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Controlled release of ferulic acid from a hybrid hydrotalcite and its application as an antioxidant for human fibroblasts

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ABSTRACT

Ferulic acid anions were intercalated in a Zn–Al layered double hydroxide. The materials generated by this process were characterised structurally and tested in two important applications: as *in vitro* drug delivery agents in a system that mimics biological conditions, for which a mathematical model describing the drug delivery profile was created, and as antioxidants for human fibroblasts.

1. Introduction

Oxidative stress resulting from the imbalance between pro- and antioxidants has been implicated in several neurodegenerative disorders, such as Alzheimer's disease, and vitagenes [1]. In this sense, the use of antioxidants has been recognised as an important counter measure against conditions under which oxidative stress is generated. Among the plethora of naturally occurring compounds, phenolic acids have been given special attention [2,3]. The antioxidant properties of ferulic acid (FA) (4-hydroxy-3-methoxycinnamic acid) in particular have been well known since the 1970s [4]; FA acts as a scavenger against hydroxyl and peroxyl radicals [5]. Other physiological functions of FA have been explored (*i.e.*, antimicrobial, anti-inflammatory, anti-thrombosis, UV-protector and anticancer properties). Due to its many health benefits as well as its antioxidant and antimicrobial activity and low toxicity, FA has been approved as a food additive and used as a natural antioxidant in foods, beverages and cosmetics in Japan.

It is the phenolic –OH group in FA that transfers hydrogen to free radicals to provide the antioxidant effect [6]. Compared to that of other phenolic acids, the antioxidant effect of FA has been shown to be equivalent to that of lecithin as observed in the peroxidation of ghee [6].

The total antioxidant activities, expressed in TEAC (*i.e.,* total equivalent antioxidant capacity) values, of phenolic acids have been reported to rank as follows: ferulic > vanillic > syringic > caffeic > *m*-coumaric > protocatechuic > gentisic > *o*-coumaric [7].

With respect to radical-scavenging activity, it has been observed that the number of hydroxyl moieties attached to phenolic acids of the benzoic and cinnamic acid families dictates their radical-scavenging activities [8].

FA is one of the most abundant phenolic acids found in plants [9,10]; nevertheless, it is rarely found in its free form, usually occurring as an ester covalently linked to polysaccharides [11]. In reality, cellular delivery involving the transfer of drugs and bioactive molecules through the cell membrane into cells is a topic that has attracted much attention because of the inefficiency and difficulty of the transfer process. Therefore, the search for efficient and safe transport agents to deliver biomolecules and drugs remains a challenge for science. In this context, inorganic agents, mainly porous or laminated agents, show promising controlled-delivery properties [12] and are alternatives to viral carriers and organic cationic carriers. Porous materials are suitable for drug and gene delivery [13–15] because of the particular interactions that occur between drug materials inside the pores of such materials. These interactions frequently occur through dipoles and partially stabilise drugs in pores, protecting them until the hybrid materials reach cells.

A wide variety of porous materials have been proposed as hosts of organic molecules [16,17]. The most recurrent among these materials are mesoporous silica, zeolites, titanium oxide and layered double hydroxides (LDHs). LDHs, also known as hydrotalcite-like compounds (HTCs), are particularly suitable for this

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application because of their good biocompatibility and potential capability for target delivery [18].

LDHs are materials consisting of positively charged layers separated by anions and water molecules. Their chemical composition is represented by the general formula $[M^{2+}_{(1-x)}M^{3+}_{x}(OH)_2]A^{n-}_{x/n}\cdot mH_2O$, where M^{2+} and M^{3+} are divalent and trivalent metal cations, respectively, and A^{n-} may be an exchangeable inorganic or organic anion. Due to this anionic exchange property, LDHs can be anionic carriers and be used instead of viral agents, which are often highly toxic to cells.

The LDH structure is reminiscent of that of brucite, where $Mg(OH)_6$ octahedral units share edges to form infinite $Mg(OH)_2$ layers. The structure of LDHs results from the partial isomorphic replacement of Mg^{2+} cations of brucite by Al^{3+} cations. As a consequence, an overall positive charge on the layers is created, which allows for the fine tuning of the material to suit specific applications by reducing or increasing the overall positive charge and thus the capacity for anions (anion exchange capacity, AEC), basicity and catalytic activity. Another interesting property of LDHs is the so-called memory effect: after relatively mild calcination, the layered structure collapses, but the layered double hydroxide structure can be regenerated after exposure to aqueous or organic solutions of anions [19,20]. This exchange reaction is regulated by the selectivity of the host for various counterions, the concentration and temperature [21]. In the case in which the counterions are organic anions with biological activity, the interlayer space behaves as a nanocontainer where these molecules are stored, protected, and later released under control by deintercalation. Thus, these intercalation compounds based on LDH can act as matrices or hosts for active molecules, yielding interesting hybrid nanocomposite materials [22]. Several pharmaceutically active drugs:antibiotics [23,24], anticarcinogens [25–27], cardiovasculars and anti-inflammatories [28], antihistamines [29], antihypertensives [30] and antifungals [31] have thus been intercalated into LDHs to obtain hybrid drug-inorganic matrix materials.

The objective of this study was to demonstrate the potential use of ferulic acid intercalated into a biocompatible ZnAl layered double hydroxide. Two *in vitro* studies were carried out to determine the viability of using this material as an antioxidant for human fibroblasts and also as a prolonged drug delivery system.

