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Black Phosphorus

Biological Effects of Black Phosphorus Nanomaterials on Mammalian Cells and Animals

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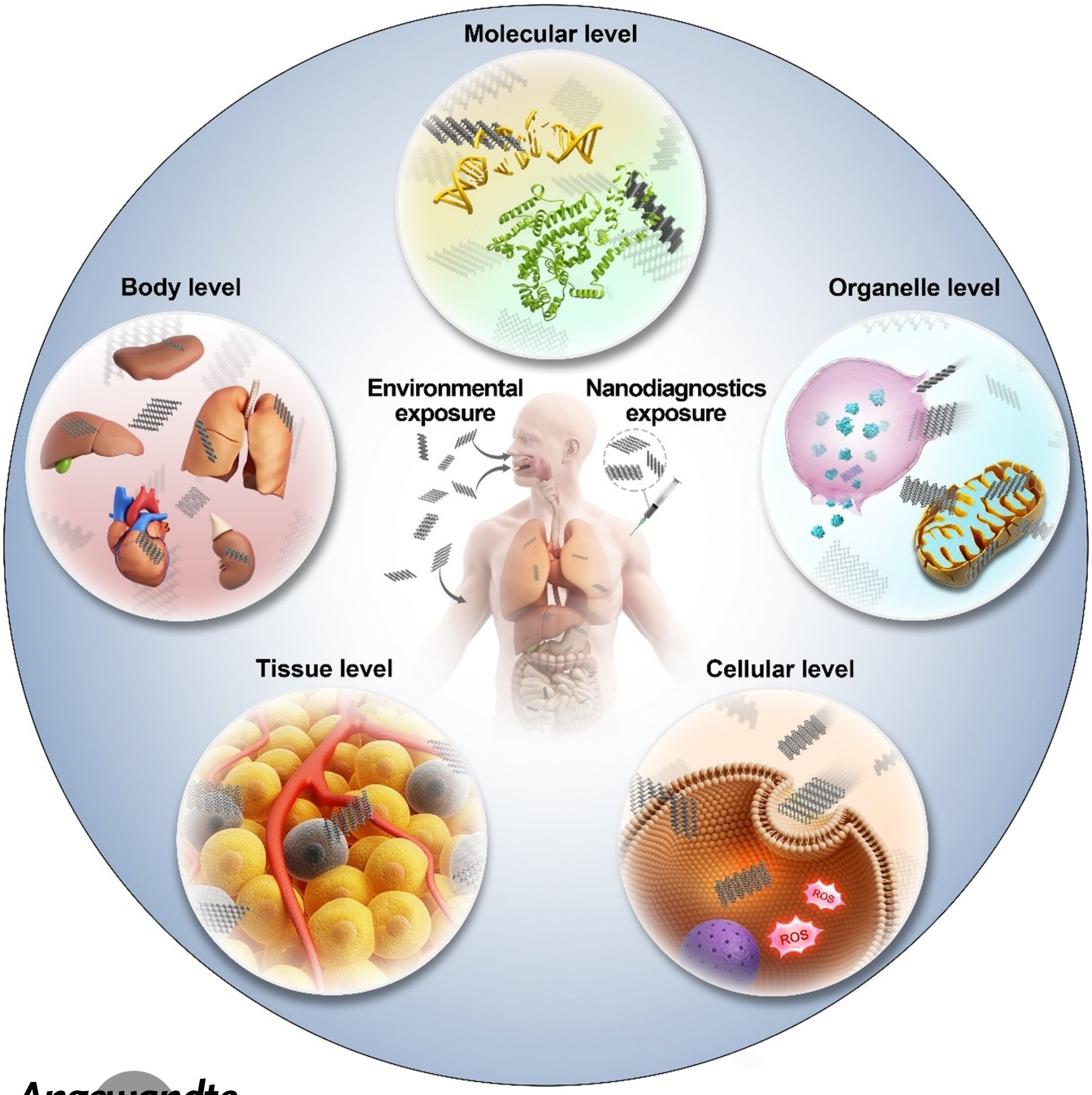

Abstract: The remarkable progress of applied black phosphorus nanomaterials (BPNMs) is attributed to BP's outstanding properties. Due to its potential for applications, environmental release and subsequent human exposure are virtually inevitable. Therefore, how BPNMs impact biological systems and human health needs to be considered. In this comprehensive Minireview, the most recent advancements in understanding the mechanisms and regulation factors of BPNMs' endogenous toxicity to mammalian systems are presented. These achievements lay the groundwork for an understanding of its biological effects, aimed towards establishing regulatory principles to minimize the adverse health impacts.

