Metal–Organic Frameworks

Synthesis, Culture Medium Stability, and In Vitro and In Vivo Zebrafish Embryo Toxicity of Metal-Organic Framework Nanoparticles


Abstract: Metal–organic frameworks (MOFs) are among the most attractive porous materials available today. They have garnered much attention for their potential utility in many different areas such as gas storage, separation, catalysis, and biomedicine. However, very little is known about the possible health or environmental risks of these materials. Here, the results of toxicity studies on sixteen representative uncoated MOF nanoparticles (nanoMOFs), which were assessed for cytotoxicity to HepG2 and MCF7 cells in vitro, and for toxicity to zebrafish embryos in vivo, are reported. Interestingly, there is a strong correlation between their in vitro toxicity and their in vivo toxicity. NanoMOFs were ranked according to their respective in vivo toxicity (in terms of the amount and severity of phenotypic changes observed in the treated zebrafish embryos), which varied widely. Altogether these results show different levels of toxicity of these materials; however, leaching of solubilized metal ions plays a main role.

Introduction

Metal–organic frameworks (MOFs) are porous materials built from the controlled crystallization of metal ions or higher nuclerity metal clusters with multifunctional organic ligands.[1–3] When assembled at the nanoscale, they are called nanoMOFs. Analogously to other classes of nanoparticles, nanoMOFs show size-dependent properties (e.g., different adsorption kinetics or better dispersibility compared with their bulk analogues),[11] which can be exploited in numerous practical applications, including traditional storage[4] and catalysis[5, 6] and in newer areas such as sensors,[7] functional membranes and thin-films,[8, 9] and in biomedical applications such as drug-delivery,[10–12] NO absorption,[13, 14] and contrast agents.[15] The ever-increasing interest in nanoMOFs (and in their bulk analogues) should ultimately lead to their widespread production and use. However, little is known about the safety of these nanomaterials to humans and to the environment. Thus, before any nanoMOF can be adopted for practical use, its Environmental Health and Safety (EHS) profile must be determined.

Prior to the work we report here, other groups had already explored the in vitro toxicity of certain bare nanoMOFs in cells. In 2008, Lin et al. assayed the cytotoxicity of amorphous disuccinato-cisplatin/TbIII nanoparticles (size: ca. 60 nm) to HT-29 human colon adenocarcinoma cells, observing no appreciable cytotoxicity.[16] Starting in 2010, Horcajada, Gref, Serre et al. evaluated the in vitro toxicity of several Fe III-based nanoMOFs (e.g., nanoMIL-53, nanoMIL-88, nanoMIL-100, and nanoMIL-101; size: 90–200 nm) to various cell lines, including mouse macrophage J774.A1, human leukemia (CCRF-CEM), human multiple myeloma (RPMI-8226) and human cervical adenocarcinoma (HeLa) cells, and low cytotoxicities were generally found.[10, 17–20] Roughly in parallel, Junior et al. assayed nanoZIF-8 (size: 200 nm) against three human cell lines (mucoepidermoid carcinoma of lung [NCI-H292], colorectal adenocarcinoma [HT-29], and promyelocytic leukemia [HL-60]), and found that at the highest tested concentration (109 m), it was not cytotoxic to any of them.[11] However, Horcajada et al. recently observed cytotoxicity of nanoZIF-8 (size: 90 nm) to HeLa and J774 cell lines, reporting IC50 values of 436 and 109 m, respectively.[18] They also reported that Zr IV-based UiO-66 (size:...
100 nm) was more toxic, showing IC₅₀ values of 239 (HeLa) and 36 μM (J774).

The aforementioned results were obtained from in vitro studies only. Importantly, the only in vivo studies on nanoMOFs reported to date were done in Wistar female rats. In these studies, the rats were given one of three Fe III-based nanoMOFs (nanoMIL-88, nanoMIL-100, or nanoMIL-101) by intravenous injection, and subsequently analyzed for various parameters (e.g., serum, enzymatic, histological, etc.). The results revealed a lack of severe acute or sub-acute toxicity.

