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Title: Anti-ulcerant kynurenic acid molecules intercalated Mg/Al-layered double hydroxide and its release study

Article Type: Research Paper

Keywords: layered double hydroxide, kynurenic acid, intercalation, in vitro drug release study, anti- ulcerant properties, simulated gastric fluid

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First Author: Ágota Deák

Order of Authors: Ágota Deák; Edit Csapó, PhD; Ádám Juhász; Imre Dékány, PhD; László Janovák, Ph.D.

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Response to Reviewers: Point-by-point response to the Reviewers' comments
The Reviewers' comments are always followed by our response highlighted in yellow.

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First of all, we would like to thank the Reviewer for his very positive evaluation of out manuscript, and for the insightful comments which truly improved the quality of the manuscript!

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Figure. The specific amount of intercalated KYNA molecules (mg KYNA/ g LDH) as a function of adsorption time (KYNA/ LDH weigh ratio: 0.5)

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Dr. Vanessa Prévot
Editors-in-Chief, Applied Clay Science
Université Blaise Pascal, Aubière cedex, France

19 January, 2018

Dear Dr. Vanessa Prévot,

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Authors: Ágota Deák, Edit Csapó, Ádám Juhász, Imre Dékány, László Janovák

Attached please find the responses to Editor and Reviewers suggestions and questions.

In the name of all co-authors I would like to thank you for the time and efforts while treating our submission.

Yours sincerely,
László Janovák
corresponding author

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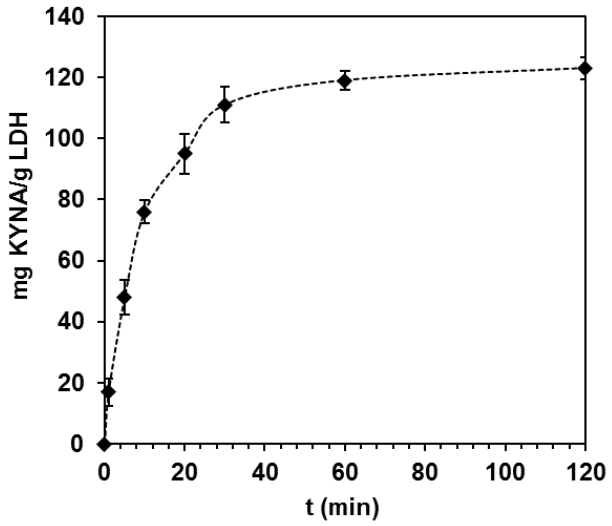

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[b]*MTA-SZTE Biomimetic Systems Research Group, University of Szeged, H-6720, Szeged, Dóm tér 8, Hungary*

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E-mail address: janovakl@chem.u-szeged.hu (L. Janovák)

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1. Introduction

LDHs are a class of anionic lamellar compounds made up of positively charged brucite- like layers (Trifiro and Vaccari, 1996). The chemical composition of the two layers of hydrotalcite-type minerals can be given by the following general formula: $[M^{2+}_{1-x}M^{3+}_{x}(OH)_2]^{b+}\cdot[A_{b/n}]^{n-}\cdot mH_2O$, where M^{2+} represents divalent and M^{3+} represents trivalent cations, the value of x may vary in the range of 0.2−0.4, and A is the anion among

the cationic layers (OH^-, Cl^-, NO^{3-}, CO_3^{2-}, and SO_4^{2-}) (Constantino and Nocchetti, 2001). LDHs have been widely exploited to create new materials for applications in catalysis (Patzkó et al., 2005; Deák et al., 2016), drug delivery and environmental remediation (Bujdosó et al., 2009; Goh et al., 2008). MgAl–LDHs are most frequently used as a LDH-based drug carrier and as evidence of its low toxicity, it is widely used as an antacid (Tarnawski et al., 2000) and the biocompatibility of this layered material was also reported in the literature (Cunha et al., 2016, Nagy et al., 2013). LDHs particularly prefer multivalent anions within their interlayer space due to strong electrostatic interaction and therefore LDHs bearing monovalent anions like nitrate or chloride ions are good precursors for exchange reactions (Choy et al., 2007). The solubility and surface charge of LDHs as hydroxides is highly pH-dependent (Bish, 1980; Deák et al., 2015).

Layered clay minerals are widely used for their capability to intercalate molecules in the interlayer space. It is also well known that beside the LDH drug carrier, the negatively charged clay minerals such as Montmorillonite [$(Na,Ca)_{0.33}$ $(Al, Mg)_2(Si_4$ $O_{10})(OH)_2$ • nH_2O] exhibit an excellent sorption property, large specific surface area, cation exchange capacity and drug-carrying capability (Joshi et al., 2009; Patel et al., 2011; Kevadiya et al., 2010). It is worth mentioning that the pioneering works of Choy' group have led to a rapid development in the research on both varied LDHs/polymers/anions hybrid systems and pharmaceutical applications of LDHs especially involving the biocompatibility and toxicity of LDHs and anti-cancer drugs intercalated LDH materials (Choy et al., 2007). Li et al. also developed anti-inflammatory drug fenbufen–LDH hybrids and showed that these drug-inorganic hybrid materials can be used as an effective drug delivery system due to their controlled release capacity (Li et al., 2004). Yang et al. reported the intercalation of vitamins A, E, and C into LDHs (Yang et al., 2003). Moreover, in addition to the intercalation of pharmaceutical drugs into layered materials causing no significant denaturation of the drug molecules, it has also

been shown to enhance the internalization of the drug into a target cell without any noticeable side effects (Oh et al., 2009). Thus, LDHs can not only play a role as a biocompatible-delivery matrix for drugs but also afford a significant increase in the delivery efficiency (Posati et al., 2012; Oh et al., 2006).

Kynurenic acid (KYNA) is a product of the tryptophan metabolism, it has a neuroprotective and neuroinhibitory properties (Marosi et al., 2010). According to this the interactions between the different model peptide fragment of human glutamate receptor and KYNA molecules has relevance in neuroscience (Juhász et al., 2016). Moreover, experimental data indicate that KYNA may be neuroprotective and it may be of therapeutic value for several neurological disorders (Varga et al., 2016). Some article also reported that the KYNA may prove useful against domoic acid induced gastropathy because it protects against gastroduodenal ulceration (Glavin et al., 1989a). Furthermore, it was also reported that KYNA protects against gastric and duodenal ulceration caused by a poisonous Atlantic shellfish (Glavin and Pinsky, 1989b). However, according to the publication of Turski et. al., the concentration of KYNA increases gradually along the gastrointestinal tract, reaching its highest value at the very end of it and the lowest concentration of KYNA was found in human gastric juice (0.01 µM) (Turski et al., 2013). Thus, the application of KYNA for the treatment of gastroduodenal ulceration is limited.

Numerous mathematical models (zero-order, first-order, Weibull, Hixone-Crowell, Korsmeyere-Peppas, etc) have been developed to describe the release properties of the drug molecules (Costa and Lobo, 2001). There has not been reported mathematical model in the literature that takes into account all the important effects, in this way we chose three models that are widely used in literature. The first-order rate model is a typically used model which describes the adsorption and/or elimination of certain drugs and states that the drug release rate depends on its concentration.

$$c_t = c_0 e^{-kt} \quad (1)$$

where C_0 is the initial concentration of drug in the drug formulation, C_t is the concentration of drug in the drug formulation at time *t*, and *k* is the first-order release constant with units of reciprocal time.

Presently, many authors utilize the semi-empirical power low model that was proposed by Korsmeyer and Peppas (Peppas and Merrill, 1977). The model was developed to specifically model the release of a drug molecule from a polymeric matrix, such as a hydrogel using the following equation:

$$c_t = c_0 k_m t^n \quad (2)$$

where C_0 is the initial concentration of drug in the drug formulation, C_t is the concentration of released drug at time *t*, k_m is the kinetic constant and n the release index, indicating the mechanism of the drug release. At $n > 0.45$, non Fickian diffusion is observed, while $n \leq 0.45$ represents the Fickian diffusion mechanism. The *n* values refer to the geometries of the particles; in the diffusion-controlled release if the value of *n* is between 0.45 and 0.43, the geometries are slab, cylinder or sphere, respectively.

Many times the drug release process can be modeled with the classical Fick's diffusion equation or with the simplified Higuchi expressions (Siepmann and Peppas, 2011). Higuchi was the first in 1961 who described the release of the drug from an insoluble matrix based on Fickian diffusion. The Higuchi model is valid for the systems where the initial drug concentration in the matrix is much higher than the solubility of the drug.

$$c_t = k_H \sqrt{t} \quad (3)$$

where C_t is the concentration of drug in the drug matrix at time *t* and k_H is the Higuchi dissolution constant.

In this article the intercalation of neuroprotective and anti- ulcerant KYNA molecules in the biocompatible MgAl–LDH drug carrier system was examined. The quantitative

characterization of intercalation and the structural properties of the prepared KYNA pillared LDH composite materials was also reported. In addition, the LDH dissolution and the KYNA drug release profile from the bioactive composite material was also presented in ~~simulated gastric juice (SGJ)~~ simulated gastric fluid (SGF).

2. Materials and methods

2.1. Reagents

For the synthesis of layered double hydroxides magnesium nitrate hexahydrate ($Mg(NO_3)_2$•$6H_2O$, 98%; Sigma-Aldrich, United Kingdom), and aluminum nitrate nonahydrate ($Al(NO_3)_3$•$9H_2O$, 99.7%; Molar Chemicals Kft., Hungary) were used as precursors. Kynurenic acid (KYNA) was obtained from Sigma-Aldrich, United Kingdom. The sodium dodecyl sulfate ($C_{12}H_{25}NaO_4S$, 98%), hydrochloric acid (HCl, 37%) were obtained from Molar Chemicals Kft., Hungary. The pH was adjusted with sodium hydroxide (NaOH, 99.80%) and hydrochloric acid (HCl, 37%) which were obtained from Molar Chemicals Kft., Hungary. The ~~SGJ~~ SGF media was prepared using pepsin (1:10000 NF; 2000 u/g activity) and hydrochloric acid (HCl, 37%) obtained from Molar Chemicals Kft., Hungary and potassium chloride (KCl, 99.5-100%) obtained from Reanal, Hungary. Furthermore, sodium chloride (NaCl, 99.98%), sodium phosphate dibasic dodecahydrate (Na_2HPO_4•$12H_2O$, 100.3%) and sodium dihydrogen phosphate monohydrate (NaH_2PO_4•H_2O, 99%) were obtained from Molar Chemicals Kft., Hungary and were used for preparing PBS buffer. All aqueous solutions were made using deionized water.

2.2. Synthesis of 2:1 Mg/Al-LDH

Mg/Al-LDH was synthesized by co-precipitation method under N_2 atmosphere to avoid or at least to minimize the contamination of LDH by atmospheric CO_2, because the adsorption

affinity of the carbonate anions derived from atmospheric CO_2 is very high for LDH (Choy et al., 2004). So, in the case of the carbonation of the LDH, the further intercalation and ion-exchange of the CO_3- LDH would be impossible. During the synthesis 25.64 g of $Mg(NO_3)_2 \cdot 6\ H_2O$ and 18.76 g of $Al(NO_3)_3 \cdot 9H_2O$ were dissolved in 300 mL of distilled water under vigorous stirring and nitrogen atmosphere at room temperature. The molar ratio of Mg:Al was 2:1. Then, 200 mL of 1.875 mol/L concentration of NaOH was added dropwise to the first solution to obtain the pH=13. The resulting mixture was vigorously stirred at 80°C temperature under nitrogen atmosphere for 17 hours and aged at 80°C for 3 days. The resulting precipitate was separated by centrifugation, washed with distilled water twice and dried in an oven at 60°C overnight.

