The acidic transformed nano-VO₂ causes macrophage cell death by the induction of lysosomal membrane permeabilization and Ca²⁺ efflux

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A B S T R A C T

Because of its outstanding thermochromic characteristics and metal-insulator transition (MIT) property, nano-vanadium dioxide (abbreviated as nano-VO₂ or nVO₂) has been applied widely in electrical/optical devices and design of intelligent window. However, the biological effect of nVO₂ is not well understood, especially when affected by environmental factors or living organisms. For VO₂ is an amphoteric oxide, we simulated pH’s influence to nVO₂’s physicochemical properties by exposure nVO₂ in water of different pH values. We found that nVO₂ transformed to a new product after exposure in acidic water for two weeks, as revealed by physicochemical characterization such as SEM, TEM, XRD, and DLS. This transformation product formed in acidic water was referred as (acidic) transformed nVO₂. Both pristine/untransformed and transformed nVO₂ displayed no obvious toxicity to common epithelial cells; however, the acidic transformed nVO₂ rapidly induced macrophage cell death. Further investigation demonstrated that transformed nVO₂ caused macrophage apoptosis by the induction of Ca²⁺ efflux and the following mitochondrial membrane permeabilization (MMP) process. A more detailed time course study indicated that transformed nVO₂ caused lysosomal membrane permeabilization (LMP) at the earlier stage, indicating LMP could be chosen as an earlier and sensitive end point for nanotoxicological study. We conclude that although nVO₂ displays no acute toxicity, its acidic transformation product induces macrophage apoptosis by the induction of LMP and Ca²⁺ efflux. This report suggests that the interplay with environmental factors or living organisms can results in physicochemical transformation of nanomaterials and the ensuing distinctive biological effects.

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1. Introduction

The widespread use of engineered nanomaterials has raised concerns about their possible adverse effects to the environment [1,2]. Engineered nanomaterials are deleterious to living organisms [3,4], and have been detected in the environment within some place at a relative high concentration [5–7]. Studies have indicated that the property and property-related toxicity of nanomaterials changed significantly after released into environment and interplayed with environmental factors and organisms. For instance, fullerene underwent surface oxidation and transformed to soluble product upon exposure to ultraviolet or sunlight [8,9]; in aquatic environment, nano-Ag was easily oxidiated and formed a sulfida-

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For nano-scaled VO\(_2\), generally asked question is that whether it induces diverse biological effects on different biological systems. Research has displayed that nVO\(_2\) induces autophagy in an epithelial cell line [17]. And nano-scaled vanadium oxide (V\(_2\)O\(_3\)) causes distinct biological effect to different cells, such as ECV304, A549, and RAW264.7 [18]. Here we asked whether nVO\(_2\)’s physicochemical properties affects its biological effects, because nVO\(_2\) is an amphoteric oxide which may easily form coordination compound on its surface under relevant environmental conditions such as pH. It has been reported that pH significantly affected the stability and dissolution of ZnO nanoparticles in aqueous solutions [19]. And the toxicity of ZnO nanoparticles to Eisenia fetida was highly influenced by pH [20].

To fully understand nVO\(_2\)’s potential risk to the organisms, we simulated pH’s influence to nVO\(_2\) by exposure nVO\(_2\) in water of different pH values. Our results demonstrated that nVO\(_2\) in acidic water formed new transformation product after two weeks. Importantly, acidic transformed nVO\(_2\) rapidly induced apoptosis in macrophages through the induction of lysosomal membrane permeabilization (LMP) and Ca\(^{2+}\) efflux. We suggest that the interplay with environmental factors or living organisms can results in physicochemical transformation of nanomaterials and the ensuing distinctive biological effects.

2. Materials and methods

2.1. Preparation of nVO\(_2\)

Synthesis of nVO\(_2\) was performed with a simple strategy, which is low-cost and large scale, by combining hydrothermal synthesis with a subsequent mild thermal treatment [11]. Endotoxin levels of nanomaterials (pristine or transformed nVO\(_2\)) were about 0.1 EU/ml (<0.25 EU/ml), measured as previously described [21].

