Chapter

Few-Layered Hexagonal Boron Nitride: Functionalization, Nanocomposites, and Physicochemical and Biological Properties

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Abstract

Hexagonal boron nitride (h-BN) is an analogue of graphite called “white graphene.” In the structure of h-BN, B and N atoms substitute C atoms. The boron and nitrogen atoms are linked via strong B-N covalent bonds and form interlocking hexagonal rings. h-BN is used in different areas due to its interesting physical and chemical properties, e.g., in electronics as an insulator and in ceramics, resins, plastics, and paints. Therefore, boron nitride (BN) is also a popular inorganic compound in cosmetic industry (the highest BN concentration up to 25% can be found in eye shadow formulation). It is also widely used in dental cement production (for dental and orthodontic applications). Boron nitride seems to be suitable for biomedical applications; therefore, the cytotoxicity in vitro and in vivo observations of h-BN nanoplates and novel few-layered h-BN-based nanocomposites are still needed. The short-time studies confirm their low cytotoxicity and suggest that BN can be used as a novel drug delivery system; however, medical application needs additional verification in long-term studies.

Keywords: boron nitride, few-layered hexagonal boron nitride, exfoliation, functionalization, hydroxyl groups, gold nanoparticles, h-BN nanocomposites, biocompatibility, cellular uptake

1. Introduction

Nanotechnology became a crucial technology in many science fields, not only in organic and inorganic chemistry, materials and surface sciences, semiconductor physics, microfabrication, and molecular engineering but also has significant impact on biological and medical science. The results of nanotechnology activity create a new reality. On the one hand, it gives the possibility to develop novel methods of diagnosis, drug delivery, and cancer treatment. On the other hand, methods implemented in nanomaterial production and development can affect human health and the state of the environment. The knowledge of the effect on
living organisms is limited due to relatively short-time in vitro and especially in vivo experiments that highlight mechanism of nanomaterials—cell-tissue-organism interactions.

Application of nanomaterials in biology and medicine has a multidirectional character. The different nanomaterials with many unique physicochemical properties are tested to develop new nanomaterial-based approaches: fluorescent labels (e.g., quantum dots), detection of pathogens and other biological samples (e.g., nucleic acids, proteins), methods of separation and purification of single biomolecules or cells, pharmacokinetic analysis, biosensing, final drug or gene delivery, cancer treatment via hyperthermia method, tissue engineering, and contrast enhancement of medical imaging technique (e.g., magnetic resonance imaging) [1].

Hexagonal boron nitride (h-BN) is one of the most unique and promising layered nanomaterial widely used as in cosmetic production. As it was stated by Fiume and co-workers [2], although the International Cosmetic Ingredient Dictionary and Handbook does not specify which crystal form/forms is/are used in cosmetics, it is presumed that the hexagonal form of boron nitride is applied for that proposes. The form of h-BN presents the most appropriate functionality in cosmetic production/properties (e.g., as a slip modifier). The use of h-BN in cosmetic formulation suggests the lack of toxicity/cytotoxicity [2–4]; thus, the new approach of h-BN, or its exfoliated form, to study its modification and functionalization to obtain a potentially interesting nanomaterial in, e.g., the context of theranostic concept is expected.

2. General information about hexagonal boron nitride (h-BN)

In recent years, 2D materials have become very attractive due to their properties. The most popular among them are graphene, graphene oxide (GO), and reduced graphene oxide (rGO). The big advantage of these materials is their potential multifunctionality, so they can be applied, for example, in transistors, sensing, energy devices [5] and biomedical devices [6], or nanomedicine [7]. Even though these materials are studied widely, there is a plenty of room to explore their properties, e.g., very complexed bio-response on many levels. Another attractive layered material, which is not fully explored, is hexagonal boron nitride. Its exfoliated form is considered as a graphene analogue.

Boron nitride is a chemical compound with equal number of boron and nitrogen atoms. Just like carbon, it occurs in amorphous and crystalline forms. The major crystalline forms are hexagonal boron nitride (h-BN) compared to graphite, sphalerite boron nitride (β-BN) similar to cubic diamond, and rhombohedral (r-BN) and wurtzite boron nitride (γ-BN), which is in hexagonal diamond form [3, 8–10]. Boron nitride nanotubes (BNNT) are also known. All the forms are electrical insulators [11]. The most popular form of BN, due to its stability, is hexagonal boron nitride. In its structure the boron and nitrogen atoms are linked with each other via strong B-N covalent bonds and form interlocking hexagonal rings [12, 13]. Atoms are bound via strong covalent bonds in-plane, and each layer is held together via van der Waals forces [12] (Figure 1).