2.3. Intercalation of KYNA molecules into LDH layers

First, the KYNA/LDH weight ratio was systematically changed in order to determine the maximal intercalation capacity of the LDH layers for the KYNA drug molecules. During this experiments a calibration series was made from 2 mM KYNA stock solution using double dilutions and the KYNA concentration was determined by fluorometric measurements. The fluorescence spectra were recorded by a Horiba Jobin Yvon Fluoromax-4 spectrofluorometer (excitation at λ= 350 nm). The KYNA concentration was quantified by the determined spectrofluorometric calibration curve between 355-550 nm emission wavelength range and at a wavelength maximum of λ_{max}= 380 nm. During the adsorption measurements, the KYNA weight ratio was 0; 0.025; 0.05; 0.1; 0.165; 0.3 0.5 referred to the LDH host lamellae. The prepared KYNA/LDH suspensions were stirred for 1 hour at room temperature (25 ° C) in order to reach the adsorption equilibrium, then were filtered through a fine filter (Millipor, 0.22 μm) than the KYNA concentration was determined from the spectrofluorometric

calibration curves. The experiments were carried out triplicate, and average values are reported. Error bars refer to the standard deviation.

In the continuation, the amount of intercalated anionic substance (KYNA) was set at 30 wt% based on the LDH mass, i.e. the anionic KYNA/LDH weight ratio was 300 mg KYNA / g LDH. During the intercalation, 30 mg of KYNA was added to 10 ml of 1 wt% LDH suspension and stirred at 25°C for 48 hours under a nitrogen atmosphere to avoid the contamination of LDH by atmospheric CO_2. The pH of the LDH suspensions was adjusted to 10.0 by dropwise addition of 1 mol/L concentration of NaOH solution. The reaction product was filtered, washed with distilled water to remove adhered KYNA molecules, and dried at 60 °C in an oven for 24 h.

2.4. Methods of sample characterization

2.4.1. PXRD measurements

The X-ray diffractograms of the powdered 2:1 Mg/Al-LDH and the KYNA intercalated LDH layers were recorded on a Philips X ray diffractometer (PXRD) with CuK_α (= 0.1542 nm) as the radiation source at ambient temperature in the 2–40° and 2.5–15° (2Θ) range applying 0.02° (2Θ) step size.

2.4.2. Determination of surface charge of LDH samples

The surface charges of the LDH samples were measured in a particle charge detector (PCD-02 MÜTEK) with manual titration. In the course of a titration process, the surface charges of the studied samples were compensated by oppositely charged sodium dodecyl sulfate (SDS) surfactants with concomitant streaming potential measurements. During the titration process, 10 mL of a 0.1% LDH (pH=10) was added to the test cell of the PCD, and was titrated with oppositely charged surfactant (SDS) solution. The equimolar amount of surfactant was

calculated from the surfactant amounts added at the charge compensation point (where streaming potential = 0 mV) and was normalized to the amount of titrated sample (meq/g).

2.4.3. Determination of specific surface area of LDH sample (BET measurement)

The specific surface area of the LDH sample was determined by BET method from N_2 adsorption isotherms at 77 ± 0.5 K (Micromeritics Gemini 2375 Surface Area Analyzer). Before the adsorption measurements the samples were evacuated (10^{-5} mmHg) at 100°C overnight.

2.4.4. ATR-FTIR spectroscopy measurements

Attenuated total reflection Fourier transform infrared (ATR-FTIR) spectroscopy measurements were performed by a Biorad FTS-60A FT-IR spectrometer by accumulation of 256 scans at a resolution of 4 cm^{-1} between 4000 and 500 cm^{-1}. Each sample was previously weighted before spectrum acquisition (about 10 ± 1 mg of powder sample) and placed onto the ATR crystal. All spectral manipulations were performed using Thermo Scientific GRAMS/AI Suite software.

2.4.5. TG measurements

The thermal behavior of the LDH drug carrier, KYNA/LDH composite and KYNA and the KYNA content of the composite were investigated with thermogravimetric (TG) analysis. During TG measurements, the samples were heated in synthetic air from 25 to 1000°C at a heating rate of 5°C/min (Mettler-Toledo TGA/SDTA 851^e Instrument).

2.5. Dissolution experiment of LDH in acidic ~~SGJ~~ SGF media

The dissolution process of the LDH drug carrier was investigated in the presence of the ~~simulated gastric juice (SGJ)~~ simulated gastric fluid (SGF) at pH= 1.5 which was prepared with a buffer mixture composed of 0.2 M HCl solution and 0.2 M KCl solution, to which pepsin was added at a ratio of 10 U/ml (Guérin et al., 2003; Cunha et al., 1997). During the process 1.491 g of KCl and 0.5 g of pepsin were dissolved in 100 mL of distilled water under vigorous stirring and 1.67 mL of 37% concentration of HCl was added dropwise to the ~~SGJ~~ SGF solution to obtain the pH at 1.5. Then, 1.0 g of LDH powder sample was added to the ~~SGJ~~ SGF solution. The concentration of LDH in ~~SGJ~~ SGF solution was 0.01 g/mL. The dissolution of LDH was followed by gravimetrically measurements and the degradation of LDH was also recorded with PXRD measurements (using Philips X ray diffractometer with CuK_{α} (= 0.1542 nm) as the radiation source at ambient temperature in the 2–30° (2Θ) range applying 0.02° (2Θ) step size.). The weight- loss measurements were carried out triplicate, and average values are reported with the calculated standard deviations.

2.6. In vitro drug release experiments

The in vitro experiments were carried out using a dialysis tubing cellulose membrane (typical molecular weight cut-off= 12-14 kDa from Sigma Aldrich). Briefly, 1 mL of KYNA solution (c= 1.6 mg/mL) or KYNA/LDH dispersion in PBS buffer (c= 1.6 mg/mL, KYNA content: ~12%), (pH=6.70) was pipetted into the tube membrane (d=1 cm, l=10 cm) then immersed into 100 mL of PBS solution (at pH= 6.70 and 1.50) in a vertical position. The 100 mL of PBS solution with the carefully closed membrane was stirred continuously with a magnetic stirrer and the release experiments were carried out at 37.0 °C using a water bath. Samples were taken every 2.5 minutes in the first 10 minutes, then were taken in the 15^{th} and 20^{th} minutes. After 20 minutes the samples were taken in each 10 minutes until the first hour and

then were taken after 30 minutes in every hour. Measurements were performed in 240 min. The presence of kynurenic acid was recorded with a diode array spectrophotometer (Ocean Optics USB2000; USA) in the λ = 250–350 nm range using a 1 cm quartz cuvette. The released kynurenic acid concentration was quantified by the previously determined spectrophotometric calibration curve at a wavelength maximum of λ_{max}= 311 nm. To determine the value of kinetic constants of the applied release kinetic models, the sum of the square of differences between the measured and predicted concentration values have been minimalized using a spreadsheet based computer application for nonlinear parameter estimation (Juhász et al., 2016).

3. Results and discussion

3.1. Characterization of the 2:1 Mg/Al-LDH

Fig. 1a illustrates the PXRD pattern of the synthesized Mg/Al-LDH sample and displays the (003) and (006) Bragg reflections characteristic to layered double hydroxides (JCPDS No. 89-0460) (Deng et al., 2015). These peaks are positioned at an angle of 11.39° (2Θ) ($d_{(003)}$= 0.77 nm) and the peak representing the secondary reflection is at an angle of 22.82° (2Θ) ($d_{(006)}$ = 0.39 nm). In addition, further characteristic reflection was observed at angle of 34.39° (2Θ) ($d_{(009)}$ = 0.26 nm) which is the (009) reflection characteristic to the sample (Costa et al., 2008). The surface charge value of LDH was determined by charge titration (Szabó et al., 2013). The streaming potentials of diluted, 0.1 wt.% LDH suspension (pH=10) was measured in a Mütek PCD02 apparatus, while adding oppositely charged NaDS surfactant solution to the system. A typical charge titration curve is presented in Fig. 1b. The measured initial streaming potential of layered LDH was positive (+704 mV). This value gradually decreased upon addition of the oppositely charged 0.1% anionic SDS solution, and reached 0 mV after the addition of 1.8 ml of surfactant solution. At this point (i.e., the charge equivalence point or

c.e.p.), the negatively charged anionic surfactant molecules compensated the positive charge of LDH in the aqueous suspension. Due to the surface adsorption of the SDS molecules on the hydrophilic LDH lamellae, the obtained hydrophobized sample was sedimented from the aqueous media after the titration process (see inserted photos). Based on the charge titration data, the specified charge of LDH at pH= 10 was +0.641 meq/g LDH. The positive surface charge of conventional LDH layers of lamellar structure is well-known in the literature (Glavin et al., 1989a), and creates the basis of its widespread utilization for the adsorption of various negatively charged molecules (Choy et al., 2007; Fudala et al., 1999).

The BET adsorption isotherm of the LDH sample reveals type II isotherm, expressing multilayer N_2 adsorption, without significant hysteresis loops between the adsorption and desorption branches (Fig. 2a). The analysis of the N_2 adsorption isotherm by the t-plot method (Fig. 2b, inset) shows that the sample has a nonporous structure. The BET- method was used to calculate the surface area of the LDH which was 114.96 ± 0.48 m^2/g.

3.2. Characterization of the intercalated kynurenic acid in LDH drug carrier

The prepared positively charged (+0.641 meq/g) LDH lamellae with relatively high specific surface area (114.96 ± 0.48 m^2/g) was suitable for the intercalation of negatively charged guest molecules. The intercalation capacity of the KYNA drug molecules into LDH layers was measured by fluorescence spectroscopy method (Fig. 3). During the experiments, the KYNA content of the samples was systematically changed from 2.5 to 50 wt.%. The obtained results presented in Fig. 3 showed that with the increasing weight ratio of KYNA/LDH the amount of intercalated KYNA is also increasing and the determined drug- loading capacity was about 120 mg KYNA/ g LDH. Considering that the measured surface charge of LDH was +0.641 meq/g LDH and the molecular weight of KYNA (189.16 g/mol), the calculated

297 theoretical intercalated amount of KYNA was 121 mg KYNA/ g LDH. This result is in good
298 agreement with the experimentally determined value (~12%).
299
300 *3.2.1. PXRD analysis of KYNA intercalation*
301 Fig. 4 illustrates the PXRD patterns for the Mg/Al-LDH intercalated with KYNA compared
302 with the initial LDH material. The intercalation of KYNA in LDH has significant influence on
303 the PXRD pattern of LDH at pH=10. The well-crystallized LDH structure with a (003)
304 diffraction peak positioned at an angle of 11.39° (2Θ) shows another (003) diffraction peak
305 centered at 2.146 nm at a lower 2Θ angle (4.11°). The interlayer spacing increases by 1.371
306 nm in comparison with initial positively charged LDH layers, which shows an interlayer
307 spacing of 0.775 nm indicating the successful intercalation of drug anions between the
308 interlayer regions.
309 As Cavani et al. (1991) mentioned, the thickness of the brucite-like layer of LDH is 0.48 nm,
310 the gallery height in the KYNA-pillared material is 1.666 nm. A gallery height of 1.666 nm
311 suggests a paraffin type monolayer arrangement (Betega de Paiva et al., 2008; Chiu et al.,
312 2014) of the intercalated KYNA, with their main axis perpendicular to the layer and the anion
313 carboxyl groups interacting with positively charged layer surfaces. The suspected structure,
314 i.e. the paraffin type monolayer arrangement of the obtained LDH/ KYNA composite is also
315 presented in Fig. 4.
316
317 *3.2.2. FT-IR spectra*
318 The FT-IR spectra of the KYNA intercalated in Mg/Al-LDH are presented in Fig. 5. The FT-
319 IR spectra of initial LDH and pure KYNA are also illustrated for comparison. Fig. 5a shows
320 the spectrum of nitrate-LDH, a broad absorption band at 3450 cm^{-1} which is due to the
321 stretching vibration of the hydroxyl groups of the LDH layers and water molecules from the

