Enhancing anticancer cytotoxicity through bimodal drug delivery from ultrasmall Zr MOF nanoparticles†

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Dual delivery of dichloroacetate and 5-fluorouracil from Zr MOFs into cancer cells is found to enhance in vitro cytotoxicity. Tuning particle size and, more significantly, surface chemistry, further improves cytotoxicity by promoting caveolae-mediated endocytosis and cytosolic cargo delivery.

Effective cell internalisation and intracellular drug release are vital characteristics of effective nanoparticulate drug delivery systems (DDSs). Nanoparticles are generally internalised through active transport mechanisms such as endocytosis, however, if they are small enough (<20 nm), nanoparticles can be internalised by passive diffusion, enabling direct release of cargo into the cytosol. Metal–organic frameworks (MOFs) – network structures composed of metal clusters linked by multidentate organic linkers with high porosity – offer the desirable combination of large cargo payloads and tunable structural features, with zirconium MOFs, which have requisite chemical stability for aqueous use, being emerging as promising potential DDSs.

We have previously shown that 50–600 nm nanoparticles of UiO-66, the zirconium 1,4-benzenedicarboxylate (bdc) MOF with ideal formula [Zr₆O₄(OH)₄(bdc)₆]ₙ, and its –Br, –NO₂, and –NH₂ functionalised derivatives (L₁–L₄ in Fig. 1a), undergo HeLa cancer cell internalisation primarily through clathrin-mediated endocytosis, while isoreticular MOFs with more hydrophobic extended linkers, such as 2,6-naphthalenedicarboxylate (L₅) and 4,4’-biphenyldicarboxylate (L₆), are partially internalised through caveolae-mediated endocytosis, with no induction of cytotoxicity at concentrations up to 1 mg mL⁻¹. Similarly, coating surfaces of UiO-66 nanoparticles with poly(ethylene glycol) can promote caveolae-mediated uptake, allowing drug-loaded MOFs to avoid the lysosomal degradation that is characteristic of clathrin-mediated endocytosis and release their cargo in the cytosol, thus enhancing therapeutic efficiency.

This example utilised the anticancer metabolic molecule, dichloroacetate (DCA), as a modulator of UiO-66 solvothermal synthesis, showing that it can be incorporated into UiO-66 nanoparticles at defects and on their surfaces (Fig. 1b), yielding regular, well-dispersed, porous nanoparticles of around 100 nm in size. Herein, we (i) investigate the DCA modulation protocol across the isoreticular series of Zr MOFs illustrated in Fig. 1, (ii) show that very small (ca. 20 nm), highly defective, DCA-containing Zr MOFs can be prepared and loaded with a second anticancer drug, and (iii) demonstrate enhanced in vitro cancer cell cytotoxicity of the dually active DDSs.

DCA is a pyruvate d-kinase inhibitor, which is over expressed in cancerous cells, and its cytotoxic effects on cancer cells depend on effective cytosolic release and mitochondrial localisation, thus making it an ideal mechanistic probe molecule for cell uptake. To promote cytotoxic release through passive diffusion, we have tuned our previously reported synthetic conditions for DCA@Zr-L₁ with the aim of obtaining smaller, DCA-loaded nanoparticles (<20 nm) of the MOFs Zr-L₁–Zr-L₆ (ESI, † Section S2). Solvothermal reaction of ZrOCl₂ with 2.5 eq. of linker and 18.2 eq. of dichloroacetic acid yields solids whose powder X-ray diffraction (PXRD) patterns (Fig. 2a) show Bragg peaks characteristic of the UiO-66 topology. When terephthalate linkers (L₁ to L₄) are used, the diffraction patterns have broad, low intensity peaks, suggesting
small and defective particles, as a consequence of DCA attachment to the Zr₆ clusters in place of linkers.

The terephthalate MOFs present type IV N₂ adsorption and desorption isotherms (77 K) with H₂ hysteresis loops typical of interconnected networks of pores with different size and shape (illustrated for DCA@Zr-L₁ small in Fig. 2d). These suggest highly defective structures, although some contribution of inter-particle space should be considered. The BET surface areas are lower than defect-free UiO-66 (1200 m² g⁻¹), but the additional, defect-induced mesoporosity results in extremely high pore volumes, ranging from 0.8 to 1.2 cc g⁻¹. DCA@Zr-L₅ (Fig. 2d) and DCA@Zr-L₆ (Fig. S7, ESI†) are also porous but do not exhibit hysteresis, in concert with PXRD and SEM analysis that indicate larger, less defective particles. The BET surface areas and pore volumes are given in Table 1.

Nuclear magnetic resonance (NMR) spectra of acid-digested samples confirm significant incorporation of DCA, while FT-IR spectra show appearance of vibration bands characteristic of DCA, but shifted to indicate its attachment at defect sites. NMR spectra of acid-digested DCA@ MOFs at (77 K) show appearance of vibration bands characteristic of DCA, while FT-IR spectra confirm significant incorporation of DCA, while FT-IR spectra show appearance of vibration bands characteristic of DCA, but shifted to indicate its attachment at defect sites.

