Efficient “green” encapsulation of a highly hydrophilic anticancer drug in metal–organic framework nanoparticles

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Abstract

Metal–organic frameworks (MOFs) are coordination polymers of interest for biomedical applications. Of particular importance, nanoparticles made of iron(III) trimesate (MIL-100, MIL standing for Material Institut Lavoisier) (nanoMOFs) can be conveniently synthesised under mild and green conditions. They were shown to be biodegradable, biocompatible and efficient to encapsulate a variety of active molecules. We have addressed here the challenges to encapsulate a highly hydrophilic anticancer prodrug, phosphated gemcitabin (Gem-MP) known for its instability and inability to bypass cell membranes. MIL-100 nanoMOFs acted as efficient “nanosponges”, soaking Gem-MP from its aqueous solution with almost perfect efficiency (>98%). Maximal loadings reached ~30 wt% reflecting the strong interaction between the drug and the iron trimesate matrices. Neither degradation nor loss of crystalline structure was observed after the loading process. Storage of the loaded nanoMOFs in water did not result in drug release over three days. However, Gem-MP was released in media containing phosphates, as a consequence to particle degradation. Drug-loaded nanoMOFs were effective against pancreatic PANC-1 cells, in contrast to free drug and empty nanoMOFs. However, an efflux phenomenon could contribute to reduce the efficacy of the nanocarriers. Size optimization and surface modification of the nanoMOFs are expected to further improve these findings.

Introduction

Nucleoside analogs (NAs) are widely used in antiviral and anticancer treatments. They act as antimetabolite agents interfering with the synthesis of cellular or viral nucleic acids. NAs are prodrugs which must be converted by cellular kinases into their pharmacologically active triphosphate form [1]. However, drug resistance and systemic toxicity might occur when this intracellular conversion is not efficient [2–4]. The direct administration of active triphosphorylated NAs is hampered by their poor stability in biological media and poor cellular uptake due to the highly hydrophilic character of these drugs [5,5–7]. To address these issues, intensive research has been carried out to protect the active triphosphorylated NAs from degradation by: (i) synthesizing bioconjugates of phosphorylated NAs and lipophilic molecules [8] and (ii) encapsulation into nanocarriers intended to deliver them inside cells [9–20].

Among the explored encapsulation strategies, of main interest was poly(ethylene glycol)–poly(ethyleneimine) (PEI) polyplex nanogels showing important loading capacities. Fludarabine 5’-triphosphate and azidothymidine 5’-triphosphate (AZT-TP) were efficiently entrapped in nanogels and drug loading reached 33 wt% [16,19]. Protonated amino groups of PEI efficiently bind the active triphosphate form of fludarabine (F-TP) leading to compact flexible nanogels allowing a sustained drug release together with a protection of F-TP against enzymatic degradation [19]. In another approach, AZT-TP loadings as high as 42 wt% could be achieved by using hybrid organic–inorganic porous nanoparticles made of metal–organic frameworks (nanoMOFs) [20].

MOFs are coordination polymers of interest for biomedical applications that: (i) can be conveniently synthesised under mild and green conditions; (ii) possess biodegradability and biocompatibility; (iii) show important loading capability of a wide range of lipophilic, hydrophilic or amphiphilic drugs and (iv) have properties as contrast agents [20–28]. Of particular interest, iron(III) trimesate (MIL-100, MIL standing for Material Institut Lavoisier) [20] nanoMOFs possessed suitable stability under physiological conditions as compared to other iron carboxylate nanoMOFs. These materials have a crystalline structure with high and regular porosity, generated by the spontaneous formation of iron(III) octahedral trimers.

Keywords

Gemcitabin, “green” synthesis, metal–organic framework, MOF, nanoparticle, nucleoside analog, prostate cancer

History

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in the presence of trimesic acid (TA) [22]. The resulting mesoporous system is based on: (i) small cages (24 Å) accessible through pentagonal microporous windows (5.6 Å) and (ii) large cages (29 Å) delimited also by both pentagonal and hexagonal apertures (8.6 Å) [22] (Figure 1).

MIL-100 nanoMOFs have shown a high AZT-TP loading capability (up to 24 wt%) together with controlled release abilities [29]. The nanoMOFs acted as “molecular sponges” quickly soaking up AZT-TP molecules from their aqueous solutions. More precisely, the small and flexible AZT-TP molecules were adsorbed within the larger mesoporous cages and interacted through the phosphate groups by crossing the MOF large hexagonal windows with diameters of around 9 Å.

Figure 1. Schematic representation of the “green” (solvent-free) procedures involved in: (1) the synthesis of MIL-100 nanoMOFs and (2) the encapsulation of Gem-MP. Microwave-assisted hydrothermal synthesis of the nanoMOFs was carried out in water using iron chloride and TA as reactants. Gem-MP was encapsulated by incubating preformed nanoMOFs in aqueous solutions of the drug, which penetrated inside the interconnected porous structure by crossing the MOF large hexagonal windows with diameters of around 9 Å.

