified Kaiser test can be used to quantify primary amino groups on NDs [31, 72]. Because of a higher isoelectric point (IEP) of ND-NH₂ compared to ND-COOH [71], the adsorption/release of drugs by these two NDs are very different [73, 74] (see section 5). In many cases the conversion of ND acyl chloride groups into amides via the reaction with diamines is not full due to the steric hindrance (figure 3(D)). This leads to a partially aminated ND derivative, in which both carboxyl and amino groups are present [31]. The exhaustive amination of the ND surface can be accomplished with less bulky molecules and by using hydroxylated ND as a starting material through the intermediate formation of tosylated- and cyano-terminated ND [75]. ND-NH₂ can be used as precursor in various biorhomboidal reactions [75], as a reactive nanofiller in polymer composites [71], etc.

ND-COOH is often used for amide bond generation via covalent conjugation of amine through the intermediate formation of ND-ester (figure 3(E)). This reaction, requiring activation with 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide (EDC) or N-hydroxysuccinimide (NHS), is performed in water at room temperature. The mild reaction conditions are suitable for covalent conjugation of delicate biomolecules such as DNA and proteins to NDs via amide bond [76–78] (figure 3(F)).

In a similar way, polyethylene glycol modified NDs can be obtained using amino-terminated PEG [79–83] (figure 3(G)). PEG shell is biocompatible and helps mitigate the adsorption of proteins, amino acids, and salts on the
nanoparticle surface [84]. The use of double functionalized PEG allows for further functionalization of ND-PEGs with various biomolecules [79, 81, 82, 85] (figure 3(G)).

The described PEGylation technique may not produce a sufficiently dense layer of a polymer, because in this approach the polymer is *grafted to* the ND surface. In contrast, when a polymer is directly assembled on the ND surface, i.e., *grafted from*, a higher polymer brush density may be achieved. For example, polyglycerol (PG) coating can be assembled on the ND surface starting from oxidized ND and glycerol [86] (figure 3(H)). PG coating provides remarkable dispersibility for ND in water (>80 mg ml⁻¹), moreover, it carries numerous hydroxyl groups (3.1 × 10⁵ glycidol units grafted from a 30 nm HPHTND particle), which can be used for further functionalization with tosylates, azido groups, carboxylic acids, active esters, hydrazones, and aluminum ion [87–90].

Other coatings, such as poly(oligo(ethylene glycol) methyl ether methacrylate) (POEGMEMA), poly(methacrylic acid) (PMAA), etc, can be assembled on the ND surface by the *grafting from* approach through radical polymerization, such as atom transfer radical polymerization and reversible addition–fragmentation chain transfer [91–94]. The synthesis and properties of the different biocompatible polymer coatings on NDs have been reviewed in [9].

Reduction of ND-COOH yields hydroxylated ND (ND-OH) [95, 96] (figure 3(I)), which can be used in acylation [97], sol-gel synthesis [6], polymer grafting [98], etc. Normally, the reduction of ND-COOH takes place in dry THF in the presence of lithium aluminum hydride (LiAlH₄), which must be quenched upon completion of the reaction. To suppress alumina formation during the hydrolysis, elaborate washing/centrifugation procedures with concentrated bases and/or Al complexing agents are recommended [6].

To improve stability in biological media, silica coatings have been formed over ND core [99–101]. For example, ND-OH has been exploited for covalent bonding of silanes (figure 3(I)) [6, 102]. Non-covalent strategies can also yield ND@SiO₂ [6, 102]. The Stoebner’s protocol [103] improves dispersion stability of NDs through silanization. In this protocol, cetyltrimethylammonium bromide (CTAB) surfactant encapsulates the nanoparticles into micelles prior silica shell formation. The thickness of the silica shell can then be tuned by varying reaction parameters including the CTAB/silane ratio [6, 102]. The silica shell of ND@SiO₂ can be further modified. For example, fluorescent HPHTND (F-HPHTND) was covered by ultrathin silica shell (∼1 nm) using tetraethoxysilane (TEOS) and 3-(trimethoxysilyl) propyl methacrylate. The introduced methacrylate moieties on the F-HPHTND@SiO₂ surface were then involved in polymerization with N-(2-hydroxypropyl) methacrylamide and N-propargylacrylamide yielding the corresponding copolymer by the *grafting from* approach. The copolymer coating enables high stability of F-HPHTND@SiO₂ in biological media due to 2-hydroxypropyl groups, while the propargyl moieties can be used for bioorthogonal reactions, for example, integration of arginylglycylaspartic acid (RGD peptide) or AlexaFluor488 fluorescent dye onto the F-HPHTND@SiO₂ surface [104] (figure 4(A)). In a few examples reported, the ultrasound assisted Stoebner’s protocol provided single digit NDs encapsulated in silica shell. For example, multilamellar liposomal vesicles (500 to 10 000 nm diameter) containing entrapped F-HPHTND and TEOs molecules have been ultrasonically disintegrated into smaller (∼100 nm diameter) composite particles (figure 4(B)). Hydrolysis of TEOs inside the liposome led to SiO₂ coated F-HPHTND. Finally, the liposome was destroyed exposing single F-HPHTND@SiO₂ nanoparticles [105]. In another example, silanization of ND-OH has been performed during BASD in THF [55]. This protocol enables ND functionalization while breaking up the ND aggregates.

In regards to surface modifications of nanodiamond, liposome-based nanodiamonds gain attention due to their better dispersibility and non-toxicity. Moreover, liposome coated nanodiamonds offer more options for labeling with proteins for specific targeting [106–108].

To minimize side reactions on ND surface biorthogonal approaches, such as Cu(I) catalyzed click reaction [54, 109–111], biotin-streptavidin recognition [75, 96, 105], and Cu-free click reaction [112] have been demonstrated. For example, Cu(I)-catalyzed azide–alkyne 1,3-dipolar cycloaddition (CuAAC), also known as the ‘click’ reaction, has been used to attach (figure 4(A), white arrow) fluorescent tags and cell penetration proteins, as well as various sugar molecules (glycans) to the ND surface [111, 113]. Azide- or alkynefunctionalized NDs can be used in this reaction, which can be produced by interaction of ND-OH with corresponding dopamine (Dop) derivative (Dop-azide or Dop-alkyne) [111, 114]. The mechanism of Dop attachment to the ND-OH surface involves non-covalent interaction between two aromatic hydroxyls of Dop and the ND-OH surface and is similar to the bonding providing mussel attachment to the sea bed [115].

The ND-glycans are proposed as alternatives to antibiotic treatment of bacterial pathogens, since bacteria have a strong affinity to sugar molecules. Monosaccharides can be easily integrated on the ND surface, yet the integration of oligosaccharides that are much more efficient in binding to bacterial pathogens, is a challenge [52, 53, 113, 116]. In this regard, the ND surface was modified in a way to easily react with any pristine sugar molecule. A selective *in situ* approach is a photochemical reaction of ND-nitrene derivative with C–H bond of sugar under UV irradiation, yielding a large array of complex glycosylated NDs including mono-, di-, and oligosaccharide-NDs [117].