1. Introduction

In 1914, bulk black phosphorus (BP) was synthesized from white phosphorus by Bridgeman for the first time.[1] BP, the most stable allotrope of phosphorus, consists of stacked triangular pyramid structured two-dimensional (2D) layers formed from sp^3-hybridized phosphorus atoms (Figure 1a),[2] connected via interlayer weak van der Waals forces.[3] Since Li et al. exfoliated bulk BP into layered BP (LBP) and reported its unique optoelectronic properties from the perspective of a 2D material in 2014,[4] research on LBP greatly accelerated. BPNMs, including LBP and BP quantum dots (BPQDs), can be derived from BP by a variety of methods, such as mechanical exfoliation,[5] liquid-phase exfoliation,[6] solvothermal reaction,[7] and microwave or laser irradiation.[8] BPNMs possess widespread applications in optoelectronics,[9] catalysis,[10] energy storage,[11] and biomedicine.[12] With the development of low-cost quantity production technology, BPNMs are currently able to be produced on a gram-scale costing between 0.25 and 1 dollar per gram, paving the way for their large-scale application.[13]

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With the ever-increasing research and industrial demand, along with the scalable production of BPNM-based materials, leakage into the environment is increasingly likely. Subsequently, the risk of human exposure through inhalation, ingestion, and dermal pathways is increasing. Once in the human body, BPNMs can be widely distributed through the blood circulation systems and accumulate in organs and tissues. Consequently, a comprehensive evaluation of BPNMs' potential impact on human health is needed. Numerous articles were retrieved from the Web of Science database (http://www.isiknowledge.com) concerning "black phosphorus" and "phosphorene", and when mapped using social network analysis VOSviewer (Figure 1b), reappearances of key phrases such as "biocompatibility" and "toxicity" are increasing. This highlights the scientific community's growing attention to BPNMs' biosafety.

In the last five years, the biological effects of BPNMs were examined both in vitro and in vivo. Initially, BPNMs were always considered to be biocompatible since they can naturally degrade into non-toxic phosphates.[14] Also in the early studies, neither LBP nor BPQDs showed cytotoxicity towards a series of mammalian cell lines, including HeLa, COS-7, 293T, and MCF-7, even at a concentration as high as 1.0 mg mL^{-1}.[15] Pumera et al. alerted the scientific community that BPNMs might react with the reagents that are used for toxicity testing, leading to the underestimation of their toxicity.[16] In our previous study, we discovered that LBP exhibited a concentration-, size-, and cell-type-dependent cytotoxicity by a label-free real-time cell analysis technique.[17] Quite a few debatable toxicological results for BPNMs have been published and neither specific conclusions nor sufficient (what type) mechanisms have been determined.[18] Besides common toxicity factors (e.g., lateral dimension, surface properties, and functionalities), BPNMs are peculiarly dependent on their chemical activity and degradation behavior. It may impact their toxicity and fate when compared with other 2D nanomaterials. Compared to normal cells, the intracellular degradation of BPNMs in cancerous cells is faster, illustrated by a higher concentration of degradation products (phosphates), which could induce a series of negative biological effects.[18e,19] Therefore, the intrinsic reactivity of BPNMs should be considered during hazard assessment.[19,20]

A consistent conclusion has been drawn that the major routes for cellular uptake of BPNMs are caveolae-dependent endocytosis and micropinocytosis.[14a,21] In addition, phagocytosis and clathrin-dependent endocytosis also play a part when the physicochemical properties (size, surface charge, functionality) of BPNMs are regulated.[22] In addi-

tion to the cellular uptake pathways, a number of studies focusing on biodistribution, cytotoxic effects and mechanisms, and excretion have been conducted. Although some reviews outline the biocompatibility and safety profiles of BPNMs at both cellular and animal levels,[23] most of them focused on their therapeutic action, where external stimuli (e.g., light and ultrasound) are employed. Nevertheless, discussions regarding the inherent biosafety of BPNMs are insufficient. Therefore, it is still a great challenge for researchers to identify the relationship between physicochemical character and toxicity of BPNMs.

In this Minireview, we provide a comprehensive summary of the reports to date on the mechanisms and regulating factors of endogenous toxicity of BPNMs both in vitro and in vivo, particularly laying out the inconclusive and controversial results. A rule-based machine learning model via association rule mining (ARM) was used to comprehensively analyze the reported toxicity data of BPNMs, which revealed that experimental methods, lateral size, and incubation time were the most important factors in determining the cell viability of BPNMs. Moreover, the interactions between BPNMs and a given biological system were discussed at the levels of the entire organism, tissues, and cells (Figure 2). Furthermore, guidelines for future in-depth studies, accompanied by the challenges in this promising field, are presented. This Minireview presents inclusive knowledge of the cytotoxicity, intracellular and in vivo fate, and other biological effects, which hopefully can not only raise awareness of this new material, but also advance the development of regulatory principles to reduce potentially detrimental exposure and adverse health impacts.