2.2. Cells and reagents

The mouse monocyte/macrophage cell line RAW264.7 and human embryonic kidney cell line HEK293T were obtained from the American Type Culture Collection (ATCC, Rockville, MD, USA). For obtain of bone marrow-derived macrophage (BMDM), bone marrow cells were isolated from femurs and tibiae of 6–8-week-old C57BL/6 mice, and cultured in 1640 complete medium containing conditional media from L929 cell culture as described previously [22]. After 24 h, non-adherent cells were transferred to a new plate and fresh L929 conditional medium was added every other day. BMDM were harvested at day 7, when they expressed common macrophage surface markers such as CD11b and F4/80. Cells were cultured and maintained in RPMI-1640 containing 10% FBS, 100 U/ml penicillin, and 100 μg/ml streptomycin (Invitrogen, San Diego, CA, USA). Mice were housed under specific pathogen-free conditions at School of Life Sciences, University of Science and Technology of China (USTC). Animal care and experimental procedures were in accordance with the experimental animal guidelines at USTC.

RAW264.7 cell line was employed to study the toxicity and cytotoxicity mechanism. HEK293T cell line and the induced primary macrophage BMDMs were employed to study the cytotoxicity.

3. Experimental procedure (nVO\(_2\)-pH treatment, characterization, and cell treatment)

As shown in the schematic diagram in Fig. 1A, the experimental procedure contains four parts:

1. nVO\(_2\) exposure to water of different pH values. We dissolved nVO\(_2\) in acidic, neutral, and alkaline water; the pH value of Milli-Q water was adjusted as previously described [23,24]. The pH values chosen here (pH 5–9) were in relevant to both the environmental and organism’s condition; other extreme pH-treated group was displayed in Fig. S1. Both nVO\(_2\)’s final concentration of 10 and 100 μg/ml displayed the same results. The exposure time was from 1 to 3 weeks, and last for half a year.

2. Obtain of pH-treated nVO\(_2\). nVO\(_2\) was take out of water by centrifuging tubes at 100,000 rpm for 5 min, washing with water for three times, and freeze-drying with a lyophilizer [11]. The dry power was used for physicochemical characterization, or quantitated by ICP-MS and dissolved to form a stock solution (100 μg/ml) for cell treatment.

3. Physicochemical characterization. The morphology, microstructure, phase structure, and composition of the nVO\(_2\) were examined, respectively, using Field-Emission Scanning Electron Microscopy (FESEM; Sirion 200), Transmission Electron Microscope (TEM; JEOL-2010), X-ray Diffraction with the Cu K\(_{\alpha1}\) line (XRD; Philips X’Pert), and Energy Dispersive X-ray (EDX, AN1085; Oxford Instruments). Dynamic Light Scattering (DLS) Size (Hydrodynamic diameters) and Zeta Potential under typical exposure conditions were measured by a Zetasizer Nano-ZS instrument (ZEN3600, Malvern Instruments). Inductively Coupled Plasma Mass Spectrometry (ICP-MS, X Series 2, Thermo fisher Scientific) was employed to determine the vanadium content in cells [11,25,26].

4. Cell treatment/toxicity study. nVO\(_2\) was diluted to 5 or 10 μg/ml in fresh medium (RPMI-1640 containing 10% FBS), and
then added to cells [27]. It takes only 24 h to determine the toxicity and toxicity mechanisms in our study. For separation with nVO₂, cells were sorted by flow cytometry (BD FACSARia III).

4. Cell viability assay

Trypan blue exclusion assay and MTT assay were performed as described [27]. Trypan blue solution (0.4%) was from Sigma–Aldrich; cells were calculated by Countstar IC1000 (Inno-Alliance Biotech). For MTT assay, MTT was added to each well after treated with nanomaterials at a final concentration of 0.5 mg/ml. Absorbance at 590 nm (A590) was determined using a microplate reader.

