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Assessing and Mitigating the Hazard Potential of Two-Dimensional Materials

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Abstract

The family of two-dimensional (2D) materials is comprised of a continually expanding palette of unique compositions and properties with potential applications in electronics, optoelectronics, energy capture and storage, catalysis, and nanomedicine. To accelerate the implementation of 2D materials in widely disseminated technologies, human health and environmental implications need to be addressed. While extensive research has focused on assessing the toxicity and environmental fate of graphene and related carbon nanomaterials, the potential hazards of other 2D materials have only recently begun to be explored. Herein, the toxicity and environmental fate of post-carbon 2D materials, such as transition metal dichalcogenides, hexagonal boron nitride, and black phosphorus, are reviewed as a function of their preparation methods and surface functionalization. Specifically, we delineate how the hazard potential of 2D materials is directly related to structural parameters and physicochemical properties, and how experimental design is critical to the accurate elucidation of the underlying toxicological mechanisms. Finally, a multidisciplinary approach for streamlining the hazard assessment of emerging 2D materials is outlined, thereby providing a pathway for accelerating their safe use in a range of technologically relevant contexts.

Table of Contents Graphic

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Author Contributions

The manuscript was written through contributions of all authors. All authors have given approval to the final version of the manuscript.

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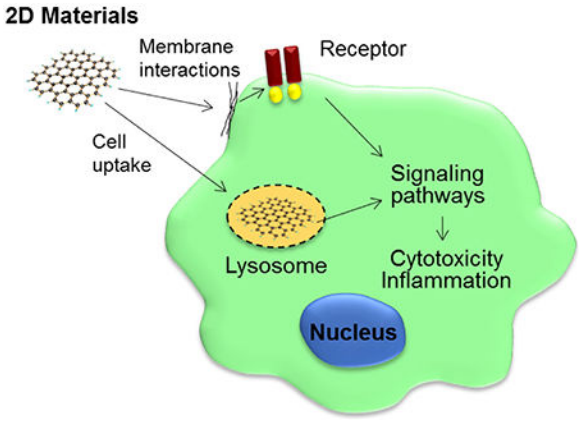

Keywords

transition metal dichalcogenide; hexagonal boron nitride; black phosphorus; toxicity; environmental fate; 2D material; risk assessment; nanotoxicology; safety

Two-dimensional (2D) materials have attracted widespread attention in the research community due to the unique physicochemical properties that emerge in the atomically thin limit, an interest first sparked by the isolation of graphene in 2004.[1–3] The 2D material family encompasses a broad range of mechanical, electrical, thermal, optical, and chemical properties that have been explored for electronic, optoelectronic, energy, and biomedical applications. However, before these promising laboratory-scale prototypes can be widely disseminated in commercialized technologies, the biological and environmental hazards of 2D materials need to be understood and mitigated. While the hazard assessment of graphene and related carbon nanomaterials have been reviewed extensively elsewhere,[4–10] here we focus on post-carbon 2D materials, such as transition metal dichalcogenides (TMDCs), hexagonal boron nitride (hBN), and black phosphorus (BP), that present a broader range of compositions, chemistries, and physicochemical properties, and thus a wider range of potential toxic responses and environmental impacts.

TMDCs are a class of 2D materials that gained significant interest in the wake of graphene due to their broadly tunable electronic band structures, which enable significant opportunities in electronic and optoelectronic applications.[11–13] Like most 2D materials, the properties of TMDCs depend on thickness, particularly in the atomically thin limit.[11,12,14–16] In the bulk, TMDCs are layered materials with a stoichiometry of MX_2, where M represents a group IV, V, or VI transition metal and X represents a chalcogen such as sulfur, selenium, or tellurium.[12] The relatively strong intralayer bonding is covalent, whereas the interlayer interactions are dictated by van der Waals forces. This weak interlayer bonding allows for relatively straightforward exfoliation of the bulk material into few-layer or monolayer form.[17] Thus far, the most widely studied TMDC is molybdenum disulfide (MoS_2). MoS_2 undergoes a transition in its electronic structure from an indirect band gap in the bulk to a direct band gap in the monolayer limit, which gives rise to qualitatively different optical properties such as photoluminescence.[18–21] The semiconducting nature of MoS_2 suggests significant potential in electronic applications, while the optical properties

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make it useful for optoelectronic as well as biomedical applications. Beyond semiconductors like MoS_2, the electronic properties of TMDCs also include metallic (*e.g.*, NdS_2) and semimetallic (*e.g.*, WTe_2) behavior.[12]

The library of TMDCs has enabled the development of a wide range of applications, specifically in the biomedical field. Due to their high surface area and chemical reactivity, these materials can be functionalized and used as drug delivery carriers.[22,23] The high surface to volume ratio also makes TMDCs highly sensitive to adsorbed molecules, enabling applications in sensing.[24–30] The intrinsic photoluminescence of some TMDCs further allows their use as biomedical imaging agents. [31–34] Other applications also make use of the optical properties of TMDCs, such as the strong absorption of light and high photothermal conversion efficiencies in a wide range of TMDCs including MoS_2, tungsten disulfide (WS_2), titanium disulfide (TiS_2), rhenium disulfide (ReS_2), and molybdenum diselenide ($MoSe_2$), enabling their use for photothermal therapies.[35–46]

Hexagonal boron nitride (hBN) is another popular layered material that has been explored extensively in recent years, especially in its 2D form.[47] Similar to graphene, the boron and nitrogen atoms are arranged in a hexagonal 2D lattice, resulting in a material with exceptionally high chemical and thermal stability. With a wide band gap of approximately 6 eV, hBN is an electrically insulating material, although it possesses relatively high thermal conductivity.[48–50] With this unique combination of properties, hBN can also be used as a component of electronic systems, concurrently acting as a thermal management material[51–53] and a dielectric.[50,54,55] In the context of biological applications, hBN has gained interest due to its lower cytotoxicity compared to its carbon analogues.[56–60] The high biocompatibility of hBN has resulted in a recent surge of studies exploring biomedical technologies based on this material, including drug delivery, fluorescent labeling in cells, and tissue engineering.[61–67]

Post-carbon elemental 2D materials are also attracting significant interest due to their chemical simplicity and unique properties. Most elemental 2D materials need to be synthesized using highly controlled bottom-up approaches because they do not occur naturally as layered materials in their bulk form.[68] Black phosphorus (BP) is a notable exception, in that the bulk form is layered and relatively stable compared to other allotropes of phosphorus.[69] As a semiconducting, anisotropic 2D material with a layer-dependent band structure, BP has been extensively studied for its use in electronics, optoelectronics, and biomedical applications.[70–77] Although BP is among the most stable forms of phosphorus, it possesses relatively high chemical reactivity compared to other 2D materials such as high environmental instability under ambient conditions and chemical degradation in the presence of oxygen and water.[78–83] While this environmental instability presents challenges for the electronic and optoelectronic applications of BP, chemical degradation pathways present potential opportunities in the biomedical field to create a biodegradable 2D nanomaterial construct. Consequently, research exploring the use of BP for biological imaging, sensing, and therapeutics has accelerated significantly in the last two years,[84–93] motivating the need for systematic exploration of the hazard potential for emerging elemental 2D materials.

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Herein, we review the studies to date that have addressed the toxicity of 2D materials, particularly emphasizing the preparation methods and resulting physicochemical properties that dictate their interactions with biological systems. In addition, due to its importance in determining exposure risk, this review summarizes key results from the literature concerning the environmental fate of 2D materials. A thorough understanding of these issues will inform ongoing efforts to realize the design of safer 2D materials.

2D Material Synthesis Methods that Impact Hazard Potential

The physicochemical properties of 2D materials are a direct result of synthesis and processing techniques. A variety of preparation methods exist for monolayer or few-layer 2D materials, which can generally be separated into either bottom-up or top-down approaches.[94] Bottom-up approaches involve the use of atomic or molecular precursors to grow the 2D material. For example, chemical vapor deposition (CVD) of MoS_2 requires solid precursors of molybdenum, usually in the form of molybdenum trioxide (MoO3), and sulfur that are vaporized by heating and subsequently deposited onto a substrate to form thin films of MoS_2.[95–97] Solution-phase synthesis methods such as hydrothermal synthesis[98] or combining precursors such as molybdic acid and thiourea at high temperature and pressure[99] can also be used to prepare TMDCs such as MoS_2. These solution-based methods produce highly polydisperse samples, both in terms of lateral dimensions and number of layers.

Top-down approaches involve the isolation of 2D materials from bulk layered crystals. The most well-known of these approaches is micromechanical cleavage, more commonly referred to as the Scotch tape method.[1,21,100,101] However, due to the low yield of micromechanical cleavage, significant research effort has been devoted to liquid phase exfoliation methods because they are more scalable and enable easier post-processing in manufacturing settings.[102–105] Liquid phase exfoliation methods often use small ionic species, such as lithium, to intercalated between the layers in the bulk material, ultimately facilitating exfoliation.[20,106,107] In particular, the addition of water reacts with intercalated lithium ions, liberating hydrogen gas that weakens the interlayer van der Waals forces, resulting in exfoliation into 2D sheets. For the case of MoS_2, while this process produces monolayer sheets in solution with high yield, the aggressive chemical conditions drive MoS_2 to undergo a phase transformation from trigonal prismatic (2H-MoS_2) to octahedral (1TMoS_2). Since 1T-MoS_2 is a metastable metallic material, it can be partially converted back to 2HMoS_2 through thermal annealing or laser-assisted phase reversion,[14,20,108] although the final structure and properties differ from pristine exfoliated 2H-MoS_2.

Another top-down liquid phase method that has been studied extensively utilizes ultrasonication or shear mixing to exfoliate 2D materials.[104,109–116] To achieve optimal exfoliation yields, the liquid needs to contain a surface energy that is well-matched to the targeted 2D material or additional additives, such as surfactants or stabilizing polymers, need to be added to the solution. While this method is amenable to scale-up and generally yields chemically pristine 2D materials, the resulting flakes are generally small in lateral dimensions and possesses high polydispersity, with the majority of the flakes being few-layer instead of monolayer.[104] Despite these drawbacks, top-down approaches are being most commonly pursued in early-stage industrial efforts due to the low costs involved.[94]

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Additionally, post-processing methods such as density gradient ultracentrifugation can be used to sort and isolate monodisperse populations of the 2D materials following liquid phase exfoliation.[54,103,112] In addition, some top-down approaches can be carried out in aqueous media, which eliminates the need for environmentally unfriendly organic solvents.[109,117–122] Consequently, aqueous and biocompatible synthesis and processing have become increasingly important for the application of 2D materials in biomedical contexts.