Hybridizing NDs with other nanoparticles, such as gold [118–123], silver [124, 125], platinum [126, 127], or amorphous carbon [127], is an attractive route towards theranostic platforms, since these materials combine multiple modalities: for example, imaging/sensing and photothermal therapy, which are of particular interest for localized temperature ablation of tumors.

Similar to polymers, nanoparticles can be also *grafted to* (a nanoparticle is attached to) [122] and *grafted from* (a nanoparticle is assembled on) [120] the ND surface. An example of *grafted from* approach is Au scroll formation over HPHTND@SiO₂ [118]. Silica coating makes the HPHTND
surface more round and thus more uniformly covered by the layer of gold. The Au scroll was produced by reduction of Au$^{3+}$ forming 2–3 nm thick gold layer, which is then used to deposit more gold. This technique produced a uniform 12.6 ± 0.3 nm layer of Au over HPHTND@SiO$_2$. The formation of a gold scroll resulted in plasmon resonance observed through a characteristic band at 675 nm. Combination of fluorescent ND with gold nanoparticles provides opportunities for simultaneous localization and thermal ablation of cancer cells.

A highly localized temperature sensing and heating at the nanoscale in biological tissues was demonstrated with HPHTND conjugates with gold nanorods (GNRs). An HPHTND with ~140 nm median size can host 3–4 GNRs attached through electrostatic attraction (figure 4(C)). Due to the non-bleaching fluorescence of ND NV centers in the wide spectral range (red peak at 550–800 nm, figure 4(D)), the HPHTND-GNRs conjugates can be tracked inside cells while at the same time they can be heated up with 808 nm radiation. The absorbance of GNRs is within the first biological window, where tissues of human body do not absorb light. This dual modality is required for nanoscale hyperthermia...
applications, e.g., precisely localized and safer therapy of cancer.

4. Toxicological effects and biocompatibility of ND in vivo and in vitro

Since the pioneering studies of ND toxicity [128, 129], various screenings have been performed to generate comprehensive toxicological profiles of NDs (table 2). These studies aimed to understand the type of toxicity and the underlying mechanisms. A general conclusion, which can be drawn from multiple toxicity studies is that ND derivatives of different origin and sizes do not damage basic functions of cells, organs, and organisms in a reasonable range of concentrations. At the same time, there are several reports of ND toxicity, which may be related to the use of poorly purified NDs. Table 2 summarizes ND purification protocols and the reported contents of impurities (when provided) along with toxicological outcomes. In most cases where significant level of toxicity was reported, NDs have been used as-received [130–136]. In those studies where NDs have been purified (for example, air or ozone oxidized and acid treated to dissolve metals or liquid oxidized) no significant toxicity was observed [128, 129, 137–149]. We assume that if metallic and other impurities were fully removed, the NDs would have shown low or no toxicity in all studies. For example, only slight apoptosis of HaCaT cells causing membrane permeability changes, caspase activation, and release of intracellular lactatedehydrogenase, was observed as a result of exposure to non-purified as-received ND at 100 μg ml⁻¹ concentration [150]. On the other hand, purified ND in same concentration did not affect basal cellular toxicity of A549 cells [141].

Another reason for induced cell death may be related to unreasonably high ND concentrations used in the tests. For example, an increased level of apoptosis has been observed in both normal and cancer cells at 200–1000 μg ml⁻¹ ND [150]. At the same time, at ND concentrations below 50 μg ml⁻¹ no evident toxicity was observed [131, 150, 151]. NDs did not induce any cytotoxicity and inflammation in concentrations up to 50 μg ml⁻¹, as has been shown through analysis of gene expression mechanisms, cell morphology, immunotoxicity, and apoptosis [151]. Analysis of other effects (size, shape, and origin of ND) has shown that the concentration played the most important role in ND induced toxicity and inflammation [151].

The immunotoxicity, which results in an increased secretion of chemokines and cytokines, has been evaluated for ND derivatives with many cell types [131, 138, 139, 142, 151]. After exposure to ND-COOH, the mesenchymal stem cells did not alter secretion of cytokines, chemokines, and growth factors [142].

Cell oxidative stress is another indicator of cellular toxicity. The oxidative stress caused by ND derivatives is cell specific [129, 131, 133, 134, 137, 145, 149]. For example, no signs of oxidative stress have been observed in neuroblastoma cells, macrophages, keratinocytes, and PC-12 cells [129, 137]. On the other hand, lymphocytes and endothelial cells have shown ND induced oxidative stress that can be partially related to the use of as-received non-purified ND [131, 133].

A concept of adsorption, distribution, metabolism, and elimination (ADME) is widely used to evaluate carbon nanomaterials bioavailability, tissue distribution, metabolism, and excretion from the body [152]. NDs labeled with 188Re, 125I, and 18F radionuclides have been used in biosafety explorative studies on mice and rats [136, 148, 153]. Similar to other nanomaterials, exposure routes to NDs can influence toxicity [136, 148, 149, 153]. Two studies of pulmonary delivery of ND report contradicting results, indicating both toxic and non-toxic properties of NDs to lungs and other organs, including cardiovascular system [136, 149]. This controversy can be traced back to a dose-dependent nature of the observed effects or to differences in purity of NDs used. In another example, the controversial ND toxicity towards blood can be explained by impurities and poor purification, since the researchers reported rupture of the membranes of white and red blood cells using non-purified as-received ND [132]. On the other hand, no signs of hematological toxicity were observed with acid purified BASD detonation NDs [144, 147].

Considerable attention has been paid to the developmental toxicity of ND. Classic test systems using embryos of Xenopus laevis, and Danio rerio (zebra fish) to determine teratogenic and embryogenic potential of NDs have been described [130, 154, 155]. Xenopus embryos turned out to be sensitive to ND surface functionalization, which sometimes resulted in a low survival rate due to developmental abnormalities [130]. Dose-dependent toxicity of ND was reported for the zebra fish model [154, 155]. On the other hand, no changes in life expectancy, reproductive potential, or stress level in response to ND have been observed for Caenorhabditis elegans invertebrate [145]. As a part of ecotoxicological monitoring, subvertebrate Corbicula fluminea mollusk [134] and Daphnia magna crustacean [135] were exposed to NDs. Particle aggregation and inability of subvertebrate species to cleanse ND from their body at higher ND concentrations can contribute to the reported ND toxicity in these cases. The fate of ND carbon in other organisms, and particularly, in animals raises questions. Unlike metal nanoparticles, NDs cannot be digested or dissolved in the human body. Granulocytes were found to bind 5 nm hydrophobic NDs followed by the formation of neutrophil extracellular traps (NETs) that immobilized and sequestered the trapped NDs [156].