2. Debatable Cytotoxicity of BPNMs

Although applications for BPNMs have been extensively investigated, the current toxicological studies are still inadequate to facilitate systematic understanding of their biosafety at cellular and organism levels. Moreover, the reported toxicity results are controversial, some of which claimed that BPNMs are biocompatible even at concentrations as high as hundreds of milligrams per milliliter, while others revealed adverse effects on the viability of a variety of cell types. For example, Zhang et al. demonstrated that only 36.6 % of HeLa cells were viable after 24 h incubation with 200 $\mu g\,mL^{-1}$ BPQDs determined by Annexin V–FITC/PI staining.[18d] In contrast, no toxic effect of BPQDs on HeLa cells was observed at 12 h up to 1.0 $mg\,mL^{-1}$ based on MTT assays.[15a] Moreover, BPQDs showed no cytotoxicity to Raw264.7 cells, while significantly decreased ATP content in J774A.1 cells according to ATP assays.[18a]

Analogous to BPQDs, LBP also displayed inconsistent effects on the cell viabilities despite keeping the key factors (lateral size, cell type, and toxicity assay) constant.[18b,c,e] These controversial results might originate from the different incubation durations and thicknesses of LBP, since 24 h incubation of LBP (lateral size of 200 nm, thickness of 5.5 nm) showed little cytotoxicity to HeLa cells,[18c] whereas extremely low IC_{50} values ($<2\,\mu g\,mL^{-1}$) were obtained for LBP with a comparable lateral size but a larger thickness of 10 nm towards HeLa cells if extending the incubation time to 48 h.[18b,e] Unexpectedly, the discrepancy even occurred for the PEGylated LBP (BP–PEG), which demonstrated biocompatibility to various cell lines (HeLa, MCF-7,

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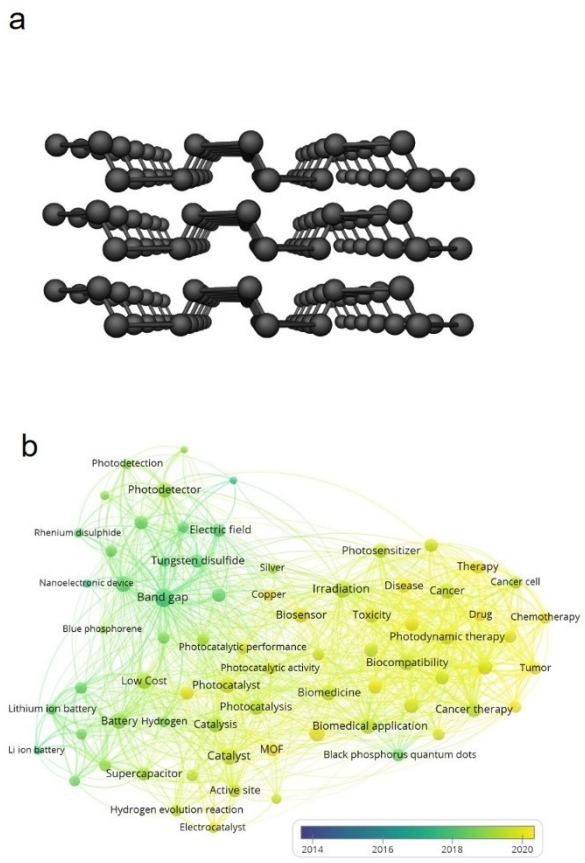

Figure 1. Network map of the research trends based on the keywords from 2016 to 2021. (The colors represent the average occurrence time of keywords.)

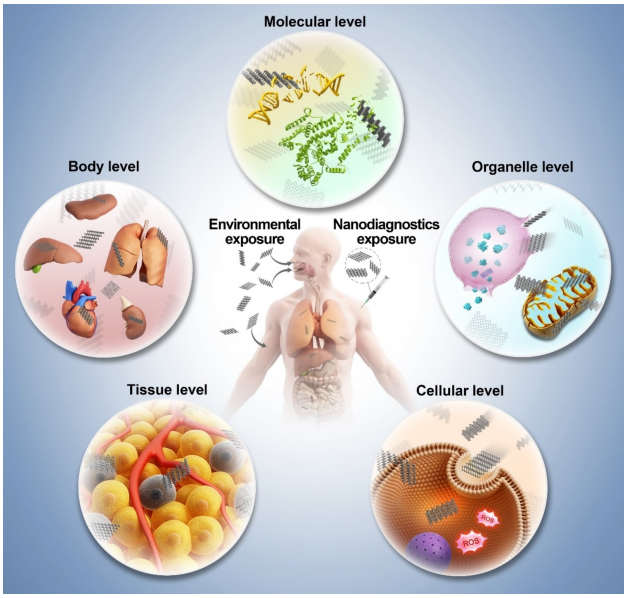

Figure 2. Schematic diagram of the interaction of BPNMs with biological systems.