4.1. Cell death assay

After treated with nanomaterials, cells (RAW264.7) were subjected to fluorescein isothiocyanate (FITC)-conjugated annexin V and propidium iodide (PI) staining according to Annexin V: FITC Apoptosis Detection Kit (556,547, BD PharmingenTM, San Diego, CA, USA), then apoptotic and dead cells were analyzed by flow cytometry.

Sub-G1 cell (apoptosis) populations were assayed using PI staining as previously described [28]. Cells were washed with PBS and fixed with 70% ethanol at 4 °C overnight. Later, cells were washed and resuspended with PBS containing 0.2% Triton X-100 and 100 μg/ml RNase A, and then stained with 50 μg/ml PI at 4 °C for 30 min.

4.2. Cytosolic calcium measurement

Rising cytosolic calcium concentration, which indicated changes in cell signals or fate, was measured by the calcium-sensitive dye FLUO3-AM [29]. Briefly, RAW264.7 cells were incubated in RPMI-1640 containing 10% FBS and pretreated with 1 μM FLUO3-AM (Invitrogen-Molecular Probes, San Diego, CA, USA) at 37 °C for 2 h. Cells were harvested at various time points (0, 6, 9, 12, 15, 18 h), and the cytosolic calcium levels were determined by flow cytometry (FLUO3-AM: excitation wavelength, 506 nm; emission wavelength, 526 nm, monitored at FL-1 channel) [29].

4.3. Assessment of ΔΨm drop

Loss of mitochondrial transmembrane potential (ΔΨm), which indicates a later ongoing mitochondrial membrane permeabilization (MMP) and cell death, was measured by employing Mitochondrial Membrane Potential Detection Kit (551,302, BD PharmingenTM, San Diego, CA, USA) [30]. Briefly, RAW264.7 cells were incubated with 5 μg/ml of JC-1 for 10 min at 37 °C; cells were assayed by flow cytometry. Cell population with a decrease in red fluorescence was gated out and regarded as the subpopulation with impaired mitochondria and ΔΨm drop [30].

4.4. Acridine orange relocation assay

Acridine orange relocation assay demonstrating lysosomal damage was performed as described [31]. Briefly, RAW264.7 cells were seeded in 6-well plates with 5 × 10⁵ cells/well and cultured for 12 h. After that, 5 μg/ml of AO (Invitrogen-Molecular Probes, San Diego, CA, USA) was added to plates and incubated for 15 min. Then, cells were washed twice with PBS and treated with nanomaterials. After treatment, cells were collected at 6 or 12 h, washed three times with PBS, fixed with 4% paraformaldehyde, and then were subjected to assessment of red (FL-3 channel) and green (FL-1 channel) AO fluorescence using flow cytometry.

4.5. Lysotracker staining

Lysotracker dye labels acidic spherical granules and will continue to stain lysosomal membranes that undergo LMP within cells [31]. RAW 264.7 cells (1 × 10⁵/ml) were incubated with 1640 complete medium containing 50 nM Lysotracker Green (Invitrogen-Molecular Probes, San Diego, CA, USA) at 37 °C for 60 min, washed twice, and then treated with nanomaterials [31]. After treatment, cells were assayed by flow cytometry.

4.6. Flow cytometry analysis

Cells were resuspended (1 × 10⁶ cells/ml) in staining buffer (PBS with 10% PBS), stained with fluorescent dye and washed with PBS. Analyses were performed on the FACScalibur (BD, San Diego, CA, USA) and analyzed with FlowJo software (Tree Star, Ashland, OR, USA).

4.7. Statistics

All data were expressed as mean ± standard error of the mean (SEM). P values were generated using two-tailed student’s t-test (*P ≤ 0.05, **P ≤ 0.01, ***P ≤ 0.001).