The multilayered form stabilizes the whole structure. Hexagonal boron nitride systems (e.g., nanotubes, flakes) are highly thermally and chemically stable, but at the same time, they are equally thermally conductive and mechanically robust. Table 1 presents the basic properties of hexagonal boron nitride. Thus, h-BN systems are widely used for durable high-temperature crucibles, antioxidation lubricants, and protective coatings and as a substrate for semiconductors, lens coatings, etc. in industry [2, 15].
Boron nitride nanosheets (BNNSs) were found to be used in polymeric film reinforcement, for example, the elastic modulus of polymethyl methacrylate (PMMA) film was increased when BN nanosheets were incorporated into the polymer [16]. It is also a popular inorganic compound in cosmetic industry used as a slip modifier [13, 17]. The data from the US Food and Drug Administration (FDA) report showed that boron nitride was used in 643 cosmetic formulations (data from 2013). The highest BN concentration (up to 25%) can be found in eye shadow formulation, up to 16% in powders and 2% in lipstick formulation [2, 13]. The successful use of BNNTs in dental adhesive and sealants has been also reported. Moreover, h-BN nanoplatelets modified by the presence of quaternary ammonium compounds (QACs) loaded on h-BN’s surface to form fillers for linear

![Hexagonal boron nitride structure](image)

**Figure 1.**
Hexagonal boron nitride structure [13].

**Table 1.**
Properties of hexagonal boron nitride [2, 14].

| Property                        | Value                          |
|---------------------------------|--------------------------------|
| Appearance                      | White powder, photostable, odorless (hexagonal, cosmetic grade) |
| Bond length                     | 1.466 Å (with interlayer spacing of 3.331 Å) |
| Molar mass                      | 24.82 g mol⁻¹                  |
| Density                         | ~2.1 g/cm³                     |
| Structure                       | Crystal; hexagonal             |
| Melting point                   | 2.973°C; sublimes              |
| Surface area                    | 0.82–30 m²/g (varies by grade) |
| Refractive index \( (n_D) \)    | 1.74                           |
| Stability                       | Chemical inert and stable      |
| Hardness                        | 1–2 on the Mohs scale          |
| Specific heat capacity \( (C) \)| 19.7 J/(K·mol)                |
| Std enthalpy of formation \( \Delta_{f}H^{o}_{298} \)| –254.4 kJ/mol                 |
| Gibbs free energy \( \Delta_{f}G^{o} \) | –22 kJ/mol                    |
| Coefficient of friction         | <0.3                           |

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low-density polyethylene (LLDPE) were tested for inhibition of growth of both *E. coli* and *S. aureus* bacteria [18–20].

Therefore, boron nitride seems to be suitable for biomedical applications as well. Several cytotoxicity studies based on boron nitride nanotubes confirmed its low cytotoxicity and suggested that BN can be used as a novel drug delivery system. In contrast, other studies showed that BNNT was cytotoxic and affected relative cell viability even at low concentrations [14, 21–24].

For the sake of discrepancies occurring in the literature, a deeper understanding of the toxicity of h-BN-based samples is crucial. Here, we will present the synthesis and cytotoxicity study on exfoliated and functionalized hexagonal boron nitride called few-layered BN.

2.1 Synthesis methods of few-layered h-BN (fBN)

There are two typical approaches to obtain exfoliated hexagonal boron nitrogen nanosheets: top-down (exfoliation methods) or bottom-up (chemical vapor deposition (CVD) or other deposition techniques).

