
REVIEWS

Carbon Nanoparticles as Promising Neuroprotectors: Pro et Contra.

I. Functionalization and Toxicity

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Abstract—Such carbon structures as fullerenes, endofullerenes, nanotubes, nanodiamonds, and graphenes, which were discovered over recent decades, possess a number of unique properties and can become the basis for the design of a new class of neuroprotective agents; however, despite years of research, this has not happened yet. In the first part of the review, the significance of the functionalization of carbon nanoparticles for their use in biology and medicine is described, and the data on their toxicity are also discussed. The second part presents the works of Russian and foreign scientists demonstrating the neuroprotective properties of carbon nanoparticles and the possibilities of their application in neurobiology and neurology. The successful experience of such experiments is described and the existing problems are indicated.

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INTRODUCTION

Advances in healthcare and the rapid development of medical technologies have led to paradoxical consequences: an increase in life expectancy contributes to an increase in the incidence of various neurodegenerative disorders. Alzheimer’s disease (AD), Parkinson’s disease (PD), and Huntington’s disease (HD), amyotrophic lateral sclerosis, and other severe diseases of the nervous system are becoming more common. They are complex and are associated with many factors such as advanced age, environmental effects, compromised immunity, and to a lesser extent genetic predisposition [1]. When analyzing the causes leading to AD development, pathogenic factors, which may be involved in the initiation of disease and complicate its course, were identified. Reactive oxygen species (ROS), metal ions, impaired processing of amyloid precursor protein, and the abnormal aggregation of amyloid peptide beta (A β), as well as many other fac-

tors have been suggested as potential ones [2]. Each of them can lead to AD both by themselves and in conjunction with others. For example, metal ions, interacting with A β , stimulate its aggregation and accumulation [3, 4], which, in turn, can lead to oxidative stress (OS) and, as a result, to neurodegeneration [5, 6]. The scheme described above is common for other neurodegenerative diseases (NDs). For example, PD is characterized by the accumulation and aggregation of α -synuclein; and HD, a mutant huntingtin protein with an increased number of polyglutamine repeats. Both diseases, like AD, are accompanied by OS and neurodegeneration. At the same time, the existing methods of treatment have limited efficacy, and the search for drugs for the treatment of NDs, which can prevent the aggregation of amyloid proteins and OS, is a priority area of research. The use of nanomaterials promises revolutionary changes in the diagnostics and treatment of many diseases. These structures are capable of interacting with biological systems at molecular and supramolecular levels and can be adapted to meet the specific conditions of cells: induce desired physiological reactions in them or minimize undesirable side effects [7]. Carbon nanoparticles (CNPs) are characterized by unique properties and have great prospects for application in neurobiology and neurology. However, it is necessary that they have good biocompatibility, are not toxic, and are not deposited in organs and tissues. Years of research have not led to a positive result, and on the basis of nanoparticles (NPs) it has not yet been possible to create efficient drugs. The reasons for this can be sought, among other things, in the

lack of systematic studies and effective analysis of data available today.