In many cases, among the three successive phosphorylation steps of NAs, the first one is the rate limiting step [31–33]. This is the case of gemcitabine (Gem), for which monophosphorylation catalyzed by the deoxycytidine kinase (dCK) is crucial for its further activation [34,35]. Gem (2,2'-difluorodeoxycytidine or dFdC, Scheme 1) is a cytidine drug against non-small cell lung and pancreatic cancer and several other solid tumors in monotherapy treatments as well as in regimens with other drugs [36–38]. Similar to other nucleoside-derived drugs, it depends on nucleoside transporters (NTs) to cross cell membranes [39,40]. Once internalized, Gem is phosphorylated by dCK into Gem monophosphate (Gem-MP), which is subsequently phosphorylated to the diphosphate (Gem-DP) and triphosphate (Gem-TP) forms [35,41]. The active Gem-TP exerts its cytotoxic effect mainly through inhibition of DNA and RNA synthesis by chain termination [42]. Gem-TP competes with the natural substrate, deoxycytidine triphosphate (dCTP), for incorporation into DNA and additionally inhibits ribonucleotide reductase [43] which catalyzes the formation of deoxyribonucleotides from ribonucleotides thus helping to reduce the dCTP concentration and resulting to higher incorporation of dFdCTP into DNA. However, more than 90% of administered Gem is converted into the inactive 2'-deoxy-2',2'-difluorouridine (dFdU) by cytidine deaminase (CDA) [35], thus lowering the activity of the drug which undergoes rapid catabolism and inactivation.

Cancer cells frequently lack dCK and/or NTs, which often results in the development of resistance to the treatment. Therefore, the direct administration of the phosphorylated active form of Gem would be a major breakthrough. However, the bioavailability of Gem is very limited, its efficacy being hampered by its short half-life [44] in vivo and extremely poor cell penetration [45]. To address this issue, we propose herein the use of MOF nanoparticles as carriers of Gem-MP, thus allowing bypassing the rate-limiting phosphorylation step toward the formation of the active Gem-TP [31–33]. The non-covalent “green” edifices are intended to efficiently entrap the challenging hydrophilic Gem-MP, protect it against degradation, thus allow increasing its efficacy against cancer cells. The proof of concept is given here with pancreatic PANC-1 cancer cells.

Methods

Chemicals

Reagents and solvents were used without further purification, otherwise indicated. Gemcitabine hydrochloride was purchased from Sequoia Research Products Ltd (Pangbourne, UK). Gemcitabine monophosphate, ammonium salt [5-3H(N)] was purchased from Moravek, Biochemicals Inc. (Brea, CA). It was packaged in sterile water solution at a concentration of 382.2 μg/mL, 2.0 mCi/mL (specific activity 2.3 Ci/mmol).

Radiochemical purity was 98.7%. Iron chloride hexahydrate (Alfa Aesar, Schiltigheim, France, 98%), 1,3,5-benzenetricarboxylic acid (Aldrich, Saint-Quentin-Fallavier, France, 98%) and absolute ethanol (Carlo Erba, Peyerin, France, 99%) were used for nanoMOF synthesis and activation. Phosphorus oxychloride, triethylamine and trimethylphosphate were from Aldrich (Saint-Quentin-Fallavier, France) and ammonium hydroxide was from Acros Organics (Geel, Belgium). 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (for MTT assay) was purchased from Life Technologies. Phosphate buffer saline (PBS, Dulbecco’s phosphate buffer...
saline without CaCl₂ and MgCl₂, 9.5 mM, Lonza, Levallois-Perret, France), RPMI (Roswell Park Memorial Institute, basal medium) were used as release or cell culture media. All solvents used were HPLC quality (Carlo Erba, Peyrin, France).

Milli-Q water was obtained from a Millipore (Guyancourt, France) apparatus equipped with a 0.22-μm filter.

### Synthesis of Gem-MP ammonium salt

The synthesis has been carried out by adapting a published procedure [46, as follows: to a solution of phosphorus oxychloride (675 μL, 7.243 mmol) in triethylphosphate (1.0 mL) at 4–5°C, gemcitabine hydrochloride (50 mg, 0.166 mmol) was added portionwise over 5 min. The reaction mixture was stirred at 5°C for 5 min, and then at 25°C for 2 h. The reaction mixture was carefully poured into a cold mixture of doubly distilled H₂O (7 mL) and Et₂O (15 mL). The aqueous layer was separated and washed with Et₂O (2 mL), decanting off the washings each time. Finally, the solid was washed with EtOH (1 mL). The pH of the aqueous layer was adjusted to pH 6.5 with conc. aq. NH₃ while chilling in an ice bath.