In this communication, we report combined in vitro (HepG2 and MCF7 cells) and in vivo (zebrafish embryos) studies on the toxicity of sixteen archetypical, uncoated nanoMOFs. As shown in Figure 1, the selected nanoMOFs comprise: MIL-100 \([\text{Fe}_2\text{O}(\text{H}_2\text{O})_2\text{Cl(btc)}_2] (1); \text{btc}: 1,3,5\text{-benzenetricarboxylic acid})\); MIL-101 \([\text{Fe}_2\text{Cl}(\text{H}_2\text{O})_2\text{O(NH}_2\text{-bdc)}_3] (2); \text{NH}_2\text{-bdc}: 2\text{-aminobenzene-1,4-dicarboxylic acid})\); MOF-5 \([\text{Zn}_2\text{O(bdc)}_3] (3); \text{bdc}: 1,4\text{-benzenedicarboxylic acid})\); and MOF-74 (also called CPO-27 family) \([\text{M}_6(\text{dhbdc})_4] (4-9); \text{M}: \text{Zn}^{II}, \text{Cu}^{II}, \text{Ni}^{II}, \text{Co}^{II}, \text{Mn}^{II}, \text{and Mg}^{II}; \text{dhbdc}: 2,5\text{-dihydroxy-1,4-benzenedicarboxylic acid})\); ZIF-7 \([\text{Zn(Ph-im)}_2] (10; \text{Ph-im}: \text{benzylimidazole})\); ZIF-8 \([\text{Zn(Me-im)}_2] (11; \text{Me-im}: \text{2-methylimidazole})\); UiO-66 \([\text{Zr}_6\text{O}_4(\text{OH})_4(\text{bdc)}_6] (12); \text{bdc}: \text{1,4-benzenedicarboxylic acid})\); UiO-66-NH₂ \([\text{Zr}_6\text{O}_4(\text{OH})_4(\text{NH}_2\text{-bdc)}_6] (13); \text{NH}_2\text{-bdc}: 2\text{-aminobenzene-1,4-dicarboxylic acid})\); UiO-67 \([\text{Zr}_6\text{O}_4(\text{OH})_4(\text{bpdc)}_6] (14); \text{bpdc}: 4\text{-carboxylic acid})\); HKUST-1 \([\text{Cu}_2(\text{btc)}_3] (15); \text{btc}: 1,3,5\text{-benzenetricarboxylic acid})\); and NOTT-100 (also called MOF-505) \([\text{Cu}_2(\text{bptc})] (16); \text{bptc}: 3,3',5,5',biphenyl-tetracarboxylic acid)\). We screened the nanoMOFs for cytotoxicity to the two aforementioned cell lines (using standard in vitro methodologies), performed in vivo studies in zebrafish (as an in vivo model appropriate for comparative studies on mammalian biology), and finally the results from each study were compared. Zebrafish is recognized by the National Institute of Environmental Health Science (NIEHS, USA) and the Institute for Environment and Sustainability (IES, Europe) as an excellent system in which to study environmental toxicity, and is accepted by the National Institutes of Health (NIH, USA) as an alternative model for exploring human diseases. Furthermore, zebrafish embryo assays do not raise major ethical questions regarding vertebrate experimentation. Based on our results, we ranked the nanoMOFs according to their in vivo toxicity (in terms of the amount and severity of phenotypic changes in the treated zebrafish embryos). We found that this ranking parallels the in vitro toxicity rankings for both cell lines, and that the toxicity depends strongly on the solubility of the nanoMOFs and on their subsequent release of metal ions.

Results and Discussion

Synthesis and characterization of the nanoMOF library

We and other groups have previously reported several methods for synthesizing nanoMOFs. By using solvo-
hydro-thermal reactions reported previously, we prepared the following nanoMOFs: nanoMIL-100 (size: 143 ± 23 nm), nanoMIL-101 (size: 170 ± 50 nm), nanoM-MOF-74s (M = Zn\textsuperscript{II}; size: 35 ± 9 nm; M = Cu\textsuperscript{II}; size: 20 ± 8 nm; M = Ni\textsuperscript{II}; size: 111 ± 38 nm; M = Co\textsuperscript{II}; size: 112 ± 26 nm; M = Mn\textsuperscript{II}; size: 51 ± 18 nm; M = Mg\textsuperscript{II}; size: 122 ± 34 nm), nanoUiO-66 (size: 66 ± 34 nm), nanoUiO-66-NH\textsubscript{2} (size: 73 ± 32 nm), and nanoUiO-67 (size: 180 ± 30 nm). We employed fast precipitation to prepare nanoZIF-8 (size: 80 ± 15 nm) in water, nanoZIF-7 (size: 170 ± 20 nm) in N,N-dimethylformamide (DMF), and nanoMIL-5 (size: 85 ± 34 nm) in DMF; the latter, by slowly adding a base into the precursor solution. Finally, we used our recently developed spray-drying technique\textsuperscript{34} to synthesize nanoHKUST-1 (size: 75 ± 28 nm) and nanoNOTT-100 (size: 45 ± 18 nm). Details on all the syntheses are provided in the Supporting Information.

Once synthesized, all the nanoMOFs were cleaned to remove any impurities (including trace amounts of toxic solvents from the syntheses), dried at 80 °C overnight, and finally redispersed in dimethyl sulfoxide (DMSO) to form stable colloids (concentrations: 25 to 100 mg/mL) for the in vitro and in vivo toxicity studies. Transmission electron microscopy (TEM; Figure 1) and X-ray powder diffraction (XRPD) of the resulting colloids demonstrated that all selected nanoMOFs were obtained as homogeneous nanoscale crystals and that their XRPD patterns were fully coincident with the simulated patterns calculated from atomic coordinates (see Figure S1 in the Supporting Information). In addition, all colloidal dispersions were characterized by dynamic light scattering (DLS) studies to confirm the crystal size measured from the TEM images, as well as the homogeneity of each sample and the absence of any aggregation of the nanocrystals in solution (see Figures S2 and S3 in the Supporting Information).

Stability of the nanoMOFs in culture medium

The stability of all the nanoMOFs in the culture medium containing 10% fetal bovine serum (FBS) was studied. Each nanoMOF colloid was separately dispersed in the medium at a final concentration of 10 mg/mL, and then incubated at 37 °C for 24 h. The resulting solids were then collected by centrifugation, dried, weighed, and finally characterized by XRPD.

The robustness of the crystal structure of each nanoMOF was evaluated by comparing the initial and final XRPD spectra (see Figure S4 in the Supporting Information). Furthermore, the XRPD spectra were used to check for any other crystalline species that might have formed in the event that the nanoMOFs had degraded. Each supernatant was also characterized by inductively coupled plasma-optical emission spectrometry (ICP-OES) to estimate the amount of metal ion that had leaked from the nanoMOF and dissolved into the culture medium. Table 1 shows all the values extracted from this study. These data clearly indicate that all the nanoMOFs were at least partially soluble in the culture medium, although the degree of solubility varied widely by structure. The data also reveal that some of the nanoMOFs had become amorphous in the culture medium, having undergone structural rearrangements and/or reactions that generated new inorganic species.