1. ON THE ROLE OF THE FUNCTIONALIZATION OF CARBON NANOPARTICLES IN BIOCOMPATIBILITY

The majority of CNPs in the intact state have low biocompatibility and this prevents their application as therapeutic agents, in particular in clinical neurology. For example, the use of fullerenes is hindered by their insolubility in water. Therefore, their purification and chemical modification are required to increase their solubility and reduce toxicity [7]. At present, quite a lot of methods have been proposed for obtaining water-soluble derivatives of fullerenes by attaching various groups and molecules to them. These derivatives are more biocompatible than native fullerenes, and at the same time, they do not lose their unique properties, such as the ability to prevent the aggregation of amyloid proteins and antioxidant activity. Depending on which groups are attached to the molecule, various structures can be formed on the surface of NPs, the number and position of which will determine their behavior in an environment and biological activity. Thus, the combination of fullerenes with biomolecules can change their function and facilitate incorporation into membranes and penetration into cell organelles. The formation of supramolecular complexes of fullerenes with therapeutic agents improves the bioavailability and pharmacokinetics of the latter, facilitating targeted drug delivery [8]. The insolubility of fullerenes in water can be overcome by synthesizing water-soluble complexes with polymers [9]. For these purposes, polyethylene glycol (PEG), dextran, polyethylenimine, etc. are used. Dextran is considered the most biocompatible [10]. Due to its properties, this polysaccharide easily binds to various functional groups and provides the required physicochemical characteristics to the complex with NPs [11]. However, hydroxylated fullerenes are most often used. In this case, not only the number, but also the different localization of hydroxyl groups on the cage surface determines the balance of hydrophilic and hydrophobic interactions of fullerenols with each other and with the environment. The preparation of individual biologically active derivatives remains a difficult task, and only a few dozen water-soluble compounds with an individual structure have been synthesized to date. According to [12], two fullerene fractions with different surface electric charges and different percentages of hydroxyl and carbonyl groups can interact differently with actin filaments in the cell. At the same time, none of the samples exhibited noticeable cytotoxicity. The degree of aggregation of fullerenes and their derivatives in biological systems is of great importance [13]. When the surface is functionalized, particle aggregation can be reduced and this improves both NP biocompatibility and colloidal stability.

Carbon nanotubes (CNTs), like fullerenes, are insoluble in most organic and aqueous solvents. In addition, they have a strong tendency to aggregation. To improve biocompatibility, the CNT surface is modified [14]; the surface chemistry plays a very important role in the interaction of CNTs with a cell and its compartments [15]. The two main methods for modifying the surface of nanotubes are the noncovalent attachment of amphiphilic molecules (lipids, polymers, etc.) to them, which makes it possible to preserve the aromatic structure of CNTs without adversely affecting their electronic characteristics, as well as covalent modification by attaching various groups directly to the carbon skeleton [16]. Functionalized CNTs can bind nucleic acids, peptides, proteins, carbohydrates, etc., while the ability to penetrate through cell membranes into the cytoplasm makes them a promising basis for targeted delivery systems for various substances [17, 18], as well as for visualization and biosensing [19]. In addition, the chemical modification of CNTs is a powerful tool for constructing substrates that can control the growth and morphology of neural cells [16].

Graphene-based materials usually aggregate in an aqueous environment containing salts, proteins, or other ions and require chemical modification to give them the desired properties. Graphene can be easily functionalized into water-soluble graphene oxide (GO). So-called reduced GO is often used; it has advantages over GO and graphene, since it has the conductive properties of the latter and, at the same time, has negatively charged GO groups [20]. The addition of PEG to GO NPs makes it possible to additionally noncovalently bind hydrophobic drugs using π - π packing [21]. The chemical structure of graphene can be modified by adding reactive functional groups such as amino, carboxyl, hydroxyl, alkyl halide, or azide groups. Peptides, antibodies, contrast agents, and pharmaceutical agents can be conjugated onto the surface of graphene modified in this way. Such conjugates are promising for detecting metabolic disorders in the brain [22].

Nanodiamonds (NDs) synthesized by various methods (for example, detonation, laser ablation, and other methods), like other CNPs, tend to form aggregates of various sizes, i.e., have low colloidal stability. Their surface is covered with functional groups, the nature of which depends on the conditions and method of synthesis, as well as purification methods [23]. For use in biology and medicine, NDs with high colloidal stability in dispersion media are obtained. In this case, additional purification technologies are used to reduce the content of metal and organic impurities on the surface [24]. One of the solutions to the problem of ND aggregation is the modification of their surface by oxidation in liquid media or ozone using singlet oxygen. To increase their dispersing ability, detonation nanodiamonds (DNDs) can be functionalized through the carboxyl group [25]. To ensure the stabil-