The aqueous layer was then concentrated under vacuum at <30°C to give a white solid residue. This residue was stirred with MeOH (7 mL) for 1 h at room temperature and then filtered to remove insoluble material. The filtrate was concentrated to dryness, and the solid residue was triturated with Et₂O (2 × 5 mL), decanting off the Et₂O. The residual white solid was washed with EtOH (1 mL) and then washed with Et₂O (3 × 2 mL), decanting off the solvent each time. The residue was slurried in MeOH (1 mL) filtered to remove insoluble material, concentrated to dryness, and the solid residue was triturated in MeOH (1 mL) filtered to remove the residual non-reacted TA. The nanoparticles’ size and morphology were characterized by dynamic light scattering (DLS; Malvern® Nano-ZS, Zetasizer Nano series, Malvern, Worcestershire, UK) and transmission electron microscopy (TEM; Darwin; 208 Philips, Guildford, UK; 60–80–100 kV; Camera AMT).

The crystallinity and purity of MIL-100 nanoMOFs were assessed. X-ray powder diffraction (XRPD) patterns were collected in a conventional high resolution (θ–2θ) D5000 Bruker diffractometer (λCu Kα, Kα₂) from 3 to 20° (2θ) using a step size of 0.02° and 4° per step in continuous mode. The nanoMOF porous surface was measured by nitrogen sorption experiments at −196°C on an ASAP 2020 (Micromeritics) after sample outgassing at 150°C for 18 h under secondary vacuum (<10⁻⁷ Torr). Obtained XRPD patterns and BET surfaces (1650 m² g⁻¹) were consistent with those previously reported [47]. The nanoparticles were stored in ethanol at room temperature and further used for in vitro assays.

### Drug loading and release studies

Suspensions of nanoMOFs (5 mg mL⁻¹ in ethanol) were prepared. Samples of 500 μL were withdrawn, centrifuged at 10 000 g, 10 min to recover sedimented nanoMOFs (2.5 mg). NanoMOFs were mixed overnight under rotational agitation, at room temperature with 0.5 mL Gem-MP solutions at concentrations c₀ ranging from 647.2 μM to 7.29 mM. Finally, the drug-loaded nanoMOFs were recovered by centrifugation (10 000 g, 10 min). The supernatants were analyzed by HPLC to determine the concentration c₁ of non-encapsulated Gem-MP.

The encapsulation efficiency (EE₁) and the drug payload (DP₁, Gem-MP wt%) were calculated according to the formulas (1) and (2), respectively:

\[
EE_1 (\%) = 100 \times \frac{c_1}{c_0}, \tag{1}
\]

\[
DP_1 (\text{wt}\%) = 100 \times \frac{m_{\text{Gem-MP}} (\text{mg})}{m_{\text{nanoMOF}} (\text{mg})}, \tag{2}
\]

where \(m_{\text{Gem-MP}}\) is the corresponding amount of Gem-MP (mg) entrapped per mg of nanoMOF.

Similar experiments were carried on with radiolabeled Gem-MP. A stock solution was prepared mixing non-radioactive Gem-MP and 1% of Gem-MP³¹H]. At the end of the impregnation, the nanoMOFs were recovered by centrifugation (10 000 g/10 min). The radioactivity present in the supernatant (S₁) was determined by scintillation counting using a Beckman Coulter apparatus (LS 6500 multipurpose scintillation counter).

The drug payload (DP₂, Gem-MP wt%) and the encapsulation efficiency (EE₂) were calculated according to the formulas (3) and (4), respectively:

\[
EE_2 = 100 \times \frac{1 - S_1 (\text{dpm})}{1 - S_0 (\text{dpm})}, \tag{3}
\]

\[
DP_2 (\text{wt}\%) = 100 \times \frac{m_{\text{Gem-MP}} (\text{mg})}{m_{\text{nanoMOF}} (\text{mg})}, \tag{4}
\]
where \( S_0 \) is the radioactivity of the mother solution and \( m_{\text{Gem-MP}} \) is the corresponding amount of radioactive Gem-MP (mg) entrapped per mg of nanoMOF.

To determine the maximum payload, 2.5 mg of MIL-100 nanoMOF was incubated with an excess Gem-MP (500 \( \mu \text{L} \)) at a concentration of 1.4 mg/mL, containing or not 1% tritiated Gem-MP). The experiments were conducted as described above.

For the release studies, the Gem-MP-loaded nanoMOFs were centrifuged (10,000 \( g \), 10 min) and the supernatants were discarded. The particles were re-dispersed in 1 mL water by vortex mixing ultrasound. Aliquots of 100 \( \mu \text{L} \) were centrifuged (10,000 \( g \), 10 min) and the supernatants were assessed by HPLC to determine the amount of released Gem-MP and trimesate to check the eventualtiy of the formation of degradation products.