<table>
<thead>
<tr>
<th>nanoMOF</th>
<th>[M] [µM]\textsuperscript{[a]}</th>
<th>Deg\textsubscript{cryst} [%]\textsuperscript{[a]}</th>
<th>XRPD analysis</th>
</tr>
</thead>
<tbody>
<tr>
<td>UO-67</td>
<td>215.6 ± 6.3</td>
<td>0.3 ± 0.0</td>
<td>amorphous</td>
</tr>
<tr>
<td>MIL-100</td>
<td>316.2 ± 46.1</td>
<td>1.1 ± 0.2</td>
<td>stable</td>
</tr>
<tr>
<td>MIL-101</td>
<td>310.4 ± 90.1</td>
<td>1.1 ± 0.3</td>
<td>amorphous</td>
</tr>
<tr>
<td>UO-66</td>
<td>1099.8 ± 105.3</td>
<td>1.8 ± 0.2</td>
<td>stable</td>
</tr>
<tr>
<td>UO-66-NH\textsubscript{2}</td>
<td>1567.5 ± 183.1</td>
<td>2.6 ± 0.3</td>
<td>stable</td>
</tr>
<tr>
<td>ZIF-7</td>
<td>448.5 ± 23.4</td>
<td>4.5 ± 0.2</td>
<td>stable</td>
</tr>
<tr>
<td>MOF-5</td>
<td>3108.6 ± 634.1</td>
<td>7.8 ± 1.6</td>
<td>new crystalline species</td>
</tr>
<tr>
<td>Mn-MOF-74</td>
<td>2651.4 ± 73.6</td>
<td>13.3 ± 0.4</td>
<td>loss of crystallinity;</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>new crystalline species</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>(Mn(CO\textsubscript{3}))</td>
</tr>
<tr>
<td>Co-MOF-74</td>
<td>3258.1 ± 58.8</td>
<td>16.2 ± 0.3</td>
<td>loss of crystallinity</td>
</tr>
<tr>
<td>ZIF-8</td>
<td>1916.2 ± 75.4</td>
<td>19.1 ± 0.8</td>
<td>stable</td>
</tr>
<tr>
<td>Zn-MOF-74</td>
<td>5442.6 ± 130.6</td>
<td>27.2 ± 0.5</td>
<td>stable</td>
</tr>
<tr>
<td>HKUST-1</td>
<td>9168.6 ± 137.5</td>
<td>30.3 ± 0.5</td>
<td>loss of crystallinity</td>
</tr>
<tr>
<td>Ni-MOF-74</td>
<td>7014.3 ± 174.9</td>
<td>31.5 ± 0.9</td>
<td>stable</td>
</tr>
<tr>
<td>NOTT-100</td>
<td>79675.7 ± 152.8</td>
<td>39.4 ± 0.8</td>
<td>loss of crystallinity</td>
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<tr>
<td>Cu-MOF-74</td>
<td>9556.8 ± 689.9</td>
<td>47.9 ± 3.4</td>
<td>loss of crystallinity</td>
</tr>
<tr>
<td>Mg-MOF-74</td>
<td>12573.7 ± 273.9</td>
<td>62.9 ± 1.4</td>
<td>loss of crystallinity</td>
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</table>

\textsuperscript{[a]} The concentration of the corresponding metal ions solubilized after the incubation of each nanoMOF at 37 °C for 24 h was determined by ICP-OES. \textsuperscript{[b]} The minimum percentage of degradation (deg\textsubscript{cryst}%) was calculated as follows: deg\textsubscript{cryst} (%) = (V\textsubscript{t} - V\textsubscript{0})/V\textsubscript{0} × 100, where V\textsubscript{t} is the volume of DMEM, S is the stoichiometric ratio of nanoMOF to metal ion, and n\textsubscript{MOF} is the number of moles of initial nanoMOF.

The most soluble nanoMOF in the culture medium was nanoMg-MOF-74 (12 573 ± 274 µM Mg\textsuperscript{2+}; which corresponds to 62.9% of its constituent Mg\textsuperscript{2+}), and the least soluble, nanoUiO-67 (216 ± 6 µM Zr\textsuperscript{IV}; which corresponds to 0.3% of its constituent Zr\textsuperscript{IV}). Despite their vastly different levels of solubility, they each lost crystallinity and became amorphous upon contact with the culture medium.

NanoZIF-7 was poorly soluble in culture medium (449 ± 23 µM dissolved Zn\textsuperscript{II} ions, corresponding to 4.5% of its constituent Zn\textsuperscript{II}), whereas nanoZIF-8 was more soluble, showing a leakage of 1916 ± 75 µM Zn\textsuperscript{II}, (19.1% of its constituent Zn\textsuperscript{II}). Interestingly, after incubation of each one in the culture medium, their respective crystal structures remained unaltered. NanoUiO-66 and nanoUiO-66-NH\textsubscript{2} were also very stable (see Figure S4), having released only 1100 ± 105 µM Zr\textsuperscript{IV} (1.8% of the constituent Zr\textsuperscript{IV}) and 1568 ± 183 µM of Zr\textsuperscript{IV} (2.6% of the constituent Zr\textsuperscript{IV}), respectively. In the case of nanoMIL-100 and nanoMIL-101, the concentrations of dissolved Fe\textsuperscript{III} ions were only 316 ± 46 µM Fe\textsuperscript{III} (1.1% of the constituent Fe\textsuperscript{III}) and 310 ± 90 µM (1.1% of the constituent Fe\textsuperscript{III}), respectively. Although upon incubation both compounds became amorphous, upon subsequent exposure to ethanol, nanoMIL-100 recovered its crystallinity, which confirmed the robustness of its framework (see Figure S4).