To conclude, ND is undoubtedly a perspective nanomaterial with less concerns regarding its toxicity as compared to other carbon materials, as well as CTAB-coated gold nanoparticles, semiconductor quantum dots, etc. Some contradictions in the experimental results obtained for NDs can be explained by the impurities content, and thus a proper purification of as-received ND is utterly important. The level of impurities as well as surface chemistry of NDs must be taken into account when evaluating the ND toxicological profile. In particular, heavy metals, graphitic and amorphous carbon, ceramic and other potentially harmful impurities have to be
<table>
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<th>Conclusion</th>
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<tr>
<td>[128]</td>
<td>100 nmF-HPHTND, liquid oxid., imp.: n.a.</td>
<td>COOH</td>
<td>293-T human kidney cells</td>
<td>Cell viability</td>
<td>20–400 μg ml⁻¹</td>
<td>No cytotoxicity</td>
</tr>
<tr>
<td>[129, 137]</td>
<td>5 nm DND, liquid oxid., imp.: 0.08 wt%</td>
<td>as-received</td>
<td>Neuroblastoma, alveolar macrophages, keratinocytes, PC-12 cells</td>
<td>Metabolic activity, mitochondrial membrane permeability, oxidative stress</td>
<td>1–100 μg ml⁻¹</td>
<td>No disruption of mitochondrial membrane, no oxidative stress</td>
</tr>
<tr>
<td>[130]</td>
<td>4.4 nm DND, imp.: n.a., used as-received</td>
<td>OH, NH₂, COOH</td>
<td>HEK293 cells</td>
<td>Metabolic activity</td>
<td>50–200 μg ml⁻¹</td>
<td>Cytoxicity decreased in the order – NH₂ ≥ –OH &gt; –COOH at 50–200 μg ml⁻¹</td>
</tr>
<tr>
<td>[130]</td>
<td>4.4 nm DND, imp.: n.a., used as-received</td>
<td>OH, NH₂, COOH</td>
<td>Xenopus laevis embryos</td>
<td>Embryogenesis, survival rate</td>
<td>2–200 μg ml⁻¹</td>
<td>Potential embroyotoxicity and teratogenicity for ND-COOH</td>
</tr>
<tr>
<td>[131]</td>
<td>5 nm DND, imp.: &lt;3 wt%, used as-received</td>
<td>as-received</td>
<td>Human lymphocytes</td>
<td>Cell death, membrane damage, oxidative stress, chromosome damage</td>
<td>1–100 μg ml⁻¹</td>
<td>Evident cytoxicity at 50 μg ml⁻¹. Genotoxicity and aneugenic effect at 1–10 μg ml⁻¹. Dose dependent oxidative stress</td>
</tr>
<tr>
<td>[132]</td>
<td>5 nm DND, imp.: n.a., used as-received</td>
<td>as-received</td>
<td>Human blood</td>
<td>Viability of red and white blood cells</td>
<td>0.125 wt%</td>
<td>Destruction of human blood cells</td>
</tr>
<tr>
<td>[133]</td>
<td>CVDND, imp.: n.a., used as-received</td>
<td>as-received</td>
<td>HUVEC-ST cells</td>
<td>Cell viability, oxidative stress</td>
<td>2–100 μg ml⁻¹</td>
<td>Toxicity reported for both DND and CVDND</td>
</tr>
<tr>
<td>[134]</td>
<td>4.4 nm DND, imp.: &lt;10 wt%, used as-received</td>
<td>as-received</td>
<td>Corbica fluminea</td>
<td>Oxidative stress, histopathology</td>
<td>0.1–10 μg ml⁻¹</td>
<td>Stress oxidative response, chronic toxicity, alteration in digestive gland cells</td>
</tr>
<tr>
<td>[135]</td>
<td>4.4 nm DND, imp.: &lt;10 wt%, used as-received</td>
<td>as-received</td>
<td>Daphnia magna crustacean</td>
<td>Survival, reproduction, tissue structure</td>
<td>0.31–5 μg ml⁻¹</td>
<td>Chronic toxicity, inhibition of reproduction</td>
</tr>
<tr>
<td>[136]</td>
<td>2–10 nm DND, imp.: &lt;5 wt% (Fe, Al, Ba, Mn, Ni), used as-received</td>
<td>OH, COOH</td>
<td>Kun Ming mice</td>
<td>Organ biodistribution, biochemical analysis of blood</td>
<td>0.8–20 mg kg⁻¹</td>
<td>Dose dependent systemic toxicity in lung, cardiovascular and hematological toxicity after intratracheal instillation</td>
</tr>
<tr>
<td>[138]</td>
<td>4–5 nm DND, liquid oxid., base treatment, imp.: n.a.</td>
<td>as-received, COOH</td>
<td>Mouse embryonic stem cells</td>
<td>p53 and MOGG-1 DNA repair proteins</td>
<td>5–100 μg ml⁻¹</td>
<td>Surface chemistry specific genotoxicity: COOH=Pristine</td>
</tr>
<tr>
<td>[139]</td>
<td>4–5 nm DND, liquid oxid., base treatment, imp.: n.a.</td>
<td>as-received, COOH</td>
<td>HepG2, HeLa cells</td>
<td>Metabolic activity, cell death, apoptosis, gene expression</td>
<td>1–500 μg ml⁻¹</td>
<td>No toxicity</td>
</tr>
<tr>
<td>[140]</td>
<td>45 nmF-HPHTND, air oxid., liquid oxid., imp.: n.a.</td>
<td>NH₂</td>
<td>Mouse (P19) and human (NT2/D1) pluripotent embryonal carcinoma</td>
<td>Membrane permeability changes, cell viability, neuronal differentiation</td>
<td>0.1–50 μg ml⁻¹</td>
<td>No cytotoxicity and apoptosis during and after differentiation</td>
</tr>
<tr>
<td>References</td>
<td>Method/Impurities content/coating</td>
<td>Surface groups</td>
<td>Biological model</td>
<td>Parameter evaluated</td>
<td>Range of ND concentrations</td>
<td>Conclusion</td>
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<tr>
<td>[141]</td>
<td>5 nm DND, liquid oxid., base treatment, imp.: n.a.</td>
<td>COOH</td>
<td>A-549, HFL-1 cells</td>
<td>Cell viability, membrane permeability changes</td>
<td>0.1–100 μg ml(^{-1})</td>
<td>No induced toxicity and apoptosis</td>
</tr>
<tr>
<td>[142]</td>
<td>270 nm HPHTND, liquid oxid., base treatment, imp.: n.a.</td>
<td>COOH</td>
<td>Mesenchymal stem cells</td>
<td>Immunotoxicity, morphology and differentiation of cells</td>
<td>1.5 μg ml(^{-1})</td>
<td>No signs of inflammation or toxicity</td>
</tr>
<tr>
<td>[143]</td>
<td>4–5 nm DND, liquid oxid., base treatment, imp.: n.a.</td>
<td>NH(_2)</td>
<td>BALB/c mice</td>
<td>Liver functions, apoptosis, tumor volume, mouse weight, biodistribution in organs</td>
<td>40–1200 μg per body weight</td>
<td>ND is biocompatible, no liver toxicity</td>
</tr>
<tr>
<td>[144]</td>
<td>4–5 nm DND, liquid oxid., base treatment, imp.: n.a.</td>
<td>as-received</td>
<td>Male CD-1:IGS rats, non-human Cynomolgus monkeys</td>
<td>Repeated dosing biocompatibility, body weight, complete blood count, serum chemistry, urinalysis</td>
<td>5 mg ml(^{-1}) (rats)</td>
<td>Therapy dosage 6.75–13.5 mg kg(^{-1}) for primates. No organ dysfunctions for both models, no apparent adverse effects for a period of 6 month</td>
</tr>
<tr>
<td>[145]</td>
<td>40 nm F-HPHTND, air oxid., liquid oxid., imp.: n.a., used as CMD/BSA coated</td>
<td>COOH</td>
<td>Caenorhabditis elegans</td>
<td>Life span, brood size, oxidative stress, stress response</td>
<td>15–25 mg kg(^{-1}) (primates)</td>
<td>No changes in longevity, reproductive potential, or stress</td>
</tr>
<tr>
<td>[146]</td>
<td>100 nm F-HPHTND, air oxid., liquid oxid., imp.: n.a., used as BSA coated</td>
<td>COOH</td>
<td>BALB/c male nude mice, male Sprague Dawley rats</td>
<td>Distribution in organs, mapping of sentinel lymph node, body weight, fodder and water consumption</td>
<td>Up to 75 mg kg(^{-1})</td>
<td>No significant toxicity on the studied models was observed</td>
</tr>
<tr>
<td>[147]</td>
<td>50 and 100 nm F-HPHTND, liquid oxid., imp.: n.a., used as BSA coated</td>
<td>COOH</td>
<td>Human/rat red blood cells</td>
<td>Cytokine production, deoxygenation/oxygenation dynamics of RBC</td>
<td>20 and 1000 μg ml(^{-1})</td>
<td>No stimulation of immune response</td>
</tr>
<tr>
<td>[148]</td>
<td>50 nm HPHTND, liquid oxid., imp.: &lt;2 wt% (Fe, Ca, W), used as BSA coated</td>
<td>COOH</td>
<td>Male ICR mice</td>
<td>Short- and long-term biodistribution</td>
<td>20 mg kg(^{-1})</td>
<td>Entraped in lung, spleen, and liver (predominant accumulation) after 28 days p.i. No symptoms of abnormality</td>
</tr>
<tr>
<td>[149]</td>
<td>5 nm DND, imp: &lt;5 wt% (S, Cl, Fe), used as-received</td>
<td>as-received</td>
<td>Male ICR mice</td>
<td>Organ indices, oxidative stress, ALP level</td>
<td>50 or 500 μg ml(^{-1})</td>
<td>No obvious pulmonary toxicity after intratracheal instillation. Mechanisms of translocation and clearance from lungs to pharynx are discovered</td>
</tr>
<tr>
<td>References</td>
<td>ND type/purification method/impurities content/coating</td>
<td>Surface groups</td>
<td>Biological model</td>
<td>Parameter evaluated</td>
<td>Range of ND concentrations</td>
<td>Conclusion</td>
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<tr>
<td>[150]</td>
<td>3.1, 7.4, and 16 nm DND, imp.: n.a., used as BSA coated</td>
<td>COOH, H</td>
<td>A-549, HaCaT cells</td>
<td>Membrane permeability changes, caspase activation, intracellular LDH release</td>
<td>10, 100, 1000 μg ml⁻¹</td>
<td>Slight signs of apoptosis in HaCaT cells. No signs of apoptosis in A-549 cells</td>
</tr>
<tr>
<td>[151]</td>
<td>5 nm DND, liquid oxid., imp.: n.a., 60, 100, 250, and 500 nm HPHT ND, liquid oxid., imp.: n.a.</td>
<td>as-received</td>
<td>Raw 264.7 murine macrophages</td>
<td>Immunotoxicity, cell morphology, cell viability, induction of cell proliferation</td>
<td>10–200 μg ml⁻¹</td>
<td>Signs of inflammation and toxicity at high concentrations from 50–200 μg ml⁻¹, no hazard below 50 μg ml⁻¹</td>
</tr>
<tr>
<td>[153]</td>
<td>5 nm DND, Fenton oxid., imp.: n.a.</td>
<td>OH</td>
<td>Female Swiss CD1, male Sprague Dawley rats</td>
<td>Biodistribution</td>
<td>n.a.</td>
<td>Retention in lungs and RES. Clearance through urine if smaller in size</td>
</tr>
<tr>
<td>[154, 155]</td>
<td>100 nm F-HPHT ND, air oxid., liquid oxid., imp.: n.a., used as BSA coated</td>
<td>COOH</td>
<td>Zebra fish (Danio rerio) embryo</td>
<td>Biocompatibility, ND movement and velocity, embryogenesis</td>
<td>1000–5000 μg ml⁻¹</td>
<td>Dose dependent toxicity reported for high concentration of ND tested</td>
</tr>
<tr>
<td>[157]</td>
<td>3, 5, and 7 nm DND, imp.: n.a., used as BSA coated</td>
<td>as-received</td>
<td>BEAS-2B cells</td>
<td>Membrane disruption mechanisms</td>
<td>25–400 μg ml⁻¹</td>
<td>Membrane disruption fades in the order 7 nm &gt; 5 nm &gt; 3 nm DND</td>
</tr>
<tr>
<td>[154]</td>
<td>5 nm DND, liquid oxid., imp.: n.a., 100 nm HPHT ND, liquid oxid., imp.: n.a.</td>
<td>as-received, COOH</td>
<td>Paramecium caudatum protozoa</td>
<td>Cell viability, cell growth, life activity</td>
<td>20 μg ml⁻¹</td>
<td>Toxicity observed due to inability of ND clearance from the protozoa</td>
</tr>
</tbody>
</table>