322 interlayer space. The band at 1383 cm^{-1} was assigned to stretching vibration of NO_3^- (Aisawa
323 et al., 2015). This strong absorption peak of the $NO_3^-$was decreased after the ion-exchanged
324 reaction, supporting that $NO_3^-$was replaced by the KYNA molecule (Fig. 5b).
325 In the spectrum of KYNA (Fig. 5c) the band at 3474 cm^{-1} was derived from the stretching
326 vibrations of –O–H bonds in the carboxyl group. The band centered at 3431 cm^{-1} is due to
327 OH bonded to the quinoline ring and the signal seen at 3076 cm^{-1} is attributed to the C–H
328 bonds of the quinoline ring. Furthermore, the band at 2968 cm^{-1} corresponds to stretch
329 vibrations of the C=H bonds of benzyl and quinoline rings and the signal at 2718 cm^{-1}
330 corresponds to the double bond N=C in the quinoline ring (López et al., 2014). The
331 asymmetric and symmetric COO stretching is obtained between 1700-1400 cm^{-1}, those at
332 1631, 1595, 1415 and 1247 cm^{-1} were attributed to ν(C=O), ν_{as}(COO), ν_s(COO) and ν(C-N),
333 respectively. (Geng et al., 2009; Ibrahim et al., 2005). After intercalation, the characteristic
334 bands of KYNA were observed at 1631, 1595, 1415, 1247 cm^{-1} in the case of KYNA/LDH
335 (Fig. 5b). So, according to the evaluation of the IR spectra, the intercalation of KYNA
336 molecules into the LDH lamellae was also proved.
337
338 *3.2.3. Thermal analysis*
339 Thermogravimetric analysis of the initial LDH clay and the synthetized composites provides
340 useful information regarding the thermal stability and thermal decomposition temperature of
341 the materials. The TG curve of powdered KYNA/ LDH pillared composite is illustrated in
342 Fig. 6. The corresponding TG curves for pure KYNA and initial Mg/Al-LDH are also
343 presented for comparison.
344 In the LDH, the thermal decomposition stages are generally overlapped and the exact
345 temperature range of each stage depends largely on LDH type, heating rate and atmosphere
346 (N_2 or O_2) (Benício et al., 2015). According to the literature data, LDH thermal behavior is

usually characterized by two main transition stages: (i) an endothermic process from room temperature to about 200 °C that corresponds to adsorbed and interlamellar water loss; this stage is reversible and occurs without lamellar structure collapse; and (ii) the second stage occurs with temperatures ranging from 200 to about 6-800 °C, and corresponds to lamellar hydroxyl group loss (dehydroxylation) as well as anions loss (Benício et al., 2015). In the case of our LDH sample, both weight losses can be observed. The interlamellar water content was 15.7 wt.% (first step up until ~245 °C), while after about 650 °C, the remaining weight was 53.7 wt.%. It can be also seen, that the heat degradation of the pure KYNA was occurred between 150 and 650 °C and after about this temperature, the mass loss of organic KYNA was complete (dashes line). Compared the corresponding TG curves, it can be determined that the remaining weigh difference between the KYNA/ LDH composite and the LDH sample was 13.9 wt.%. Fig. 3 shows that the maximum adsorption capacity of the LDH clay lamellae was about 120 mg KYNA/ g LDH. The thermogravimetric results were confirmed this ~12 wt.% KYNA content of the KYNA pillared composite material.

3.3. Dissolution properties of LDH clay in ~~SGJ~~ SGF media

The dissolution properties of the LDH drug carrier in acidic pH was investigated gravimetrically and the acidic dissolution, i.e. the destruction of lamellae was also followed up by PXRD measurements (Fig. 7) The dissolution profile of LDH at pH= 1.5 was exhibited a very fast process: after about 30 minutes, more than 70 wt.% of the initial LDH weight was disappeared and at the end of the experiment, near 83% of LDH were completely dissolved in the applied ~~SGJ~~ SGF media after 360 minutes (Fig. 7a). The corresponding PXRD patterns of the LDH is presented in Fig. 7b and gives information about the LDH structure during the dissolution experiment. It can be observed that the intensity of the characteristic peaks of the well-crystallized LDH was continuously decreased, i.e. the (003) diffraction peak positioned

at an angle of 11.39° (2Θ) was shown decreasing intensity. This phenomenon is obviously due to dissolution of LDH drug carrier at pH= 1.5.

3.4. *In vitro* drug release properties of the samples

The release properties of KYNA were studied at the pH of human saliva (Baliga et al., 2013; Pietrzyńska and Voelkel, 2017) at a value of 6.70 and at pH of ~~SGJ~~ SGF (pH=1.50), as well. The release study of the KYNA/LDH have also been performed at pH=1.5. Fig. 8 presents the drug release profiles which were carried out over a period of 240 min and at body temperature (37 °C) and in physiological saline. The released concentration of drug were determined spectrophotometrically. The solubility and thus the release rate of anionic KYNA depends on pH (Varga et al., 2016). Thus, on Fig. 8a, a significant difference can be seen on the release rate of KYNA between pH= 6.70 and 1.50 under the same experimental condition. Due to the higher solubility of the KYNA molecules at pH= 6.70, the percentage amount of the released drug molecules were continuously increased up to 180 min and after three hours it was reached the 90% plateau value. However, at low pH (=1.50), the release of the KYNA was obviously slower process and the measured maximum value was only about 40%, because in this pH the KYNA molecules were formed heterogeneous precipitate instead of a clear homogenous solution (pH=6.70) (see inserted photos). In the case of KYNA release from concentrated solutions via the fitted (Fig. 8b) first-order rate model (Eq. 1) the following rate constant values (k) were provided: $4.74 \cdot 10^{-5}$ s^{-1} and $5.91 \cdot 10^{-5}$ s^{-1} at pH=6.70 and pH=1.50, respectively. As regarding the composite sample, drug release profile for KYNA/LDH at pH= 1.5 was exhibited a delayed drug release rate compared to the pure KYNA at the same pH. This is because the kinetic of the release process is controlled by the LDH, so the diffusion rate is significantly decreased. The determined k value was $1.49 \cdot 10^{-5}$ s^{-1} in this case. The corresponding half-life values ($t_{1/2}$ = 4.1 and 3.3 h for KYNA at pH=6.70 and 1.50,

respectively) are smaller than the half-life value of composite sample ($t_{1/2}$ = 12.9 h) at pH=1.50. The kinetic constant, release index and correlation coefficients (R^2) values, which are summarised in Table 1 indicate what processes are controlled by the suggested release mechanism. Based on the values of correlation coefficients release of the KYNA from the KYNA/LDH composite was well described by the first-order rate model, while the drug release from concentrated solutions agreed with the Korsmeyer–Peppas model. The observed low release index values (n = 0.43 and 0.38 for KYNA at pH=6.70 and 1.50, respectively) suggest the existing of Fickian diffusion mechanism which absolutely corresponds to the experimentally arranged conditions. Moreover, this model clearly shows the appearance of non Fickian diffusion (n = 0.46 for KYNA/LDH at pH = 1.50) in the case of the composite where combination of drug diffusion and carrier erosion result the anomalous process.

This result allows to conclude that the rate determining step for release of KYNA from the KYNA/LDH composite may be depending on the following conditions: (i) dissolution of LDH lamellae; (ii) ion-exchange reaction between KYNA and LDH and (iii) release of KYNA from LDH host lamellae. These data indicate that the Mg/Al-LDH could be an adequate drug carrier system for the neuroprotective and anti- ulcerant KYNA molecules at low pH.

4. Conclusions

This study demonstrated the intercalation of anti- ulcerant KYNA molecules into a layered inorganic host, LDH, which was carried out with a simply ion- exchange reaction. The PXRD studies showed a paraffin type monolayer arrangement for anti-ulcerant drug molecules into the synthetized KYNA/ LDH hybrid material. The drug loading capacity of the 2:1 Mg/Al-LDH was studied by fluorescence spectroscopy method and the obtained ~12% KYNA content was in good agreement with the theoretical value calculated from the +0.641 meq/g

surface charge of the LDH lamellae. Moreover, this ~12% KYNA content of the hybrid materials was also confirmed by thermogravimetric (TG) measurements.

Next, it was also demonstrated by gravimetric and PXRD measurements, that the prepared LDH was almost completely dissolved (~83 wt.%) in the applied simulated gastric ~~juice (SGJ)~~ fluid (SGF) media at pH=1.5. The *in vitro* release studies showed that due to the higher solubility of the KYNA molecules at pH= 6.70 a significant difference can be seen on the release rate of KYNA between pH= 6.70 ($t_{1/2}$ = 4.1 h) and 1.50 ($t_{1/2}$ = 3.3 h) under the same experimental condition. The drug release from KYNA/ LDH composite material at pH= 1.5 was observed a delayed drug release profile ($1.49 \cdot 10^{-5}$ s^{-1}) compared to the pure KYNA ($t_{1/2}$ = 12.9 h) at the same pH. The obtained results gives a proof to us that the chosen LDH host molecule could be an adequate drug carrier system for the neuroprotective and anti- ulcerant KYNA molecules at gastric pH, used for peptic ulcer diseases.

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Figure 1. PXRD pattern of the synthetized 2:1 Mg/Al LDH powder (a) and the charge titration curve of 0.1 wt.% cationic LDH suspension (pH=10) with 0.1 wt.% of anionic sodium dodecyl sulfate (NaDS) surfactant solution (b).

Figure 2. Adsorption isotherms of N_2 at 77 K on LDH powder sample (a). The inserted figure shows the corresponding deBoer *t*-plot of the same sample (b).

Figure 3. The effect of the KYNA weight ratio on the intercalation capacity of the KYNA molecules loaded LDH layers

Figure 4. PXRD patterns of the initial LDH lamellae and KYNA molecules intercalated into the LDH layers and the proposed structure of KYNA-LDH composite

Figure 5. FT-IR spectra for (a) LDH drug carrier, (b) KYNA-LDH composite and (c) KYNA powder samples

Figure 6. TG profiles for LDH drug carrier, KYNA-LDH composite and initial KYNA

Figure 7. The gravimetrically measured percentage weight loss of the LDH drug carrier in ~~SGJ~~ SGF media at pH= 1.5 (a) and the corresponding PXRD patterns of LDH sample during the dissolution process (b).

Figure 8. The percentage release profile of anionic KYNA molecules at two different pH values (pH= 6.70 and 1.50) and the KYNA release from LDH lamellae at acidic (pH= 1.50) pH, as well as the measured KYNA concentration values (C_t, mg/ mL) as a function of release time with the fitted curves calculated by the first-order rate model

Table 1. Interpretation of the release experiments using different various models.