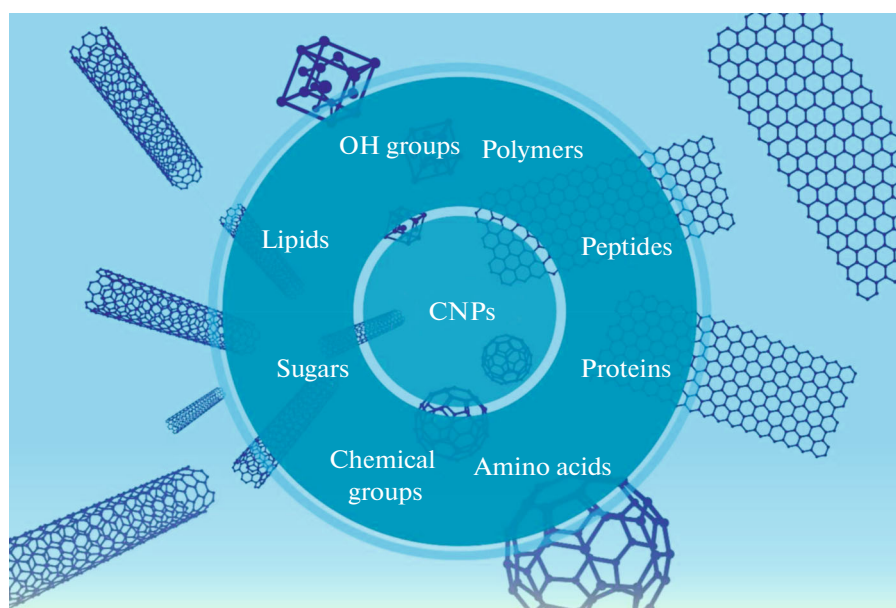


Fig. 1. Functionalization of carbon nanoparticles. The functionalization of CNPs is carried out by attaching various chemical groups and molecules to them. These modifications make CNPs soluble and biocompatible, and therefore more suitable for use in biology and medicine.

ity of dispersions in water, the surfaces of diamond single crystals were functionalized using hydrophilic groups [26]. Positively charged hydrogenated NDs, as well as NDs coated with a polymer shell can form complexes with nucleic acids. In general, the results of biomedical studies testify in favor of the biocompatibility and low toxicity of diamond NPs. For example, it has been shown that NDs are more biocompatible than most other carbon materials [27]. The chemical composition of their surface makes their conjugation with biomolecules possible, and the unique optical, mechanical, and thermal properties make NDs promising candidates for a wide range of applications in drug delivery [28].

Thus, all CNPs require functionalization to increase bioavailability, biocompatibility, and impart the necessary qualities to them (Fig. 1). At the same time, NPs can lose their original properties and become either less or, conversely, more toxic. That is, the functionalization of NPs and their properties are closely related to each other.

2. TOXICITY OF CARBON NANOPARTICLES

Evaluation of the toxicity of NPs and various derivatives is a paramount task in the design of new drugs. By interaction with structures inside the cell, NPs are capable of both therapeutic and harmful effects. Their contribution to the toxicity of complex preparations is also important. In addition, the widespread use of NPs increases the likelihood of adverse environmental impacts [29]. Their use of intracellular pathways inac-

cessible to larger particles, for example, endocytosis, contributes to toxicity [30].

2.1. Toxicity of Carbon Nanoparticles in *In Vitro* Studies

Currently available data on the toxicity of NPs are very ambiguous. This is due to the fact that very different preparations made in different ways are used in the studies. In addition, different evaluation methods and test systems are used. However, even these segmental, but rather numerous data allow us to draw certain conclusions. For example, the cytotoxic effects of fullerenes are associated with the release of ROS, direct oxidation of the membrane, its destruction, disruption of the structure and functions of membrane proteins, and ROS production inside the cell, which leads to the damage of proteins and organelles [31]. Fullerenols and some of their derivatives have been shown to affect cell homeostasis and are capable of blocking the ion channels of membranes [32]. However, more and more facts have recently appeared confirming the thesis that the C_{60} -type fullerene itself is practically harmless [33–35]. The toxicity of preparations is often associated with imperfection of the methods used to obtain them. In particular, to purify fullerenes and obtain, for example, fullerenols, organic solvents are used, the residues of which are able to remain in the structure of fullerenols in the adsorbed state or in the form of fragments and functional groups chemically bonded to the fullerene framework [29]. For example, several studies have confirmed that the increase in mortality from exposure to C_{60} dispersed with tetrahydrofuran is caused by