*ND—detonation ND, HPHTND—high temperature high pressure ND, F-HPHT ND—fluorescent HPHT nanodiamond, CVDND—chemical vapor deposition nanodiamond, n.a.—not indicated, imp.—impurities ROS—reactive oxygen species, CMD—carboxymethyl dextran, BSA—bovine serum albumin, p.i.—post injection, ALP—alkaline phosphatase, IL-6—interleukin 6, RES—reticulendothelial system, RBC—red blood cells, LDH—lactate dehydrogenase.
removed. The methods to purify and deaggregate NDs were discussed in section 2 of this review.

5. ND for anticancer therapy

Controversies and the need for further progress with targeted nanoparticle-mediated drug delivery were emphasized by a recent review that analyzed data published over the past 10 years and concluded that only 0.7% (median) of the administered nanoparticle dose is found to be delivered to a solid tumor [158]. In this regard, it should be mentioned that the drug can still be delivered and accumulated in the tumor via other mechanisms that are directly or indirectly mediated or affected by the nanoparticles. An example of the latter is passive tumor targeting, also known as the enhanced permeability and retention (EPR) effect. EPR can be used at later stages in tumorigenesis as it relies upon a well-formed tumor vasculature. However, recently it was demonstrated that ND may induce the enhanced vascular permeability independent of tumor-induced EPR [10]. Interestingly, among NDs with different but well defined surface chemistries, ND-NH$_2$ has induced the highest degree of vascular leakiness compared to ND-COOH and as-received ND. The proposed mechanism involves a ND-triggered cascade of biochemical processes, resulting in opening of tight junctions between the endothelial cells. This effect is reversible and the tight junctions are restored to normal when the ND treatment ceased. The observed ND-triggered endothelial tissue leakiness and its application to kill the tumor cells were demonstrated in vitro using the Transwell model with a cancer cell layer grown in the bottom well and a vascular barrier formed by the endothelial cells in the top well (figure 5(A)). When not treated with ND, the vascular barrier well protected the cancer cells in the bottom well from doxorubicin (DOX) added to the top well (figures 5(A)–(B)). However, pre-treatment with NDs rendered the vascular barrier transparent to DOX resulting in a significantly higher cancer cell death rate (up to 140%) compared to ~6% death rate observed in the control (not exposed to NDs) vascular barrier model (figures 5(A), (C)).