Anti-ulcerant kynurenic acid molecules intercalated Mg/Al-layered double hydroxide and its release study

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Abstract

Kynurenic acid (KYNA) is a product of the tryptophan metabolism and it possess also anti-ulcerant properties, however, the application of KYNA for the treatment of gastroduodenal ulceration is limited, because the concentration of KYNA is very low in human gastric fluid (0.01 µM). The intercalation of KYNA molecules into biocompatible Mg–Al layered double hydroxides (LDH) lamellae could solve this problem. For this purpose Mg–Al LDH with 114.96 ± 0.48 m^2/g BET surface area and +0.641 meq/g specific surface charge was synthesized. The intercalation of the anionic target molecules into positively charged LDH layers was carried out with simply ion- exchange reaction. The structure of the obtained KYNA/ LDH hybrid materials were studied by powdered X-ray diffraction (PXRD) and Attenuated total reflection Fourier transform infrared (ATR-FTIR) spectroscopy verifying that the KYNA molecules prefer creating a paraffin type monolayer arrangement. Due to the

intercalation process the (003) reflection peaks of initial LDH (2Θ= 11.39°, $d_{(003)}$= 0.775 nm) shift to lower angles (2Θ= 4.11°, *d*= 2.146 nm). That means, that the basal space value (Δd_L) of the KYNA-LDH sample was 1.436 nm. The total amount of the intercalated KYNA molecules into LDH layers was measured by fluorescence spectroscopy method. According to the results the drug- loading capacity was about 120 mg KYNA/ g LDH. This ~12% KYNA content of the hybrid materials was also evidenced by thermogravimetric measurements, because the thermal decomposition of the bio-hybrid materials was examined by thermogravimetry (TG) analysis. Our experimental data confirm that the anti- ulcerant KYNA molecules can be safely loaded and stored into LDH's layers forming a new bio-active hybrid material. In addition we also presented by PXRD and gravimetric measurements that prepared LDH layers were almost completely dissolved (~83 wt.%) in the applied simulated gastric fluid (SGF) media (pH=1.5) under 60 min and the encapsulated KYNA molecules released from the destroyed interlayers. Finally, the measured KYNA drug release profile from the bioactive composite material was also presented in SGF media. According to the results 18% of the loaded KYNA molecules were released during 6 hours.

Keywords: layered double hydroxide, kynurenic acid, intercalation, *in vitro* drug release study, anti- ulcerant properties, simulated gastric fluid

1. Introduction

LDHs are a class of anionic lamellar compounds made up of positively charged brucite- like layers (Trifiro and Vaccari, 1996). The chemical composition of the two layers of hydrotalcite-type minerals can be given by the following general formula: $[M^{2+}_{1-x}M^{3+}_{x}(OH)_2]^{b+}\cdot[A_{b/n}]^{n-}\cdot mH_2O$, where M^{2+} represents divalent and M^{3+} represents trivalent cations, the value of x may vary in the range of 0.2−0.4, and A is the anion among

the cationic layers (OH^-, Cl^-, NO^{3-}, CO_3^{2-}, and SO_4^{2-}) (Constantino and Nocchetti, 2001). LDHs have been widely exploited to create new materials for applications in catalysis (Patzkó et al., 2005; Deák et al., 2016), drug delivery and environmental remediation (Bujdosó et al., 2009; Goh et al., 2008). MgAl–LDHs are most frequently used as a LDH-based drug carrier and as evidence of its low toxicity, it is widely used as an antacid (Tarnawski et al., 2000) and the biocompatibility of this layered material was also reported in the literature (Cunha et al., 2016, Nagy et al., 2013). LDHs particularly prefer multivalent anions within their interlayer space due to strong electrostatic interaction and therefore LDHs bearing monovalent anions like nitrate or chloride ions are good precursors for exchange reactions (Choy et al., 2007). The solubility and surface charge of LDHs as hydroxides is highly pH-dependent (Bish, 1980; Deák et al., 2015).

Layered clay minerals are widely used for their capability to intercalate molecules in the interlayer space. It is also well known that beside the LDH drug carrier, the negatively charged clay minerals such as Montmorillonite [$(Na,Ca)_{0.33}$ $(Al, Mg)_2(Si_4$ $O_{10})(OH)_2$ • nH_2O] exhibit an excellent sorption property, large specific surface area, cation exchange capacity and drug-carrying capability (Joshi et al., 2009; Patel et al., 2011; Kevadiya et al., 2010). It is worth mentioning that the pioneering works of Choy' group have led to a rapid development in the research on both varied LDHs/polymers/anions hybrid systems and pharmaceutical applications of LDHs especially involving the biocompatibility and toxicity of LDHs and anti-cancer drugs intercalated LDH materials (Choy et al., 2007). Li et al. also developed anti-inflammatory drug fenbufen–LDH hybrids and showed that these drug-inorganic hybrid materials can be used as an effective drug delivery system due to their controlled release capacity (Li et al., 2004). Yang et al. reported the intercalation of vitamins A, E, and C into LDHs (Yang et al., 2003). Moreover, in addition to the intercalation of pharmaceutical drugs into layered materials causing no significant denaturation of the drug molecules, it has also

been shown to enhance the internalization of the drug into a target cell without any noticeable side effects (Oh et al., 2009). Thus, LDHs can not only play a role as a biocompatible-delivery matrix for drugs but also afford a significant increase in the delivery efficiency (Posati et al., 2012; Oh et al., 2006).

Kynurenic acid (KYNA) is a product of the tryptophan metabolism, it has a neuroprotective and neuroinhibitory properties (Marosi et al., 2010). According to this the interactions between the different model peptide fragment of human glutamate receptor and KYNA molecules has relevance in neuroscience (Juhász et al., 2016). Moreover, experimental data indicate that KYNA may be neuroprotective and it may be of therapeutic value for several neurological disorders (Varga et al., 2016). Some article also reported that the KYNA may prove useful against domoic acid induced gastropathy because it protects against gastroduodenal ulceration (Glavin et al., 1989a). Furthermore, it was also reported that KYNA protects against gastric and duodenal ulceration caused by a poisonous Atlantic shellfish (Glavin and Pinsky, 1989b). However, according to the publication of Turski et. al., the concentration of KYNA increases gradually along the gastrointestinal tract, reaching its highest value at the very end of it and the lowest concentration of KYNA was found in human gastric juice (0.01 μM) (Turski et al., 2013). Thus, the application of KYNA for the treatment of gastroduodenal ulceration is limited.

Numerous mathematical models (zero-order, first-order, Weibull, Hixone-Crowell, Korsmeyere-Peppas, etc) have been developed to describe the release properties of the drug molecules (Costa and Lobo, 2001). There has not been reported mathematical model in the literature that takes into account all the important effects, in this way we chose three models that are widely used in literature. The first-order rate model is a typically used model which describes the adsorption and/or elimination of certain drugs and states that the drug release rate depends on its concentration.

$$c_t = c_0 e^{-kt} \quad (1)$$

where C_0 is the initial concentration of drug in the drug formulation, C_t is the concentration of drug in the drug formulation at time *t*, and *k* is the first-order release constant with units of reciprocal time.

Presently, many authors utilize the semi-empirical power low model that was proposed by Korsmeyer and Peppas (Peppas and Merrill, 1977). The model was developed to specifically model the release of a drug molecule from a polymeric matrix, such as a hydrogel using the following equation:

$$c_t = c_0 k_m t^n \quad (2)$$

where C_0 is the initial concentration of drug in the drug formulation, C_t is the concentration of released drug at time *t*, k_m is the kinetic constant and n the release index, indicating the mechanism of the drug release. At $n > 0.45$, non Fickian diffusion is observed, while $n \leq 0.45$ represents the Fickian diffusion mechanism. The *n* values refer to the geometries of the particles; in the diffusion-controlled release if the value of *n* is between 0.45 and 0.43, the geometries are slab, cylinder or sphere, respectively.

Many times the drug release process can be modeled with the classical Fick's diffusion equation or with the simplified Higuchi expressions (Siepmann and Peppas, 2011). Higuchi was the first in 1961 who described the release of the drug from an insoluble matrix based on Fickian diffusion. The Higuchi model is valid for the systems where the initial drug concentration in the matrix is much higher than the solubility of the drug.

$$c_t = k_H \sqrt{t} \quad (3)$$

where C_t is the concentration of drug in the drug matrix at time *t* and k_H is the Higuchi dissolution constant.

In this article the intercalation of neuroprotective and anti- ulcerant KYNA molecules in the biocompatible MgAl–LDH drug carrier system was examined. The quantitative

characterization of intercalation and the structural properties of the prepared KYNA pillared LDH composite materials was also reported. In addition, the LDH dissolution and the KYNA drug release profile from the bioactive composite material was also presented in simulated gastric fluid (SGF).

2. Materials and methods

2.1. Reagents

For the synthesis of layered double hydroxides magnesium nitrate hexahydrate ($Mg(NO_3)_2 \cdot 6H_2O$, 98%; Sigma-Aldrich, United Kingdom), and aluminum nitrate nonahydrate ($Al(NO_3)_3 \cdot 9H_2O$, 99.7%; Molar Chemicals Kft., Hungary) were used as precursors. Kynurenic acid (KYNA) was obtained from Sigma-Aldrich, United Kingdom. The sodium dodecyl sulfate ($C_{12}H_{25}NaO_4S$, 98%), hydrochloric acid (HCl, 37%) were obtained from Molar Chemicals Kft., Hungary. The pH was adjusted with sodium hydroxide (NaOH, 99.80%) and hydrochloric acid (HCl, 37%) which were obtained from Molar Chemicals Kft., Hungary. The SGF media was prepared using pepsin (1:10000 NF; 2000 u/g activity) and hydrochloric acid (HCl, 37%) obtained from Molar Chemicals Kft., Hungary and potassium chloride (KCl, 99.5-100%) obtained from Reanal, Hungary. Furthermore, sodium chloride (NaCl, 99.98%), sodium phosphate dibasic dodecahydrate ($Na_2HPO_4 \cdot 12H_2O$, 100.3%) and sodium dihydrogen phosphate monohydrate ($NaH_2PO_4 \cdot H_2O$, 99%) were obtained from Molar Chemicals Kft., Hungary and were used for preparing PBS buffer. All aqueous solutions were made using deionized water.

2.2. Synthesis of 2:1 Mg/Al-LDH

Mg/Al-LDH was synthesized by co-precipitation method under N_2 atmosphere to avoid or at least to minimize the contamination of LDH by atmospheric CO_2, because the adsorption

affinity of the carbonate anions derived from atmospheric CO_2 is very high for LDH (Choy et al., 2004). So, in the case of the carbonation of the LDH, the further intercalation and ion-exchange of the CO_3- LDH would be impossible. During the synthesis 25.64 g of $Mg(NO_3)_2 \cdot 6\ H_2O$ and 18.76 g of $Al(NO_3)_3 \cdot 9H_2O$ were dissolved in 300 mL of distilled water under vigorous stirring and nitrogen atmosphere at room temperature. The molar ratio of Mg:Al was 2:1. Then, 200 mL of 1.875 mol/L concentration of NaOH was added dropwise to the first solution to obtain the pH=13. The resulting mixture was vigorously stirred at 80°C temperature under nitrogen atmosphere for 17 hours and aged at 80°C for 3 days. The resulting precipitate was separated by centrifugation, washed with distilled water twice and dried in an oven at 60°C overnight.