2D Material Hazard Assessment

The safety of 2D materials is an emerging topic that assesses the potential adverse interactions of these materials at the nano-bio interface. In this section, we highlight several of these initial studies and discuss how their conclusions can inform the design and implementation of improved toxicity screening methods in the future. Specifically, we have categorized the most important characteristics for the hazard assessment of 2D materials including composition, lateral size and thickness, surface functionalization, and crystal structure (Figure 1).

Composition

Chemical composition is one of the most important considerations for determining the biological interactions and fate of 2D materials *in vivo*. The surface chemistry and the dissolution of the material will be determined by its chemical composition, which will in turn affect the cellular interactions, uptake, and biodistribution.[123,124] For 2D materials, several studies have assessed the cytotoxicity of TMDCs according to composition.[125–128] For example, Teo *et al.* investigated the toxicity of chemically exfoliated MoS_2, WS_2, and WSe_2 in human lung carcinoma epithelial cells (A549).[125] While low toxicity of MoS_2 and WS_2 was observed, a dose-dependent toxicity of WSe_2 was detected, indicating that the presence of selenium plays an important role in the toxicity of TMDCs. However, it is unclear whether this toxicity can be attributed to the nano-bio interface interactions caused by the presence of selenium at the surface of the TMDC or the toxicity related to dissolved selenium. A similar study by the same group was recently published on the cytotoxicity of vanadium ditelluride (VTe_2), niobium ditelluride ($NbTe_2$), and tantalum ditelluride ($TaTe_2$) in the same cell line (A549), allowing for direct comparison to the cytotoxicity of MoS_2, WS_2, and WSe_2 from the previous study.[127] High cytotoxicity was observed after exposure to VTe_2, whereas $NbTe_2$ and $TaTe_2$ showed reduced cytotoxicity. Overall, the ditellurides exhibited higher cytotoxicity than the disulfide materials, while WSe_2 exhibited similar cytotoxicity to the ditellurides. To further investigate the role of the chalcogen, the same group ran an identical study comparing the cytotoxicity of vanadium dichalcogenides: VS_2, VSe_2, and VTe_2.[126] VS_2 showed the least cytotoxic effect, whereas VSe_2 and VTe_2 showed similarly higher cytotoxicity, further suggesting that the chalcogen significantly affects the toxicological response.

From these initial studies comparing the cytotoxicity of varying TMDCs, it is clear that the identity of the chalcogen atom plays an important role in the overall cytotoxicity. This observation is most likely due to the differences in chemical reactivity of different chalcogenides, where the more reactive TMDCs result in the release of the chalcogen atoms,

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resulting in higher toxicity. These cytotoxicity results as well as those discussed in the following sections are summarized in Table 1. Due to their relatively infrequent study thus far in the field of toxicology, cytotoxicity has been the most widely used metric for gauging the biocompatibility of emerging 2D materials. This empirically reported cytotoxicity provides some insight, but a deeper mechanistic understanding is required to fully assess the hazard potential.

Exfoliation

As layered materials are exfoliated, the fundamental properties of the material change.[17,75] Similarly, biological interactions with layered materials vary as a function of exfoliation state. For example, Chng *et al.* investigated the effects of exfoliation on the cytotoxicity of MoS_2.[129] Using three different lithium sources—methyllithium, n-butyllithium, and tert-butyllithium—MoS_2 was chemically exfoliated, and the exfoliation yield was deduced using Raman spectroscopy. Higher exfoliation yield was achieved with tert-butyllithium and n-butyllithium, which corresponded to higher cytotoxicity. Based on this observation, the study concluded that the increase in surface area and active edge sites leads to higher toxicity in more highly exfoliated MoS_2. However, limited material characterization left open the possibility that other physical properties of these MoS_2 samples led to the differential cytotoxicity. A more thorough investigation of the toxicity of MoS_2 by Wang *et al.* compared two forms of exfoliated MoS_2 to an aggregated form of MoS_2 (Figure 2).[130] The exfoliated forms of MoS_2 were prepared by chemical exfoliation with nbutyllithium and ultrasonication-assisted liquid phase exfoliation with the aid of the surfactant Pluronic F87. The toxicity of these materials was examined *in vitro* and in the mouse lung *in vivo*. Aggregated MoS_2 induced significantly increased production of pro-inflammatory cytokines, indicating a pro-inflammatory response *in vitro*, whereas the lithiated and Pluronic-dispersed MoS_2 showed reduced effects, which was attributed to the bioavailability and cellular uptake of these MoS_2 materials. *In vivo* studies revealed focal areas of inflammation in the lung after acute exposure to aggregated MoS_2 but no significant inflammatory response was observed for any of the MoS_2 materials following sub-chronic exposure. Overall, effective exfoliation reduced the toxic response of MoS_2 compared to its aggregated state, suggesting that stable dispersions of exfoliated MoS_2 lead to higher biocompatibility. Negligible toxicity of exfoliated MoS_2 was also observed by Shah *et al.* in rat pheochromocytoma cells and adrenal medulla endothelial cells.[131] In this work, the MoS_2 materials were also chemically exfoliated, further supporting this synthetic pathway to biocompatible MoS_2.

The cytotoxicity of MoS_2 by different exfoliation and preparation methods has also been explored in a series of recent studies. For instance, a comparative study of the cytotoxicity and genotoxicity in epithelial kidney cells was performed for mechanically exfoliated MoS_2 *versus* MoS_2 that was grown by chemical vapor deposition (CVD).[132] Similar to exfoliated MoS_2 prepared by solution processing methods, both mechanically exfoliated MoS_2 and CVD-grown MoS_2 showed minimal impact on cell morphology in addition to non-significant reactive oxygen species (ROS) generation and negligible effects on cell viability. Furthermore, exfoliated MoS_2 did not induce measurable genetic changes. Similar results for CVD-grown MoS_2 were observed in both mouse embryo fibroblasts (NIH-3T3) and human

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adipose-derived mesenchymal stem cells (HAMSCs).[133] In particular, no significant loss of cell viability was observed for MoS_2 concentrations up to 50 μg mL^{-1} and 300 μg mL^{-1} in NIH-3T3 and HAMSCs, respectively. While most studies have found a high degree of biocompatibility for MoS_2, a recent study by Liu *et al.* indicated that exposure to MoS_2 induces a loss in cell viability in HepG2 cells at concentrations as low as 30 μg mL^{-1}.[134] In this case, MoS_2 induced a significant increase in intracellular ROS at doses greater than 2 μg mL^{-1} in addition to membrane damage at doses greater than 4 μg mL^{-1}. The study also found similar results for hBN. Thus, the observed toxicity was attributed to membrane damage caused by the 2D sheet-like structure of the materials. However, the study lacks proper material characterization to rule out other mechanistic possibilities. Overall, the cytotoxicity results for exfoliated MoS_2 have been highly contradictory, resulting from inconsistent material preparation methods, inadequate material characterization, and varying cytotoxicity assays in a wide range of different biological systems as illustrated in Table 1.

For the case of black phosphorus, a study by Latiff *et al.* investigated the cytotoxicity of bulk BP crystals in human lung carcinoma epithelial cells (A549), which showed a dose-dependent toxicity and reduced cell viability at BP concentrations of 50 μg mL^{-1}.[135] In contrast, a later study by Mu *et al.* investigated the toxicity of BP quantum dots (BPQDs) both *in vitro* in HeLa cells and *in vivo* in mice.[136] At high BPQD concentrations of 200 μg mL^{-1}, significant cytotoxicity and apoptotic effects were observed, which were attributed to oxidative stress. Weight loss in the mice after exposure indicated acute toxicity, but the mice eventually recovered, indicating that BPQDs did not induce long-term inflammatory responses or injury. Thus, the exfoliation of BP may play a role in the toxicological response, but additional studies are needed to gain mechanistic insight.

Lateral Size and Thickness

The biological response to a 2D geometry is unique, and the physical interactions of 2D materials with cells are expected to vary based on their aspect ratio and mechanical properties.[137] Thus, the characterization of lateral size and thickness of 2D materials is critical in order to quantify the aspect ratio and its effects on the biological response. Towards this end, Moore *et al.* investigated the role of lateral size of MoS_2 flakes in the cytotoxicity, cellular uptake, and inflammatory response.[138] Using liquid cascade centrifugation, MoS_2 flakes dispersed with sodium cholate of varying lateral sizes were isolated. The response of three separate MoS_2 samples with mean lateral sizes of 50 nm, 117 nm, and 177 nm were studied in A549, AGS, and THP-1 cell lines, chosen specifically to assess risk *via* different exposure routes. At 1 μg mL^{-1} dose, all samples showed minimal cytotoxicity and invariant cell morphology, although an inflammatory response was observed. Assays revealed a size-dependent increase in cytokine production (IL-6, IL-10, IL-13, TNF-α, and IL-1β) in THP-1 cells, with the smallest MoS_2 flake sizes inducing the largest increases in cytokine production. However, the MoS_2 samples were prepared under conditions that suffered from endotoxin contamination, making it difficult to determine if the size-dependent toxicity resulted from the material itself or the increased surface area allowing for increased endotoxin levels. In this case, it is most likely the increase in effective surface area of the 2D material that results in higher endotoxin adsorption and subsequently higher production of inflammatory cytokines. Thus, as the material becomes thinner and

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smaller, the available surface area increases, resulting in higher cytotoxicity. To elucidate the fundamental effects of 2D material lateral size on the biological response, the material processing needs to be performed in a manner where other variables, such as endotoxin contamination, are held constant.

More recently, BP has also been used as a model 2D material to probe the effects of lateral size on the cytotoxic response.[139,140] Fu *et al.* prepared BP nanosheets by liquid phase exfoliation and then isolated three different lateral size distributions through varying centrifugation speeds.[139] In human hepatocyte cells (LO2), a small dose-dependent loss of viability was observed, but even at concentrations of 50 μg mL^{-1}, cell viability still exceeded 80%. In a more mechanistic study, three different sizes of BP were prepared by aqueous liquid phase exfoliation and fractional centrifugation (Figure 3).[140] The three samples varied in both lateral size and thickness with BP-1 containing flakes of the largest lateral size and thickness and BP-3 containing flakes of the smallest lateral size and thickness. The cytotoxicity was tested in three cell lines: mouse fibroblasts (NIH-3T3), human colonic epithelial cells, and human embryonic kidney cells (293T). The cytotoxic response was found to be dependent on concentration, size, and cell type, with the 293T cells being the most sensitive and the epithelial cells being the least sensitive. Unlike MoS_2, BP flakes with the largest lateral size and thickness showed the highest levels of cytotoxicity. However, it should be noted that the smallest BP sample in this study was similar in size to the largest MoS_2 sample in the previous study, while the largest BP sample is an order of magnitude larger. Thus, it is likely that at these different length scales, different mechanisms of toxicity will dominate. Additional assays showed ROS generation, but the response was not size-dependent. Further investigation into the interaction of BP with model cell membranes using a quartz crystal microbalance with dissipation monitoring revealed that the largest BP samples disrupted the integrity of the cell membrane, implicating this pathway as the most likely size-dependent toxicity mechanism. Consequently, it appears that the BP flakes induce physical damage to the cell membrane, an interaction that is more extreme with larger sized flakes, resulting in higher cytotoxicity of the larger BP flakes compared to smaller flakes. However, the study did not account for the environmental instability of exfoliated BP, and no stability or long-term (> 24 hours) cytotoxicity data were presented.