5. Results

5.1. The physicochemical characterization of nVO₂ transformation in acidic water

Metal oxide nanoparticles have been used in all aspects of human life and social production, leading to an increased interest of their behavior affected by environmental factors and organisms’ process [32]. pH, an important factor, could influence the stability, aggregation, and dissolution of nano metal oxides, both under environmental and organisms’ condition [19]. In order to reveal the influence of pH to nVO₂’s physicochemical properties and biological effects, we first simulated pH’s impact on nVO₂ by exposure nVO₂ in water of different pH values. The experimental model was displayed in schematic diagram (Fig. 1A). nVO₂ was kept in water at different pH values for different time (Fig. 1B). We found that nVO₂ in acidic water (10–100 μg/ml) formed flocculent unapparently at the beginning of first 10 days, and there was obvious aggregated mass of flocculent material suspended in the acidic water after two weeks (Fig. 1B) (For visible to the unaided eye, experiment on 100 μg/ml was displayed here).

We characterized the acidic transformed nVO₂ using different methods. SEM study displayed that the morphology was affected after exposure to acidic or neutral water for two weeks (Fig. 2A), and eight months’ exposure displayed that nVO₂ transformed to colloid-like V₂O₅ (Fig. S1). Using TEM to further study the mass of flocculent material and determined the microstructure and surface change, we revealed that nVO₂ aggregated together to form a new transformation product after exposure in acidic water for two weeks (Fig. 2B). For short-term exposure (less than one week), we did not observe this transformational change. And DLS assay demonstrated that the acidic transformed nVO₂ displayed distinct particle size and Zeta potential (Fig. 3C and D).

Next, using XRD and EDX, we revealed that the phase structure, elementary composition, and vanadium-oxygen ratio of acidic transformed nVO₂ (exposure for two weeks) had no obviously change, indicating that the acidic transformed nVO₂ was not oxidized to V₂O₅ (Fig. 3A and B). Therefore, nVO₂ undergoes physicochemical transformation after two weeks’ exposure in acidic water.
Fig. 2. Characterization of nVO$_2$'s transformation products by SEM and TEM. The surface morphology and microstructural characterization of nVO$_2$'s transformation products (exposure for two weeks) were performed on SEM and TEM, respectively.
5.2. The acidic transformed nVO₂ rapidly induced macrophage cell death mainly by apoptosis

To explore the biological effect of the acidic transformed nVO₂, we first measured its toxicity to different types of cell lines. Although neither pristine nor transformed nVO₂ was obviously toxic to HEK293T cells after treated with different doses for 24–48 h (Fig. S2A and Fig S3A), the transformed nVO₂ displayed an acute toxicity to macrophage RAW264.7 cells and BMDMs (Fig. 4, Fig. S2B, and Fig. S3B). The differences among cell lines might be due to cell sensitivity, phagocytosis, and other immune characteristics of macrophage [21]. In trypan blue exclusion assay, after exposure to nVO₂ for 24 h, there was a sharp decrease in live cell number only after exposure to acidic transformed nVO₂ (5 μg/ml or 10 μg/ml). MTT assay was also explored to study the toxic change of nVO₂ after transformation. Data in Fig. 4B demonstrated that the toxicity of transformed nVO₂ increased with treatment time.

Next, we distinguished the cell death pattern using FITC-conjugated annexin V and PI staining as previously described [33]. As shown in Fig. 4C, at the time point of as earlier as 12 h, in the group that treated with transformed nVO₂, there was an obvious increase in the population labeled as annexin V⁺ PI⁻. Furthermore, PI staining on fixed cells, which represents a late phase detection index of the progress of apoptosis, also displayed that sub-G₁ cell (apoptosis) populations increased in the group treated with transformed nVO₂ (Fig. 4D). These results demonstrated that the macrophage death induced by transformed nVO₂ was mainly apoptosis.

The acidic transformed nVO₂ induced Ca²⁺ enflux and loss of mitochondrial transmembrane potential (ΔΨₘ) that parallels the progression of apoptosis.