Due to its large and fully accessible surface area (all external, no pores in contrast to activated carbons), ND was studied for adsorption and triggered release of many anticancer drugs, including 4-hydroxytamoxifen [45], tetracyclines [31, 73, 159–162], and paclitaxel [163]. When entering the cell in the form adsorbed on ND, the drugs cannot be easily ejected by cell efflux mechanisms, instead they are slowly desorbed from ND inside the cell maintaining therapeutic concentrations, an effect that was used to reverse the drug resistance of cancer cells to traditional chemotherapeutics [143]. Therapeutic efflux is the most common mechanism of tumor chemoresistance that limits the effectiveness of cytotoxic drugs. ND-drug complexes have been shown to bypass the cell protective mechanisms and deliver the anticancer cargo to the cytoplasm through the endosomal release [161]. The endosomal release is triggered by low pH in the endosome that, provided the ND surface chemistry has been rationally designed, will favor desorption of the drug from ND [162]. Considerably higher IC50 for ND-drug complexes compared to the drugs alone are associated with sustained release of the drug and are considered beneficial for clinical use, resulting in lower systemic doses and preventing systemic apoptosis and myelosuppression caused by cytotoxic therapy [161]. A large size of ND-drug complex results in a prolonged retention time in a cancer cell. For example, ND-epirubicin complex gives a fluorescence signal inside LT2-MYC cells after 12 h post-treatment, compared to epirubicin alone or liposomal epirubicin formulation where no fluorescence was observed after the same period of time [161].

Adsorption on 5 nm ND particles may also increase bioavailability of poorly soluble drugs, exposing them to a physiological environment in the form of a molecule-thick monolayer—the finest dispersion state achieved in principle for poorly soluble molecules. Rational design of the ND surface is paramount in developing and optimizing drug adsorption/release strategies. Adsorption/desorption mechanisms of drugs with differently modified NDs have been studied [73, 74].

Combination chemotherapy using drug cocktails is currently the most effective treatment of mutated and multidrug resistant tumors [164]. As a step toward clinical implementation of ND chemotherapeutic platform, the Feedback System Control (FSC—a technology for rapid search of optimal compositions among numerous combinations of drugs in different concentrations) was recently utilized to optimize millions of possible formulations with ND-DOX, ND-bleomycin, ND-mitoxantrone, and unmodified paclitaxel [165]. The FSC picked up 57 different optimal combinations, which were tested on three different breast cancer cell lines in order to determine the global optimum of ND-drugs ratios (figure 5(D)). It turned out that these optimized ND-drug combinations outperformed single drugs in every cell line tested.

The migration of cancer stem cells is the main reason of metastasis. As a rule, cancer stem cells are chemoresistant to conventional drug treatment. ND-epirubicin complex has been shown to kill cancer stem cells in vitro and in vivo proving a higher efficiency of ND-based therapeutics in comparison to neat epirubicin [161]. The ND-epirubicin complex showed no toxicity to chemoresistant tumor bearing mice 12 days post treatment, while at the same time eliminating tumor cells including cancer stem cells. On the contrary, epirubicin alone was toxic to mice, resulting in body weight loss and lower percent of survival.

ND-DOX was efficient in killing tetracycline-resistant leukemia cells K562 [162] and brain tumors in C6 and U251 MG orthotopic xenograft mice [166]. Normally, to get the ND-DOX across the blood–brain barrier (BBB), the material is injected intracranially [166]. However, it will be more exciting in the future to see ND-mediated DOX delivery to brain by direct penetration through the BBB. For example, in a recent work researchers have shown the potential of F-HPHTND-alum (where alum is aluminum oxyhydroxide) complex of 2930 ± 230 nm to penetrate through the BBB [167]. A small fraction of injected FND-alum was observed in mice brain along with a larger fraction found in liver, spleen,
and lymph nodes. ND-mediated drug delivery across the BBB can be used to treat a broader spectrum of central nervous system diseases besides cancer. In particular, ND was shown to suppress the activity of the Alzheimer disease–associated amyloid-β, BACE-1, and p-tau receptors [90].

Apart from desorption, several publications studied the covalent bond cleavage mediated release in response to environment pH. In particular cis-platin [89], DOX [88], and paclitaxel [168] released from their covalent conjugates with NDs through this mechanism suppressed proliferation of...
Various cancer cells. To further improve in vivo physiological stability, silica, polymer or silica/polymer ND composites have been designed (see section 3). These composites inhibit cancer cell proliferation in vitro and in vivo, desorbing the anticancer drug inside the cells in response to a slightly acidic pH in endosomes [67, 70, 77, 79, 83, 104].

Another recent research topic in this area is the use of ND-metal hybrid particles for photothermal ablation of tumors. The photothermal effect, also known as hyperthermia, is conversion of the energy of light into heat achieved with certain nanomaterials, e.g., gold nanoparticles. As a result, high temperatures can be reached locally, leading to cell death. A new generation of theranostic photothermal agents combines fluorescent HPHTiND and gold nanorods [118, 120, 121]. Due to its fluorescence, ND in these materials helps locate the photothermal particles [122, 124]. After the particles are localized in the tumor, precise and tuned laser treatment can be applied to selectively destroy tumor lesions.

To conclude, ND-based anticancer therapies demonstrate a great promise, opening many new exciting avenues. For example, recent progress in inducing vascular leakiness will potentially help to deliver higher concentrations of chemotherapeutics to tumors in early stages of tumorigenesis, when the EPR effect has not yet been developed. Great progress was achieved in delivering poorly water-soluble anticancer therapeutics in response to pH change and other stimuli. ND-drug adsorption complexes have shown great promise in killing drug resistant cancer, bypassing drug efflux and reducing side effects of anticancer drugs. Also, ND-drug adsorption complexes have been used in combination therapy as a part of efficient drug cocktails to treat multidrug resistant tumors and fight migrating cancer stem cells causing metastases.

There is great hope that these exciting successes of ND in fighting cancer can soon be translated to clinics. Taking into account the negligible toxicity of ND, low cost and available commercial production, ND meets all criteria to an excellent anticancer theranostic platform.

6. Application of ND in gene delivery

Gene delivery is the introduction of genetic material or gene therapeutics into cells, aiming to replace the ‘impaired’ gene to regain biological function or add a new gene to trigger additional functions [169]. Long ago viruses have been discovered as primitive and smart enough to transspire their genetic material into a genome of cells [170]. Since then the delivery of genetic material via viruses (viral vectors) has been widely pursued in clinics to irreversibly change cell functions—a permanent transfection. Although viral vectors have high gene transfection efficiency they give rise to serious safety concerns. This is why non-viral delivery is also actively pursued [169]. Non-viral strategies are good to deliver genetic material exclusively to cytoplasm—transient transfection. The transiently transfected genetic material residing in cytoplasm, does not replicate, and is gradually lost when the cells divide. Transposing DNA in chromosome by non-viral vectors is much less efficient compared to viral vectors [171]. Genetic material can be delivered to the nucleus by means of passive diffusion of non-viral vectors (nanoparticles) through the nuclear pore complex (NPC) [172]. The passive diffusion through NPC strongly depends on the net size of the vector and genetic cargo (preferentially less than 5 nm in diameter).