2.3. Intercalation of KYNA molecules into LDH layers

First, the KYNA/LDH weight ratio was systematically changed in order to determine the maximal intercalation capacity of the LDH layers for the KYNA drug molecules. During this experiments a calibration series was made from 2 mM KYNA stock solution using double dilutions and the KYNA concentration was determined by fluorometric measurements. The fluorescence spectra were recorded by a Horiba Jobin Yvon Fluoromax-4 spectrofluorometer (excitation at λ= 350 nm). The KYNA concentration was quantified by the determined spectrofluorometric calibration curve between 355-550 nm emission wavelength range and at a wavelength maximum of λ_{max}= 380 nm. During the adsorption measurements, the KYNA weight ratio was 0; 0.025; 0.05; 0.1; 0.165; 0.3 0.5 referred to the LDH host lamellae. The prepared KYNA/LDH suspensions were stirred for 1 hour at room temperature (25 ° C) in order to reach the adsorption equilibrium, then were filtered through a fine filter (Millipor, 0.22 μm) than the KYNA concentration was determined from the spectrofluorometric

calibration curves. The experiments were carried out triplicate, and average values are reported. Error bars refer to the standard deviation.

In the continuation, the amount of intercalated anionic substance (KYNA) was set at 30 wt% based on the LDH mass, i.e. the anionic KYNA/LDH weight ratio was 300 mg KYNA / g LDH. During the intercalation, 30 mg of KYNA was added to 10 ml of 1 wt% LDH suspension and stirred at 25°C for 48 hours under a nitrogen atmosphere to avoid the contamination of LDH by atmospheric CO_2. The pH of the LDH suspensions was adjusted to 10.0 by dropwise addition of 1 mol/L concentration of NaOH solution. The reaction product was filtered, washed with distilled water to remove adhered KYNA molecules, and dried at 60 °C in an oven for 24 h.

2.4. Methods of sample characterization

2.4.1. PXRD measurements

The X-ray diffractograms of the powdered 2:1 Mg/Al-LDH and the KYNA intercalated LDH layers were recorded on a Philips X ray diffractometer (PXRD) with CuK_α (= 0.1542 nm) as the radiation source at ambient temperature in the 2–40° and 2.5–15° (2Θ) range applying 0.02° (2Θ) step size.

2.4.2. Determination of surface charge of LDH samples

The surface charges of the LDH samples were measured in a particle charge detector (PCD-02 MÜTEK) with manual titration. In the course of a titration process, the surface charges of the studied samples were compensated by oppositely charged sodium dodecyl sulfate (SDS) surfactants with concomitant streaming potential measurements. During the titration process, 10 mL of a 0.1% LDH (pH=10) was added to the test cell of the PCD, and was titrated with oppositely charged surfactant (SDS) solution. The equimolar amount of surfactant was

calculated from the surfactant amounts added at the charge compensation point (where streaming potential = 0 mV) and was normalized to the amount of titrated sample (meq/g).

2.4.3. Determination of specific surface area of LDH sample (BET measurement)

The specific surface area of the LDH sample was determined by BET method from N_2 adsorption isotherms at 77 ± 0.5 K (Micromeritics Gemini 2375 Surface Area Analyzer). Before the adsorption measurements the samples were evacuated (10^{-5} mmHg) at 100°C overnight.

2.4.4. ATR-FTIR spectroscopy measurements

Attenuated total reflection Fourier transform infrared (ATR-FTIR) spectroscopy measurements were performed by a Biorad FTS-60A FT-IR spectrometer by accumulation of 256 scans at a resolution of 4 cm^{-1} between 4000 and 500 cm^{-1}. Each sample was previously weighted before spectrum acquisition (about 10 ± 1 mg of powder sample) and placed onto the ATR crystal. All spectral manipulations were performed using Thermo Scientific GRAMS/AI Suite software.

2.4.5. TG measurements

The thermal behavior of the LDH drug carrier, KYNA/LDH composite and KYNA and the KYNA content of the composite were investigated with thermogravimetric (TG) analysis. During TG measurements, the samples were heated in synthetic air from 25 to 1000°C at a heating rate of 5°C/min (Mettler-Toledo TGA/SDTA 851^e Instrument).

2.5. Dissolution experiment of LDH in acidic SGF media

The dissolution process of the LDH drug carrier was investigated in the presence of the simulated gastric fluid (SGF) at pH= 1.5 which was prepared with a buffer mixture composed of 0.2 M HCl solution and 0.2 M KCl solution, to which pepsin was added at a ratio of 10 U/ml (Guérin et al., 2003; Cunha et al., 1997). During the process 1.491 g of KCl and 0.5 g of pepsin were dissolved in 100 mL of distilled water under vigorous stirring and 1.67 mL of 37% concentration of HCl was added dropwise to the SGF solution to obtain the pH at 1.5. Then, 1.0 g of LDH powder sample was added to the SGF solution. The concentration of LDH in SGF solution was 0.01 g/mL. The dissolution of LDH was followed by gravimetrically measurements and the degradation of LDH was also recorded with PXRD measurements (using Philips X ray diffractometer with CuK_{α} (= 0.1542 nm) as the radiation source at ambient temperature in the 2–30° (2Θ) range applying 0.02° (2Θ) step size.). The weight- loss measurements were carried out triplicate, and average values are reported with the calculated standard deviations.

2.6. In vitro drug release experiments

The in vitro experiments were carried out using a dialysis tubing cellulose membrane (typical molecular weight cut-off= 12-14 kDa from Sigma Aldrich). Briefly, 1 mL of KYNA solution (c= 1.6 mg/mL) or KYNA/LDH dispersion in PBS buffer (c= 1.6 mg/mL, KYNA content: ~12%), (pH=6.70) was pipetted into the tube membrane (d=1 cm, l=10 cm) then immersed into 100 mL of PBS solution (at pH= 6.70 and 1.50) in a vertical position. The 100 mL of PBS solution with the carefully closed membrane was stirred continuously with a magnetic stirrer and the release experiments were carried out at 37.0 °C using a water bath. Samples were taken every 2.5 minutes in the first 10 minutes, then were taken in the 15^{th} and 20^{th} minutes. After 20 minutes the samples were taken in each 10 minutes until the first hour and

then were taken after 30 minutes in every hour. Measurements were performed in 240 min. The presence of kynurenic acid was recorded with a diode array spectrophotometer (Ocean Optics USB2000; USA) in the λ = 250–350 nm range using a 1 cm quartz cuvette. The released kynurenic acid concentration was quantified by the previously determined spectrophotometric calibration curve at a wavelength maximum of λ_{max}= 311 nm. To determine the value of kinetic constants of the applied release kinetic models, the sum of the square of differences between the measured and predicted concentration values have been minimalized using a spreadsheet based computer application for nonlinear parameter estimation (Juhász et al., 2016).

3. Results and discussion

3.1. Characterization of the 2:1 Mg/Al-LDH

Fig. 1a illustrates the PXRD pattern of the synthesized Mg/Al-LDH sample and displays the (003) and (006) Bragg reflections characteristic to layered double hydroxides (JCPDS No. 89-0460) (Deng et al., 2015). These peaks are positioned at an angle of 11.39° (2Θ) ($d_{(003)}$= 0.77 nm) and the peak representing the secondary reflection is at an angle of 22.82° (2Θ) ($d_{(006)}$ = 0.39 nm). In addition, further characteristic reflection was observed at angle of 34.39° (2Θ) ($d_{(009)}$ = 0.26 nm) which is the (009) reflection characteristic to the sample (Costa et al., 2008). The surface charge value of LDH was determined by charge titration (Szabó et al., 2013). The streaming potentials of diluted, 0.1 wt.% LDH suspension (pH=10) was measured in a Mütek PCD02 apparatus, while adding oppositely charged NaDS surfactant solution to the system. A typical charge titration curve is presented in Fig. 1b. The measured initial streaming potential of layered LDH was positive (+704 mV). This value gradually decreased upon addition of the oppositely charged 0.1% anionic SDS solution, and reached 0 mV after the addition of 1.8 ml of surfactant solution. At this point (i.e., the charge equivalence point or

c.e.p.), the negatively charged anionic surfactant molecules compensated the positive charge of LDH in the aqueous suspension. Due to the surface adsorption of the SDS molecules on the hydrophilic LDH lamellae, the obtained hydrophobized sample was sedimented from the aqueous media after the titration process (see inserted photos). Based on the charge titration data, the specified charge of LDH at pH= 10 was +0.641 meq/g LDH. The positive surface charge of conventional LDH layers of lamellar structure is well-known in the literature (Glavin et al., 1989a), and creates the basis of its widespread utilization for the adsorption of various negatively charged molecules (Choy et al., 2007; Fudala et al., 1999).

The BET adsorption isotherm of the LDH sample reveals type II isotherm, expressing multilayer N_2 adsorption, without significant hysteresis loops between the adsorption and desorption branches (Fig. 2a). The analysis of the N_2 adsorption isotherm by the t-plot method (Fig. 2b, inset) shows that the sample has a nonporous structure. The BET- method was used to calculate the surface area of the LDH which was 114.96 ± 0.48 m^2/g.

3.2. Characterization of the intercalated kynurenic acid in LDH drug carrier

The prepared positively charged (+0.641 meq/g) LDH lamellae with relatively high specific surface area (114.96 ± 0.48 m^2/g) was suitable for the intercalation of negatively charged guest molecules. The intercalation capacity of the KYNA drug molecules into LDH layers was measured by fluorescence spectroscopy method (Fig. 3). During the experiments, the KYNA content of the samples was systematically changed from 2.5 to 50 wt.%. The obtained results presented in Fig. 3 showed that with the increasing weight ratio of KYNA/LDH the amount of intercalated KYNA is also increasing and the determined drug- loading capacity was about 120 mg KYNA/ g LDH. Considering that the measured surface charge of LDH was +0.641 meq/g LDH and the molecular weight of KYNA (189.16 g/mol), the calculated

theoretical intercalated amount of KYNA was 121 mg KYNA/ g LDH. This result is in good agreement with the experimentally determined value (~12%).

3.2.1. PXRD analysis of KYNA intercalation

Fig. 4 illustrates the PXRD patterns for the Mg/Al-LDH intercalated with KYNA compared with the initial LDH material. The intercalation of KYNA in LDH has significant influence on the PXRD pattern of LDH at pH=10. The well-crystallized LDH structure with a (003) diffraction peak positioned at an angle of 11.39° (2Θ) shows another (003) diffraction peak centered at 2.146 nm at a lower 2Θ angle (4.11°). The interlayer spacing increases by 1.371 nm in comparison with initial positively charged LDH layers, which shows an interlayer spacing of 0.775 nm indicating the successful intercalation of drug anions between the interlayer regions.

As Cavani et al. (1991) mentioned, the thickness of the brucite-like layer of LDH is 0.48 nm, the gallery height in the KYNA-pillared material is 1.666 nm. A gallery height of 1.666 nm suggests a paraffin type monolayer arrangement (Betega de Paiva et al., 2008; Chiu et al., 2014) of the intercalated KYNA, with their main axis perpendicular to the layer and the anion carboxyl groups interacting with positively charged layer surfaces. The suspected structure, i.e. the paraffin type monolayer arrangement of the obtained LDH/ KYNA composite is also presented in Fig. 4.