residual products of solvent decomposition, and not by the fullerene itself [35–38]. If fullerenols are obtained by a method that allows the production of an especially pure product, they are absolutely harmless [39]. There is also an opinion that the toxicity of fullerenes and their derivatives is a manifestation of the characteristics of the attached functional groups [40], and depending on the specific characteristics, the biological effect varies from cytoprotective to cytotoxic [32]. For example, the toxicity of cationic fullerene derivatives [40, 41] can be explained by their interaction with negatively charged nucleic acids. At the same time, the carboxyl derivatives of fullerene [41] and hydroxyfullerenes [42] are practically harmless. In the previous section, we indicate that dextran is the most biocompatible agent for fullerene functionalization. However, when several assessment methods were used, it was demonstrated that the dextran-containing C60 Dex-C₆₀ preparation has a dose-dependent toxic response in glial cells [29]. The toxicity of fullerene solutions can also be associated with some of their properties. Thus, it is believed that it correlates with the sizes of particle clusters [43, 44], since a difference in the sizes and geometry leads to different interactions with biological structures. Smaller aggregates are considered to be more toxic [43, 45]. However, when studying the relationship between the toxicity of fullerene solutions and their physical characteristics under various conditions, it has been shown that the main negative factor is not the cluster size, but the use of N-methylpyrrolidone as a solvent, which makes it possible to reduce aggregates to 10 nm. With a decrease in the concentration of fullerenes, the toxic effects of residual N-methylpyrrolidone also significantly decreased [13]. Thus, for use in medicine, it is necessary to standardize the purity criteria for fullerene-based preparations and determine the quality criteria [33].

The possible toxicity of CNTs is being actively discussed. According to [17, 46–48], the geometry, degree of aggregation, and functionalization play an important role in this case. The presence of impurities is much less important. However, it has been shown that the toxicity of CNTs is affected by the presence of impurities of various metals (Co, Ni, Fe, and rare-earth elements), as well as graphite and amorphous carbon that are formed during the production process. For example, the purification of commercial CNTs from iron impurities led to a decrease in the formation of free radicals [49]. It was demonstrated that purified CNTs do not produce free radicals, but, on the contrary, reduce the activity of hydroxyl and superoxide radicals [50]. In this case, the complete removal of impurities can lead to the opposite effect due to an increase in the surface area [51] and a high density of carboxyl and hydroxyl groups formed on it during acid treatment [48, 52]. Based on the analysis of collected data on the cytotoxicity of various types of CNTs towards different mammalian cells, it has been con-

cluded that toxicity often depends on the dose of the agent [53]. Thus, as in the case of fullerenes, the main factors determining the cytotoxicity of CNTs are their hydrophobicity, susceptibility to aggregation, and the presence of impurities. An important role is played by functional groups conjugated with them and molecules adsorbed on their surface [53].

The toxicity of graphene-based nanomaterials is poorly understood. It is believed that graphene is able to overcome the cell membrane and interact with both the plasma membrane itself and cytoplasmic organelles. When it enters the nucleus, it interacts with DNA, thereby posing a threat to both the genome and the epigenome [54]. It is believed that the main mechanism of graphene toxicity is associated with the generation of ROS inside the cell [55]. Difficulties in assessing its effects arise due to differences in the morphology and size of NPs. Toxicity may depend on the charge, the functional groups present, the methods of synthesis, the route of administration, the dose and time of exposure, and residual contaminants, and all this is the reason for discrepancies in the data of various authors [56]. Studies on graphene toxicity are systematized depending on the parameters described above in reviews [56, 57]; a complex relationship between the physico-chemical properties of graphene and the resulting biological effects is shown. From the data presented, it can be concluded that the toxicity of graphene does not depend much on the time of incubation with the drug, but greatly depends on the dose used, functional groups (functionalization), cell type, and, possibly, particle size. All this should be taken into account when designing graphene-based medicinal agents.