Many different nanocarriers are studied as gene delivery agents, including gold [173, 174] and magnetic iron oxide nanoparticles (magnetofection) [175]. ND-based gene delivery platforms are attractive because NDs are biocompatible [32], and have a rich surface chemistry, amenable to various modifications to help cell entry and ferry a gene [5]. ND particle size (2 to 5 nm in diameter) meets the criteria for a passive diffusion into the nucleus. Unprecedented examples of ND particle nuclear entry have been demonstrated a few years ago with Fenton treated ND [176]. The Fenton oxidation leads to ND free of amorphous carbon and of much smaller size (on average 4.4 nm after the oxidation of 7 nm NDs contained in the soot), small enough that it can passively penetrate into HeLa cells nuclei [172, 176] (figure 6). The reported ability of ND to easily escape from the endosomes [177] is also important for delivery of genetic material into nuclei. Fast escape from endosome helps to preserve genetic material from digesting enzymes.

Perhaps, the most studied application of ND for gene delivery is based on the non-covalent integration of poly-cationic molecules onto the ND surface followed by association with negatively charged nucleic acids [69, 87, 152, 178–183] (inset, figure 6). For example, pEGFPLuc plasmids encoding Luciferase and green fluorescent protein (GFP) have been successfully delivered in cytoplasm by means of aa ND-PEI vector [178]. Positively charged PEI-ND has significantly enhanced transfection when compared to PEI or ND alone, probably, due to a faster endosomal release. Since high molecular weight cationic vectors show high cellular toxicity [178], the authors have optimized the ratio PEI:ND:DNA to achieve high transfection rates, while minimizing toxicity. It is important to maintain the right balance between the quantity of DNA on the surface of the vector and the DNA-induced reduction of the positive charge of the structure, which is needed for the efficient endosomal release and transcription of genetic material. It was calculated that 4.1 nm ND particle binds on average 70 branched 800 Da PEI molecules [184]. Studies have demonstrated that siRNA and ND-PEI ratios (1 to 75 w/w siRNA to ND-PEI, respectively) can be tuned to knock down GFP and EWS-Fli1 genes more efficiently than the well-known liposomal vector Lipofectamine [179, 180]. The ability of ND-PEI to release siRNA in the cytoplasm much faster than other vectors (including other ND-polycationic complexes) is due to the fast endosomal release of the vector confirmed by TEM [180, 185]. The large number of primary and tertiary amino groups (at least 216 μmol g−1) on the ND-PEI surface results in osmotic influx of counter-ions through the endosome membrane to protonated ND-PEI complex leading to endosome swelling and disruption [180].
In addition to ND-PEI complexes, hydrogenated detonation NDs with zeta potential $+55$ mV have been studied to electrostatically bind negatively charged siRNA [186]. The estimated number of siRNA molecules is 37 per one 7 nm ND particle. The air oxidized ND-COOH (zeta potential $-50$ mV) did not demonstrate any non-specific binding of siRNA as expected [186].

Carboxylated derivatives of larger, 20 nm HPHTNDs have been exploited in a covalent reaction with amino-modified nucleic acid through EDC/NHS chemistry [187]. Another covalent DNA binding technique has been recently demonstrated via a copper-free coupling of dibenzocyclooctin-modified nucleic acid to azido-functionalized 100 nm HPHTND [188].

7. ND-based antimicrobial agents

Infectious diseases are among the leading causes of death world-wide, with bacterial infections contributing substantially to the high rate of mortality. With the first case in the United States of a person carrying a ‘superbug’, an $E.~coli$ bacteria strain resistant to antibiotics of last resort [1], it becomes obvious that alternative strategies are urgently needed to combat bacterial infections [189]. Bactericidal nanoparticles can provide a viable alternative to current antibiotic therapies [190]. The development of bactericidal nanoparticles requires rational design of the particle, an efficient synthetic approach, and understanding of their biocompatibility and toxicity [191, 192]. Comprehending bacterial pathogenesis provides another key to bacterial inhibition without altering gut microbiome or inducing undesirable bacterial resistance [193, 194]. Among the emerging antibacterial particles, NDs have been tested in combination with carbohydrates [111, 113, 195], proteins [196–199], and antibiotics [44, 73], with very recent exciting application of ND-Gutta percha-amoxicillin composite as a tooth root canal filler that is in clinical trials now [200] (figure 7(A)). Various protocols to modify the ND surface followed by grafting of antimicrobial therapeutics have been studied [52, 53, 110, 117]. However, the use of ND is not limited to its role as a carrier of antibacterial therapeutics. Surprisingly, ND itself may induce bacterial death [201, 202]. Below we analyze the ND potential in preventing bacterial infections with emphasis on uropathogenic infections.

In spite of extensive in vivo and in vitro studies conducted with ND derivatives using eukaryotic cells, only a handful of studies have been published on ND toxicity toward prokaryotic cells [154, 201, 202]. On the contrary, interactions of other carbonaceous materials with bacteria have been extensively studied. Crucial properties of graphene-based materials [203–205], CNT [206, 207], and fullerenes [208, 209], such as dispersibility, size, and oxidation capability have been evaluated with regard to bacterial toxicity. ND has emerged as a toxic nanomaterial toward Gram-negative $Escherichia~coli$ and Gram-positive $Bacillus~subtilis$ strains (figure 7(B)) [201]. The authors propose that the antibacterial ND activity is likely linked to the reactive oxygen-containing groups on the ND surface, although more studies are needed to support this proposition. It is likely that ND antibacterial properties, similarly to other carbon materials, are related to: (i) direct attachment to the bacterial wall through the surface groups (i.e. charge interaction, hydrogen bonds), (ii) membrane stress (physical damage of the membrane), (iii) inhibition of metabolic processes (i.e. inability to

Figure 6. The mechanisms of permanent and transient transfections. The inset shows a schematic structure of a genetic cargo delivery system (ND-PEI-DNA complex): the negatively charged surface of ND is overcoated with positively charged PEI, which in turn attracts negatively charged DNA molecules.
produce antioxidants in response to oxidative stress.