3.2.2. FT-IR spectra

The FT-IR spectra of the KYNA intercalated in Mg/Al-LDH are presented in Fig. 5. The FT-IR spectra of initial LDH and pure KYNA are also illustrated for comparison. Fig. 5a shows the spectrum of nitrate-LDH, a broad absorption band at 3450 cm^{-1} which is due to the stretching vibration of the hydroxyl groups of the LDH layers and water molecules from the

322 interlayer space. The band at 1383 cm^{-1} was assigned to stretching vibration of NO_3^- (Aisawa
323 et al., 2015). This strong absorption peak of the $NO_3^-$was decreased after the ion-exchanged
324 reaction, supporting that $NO_3^-$was replaced by the KYNA molecule (Fig. 5b).
325 In the spectrum of KYNA (Fig. 5c) the band at 3474 cm^{-1} was derived from the stretching
326 vibrations of –O–H bonds in the carboxyl group. The band centered at 3431 cm^{-1} is due to
327 OH bonded to the quinoline ring and the signal seen at 3076 cm^{-1} is attributed to the C–H
328 bonds of the quinoline ring. Furthermore, the band at 2968 cm^{-1} corresponds to stretch
329 vibrations of the C=H bonds of benzyl and quinoline rings and the signal at 2718 cm^{-1}
330 corresponds to the double bond N=C in the quinoline ring (López et al., 2014). The
331 asymmetric and symmetric COO stretching is obtained between 1700-1400 cm^{-1}, those at
332 1631, 1595, 1415 and 1247 cm^{-1} were attributed to ν(C=O), ν_{as}(COO), ν_s(COO) and ν(C-N),
333 respectively. (Geng et al., 2009; Ibrahim et al., 2005). After intercalation, the characteristic
334 bands of KYNA were observed at 1631, 1595, 1415, 1247 cm^{-1} in the case of KYNA/LDH
335 (Fig. 5b). So, according to the evaluation of the IR spectra, the intercalation of KYNA
336 molecules into the LDH lamellae was also proved.
337
338 *3.2.3. Thermal analysis*
339 Thermogravimetric analysis of the initial LDH clay and the synthetized composites provides
340 useful information regarding the thermal stability and thermal decomposition temperature of
341 the materials. The TG curve of powdered KYNA/ LDH pillared composite is illustrated in
342 Fig. 6. The corresponding TG curves for pure KYNA and initial Mg/Al-LDH are also
343 presented for comparison.
344 In the LDH, the thermal decomposition stages are generally overlapped and the exact
345 temperature range of each stage depends largely on LDH type, heating rate and atmosphere
346 (N_2 or O_2) (Benício et al., 2015). According to the literature data, LDH thermal behavior is

usually characterized by two main transition stages: (i) an endothermic process from room temperature to about 200 °C that corresponds to adsorbed and interlamellar water loss; this stage is reversible and occurs without lamellar structure collapse; and (ii) the second stage occurs with temperatures ranging from 200 to about 6-800 °C, and corresponds to lamellar hydroxyl group loss (dehydroxylation) as well as anions loss (Benício et al., 2015). In the case of our LDH sample, both weight losses can be observed. The interlamellar water content was 15.7 wt.% (first step up until ~245 °C), while after about 650 °C, the remaining weight was 53.7 wt.%. It can be also seen, that the heat degradation of the pure KYNA was occurred between 150 and 650 °C and after about this temperature, the mass loss of organic KYNA was complete (dashes line). Compared the corresponding TG curves, it can be determined that the remaining weigh difference between the KYNA/ LDH composite and the LDH sample was 13.9 wt.%. Fig. 3 shows that the maximum adsorption capacity of the LDH clay lamellae was about 120 mg KYNA/ g LDH. The thermogravimetric results were confirmed this ~12 wt.% KYNA content of the KYNA pillared composite material.

3.3. Dissolution properties of LDH clay in SGF media

The dissolution properties of the LDH drug carrier in acidic pH was investigated gravimetrically and the acidic dissolution, i.e. the destruction of lamellae was also followed up by PXRD measurements (Fig. 7) The dissolution profile of LDH at pH= 1.5 was exhibited a very fast process: after about 30 minutes, more than 70 wt.% of the initial LDH weight was disappeared and at the end of the experiment, near 83% of LDH were completely dissolved in the applied SGF media after 360 minutes (Fig. 7a). The corresponding PXRD patterns of the LDH is presented in Fig. 7b and gives information about the LDH structure during the dissolution experiment. It can be observed that the intensity of the characteristic peaks of the well-crystallized LDH was continuously decreased, i.e. the (003) diffraction peak positioned

at an angle of 11.39° (2Θ) was shown decreasing intensity. This phenomenon is obviously due to dissolution of LDH drug carrier at pH= 1.5.

3.4. *In vitro* drug release properties of the samples

The release properties of KYNA were studied at the pH of human saliva (Baliga et al., 2013; Pietrzyńska and Voelkel, 2017) at a value of 6.70 and at pH of SGF (pH=1.50), as well. The release study of the KYNA/LDH have also been performed at pH=1.5. Fig. 8 presents the drug release profiles which were carried out over a period of 240 min and at body temperature (37 °C) and in physiological saline. The released concentration of drug were determined spectrophotometrically. The solubility and thus the release rate of anionic KYNA depends on pH (Varga et al., 2016). Thus, on Fig. 8a, a significant difference can be seen on the release rate of KYNA between pH= 6.70 and 1.50 under the same experimental condition. Due to the higher solubility of the KYNA molecules at pH= 6.70, the percentage amount of the released drug molecules were continuously increased up to 180 min and after three hours it was reached the 90% plateau value. However, at low pH (=1.50), the release of the KYNA was obviously slower process and the measured maximum value was only about 40%, because in this pH the KYNA molecules were formed heterogeneous precipitate instead of a clear homogenous solution (pH=6.70) (see inserted photos). In the case of KYNA release from concentrated solutions via the fitted (Fig. 8b) first-order rate model (Eq. 1) the following rate constant values (k) were provided: $4.74 \cdot 10^{-5}$ s^{-1} and $5.91 \cdot 10^{-5}$ s^{-1} at pH=6.70 and pH=1.50, respectively. As regarding the composite sample, drug release profile for KYNA/LDH at pH= 1.5 was exhibited a delayed drug release rate compared to the pure KYNA at the same pH. This is because the kinetic of the release process is controlled by the LDH, so the diffusion rate is significantly decreased. The determined k value was $1.49 \cdot 10^{-5}$ s^{-1} in this case. The corresponding half-life values ($t_{1/2}$ = 4.1 and 3.3 h for KYNA at pH=6.70 and 1.50,

respectively) are smaller than the half-life value of composite sample ($t_{1/2}$ = 12.9 h) at pH=1.50. The kinetic constant, release index and correlation coefficients (R^2) values, which are summarised in Table 1 indicate what processes are controlled by the suggested release mechanism. Based on the values of correlation coefficients release of the KYNA from the KYNA/LDH composite was well described by the first-order rate model, while the drug release from concentrated solutions agreed with the Korsmeyer–Peppas model. The observed low release index values (n = 0.43 and 0.38 for KYNA at pH=6.70 and 1.50, respectively) suggest the existing of Fickian diffusion mechanism which absolutely corresponds to the experimentally arranged conditions. Moreover, this model clearly shows the appearance of non Fickian diffusion (n = 0.46 for KYNA/LDH at pH = 1.50) in the case of the composite where combination of drug diffusion and carrier erosion result the anomalous process.

This result allows to conclude that the rate determining step for release of KYNA from the KYNA/LDH composite may be depending on the following conditions: (i) dissolution of LDH lamellae; (ii) ion-exchange reaction between KYNA and LDH and (iii) release of KYNA from LDH host lamellae. These data indicate that the Mg/Al-LDH could be an adequate drug carrier system for the neuroprotective and anti- ulcerant KYNA molecules at low pH.

4. Conclusions

This study demonstrated the intercalation of anti- ulcerant KYNA molecules into a layered inorganic host, LDH, which was carried out with a simply ion- exchange reaction. The PXRD studies showed a paraffin type monolayer arrangement for anti-ulcerant drug molecules into the synthetized KYNA/ LDH hybrid material. The drug loading capacity of the 2:1 Mg/Al-LDH was studied by fluorescence spectroscopy method and the obtained ~12% KYNA content was in good agreement with the theoretical value calculated from the +0.641 meq/g

surface charge of the LDH lamellae. Moreover, this ~12% KYNA content of the hybrid materials was also confirmed by thermogravimetric (TG) measurements.

Next, it was also demonstrated by gravimetric and PXRD measurements, that the prepared LDH was almost completely dissolved (~83 wt.%) in the applied simulated gastric fluid (SGF) media at pH=1.5. The *in vitro* release studies showed that due to the higher solubility of the KYNA molecules at pH= 6.70 a significant difference can be seen on the release rate of KYNA between pH= 6.70 ($t_{1/2}$ = 4.1 h) and 1.50 ($t_{1/2}$ = 3.3 h) under the same experimental condition. The drug release from KYNA/ LDH composite material at pH= 1.5 was observed a delayed drug release profile ($1.49 \cdot 10^{-5}$ s^{-1}) compared to the pure KYNA ($t_{1/2}$ = 12.9 h) at the same pH. The obtained results gives a proof to us that the chosen LDH host molecule could be an adequate drug carrier system for the neuroprotective and anti- ulcerant KYNA molecules at gastric pH, used for peptic ulcer diseases.

Acknowledgements

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569

570 **Figure 1.** PXRD pattern of the synthetized 2:1 Mg/Al LDH powder (a) and the charge
571 titration curve of 0.1 wt.% cationic LDH suspension (pH=10) with 0.1 wt.% of anionic
572 sodium dodecyl sulfate (NaDS) surfactant solution (b).
573 **Figure 2.** Adsorption isotherms of N_2 at 77 K on LDH powder sample (a). The inserted figure
574 shows the corresponding deBoer *t*-plot of the same sample (b).
575 **Figure 3.** The effect of the KYNA weight ratio on the intercalation capacity of the KYNA
576 molecules loaded LDH layers
577 **Figure 4.** PXRD patterns of the initial LDH lamellae and KYNA molecules intercalated into
578 the LDH layers and the proposed structure of KYNA-LDH composite
579 **Figure 5.** FT-IR spectra for (a) LDH drug carrier, (b) KYNA-LDH composite and (c) KYNA
580 powder samples
581 **Figure 6.** TG profiles for LDH drug carrier, KYNA-LDH composite and initial KYNA
582 **Figure 7.** The gravimetrically measured percentage weight loss of the LDH drug carrier in
583 SGF media at pH= 1.5 (a) and the corresponding PXRD patterns of LDH sample during the
584 dissolution process (b).
585 **Figure 8.** The percentage release profile of anionic KYNA molecules at two different pH
586 values (pH= 6.70 and 1.50) and the KYNA release from LDH lamellae at acidic (pH= 1.50)
587 pH, as well as the measured KYNA concentration values (C_t, mg/ mL) as a function of release
588 time with the fitted curves calculated by the first-order rate model
589 **Table 1.** Interpretation of the release experiments using different various models.