The two Cu\textsuperscript{II}-based nanoMOFs (nanoHKUST-1 and nanoNOTT-100) were relatively soluble, releasing 9169 ± 138 µM Cu\textsuperscript{II}, (30.3% of its constituent Cu\textsuperscript{II}) and 7968 ± 153 µM Cu\textsuperscript{II}, (39.4% of its constituent Cu\textsuperscript{II}). This degradation was clearly accompanied by a loss of crystallinity, which, in the XRPD patterns, is evidenced by the disappearance of most of the char-
acteristic peaks. Similarly, the nanoM-MOF-74 family exhibited moderate to high solubility: nanoCu-MOF-74 released 9557 ± 690 μM Cu II (47.9% of its constituent Cu II); nanoNi-MOF-74, 7014 ± 175 μM Ni II (35.1% of its constituent Ni II); nanoZn-MOF-74, 5443 ± 131 μM Zn II (27.2% of its constituent Zn II); nanoCo-MOF-74, 3258 ± 59 μM Co II (16.2% of its constituent Co II); and nanoMn-MOF-74, 2651 ± 74 μM Mn II (13.3% of its constituent Mn II). Upon incubation in the culture medium, all of these nanoMOFs suffered a loss of crystallinity, with nanoCu-MOF-74, nanoCo-MOF-74, and nanoMn-MOF-74 exhibiting the greatest loss.

We would like to note that the proportion of metal ion (relative to the constituent amount of the tested nanoMOF) found in solution cannot always be directly related to the degradation of the nanoMOF. This is because degradation sometimes leads to formation of new, insoluble species. In our study, such species—provided that they were crystalline—were detectable by XPRD. Such was the case with nanoM-MOF-74, the XPRD spectrum of which after incubation in culture medium indicated the formation of MnCO 3 (see Figure S5 in the Supporting Information). The formation of MnCO 3 was further studied by analyzing the powder resulting from the incubation, which confirmed the generation of new (rod-like) particles (see Figure S5). Electron-diffraction analysis (by TEM) of one of these particles revealed a diffraction that was pattern identical to that expected for MnCO 3 (see Figure S5). Formation of new species was also observed in the XRPD patterns for nanoCo-MOF-74, nanoMg-MOF-74, and nanoMn-MOF-5. Unfortunately, in those cases, the new species could not be identified. NanoMOF-5 released 3108 ± 634 μM Zn II (7.8% of its constituent Zn II), but its XRPD spectrum revealed the formation of a new, insoluble crystalline species, and lacked the characteristic peak of nanoMOF-5 itself (see Figure S4).

Given the above findings, we reasoned that the percentage of solubilized metal ions relative to the constituent amount of the tested nanoMOF represents the minimum percentage of degradation, because the ions might have further reacted to form insoluble species in the culture medium. Here, we would like to mention that four of the most structurally robust nanoMOFs (nanoMIL-100, nanoMIL-101, nanoUio-66, and nanoUio-66-NH 2 ) actually underwent greater degradation in culture medium than that detected by analyzing the solubilized metal ions. In these cases, the weight-loss values (calculated by comparing the post- and pre-weight values, and expressed as a percentage) were much higher than were the corresponding values for relative percentage of solubilized metal ions: 25.8 ± 2.5% for nanoMIL-100 (1.1%); 10.3 ± 4.1% for nanoMIL-101 (1.1%); 14.7 ± 0.3% for nanoUio-66 (1.8%); and 10.1 ± 0.8% for nanoUio-66-NH 2 (2.6%). We have tentatively attributed these differences to insoluble, amorphous metal-containing species resulting from the reaction of the released metal ions in the cell culture media. In fact, this phenomenon has previously been observed for Zn II metal ions in cell culture media: for instance, ZnO nanoparticles have been reported to release Zn II into cell culture media or serum, which then rapidly reacts to form a poorly soluble, amorphous nanostructured Zn II-carbonate-phosphate precipitate. 35,36

In vitro cytotoxicity of the nanoMOF components

The cytotoxicity of each organic ligand and each metal ion (as a chloride salt; except for MgSO 4 ) used to build the nanoMOFs was individually evaluated in HepG2 cells, using the XTT assay. The cells were exposed to a single organic ligand or metal salt at doses ranging from 1 to 200 μM for 24 h (see Figure S6 and S7). None of the organic ligands showed any significant cytotoxicity, even at the highest dose, nor did the Co II, Ni II, Zn II, or Mg II salts: cell viabilities were greater than 75% in all cases. In contrast, the Cu II and Mn II salts exhibited high cytotoxicity, even at low doses (5 to 10 μM); and Fe III showed moderate to high cytotoxicity from 25 to 200 μM, respectively.