Uropathogenic *E. coli* (UPEC) Gram-negative bacteria are responsible for 90% of urinary tract infections (UTIs). In spite of numerous defensive mechanisms of the innate immune system (production of antimicrobial factors, cytokine triggered recruitment of neutrophils followed by exfoliation of superficial infected tissue), some bacteria remain undeterred even after antibiotic treatment [194] hidden in intercellular reservoirs of bladder epithelial cells (BECs) [211, 212]. Nanodiamond may provide an interesting opportunity to rupture BEC membranes followed by their exfoliation due to its superior hardness and large number of sharp edges (HPHTND), which was reported to facilitate its transport through cell membrane with subsequent fast endosomal escape [177]. UPEC establishment in the bladder heavily relies upon type I pili virulence factor, *FimH*, which binds to uroplakin mannosylated proteins on the surface of BECs [195, 213]. Thus, a possible line of defense is focused on preventing UPEC adherence to the bladder cells [214]. Mannose molecules very strongly adhere to *FimH* lectin. Although monosaccharides possess low affinity to *FimH*, multivalent mannose ligands provide high strength of binding for *FimH* lectin. However, synthetic protocols toward multivalent mannose ligands are sometimes too elaborate [52, 113, 116]. Recently, a new way of integration of polymannose ligands onto the ND surface has been proposed [117] via photoactivation of ND-tetrafluoroazide leading to a ND-nitrene radical that reacts with sugar molecules through C-H insertion. Preserved activity of sugar units after the reaction with nitrenes was demonstrated by effective binding of ND-glycans to plant fluorescent lectins. A recent review [215] covers many applications of ND-glycan particles against UTIs. ND-glycans have been tested as *E. coli* antiadhesives [53, 111, 116], biofilm inhibitors for Gram-positive and Gram-negative bacteria [113, 216], and promoters of agglutination [52, 117].

8. ND for bone and tissue implants

Bone tissue engineering (BTE) emerged ~30 years ago and is based on seeding the artificial scaffold with stem cells, which are then differentiated into bone tissue. This approach in principle allows one to restore damaged or fractured bones and can be used in bone surgery. After bone is formed, there is no need for the scaffold and in most cases it can be surgically removed or (preferentially) degraded without additional surgery. Ideally, the scaffold degradation should be synchronized with the bone tissue regeneration rate. Because bones support significant mechanical loads, the scaffolds for bone surgery need to have high mechanical strength. This is

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**Figure 7.** (A) Schematic of ND-Gutta percha embedded with ND-amoxicillin conjugates. The upper part shows synthesis of ND-Gutta percha: a polyisoprene solution was prepared by dissolving trans-1,4-polyisoprene in chloroform with subsequent addition of ZnO, BaSO\(_4\), and wax. ND-amoxicillin was then mixed into the polyisoprene solution, and the final mixture was lyophilized to obtain solid ND-Gutta percha. Lower part shows the antibacterial effect of ND-Gutta percha implant: bacterial infection of the root canal is suppressed due to antimicrobial properties of both ND and amoxicillin embedded into polymer. Adapted with permission from [44]. Copyright 2014 American Chemical Society. (B) NDs exhibit antibacterial properties depending on purity and surface chemistry: NDs and NDs annealed in air at 450 °C cause bacterial death. Hydrogenated NDs cause bacterial death only at high concentrations. Extra-pure NDs (>99% purity) independent of surface chemistry did not show any bactericidal effects. Adapted with permission from [201]. Copyright 2014 American Chemical Society.
the main reason why bone surgery fixation devices are currently made of metals. However, metals normally require extraction surgery to be removed from the body, while bioresorbable polymers, which would be more beneficial in this respect, cannot be used due to their poor mechanical properties. Also, the chemical properties of the scaffolds are important to diminish immune response and minimize other adverse effects [217].

Superior hardness and Young’s modulus of ND, as well as rich surface chemistry and chemical stability of the ND core are beneficial for improving mechanical and chemical properties of bioresorbable polymer scaffolds. It was reported that octadecylamine (ODA) functionalized NDs [65] embedded in poly L-lactic acid (PLLA) resulted in 200% higher Young’s modulus and 800% higher hardness compared to neat PLLA [218]. Murine osteoblast growth was demonstrated on the ND-ODA/PLLA matrix for up to one week. The solubility of ND-ODA in organic solvent (e.g. chloroform) facilitates the dispersion of the nanofiller in the hydrophobic PLLA matrix.

The ND-adsorbed phospholipid complex was used to obtain a stable dispersion of ND particles in poly(lactic-co-glycolic acid) (PLGA) [219]. Compared to neat PLGA, 10 wt% ND/PLGA showed 100% increase in Young’s modulus and 550% increase in hardness. Moreover, the ND inclusion into PLGA matrix slowed down its biodegradation in vivo, thus enabling a robust growth of hFOB1.19 osteoblasts. The in vivo testing of ND/PLGA matrix over eight weeks has shown acceptable immune response and no toxicity.

Stem cells differentiation towards osteoblasts is controlled by growth factors. One of them, bone morphogenetic protein (BMP), has been widely used in BTE, however, concerns regarding its tumorigenic potential have recently been raised. The improved delivery system based on poly[(L-lactide)-co-(ε-caprolactone)] (PLCL) scaffold and ND has been demonstrated to release BMP-2 locally [220]. Authors claim that the ND surface chemistry plays an important role in sustained release of low doses of adsorbed BMP-2. The ND/PLCL implant degrades in vivo over six months to 10% of its initial weight while minimizing anti-inflammatory response due to its low toxicity [221]. ND/PLCL scaffolds have been recently reported to decrease the tumorigenic potential of early neoplastic dysplastic oral keratinocytes. The anticancer activity was associated with the presence of ND in these scaffolds through the mechanisms, which are currently under investigation [222]. This result is a spectacular demonstration of anticancer activity of ND containing bone implants.

9. Recent advances in ND for biomedical imaging

Due to an immense popularity of HPHTND in bioimaging, this topic has been covered in many reviews [11, 223–227]. Fluorescence associated with ND NVN and NV defects is most widely used in bioimaging. Both defects are produced by high-energy particles irradiation of NDs, followed by annealing [226]. The advantages of NVN and NV defects for bioimaging are related to their stable photoemission in the 500–800 nm spectral range (figure 8(A)) [226] and were used in many in vivo and in vitro studies [67, 89, 100, 101, 104, 118, 122, 123, 145, 146].

Other defects in nanodiamond also demonstrate interesting optical properties [228]. For example, europium-vacancy (EuV) [229], and silicon-vacancy (SiV) defects [230] can be potentially exploited as bright biomarkers with
excellent photostability, which is not compromised by tissue autofluorescence (figure 8(A)). Given the low probability of existence of NV centers in NDs smaller than 40 nm [231], the possibility to create SiV defects in the ND crystals less than 10 nm in diameter seems to be a great step forward [232, 233]. SiV defects can be created in NDs for example by plasma CVD growth on a silicon substrate [232, 234]. Shorter excited state lifetime, weak and narrow vibronic side band, and zero phonon (ZP) emission maximum at 739 nm (figure 8(A)) with little overlap with broad photoluminescence of ND (450–650 nm), are the advantages of SiV over NV defects.

In addition to widely studied ‘intrinsic’ fluorescence associated with diamond structure defects, the so-called ‘extrinsic fluorescence’ associated with ND surface functional groups has also been analyzed recently. The intensity of the ‘extrinsic fluorescence’ depends on the type of ND functional groups and varies from very high (e.g., for octadecyl amine terminated ND) to low (many other types of surface termination). Octadecyl amine [65], hydroxyl, ketone, and ester groups [235] have a significant effect on the fluorescent properties of DND (figure 8(B)). The ‘extrinsic fluorescence’ of DND with simultaneously present hydroxyl, ester, and ketone groups is wavelength-dependent. However, when the surface is covered by mainly one type of functional groups, for example, hydroxyl, the fluorescence becomes independent on the excitation. Importantly, each type of functional groups results in slightly different emission wavelength (figure 8(B)) potentially allowing multicolor imaging using DNDs with well-defined surface chemistry.

