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Modulation of folic acid bioaccesibility by encapsulation in pH-responsive gated mesoporous silica particles

Édgar Pérez-Esteve[a], Ana Fuentes[a], Carmen Coll[b,c], Carolina Acosta[a], Andrea Bernardos[a,b], Pedro Amorós[d], María D. Marcos,[b,c], Félix Sancenón [b,c,], Ramón Martínez-Máñez[b,c,]*, José M. Barat[a]

[a]Grupo de Investigación e Innovación Alimentaria, Universitat Politècnica de València. Camino de Vera s/n, 46022, Spain

[b]Centro de Reconocimiento Molecular y Desarrollo Tecnológico (IDM), Unidad Mixta Universitat Politècnica de València – Universidad de Valencia. Departamento de Química Universitat Politècnica de València, Camino de Vera s/n, 46022, Valencia, Spain

[c]CIBER de Bioingeniería, Biomateriales y Nanomedicina (CIBER-BBN)

[d]Institut de Ciència dels Materials (ICMUV), Universitat de València, P.O. Box 2085, 46071, Valencia, Spain

* Corresponding author: Tel.: +34 963877343; E-mail address: rmaez@qim.upv.es (R. Martínez-Máñez).

ABSTRACT

A study on the controlled release of Folic Acid (FA) from pH-responsive gated mesoporous silica particles (MSP) is reported. The MCM-41 support was synthesized using tetraethyl orthosilicate (TEOS) as hydrolytic inorganic precursor and the surfactant hexadecyltrimethylammonium bromide (CTAB) as porogen species. Calcination of the mesostructured phase resulted in the starting solid. This solid was loaded with FA to obtain the initial support **S0**. Moreover, this FA-loaded material was further functionalized with 3-[2-(2-aminoethylamino)ethylamino]propyltrimethoxysilane (N3) in order to obtain the gated polyamine-functionalised material **S1**. Solids **S0** and **S1** were characterized using standard solid state procedures. It was found that the functionalization process and the inclusion of FA on the pores do not modify the mesoporous structure of the starting material. FA delivery studies in water with solids **S0** and **S1** were carried out in water at pH 2 and 7.5. **S0** was not able to completely inhibit FA delivery at acidic pH yet a rapid FA release at neutral pH was observed in few minutes. In contrast, **S1** was tightly capped at pH 2 and displayed a sustained delivery of FA when the pH was switched to 7.5. In the second part of the study, FA loading and functionalization of **S1**-like supports was optimized. In particular, solids loaded with FA in phosphate buffered saline (PBS) and capped with N3 in acetate buffer at pH 2 exhibited a delivery capacity up to 95 μg FA/mg solid. Finally, FA release from the selected optimized supports was studied following an *in vitro* digestion procedure. The results showed that amine-capped MSP were not only able to hinder the release of the vitamin in gastric fluids (pH 2), but were also capable of deliver progressively the FA in presence of a simulated intestinal juice (pH 7.5) offering a suitable mechanism to control the bioaccessibility of the vitamin.

Keywords: Folic acid; bioaccessibility; loading optimization; controlled release; mesoporous silica particles

1. Introduction

Folate is a generic term by which is known a group of water-soluble compounds with B9 vitamin activity and with chemical structures similar to synthetic pteroyl monoglutamic acid (PGA), commonly known as folic acid (FA). Folates are essential to numerous bodily functions, including DNA synthesis and repair, cell division and cell growth [1]. Main folate sources include green leafy vegetables, yeast extracts, liver, kidney, and citrus fruit. Despite this wide distribution, folate deficiency is a common finding that can be caused by a variety of factors such as malabsorption of folate from the diet, an increased utilization by the body or a significant loss up to 50% during cooking processes [2]. FA deficiency is such important in humans that it can cause neural tube defects in developing embryos [3], is associated with elevated plasma homocysteine (an emerging risk factor for vascular diseases), with cognitive decline and neurodegenerative diseases such as Alzheimer and also is risk factor for certain tumours (acute lymphoblastic leukaemia, breast cancer, and gastric cancer) [4,5].

To prevent the occurrence of these and other diseases, food supplementation with folates is mandatory in certain countries such as USA [6], Canada [7] or UK [8] with the objective of ensure a 400 μg intake for adults and an additional 200 μg for pregnant women [9]. To achieve this supplementation, the most employed molecule is FA, due to its high stability and bioavailability [10].