**Table 1** Pertinent physical characteristics of the DCA@MOFs

<table>
<thead>
<tr>
<th>Size/nm (SEM)</th>
<th>% DCA w/w</th>
<th>BET SA/m² g⁻¹</th>
<th>Pore vol/cc g⁻¹</th>
<th>% 5-FU w/w</th>
</tr>
</thead>
<tbody>
<tr>
<td>DCA@Zr-L₁ small</td>
<td>12.8 ± 3.6</td>
<td>26.2</td>
<td>891</td>
<td>0.87</td>
</tr>
<tr>
<td>DCA@Zr-L₂ small</td>
<td>30.2 ± 7.9</td>
<td>19.3</td>
<td>639</td>
<td>0.81</td>
</tr>
<tr>
<td>DCA@Zr-L₃ small</td>
<td>21.7 ± 5.3</td>
<td>21.5</td>
<td>901</td>
<td>1.12</td>
</tr>
<tr>
<td>DCA@Zr-L₄ small</td>
<td>26.5 ± 2.9</td>
<td>26.4</td>
<td>990</td>
<td>1.21</td>
</tr>
<tr>
<td>DCA@Zr-L₅</td>
<td>232 ± 30</td>
<td>14.1</td>
<td>764</td>
<td>0.42</td>
</tr>
<tr>
<td>DCA@Zr-L₆</td>
<td>196 ± 32</td>
<td>6.6</td>
<td>2241</td>
<td>0.99</td>
</tr>
</tbody>
</table>

* Determined after postsynthetically loading 5-FU into the DCA@MOF sample.
ones determined by SEM for the positively charged DCA@Zr-L4 small aggregates of ~100 nm for the rest of the smaller MOFs. Aggregation of DCA@Zr-L6 (~200 nm) also decreased.

The cytotoxicity of the DCA@MOFs towards MCF-7 breast cancer cells was assessed using the MTS cell proliferation assay (ESI† Section S5). Free DCA has little effect on cell proliferation, as its hydrophilic nature results in poor internalisation;17 a decrease in cell viability was observed only for concentrations >3 mg mL⁻¹ (Fig. S28, ESI†). To examine the effect of ligand functionality, the cytotoxidades of the larger DCA-containing terephthalate derivatives (ca. 70–130 nm) were compared with DCA@Zr-L5 and DCA@Zr-L6 (ca. 200 nm), and plotted against DCA concentration (Fig. 3a). DCA@Zr-L5 and DCA@Zr-L6 are most therapeutically active, decreasing MCF-7 viabilities to around 35% when delivering <0.1 mg mL⁻¹ of DCA. These results correlate well with the enhanced cytotoxicity towards HeLa cancer cells of Zr-L6 when delivering the anti-cancer drug 5-cyano-4-hydroxycinnamic acid,6 likely as a consequence of the preference of Zr-L5 and Zr-L6 for caveolae-mediated endocytosis promoting efficient cytosolic cargo release,7 rather than size, as empty Zr-L5 and Zr-L6 samples of varying size were found to not be cytotoxic towards HeLa cells.8

DCA@Zr-L1, in contrast, shows no cytotoxicity towards MCF-7, likely due to clathrin-mediated endocytosis leading to lysosome localisation; our previous work showed that a similar material has no cytotoxicity towards HeLa cells at similar concentrations.9 DCA@Zr-L2 and DCA@Zr-L3 only reduce proliferation to 61 ± 16% and 81 ± 15%, respectively, at the highest delivered DCA concentrations, while the enhanced therapeutic effect of DCA@Zr-L4, with cell viabilities similar to DCA@Zr-L5, could be a result of the positive surface charge of protonated amino units in L4 enhancing internalisation efficiency.18 The effect of particle size was assessed by comparing the cytotoxidades of the DCA@Zr-LX small derivatives (~20 nm) towards MCF-7 cells with their larger DCA@Zr-LX analogues (~100 nm), and generally the smaller nanoparticles showed enhanced cytotoxicity when plotted against DCA concentration, suggesting enhanced internalisation and cell uptake by passive diffusion resulting in cytosolic release.18† Fig. 3b shows the more pronounced cytotoxicity of DCA@Zr-L3 small compared to its larger analogue, which shows no appreciable deleterious effects, with similar trends observed for DCA@Zr-L1 small and DCA@Zr-L2 small (Fig. S31, ESI†). Only DCA@Zr-L4 small (ca. 13 nm) was less efficient than its larger analogue DCA@Zr-L6 (ca. 86 nm), but both samples still reduced cell proliferation, again likely due to their surfaces having significant positive charge. It has been reported that DCA enhances the cytotoxic activity of anticancer drugs such as 5-fluorouracil (5-FU) while reducing cancer cells' resistance towards them.17,19 As such, the smaller, DCA-loaded terephthalate MOF samples that had shown generally enhanced cytotoxicity, along with DCA@Zr-L5 and DCA@Zr-L6, were postsynthetically loaded with 5-FU to generate multimodal DDSs (ESI† Section S6). Thermogravimetric analysis cannot distinguish between loaded DCA and 5-FU, although it suggests some loss of DCA during 5-FU loading for the small terephthalate MOFs. The loading of 5-FU, shown in Table 1, was calculated by UV-Vis spectroscopy, and found to range from 1.5–4.3% w/w.