In vitro cytotoxicity of the nanoMOFs

The effect of each nanoMOF on cell viability was tested in HepG2 cells and in MCF7 cells using the XTT assay, at doses ranging from 25 to 200 μM, for 24 h and 72 h. Firstly, prior to the assay, the DMSO colloid of a given nanoMOF was mixed with the cell culture medium. We would like to mention that the formation and use of these colloids is an intermediate but necessary step for minimizing any possible aggregation of nanoMOFs in the cell-culture medium. However, despite this step, many of the nanoMOFs still gradually agglomerated in both media, forming soft agglomerates. Thus, under these conditions, we were unable to differentiate between toxicity arising from single nanocrystals of each nanoMOF and toxicity arising from their corresponding agglomerates. For most of the nanoMOFs, their respective cytotoxicity depended on the cell type and on the concentration (see Figure 2 and Figure S8 in the Supporting Information). Interestingly, some of the nanoMOFs (Co-MOF-74, Mg-MOF-74, Uio-66, and Uio-67) showed little or no cytotoxicity, even at the highest dose (200 μM). Within the nanoMOF-74 family, the cytotoxicity of each member varied according to the metal component (Figure 2): those containing Co, Ni or Mg showed no marked cytotoxicity to either of the cell lines after 24 h of incubation, whereas those containing Cu, Mn or Zn showed high levels of cytotoxicity at the highest dose (200 μM). The viability levels observed in the cells exposed to the latter were 20.9 ± 2.7% (HepG2) and 38.2 ± 0.3% (MCF7) for nanoCu-MOF-74; 18.3 ± 3.0% (HepG2) and 32.4 ± 3.8% (MCF7) for nanoMn-MOF-74; and 38.8 ± 3.6% (HepG2) and 57.6 ± 0.6% (MCF7) for nanoZn-MOF-74. These results were generally consistent with those observed at 72 h (see Figure S8), although some differences were identified. For example, the cytotoxicity of nanoNi-MOF-74 to MCF7 cells was higher after 72 h, whereas that of nanoCu-MOF-74 to the same cells, and that of nanoZn-MOF-74 to HepG2 cells, were each lower after 72 h. NanoMIL-100 and -101 showed little or moderate toxicity to each cell line after 24 and 72 h. However, at its highest dose (200 μM) and 24 h incubation, nanoMIL-100 provoked a substantial decrease (53.2 ± 1.0%) in the viability of the HepG2 cells. After 72 h, nanoMIL-101 also led to a decrease in the viability (41.8 ± 7.3%) of HepG2 cells. None of the nanoUio MOFs exhibited substantial cytotoxicity at either incubation time, except for
nanoUiO-66-NH₂: at the highest dose (200 μM) and 24 h incubation, this material showed moderate cytotoxicity to HepG2 cells (47.2 ± 9.4% viability). The results for the nanoZIF family of MOFs varied widely: nanoZIF-7 was not cytotoxic to the HepG2 cells and moderately cytotoxic to the MCF7 cells, whereas nanoZIF-8 was highly cytotoxic to both. Both nanoHKUST-1 and nanoNOTT-100 were highly cytotoxic to both cell lines at both incubation times; for example, at 200 μM and 24 h incubation, nanoHKUST-1 was highly toxic to HepG2 cells (17.4 ± 0.5% viability). The only exception was nanoNOTT-100 at 200 μM after 24 h incubation in MCF7 cells (94.2 ± 4.0% viability). Lastly, at 200 μM and 24 or 72 h incubation time, nanoMOF-5 was highly cytotoxic to both cell lines.

The toxicity of nanomaterials is widely accepted to derive from their chemical composition, through mechanisms such as dissolution and consequent release of toxic components (e.g., metal ions) or generation of reactive oxygen species, and/or to stress or stimuli caused by their surface reactivity, their size and/or their shape. Although distinguishing between these mechanisms is not trivial, our results indicate that the toxicity of nanoMOF crystals is strongly related to their solubility and, therefore, to the release of solubilized (toxic) metal ions. The solubility tests on the sixteen nanoMOFs confirmed that they are all at least partially soluble in cell culture media and show minimum percentage of degradation in solution, ranging from 0.3% (nanoUiO-67) to 62.9% (nanoMg-MOF-74). This degradation induces the release of their constituent metal ions and organic ligands into the media.

Given that free metal ions, rather than free organic ligands, have previously been imputed as the toxic agents in MOFs, we focused our attention on the metal ions solubilized in the media. Indeed, we found that the most cytotoxic nanoMOFs in our assays were those that released sufficiently high amounts of soluble metal ions known to be moderately or highly cytotoxic in their free form (e.g., Cu²⁺, Mn²⁺, and Fe³⁺). For example, nanoCu-MOF-74, nanoHKUST-1, and nanoNOTT-100, all of

Figure 2. In vitro cytotoxicity of nanoMOFs to human cells. Cell viability of MCF7 or HepG2 cells after 24 h incubation with a single nanoMOF at one of various concentrations (25 to 200 μM). Untreated cells were used as controls (marked as C). Data represent the mean ± standard error of the mean (SEM) of three independent experiments. Significant differences from the control are marked accordingly (*p < 0.05, **p < 0.01, ***p < 0.005).
which are built from Cu\textsuperscript{2+} ions, were highly soluble and toxic in our tests. We believe that this toxicity, similarly to that observed with other soluble nanomaterials,\textsuperscript{[36-40]} might be attributed to the release and subsequent cellular uptake of Cu\textsuperscript{2+} ions. For example, nanoCu-MOF-74 was highly cytotoxic and provoked a rapid toxic effect: at nanoCu-MOF-74 concentrations of 50 and 100 \textmu M, and 24 h of incubation, the viability of treated HepG2 cells had decreased to approximately 40 and approximately 30\%, respectively. This result is consistent with our cytotoxicity studies on free Cu\textsuperscript{2+} ions (see Figure S6): for example, at a Cu\textsuperscript{2+} concentration of 50 \textmu M and 24 h of incubation, the viability of treated HepG2 cells had dropped to 23.9\%. Assuming a similar rate of release of Cu\textsuperscript{2+} over the entire range of concentrations, this viability value for Cu\textsuperscript{2+} alone corresponds to approximately 50 \textmu M of nanoCu-MOF-74 (47.9\% of constituent Cu\textsuperscript{2+} lost; 38.7\% cell viability for the same incubation time), approximately 55 \textmu M of nanoHKUST-1 (30.3\% of constituent Cu\textsuperscript{2+} lost; cell viability: <60\%) and approximately 63 \textmu M of nanoMOFs in our studies on free Mn\textsuperscript{2+} ions and on nanoMn-MOF-74. At the lowest concentration (25 \textmu M) and 24 h of incubation, this nanoMOF caused the viability of HepG2 cells to decrease to 49.9\%. At this concentration, a release of approximately 3 \textmu M Mn\textsuperscript{2+} (13.3\% of the constituent Mn\textsuperscript{2+}) would be expected, which would correlate to cell viabilities of between 30 and 70\%.