NV defects of bulk diamond have been exploited in the areas spanning beyond conventional fluorescence techniques. Being sensitive to weak magnetic fields (nanotesla), these defects enable nanometer-scale resolution sensing and monitoring of proteins and nucleic acids in their natural environment [105, 236, 237]. For example, tracking of the motion of a segment of a single protein molecule can be achieved with the nitroxide nuclear spin label detection by diamond located 10 nm away [237]. This approach helps to understand the structure and dynamics of proteins in their natural environment. The exceptional photostability and non-blinking fluorescence of NV defects resulted in progress in stimulated emission depletion (STED) microscopy and other sub-diffraction resolution techniques [100, 238, 239]. For example, NV center electron spin resonance probed with and switched on and off by microwave radiation, was superimposed with their optical emission leading to subdiffraction limited resolution of NV centers in 2 NDs separated by only 22 nm within 7 × 9 μm² field of view (see figure 3 in [240]).

NV defects in HPHT NDs and bulk diamonds have been used as precise temperature probes with potential in vivo applications [241, 242].

In addition to fluorescence, NDs provide many other modalities, enabling their use in a broad array of biomedical imaging techniques from high resolution confocal fluorescent microscopy to nanoscale magnetometry and magnetic resonance imaging including clinically accessible imaging protocols, such as CT, MRI, and PET [243]. These modalities combined with low production cost, facile surface modification, and low to no toxicity, open exciting avenues for ND particles as future theranostic platforms.

10. Conclusions and outlook

In the previous decade we have witnessed significant growth of interest to biomedical applications of NDs, which now become one of the hottest research areas. Main factors behind such a rapid progress include our better understanding of NDs at the fundamental level [5, 23, 27, 32, 244], successes in their purification [16, 25, 244], dispersion into stable single-digit colloidal state [32, 34, 41, 42], and development of robust techniques to control surface chemistry of these materials [37, 54, 55, 65, 71, 72, 96, 245]. Pure, well characterized, and dispersed NDs are now available for biological studies revealing the true potential of ND as a unique theranostic platform. Researchers developing biomedical applications work closely with materials scientists and chemists and they are now aware of the need for careful characterization, purification, and control of surface chemistry to get the full benefits of NDs in biology and medicine. A vast array of ND surface groups composed primarily of organic carbon are convenient for a chemist to work with. Moreover, the ND surface can be coated by silica and polymer shells enhancing solubility and biological stability [86, 101]. Enormous progress has been achieved in ND deaggregation [37, 40, 42]. Various protocols are now available to deaggregate NDs into single-digit colloidal particles. Some of these protocols are especially attractive for biomedical applications since they use only sodium chloride crystals and ultrasound [42].

Size of the nanoparticles plays a role in determining their behavior in the biological system. For example, passive transport through nucleolemma is only possible for nanoparticles of 5 nm or less. Single-digit detonation ND meets this requirement and potentially can passively deliver genetic material into the nucleus, not just into cytoplasm [176].

Both, neat ND and ND combined with antibiotics demonstrate antibacterial properties. The ND-amoxicillin complex in Gutta percha matrix is now in clinical studies as a root canal tooth implant [200]. Due to their hardness and sharp edges [177], certain ND particles can potentially be used as exfoliating agents, exposing difficult to reach uropathogenic bacterial seeds from intracellular reservoirs in bladder epithelium.

In novel cancer treatment strategies NDs with tailored surface chemistry can be used to controllably trigger the vasculature leakiness [10]—an effect similar to EPR that may help to kill tumors in early stages of tumorigenesis. Versatile surface characteristics enable the use of ND as a platform to deliver different types of anticancer drugs [45]. These ND-drug adsorption complexes provide a long intracellular residence time and sustained drug release, thus overcoming drug resistance of cancer cells, while reducing harmful side effects of chemotherapy [161]. As the next step in anticancer therapy, NDs have recently been proposed as combinatorial agents to
fight multidrug resistant tumors, allowing the lowest possible dose of the drug in a cocktail to kill tumors [165].

Biology and medicine will benefit from bright and non-photobleaching fluorescence of NDs [226]. Different ND defects, such as NV, SIV, and EuV are explored for imaging in different wavelength ranges, in particular, in the near infrared, which is of special interest for in vivo imaging [230]. Being non-toxic, NDs provide a highly attractive alternative to semiconductor quantum dots in bioimaging. An unusually long spin coherence time of NV centers and our ability to controllably switch their fluorescence on and off by applying microwave radiation, enables NV ND applications as nanoscale sensors of proteins and nucleic acids, nanoscale thermometers, and in super resolution sub-diffraction optical imaging [240]. Combining ND particles with other particles and traditional imaging agents such as Gd [246] is beneficial in different imaging and hyperthermia applications [122].

The prospects of NDs in biology and medicine are bright. However, many questions remain. The fate of these little gems in the human body is one of them. Diamond structure is chemically inert and can hardly be digested by enzymes. Will NDs exit the body through the kidney or other natural routes or will they accumulate? If they accumulate in the body, then the studies of their long-term effects become very important. What happens when NDs are exposed to myriads of protein, lipid, carbohydrate, and other molecules in blood or inside the cells? To what extent do the NDs aggregate when they circulate in the blood stream and how does their aggregation impact performance? Can NDs deliver drugs over the blood–brain barrier as some other nanoparticles do? If so, how does ND interfere with functions of the central neuronal system? Can we use NDs to design multifunctional protein mimics [247], for example, mimicking hystones or enzymes? Will it be possible to achieve bright and stable NV center fluorescence in the smallest NDs (5 nm diameter or less)? Or maybe we should focus on other defects in these smallest NDs that are most attractive for biomedical imaging? Antibacterial properties of NDs are intriguing. What is the mechanism of their antibacterial activity? Will ND become an essential weapon in our arsenal against antibiotic resistant microbes, superbugs, which emerge as a new threat? Or perhaps NDs will help to deliver poorly soluble antibiotics in lowest effective doses exactly to the location where they are needed, in this way helping to minimize overdosing and suppressing development of bacterial resistance to antibiotics when treating wounds and other infections? The search for answers to these and other important questions will keep the nanodiamond community busy for the years ahead.

Last but not least, it is very important to pursue translational research aimed at bringing NDs into clinical practice. In many cases researchers have already accumulated a critical mass of results to support unique advantages of NDs. Translation of these advantages into clinics has been pioneered by D Ho’s group with others now increasingly realizing the importance and timeliness of this step. Although translational research is very expensive and time- and resource-demanding, this is a necessary and only way to bring the promises of NDs in biomedical applications to reality. Only with the help and participation of medical doctors and physicians working in a clinical environment, as well as continued financial support of this research, can these little gems make a big difference in our approach to provide a better healthcare for all.

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