Overall, it is clear that the lateral size and thickness of 2D materials affect the overall biological response. The aspect ratio and resulting mechanical properties will also influence cellular interactions and thus biological outcomes, but additionally the lateral size and thickness will determine their biodistribution in tissues. Furthermore, at varying size scales of the 2D materials, different mechanisms of toxicity will play a more dominant role. Thus far, there are no comprehensive studies focusing on the size effects of the biodistribution of 2D materials, and therefore further *in vivo* investigations of this nature are needed in the future.

Structural Forms

Most toxicity studies to date have focused on dispersions of 2D materials because many biological applications, such as drug delivery and imaging agents, call for such a format.[72,141–145] However, 2D materials are also being explored in a myriad of other applications in

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which the qualitatively different physical form factors are needed including thin films, three-dimensional constructs, and composites.[146–151] In these cases, biological interactions will fundamentally differ from those of a well-dispersed 2D material in solution. Consequently, some recent studies have begun to specifically probe the toxicity of 2D materials in solid-state formats such as thin films, foams, and composites. One such study investigated the toxicity of MoS_2, WS_2, and hBN coated glass slides, seeding adipose-derived human mesenchymal stem cells (hMSCs) onto these thin films and then studying the cellular response (Figure 4).[152] None of the 2D materials resulted in a significant loss in cell viability up to concentrations of 5 μg mL^{-1}, but rather the cells showed higher cellular adhesion and enhanced proliferation compared to an uncoated glass slide. Furthermore, the presence of the 2D materials contributed to enhanced adipogenesis in the hMSCs.

The biocompatibility of thin films generated from printed 2D material inks was explored by McManus *et al.*[153] In this study, a range of 2D materials, including graphene, MoS_2, WS_2, and hBN, were exfoliated in water in the presence of pyrene sulfonic acid derivatives. To achieve printable viscosities of the exfoliated dispersions, Triton X-100 and propylene glycol were added to the solutions following exfoliation. Cytotoxicity of the 2D inks was then determined using an LDH assay in human lung epithelial cells (A549) and human keratinocyte cells (HaCaT). No loss of cell viability was observed for any of the materials up to concentrations of 100 μg mL^{-1}. Furthermore, cells seeded onto the 2D material thin films showed strong interactions with the substrates, similar to what was observed in the previous study by Suhito *et al.*[152] This favorable cell attachment and high biocompatibility have prompted further studies on the biological interactions of nanostructured films of 2D materials with different cell types, such as differentiation of stem cells.[154] Similar biocompatibility has also been reported for a number of polymer composites containing 2D materials.[64,155–157] Like 2D material thin films, these composites have shown high cell adhesion, low cytotoxicity, and accelerated cell proliferation and growth. While studies exploring these varying structural forms of 2D materials have been limited thus far, it is important to note that by changing the form of the nanomaterial from a dispersion to a thin film or 3D dimensional network, the interface with the biological system fundamentally changes, resulting in different biological outcomes.

Surface Functionalization

Extensive studies on graphene and related carbon nanomaterials have shown that surface functionalization plays a major role in the toxicological response.[158–161] Analogous studies on the surface functionalization of post-carbon 2D materials have begun to be undertaken with an eye toward achieving specific biological outcomes and minimizing toxic response.[162–165] For example, Qu *et al.* showed that functionalization of BPQDs with a titanium sulfonate ligand (TiL_4) resulted in lower cytotoxicity and reduced inflammatory response (Figure 5).[164] In this study, the cytotoxicity of bare BPQDs was compared to that of TiL_4-modified BPQDs in two different macrophage cell lines. Both BPQD samples showed minimal effect on the viability of RAW264.7 macrophage cells. On the other hand, in J774A.1 macrophage cells, bare BPQDs showed a significant loss of viability, while this effect was reduced in the case of TiL_4-modified BPQDs. The proinflammatory response of the BPQDs was also tested by measuring the cytokine (TNF-α) production in RAW264.7

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cells. TiL_4-modified BPQDs showed reduced inflammatory response compared to bare BPQDs, which correlated with the cellular uptake of the materials, namely that the intracellular level of bare BPQDs was significantly higher than that of TiL_4-modified BPQDs. Further *in vivo* studies revealed that bare BPQDs showed an acute inflammatory response at 24 hour post-exposure, as evidenced by the significant increase of neutrophils in the blood and concentration of cytokines (TNF-α, eotaxin, IL-6, MCP-1, KC, MIP-1, MIG, VEGF) in the mice serum, whereas TiL_4-modified BPQDs did not show this response. Although neither material demonstrated chronic inflammation, the differences in acute toxicity confirmed that surface functionalization of BP can be used to minimize toxicity by altering cellular uptake. Surface functionalization techniques can also be used to minimize toxicity by changing the degradation behavior of the 2D material. For example, chemically exfoliated MoS_2 functionalized with 2-iodoacetamide showed a higher resistance to enzymatic degradation than non-functionalized chemically exfoliated MoS_2.[165] Upon exposure to the degradation products of both MoS_2 samples, both HeLa and RAW264.7 cells showed a higher loss of viability upon exposure to the degradation products of non-functionalized MoS_2, thus providing evidence that the degradation products at elevated concentrations can induce a toxic response. More generally, the surface functionalization of 2D materials will dictate the dispersion stability, the surface reactivity, and the degradation behavior, all of which will contribute to the overall toxicity of the material.

Environmental Stability and Chemical Dissolution

Although most TMDCs are considered highly stable, some studies have shown that these materials undergo environmental transformations. In particular, the dissolution of TMDCs has been shown to vary as a function of composition due to differences in the strength and reactivity of the bonds formed between metal and chalcogen atoms. By varying the metal atom but otherwise maintaining analogous TMDC structure and processing, Hao *et al.* investigated the *in vitro* cytotoxicity and *in vivo* biodistribution and toxicity of MoS_2, WS_2, and TiS_2 nanosheets (Figure 6).[166] The nanosheets were prepared using a high-temperature solution-phase synthesis method in which bulk TMDCs were ultrasonicated in *N*-methyl-2-pyrrolidone (NMP) and then redispersed in water with the aid of lipoic acid-conjugated polyethylene glycol (PEG). An MTT viability protocol revealed no cytotoxicity up to 200 μg mL^{-1}, and further assays showed no damage to the cell membrane and no increase in ROS for this set of materials. *In vivo* biodistribution after intravenous injection into mice showed accumulation of the TMDCs mainly in the liver and spleen after 1 day, indicating that the TMDCs were most likely taken up by Kupffer cells and spleen macrophages. Despite these apparent similarities among the TMDC samples, other responses varied as a function of the metal atom such as MoS_2 showing the fastest metabolic rate and excretion from the mice, with significant amounts of Mo detected in the urine and feces. *In vitro* degradation of the TMDCs was further used to characterize biodistribution and metabolism. When stored in PBS, WS_2 showed the highest stability, due to the strong W-S covalent bond, while MoS_2 degraded into soluble Mo in the form of MoO_4^{2-}. TiS_2 also oxidized, which resulted in an insoluble precipitate of TiO_2. These differences in chemical reactivity also explain the *in vivo* excretion behavior where MoS_2 is most easily cleared from the system as it degrades to soluble MoO_4^{2-}, whereas WS_2 and TiO_2 persist and accumulate in organs. In a separate study, vanadium disulfide (VS_2) nanosheets showed a similar excretion profile to MoS_2,

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accumulating mainly in the liver and spleen of mice, but then degrading into a soluble vanadium oxide species that was easily cleared from the body and detected in the urine and feces.[167]

A study by Song *et al.* found a similar dissolution and biodistribution of molybdenum oxide (MoO_x) nanosheets, but further investigated the role of pH on the dissolution characteristics.[168] In this study, MoO_x nanosheets functionalized with PEG were prepared by a one-pot hydrothermal method and investigated for their capabilities as a photothermal therapeutic agent. The resulting MoO_x nanosheets, while stable in acidic pH, degrade quickly at physiological pH. This pH-dependent degradation was exploited to efficiently accumulate the MoO_x nanosheets in relatively acidic tumor cells, whereas MoO_x nanosheets elsewhere in unaffected cells were excreted quickly and efficiently to avoid long-term retention. Similar to studies on MoS_2, the MoO_x nanosheets accumulated mainly in the liver and spleen but were eventually cleared due to dissolution into soluble molybdate anions. Thus, in the case of environmentally unstable 2D materials, the degradation products need to be identified and their behavior and fate in the biological system need to be well understood in order to accurately assess the hazard of these materials and design schemes to mitigate any potential risks.

Furthermore, the risk associated with 2D materials will be highly dependent on their transport and transformation in the environment. For example, Lanphere *et al.* investigated the transport of two different MoS_2 dispersions in aquatic environments, in which chemically exfoliated MoS_2 was compared to a sample of MoS_2 exfoliated by ultrasonication in the presence of Pluronic F87 as a surfactant.[169] While the Pluronic-dispersed MoS_2 demonstrated high dispersion stability in aqueous solution, the material was more likely to bind irreversibly to the quartz porous media than the chemically exfoliated MoS_2, which was highly mobile in the sand columns. In a more recent study, Zou *et al.* explored the environmental transformation of MoS_2 after exposure to humic acid in an aqueous environment.[170] The chemical dissolution of MoS_2 increased significantly upon exposure to humic acid, and the resulting humic acid-MoS_2 complex showed higher peroxidase-like catalytic activity. In order to better assess their ecological and biological risks, further study is required to understand the environmental transformations of 2D materials, which will in turn affect their biological interactions and outcomes.