Unlike the aforementioned cases, the nanoMOFs that release significant amounts of soluble metal ions, but whose free constituent metal ion did not show cytotoxicity in the free form at the working concentrations (Co\textsuperscript{2+}, Ni\textsuperscript{2+}, or Mg\textsuperscript{2+}), showed little or no toxicity. These include nanoMg-MOF-74, nanoCo-MOF-74, and nanoNi-MOF-74. Similarly, those nanoMOFs that do not release significant amounts of soluble metal ions showed low cytotoxicity. These include nanoMIL-100, nanoMIL-101, nanoUIO-66, nanoUIO-66-NH\textsubscript{2}, nanoUIO-67, and nanoZIF-7. The only exceptions to this trend were three of the Zn\textsuperscript{2+}-based nanoMOFs: nanoZn-MOF-74, nanoZIF-8, and nanoZIF-5. In all three cases, we expected the concentration of Zn\textsuperscript{2+} ions released from the nanoMOF to be lower than the maximum working concentration of 200 \textmu M (at which free Zn\textsuperscript{2+} ions did not show any significant cytotoxicity; in fact, the in vitro toxic concentration of Zn\textsuperscript{2+} ions has been reported to be ca. 400 \textmu M).\textsuperscript{[41]} However, all three nanoMOFs caused high cytotoxicity at 200 \textmu M. Interestingly, these results are consistent with recent studies on nanoMRFM-3 that showed cytotoxicity to PC12 cells; in that study, cell viability was approximately 55\% at 122 \textmu M and <20\% at 488 \textmu M.\textsuperscript{[42]} They are also consistent with findings from studies on ZnO or Zn nanoparticles, the toxicity of which could not be imputed solely to their release of Zn\textsuperscript{2+} ions into solution.\textsuperscript{[43]} In fact, several authors have suggested that Zn nanoparticles might generate reactive oxygen species that induce oxidative stress\textsuperscript{[36,44-46]} or that exhibit body burden or size effects; any of these factors might have contributed to the cytotoxicity that we observed in the Zn\textsuperscript{2+}-based nanoMOFs.

In vivo toxicity in zebrafish embryos

We then evaluated the in vivo toxicity of nine of the sixteen nanoMOFs in zebrafish (Danio rerio) embryo, and subsequently compared the results to those corresponding to the in vitro assays. Zebrafish embryos have been used extensively to assay the in vivo toxicity of nanoparticles because they are comparable to mammalian systems and amenable to medium-to-high-throughput screening.\textsuperscript{[47-50]} Zebrafish embryos exposed to different concentrations (1 to 200 \textmu M) of a suspension of a single nanoMOF were assessed for several toxicity parameters every 24 h until 120 h post-fertilization (hpf); these include mortality, hatching rate, and appearance of abnormal phenotypes (e.g., low pigmentation, pericardial/yolk-sac edema, delayed development, bent spine, etc.).

We chose nine nanoMOFs for in vivo evaluation to represent a wide spectrum of cytotoxicity from the in vitro assays, including nontoxic or barely cytotoxic (nanoUIO-66, nanoUIO-67, and nanoCo-MOF-74, and nanoMg-MOF-74), moderately cytotoxic (nanoZIF-7, nanoMIL-100, and nanoMIL-101), and highly cytotoxic (nanoZIF-8 and nanoHKUST-1). To rule out any effects provoked by the 0.5\% DMSO present in the three nanoMOF suspensions (1 \textmu M, 20 \textmu M and 200 \textmu M), we also assessed its toxicity. At a final concentration of 0.5\% in E3 medium, DMSO was found to have no visible effects on normal larvae development after 120 h exposure: no differences in survival rate, hatching rate, or phenotype were observed between the DMSO-treated group of larvae and the negative (untreated) control group.

Figure 3a shows the cumulative mortality of embryos from the assays. We found that at 120 h post fertilization (hpf), the vast majority of nanoMOFs had not altered embryo viability. Hence, the viabilities of the embryos exposed to nanoCo-MOF-74, nanoMg-MOF-74, nanoUIO-66, nanoUIO-67, and nanoMIL-100, and nanoMIL-101 were not significantly different to those of the control (DMSO-treated) group. In contrast, nanoZIF-7, nanoZIF-8, and nanoHKUST-1 provoked significant decreases in embryo survival (Figure 3 and S9). NanoZIF-7 was slightly toxic at 200 \textmu M (embryo viability at 120 hpf: 79.2\%); nanoZIF-8 was more toxic at the same concentration (embryo viability at 120 hpf: 33.3\%); and nanoHKUST-1 was extremely toxic at this concentration (viability: 0\%); in fact, even at 20 \textmu M nanoHKUST-1, none of the embryos treated with this nanoMOF had survived by 48 hpf (viability: 0\%).

We quantified the hatching rate of the embryos in the nanoMOF-treated group and the control group. As shown in Figure 3b and c, some of the nanoMOF-treated group exhibited a significant concentration-dependent hatching delay. Many previous studies have reported that fish embryos exposed to nanoparticles exhibit delayed hatching,\textsuperscript{[40,47,51]} although whether this delay is caused by the whole nanoparticles themselves or by their released constituent materials remains unknown.\textsuperscript{[52,53]} Some researchers have proposed that the delay might be caused by interactions between the nanoparticles and the zebrafish hatching enzyme, which is crucial for digesting the inner layer of the chorion (the membrane surrounding the embryo).\textsuperscript{[52,54]}

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When incubated under normal conditions, control embryos hatched from their chorion between 48 and 72 hpf (Figure 3c), exhibiting slightly accelerated hatching, similar to an effect that has been previously reported. However, the embryos incubated with nanoZIF-7 or nanoHKUST-1 hatched significantly later than did the control embryos (Figure 3b and c, and Figure S10 in the Supporting Information), and of these two treated groups, only the nanoZIF-7 group reached 100% hatching rate. In the case of nanoHKUST-1, the hatching delay was only quantified at a nanoHKUST-1 dose of 1 μM, because no embryos survived at the higher doses. Interestingly, embryos exposed to the highest dose (200 μM) of nanoZIF-8 or nanoMIL-101 did not hatch at all by 120 hpf. It is important to mention here that nanoZIF-8 has an effect on the survival at this highest dose (200 μM). No significant hatching delay was observed in the groups exposed to nanoCo-MOF-74, nanoMg-MOF-74, nanoUiO-66, nanoUiO-67, or nanoMIL-100.