HPLC analyses were performed on a Waters (Guyancourt, France) system (717 Autosampler, Waters 2996 Photodiode array detector and a Waters 600 pump). Experiments were analyzed by using a ZORBAX Eclipse XDB-C18 Agilent (Les Ulis, France) reverse phase column. For the simultaneous detection of Gem, Gem-MP and TA (nanoMOF ligand) in water and PBS, a mobile phase consisting of 84% water (0.2 M TEAA):16% MeOH was used. In all cases, injected volumes were 50 \( \mu \text{L} \), 10 min) and the supernatants were assessed by HPLC to determine the amount of released Gem-MP and trimesate to check the eventualtiy of the formation of degradation products.

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**In vitro interaction with PANC-1 cells**

Panc-1 cells obtained from ATCC were grown in DMEM/F12 GlutaMAXTM supplemented with 10% (v/v) heat-inactivated (56°C, 30 min) FBS, penicillin (100 \( \text{U/mL} \)) and streptomycin (100 \( \mu \text{g/mL} \)) (DMEM/F12 + 10%SBF). Cells were maintained in a humid atmosphere at 37°C with 5% \( \text{CO}_2 \).

To quantitatively measure the cell internalization of nanoMOFs loaded with Gem-MP compared to free Gem-MP at 1 \( \mu \text{M} \) concentration, PANC-1 cells were cultured on six well plates for 24 h to achieve 60–80% confluence. Before the experiments, the media were discarded and replaced with saline containing 2% FBS at osmolar conditions. NanoMOFs (100 \( \mu \text{L} \) loaded with radiolabeled Gem-MP (1% of the total amount of the drugs) were added to the wells and incubated up to 5 h. After incubation, the supernatants and the cells were collected at different time intervals (i.e. 0.5, 1, 2.5 and 5 h) for measurement of Gem-MP levels by using two methods (direct counting of radioactivity and HPLC assessment of non-entrapped Gem-MP). Then, the cells were lysed and radioactivity was counted both in the supernatants and in the cells.

All experiments were performed in triplicate, except where noted. Data were reported as mean ± standard deviation and analyzed by single-factor ANOVA, setting the level of significance at \( p<0.05 \).

**Cell proliferation assay**

The antiproliferative activity of Gem-MP-loaded nanoMOFs on pancreatic (PANC-1) cancer cells was investigated using a MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide) assay. Briefly, \( 5 \times 10^5 \) of PANC-1 cancer cells per well were incubated in 200 \( \mu \text{L} \) medium containing 10% (v/v) FBS in 96-well plates for 24 h. The cells were then incubated with increasing concentrations of tested compounds, i.e. Gem, Gem-MP, empty nanoMOFs and Gem-MP-loaded nanoMOFs (loading of 7.4 wt%) in NaCl-based media containing 2% SVF at 37°C for 1 or 5 h with further incubation in a new medium (200 \( \mu \text{L/well} \)) without any drug up to 72 h, washed with PBS. Then, 100 \( \mu \text{L} \) of MTT solution (0.5 \( \text{mgmL}^{-1} \) and 10% (v/v) FBS) were added to each well. The plates were incubated for 2 h at 37°C and 100 \( \mu \text{L} \) of SDS solution (10% in PBS) were then added to each well. After incubating the plates for 24 h at 37°C, absorbance was measured at 570 nm using a plate reader (Metertech Σ 960, Fisher Bioblock, Illkirch, France).

The percentage of surviving cells was calculated as the absorbance ratio of treated to untreated cells. The inhibitory concentrations 50% (IC50) were determined from the dose–response curve. All experiments were set up in quadruplicate to determine means and SDs. No cytotoxic effects were observed for control empty nanoMOFs.

**Results and discussion**

**Synthesis and molecular characterization of Gem-MP**

Gem-MP (Scheme 1) was prepared according to a published method [46] using a modified procedure for product isolation. Gem-MP, obtained in the form of bisammonium phosphate salt, was characterized by \( ^1\text{H} \) (Figure S1a) as well as \( ^3\text{P} \) NMR spectra (Figure S1b). The latter, with a signal at 1.31 ppm [48], showed that the monophosphate at CS2 of Gem was successfully obtained.

The obtained data clearly showed the successful obtention of monophosphate at CS2 with a signal at 1.31 ppm [48]. Furthermore, the \( ^{19}\text{F} \) NMR spectrum of Gem-MP displayed an AB pattern comprising four signals with two doublets (\( -118.4 \) and \( -118.9 \text{ppm} \)) each with typical geminal \( J_{FF} = 240 \text{Hz} \) [49], assigned to the equatorial (a) and axial (a) fluorine atoms, respectively. The components of the Fe doublet were broadened compared to those of Fe due to non-negligible \( J_{HH} \) coupling, thus confirming the assignments. The \( ^{19}\text{F} \) NMR pattern (Figure S1c) was distinctly different from that of Gem in the same buffer, very much affected by the pH [49] compared to the highly resolved spectrum in unbuffered D2O (Figure S2).