Crystal Structure

In addition to the composition, the crystal structure of the material can also play a role in dissolution and biological fate. For example, MoS_2 has two common polytypes: trigonal prismatic (2H) and octahedral (1T). Naturally occurring bulk MoS_2 has a 2H structure, which is maintained following ultrasonication-based exfoliation in solution.[120] However, lithiation-based chemical exfoliation of MoS_2 results in a phase change of the material to 1T.[20] The chemical dissolution pathways of these two polytypes of MoS_2 in biological and environmental media was investigated by Wang *et al.* (Figure 7).[171] 1T-MoS_2 (or ce-MoS_2) was prepared by chemical exfoliation using n-butyllithium, whereas 2H-MoS_2 (or ue-MoS_2) was prepared using ultrasonication in an aqueous solution containing sodium cholate. Rapid oxidation and degradation of the ce-MoS_2 was observed, while the ue-MoS_2 showed a

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significantly lower rate of degradation. This preferential degradation of ce-MoS_2 indicates that ce-MoS_2 samples will contain a mixture of soluble ions (*e.g.*, MoO_4^{2-}) and nanosheets during exposure and cellular uptake. This could impact the biological interactions and final biodistribution of the material. In the same study, chemically exfoliated $MoSe_2$ was also investigated and showed a similar oxidative dissolution to ce-MoS_2. However, unlike ce-MoS_2, the chalcogen product in this case will be insoluble, having different implications for its biological interactions and fate. Additionally, the bulk form of MoS_2 did not show significant dissolution, indicating that the dissolution of MoS_2 is accelerated as its thickness approaches the 2D limit.

The environmental stability of 2D materials has clear implications for toxicity and fate, and thus needs to be considered when assessing hazard potential. Additionally, a greater understanding of the material properties and environmental factors that influence stability and dissolution of 2D materials provides opportunities for engineering biomedical constructs with desirable degradation properties. This concept of designing biodegradable 2D materials for biomedical applications has grown in recent years due to the ambient instability of black phosphorus in the presence of water and oxygen.[85,90,172] While many BP constructs have been proposed for biological imaging, drug delivery, sensing, and theranostics, limited research has been devoted to the understanding the degradation of BP and the resulting byproducts, which will be imperative to safely utilizing this 2D material in biomedical contexts. With the emergence of other environmentally unstable 2D materials such as indium selenide (InSe), the field of biodegradable 2D materials is likely to expand in the future.[173]

Future Outlook

The growing interest in the use of 2D materials in electronics, optoelectronics, energy capture and storage, and biomedical applications necessitates the need to understand their health and environmental implications. The interactions of nanomaterials with a biological system are dictated by the physicochemical properties of the material.[174–179] In this review, we presented several important factors that play a role at the nano-bio interface including composition, exfoliation method, flake size and thickness, surface functionalization, crystal structure, and chemical dissolution. More comprehensive material characterization is necessary to integrate ongoing toxicity studies of 2D materials into a meaningful hazard assessment. Similarly, since the synthesis and processing protocols significantly affect the resulting structure and properties of 2D material, these details should always be included in toxicological reports. Finally, a detailed understanding of how 2D material structure and properties and behavior change in biological media is critical for determining hazard potential in biomedical contexts. Specifically, the dispersion stability, particle size, and dissolution rate can vary significantly in biologically relevant media due to changes in pH and the presence of salts and proteins, which have the potential to fundamentally alter how 2D materials interact with or are taken up by cells.[180]

In addition to thorough material characterization, thoughtful design of toxicity studies is required for 2D materials. When investigating *in vitro* cytotoxicity of these materials, the cell line selection will play a large role in the measured toxicological response, especially since certain cell lines have already shown different sensitivities to 2D material exposure.

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[140,164] Thus, it is important to resist the temptation to generalize cytotoxicity results for different cell lines based on any single study. To date, cytotoxicity of emerging 2D materials has focused mainly on biomedical exposure due to the obvious implications for biomedical technologies.[137,181,182] However, because 2D materials are being explored for a wide range of applications and industries, occupational and environmental exposure also merit investigation. Understanding the effects of all potential exposure routes will provide the most comprehensive picture of the hazard potential associated with 2D materials.

The lack of thorough material characterization, consistent design of *in vitro* assays, and mechanistic understanding of the cytotoxic response has led to contradictory reports of the biocompatibility of many 2D materials. Additionally, the cytotoxicity of the 2D materials in most cases is reported as a simple quantification of cell viability, without details of the characterization of cell growth or cell morphology that would enable better comparison across studies. To provide a more comprehensive understanding of the toxicity mechanisms of 2D materials, we thus recommend an interdisciplinary approach that relates the physicochemical properties of the materials to specific biological endpoints (Figure 8). Nanomaterial libraries should also be used to systematically vary the physicochemical properties of the 2D material of interest. Ideally, thoroughly characterized and monodisperse populations of 2D materials should be used to isolate the effects of specific characteristics (*e.g.*, lateral size, thickness, surface functionalization, and electronic structure) on biological outcomes. Using *in vitro* high throughput screening (HTS) assays, cellular interactions can be elucidated by quantifying the biodistribution, inflammation, fibrosis, and metabolic functions as a result of exposure to 2D materials. These results can then be validated by limited, carefully designed *in vivo* assays. In addition to the acute exposure testing, the long-term biodistribution and effects need to be investigated. From the initial toxicity studies of TMDCs, it is clear that some of these materials persist in organs and tissues, and thus bioaccumulation of 2D materials may present long-term repercussions.[166]

The field of 2D material research is still accelerating, with continual discovery of additional 2D materials and phenomena associated with their unique structures and compositions.[173,183,184] With a constantly expanding library of 2D materials, the ability to predict toxicological outcomes is of critical importance. Recent 2D materials of interest, such as black phosphorus, indium selenide, and germanium sulfide, demonstrate high environmental instability.[185–187] In these cases, a firm understanding of the dissolution behavior in addition to the temporal evolution of material properties is paramount for both their biomedical application as well as understanding the health and environmental risks of these materials. While some studies have begun to probe the toxicological effects of structural parameters such as lateral size, thickness, and surface functionalization, the effects of the electronic structure of 2D materials remain largely unexplored. In the case of MoS_2, it has been observed that the crystal structure and resulting change in electronic properties results in a change in the dissolution behavior of the material,[171] thus motivating further studies along these lines.[188] Overall, a comprehensive assessment of the hazard potential of 2D materials requires an interdisciplinary approach to elucidate the relationship between the physicochemical properties of the materials and the resulting biological outcomes. In this manner, predictive toxicological models can be developed that will ultimately enable the

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development of mitigation schemes that will accelerate the safe use of 2D materials in widely used technological applications.

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VOCABULARY

Two-dimensional material

Planar material that is generally layered in structure with relatively weak interlayer bonding that allows for facile exfoliation down to the monolayer limit

Liquid phase exfoliation

Solution-based process in which energy is added to the system through sonication or shear mixing to exfoliate two-dimensional materials

Cytotoxicity

Injury or death of a cell

Cytokine

Small proteins or biomolecules secreted by the cell in the immune response

Reactive oxygen species

Oxygen-containing molecules or materials that are reactive in nature and can result in cellular damage at high levels

Biodistribution

Movement and fate of materials within a biological system

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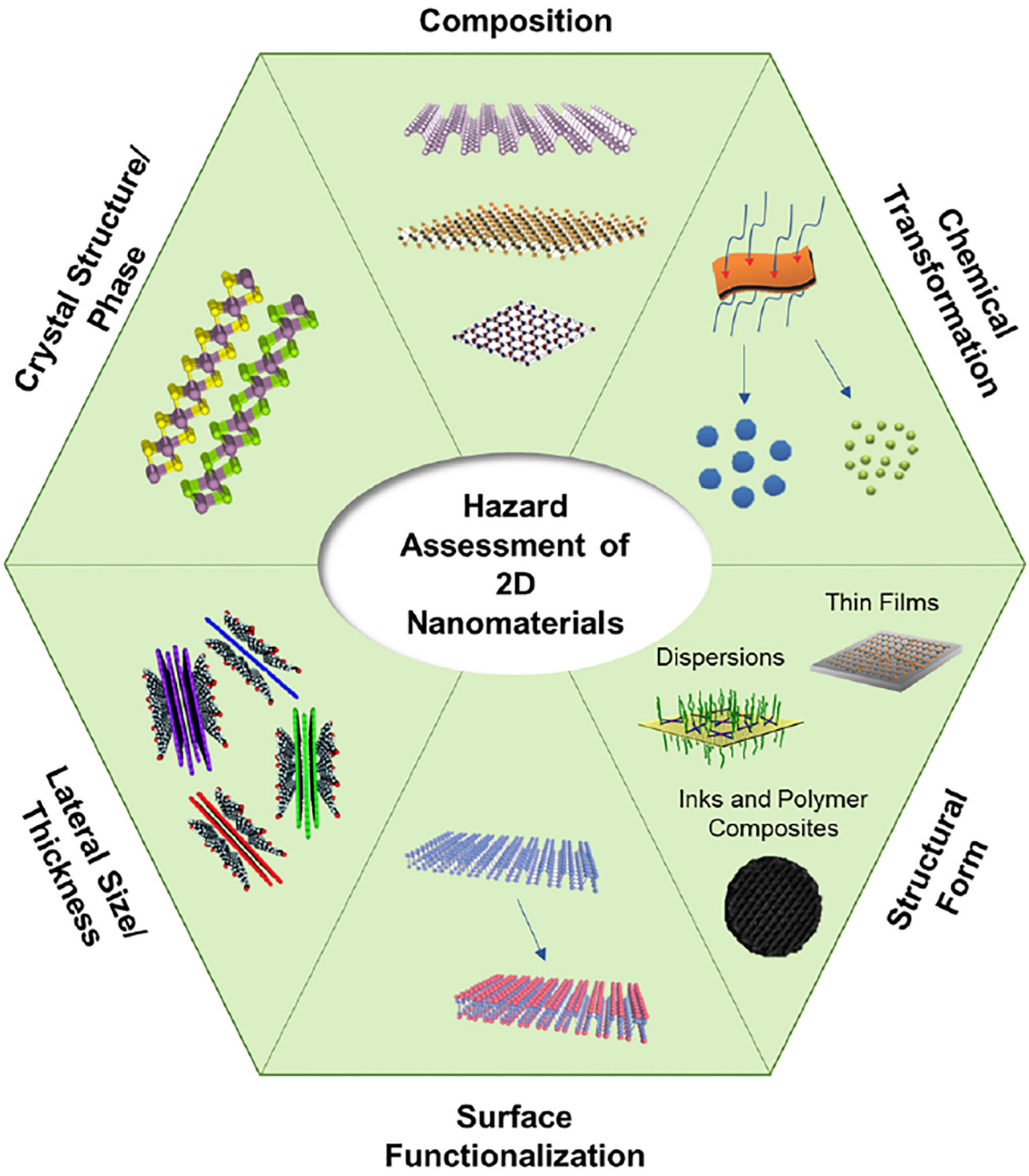

Figure 1.