An example of top-down had been described by Liu et al. [25]. They used one-pot solvothermal synthesis involving mixing bulk h-BN, ethanol, and sodium hydroxide in Teflon autoclave. In this method they obtained boron nitride nanosheets and quantum dots at the same time. Marsh with co-workers found even a simpler method [26]. They produced boron nitride nanosheets (BNNSs) from bulk h-BN powders using a simple cosolvent approach. Authors used common organic solvents and water to create a mixture. It was more efficient than using the individual components to get h-BN exfoliated and suspended. They maintain that cosolvent system is inexpensive, safe to work with, and completely scalable. Han et al. [27] used 1,2-dichloroethane solution of poly[(m-phenylenevinylene)-co-(2,5-dioctoxy-p-phenylenevinylene)] to disperse and break up van der Waals forces between h-BN layers, while Zhi et al. [28] reported the large-scale fabrication of 2D h-BN nanosheets by vigorous sonication of h-BN in dimethylformamide (DMF). The choice of solvent should be optimized to overcome van der Waals forces.

The optimization to use two-step exfoliation technique combining chemical and mechanical exfoliation was also reported [29]. Chemical exfoliation of h-BN was carried out by a modified Hummer’s method. h-BN was additionally delaminated mechanically. Mechanical exfoliation was performed using a tip sonicator. Chemically exfoliated h-BN was added into 1-methyl-2-pyrrolidinone (NMP) in a volume ratio of 0.5%. After the sonication, the mixture was left to evaporate the solvent. This method is simple and fast.

The most important representative of bottom-up method is chemical vapor deposition. In general, it can be divided into two types: one that requires a substrate and another which does not need it. A lot of optional substrates have been used in the process. CVD can be carried out on metals (Cu [30], Ni [31], Co [32], etc.) as well as on metal oxides (Al₂O₃) [33] or graphite [34]. The precursors can also be in different forms. Most popular are borazine [35], ammonia borane [36], and diborane [37].

Each method has its advantages and disadvantages. Exfoliation techniques ensure higher crystallinity of the material, but high-scale synthesis is very difficult. In contrast, the materials obtained from CVD give the possibility to control over the thickness or size of the sheets, but their crystallinity is lower. Therefore, the technique of material synthesis should be adapted to the requirements of a specific application.
2.2 Characterization of few-layered h-BN (fBN)

Depending on the synthesis method, a material with different properties can be expected. CVD is more suitable for large-scale synthesis in industry, but exfoliation methods have been also used for various applications, especially on a laboratory scale. Therefore, these materials cannot be clearly compared. Table 2 presents a summary of properties of fBN such as flake size and material thickness in relation to the method of synthesis.

Following data from state of the art, it can be concluded that chemical vapor deposition provides thinner and larger layers of h-BN than in the case of exfoliation. It should be noted that in the case of exfoliation, the starting bulk material is used, while in the case of CVD, a completely new material is obtained from various molecular precursors. Mostly, the number of the obtained h-BN layers is strongly related with the used substrates. It can be also concluded that the combination of

| Method         | Substrates                                               | Thickness [nm] | Flake size [μm] | Ref.               |
|----------------|----------------------------------------------------------|----------------|-----------------|--------------------|
| Exfoliation    | Potassium permanganate                                    | 5              | 0.3–0.6         | This work          |
|                | Sulfuric acid                                             |                |                 |                    |
|                | 1-Methyl-2-pyrrolidinone                                  |                |                 |                    |
| Exfoliation    | Ethanol                                                  | ~1             | ~1.2            | [25]               |
|                | Sodium hydroxide                                          |                |                 |                    |
| Exfoliation    | Methanol ethanol                                          | 6–10           | —               | [26]               |
|                | 1-Propanol                                               |                |                 |                    |
|                | 2-Propanol acetone                                        |                |                 |                    |
|                | Tert-butanol (cosolvents)                                 |                |                 |                    |
| Exfoliation    | 1,2-Dichloroethane poly[(m-phenylenevinylene)-co-(2,5-dictoxy-p-phenylenevinylene)] | ~1.2           | Several         | [27]               |
| Exfoliation    | Dimethylformamide                                         | 3–7            | Smaller than pristine material | [28]               |
| Exfoliation    | Octadecylamine                                            | 1–2            | 0.3–0.5         | [38]               |
| CVD (LPCVD)    | Cu foil, Borazine                                         | 0.42           | 0.05–0.1        | [30]               |
| CVD (LPCVD)    | Co film, Ammonia borane                                   | ~1             | > 5             | [32]               |
| CVD (LPCVD)    | Al₂O₃ substrate Borazine                                  | Temperature depending on | — | [33]               |
|                |                                                          | 40–228         |                 |                    |
| CVD            | Fe foil, Borazine                                         | 5–15           | 10 × 10         | [35]               |
| CVD (AP-CVD)   | Ag foil, Borazine                                         | 0.7–1.3        | 0.1             | [27]               |
| CVD (AP-CVD)   | Pt foil, Borazine                                         | 0.32–0.809     | 1–2             | [40]               |

