Folate-intercalated layered double hydroxide as a vehicle for cyclophosphamide, a non-ionic anti-cancer drug

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Layered double hydroxides (LDH) are matrices with interlayer anions that can be exchanged with several types of organic or inorganic anions. Due to the anion-exchange capability, hundreds of new materials have been prepared in the past two decades. Conversely, attempts to intercalate neutral molecules (and increase the range of applications) have been achieved by expanding the interlayer space with long-chain surfactants, thus allowing to exclusively retain highly hydrophobic molecules. This work describes a folate-intercalated LDH structure, where folate pillars are capable of forming both hydrogen bonds and hydrophobic interactions with neutral molecules. Infrared spectroscopy and X-ray diffraction data indicated that imidazole, urea and cyclophosphamide were successfully intercalated. This evidence increases the opportunity to prepare more novel materials with neutral molecules in LDH. The cyclophosphamide-LDH product here obtained represents a remarkable example of an LDH-based vehicle for a non-ionic anti-cancer drug used in current chemotherapies.

1. Introduction: Layered double hydroxides (LDH) are synthetic layered compounds widely known for their positively charged layers as a result of the isomorphic substitution of divalent cations located in octahedral sites of brucite-like layers by trivalent cations [1]. The positive charge is neutralised by interlayer anions, which can be exchanged with a large number of anions. This anion-exchange property is the most remarkable feature of these structures [2, 3] because even organic anions can be inserted between the layers by the exchange process to produce new hybrid materials such as gene and drug vehicles [4, 5], polymer flame retardants [6], enzyme supports [7, 8] or nutraceutical and cosmeceutical carriers [9, 10]. Due to the ease of intercalating different types of anions into LDH, hundreds of scientific articles are published every year. Unusual cases of neutral molecules intercalated in LDH have been reported with ribose and curcubituril [11, 12] and attempts to improve this intercalation involves pre-expansion of LDH with long chain anions. Examples of this is the intercalation of iphosphamide into a pre-expanded LDH with dodecylsulphate (DDS); the intercalation of ultraviolet (UV)-absorbers after expansion of the interlayer space with DDS or dodecylbenzenesulphonate [13]; and the intercalation of curcubituril into a pre-expanded LDH with DDS [12].

This strategy has been limited to increase the size of interlayer galleries with long chain hydrophobic anions (surfactants) and consequently, the neutral species intercalated further required a high hydrophobic character. In fact, we have observed our own experiments that urea (a highly polar molecule) does not intercalate into surfactant pre-expanded LDH.

On the other hand, considering that folic acid, by itself, can retain aromatic molecules like anthracene due to an attraction with π bonds [14], the present Letter reports the use of a pre-expanded LDH structure with folate anions where the aromatic rings allow retention of hydrophobic neutral molecules, while the amino, carbonyl and carboxylate groups favour the retention of polar neutral molecules through formation of hydrogen bonds. The wider range of neutral molecules intercalated in folate-pre-expanded LDH could increase the number of materials and applications of this anionic layered structure; in this report the insertion of urea, imidazole and cyclophosphamide into folate-modified LDH is demonstrated. Specially, the cyclophosphamide product obtained here is an example of the valuable contribution of folate ions in the LDH. The product obtained becomes a model to design vehicles for non-ionic anti-cancer drugs.

2. Materials and methods: The LDH was prepared by dissolving 1.00×10^{-2} mol of $Zn(NO_3)_2$ and 4.03×10^{-3} mol of $Al(NO_3)_3$ in 150 ml of deionised water. pH 8.0 was adjusted with an ammonia solution and the LDH powder that was produced was washed and dried. The intercalation of folate was achieved by dispersing 0.160 g of LDH in 50 ml of solution containing 0.320 g of folic acid (previously adjusted at pH 7). The suspension was stirred for 24 h and the recovered powder was labelled as layered matrix hybridised with folate ions (LDH-Fol). Intercalation of imidazole and cyclophosphamide was conducted in glass vials with screw caps containing 0.050 g of LDH-Fol and 0.050 g of imidazole or 0.050 g of cyclophosphamide dispersed in 5.0 ml of deionised water. The suspensions were stirred for 5 days at room temperature. Urea was intercalated by a solid state reaction mixing 0.206 g of LDH-Fol and 0.401 g of urea finely ground in a mortar. The powder was pressed to form a pellet at 4 ton cm^{-2} for 7 days. The resulting pellet was ground, and washed with water. The powder products were washed three times with 20 ml of water and dried at 70°C and identified as LDH-Fol-imidazole (LDH-Fol-I), LDH-Fol-cyclophosphamide (LDH-Fol-C) and LDH-Fol-urea (LDH-Fol-U) according to the reactions with imidazole, cyclophosphamide and urea, respectively.