The toxicity of NDs, among other CNPs, was described in detail in review [58]. It is impossible to draw unambiguous conclusions from the published data presented in the work. Thus, a number of studies have shown that NDs are not toxic or have low toxicity for various transplanted cell cultures. Other studies have demonstrated the opposite for both transplanted lines and cultured human blood cells, the effects being dose dependent. However, an analysis of the literature suggests that the *in vitro* toxicity of NDs is either negligible or absent. Here we present only some data. It was shown that NDs did not significantly affect cell proliferation and differentiation, cell cycle, and protein expression [59]. In other experiments, NDs were not toxic to HeLa cells in complete cell culture medium, and their cytotoxicity was associated with the absence of serum [60]. Similar results were obtained with human peripheral blood lymphocytes and transplanted tumor lines A549 and HT29; moreover, the effect of purification and functionalization of NDs on cytotoxicity was demonstrated [61]. To understand how particles behave inside a cell, NDs without surface functionalization were used. It has been shown that NDs are localized in separate groups in early endosomes, lysosomes, and near the plasma mem-

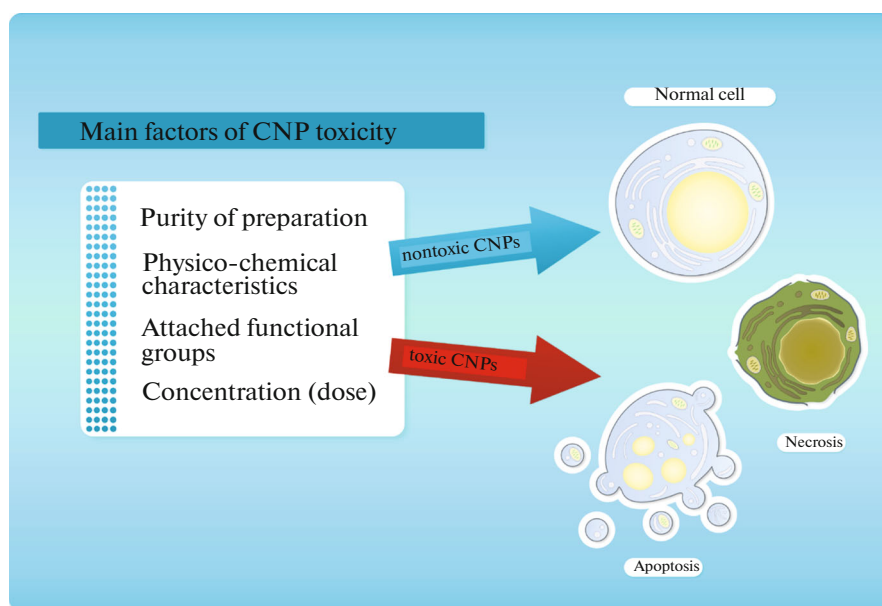


Fig. 2. Toxicity of carbon nanoparticles. The toxicity of CNPs is associated with a number of factors that must be taken into account in order for the risk-to-benefit ratio in their use in biology and medicine to be optimal.

brane. Presumably, NDs enter the cell through continuous endocytosis and, as shown by the cocultivation method, do not accumulate in the cell, but are removed from it by exocytosis. NDs were not identified in the nucleus, mitochondria, and Golgi apparatus, and had no negative effect [62]. The study of the effects of NDs on urothelial cells also showed their localization in endosomes and the absence of toxicity [63]. According to review [64], NDs have low toxicity towards various types of cells and do not lead to the noticeable generation of ROS. The same conclusions can be drawn from [27, 65–67]. In addition, NDs may contribute to the reduction of adverse drug reactions. For example, it has been shown that Fe-doped NDs have very little effect on living cells, despite the fact that the toxicity of Fe is well known [68].