Anti-ulcerant kynurenic acid molecules intercalated Mg/Al-layered double hydroxide and its release study

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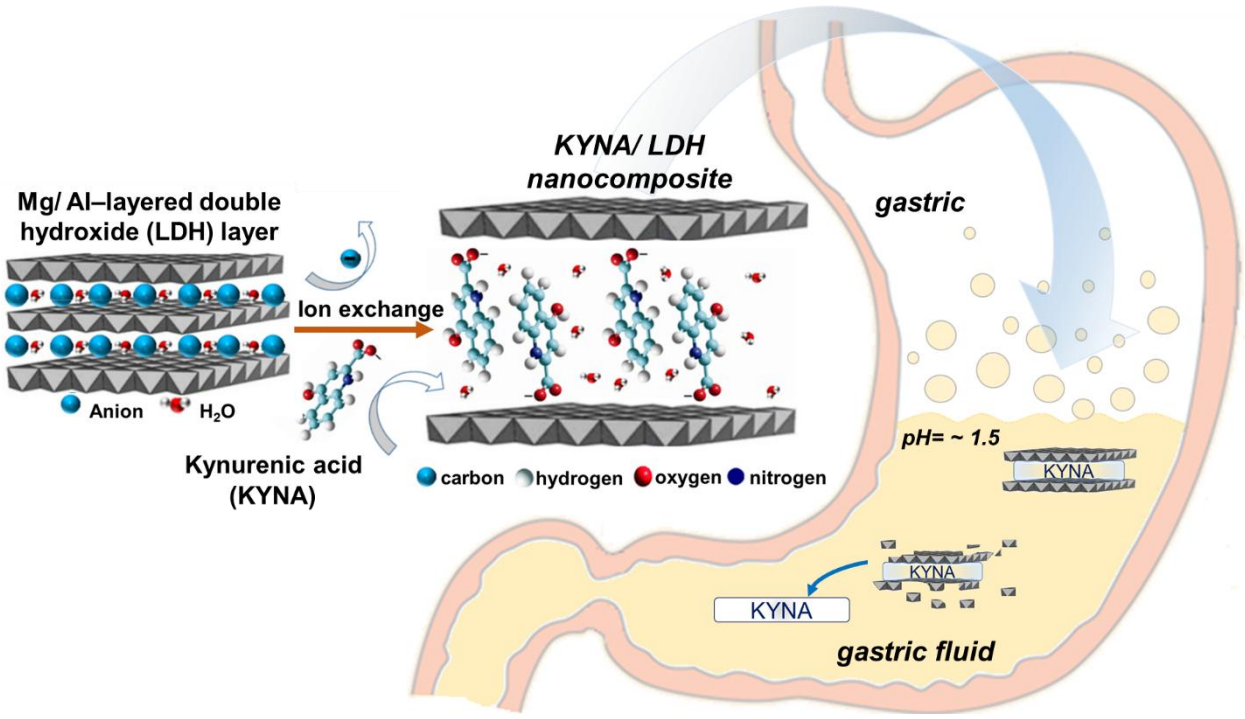
Graphical abstract



Highlights

- LDH was synthetized as inorganic drug carrier system for kynurenic acid molecules
- KYNA molecules exhibited paraffin type monolayer arrangement in LDH lamellae
- LDH layers were almost completely dissolved at gastric pH during the *in vitro* study
- The anti-ulcerant KYNA molecules were released from the destroyed interlayers

Table 1

Table 1

	KYNA pH= 6.70		*KYNA pH= 1.50*		*KYNA/LDH pH= 1.50*	
	37°C		37°C		37°C	
Kinetic models	*k*	R^2	*k*	R^2	*k*	R^2
First order Model (s^{-1})	4.75×10^{-5}	0.9955	5.91×10^{-5}	0.8363	1.49×10^{-5}	0.9045
Korsmeyer–Peppas Model (s^{-n})	1.62×10^{0}	0.9524	1.26×10^{0}	0.9506	1.90×10^{-1}	0.8361
Higuchi Model ($s^{-1/2}$)	8.88×10^{-3}	0.9389	8.51×10^{-1}	0.9261	8.57×10^{-1}	0.8467

Figure 1
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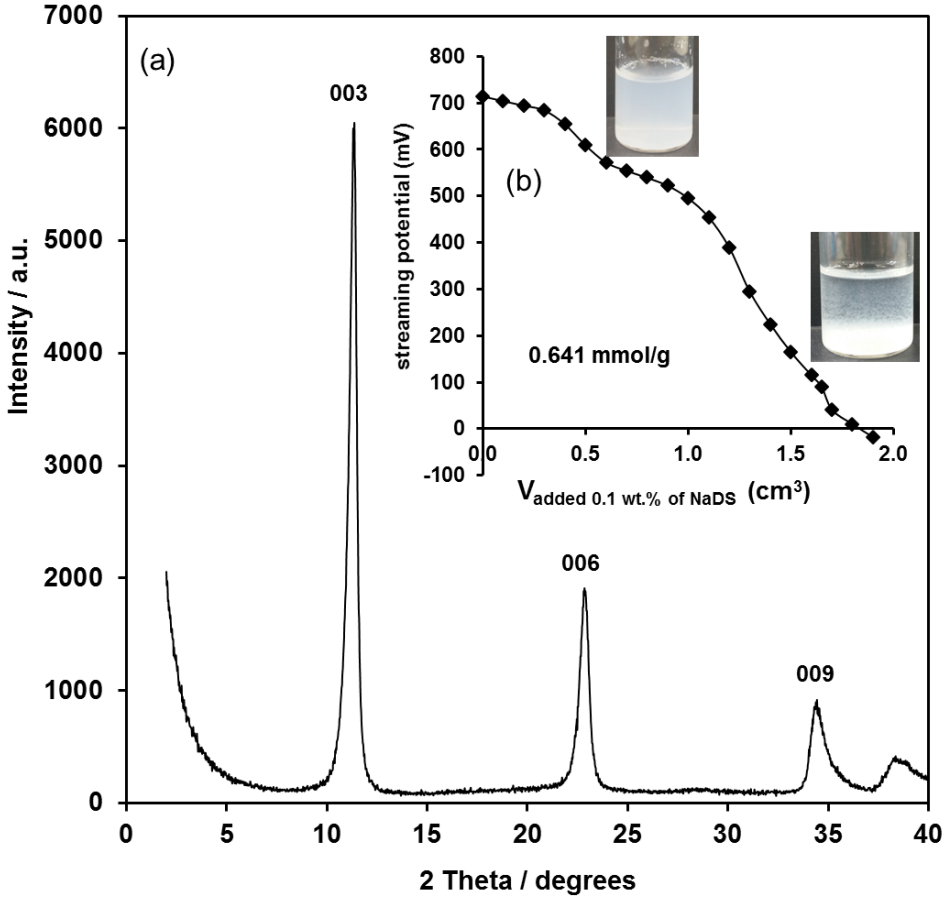

Figure 1.

Figure 2
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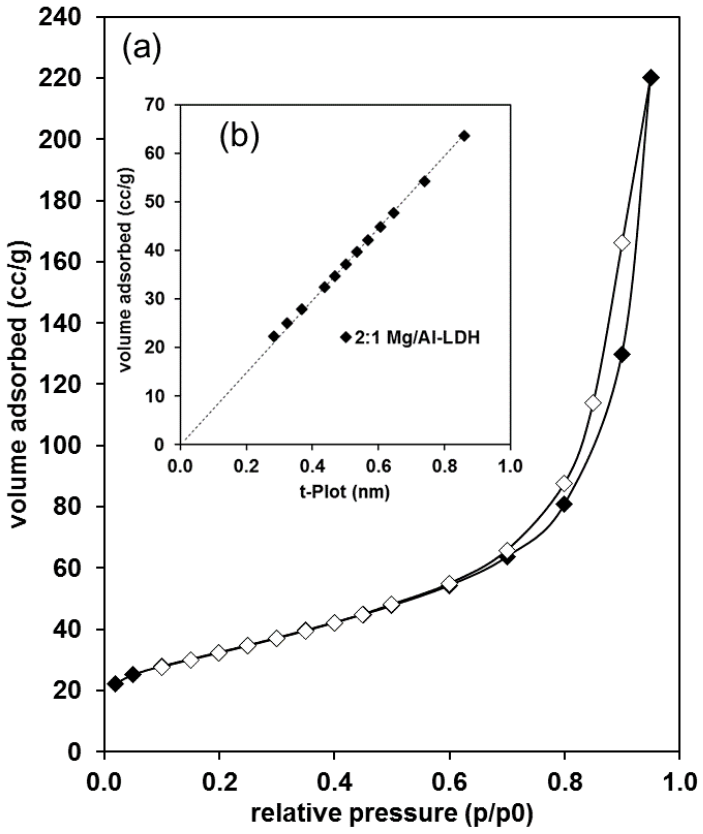

Figure 2.

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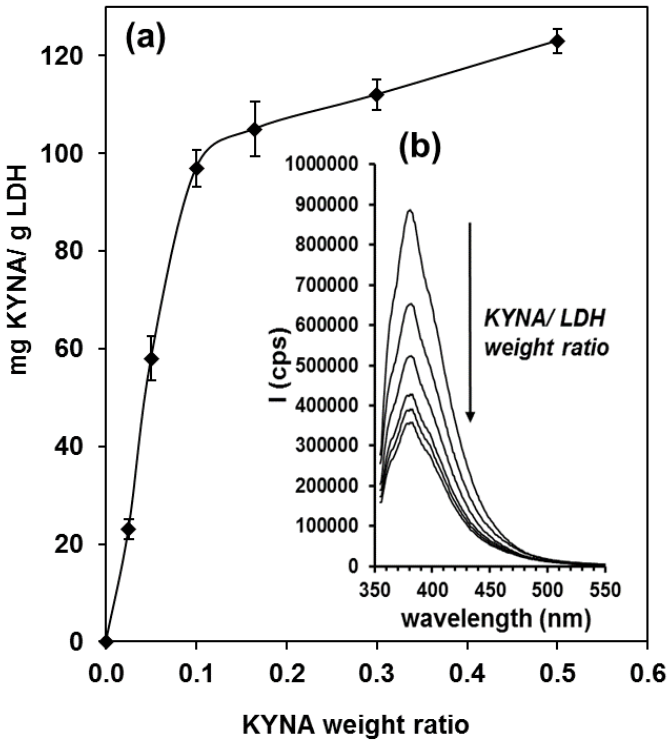

Figure 3.

Figure 4
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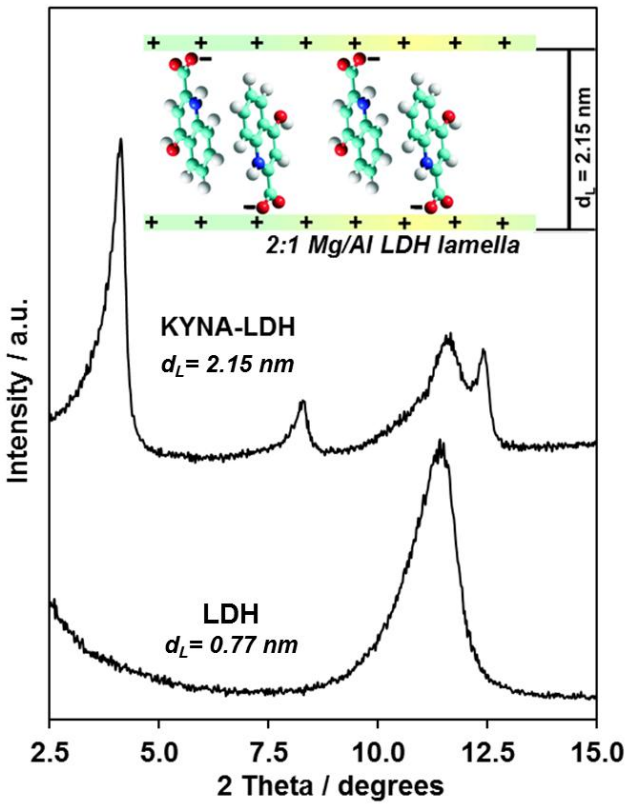

Figure 4.