HepG2, PC3, A375) up to 100 μg mL^{-1} by MTT or CCK-8 assays,[14b] but was selectively toxic to HeLa cells rather than D551 cells under higher concentrations (>200 μg mL^{-1}) by Alamar Blue™ assay.[19] Therefore, in addition to the already-known factors that are important to the cytotoxicity results, we infer the toxicity assessment techniques also have a significant impact on the results.

Indeed, Pumera et al. compared five commonly used toxicity assays, including three tetrazolium-salt-based assays (MTT, WST, XTT) and two non-tetrazolium assays (lactate dehydrogenase (LDH) and Multi-Tox Glo), for the cytotoxicity evaluation of LBP.[24] By using Annexin V–FITC/PI staining to verify the accuracy of these assays, they found that LBP could induce a concentration-dependent interference on the background signals of tetrazolium-salt-based assays. The interference could be derived from the reduction or adsorption of assay reagents by LBP.[16,25] This phenomenon has also been observed on carbon-based nanomaterials,[26] noble metal nanoparticles,[27] silica nanoparticles,[28] and quantum dots.[29] In our recent study, a label-free real-time cell analysis technique with no need of fluorescent or colorimetric reagents was introduced to perform the cytotoxicity evaluation of LBP.[17] We found that the cytotoxicity of LBP displayed a time-, size-, concentration-, and cell-type-dependent profile.

According to literature, surface modifications reduce the cytotoxicity of BPNMs.[18a,30] For example, TiL_4 modification (TiL_4@BPs) decreased the toxicity of BPQDs in Raw264.7 cells due to the reversed surface charge and higher stability of TiL_4@BPs, which lowered cellular uptake and intracellular reactive oxygen species (ROS) generation.[18a] Functionalization with polyglycerol significantly decreased the toxicity of BPNMs.[31] Moreover, Pumera et al. presented the effect of synthetic methods on the toxicity profiles of BPNMs by influencing their properties, i.e., exfoliation degree and oxidation degree.[32] They claimed that thinner structures and higher oxidation content led to higher toxicity.

Until now, it has been hard to draw a general conclusion of BPNMs' biosafety due to the diversity and complexity of cytotoxicity results. Thus, we used a rule-based machine learning model via ARM to make a comprehensive analysis of the BPNM-related toxicity data. From forty articles regarding the cytotoxicity of BPNMs published from 2017 to 2021, 1257 instances reflecting the material properties, cell type, and experimental conditions were extracted. The cell viability data was classified into low (<50 %), medium (50–85 %), and high (>85 %) viability. The a priori algorithm was executed with the mlxtend module in the Python software, and association rules were extracted according to three parameters: support, confidence, and lift. Given a set of combinations A (antecedent) and B (consequent), support is a probability of a combination in the dataset; Confidence is the proportion of a combination in the dataset including the antecedent A that also includes consequent B; Lift is the confidence adjusted by the relative support of combination B.[33] The most important factors in determining the cell viability are experimental methods, lateral size, and incubation time (Table 1). MTT assay generally yields

Table 1: Support, confidence, and lift values of association rules between factors and cell viability (significant associations are shown based on ranked lift values with support values ≥ 0.1).

Antecedent	Consequent	Support	Confidence	Lift
Source/Synthetic method = Smart-Elements	high viability	0.107	0.854	1.76
Lateral size [nm] = 200, materials = LBP	low viability	0.110	0.421	1.73
Incubation time [h] = 48, materials = LBP	low viability	0.104	0.406	1.67
Lateral size [nm] = 200	low viability	0.110	0.390	1.60
Method = MTT	high viability	0.169	0.700	1.45
Materials = LBP	low viability	0.233	0.312	1.28
Incubation time [h] = 48	low viability	0.106	0.297	1.22
Method = CCK-8	medium viability	0.140	0.326	1.19
Incubation time [h] = 48	medium viability	0.114	0.319	1.17
Materials = LBP, method = CCK-8	medium viability	0.115	0.312	1.15
Materials = LBP, incubation time [h] = 24	low viability	0.111	0.271	1.12
Incubation time [h] = 24, method = CCK-8	high viability	0.112	0.516	1.07
Method = CCK-8	high viability	0.216	0.502	1.04
Incubation time [h] = 24	high viability	0.258	0.500	1.03
Materials = LBP, method = CCK-8	high viability	0.181	0.491	1.02

high viability (confidence = 70 %, lift = 1.45). When the lateral size is 200 nm (confidence = 42 %, lift = 1.73) or the incubation time is 48 h (confidence = 40.6 %, lift = 1.67), the cell viability of LBP is observed to be low. Noticeably, the manufacturer also plays a part, as BP crystals sourced from Smart-Elements produce highly viable BPNMs (confidence = 85 %, lift = 1.76). In spite of the abundant cytotoxicity data, the question how these factors affect the cytotoxicity of BPNMs still requires in-depth exploration in future studies.