When looking at studies demonstrating the harmful effects of NDs, there are several hypotheses that explain this phenomenon. Thus, when comparing different groups of NDs, it turned out that NDs–NH₃⁺ have a higher cytotoxicity than NDs–COOH and NDs–PEG. It is assumed that this may be associated with the ability of positively charged particles to pass through the cell membrane more easily due to the properties of the membrane itself: the inner part of its lipid bilayer is hydrophobic and negatively charged [69]. At the same time, NDs–NH₂ and NDs–COOH, in contrast to NDs–PEG, caused dose-dependent toxicity; moreover, the toxicity of NDs with an amino was higher than that of NDs with a carboxyl group under the same exposure conditions [70]. This suggests that, as in the case of fullerenes, the biological properties of NDs are closely related to the character-

istics of the attached functional groups. It is possible that the particle size is also important (small particles are more toxic), but despite this, the toxicity of NDs is primarily associated with the presence of impurities (for example, carbon) in the sample [71].

Thus, the *in vitro* toxicity of all described CNPs is associated with the same factors: the purity of preparation, dose, NP size, and attached functional groups (Fig. 2). Toxicity at the organism level (*in vivo*) may be related to additional causes.

2.2. Toxicity of Carbon Nanoparticles in *In Vivo* Studies

The use of NPs as medicinal agents, components of pharmaceutical or diagnostic agents implies the need to take into account the reaction of the whole organism and should be preceded by an assessment of their effect at all stages of exposure: from initial administration to penetration into the tissue endothelium, into the intercellular space and through the cell membrane into the cell, into its compartments, and further into the nucleus [72]. It is important how this drug is distributed inside the body, in which organs and tissues it accumulates, how it is excreted, how the immune system reacts, whether there will be an inflammatory reaction to it, etc. In this regard, the task of assessing the direct and long-term consequences of the administration of NPs *in vivo* arises.

In 2012, it was shown that C₆₀ fullerene dissolved in olive oil, when administered orally to rats (1.7 mg/kg), doubled their lifespan [73]. Fullerene derivatives showed very low cytotoxicity as well as low acute toxicity in mice. For one of these compounds, it was 4 times less than that of aspirin and comparable to that

of table salt or sugar [74]. A harmful effect of fullerenes was also not found in [75]. The study of the effect of $C_{60}(OH)_{30}$, $C_{70}(OH)_{30}$, and $C_{120}(OH)_n$ fullerlenols on *Drosophila melanogaster* showed that even very high doses of preparations (2 mg/mL) did not change the lifespan and behavior of flies. No histopathological changes were found in the respiratory organs of rats after the inhalation of C_{60} particles of different sizes [76]. A number of studies have shown that fullerenes and their derivatives are not only not highly toxic in vivo, but can also have some protective functions [77–80]. Such a response of the body may be associated with the low absorption and efficient excretion of these particles from the gastrointestinal tract. For example, it has been demonstrated that water-soluble fullerene derivatives after intravenous administration are widely distributed in tissues, detected in the liver, kidneys, spleen, and bones, and then excreted in the urine or feces [81–83].

However, the report on the formation of antibodies to fullerene suggests that an immune reaction may follow administration of the preparation [84]. There is evidence of a dose-dependent toxic response of the body to the administered preparation [85]. It is likely that the response in vivo, as well as in vitro, can be influenced by the way in which the preparations were obtained and their characteristics.