**NanoMOF synthesis and characterization**

Mesoporous MIL-100 nanoMOFs are built up by the association of oxo-centered trimers of iron(III) octahedra and trimesate linkers into hybrid supertetrahedral (ST) units. The ST units further assemble into a highly porous MTN zeotypic architecture. The resulting tridimensional network is composed of two types of mesoporous cages with an open...
porosity (Figure 1). Several methods have been described for
the synthesis of MIL-100 nanoMOFs [20,23,29,47].
Microwave-assisted techniques were effective allowing to
obtain rapidly high yields of nanoMOFs with controlled sizes.
In some cases, hydrofluoric acid (HF) was used as
mineralizing agent. More recently, the use of the toxic HF
catalyst has been avoided [29,50,51]. In this optimized
synthesis method, crystalline nanoMOFs were obtained
replacing the iron source (Fe⁰) by a salt (FeCl₃·6H₂O).
This “green” synthesis method has been used here to
produce nanoMOFs. Iron(III) trimesate MIL-100 nanoMOFs
of mean diameters of 196 ± 15 nm have been successfully
prepared by a microwave-assisted hydrothermal process
(Figure 1). NanoMOFs were not spherical but presented a
typical facetted structure (Figure 2A). According to X-ray
investigations, they were crystalline (Figure 3A) and the
obtained diffraction patterns were in agreement with data in
the literature on the same type of materials [29,30]. Iron
trimesate nanoMOFs were porous, displaying BET surfaces of
1650 ± 50 m² g⁻¹.

The encapsulation of low molecular weight hydrophilic
molecules such as NAs in nanoparticulate drug carriers is
particularly challenging [28,29,52] Indeed, these molecules
have poor affinity for hydrophobic polymer matrices and tend
to rapidly leak out, whereas encapsulation into the aqueous
core of liposomes often leads to low loadings. As described in
the “Introduction” section, this challenge was addressed by
chemical linkage with squalene [8] or by encapsulation,
taking advantage of electrostatic interactions [16,18,19,53,54]
or coordination interactions [19,28,29].

Similar to the synthesis procedure, Gem-MP loading was
achieved here by a convenient one-step procedure,
particularly suitable for biomedical applications, simply
based on the nanoMOFs impregnation in drug aqueous
solutions. The interconnected porous structure of the
nanoMOFs allows them to adsorb a large number of
therapeutic molecules, including anticancer and antiviral
drugs, which, according to their nature (hydrophilic, hydro-
phobic or amphiphilic) were able to interact with the organic
ligands and/or with the metal centers of the nanoMOFs
[19,20,22,28,29]. Moreover, each iron trimer possesses two
accessible coordinatively unsaturated metal sites (CUS), able
to coordinate a wide range of polar or quadrupolar species
[21, 55,56].

First, the capacity of MIL-100 nanoMOFs to encapsulate
Gem and Gem-MP was studied. These molecules were
encapsulated by incubating the nanoMOFs with aqueous solutions of each drug. The Gem encapsulation efficiency (EE) did not exceed 10% and only poor loadings (up to 1 ± 0.3 wt%) were achieved, whatever the incubation time (up to 72 h). This clearly shows that Gem does not efficiently interact with the nanoMOFs. This finding is in agreement with previously reported data showing a low interaction of MIL-100 MOFs with unmodified NAs such as AZT [29,30].

Two methods were used to determine Gem-MP loadings: (i) by determining the amount of non-encapsulated Gem-MP by HPLC and (ii) by radioactivity counting (see “Methods” section for details). Both methods gave very similar results (less than 5% differences). In contrast to Gem, Gem-MP was efficiently encapsulated, reaching a maximal loading of 30.7 ± 0.8 wt%. These loadings are among the highest ones reported in the literature with solid nanoparticulate systems [57,58]. Noteworthy, encapsulation efficiencies (EEs) were higher than 98%.

Similar findings were reported with nanoMOFs in the case of the encapsulation of the antiviral phosphated drugs, AZT-MP and AZT-TP [30]. It has been shown that the number of phosphate groups per NA has a high impact on the drug-loading capacity, and their interaction with the Lewis acid sites from the nanoMOFs. Significantly larger loadings of AZT-MP than of AZT-TP were obtained (36 wt% as compared to 25 wt%) in agreement with predictions issued from molecular simulations. Whereas both molecules could access only the large mesoporous cages and filled them almost completely, the molecular volume of AZT-MP was significantly lower than that of its triphosphorylated analog (263 versus 356 Å³). As a result, both AZT-MP and AZT-TP filled the available free volume in the large Mill-100 large cages, but more AZT-MP than AZT-TP molecules fit in, leading to increased loadings.