Physicochemical properties and related factors for 2D materials that affect their toxicity. Complete characterization of these properties is needed to accurately assess the hazard potential for each 2D material. Figure adapted from references[103,120,130,152,156,164,166,189,190]

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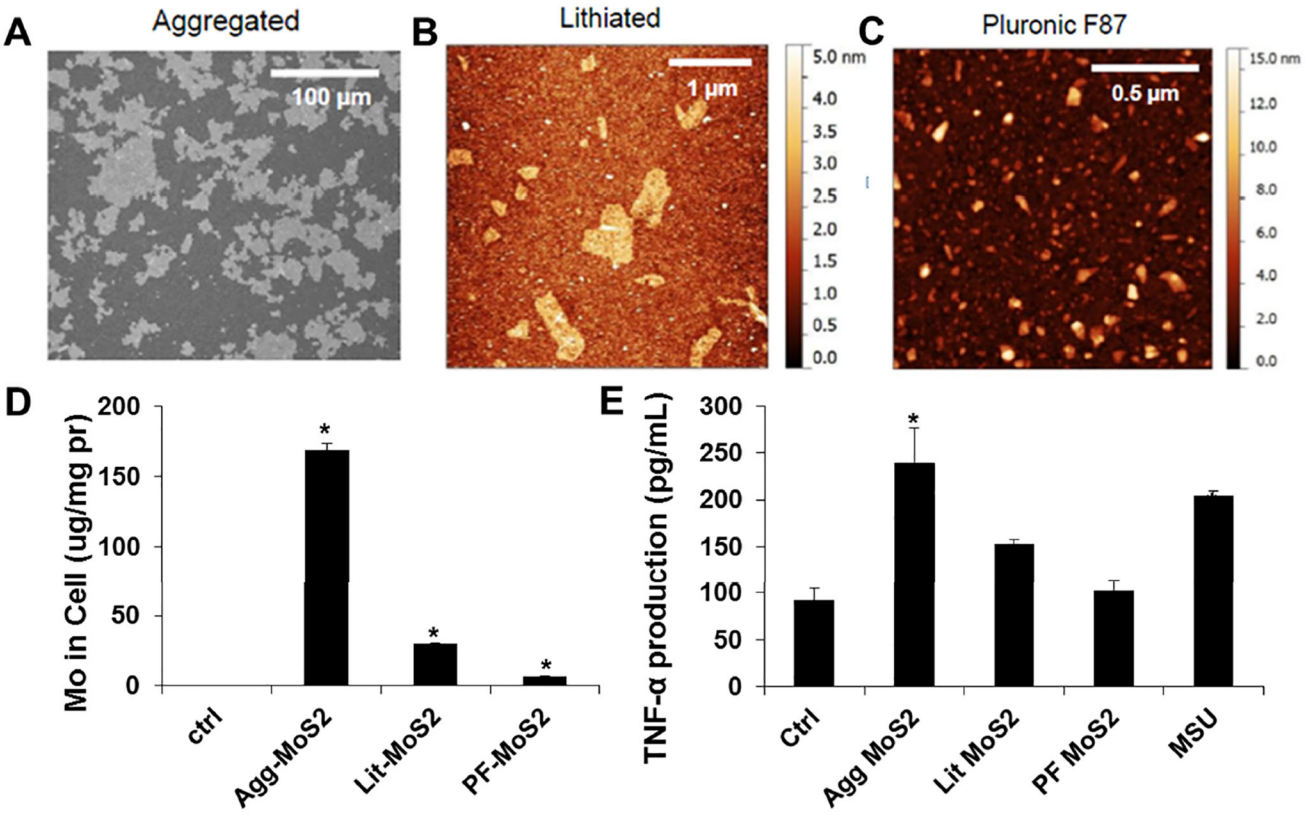

Figure 2.

Effects of aqueous exfoliation methods on the pulmonary hazard potential of molybdenum disulfide (MoS_2). (A) Scanning electron micrograph of aggregated MoS_2. (B) Atomic force microscopy (AFM) image of chemically exfoliated MoS_2 using a lithiation process. (C) AFM image of MoS_2 exfoliated by ultrasonication in the presence of Pluronic F87. The representative AFM images show the differences in lateral size and thickness of the MoS_2 materials as a function of exfoliation method. (D) Cellular content of molybdenum (Mo) and (E) TNF-α production in THP-1 cells 24 hours after exposure to 50 μg mL^{-1} of the MoS_2 samples. The cellular association and uptake correlates well with the proinflammatory effects observed for the MoS_2 materials, indicating that bioavailability plays a key role in the production of cytokines and chemokines. Figure adapted from reference[130]

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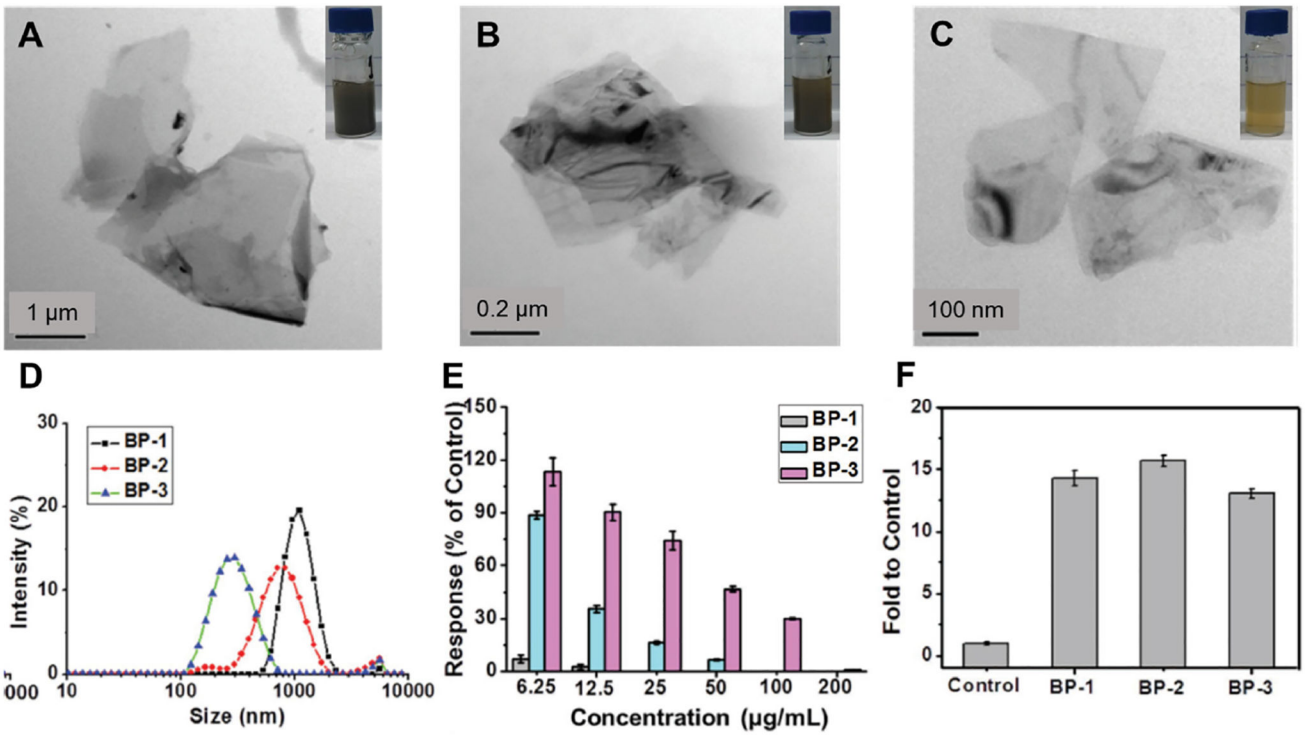

Figure 3.

Effects of lateral size and thickness on the hazard potential of exfoliated black phosphorus (BP). Representative transmission electron micrographs of flakes from three different sized BP dispersions, namely, BP-1 (A), BP-2 (B) and BP-3 (C), and the corresponding photographs of the dispersions (insets). (D) Size measurements of the three BP dispersions in 10% FBS supplemented cell culture medium as determined by dynamic light scattering. (E) Cell viability of NIH-3T3 cells after 24 hours exposure to the BP samples. (F) Intracellular detection of reactive oxygen species in NIH-3T3 cells after exposure to 10 μg mL^{-1} of the BP samples for 4 hours. Figure adapted from reference[140]

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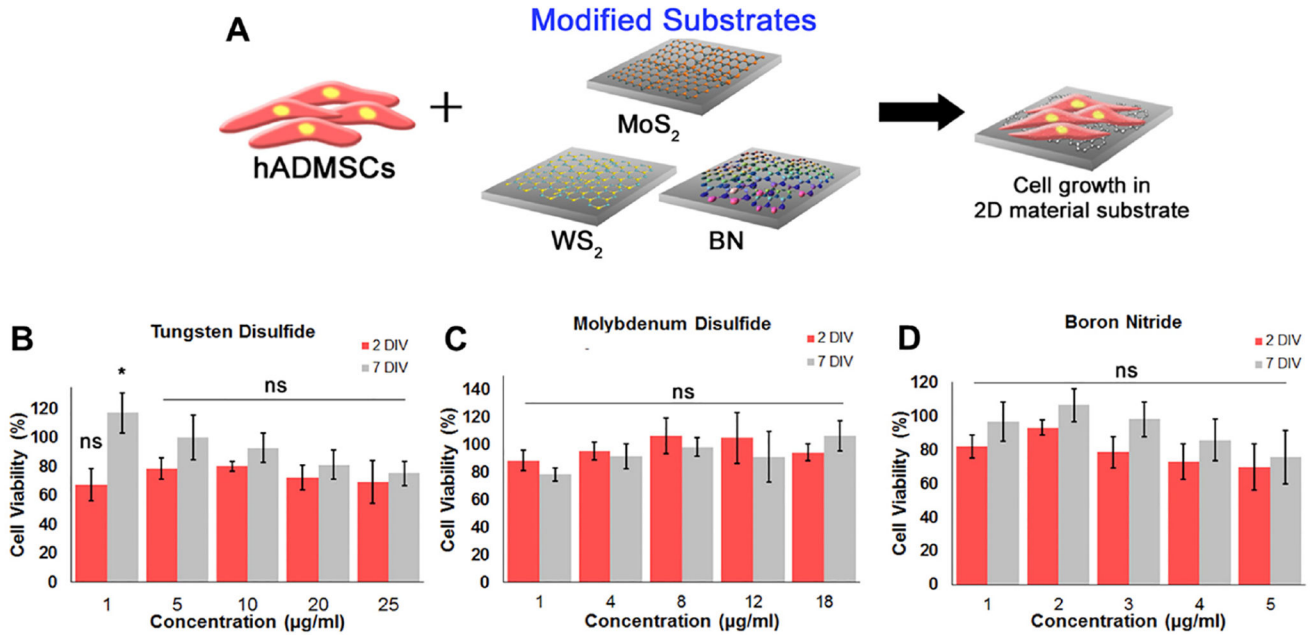

Figure 4.