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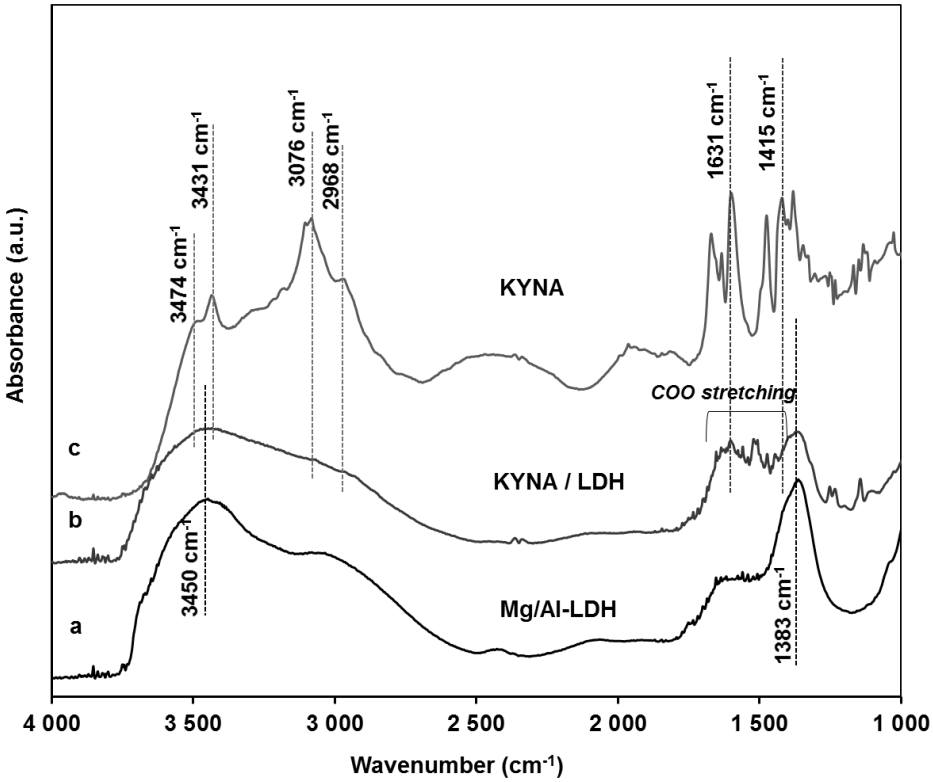

Figure 5.

Figure 6
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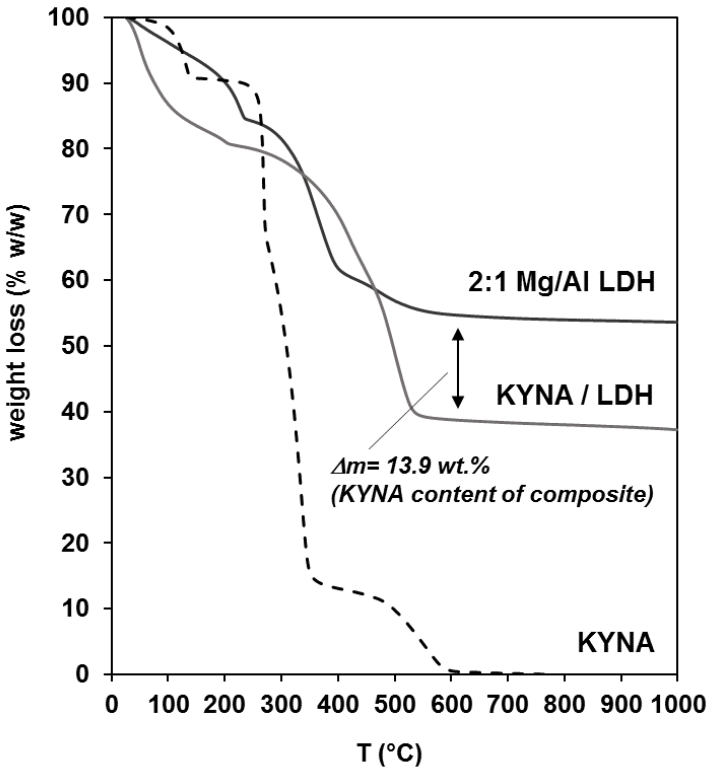

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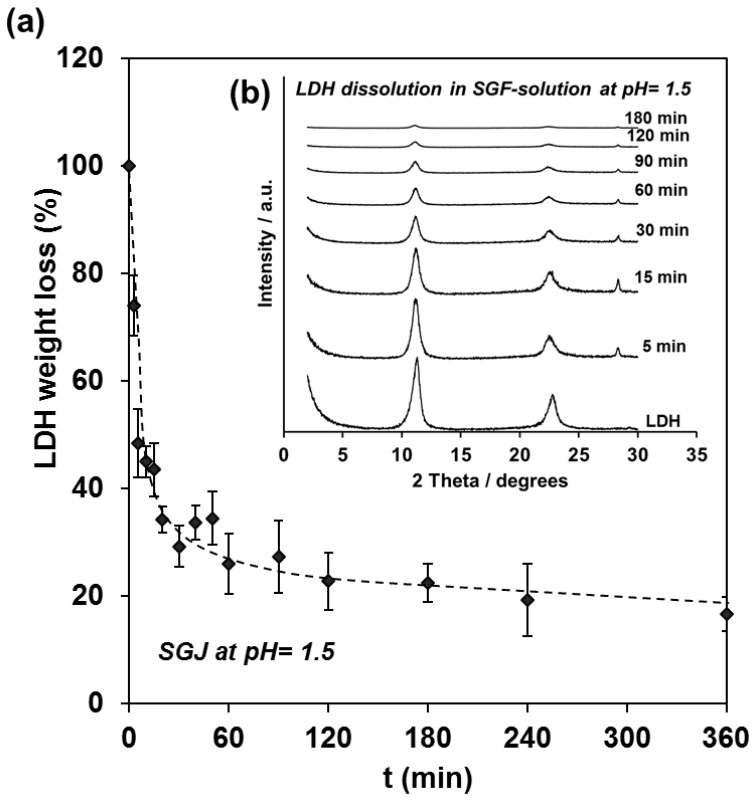

Figure 7.

Figure 8
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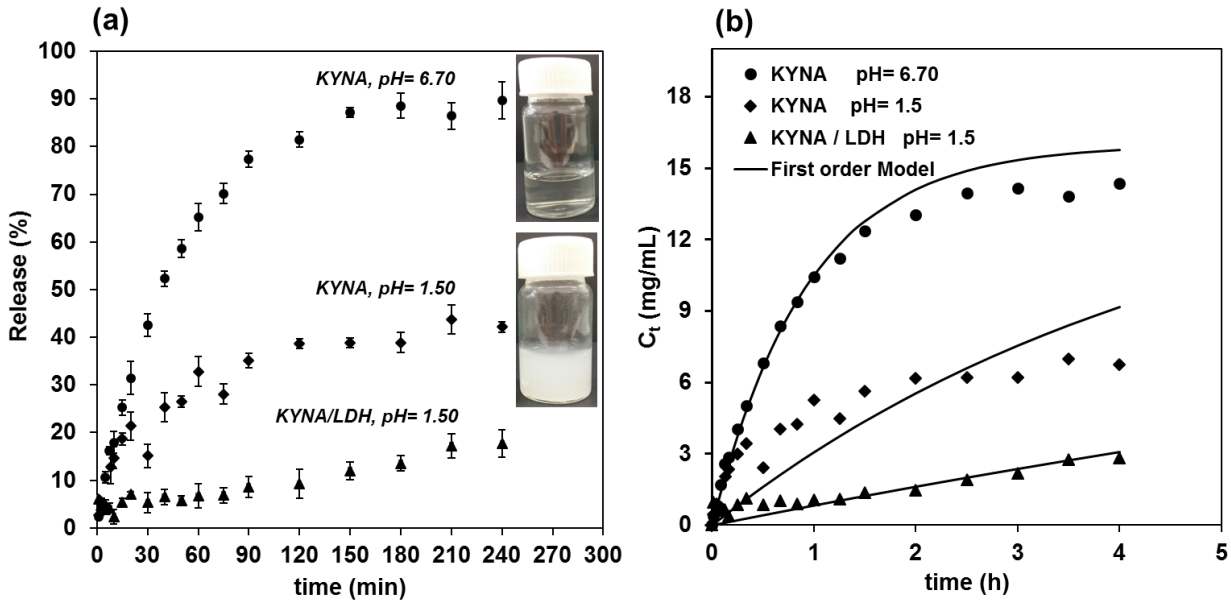

Figure 8.

Anti-ulcerant kynurenic acid molecules intercalated Mg/Al-layered double hydroxide and its release study

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Abstract

Kynurenic acid (KYNA) is a product of the tryptophan metabolism and it possess also anti-ulcerant properties, however, the application of KYNA for the treatment of gastroduodenal ulceration is limited, because the concentration of KYNA is very low in human gastric fluid (0.01 µM). The intercalation of KYNA molecules into biocompatible Mg–Al layered double hydroxides (LDH) lamellae could solve this problem. For this purpose Mg–Al LDH with 114.96 ± 0.48 m^2/g BET surface area and +0.641 meq/g specific surface charge was synthesized. The intercalation of the anionic target molecules into positively charged LDH layers was carried out with simply ion- exchange reaction. The structure of the obtained KYNA/ LDH hybrid materials were studied by powdered X-ray diffraction (PXRD) and Attenuated total reflection Fourier transform infrared (ATR-FTIR) spectroscopy verifying that the KYNA molecules prefer creating a paraffin type monolayer arrangement. Due to the

intercalation process the (003) reflection peaks of initial LDH (2Θ= 11.39°, $d_{(003)}$= 0.775 nm) shift to lower angles (2Θ= 4.11°, *d*= 2.146 nm). That means, that the basal space value (Δd_L) of the KYNA-LDH sample was 1.436 nm. The total amount of the intercalated KYNA molecules into LDH layers was measured by fluorescence spectroscopy method. According to the results the drug- loading capacity was about 120 mg KYNA/ g LDH. This ~12% KYNA content of the hybrid materials was also evidenced by thermogravimetric measurements, because the thermal decomposition of the bio-hybrid materials was examined by thermogravimetry (TG) analysis. Our experimental data confirm that the anti- ulcerant KYNA molecules can be safely loaded and stored into LDH's layers forming a new bio-active hybrid material. In addition we also presented by PXRD and gravimetric measurements that prepared LDH layers were almost completely dissolved (~83 wt.%) in the applied simulated gastric fluid (SGF) media (pH=1.5) under 60 min and the encapsulated KYNA molecules released from the destroyed interlayers. Finally, the measured KYNA drug release profile from the bioactive composite material was also presented in SGF media. According to the results 18% of the loaded KYNA molecules were released during 6 hours.

Keywords: layered double hydroxide, kynurenic acid, intercalation, *in vitro* drug release study, anti- ulcerant properties, simulated gastric fluid