The powder X-ray diffraction (XRD) patterns were acquired with a Panalytical-Empyream diffractometer using CuKα radiation (0.15418 nm) obtained at 45 kV and 40 mA. The samples were analysed with steps of 0.02° and 30 s of exposition per step.

Infrared (IR) spectra were collected in the reflectance mode with a ThermoScientific spectrometer model NICOLET iS5 iD5 ATR using resolution of 4 cm^{-1} and 16 scans. High-performance liquid chromatography (HPLC) was conducted in an Agilent Technologies apparatus, 1260 series, with a diode array detector

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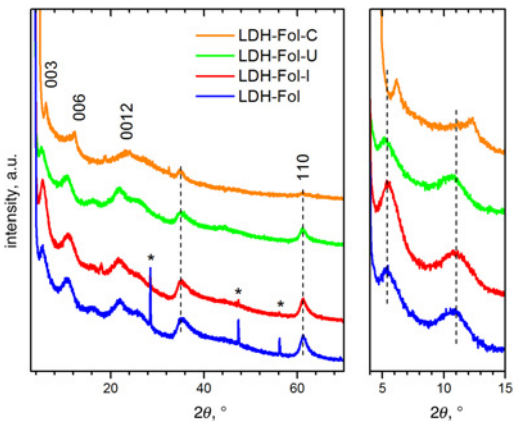

Fig. 1 *XRD patterns of the LDH-Fol and the subsequent products with LDH-Fol-I, LDH-Fol-U and LDH-Fol-C as guest molecules*

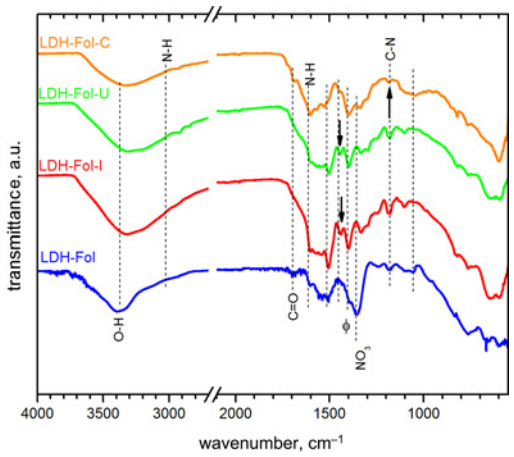

Fig. 2 *IR spectra of the LDH-Fol and the subsequent products with LDH-Fol-I, LDH-Fol-U and LDH-Fol-C as guest molecules*

set to a detection wavelength of 195 nm. The analysis was run using an Agilent Zorbax SB-C18 column (150 × 4.6 mm, 5 µm), and a water/acetonitrile (60:40 v/v) mixture flowing at 1 ml min^{-1} at room temperature. The LDH-Fol-C sample was treated by adding 0.002 g of the powder sample to 1 ml of 0.10 M HCl. The mixture was stirred in a vortex apparatus for 5 min, then the solution formed was injected to the HPLC equipment.

3. Results: Products of intercalation in the LDH-Fol host matrix were analysed by XRD to assess any change in the interlayer space. All of the patterns in Fig. 1 present the typical profile of LDH structures intercalated with large anions [15]. The 003 plane (or basal plane) reflection at 5.3° (2θ) have been previously observed and reported in the literature [16]. This reflection is not altered after the reaction with imidazole, but this has been slightly shifted after the solid state reaction with urea and the stirring with cyclophosphamide. Such a shift is better visualised on the right side of the same figure and data from those reflections are listed in Table 1. The basal distances in these compounds range from 14.4 to 16.4 Å, suggesting that folate ions determine the height of the interlayer space, with a probable rotation of the amino bond between the pteroyl and aminobenzoic rings producing the basal distance changes. The size of the galleries ($d_{interlayer}$), estimated by subtracting the metal hydroxide layer thickness (4.8 Å [17]) from the basal distance are between 9.6 and 12.3 Å, indicating that there is enough room to allocate imidazole, urea or cyclophosphamide molecules. The presence of these molecules in the powder products is evidenced in the IR spectra.

The IR spectrum of the LDH-Fol matrix in Fig. 2 has the profile of a folate-intercalated LDH as previously observed [16]. This spectrum is composed by the hydroxyl absorption region between 3600 and 3300 cm^{-1} and a set of bands from the folate ion corresponding to vibrations of the following groups of atoms: C=O (1694 cm^{-1}) [18], N–H (1605 cm^{-1}) [18, 19], -OH phenyl (1411 cm^{-1}) [18, 19], phenyl ring (observed here at 1503 cm^{-1}, and reported in the literature at 1485 cm^{-1}) [18, 19], out-of plane and in-plane vibrations of -NH_2, and C–N (824, 853, 1192 and 1297 cm^{-1}) [20], C–O (1300 cm^{-1}) [21], and C–N (1188 cm^{-1}) [21]. A band at 1350 cm^{-1} indicates that nitrate ions co-exist with folate allowing the formation of voids in the interlayer space, which would be surrounded by diverse functional groups from folate ions capable of forming hydrogen bonds (with amino, carbonyl and carboxylate groups) and hydrophobic adsorption (due to the aromatic rings) with neutral molecules. Complexation of highly hydrophobic molecules like anthracene, phenanthrene and naphthalene with the aromatic rings of folic acid has already been described in the literature [14, 22, 23].