Cytotoxicity of 2D material modified glass substrates. (A) Schematic of the experimental design. Human adiopose-derived mesenchymal stem cells (hADMSCs) were seeded onto glass substrates that were modified with a thin film of various 2D materials including molybdenum disulfide (MoS_2), tungsten disulfide (WS_2), and hexagonal boron nitride (BN). (B-D) Cell viability of the modified substrates after 2 days and 7 days as determined by growth rate using a CCK-8 assay. Figure adapted from reference[152]

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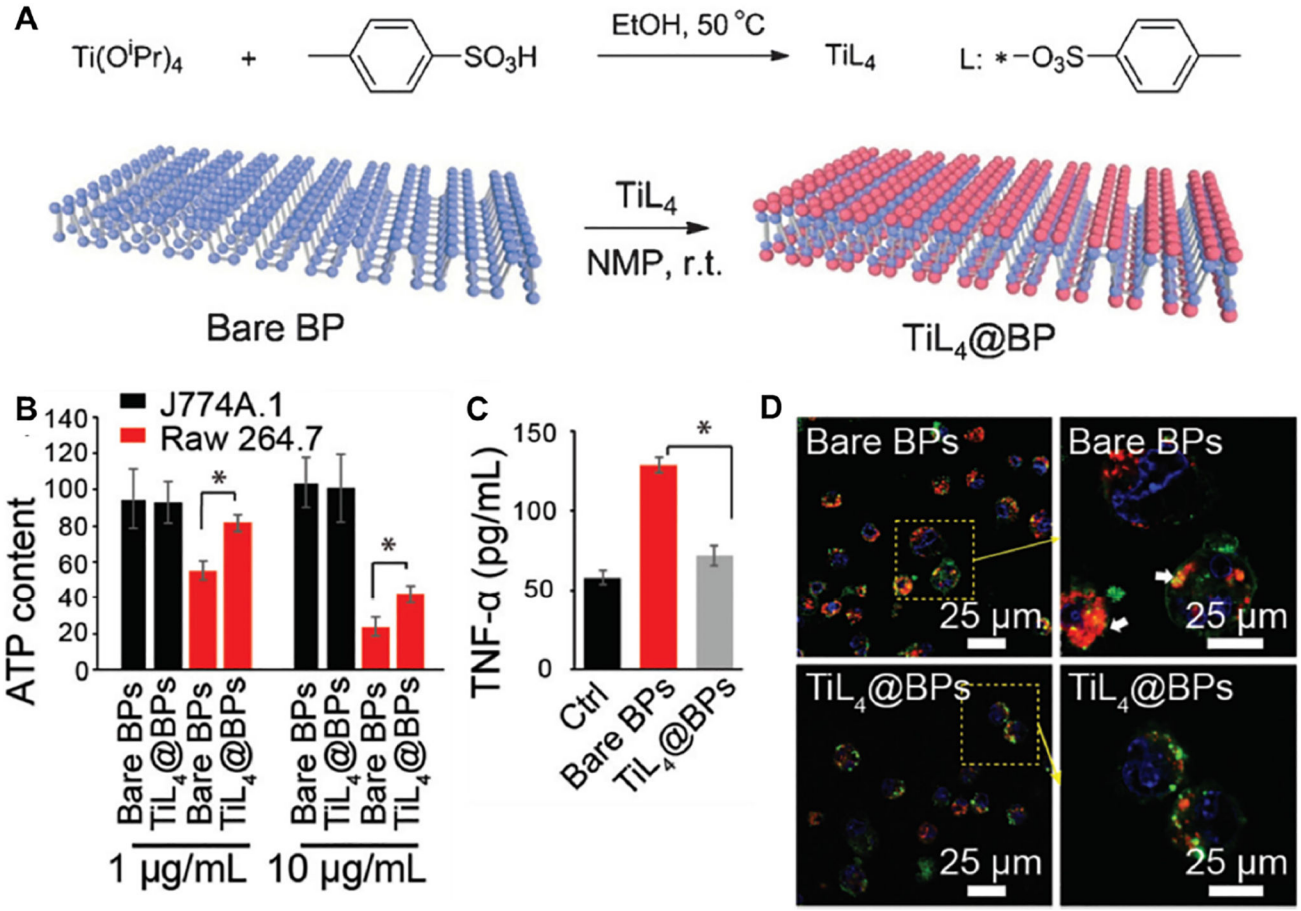

Figure 5.

Effects of chemical modification on the toxicity of black phosphorus (BP). (A) Synthesis scheme and illustration of the surface coordination of the titanium sulfonate ligand (TiL_4) to BP to generate TiL_4@BP. (B) Cell viability of raw 264.7 and J774A.1 cells after 24 hours exposure to bare BP and TiL_4@BP as determined by an ATP assay. (C) TNF-α production from raw 264.7 cells after 12 hours exposure to 10 μg mL^{-1} of the BP samples. (D) Confocal microscopy images of J774A.1 cells stained with Magic Red to show Cathepsin B location after exposure to the BP samples for 6 hours. In the control cells, cathepsin B appears as concentrated dots, localized within the lysosomes. After exposure to bare BP, the lysosomes appear swollen, while this inflammatory effect is not observed after exposure to TiL_4@BP. Figure adapted from reference[164]

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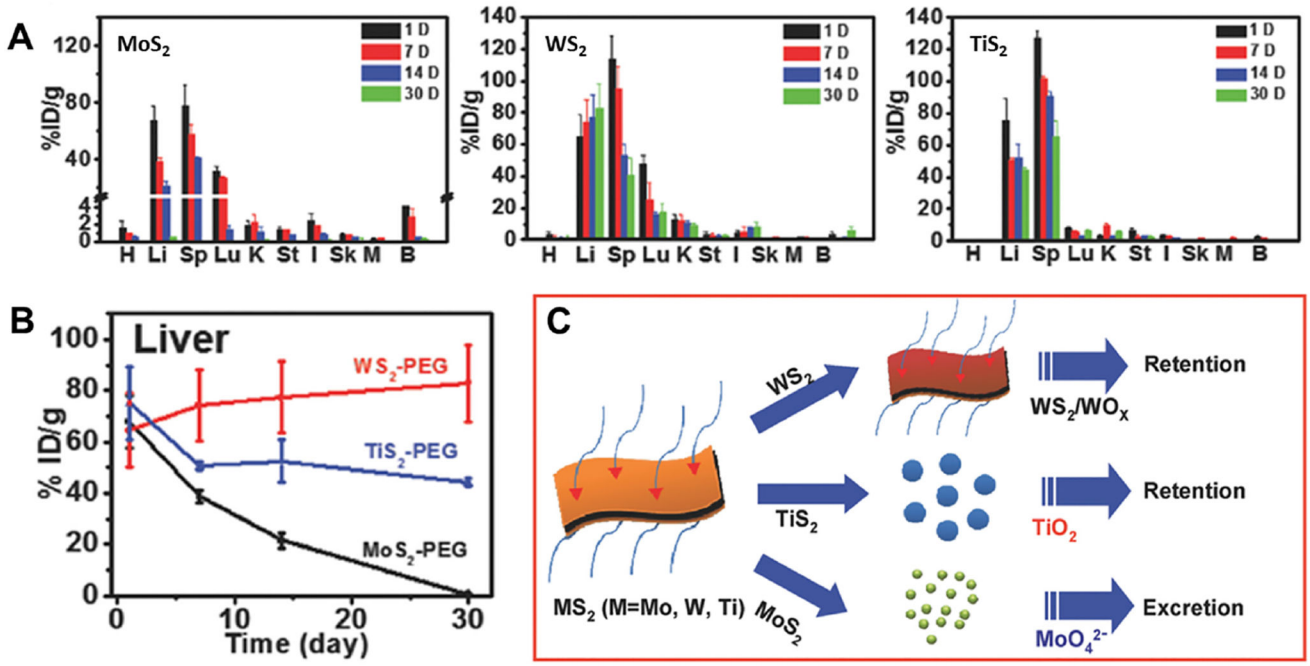

Figure 6.

Biodistribution and fate of PEGylated transition metal dichalcogenides (TMDCs). (A) *In vivo* biodistribution of PEGylated molybdenum disulfide (MoS_2), tungsten disulfide (WS_2), and titanium disulfide (TiS_2) in major organs of mice after intravenous injection for up to 30 days. All materials accumulated mainly in the liver and spleen, but after 30 days MoS_2 was cleared from the system while WS_2 and TiS_2 persisted. (B) Clearance of the PEGylated TMDCs from the liver over 30 days. (C) Schematic illustrating the chemical transformation of the PEGylated TMDCs. MoS_2 dissolves and forms soluble MoO_4^{2-}, which is excreted. TiS_2 oxidizes to form TiO_2, which is insoluble in aqueous solution and the aggregates are retained. WS_2 undergoes some oxidation, but its chemical stability leads to its retention in the organs. Figure adapted from reference[166]