3. Cytotoxicity Mechanisms of BPNMs

3.1. Local Disturbance of Plasma Membrane

The plasma membrane is the first interface between nanomaterials and cells, making it the main obstacle for them to enter cells.[34] The plasma membrane is composed of lipids, proteins, and carbohydrates, with a thickness of 5–10 nm.[35] The maintenance of membrane integrity is a key function of plasma membrane. When nanomaterials encounter the plasma membrane, the membrane integrity can be altered, inducing lysis. LDH leakage is an indicator of plasma membrane integrity and consequently cell viability. Both LBP and BPQDs exposure could increase the release of LDH in a concentration-dependent manner, indicating the membrane disturbance.[22]

Due to the intrinsic 2D morphology, LBPs can penetrate the plasma membrane. In our previous study, the model cell membrane based on zwitterionic 1,2-dioleoyl-*sn*-glycero-3-phosphocholine (DOPC) vesicles was fabricated to detect the interactions of LBPs with lipid membrane by quartz crystal microbalance with dissipation (QCM-D).[17] Larger LBP (lateral size of ≈900 nm) induced a sustained frequency increase and dissipation decrease due to the disruption of model cell membrane (Figure 3a). In contrast, small BPs (lateral size of ≈400 and ≈200 nm) only slightly altered the signals indicating mild damage of model membrane (Figure 3b, c). The size-dependent membrane damage could be responsible for the higher cytotoxicity of large LBPs. Furthermore, Zhang et al. revealed the molecular mechanisms of the interactions between phosphorene or phosphorene oxide (PO) and lipid membrane by large-scale molecular dynamics (MD) simulations.[18b,36] The results showed that both BPNMs could penetrate into the cell membrane and extract abundant phospholipids (Figure 3d). The oxidation level was inversely proportional to the extraction ability (Figure 3e). Yu et al. detected no LDH leakage after 24 h exposure to LBP, although the duration was adequate for its internalization. After 48 h, LDH leakage was observed, indicating that the intracellular bioactivities of BPs were responsible for the antiproliferation effect of LBP instead of the physical damage of plasma membrane.[18e]

3.2. Oxidative Stress

High levels of oxidative stress lead to cytotoxicity. Once internalized by the cell, BPNMs can promote intracellular oxidative stress by inducing the overproduction of ROS. In our previous study, we reported that the cytotoxicity of LBP partially relied on the ROS generation.[17] However, BP-mediated ROS were not size-dependent, unlike the results of cytotoxicity. BP was investigated for photodynamic therapy as a binder and photoinducer for ROS.[37] Several cell organelles can participate in ROS generation. Zhao et al. found that the adsorption of plasma protein on BPNMs could increase the BP-induced ROS production in macrophages from 143.7 % to 185.8 % for BPQDs and from 159.9 % to 215.2 % for LBP, respectively.[30a] Other important cell organelles that are the main source of intracellular ROS are mitochondria.[38] When the concentration of LBP increased to 100 $\mu g\,mL^{-1}$, the integrity of mitochondria was destroyed as indicated by the decreased mitochondrial membrane potential (MMP) and the intracellular ROS level elevated ≈2.5-fold.[39] The damaged mitochondria might either be the result of the overproduced ROS in the intracellular matrix, or the source of the elevated intra-

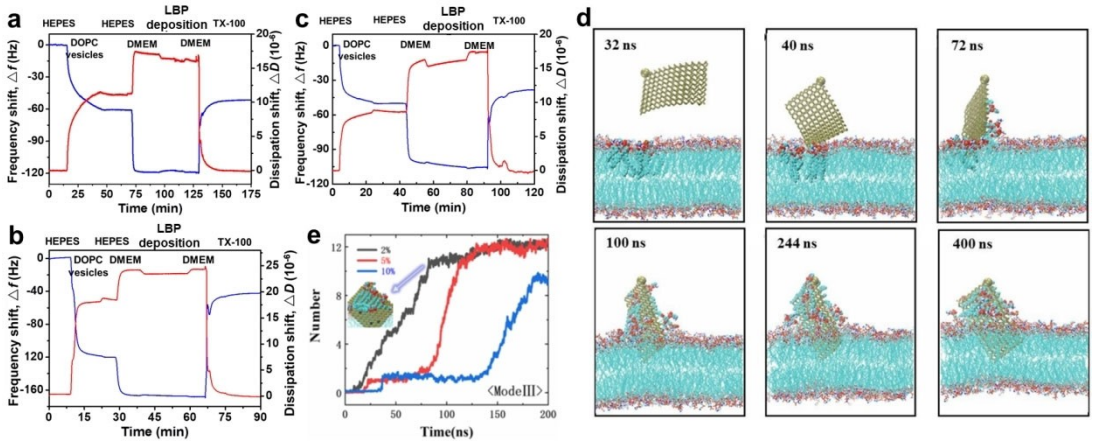

Figure 3. Changes of frequency (blue) and dissipation (red) of DOPC vesicles treated with cell culture media suspended LBP with different lateral sizes: ≈900 nm (a), ≈400 nm (b), and ≈200 nm (c) monitored by QCM-D.[17] Copyright 2017, John Wiley & Sons. d) Snapshots of the process of lipid extraction and membrane insertion by phosphorene based on MD simulations.[36] Copyright 2020, Royal Society of Chemistry. e) Time-dependent extraction of phospholipid molecules by PO with the oxidation levels of 2%, 5%, and 10%.[18b] Copyright 2021, American Chemical Society.