The molecular volume of the energetically lowest Gem-MP conformer, optimized [59] at the PM7(COSMO) level of theory, was calculated to be 322 Å³, whereas at the DFT B3LYP/6-31 G(d) level was 334 Å³, both values in between the ones of AZT-MP and AZT-TP. Consistently, the obtained maximal loading of Gem-MP (30.7 wt%) was between 25 and 36 wt%, corresponding to the maximal loadings of the phosphate drugs AZT-MP and AZT-TP. In a nutshell, these data corroborate previous findings proving that MIL-100 nanoMOFs act as efficient “nanosponges”, soaking Gem-MP with almost perfect efficiency (EE >98%) from aqueous solutions to reach loadings as high as ~30 wt%, reflecting the high interactions between the phosphate drug and the iron trimesate matrices.

It is worth mentioning that a reported advantage of encapsulating the monophosphorylated form as compared to the triphosphorylated one was that this resulted in low matrix degradation [30]. Indeed, only negligible amounts of trimesate ligand (1–2%) were displaced from the matrices during encapsulation of AZT-MP at the maximum payload (36 wt%), whereas larger amounts of ligands (up to 10%) were found in the case of AZT-TP. This was explained by the fact that nanoMOFs exhibited weaker interactions with AZT-MP as compared to AZT-TP. However, the interaction between AZT-MP and the nanoMOFs was sufficient to allow excellent EEs (>98% after 30 min) in agreement with the good affinity of AZT-MP for the MOF system.

Encapsulation of Gem-MP showed similar low degradation of the MIL-100 nanoMOF matrices during the encapsulation process, with lower than 1% trimesate loss, as assessed by HPLC experiments. Furthermore, Gem-MP loading did not affect neither the aspect nor the crystallinity of the nanoMOF (Figure 2A and B). Moreover, during storage in water of the drug-loaded nanoMOFs, there was neither crystallinity, nor size and shape changes (Figure 2C). Up to one day storage, the amount of trimesate ligand in the suspension media did not exceed 1% of the initial amount. No detectable (<1%) Gem-MP release was found during this time. Thus, the Gem-MP-loaded nanoMOFs neither degrade nor release their encapsulated cargo over storage in water.

In contrast, release studies conducted in PBS clearly showed nanoMOF erosion as shown by: (i) constitutive ligand (trimesate) release in the suspension media (Figure 4); (ii) crystallinity loss after 4 h (Figure 3B) and (iii) microscopic investigations (Figure 2D). Furthermore, it was observed that ligand release was faster at maximal Gem-MP loading (30.7 wt%) than at lower one (5 wt%) (Figure 4). After 4 h incubation in PBS at 37 °C, more than 60% of trimesate was released in the first case, whereas around 50% was released in the second case. These findings show that the drug encapsulated in high amounts possibly weakens the supramolecular structure of the nanoMOFs by forming numerous coordination interactions, leading to more pronounced matrix dissociation. Interestingly, in spite of the important (~50%) ligand loss in PBS, the global shape of the nanoMOFs was maintained under the same experimental conditions (Figure 2D). More precisely, the nanoMOFs lost their faceted structure and eroded surfaces were observed, but the mean diameters were similar as assessed by TEM and DLS studies. In agreement with these visual observations, X-ray diffractograms (Figure 3B) clearly show that the crystalline structure was affected during incubation in PBS. Indeed, peaks at low 2 Theta scale, presumably corresponding to a more long-range structural organization (links between CUS or cages) progressively disappeared.

Gem-MP release was fast in PBS media (Figure 4) and a “burst effect” was observed irrespective of the drug loading. However, drug release was strongly dependent upon the
nanoMOF concentration in the release media and on the composition of the release media (Figure 5). The higher the concentration of phosphates in the release media, the higher the drug release (Figure 5A). These findings are consistent with previously reported ones [29]. It was suggested that the encapsulated phosphate AZT could only be displaced by free phosphates, present in PBS, and not by water or other buffers such as Tris buffer.

Presumably, Gem-MP release is governed by a competition of coordination between MP of Gem-MP and free phosphates in PBS for the iron(III) Lewis acids of MIL-100 nanoMOFs. Interestingly, the higher was the nanoMOF concentration in PBS, the lower was the drug release (Figure 5B). This could be explained by the lower nanoMOF erosion at higher nanoMOF concentration, due to the fact that trimethylene, resulting from the nanoMOF dissociation, has a low solubility in the suspension media, thus hampering nanoMOF erosion [29]. Noteworthy, HPLC enabled to ascertain that encapsulated and released Gem-MP was intact. Indeed, in all cases, less than 1% non-phosphorylated Gem was detected, showing that there was no degradation in the non-phosphated form.