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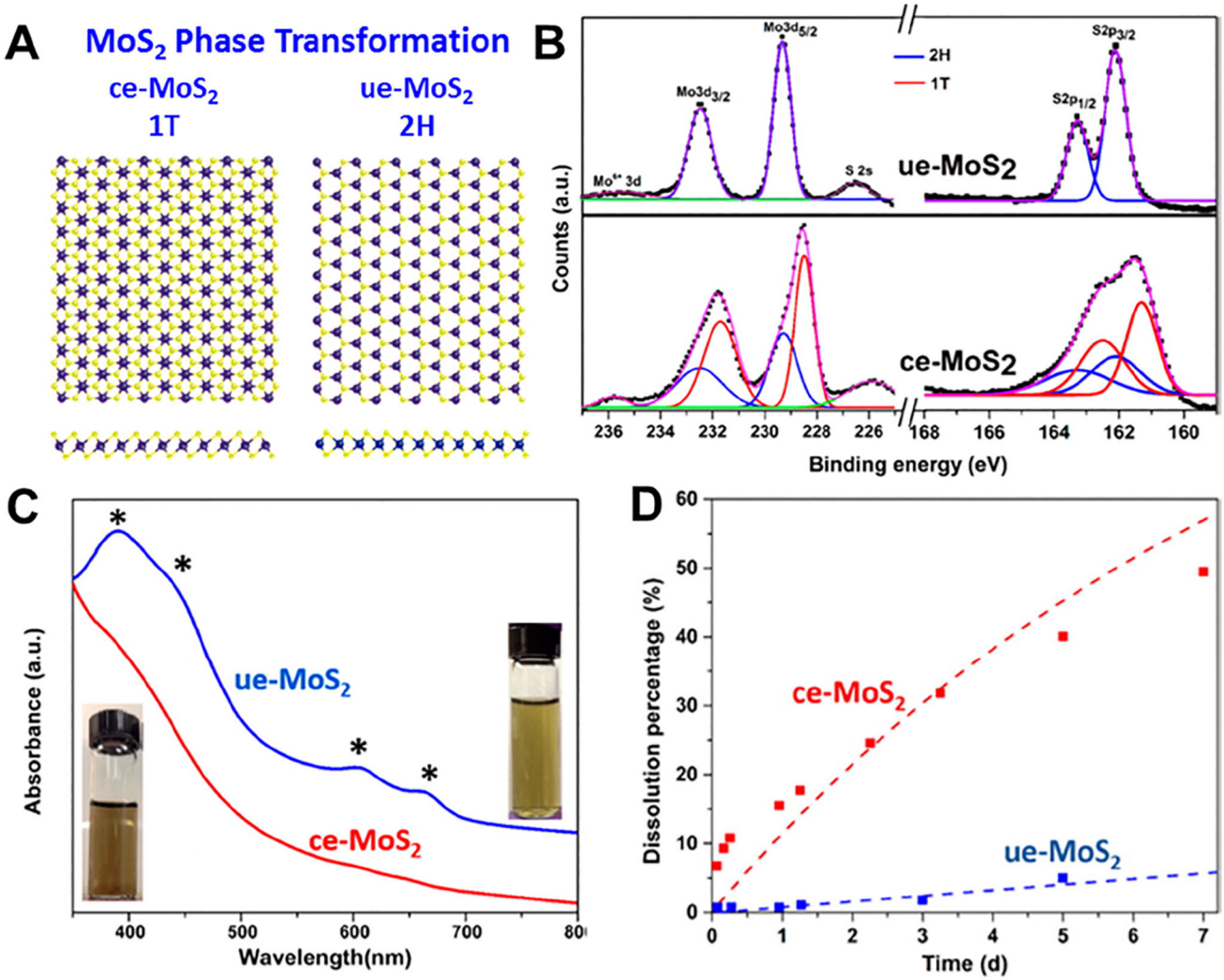

Figure 7.

Stability and dissolution of 1T and 2H phases of molybdenum disulfide (MoS_2). (A) Illustration of the 1T and 2H phases of MoS_2. Chemical exfoliation of MoS_2 (ce-MoS_2) results in a phase change to the 1T phase while ultrasonication-assisted exfoliation (ue-MoS_2) maintains the 2H phase. (B) XPS spectra and fitting of the Mo 3d and S 2p peaks of ce-MoS_2 and ue-MoS_2. ue-MoS_2 is in the 2H phase, while ce-MoS_2 contains both 1T and 2H phase materials. (C) UV-Vis spectra and pictures of ue-MoS_2 and ce-MoS_2. ue-MoS_2 shows the exciton peaks intrinsic to the 2H phase of the material. (D) Dissolution of ce-MoS_2 and ue-MoS_2 in HEPES buffer (pH 7), showing a significantly faster rate of dissolution for ce-MoS_2. Figure adapted from reference[171]

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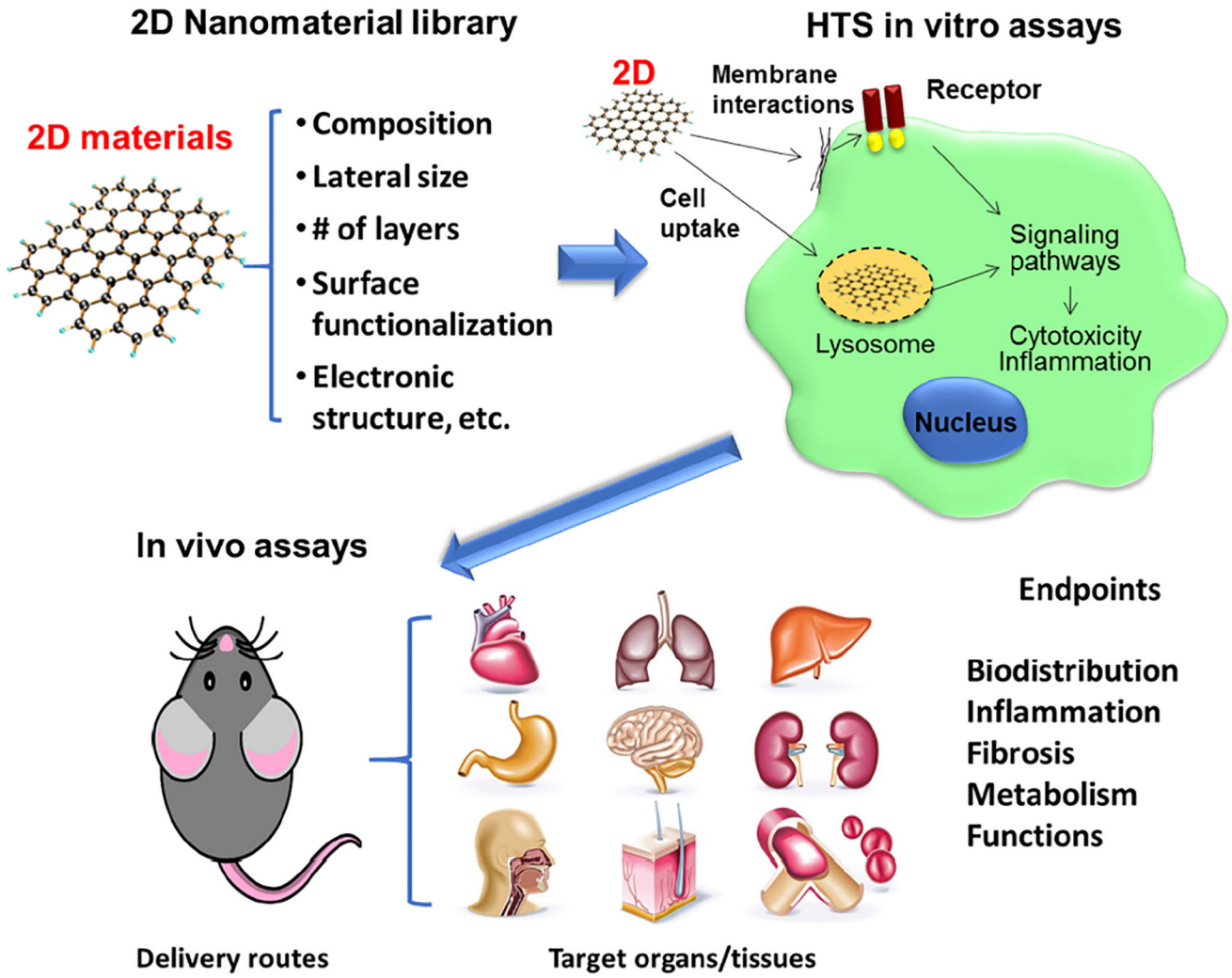

Figure 8.

A proposed approach for the hazard assessment of 2D materials. A library of 2D materials where the physicochemical properties are systematically varied and extensively characterized can be used to relate specific properties to biological outcomes. *In vitro* high throughput screening (HTS) assays can further be used to quantify a response for specific biological endpoints. From these assays, carefully selected *in vivo* assays can then be employed to validate the *in vitro* results and confirm the hazard potential of the 2D material.

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Table 1.

Cytotoxicity evaluations of 2D materials. Reported cytotoxicity results for 2D materials are highly dependent on synthesis and preparation methods, material properties, and experimental parameters.

Composition (Phase)	Synthesis/Exfoliation	Size (Method)	Surface modification	Cytotoxicity cell system (Assay, time)	Cytotoxicity results	Additional toxicity assays	Ref
MoS_2 (1T)	Chemical exfoliation	Thickness: 5 nm (AFM) Lateral Size: 100 nm - 1 μm (SEM)	-	A549 (WST-8, 24 h)	≥ 80 % viability up to 200 μg/mL	-	125
MoS_2 (2H)	Bulk	Not exfoliated (Raman)	-	A549 (MTT and WST-8, 24 h)	64–82% cell viability between 200 and 400 μg/mL	-	189
MoS_2 (1T)	Chemical exfoliation (methyllithium)	Not exfoliated (Raman)	-	A549 (MTT and WST-8, 24 h)	64–82% cell viability between 200 and 400 μg/mL	-	129
MoS_2 (1T)	Chemical exfoliation (n-butyllithium)	Exfoliated (Raman)	-	A549 (MTT and WST-8, 24 h)	53–55% cell viability between 200 and 400 μg/mL	-	129
MoS_2 (1T)	Chemical exfoliation (tert-butyllithium)	Exfoliated (Raman)	-	A549 (MTT and WST-8, 24 h)	53–55% cell viability between 200 and 400 μg/mL	-	129
MoS_2 (2H)	Bulk	1–100 μm (SEM)	-	THP-1 and BEAS-2B (MTS, 24 h)	≥ 80 % viability up to 50 μg/mL	Cellular uptake, *in vitro* proinflammatory cytokine production, *in vivo* toxicity, acute and sub-chronic *in vivo* inflammatory effects	130
MoS_2 (1T)	Chemical exfoliation	Thickness: < 3 nm Lateral Size: 50 – 600 nm (AFM)	-	THP-1 and BEAS-2B (MTS, 24 h)	≥ 80 % viability up to 50 μg/mL	Cellular uptake, *in vitro* proinflammatory cytokine production, *in vivo* toxicity, acute and sub-chronic *in vivo* inflammatory effects	130
MoS_2 (2H)	Liquid phase exfoliation (aqueous)	Thickness: 3 – 10 nm Lateral Size: 10 – 150 nm (AFM)	Pluronic F87	THP-1 and BEAS-2B (MTS, 24 h)	≥ 80 % viability up to 50 μg/mL	Cellular uptake, *in vitro* proinflammatory cytokine production, *in vivo* toxicity, acute and sub-chronic *in vivo* inflammatory effects	130
MoS_2 (2H)	Liquid phase exfoliation (organic)	Thickness: 2–3 layers (TEM)	-	RAMEC and PC12 (SRB, 24 h)	≥ 90 % viability up to 50 μg/mL	Cell morphology and proliferation	131
MoS_2 (2H)	Mechanical exfoliation	Thickness: 3 – 4 layers (Raman) Lateral Size: 8 nm - 10 μm (SEM)	-	HEK293f (Calcein AM/ Ethidium homodimer-1, 4 – 48 h)	≥ 80 % viability up to 48 hr	Cell morphology, ROS, and mutagenicity assays	132