cellular ROS level.[40] The antioxidant defence system is responsible for the elimination of excess ROS. BPNMs could impair the antioxidant defence system by inhibiting the superoxide dismutase activity or altering the Nrf2/HO-1 antioxidant pathway, leading to the intracellular oxidative stress.[19,25] The overproduced ROS activated caspase-3 and subsequently induced cell apoptosis.[39]

3.3. Endoplasmic Reticulum Stress

Endoplasmic reticulum (ER) is a eukaryotic cell organelle and the maintenance of ER internal homeostasis is important for regulating ions, basic metabolism, and generally, for the normal physiological function of cells.[41] The ER is activated by oxidative stress, calcium imbalance, toxin invasion, and other stress events as a defense mechanism to reduce cellular damage.[42] However, sustained ER stress activated by nanomaterials can lead to cell death.[43] Recently, Zuo et al. demonstrated that BPQDs caused renal toxicity through the ER stress pathway.[44] They proved the nephrotoxicity of BPQDs at the levels of cell, tissue, and organism by using HK2 human renal tubular epithelial cells, kidney organoids, and BALB/c mice.

3.4. Different Types of Cell Death Modes

It is important to consider the different cell death pathways during BPNMs' cytotoxicity assessment. LBP could trigger three major forms of programmed cell death in mammalian cells, including apoptosis, autophagy, and ferroptosis. LBP impairs the integrity of mitochondria, resulting in the elevation of the intracellular ROS level, which could activate the cell-apoptosis-related downstream proteases, e.g., caspase-3.[39] Mei et al. proved the occurrence of autophagy in cancer cells under the treatment with BP–PEG.[14b] Later on, Yu's team further confirmed that LBP could not only induce autophagy, but also disturb the autophagic flux, leading to the cell death.[18e] In addition, BPQDs induced ferroptosis in a cell-type-dependent manner via the inhibition of demethylase (ALKBH5) expression.[45]

4. In Vivo Toxicity

Despite the extensive investigations and applications, systematic investigations of the toxicity of BPNMs, especially on the in vivo biosafety, are still in the early stages. In this section, we will summarize the latest research progress on the in vivo biological effects of BPNMs, with regard to intracorporal accumulation and biotoxicity.

4.1. Biodistribution, Pharmacokinetics, and Clearance of BPNMs

A major clearance pathway of nanoparticles in blood is through the important immune mononuclear phagocyte system, consisting of the liver and spleen.[46] Among modern imaging techniques, fluorescence and photoacoustic imaging have been widely applied to visualize the in vivo biodistribution of BPNMs. They use the labeling with near-infrared (NIR) fluorescence dyes (Cy5, Cy5.5, and Cy7), or directly take advantage of BPNMs' intrinsic NIR absorption.[14b,19,47] In addition, single-emission computed tomography/computed tomography imaging can quantitatively analyze the in vivo biodistribution and pharmacokinetics with high resolution by labeling BPNMs with radioactive technetium-99m.[48] Moreover, Raman scattering mapping is an additional way to directly examine the ex vivo tumor accumulation of BPNMs without labeling.[49]

As expected, liver and spleen were the main targets for the in vivo accumulation of BPNMs, since the nanomaterials

can be spontaneously arrested by macrophages in these organs.[14b,18d,19,48,49] Apart from the liver and spleen, tumor tissues could also sequester BPNMs due to the enhanced permeation and retention (EPR) effect (Figure 4a).[14b,19,49] Interestingly, the similar morphology of LBP with DNA rectangular origami nanostructures endowed LBP with kidney targeting capacity (Figure 4b).[47] Besides the above major target organs, slight distribution of BPNMs in lung and heart was also detected.[47–49]

Due to the considerable intrinsic phosphorus background in animals, it is difficult to directly quantify the in vivo phosphorus content. At present, researchers obtain the pharmacokinetics of BPNMs by monitoring the fluorescence intensity or radioactivity of fluorophore- or radioisotope-labeled BPNMs.[47–49] Currently, BPNMs follow a two-compartment model for cleaning the circulatory system. The first phase (distribution phase) showed a rapid decline with a circulation half-life from 1.3 min to 1.16 h. The second phase (elimination phase) representing the clearance of BPNMs from blood had a longer half-life from 125.7 min to 18.45 h.