**In vitro efficacy on pancreatic cancer cells**

The nanoMOFs loaded with intact Gem-MP were further evaluated on PANC-1 cancer cell line. As previously shown, nanoMOFs tend to rapidly degrade and release their content in PBS and other media containing phosphates. Phosphate is a predominantly intracellular anion, mostly complexed or bound to proteins and lipids. Intracellular phosphate is essential for most, if not all, cellular processes. Because the intracellular concentration of phosphate is greater than the extracellular concentration, phosphate entry into cells requires a facilitated transport process. A cell culture media devoid of phosphates was chosen to investigate the effect of entrapped Gem-MP on PANC-1 cancer cells. Thus, it was intended to minimize drug release outside the cells.

Around 50% of the encapsulated drug was released within the first minutes of incubation, but this amount was unchanged up to 20 h of incubation at 37°C in the same conditions as in the cell culture experiments with nanoMOFs.

NanMOFs were loaded with Gem-MP containing 1% radiolabeled (3H) Gem-MP. As previously indicated in this study, there was a perfect agreement between the loading determined by using two methods (direct counting of radioactivity and HPLC assessment of non-entrapped Gem-MP). Therefore, it can be concluded that radioactive Gem-MP perfectly replaced the non-radioactive one inside the porous MOF structure.

The amount of Gem-MP inside the cells was comparatively assessed using the free form of the drug (Gem-MP) and same amounts of encapsulated drug (Figure 6). As expected, Gem-MP did not penetrate inside the cells as less than 0.7% of the drug was found inside the cells even after 5 h incubation. In contrast, when encapsulated in NanoMOFs, more than 6% of the drug was inside the cells after only 1 h incubation, representing a more than ninefold increase of the amount of the drug inside the cells. However, this amount progressively declined from 1 to 5 h, to finally reach a value corresponding to 3.3% of encapsulated drug inside the PANC-1 cells. Most probably, the intracellular drug accumulation is the result of two opposite phenomena: (i) nanoMOF interaction with the cells followed by drug release inside the cells and (ii) drug efflux from the cells. It can be hypothesized that at later

![Figure 5. Effect of the release medium composition and nanoMOF concentration on Gem-MP release. (A) PBS 9 (○), 3 (+) and 1 (△) mM and water (×). Experiments for different molarity of PBS were carried out at 7.4% dFdCMP loaded with 2.5 mg/mL of nanoMOFs by triplicate for the experiment at 9.5 mM of PBS and duplicate for experiments at 3.16 and 1.05 mM of PBS. The experiment of water was carried out at 5.3% dFdCMP loaded with 1 mg/mL of nannMOFs without duplicates. (B) Effect of nanoMOF concentration (50 μg/mL, □ and 2.5 mg/mL, ◇) on Gem-MP release in 9.5 mM PBS pH 7.4, at 37°C. The loading was 7.4%.](image)

![Figure 6. Amount of radioactive Gem-MP inside PANC-1 cells at different times of incubation. Comparison between the free Gem-MP (left) and encapsulated Gem-MP (right). Experiments were conducted in triplicate.](image)
incubation times (5 h), the drug is substantially effluxed out of the cells, resulting in decreased amounts of drug inside the cells (3.3%) as compared to early incubation times (1 h, 6%).

The cell proliferation was assessed by measuring the half maximal inhibitory concentration of cell proliferation by MTT assay. Results showing the concentrations required to inhibit cell growth by 50% (IC_{50} values) are presented in Table 1. As a general trend, Gem-MP loaded nanoMOFs showed an impressive activity in vitro on the PANC-1 cancer cell line as compared to free Gem-MP or Gem. Indeed, after 1 h incubation, only the Gem-MP-loaded nanoMOFs had an effect on cell viability. Control empty nanoMOFs, free Gem-MP or even free Gem had no detectable impact on cell viability. IC_{50} of Gem-MP-loaded nanoMOFs reached 450 nM. This value was further improved after 5 h incubation reaching a value as low as 45 nM. At 5 h incubation time, IC_{50} of both free drugs (Gem and Gem-MP) was only in the μM range (17 and 28 μM, respectively). Results gathered in Table 1 show that probably, there is a necessary lapse of time for Gem and Gem-MP to exert their biological effect on PANC-1 cells. It can be speculated that these results could be further improved by optimizing the nanoMOF size and surface characteristics, for example, by decoration with specific ligands for targeting cancer cells.

**Conclusion**

MIL-100 nanoMOFs synthesized by a “green” procedure acted as efficient “nanosponges”, soaking Gem-MP from its aqueous solution with almost perfect efficiency (>98%). Maximal loadings reached ~30 wt% reflecting the strong interaction between the drug and the iron trimesate matrices. Neither degradation nor loss of crystalline structure was observed after drug loading or during particle storage in water. However, nanoMOFs degrade in media containing phosphates, releasing their intact Gem-MP cargo. Drug-loaded nanoMOFs were nine times more effective against pancreatic PANC-1 cells than free drugs. Size optimization and surface modification are expected to improve cell internalization, and thus nanoMOF efficacy. With further studies, these results pave the way toward an improved use of phosphated NAs in cancer therapy.