The enhanced cytotoxicity (ESI† Section S7) of all the 5-FU@DCA@MOFs towards MCF-7 cells compared to their DCA@MOF precursors, despite the decrease in DCA content, is clearly observed when cell proliferation is plotted against MOF concentration (Fig. 4a) suggesting successful intracellular deliver of 5-FU. Of the smaller MOF species, 5-FU@DCA@Zr-L1 small exhibits a more significant dose–response effect than its precursor, decreasing cell viability with concentration down to 21 ± 7% at 1 mg mL⁻¹. The cytotoxicity of 5-FU@DCA@Zr-L2 small increases only slightly compared to its precursor, whereas 5-FU@DCA@Zr-L3 small and 5-FU@DCA@Zr-L4 small have more notable enhancements, with cell viabilities of 19 ± 7% and 33 ± 8%, respectively, when MCF-7 cells were incubated with 0.5 mg mL⁻¹ of the MOFs.

Fig. 3 (a) MCF-7 cell proliferation on incubation with the larger DCA@MOFs. Exact cell viability values are tabulated in the supporting information alongside plots against MOF concentration. (b) Comparison of MCF-7 cell proliferation for smaller and larger DCA@Zr-L3 and DCA@Zr-L4 samples.
The most effective of the DCA@MOFs also showed further enhancements in cytotoxicity towards MCF-7 cells when loaded with 5-FU; cell viability drastically decreases to values of 7 ± 6% and 4 ± 6% when cells were incubated with just 0.5 mg mL\(^{-1}\) of 5-FU@DCA@[Zr-L5] and 5-FU@DCA@[Zr-L6], respectively.

Free 5-FU itself also has significant dose-responsive cytotoxic behaviour (Fig. 4b), with an IC\(_{50}\) of 0.015 ± 0.001 mg mL\(^{-1}\), but plotting cytotoxicity of the 5-FU@DCA@MOF samples against 5-FU concentration shows they have a greater effect than the free drug at lower concentrations, which might be a consequence of more efficient or faster internalisation, or a synergistic effect of DCA and 5-FU delivered in tandem, given that the cytotoxicity of free 5-FU is not enhanced when administered with DCA (Fig. S36, ESI). At higher concentrations 5-FU@DCA@[Zr-L1\text{small}] and 5-FU@DCA@[Zr-L4\text{small}] continue to exhibit greater cytotoxic effects than the free drug, while 5-FU@DCA@[Zr-L3\text{small}] has no notable enhancement and 5-FU@DCA@[Zr-L2\text{small}] has a poorer performance than free 5-FU. Again, the larger samples, 5-FU@DCA@[Zr-L5] and 5-FU@DCA@[Zr-L6] have the most pronounced cytotoxic effects, significantly enhancing the efficacy of free 5-FU and killing nearly all cells at all measured concentrations, suggesting that it is the surface chemistry of the MOFs that influences cellular uptake, and thus cytotoxicity, to a greater extent.

We have shown that incorporation of DCA at defect sites during the modulated synthesis of Zr MOFs offers (i) particle size control in the assembly of highly defective ~20 nm nanoparticles of hierarchically porous materials, (ii) high loading (15–25% w/w) of the anticancer probe molecule DCA, and (iii) porous MOFs into which further medicinal cargo can be loaded. On the whole, the smaller (~20 nm) DCA-loaded particles exhibit greater cytotoxicity towards MCF-7 cancer cells than their larger (~100 nm) analogues; we hypothesise that partial internalisation of the smaller MOFs through passive diffusion allows DCA release directly into the cytosol to enhance its therapeutic effects. However, the surface chemistry of the MOFs has a greater effect, with DCA@[Zr-L5] and DCA@[Zr-L6] the most therapeutically efficient MOFs, despite their bigger size, in agreement with our recent study on endocytosis mechanisms.\(^7\) Concurrent delivery of two drugs from the 5-FU@DCA@MOFs further enhances cytotoxicity compared to precursor DCA@MOFs and the free drugs. Delivery of multiple drugs from one DDS has the potential to overcome issues with resistance and poor efficacy, and is enabled by utilisation of different loading protocols; defect-loading of cargo into Zr MOFs is possible for any carboxylic acid containing drug.

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Conflicts of interest

There are no conflicts to declare.