The signals of BPNMs in the major organs increased in the first few hours post injection to reach the maximum, and then gradually decreased due to elimination. The in vivo experiments demonstrated that renal clearance was the major route for the excretion of BPNMs in the form of urine containing degraded products (PO_4^{3-} species) (Figure 4c).[18d,50] The accumulation of LBP in kidney was prolonged due to the suppression of renal clearance in the acute kidney injury (AKI) of mice. It demonstrated the ROS scavenging ability for the therapeutic treatment of AKI via alleviating oxidative-pressure-induced cell apoptosis in tissues (Figure 4d).[47] However, the current methods for in vivo quantification of BPNMs are greatly limited, considering the stability and integrity of the labeled BPNMs. Therefore, the development of more accurate and reliable quantitative techniques for monitoring the biodistribution and clearance of BPNMs is highly desirable.

4.2. Biochemical, Hematological and Histological Changes by BPNMs

Although numerous in vitro studies have provided preliminary information for the biosafety evaluation of BPNMs, these results cannot be extracted for all the cell types and organs. Accordingly, it is essential to assess the toxicity of BPNMs in animal models, in particular, in terms of the changes in biochemical, hematological, and histological parameters.

Both BPQDs and LBP induced transient toxicological responses in vivo without long-term impact.[18a,d,51] At 1 day post-injection, BPQDs caused a series of toxic responses in male C57BL/6 mice, including lipid peroxidation, reduced catalase activity, DNA damage, and decreased bone marrow nucleated cells. These adverse effects strongly decreased after 7 days and nearly returned to the normal levels after 30 days. In an analogous study, Yang et al. discovered the time-dependent alternation of inflammatory cytokines (TNF-α and IL-1β) by BPNMs, following the process of elevation (1 day), decrease (3 day), and recovery (7 day).[51] However, the accumulation of $CD68^+$ cells (referring to macrophages and white blood cells) was observed in lungs even after 7 and 28 days, indicating the sensitivity of lungs to

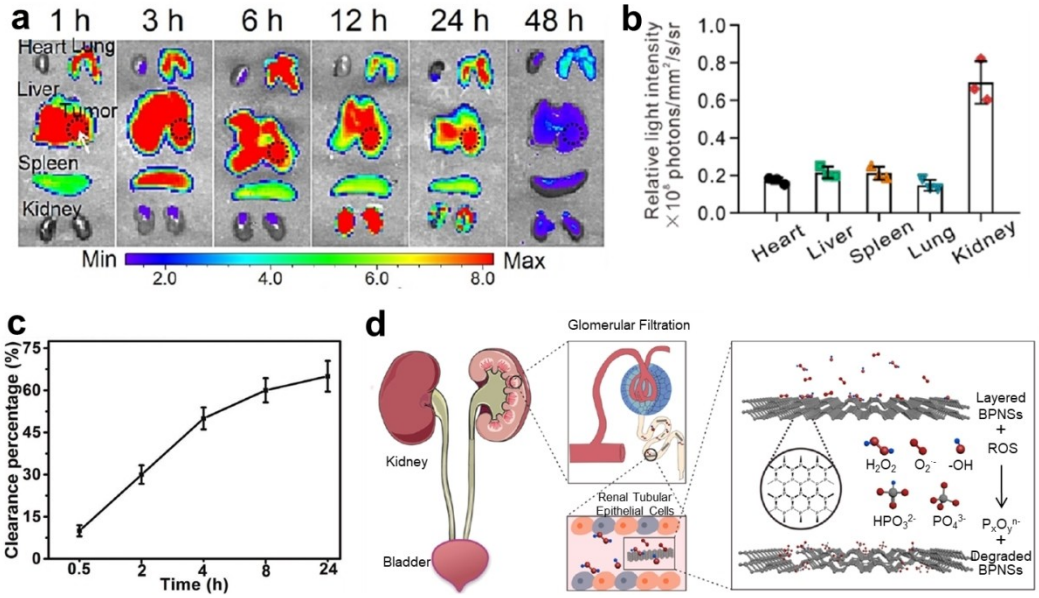

Figure 4. a) Ex vivo fluorescence images of the major organs in the orthotopic liver tumor-bearing nude mice treated with Cy5.5-labeled LBP.[49] Copyright 2020, Ivyspring International Publisher. b) The quantitative fluorescence signal of Cy5-labeled LBP in mice.[47] Copyright 2020, American Chemical Society. c) Renal clearance of BPQDs determined by the time-dependent phosphorus content in urine.[50] Copyright 2018, John Wiley & Sons. d) Schematic diagram of the renal accumulation and AKI curing by LBP through ROS scavenging.[47] Copyright 2020, American Chemical Society.