Table 2.
Properties of fBN (flake size, material thickness) in relation to the synthesis method.
chemical and mechanical exfoliation is a very effective and repeatable method. Figure 2A presents the bulk hexagonal boron nitride. The multilayered material is clearly visible. Many flakes are aggregated and connected to each other. After the exfoliation process, even individual flakes of the material are detected (Figure 2B).

The exfoliation efficiency was also confirmed by atomic force microscopy (AFM). The thickness of bulk nanomaterial was estimated to be ~40 nm (Figure 3A). After chemical and mechanical exfoliation, the number of layers had been greatly reduced. The obtained thickness was ~5 nm, which corresponded to several layers of h-BN (Figure 3B).

Scanning electron microscope (SEM) was also used to analyze the flake size of fBN in greater details (Figure 4). The observation revealed that most of the flakes were in a size range of 0.3–1.2 μm.

Concluding this observation, using various methods the materials with similar parameters can be obtained. The issue of choosing the method of preparation should be adapted to the properties that the material should exhibit to perform the best in required applications.

2.3 Functionalization of few-layered h-BN (fBN)

Functionalization process is undertaken to provide additional/new properties. In the case of hexagonal boron nitride, the biggest challenge is to raise the materials’ water solubility/dispersibility. This can be achieved by introducing the functional groups on fBN surface. One of the simplest routes is functionalization with hydroxyl groups. At the same time, as it was proved, the presence of -OH groups determines the stability of dispersion in water-based solution [41].

The procedure was easy and repeatable. Chemically and mechanically exfoliated hexagonal boron nitrides were refluxed in hydrogen peroxide for a longer time. To confirm the functionalization, FT-IR spectra were analyzed (Figure 5). Except for the peaks characteristic for hexagonal boron nitride (810 and 1370 cm⁻¹), clearly visible new bonds corresponding to hydroxyl groups at 2525 and 3400 cm⁻¹ are detected [42].

Pristine BN nanomaterials exhibit notable hydrophobicity when interacting with water or aqueous solutions. Therefore, this functionalization allows stable dispersion in phosphate buffer solution, which is crucial for biological application [41].
Other methods for obtaining hydroxylated hexagonal boron nitride are also known. Sainsbury et al. [43] used boron nitride nanosheets with tert-butoxy groups on the surface. To induce hydroxyl groups, they mixed and sonicated piranha solution.
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(H_2SO_4:H_2O_2, 3:1) [43]. Moreover, the authors carried out further functionalization. They used hydroxylated material to obtain isocyanate-functionalized BNNSs. A completely different approach to obtain hydroxylated BN has been shown by Pakdel et al. [44]. Boron nitride nanostructure films were subjected to direct ion/electron bombardment in a plasma generator device. In this way they aimed to control the wetting properties of nanomaterial.

It turned out that hydroxylated h-BN can also be obtained by ball milling. Lee with co-workers [45] presented a simple ball milling of BN powders in the presence of sodium hydroxide. They connected the synergetic effect of chemical peeling and mechanical shear forces. There are many other functionalities that allow h-BN to be used in even more applications. For example, it is possible to decorate hexagonal boron nitride with metal nanoparticles. There are reports, for example, of platinum, silver, and gold decoration. Anna Harley-Trochimczyk et al. [46] described crystalline boron nitride aerogel loaded with crystalline platinum nanoparticles. They found out that this material can be used in catalytic gas sensing. Dai et al. [47] produced Ag nanoparticle coverage on porous BN microfibers and examined it for a novel pollutant-capturing surface-enhanced Raman scattering (SERS) substrate. There are already several reports on BN materials functionalized with gold; however, such a composite had not been previously tested for biological response as described below. Until now, such nanocomposite has found application in electrocatalysis [48] or hydrogen peroxide detection [49].