Apoptosis can be triggered by several pathways: the extrinsic pathway signaling through death receptors such as Fas/CD95 and TNFR [34], the intrinsic pathway characterized by mitochondrial membrane permeabilization (MMP), and pathway mediated by flux of Ca²⁺ from endoplasmic reticulum (ER) stores [35]. Several (immune) cell death receptor can initiate death signaling in macrophages [34], here, we checked the TNF-α/TNF-α receptor...
Fig. 4. The acidic transformed nVO2 in acidic water induced macrophage cell death mainly by apoptosis. (A) Viability of macrophages treated with nVO2 (pristine or transformed) at 5 to 10 μg/ml for 24 h was estimated by trypan blue exclusion assay; live cell number was counted. (B) Different pH-treated nVO2 (for 1–3 weeks) were obtained and exposed to RAW 264.7 cells. Cell viability was assayed by using MTT. (C) Annexin V/PI staining was performed for displaying the apoptotic or dead cells after exposure to either pristine or transformed nVO2 (10 μg/ml for 12 h). (D) After exposure, fixed cells were stained with PI and/or subdiploid cells was shown. Representative of at least 3 independent experiments (n ≥ 3).

Fig. 5. Acidic transformed nVO2 induced an increase in cytosolic Ca2+ concentration and a loss of mitochondrial transmembrane potential (Δψm) that parallels the progression of apoptosis. (A) RAW 264.7 cells were exposed to either pristine or transformed nVO2 (at 10 μg/ml) from 0 to 18 h, stained with FLUO3-AM, and the fluorescence was quantified by flow cytometry. A 12 h-experiment was presented on left, and cells with increased cytosolic Ca2+ were gated. The time-dependent statistical result was shown on the right side. (B) Macrophages that exposure to 10 μg/ml of either pristine or transformed nVO2 for 12 h were stained with JC-1 reagent, and assayed by flow cytometry. Cells attenuated in red fluorescence were gated, and the statistical result was shown on the right. Representative of at least 3 independent experiments (n ≥ 3).

pathway, a common cell death route in macrophage, in the initial stage of macrophage apoptosis. Fig. S4 showed that the cell death caused by new transformation product was not attenuated upon pretreatment with the antagonist of TNF-α/TNF-α receptor (the WP9QY peptide) after exposure to nVO2 for 12 h, suggesting that the TNF-α/TNFR did not participate in the transformation product induced apoptosis.

Next, we investigated whether the new transformation product modulated the cytosolic Ca2+ in macrophages using the calcium-sensitive dye FLUO3-AM. As shown in Fig. 5A, after 12 h exposure to different pH-treated nVO2, a significant increase of cytosolic Ca2+ concentration was observed in cells exposed to transformed nVO2 (in acidic water). A detailed experiment from 6 to 18 h displayed a time-dependent increase in the percentage of cells with
high cytosolic Ca$^{2+}$ concentration. These results indicated that the cytosolic Ca$^{2+}$ concentration increased in parallel with apoptosis.

To further find out whether efflux of cytosolic Ca$^{2+}$ to mitochondria induced the loss of mitochondrial transmembrane potential (Δψm) and later MMP with the release of proapoptotic molecules [35]. Δψm was measured using the JC-1 reagent. As shown in Fig. 5B, upon treatment with acidic transformed nVO₂, the gated cell population displayed a decrease in red fluorescence, implying an impairment of mitochondria and Δψm drop. All together, these results demonstrated that the transformed nVO₂ induced cell death at least by flux of Ca$^{2+}$ from ER stores and MMP.

5.3. The acidic transformed nVO₂ caused macrophage cell death by the induction of lysosomal membrane permeabilization (LMP)

Lysosome can be affected before cells undergo apoptosis, especially for macrophages exposure to toxicants [36]. Studies showed that toxicants, such as anthrax lethal toxin, could induce LMP and cytosolic cathepsin release in macrophage [31], and lysosome-initiated apoptosis was followed by MMP [30]. To find whether lysosomes were impaired upon exposure to the transformation product of nVO₂, lysosomal acidity was analyzed using acidine orange (AO) staining [31]. As shown in Fig. 6A, LMP occurred in macrophage upon exposure to this transformation product as early as within 6 h (Fig. 6A, the subpopulation with low red and high green (LR/HG) fluorescence represented cells that underwent LMP), indicating that LMP was the initiating step during the progression of cell death. A longer time-point study at 12 h showed that LMP intensified in macrophages exposure to transformed nVO₂ (Fig. 6B). Additionally, experimental groups treated with pristine nVO₂ displayed a significant increase in the percentage of AVO high cells (Fig S5, Fig. 6C). This finding is consistent with previous study, both indicating that pristine nVO₂ induces autophagy [17].