LBP exposure.[18a] In contrast to the above conclusion that LBP has reversible in vivo toxic effects, Tao et al. discovered the in vivo selective killing of cancer cells by BP–PEG without damaging other organs.[19] The tumor killing effect was attributed to the excess amount of ROS generated by BP–PEG in tumors, which caused severe irreparable DNA damage and apoptosis.[18b]

For histological evaluation, the major organs, such as the heart, liver, spleen, lungs, and kidneys, were stained with hematoxylin and eosin (H&E). Most of the current studies demonstrated that BP administration did not produce obvious damage or inflammatory lesion to the major organs.[14b,18c,d,39,47,49] Also, there were some different results. For example, BPQDs could cause kidney impairment in mice as elevated neutrophil infiltration and tubule degeneration were observed in the H&E staining images.[44] In addition, the stained images showed that BPNMs appeared in the lungs, which led to cell apoptosis as indicated by the results of terminal deoxynucleotidyl transferase mediated deoxyuridine triphosphate nick end labeling.[25]

Animal studies that evaluate the biosafety of BPNMs have not come to consistent and definitive conclusions regarding their in vivo biological effects, e.g., how the synthetic process, physicochemical properties (size, morphology, and surface modification), dosage, and way of exposure affect the in vivo behavior of BPNMs. Long-term evaluations are necessary, especially for future therapeutic applications.

4.3. Other Biological Effects

In addition to the direct toxicity to mammalian cells, BPNMs can also disturb the normal physiological functions and elicit certain biological effects. It is rational that kidneys are target organs of BPQDs since renal clearance is the main route for the excretion of ultrasmall particles.[52] However, the protein corona on BPQDs significantly increased the diameter from 5.6 to 362.5 nm due to the formation of bulky particles,[30a] leading to the accumulation of BPQDs in the kidney. Therefore, Zuo et al. explored the impairment of the kidney function by BPQDs with HK2 cells.[44]

5. Future Perspectives

Considering the tremendous progress in the research and manufacturing of BPNMs, a growing possibility of the environmental and occupational exposure raises concerns for their health implications and necessitates the evaluation of the biological effects of BPNMs. Currently, two opposite opinions regarding the toxicity of BPNMs have been proposed. In the initial stage of emergence, BPNMs were always considered to be non-toxic, in particular in the biomedicine-related studies. In contrast, several other reports declared their cytotoxicity and adverse biological impacts to be significant. Several factors may contribute to the debatable toxicity, such as unstandardized testing protocols, various cell or animal models, and discrepant physicochemical properties of BPNMs deriving from different productive processes. In consequence, no definitive conclusion about the biosafety of BPNMs has been reached until now.

Here, we provide a comprehensive overview of the biological responses at the levels of body, tissue, cell, and molecules, stemming from the intrinsic character of BPNMs and their interactions with biological systems. The dynamic process of BPNMs' dimensional changes should also be related with their biological effects. In accordance, understanding the influence of BPNMs' biodegradation process and products on their biological effects should be of great concern. The current obstacle is the lack of accurate and real-time technology for monitoring the intracellular biodegradation process of BPNMs.

The intracellular journey of BPNMs elicits a series of cellular responses, including morphological changes, plasma membrane disturbance, oxidative stress, lipid peroxidation, ER stress, MMP decrease, lysosome leakage, Ca^{2+} flux, caspase activation, cell cycle arrest, autophagy dysfunction, proinflammatory effects, DNA damage, and cell death. However, the relationship of BPNMs' properties and cell types with the biological outcomes is still poorly understood.

In addition to the in vitro evaluations, the in vivo behaviors (biodistribution, pharmacokinetics, and excretion) and toxicological effects (impact on blood biochemistry, tissue or organ pathology, and immune system) of BPNMs have also started to be acknowledged. Throughout the whole body, BPNMs accumulate in the liver and spleen due to the immune clearance. Further, bioaccumulation in tumor issues due to the EPR effect suggests BPNMs could be a potential anticancer agent. Although BPNMs' pharmacokinetics follows a typical two-compartment model, and renal clearance is their major excretion route, the intracorporal transformation process of BPNMs remains undetermined due to technological limits. Also, several controversial results regarding their in vivo toxicological effects have been reported. Notably, most of the current animal experiments were conducted by intravenous injection of BPNMs. However, the portal of entry into the body is a key factor for determining the biological environment (pH, biomolecules, and cell population) for the biotranformation of BPNMs, which may influence their fate. Hence, the exposure routes should not be overlooked during the in vivo bio-safety evaluations of BPNMs.

Although research on the in vitro and in vivo biological behavior and toxicological information is still in its infancy, the potential impact of BPNMs on human health has already been observed, which deserves a more systematic exploration, not only from the aspect of biomedicine, but also for the sake of safe material design and applications.

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Conflict of Interest

The authors declare no conflict of interest.

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