In research [29] simple and repeatable Au-fBN nanocomposite synthesis method was demonstrated. Briefly, exfoliated h-BN was sonicated in distilled water. The mixture was heated with gold(III) chloride trihydrate under the reflux. After a few minutes, trisodium citrate was added to the boiling mixture. The whole system was heated. Figure 6 shows transmission electron microscope (TEM) images of the obtained nanocomposite (Au-fBN).

Based on TEM micrographs, the size of gold nanoparticles was determined (Figure 7). The nanoparticle size distribution was in the range from 6 to 25 nm with a majority of ~16 nm.

The presence of gold nanoparticles was confirmed by Raman spectroscopy (Figure 8). The peak at 1366 cm\(^{-1}\) is the most characteristic for hexagonal boron nitride. It is resulting from the E2g phonon mode and is an analogue of the G peak in graphene [50]. It is very clearly visible in the spectrum of pure material and much

Figure 6.
TEM images of Au-fBN nanocomposite.
less intense in the nanocomposite. This is due to the presence of gold nanoparticles with characteristic peaks in the region from 1300 to 1600 cm\(^{-1}\) [51]. There is another intense peak in the spectrum of gold nanoparticles. It occurs at 2130 cm\(^{-1}\), and it can also be clearly seen in the nanocomposite spectrum, which additionally ensures efficient functionalization.

Nanomaterials prepared in the method described above (fBN-OH and Au-fBN) have been subjected to biological tests, which will be discussed in further parts of the chapter.

3. The effect of few-layered BN-OH (fBN-OH) and Au-fBN nanocomposites on cellular viability

The biocompatibility of h-BN-based nanocomposites synthesized in studies [29, 41] was determined in three main steps.
The first step of the in vitro study was based on morphological cell analysis. This step often is omitted by the researchers, although the morphology observation is the simplest and direct method that gives possibility to identify the changes of cellular shape and adhesion ability. Both of them may be changed upon specific environmental stress [52]. Figure 9 presents the cellular morphology and distribution of L929 and MCF-7 cells after 48-hour incubation with fBN-OH and Au-fBN nanocomposites (analysis was conducted using phase contrast microscopy, ×100, Nikon TS-100 microscope). Both cell lines grown in monolayers display typical morphology—the cells did not change shape. The cells did not show a tendency to form clusters, and the adhesion process was not impaired after 48 hours.

Moreover, in the second step, the cellular uptake and distribution of fBN-OH and Au-fBN nanocomposites were analyzed using confocal microscopy (Figure 10). The nanomaterials were labeled with FITC, and the presence of nanocomposites in cells was confirmed by green fluorescent signal. The internalized h-BN-based nanocomposites were accumulated in the peripheral cytoplasm and the perinuclear region, but the presence of fBN-OH and Au-fBN was not confirmed in the nucleus. The labeled nanocomposites formed small intercellular aggregates. The uptake
efficiency process of hexagonal boron nitride nanocomposites by normal and cancer cells was established at a similar level.

In the third step of the study, a comparison of biocompatibility results of the fBN-OH and Au-fBN nanocomposites at 3.125, 6.25, 10.0, 12.5, 25.0, 50.0, 100.0, and 200.0 μg mL⁻¹ concentrations was determined using Cell Counting Kit-8 (CCK-8), neutral red uptake (NRU), and lactate dehydrogenase leaking (LDH) assays (Figure 10) [29]. The highest reduction of the cell viability was recorded at the concentrations of 100.0–200.0 μg mL⁻¹ for fBN-OH and 200.0 μg mL⁻¹ for Au-fBN in L929 cell cultures incubated for 48 hours (Figure 10). The MCF-7 cell cultures exhibited higher reduction of cell viability for fBN-OH at the concentration range between 12.5 and 200.0 μg mL⁻¹ (Figure 10). The cell viability of MCF-7 cells incubated with nanocomposites loaded with gold NPs was reduced at the concentration of 10.0 and 200.0 μg mL⁻¹, in comparison to free-grown MCF-7 control culture (Figure 10).

In contrast to CCK-8 assay results, NRU assay showed higher reduction of the viability of both cell lines (Figure 10). Both cell lines showed higher sensitivity of

![Figure 10. Uptake of tested nanomaterials after 48-hour incubation.](image-url)
neutral red dye uptake based on functional lysosomes in the presence of few-layered h-BN-based nanocomposites (Figure 10). In the case of fBN-OH, the L929 and MCF-7 responded to the presence of NPs in a dose-dependent manner, but MCF-7 seems to be more sensitive than L929 cell cultures. The presence of Au-fBN affected the cell viability less than the fBN-OH.