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Composition (Phase)	Synthesis/Exfoliation	Size (Method)	Surface modification	Cytotoxicity cell system (Assay, time)	Cytotoxicity results	Additional toxicity assays	Ref
MoS_2 (2H)	Chemical vapor deposition	Thickness: 2 – 3 layers (Raman)	-	HEK293f (Calcein AM/ Ethidium homodimer-1, 4 – 48 h)	≥ 80 % viability up to 48 hr	Cell morphology, ROS, and mutagenicity assays	132
MoS_2	No details provided	441.2 nm (DLS)	-	HepG2 (WST-8, 24 h)	≥ 80 % viability up to 30 μg/mL	Cellular uptake, ROS, mitochondrial membrane potential, membrane damage, membrane transport, chemosensitive effects, and gene expression assays	134
MoS_2 (2H)	Liquid phase exfoliation (aqueous)	Lateral Size: 50 nm, 117 nm, 177 nm (TEM)	Sodium cholate	A549, AGS, THP-1 (Calcein AM/ Ethidium homodimer-1, 24 h)	No loss of viability up to 10 μg/mL (A529) < 50% viability at 10 μg/mL (AGS and THP-1)	Endotoxin detection, cytokine production, and dendritic cell activation	138
MoS_2 (2H)	Liquid phase exfoliation	Thickness: 1 – 8 monolayers Lateral Size: 100 – 400 nm (Supplier)	-	hADMSCs (WST-8, 2 – 7 d)	* Thin film No loss of viability up to 18 μg/mL	-	152
MoS_2 (2H)	Liquid phase exfoliation (aqueous)	Lateral Size: 50 – 350 nm (AFM)	1-pyrenesulfonic acid sodium salt	A549 and HaCaT (PI/ Annexin V staining, 24 h)	No loss of viability up to 100 μg/mL	-	153
MoS_2 (1T)	Chemical exfoliation	Lateral size: 50 – 500 nm (TEM)	-	HeLa, RAW 264.7 and hMDM (PI/ Annexin V staining, 24 h)	No loss of viability in any of the cell lines	Enzymatic degradation, cytotoxicity of degradation products, cellular interaction and uptake, and cytokine production	165
MoS_2 (1T)	Chemical exfoliation	Lateral size: 50 – 500 nm (TEM)	2-iodoacetamide	HeLa, RAW 264.7 and hMDM (PI/ Annexin V staining, 24 h)	No loss of viability in HeLa and hMDM cells ~20% loss of viability at 100 ^g/mL in RAW 264.7	Enzymatic degradation, cytotoxicity of degradation products, cellular interaction and uptake, and cytokine production	165
MoS_2	High temperature solution phase synthesis	91 nm (DLS)	lipoic acid conjugated 5 kDa PEG	RAW 264.7, 293T and 4T1 (MTT, 24 h)	≥ 70 % viability up to 200 μg/mL	*In vitro* cell membrane damage and ROS; *in vivo* biodistribution, clearance, and toxicology	166
MoS_2 (1T)	Chemical exfoliation	Thickness: ~1.2 nm Lateral Size: ~250 nm	-	TIB-67 and HTB-177 (WST-8, 24 – 72 h)	≥ 80 % viability up to 80 μg/mL	-	171

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Composition (Phase)	Synthesis/Exfoliation	Size (Method)	Surface modification	Cytotoxicity cell system (Assay, time)	Cytotoxicity results	Additional toxicity assays	Ref
$NbTe_2$	Bulk	500–1000 nm (DLS)	-	A549 (WST-8, 24 h)	≥ 60 % viability up to 200 μg/mL	-	127
$TaTe_2$	Bulk	500–1000 nm (DLS)	-	A549 (WST-8, 24 h)	≥ 60 % viability up to 200 μg/mL	-	127
TiS_2	High temperature solution phase synthesis	102 nm (DLS)	lipoic acid conjugated 5 kDa PEG	RAW 264.7, 293T and 4T1 (MTT, 24 h)	≥ 70 % viability up to 200 μg/mL	*In vitro* cell membrane damage and ROS; *in vivo* biodistribution, clearance, and toxicology	166
VS_2	Chemical exfoliation	Lateral Size: 10 nm - 1 μm (SEM)	-	A549 (MTT and WST-8, 24 h)	43% viability at 50 μg/mL	-	126
VSe_2	Chemical exfoliation	Lateral Size: 10 nm - 1 μm (SEM)	-	A549 (MTT and WST-8, 24 h)	9% viability at 50 μg/mL	-	126
VTe_2	Chemical exfoliation	Lateral Size: 10 nm - 1 μm (SEM)	-	A549 (MTT and WST-8, 24 h)	8% viability at 50 μg/mL	-	126
VTe_2	Bulk	500–1000 nm (DLS)	-	A549 (WST-8, 24 h)	< 50% viability at 25 μg/mL < 10% viability at 200 μg/mL	-	127
WS_2 (2H)	Mechanical exfoliation	Thickness: 5 – 8 layers (Raman) Lateral Size: 8 nm - 10 μm (SEM)	-	HEK293f (Calcein AM/ Ethidium homodimer-1, 4 – 48 h)	≥ 80 % viability up to 48 hr	Cell morphology, ROS, and mutagenicity assays	132
WS_2 (2H)	Liquid phase exfoliation (aqueous)	Lateral Size: 50 – 350 nm (AFM)	1-pyrenesulfonic acid sodium salt	A549 and HaCaT (PI/ Annexin V staining, 24 h)	No loss of viability up to 100 μg/mL	-	153
WS_2	High temperature solution phase synthesis	72 nm (DLS)	lipoic acid conjugated 5 kDa PEG	RAW 264.7, 293T and 4T1 (MTT, 24 h)	≥ 70 % viability up to 200 μg/mL	*In vitro* cell membrane damage and ROS; *in vivo* biodistribution, clearance, and toxicology	166
WS_2 (1T)	Chemical exfoliation	Thickness: 20 nm (AFM) Lateral Size: 100 nm - 1 μm (SEM)	-	A549 (WST-8, 24 h)	≥ 80 % viability up to 400 μg/mL	-	125
WS_2 (2H)	Liquid phase exfoliation	Thickness: 1 – 4 monolayers Lateral Size: 50 – 150 nm (Supplier)	-	hADMSCs (WST-8, 2 – 7 d)	* Thin film No loss of viability up to 25 μg/mL	-	152

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Composition (Phase)	Synthesis/Exfoliation	Size (Method)	Surface modification	Cytotoxicity cell system (Assay, time)	Cytotoxicity results	Additional toxicity assays	Ref
WSe_2 (1T)	Chemical exfoliation	Thickness: 7 nm (AFM) Lateral Size: 100 nm - 1 µm (SEM)	-	A549 (WST-8, 24 h)	< 50% viability at 100 µg/mL	-	125
hBN	Liquid phase exfoliation (aqueous)	Lateral Size: 50 – 350 nm (AFM)	1-pyrenesulfonic acid sodium salt	A549 and HaCaT (PI/ Annexin V staining, 24 h)	No loss of viability up to 100 µg/mL	-	153
hBN	Purchased	472.7 nm (DLS)	-	HepG2 (WST-8, 24 h)	≥ 80 % viability up to 30 µg/mL	Cellular uptake, ROS, mitochondrial membrane potential, membrane damage, membrane transport, chemosensitive effects, and gene expression assays	134
hBN	Liquid phase exfoliation	Thickness: 1 – 5 monolayers Lateral Size: 50 – 200 nm (Supplier)	-	hADMSCs (WST-8, 2 – 7 d)	* Thin film No loss of viability up to 5 µg/mL	-	152
BP	Bulk	> 20 µm (SEM)	-	A549 (WST-8 and MTT, 24 h	48% viability (WST-8) 34% viability (MTT) at 50 µg/mL	-	135
BP	Ground by hand in a mortar in NMP followed by ultrasonication	Thickness: 1.5 ± 0.8 nm Lateral Size: 2.7 ± 0.7 nm (AFM)	-	HeLa (Annexin V staining, 24 h)	36.6% viability at 200 µg/mL	*In vitro* ROS, DNA damage, *in vivo* toxicity, and biodistribution	136
BP	Liquid phase exfoliation (DMSO)	Thickness: 2 – 3 nm (S), 5.5 – 6.5 nm (M), 15 – 20 nm (L) Lateral Size: 4.5 0.6 nm (S), 118 ± 22 nm (M), 394 ± 75 nm (L) (AFM/SEM/TEM)	-	LO2 (WST-8, 12 – 48 h)	≥ 80 % viability up to 48 hr	Cell apoptosis and necrosis assay	139
BP	Liquid phase exfoliation (aqueous)	Thickness: 91.9 ± 32.0 nm (BP-1), 27.0 ± 12.0 nm (BP-2), 17.4 ± 9.1 nm (BP-3) Lateral Size: 884.0 ± 102.2 nm (BP-1), 425.5 ± 78.8 nm (BP-2), 208.5 ± 46.9 nm (BP-3) (AFM)	-	NIH 3T3, HCoEpiC, and 293T (RTCA, 12 – 48 h)	Size dependent toxicity for all cell lines: BP-1 > BP-2 > BP-3	Cell morphology, ROS, and cell membrane integrity	140
BP	Liquid phase exfoliation (NMP)	Thickness: 2.0 ± 0.6 nm Lateral size: 2.8 ± 1.5 nm	TiL_4	J774A.1 and RAW 264.7 (ATP, 24 h)	No loss in viability in J774A.1 < 30% viability for bare BP	*In vitro* inflammatory effects, cellular uptake, and *in vivo* inflammatory effects	164

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Composition (Phase)	Synthesis/Exfoliation	Size (Method)	Surface modification	Cytotoxicity cell system (Assay, time)	Cytotoxicity results	Additional toxicity assays	Ref
					< 50% viability for TiL4@BP at 10 μg/mL in raw 264.7		