The response to the administration of nanotubes can also be different: if NPs that enter the body of animals through the respiratory tract can cause an inflammatory reaction as a result of OS, then orally administered NPs are mainly excreted in the feces [86]. The data available in publications on their toxicity are ambiguous. The difference between the results of different authors, as in the case of fullerenes, may be due to the fact that each type of nanotubes should be considered as a separate substance with an individual assessment of hygienic standards. Data on the in vivo study of CNTs are collected in [72]. They are not numerous and mostly associated with the effect of CNTs on the respiratory organs. As the analysis shows, most of the works demonstrating the toxicity of CNTs describe nonfunctionalized nanotubes that enter the body through the respiratory tract. The biodistribution of NPs is of great interest. When studying CNTs labeled with radioactive isotopes, it has been shown that they are easily distributed over the organs and tissues of mice, accumulate in the stomach, kidneys and bones, and are then excreted with urine and feces. No tissue damage was found [87]. Nanotubes functionalized with diethylenetriaminepentaacetic acid, after intravenous injection, were not retained in any of the organs of the reticuloendothelial system (liver and spleen) and were quickly removed from the circulatory system through the kidneys. The electron microscopic analysis of urine samples showed that nanotubes were isolated in an intact form [88]. According to some

data, CNTs do not have intrinsic immunogenicity [89].

The above-mentioned reviews on graphene-based nanomaterials [56, 57] present data on its in vivo toxicity. The results of studies of the intravenous injection of various graphene derivatives to experimental animals are extremely diverse. A number of studies have shown severe pulmonary thromboembolism and weight loss after exposure to the preparations. The preparation accumulated in the spleen and liver, entered the lungs, stomach, kidneys, and passed through the blood-brain barrier. In other cases, no pathological changes were noted. The dependence of the effect on the dose and functional groups of the preparation was shown. In most cases, no adverse effects were observed with intraperitoneal administration. When administered orally to females, a significant reduction in body weight and other changes, as well as short-term behavioral disorders, were observed in the offspring.

According to [90, 91], the in vivo behavior of NDs is similar to that of fullerenes and nanotubes. The problem may lie in their high propensity to aggregate under conditions of increased ionic strength after intravenous injection. This process is uncontrollable and can affect their ability to penetrate into cells and their viability [26]. In this regard, it is very important to use NDs stabilized by the adsorption of a biocompatible polymer or a surfactant that can also absorb and desorb pharmaceuticals. At the same time, the results of studies of the biocompatibility of NDs, which were carried out on primates and rats, have shown that they are well tolerated at clinically relevant doses. The study in rats lasted two weeks and included histological analysis and blood and urine tests. Primates were comprehensively tested for 6 months, analyzing blood serum, urine, the histological pattern of tissues, and taking into account body weight. In both cases, several doses of drugs were used [92]. The ND biodistribution was analyzed using a radioactive label (^{188}Re) [90]. In this study, NPs were administered intracheally. It turned out that most of the NDs accumulated in the lungs, from which they were excreted very slowly. They entered the spleen, liver, bones, and heart and exhibited acute toxicity in Kunming mice. However, according to [93], NDs have low pulmonary toxicity, since their number in the alveoli decreases over time with the involvement of pulmonary macrophages in this process. When applied to the skin, NDs did not cause allergic reactions, and in the case of subcutaneous injections, they were localized at the injection site and formed clusters in the intercellular spaces; in this case, cell destruction was not observed [94]. It should be noted that the metabolism of NDs in the body has not been sufficiently studied; nevertheless, there are already data on their use in the clinic. A randomized trial of recovery after root canal treatment was conducted by comparing material (polymer) without NDs and with NDs [95]. From the report it

follows that when NDs were used, healing of the lesion, reduction in postoperative pain, and the absence of re-infection were observed. After 3 and 6 months, no side effects were observed.

CONCLUSIONS

From the analysis of numerous data on the toxicity of CNPs, it can be concluded that the response of a cell or an organism to their administration can be very different, and at present there is still no sufficient understanding of the relationship between the physico-chemical properties of the materials under study and biological processes. In addition, there is no proper quality control during synthesis processes. At the same time, it is clear that the purity of the synthesized CNPs and their correct functionalization play a paramount role on the way to the design of medicinal agents based on them.

CONFLICT OF INTEREST

We declare that we have no conflicts of interest.

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