In order to determine that the decrease in acidic compartments measured by AO was not caused by other processes [26,32,33], we employed the lysotracker dye, which labels acidic spherical granules within cells (but not lysosome-specific). Basically, when added prior to stimuli, lysotracker will continue to stain lysosomal membranes that undergo LMP; thus the fluorescence will change when lysosomal loss or fusion occurs during exocytosis, autophagy, or disintegration of lysosomal membranes [37]. Data in Fig. 6D showed that transformed nVO₂ did not induce a decrease in the lysotracker fluorescence intensity after 12 h treatment. This result
indicated that decrease in acidic compartments measured by AO was not influenced by lysosomal loss or fusion.

Following permeabilization (LMP), lysosomal proteins such as effector protease cathepsins, leak into the cytosol and induce cell death [30]. Alkalinization of lysosomes with NH₄Cl can reduce the activity of lysosomal enzymes such as cathepsins [38]. Therefore, we wondered whether NH₄Cl pretreatment attenuated cell death in our experiment. As expected, pretreatment of cells with NH₄Cl (10 mM) for 1 h dramatically decreased cell death induced by transformed nVO₂ (Fig. 6E). This result demonstrated that the transformed nVO₂-induced macrophage cell death was mainly caused by LMP and the leakage of lysosomal effector proteases.

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**Fig. 7.** Cellular uptake of pristine or transformed nVO₂ by RAW264.7 displayed no difference. (A) Supernatant or transformed nVO₂ itself was exposed to RAW264.7 cells for 24 h, (the supernatant of 1 mg/ml washed transformed nVO₂ was diluted to 1:10 or 1:20 for treatment, and 1 mg/ml transformed nVO₂ was added 1:100 to culture for obtain of a final concentration of 10 μg/ml). Live cell was estimated by trypan blue exclusion assay. (B) RAW264.7 cells were treated with 10 μg/ml of pristine or transformed nVO₂ for 12 h. Cells were assayed by flow cytometry. The elliptical gate displayed the main cell group while the rectangular displayed cell debris containing nVO₂. (C) SSC of cells treated with pristine or transformed nVO₂ for 12 h was displayed. (D) After treated with 10 μg/ml of pristine or transformed nVO₂ for 12 h, cells were sorted by flow cytometry, and their vanadium content was determined by ICP-MS.

**Fig. 8.** Schematic of the toxicity mechanism of nVO₂'s acidic transformation product to macrophage was displayed. The acidic transformed nVO₂ induces macrophage apoptosis by the induction of LMP and Ca²⁺ efflux.
5.4. Dissociated ions and cellular uptake level were not responsible for the acidic transformed nVO₂-induced cell death

To find whether the differences in toxicity induced by pristine or transformed VO₂ were caused by the dissociated ions, we exposed the supernatant (which contain dissociated ions) or transformed nVO₂ itself to RAW264.7 cells for 24 h, respectively (Fig. 7A). No obvious toxicity was observed in the supernatant-treated groups. Thus, the acidic transformed nVO₂-induced cell death was not due to any residual dissociated ions, as even a large amount of the supernatant of the transformed nVO₂ was non-toxic to RAW264.7 cells. Next, we investigated the cellular uptake level of pristine or transformed VO₂ by RAW264.7 cells using flow cytometry (Fig. 7B). The side scatter (SSC) of cells treated with pristine or transformed nVO₂ for 12 h changed significantly in relative to control cells; however, groups between pristine and transformed nVO₂ displayed no difference (Fig. 7C). The cellular uptake level of pristine and transformed nVO₂ had no significant change as determined by ICP-MS (Fig. 7D). Therefore, cellular uptake level of nVO₂ was not responsible for the acidic transformed nVO₂-induced cell death.