To further characterize the in vivo toxicity of nanoMOFs, we also quantified the morphological defects observed on the treated embryos after they had hatched (Figure 4 and Figure S11 in the Supporting Information). No significant malformations were found in the embryos that had been exposed to nanoMg-MOF-74; significant levels of yolk sac edema were found in the nanoCo-MOF-74, nanoUiO-66, nanoUiO-67, and nanoMIL-100 groups; concentration-dependent yolk sac edemas and significant pericardial edema was found in the nanoMIL-100 group. No further putative morphological defects could be detected in the groups exposed to 200 μM of nanoZIF-8 or...
nanoMIL-101, due to their inability to hatch, although some morphological malformations were observed in the groups exposed to lower doses of nanoZIF-8 (pericardial edema, yolk-sac edema and bent spine) or nanoMIL-101 (yolk-sac edema) (see Figure S11). Interestingly, significant levels of pericardial edema, yolk-sac edema, and bent spine were observed in the group that had been exposed to nanoHKUST-1 at the low dose of 1 μM; however, the malformations in the groups exposed to higher doses were not characterized because none of the embryos had survived.

Ranking of the in vivo toxicity of nanoMOFs

To compare the in vivo toxicity of the nine studied nanoMOFs, we quantified their in vivo effects on the zebrafish embryos using a scoring system that was first described by Peterson et al.\(^\text{[56, 30]}\) and subsequently modified by Nel et al.\(^\text{[47]}\). Thus, we scored the amount and severity of the phenotypic changes, on a scale from 0 to 4, whereby 0 = normal phenotype; 1 = a minor phenotypic change; 2 = multiple moderate alterations; 3 = severe embryo deformation; and 4 = no survival (see Table 2). The nanoMOFs scored as follows: 0 (nanoMg-MOF-74); 1 (nanoCo-MOF-74, nanoUiO-66, and nanoUiO-67); 2 (nanoMIL-100 and nanoZIF-7); 3 (nanoZIF-8 and nanoMIL-101); and 4 (nanoHKUST-1).

Comparison of the in vitro toxicity and the in vivo toxicity results for the nanoMOFs

We found a strong correlation between the in vitro toxicity results and the in vivo toxicity results for the nine nanoMOFs that were tested in both assays (Table 3). The only deviation from this trend was that observed for nanoMIL-101, which was

Table 2. Ranking of nanoMOFs by in vivo toxicity, according to a semiquantitative scoring system (adapted from Nel et al., 2011\(^\text{[47]}\); and Furgerson et al., 2009\(^\text{[30]}\)).

<table>
<thead>
<tr>
<th>Score</th>
<th>Attributes</th>
<th>nanoMOF</th>
<th>Morphological defects</th>
<th>Physiological defects</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>no morphological or physiological defects</td>
<td>nanoMg-MOF-74</td>
<td>yolk sac edema</td>
<td>mortality, reduced hatch-</td>
</tr>
<tr>
<td>1</td>
<td>single morphological/physiological defect</td>
<td>nanoCo-MOF-74, nanoUiO-66 and nanoUiO-67</td>
<td>pericardial and yolk sac edema, bent spine</td>
<td>ing rate, mortality, embryos failed to hatch</td>
</tr>
<tr>
<td>2</td>
<td>multiple morphological and physiological defects</td>
<td>nanoMIL-100 and nanoZIF-7</td>
<td>pericardial and yolk sac edema, bent spine</td>
<td></td>
</tr>
<tr>
<td>3</td>
<td>severe multiple morphological and physiological defects</td>
<td>nanoMIL-101 and nanoZIF-8</td>
<td>disintegrated embryo</td>
<td></td>
</tr>
<tr>
<td>4</td>
<td>embryos do not survive</td>
<td>nanoHKUST-1</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
more toxic to zebrafish embryos (adverse effect on hatching) than it was to HepG2 or MCF7 cells.

Conclusion

We have assessed the in vitro toxicity and in vivo toxicity of a series of representative, uncoated, nanoscale metal-organic frameworks (nanoMOFs). We first screened sixteen nanoMOFs against two human cell lines (HepG2 and MCF7), and then screened a diverse set of nine of these nanoMOFs in zebrafish embryos. We found a strong correlation between the in vitro toxicity results and the in vivo toxicity results, with nanoMg-MOF-74 being the least toxic in both assays. The only exception to this trend was nanoMIL-101, which was more toxic to embryos than to cell cultures. Our findings suggest that degradation of nanoMOFs in solution generates metal ions that strongly determine the toxicity of these nanomaterials. However, other factors might also influence the toxicity of nanoMOFs, including the formation of other species upon degradation, or certain crystal parameters (e.g., size, shape, charge, etc.). We affirm that more studies on the possible environmental and health risks of nanoMOFs must be performed before these new nanomaterials can be exploited for practical use.

Experimental Section

Materials

All reagents and solvents used in the nanoMOF syntheses were purchased from Sigma-Aldrich and Romil, respectively, and were used without any further purification.

NanoMOF synthesis

The nanoMOFs were prepared by using modified versions of reported methods. The general procedures are detailed in the Supporting Information.