2. Experimental

2.1. Synthesis

The urea hydrolysis method was used to synthesise pure ZnAl–NO_3 ($Zn^{2+}/Al^{3+} = 2$) layered double hydroxide [32]. All reagents were purchased from Sigma–Aldrich. Briefly, $Zn(NO_3)_2{\cdot}6H_2O$ and $Al(NO_3)_3{\cdot}9H_2O$ were dissolved in CO_2-free distilled and deionised water at room temperature. Then, a mixture of urea and ammonium nitrate was added, and the mixture was magnetically stirred in a three-necked 500-mL round-bottom flask equipped with a reflux condenser at 90 °C for 10 h. The system was purged of CO_2 by bubbling argon gas for 1 h. The molar concentrations in the final solution were 0.335, 0.165, 1.65 and 1 mol L^{-1} for Zn^{2+}, Al^{3+}, urea and NH_4NO_3, respectively, resulting in a NO_3^-/urea molar ratio equal to 1.312. The white precipitate was centrifuged for 15 min, washed with hot deionised and CO_2-free water and finally dried at 120 °C for 12 h in an oven.

After the ZnAl–NO_3 LDH was prepared, the memory effect was used to produce an LDH containing intercalated FA (ZnAl–FA). Briefly, ZnAl–NO_3 LDH was first heat-treated in flowing N_2 (heating rate of 5 °C min^{-1}) to 500 °C, where it was maintained for 5 h to obtain the mixed oxide ZnAl(O). After cooling to room temperature, the mixed oxide (0.37 g) was suspended in a CO_2-free aqueous solution of ferulate sodium salt at room temperature under an argon atmosphere. The resulting suspension was magnetically stirred for 7 days. FA is poorly soluble in water; thus, to generate a soluble anionic species, an aqueous solution of ferulate anion was freshly prepared by adding 0.1 M NaOH to a 30-mL suspension containing 4.8 mmol of FA until the pH reached 9. The ZnAl–FA solid was then separated by centrifugation, washed several times with deionised water and dried in an oven at 80 °C overnight.

2.2. Materials characterisation

X-ray powder diffraction patterns were obtained using CuK_α radiation (λ = 1.54 Å) on a Philips X'Pert Pro instrument operating at 45 kV and 40 mA in the 2θ range of 4–80°, with a step size of 0.02° and step scan of 0.4 s.

Infrared spectra were recorded in the spectral window 4000–400 cm^{-1} using an FTIR Nicolet Magna IR 750 infrared spectrophotometer. Samples were diluted with KBr and handled as pellets.

Nitrogen adsorption isotherms were determined at −196 °C using a volumetric adsorption BELSORP apparatus (BEL Japan). Prior to the adsorption of N_2, all of the samples were outgassed at 90 °C for 12 h.

The morphology of the samples was studied with a SEM JEOL 7600 scanning electron microscope.

Solid-state ^{27}Al and ^{13}C magic angle spinning-nuclear magnetic resonance (MAS-NMR) experiments were performed on a Bruker Avance II spectrometer at frequencies of 104.2 and 100.58 MHz, respectively. ^{13}CP MAS-NMR spectra were acquired using a 4-mm cross-polarisation (CP) MAS probe spinning at a rate of 5 kHz. Typical ^{13}C CP MAS-NMR conditions for the ^1H-^{13}C polarisation experiment, included a $\pi/2$ pulse of 4 μs, contact time of 1 ms and delay time of 5 s. Chemical shifts were referenced to a solid shift at 38.2 ppm relative to TMS. ^{27}Al MAS-NMR spectra were acquired using short single pulses ($\pi/12$) and a delay time of 0.5 s. The samples were spun at 10 kHz, and the chemical shifts were referenced to an aqueous 1 M $AlCl_3$ solution.

2.2.1. In vitro studies

2.2.1.1. Drug release. Drug release studies were performed in a dialysis bag (the dialysis bag consisted of regenerated cellulose, 12,000–14,000 Da, 25-Å pore diameter, SERVA). Vessels were kept in a thermostatically controlled circulating water bath at 37.0 ± 0.5 °C with a rotational speed of 100 rpm. The dissolution medium was phosphate buffer with a pH of 7.5 (50 mM disodium hydrogen phosphate). The release studies were performed by placing 20 mg of ZnAl–FA in 50 mL of medium under sink conditions. Samples of 100 μL were withdrawn at predetermined intervals, followed by replenishment after each withdrawal with the same volume of fresh medium equilibrated at 37.0 ± 0.5 °C. Samples were appropriately filtered and analysed by UV spectrophotometry (Nanodrop 2000, Thermo Scientific, DE, USA) at λ_{max} = 310 nm according to a previously determined calibration curve (ranging from 5 to 150 μg/mL, $y = 0.007x + 0.003$, $r = 0.999$). The percentage released at each time point was expressed as a fraction of the total amount of FA. Drug release was monitored for 10 h; the FA concentration was reported as the average of 3 determinations. All recordings were within the range of the calibration curve.

2.2.1.2. Antioxidant activity in human cells. The procedure followed to measure antioxidant activity in human cells can be summarised in three steps: (i) *Antioxidant preparation*. LDH loaded with ferulic acid (ZnAl–FA) or control materials (ZnAl–NO_3, FA or a mixture of ZnAl–NO_3 plus FA) were dispersed in water at concentrations equivalent to 50 μM of FA. To prevent FA from being released from the LDH during the sterilisation procedure (autoclave), ZnAl-FA