6. Discussion

Though the distinctive physicochemical properties of nanomaterials enable them to play an important role in all aspects of human life and social production, they also lead to adverse effects correlating with the small size-effect and surface-effect [39,40]. Nanomaterials change easily upon releasing into environment, especially in water systems. For instance, fullerene underwent surface oxidation and transformed to more soluble product in water; after entering into water systems, nano-Ag could be easily oxidized, resulting in increased toxicity to living system. These environmental transformations strongly affected biological effects of nanomaterials. For example, the toxicity of nano-ZnO to Eisenia fetida was highly influenced by pH [20]. Nano amphoteric oxides could easily transform under environmental factors, and the transformation products displayed distinctive biological effects [10].

nVO₂ has been widely applied in electrical/optical devices because of its outstanding thermochromic characteristics and MIT property [13,37]. Research demonstrated that nVO₂ induced autophagy in Hela cells [17]: however, there is little study concerning the toxicity of nVO₂ affected by environmental factors. Here, we demonstrate that nVO₂ aggregates together to form a new transformation product under acidic water condition for two weeks. And acidic transformed nVO₂ causes an acute toxicity to macrophages but not to epithelial cells. The specialized toxicity of transformed nVO₂ to macrophage suggests that immunotoxicity of nanoparticles is strongly influenced by environmental factors [41].

Several studies displayed that nanomaterials activated different signaling pathways to initiate cell death [27]. For instance, graphene oxide targeted TLR4 and resulted in TNF-R-induced programmed necrosis in macrophages, and nano-ZnO quickly initiated ER stress response before inducing apoptosis [21,42]. In order to clarify the underlying mechanism of macrophage cell death mediated by transformed nVO₂, we checked several pathways triggering cell death. We found that transformed nVO₂ induced an increase in cytosolic Ca²⁺ concentration and loss of ΔΨₚ, that parallels the progression of apoptosis. These results indicated that ER was suffered at the initiate stage during transformed nVO₂-induced cell death. The finding is consistent with the report displaying that nano-ZnO could interrupt ER homeostasis before inducing apoptosis [27].

Nanomaterials enter cytoplasm and different organelles where they cause damage [27,42,43]. In the present study, we showed that besides ER and mitochondria, lysosome was suffered upon exposure to transformed nVO₂ [31,44]. LMP is an initiating step during the progression of cell death under several conditions. For example, lysosomal rupture is an early event in p53-induced apoptosis [45]. Lysosome also suffered by cytotoxic stimulus: silica, or even ROS, can modulate lysosomal enzyme activity and induce apoptosis in macrophages [38,46]. In this work, by employing AO and lycosticker staining, we confirmed that lysosomal function was impaired and LMP occurred at the early stage upon exposure to transformed nVO₂. This process was earlier than Ca²⁺ efflux and MMP, indicating that the lysosome-initiated apoptosis later initiated MMP [30]. Besides, alkalization of lysosomes by pretreating cells with NH₄Cl, which can reduce the activity of lysosomal enzymes such as cathepsins, attenuated the cell death caused by transformed nVO₂. Therefore, we indicated that LMP was an initiating signal that induced Ca²⁺ efflux and loss of ΔΨₚm in our study, and leakage of lysosomal proteins into the cytosol might also promote cell death (Fig. 8).

In conclusion, we confirm that nVO₂ transforms during exposure to acidic water condition and its transformation product rapidly induces macrophage apoptosis by the induction of LMP and Ca²⁺ efflux. These findings indicate that pH affects nVO₂’s physicochemical properties, thereby altering their toxicity. This work suggests that more attention should be paid to the influence of environmental factors when assessing the risk of metal oxide nanoparticles.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.toxrep.2015.06.005

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