NanoMOF characterization

We characterized the synthesized nanoMOFs by XRPD, to determine their purity, and by transmission electron microscopy (TEM), field-emission scanning electron microscopy (FESEM) and dynamic light scattering (DLS), to determine their size distribution.

Table 3. Qualitative comparison between the in vitro and in vivo toxicity of the nanoMOFs studied in both assays.

<table>
<thead>
<tr>
<th>Grade of toxicity</th>
<th>In vitro</th>
<th>In vivo</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>−</td>
<td>nanoMg-MOF-74, nanoCoMOF-74, nanoUIO-66 and</td>
<td>(0) nanoMg-MOF-74</td>
</tr>
<tr>
<td>+</td>
<td>nanoUIO-67</td>
<td>(1) nanoCoMOF-74, nanoUIO-66 and nano-</td>
</tr>
<tr>
<td>+ +</td>
<td>nanoMIL-100, nanoZIF-7 and nanoMIL-101</td>
<td>(2) nanoMIL-100 and nanoZIF-7</td>
</tr>
<tr>
<td>+ + +</td>
<td>nanoZIF-8</td>
<td>(3) nanoZIF-8 and nanoMIL-101</td>
</tr>
<tr>
<td>+ + + +</td>
<td>nanoHKUST-1</td>
<td>(4) nanoHKUST-1</td>
</tr>
</tbody>
</table>

Evaluation of the in vitro toxicity of the nanoMOFs and their constituent components to HepG2 and MCF7 cells

Human hepatocyte (HepG2) and breast cancer (MCF7) cell lines were separately incubated with a single nanoMOF, and then the effects of the nanoMOFs on cell viability were assessed by using the XTT (2,3-bis-(2-methoxy-4-nitro-5-sulphophenyl)-2H-tetrazolium-5-carboxanilide) cell viability assay after 24 and 72 h incubation time. The cells were cultured in either DMEM (Dulbecco’s modified Eagle’s medium; Invitrogen) for HepG2 cells or DMEM F12 (for MCF7 cells), containing GlutaMax 1 and supplemented with 10% fetal bovine serum (FBS) (Invitrogen) at 37 °C, 5% CO2, and 95% humidity. The cells were seeded into 96-well plates (cell density: 4.0 × 10^3 cells/well), incubated for 24 h, and then exposed to fresh medium containing a DMSO suspension of the desired nanoMOF (concentrations: 25 to 200 μM). At 24 and 72 h incubation, aliquots of 20 μL of XTT solution were added to each well, and the resulting color was quantified (λ = 450 nm) in a spectrophotometric plate-reader (PerkinElmer Victor3V). Cell viability was expressed as a percentage of the control level. All the measurements were performed in triplicate.

Stability studies

A carefully weighed sample of each nanoMOF was separately dispersed in 10 mL of DMEM medium with 10% FBS to achieve a final concentration of 10 mM. Each mixture was incubated at 37 °C for 24 h with gentle stirring, and then centrifuged for 5 min at 10,000 rpm (Alegria 64R, Beckman Coulter). Each supernatant was characterized by ICP-OES with an Optima 4300 DV unit (Perkin-Elmer) to determine the amount of solubilized metal ions. In parallel, each pellet was washed with 10 mL of deionized water to remove buffer salts, and then centrifuged. This process was repeated with methanol, and the resulting solids were dried in an oven at 90 °C overnight, weighed and finally, characterized by XRPD. All the experiments were performed in triplicate.

Exposure of zebrafish embryos to nanoMOFs

Adult zebrafish (Danio rerio) were maintained in tanks with recirculating water under a 14 h light/10 h dark cycle at 28 °C. Male and female zebrafish were set up in pairs for breeding in breeding tanks. A grid insert in the tanks enabled the resulting embryos to fall to the bottom, avoiding parental predation. The embryos were collected in E3 medium (5 mM NaCl, 0.17 mM KCl, 0.33 mM CaCl2, 0.33 mM MgSO4, and 0.1% Methylene Blue), rinsed carefully to remove debris, and kept at 28 °C in an incubator. The embryos were visually assessed with a microscope (Olympus, CXX31, Japan) for viability and developmental stage, and the selected healthy specimens were plated into 96-well plates at 1 embryo/well. Starting at 5 h post-fertilization, the embryos were exposed to 200 μL of a solution of a single nanoMOF at different concentrations.
(1, 20, and 200 μm; final concentration of DMSO in E3 medium: 0.5%), or to a DMSO control solution (0.5% DMSO in E3 medium), or to E3 medium alone (negative control). The embryos were assessed for hatching rate, cumulative mortality and malformations at 24, 48, 72, 96, and 120 hpf. Screening for morphological defects included the assessment of abnormally developed eyes; lack of somite formation; delayed development; pericardial edema; yolk-sac edema; and bent spine. The most frequently found malformations (pericardial edema, yolk-sac edema, and bent spine) were quantified. Embryos with abnormal morphologies were anaesthetized with ethyl 3-aminobenzoate methanesulfonate (160 ppm MS-222, Sigma), transferred onto microscope slides, and then photographed in a Leica Stereomicroscope MZ FLIII. Each condition was tested in 24 individuals. All experimental procedures were submitted to the Ethical Committee of the Universitat Autonoma de Barcelona, and the procedures follow the International Guiding Principles for Research Involving Animals.

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Metal–Organic Frameworks

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Synthesis, Culture Medium Stability, and In Vitro and In Vivo Zebrafish Embryo Toxicity of Metal-Organic Framework Nanoparticles

Relating in vivo to in vitro: In vitro and in vivo zebrafish embryo toxicity studies on sixteen synthesized nano-metal–organic frameworks (MOFs) are reported (see figure). It was demonstrated that there is a strong correlation between their in vitro toxicity and their in vivo toxicity. The nanoMOFs were ranked according to their respective in vivo toxicity.
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