The integrity of cellular plasma membranes (analyzed via LDH leakage assay) was impaired less than the other cell’s features. L929 cells as well as MCF-7 showed minimal changes in lactate dehydrogenase leakage even at the highest concentrations of fBN-OH and Au-fBN (Figure 10). The reduction of cell membrane integrity was the highest (to 80% vs. control cultures) for L929 and MCF-7 at concentrations of 200.0 μg mL⁻¹ fBN-OH (Figure 11).

Pristine bulk h-BN is known to be poorly soluble in water-based solutions. Thus, the h-BN preparation (e.g., exfoliation, functionalization) should be optimized to obtain a nanomaterial that exhibits suitable properties in required applications (e.g., higher hydrophobicity that allows stable dispersion in aqueous solutions) [41]. The functionalization of the h-BN by hydroxyl groups improve h-BN hydrophobicity and allows to obtain stable dispersion in phosphate buffer solution [41] or in phosphate buffer solution supplemented with dispersant Pluronic F-127 [29]. This is crucial for cytotoxicity experimentations and biological/medical applications.

Another crucial factor in biocompatibility analysis is experiment in short- and long-term studies. In studies it was demonstrated that the effect of the fBN-OH on cells may vary depending on the species, type of cells tested, their function, and time of exposure of cells to these nanoparticles. The short-term in vitro study on L929 cell cultures and human erythrocytes as well as in vivo study on insect (T. molitor) hemocytes demonstrated a low cytotoxicity of this fBN-OH (dispersed in PBS), whereas a long-term study in T. molitor has shown a significant effect of fBN-OH on the behavior of immunocompetent cells and their function during the immune response [41].

In the preliminary study based on hexagonal boron nitride (exfoliated and functionalized with Au nanoparticles), it was found that Au-fBN nanoflakes

![Figure 11. Biocompatibility of fBN-OH and Au-fBN nanoplates incubated for 48 hours—L929 cell cultures (A, B) and MCF-7 cell cultures (C, D). Bars represent standard deviation.](image-url)
did not affect the cellular metabolism (CCK-8) and membrane integrity (LDH assays). However, the function of lysosomes in both normal and cancer cell lines during 24-hour exposition was modified. Longer incubation, for 48 and 72 hours, affected the cell relative viability and proliferation activity of the MCF-7 cancer cell line in comparison to normal L929 cell line after 72-hour incubation period [29]. Additionally, fBN-based nanocomposites have been tested as a platform for drug delivery. Both the hexagonal boron nitride nanocomposites (fBN-OH and Au-fBN) were loaded chemically with an anticancer drug—10-hydroxycamptothecin (HCPT)—and were tested against human breast adenocarcinoma cells [53]. It was found that both nanocomposites conjugated with HCPT were effectively internalized and cumulated inside the cell cytoplasm, but not in the nuclear region of cells. Both the tested boron nitride nanocomposites loaded with 10-hydroxycomptothecin significantly reduced relative cell viability of MCF-7 cells. The slightly higher reduction was observed for Au-fBN-HCPT against human breast adenocarcinoma cells [53].

4. Conclusions

The hexagonal boron nitride (h-BN) is an attractive layered material that can be used in different industry sectors (due to its interesting physical and chemical properties). Its exfoliated form is considered as a graphene analogue. The pristine bulk h-BN is poorly soluble and exhibits a hydrophobic character in water/aqueous solutions. That is why the h-BN preparation (e.g., exfoliation, functionalization) is crucial to obtain a nanomaterial that exhibits the best properties that are required especially in in vitro and/or in vivo applications. The effect of the presented few-layered h-BN-based nanocomposites on biological environment may vary depending on the type of cells tested, their function, and time of exposure of cells to these nanoparticles. Boron nitride seems to be suitable for biomedical applications; therefore, the cytotoxicity in vitro and in vivo observations of novel few-layered h-BN-based nanocomposites are needed. The short-time studies confirmed their low cytotoxicity and suggest that h-BN can be used as a novel drug delivery system. However, medical applications need additional verification in long-term studies.

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Conflict of interest

The authors declare no conflict of interest.
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