The spectra of the imidazole, urea and cyclophosphamide products contain two types of changes: (i) new signals from neutral molecules and (ii) modifications in folate signals due to an interaction with neutral molecules. For instance, the intercalation products present a wider and more intense band of N–H vibration at 3100 cm^{-1} overlapped with the band from OH-groups. A simultaneous increase of intensity and width in the band at 1605 cm^{-1} (also related to N–H) reinforces the evidence of the neutral molecules in the powders.

On the other hand, the band formerly present in LHD-Fol of C=O in the pteroyl ring or amide groups overlapped and formed a shoulder in the imidazole and urea product, suggesting that this group interacted with these neutral molecules.

In the case of the cyclophosphamide product, the carbonyl of folate does not interfere with retaining this molecule once this signal at 1694 cm^{-1} remains in the same wavelength. Conversely, the intensity of the band at 1503 cm^{-1} related to double bonds in

Table 1 Basal (d_{003}) and interlayer ($d_{interlayer}$) distances in LDH-Fol and the intercalation products with imidazole (LDH-Fol-I), urea (LDH-Fol-U) and cyclophosphamide (LDH-Fol-C)

Material	2θ angle, deg	d_{003}, Å	$d_{interlayer}$, Å
LDH-Fol	5.4	16.4	11.6
LDH-Fol-I	5.4	16.4	11.6
LDH-Fol-U	5.2	17.1	12.3
LDH-Fol-C	6.1	14.4	9.6

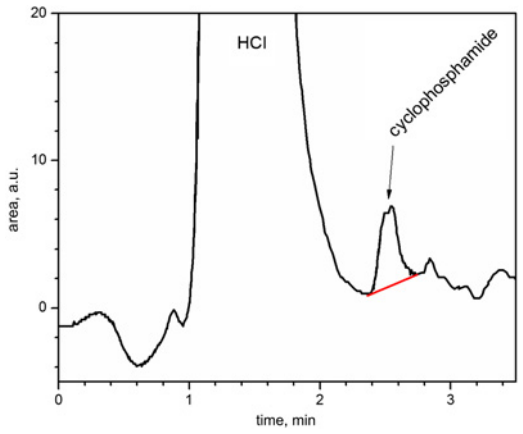

Fig. 3 *HPLC elution profile of the LDH-Fol-C product*

the pteroyl ring decreases due to the probable retention of cyclophosphamide in this ring. The pteroyl ring is capable to retain double bond-rich molecules through π–π attraction [14, 22, 23] and the same effect has been observed between the benzyl ring of dodecylbenzene sulphonate and carotene [24], therefore, the pteroyl ring is very likely retaining cyclophosphamide with π–π interaction. This fact is reinforced by the decrease of the band at 1188 cm^{-1}, associated to C–N of the same ring.

The LDH-Fol-C product is relevant, once LDH are biocompatible inorganic nanoparticles widely used as vehicles for biologically active compounds for biomedical applications, all of them with anionic character [4, 9, 25]. The cyclophosphamide product here obtained demonstrates that neutral drugs can be loaded in LDH particles, and this increases the ranges of formulations that could be prepared with LDH and non-ionic anti-cancer drugs.

A clearer qualitative fact proving the presence of cyclophosphamide is the HPLC analysis where the peak corresponding to the drug was clearly observed (Fig. 3). In addition to demonstrate that the LDH-Fol can retain neutral molecules, the LDH-Fol-C product becomes of interest in chemotherapy studies once this non-ionic drug could be delivered to a specific target considering that folate ions are capable to recognise specific damaged tissues [18, 20].

4. Conclusion: An LDH structure with folate interlayer anions was prepared by co-precipitation. The space between the inorganic layers was 11.6 Å. The presence of residual nitrate ions suggested that interlayer voids exist among pillars formed by folate ions. Unlike reports of pre-expanded LDH with long-chain surfactant anions to retain hydrophobic molecules, the current LDH-Fol product contains galleries to intercalate hydrophobic and hydrophilic non-ionic molecules due to the ability to form hydrogen bonds with the amino, carbonyl and carboxylic groups or π–π interactions with the aromatic ring of the folate pillars. This ability was proven with imidazole, urea and cyclophosphamide molecules. Specially, the cyclophosphamide-loaded LDH are of interest for biomedical and chemotherapy studies and demonstrates that a new range of LDH formulations can be designed with neutral molecules.

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