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**Declaration of interest**

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**References**

1. Furman PA, Fyfe JA, St Clair MH, et al. Phosphorylation of 3’-azido-3’-deoxythymidine and selective interaction of the 5’-triphosphate with human immunodeficiency virus reverse transcriptase. Proc Natl Acad Sci USA 1986;83:3333–7.
2. Balzarini J, Herdevijn P, De Clercq E. Differential patterns of intracellular metabolism of 2’,3’-Didehydro-2’,3’-dideoxynucleosidem and 3’-azido-2’,3’-dideoxynucleoside, two potent anti-human immunodeficiency virus compounds. J Biol Chem 1989;264:6127–33.
3. Kukhanova M, Krayevsky A, Prusoff W, Cheng YC. Design of anti-HIV compounds from nucleoside to nucleoside 5-triphosphates: problems and perspectives. Curr Pharm Des 2000;6:585–98.
4. Törnevik Y, Ullman B, Balzarini J, et al. Cytotoxicity of 3’-azido-3’-deoxythymidine correlates with 3’-azidothymidine-5’-monophosphate (AZTMP) levels, whereas antihuman immunodeficiency virus (HIV) activity correlates with 3’-azidothymidine-5’-triphosphate (AZTTP) levels in cultured CEM T-lymphoblastoid cells. Biochem Pharmacol 1995;49:829–37.
5. Li X, Chan WK. Transport, metabolism and elimination mechanisms of anti-HIV agents. Adv Drug Deliv Rev 1999;39:81–103.
6. Loke SL, Stein CA, Zhang XH, et al. Characterization of oligonucleotide transport into living cells. Proc Natl Acad Sci USA 1989;86:3474–8.
7. Diab R,Degobert G, Hamoudeh M, et al. Nucleoside analogue delivery systems in cancer therapy. Expert Opin 2007;4:513–31.
8. Caron J, Lepeltier E, Harivardhan Reddy L, et al. Squalenoyl gemcitabine monophosphate: synthesis, characterisation of nanocapsules and biological evaluation. Eur J Org Chem 2011;14:2615–28.
9. Hillaireau H, Covreur P. Nonoencapsulation of antiviral nucleotide analogs. J Drug Deliv Sci Technol 2009;19:385–90.
10. Hillaireau H, Le Doan T, Appel M, Covreur P. Hybrid polymer nanocapsules enhance in vitro delivery of azidothymidine-triphosphate to macrophages. J Control Release 2006;116:346–52.
11. Hillaireau H, Le Doan T, Besnard M, et al. Encapsulation of antiviral nucleotide analogues azidothymidine-triphosphate and cidofovir in poly(iso-butylcyanoacrylate) nanocapsules. Int J Pharm 2006;324:37–42.
12. Hillaireau H, Le Doan T, Chacun H, et al. Encapsulation of mono- and oligo-nucleotides into aqueous-core nanocapsules in presence of various water-soluble polymers. Int J Pharm 2007;331:148–52.
13. Kohli E, Han HY, Zeman AD, Vinogradov SV. Formulations of biodegradable nanogel carriers with 5’-triphosphates of nucleoside analogs that display a reduced cytotoxicity and enhanced drug activity. J Control Release 2007;121:19–27.
14. Saiyed ZM, Gandhi NH, Nair MPN. AZT 5’-triphosphate nanof ormulation suppresses human immunodeficiency virus type 1 replication in peripheral blood mononuclear cells. J Neurovirol 2009;15:343–7.
15. Saiyed ZM, Gandhi NH, Nair MPN. Magnetic nanof ormulation of azidothymidine 5’-triphosphate for targeted delivery across the blood–brain barrier. Int J Nanomed 2010;5:157–66.
16. Vinogradov SV. Polymeric nanogel formulations of nucleoside analogs. Expert Opin Drug Deliv 2007;4:5–17.
17. Vinogradov SV, Kabanov AV. Synthesis of nanogel carriers for delivery of active phosphorylated nucleoside analogues. Polymer Prepr 2004;22:296.
18. Vinogradov SV, Poluektova LY, Makarov E, et al. Nano-NRTIs: efficient inhibitors of HIV type-1 in macrophages with a reduced mitochondrial toxicity. Antivir Chem Chemother 2010;21:1–14.
19. Vinogradov SV, Zeman AD, Batrakova EV, Kabanov AV. Polypexyl nanogel formulations for drug delivery of cytotoxic nucleoside analogs. J Control Release 2005;107:143–57.