Although there are irrefutable evidences about the benefits of FA supplementation to prevent some diseases, recent studies suggest that the margin of the benefit is very narrow, and despite the necessity of the supplementation, a massive exposition to high bioavailable FA could be a double-edged sword [8]. In particular it has been reported that biotransformation processes of FA are saturated at doses of 266-400 μg of FA and up to this amount unmetabolized FA is found in plasma, which could be correlated with the increase of cancer risk, insulin resistance, preneoplastic and neoplasctic lesions [11]. In this context, the design of systems to dosage FA along the digestion and therefore modulating its bioaccessibility and bioavailability is a challenge for current nutritional science.

The bioaccessibility of a nutrient is defined as the amount of the nutrient that is released from a food matrix during digestion and made accessible for absorption into the intestinal mucosa [12]. One possibility to modulate bioaccessibity of a molecule consists of its encapsulation and later controlled release under suitable stimuli. Organic-based ensembles using lipids or carbohydrates have been reported as classical encapsulation systems [13]. However, they have some important drawbacks, such as the difficulty for a scale production [14, 15], low stability of the structure during food processing and storage, difficulty of controlling the release rate and also a poor capability to protect the encapsulated substance through the harsh stomach conditions [16]. As an alternative, systems based in polyalcohols, polyamides, celluloses [17] or in mesoporous

inorganic materials [18], have been recently developed as suitable systems for controlled delivery applications. In particular, mesoporous silica particles (MSP) exhibit unique features as supports for controlled release, such as high stability [19], biocompatibility [20], no apparent toxicity [21], large load capacity [22], and the possibility to include gate-like scaffoldings on the external surface. This last characteristic allows the design of carriers for on-command delivery in the presence of target physical (such as light, temperature) [23, 24], chemical (pH-changes, redox potencial) [25-29] and biomolecules (enzymes, antibodies, DNA) [30-33] stimuli. Of those, several gated MSP have proved to show "zero" delivery yet are able to release the cargo under digestive stimuli [34, 35]. In particular, gated ensembles based in amines have been reported to be suitable systems for cargo delivery upon pH changes.

The purpose of this study is, on one hand, to evaluate the use of MSP capped with amines as suitable pH-responsive systems capable to modulate FA delivery and bioaccesibility in *in vitro* digestion assays and on the other hand, to optimize the loading process in these materials to achieve the release of the recommended dietary intake of folic acid using the minimum amount of inorganic matrix. As far as we know, this is the first time that an optimization of folic acid loading in MSP and a study of *in vitro* delivery from MSP of this molecule of nutritional interest is reported.

2. Materials and methods

2.1 Chemicals

Tetraethylorthosilicate (TEOS), n-cetyltrimethylammonium bromide (CTABr), sodium hydroxide (NaOH), triethanolamine ($TEAH_3$), the organosiloxane derivative 3-[2-(2-aminoethylamino)ethylamino]propyl (N3), NaH_2PO_4, Na_2HPO_4 and Tetrabutylammonium hydrogen 3ulphate (TBAHS) and all chemicals for the digestive fluids were provided by Sigma-Aldrich (Poole, Dorset, UK). Folic acid was purchased from Schircks Laboratories (Jona, Switzerland). Acetonitrile HPLC grade was provided by Scharlau (Barcelona, Spain).

2.2 FA molecular structural mechanics simulations

Theoretical calculations of the structure of FA were carried out by using HyperChem 8.0.6 Molecular Modeling System (Hypercube Inc., Gainesville, FL, USA). The calculation of geometry was performed using molecular mechanics MM+ in a first step and AMBER in a second step. Full geometry optimizations were carried out in vacuum employing the Polak-Ribiere conjugate gradient method until an RMS gradient of 0.1 kcal/mol was reached. The final 3D structure was refined by optimization of the geometry using molecular dynamics methods at a simulation temperature of 300 K. QSAR properties of the vitamin were determined.

2.3 Synthesis of MCM-41

The mesoporous MCM-41 support was first synthesized using the so-called "atrane route" in which 4.68g of CTAB were added at 118°C to a solution of $TEAH_3$ (25.79 g) containing 0.045 mol of a silatrane derivative (11 mL of TEOS). Next, 80 mL of water was slowly added with vigorous stirring at 70 °C. After few minutes, a white suspension was formed. This mixture was aged at

room temperature overnight. The resulting powder was collected by filtration and washed with water and ethanol. Finally, the solid was dried at 70 °C. To prepare the final mesoporous material, the as-synthesized solid was calcined at 550 °C using an oxidant atmosphere for 5 h in order to remove the template phase.

*2.4 Synthesis of **S0** and **S1***

100 mg of MCM-41 and 0.035 g (0.08 mmol) of FA were suspended in 7 mL of phosphate buffered saline (PBS) inside an amber round-bottom flask in an inert atmosphere. The mixture was stirred for 24h at room temperature to achieve the maximum loading in the pores of the MCM-41 scaffolding. The loaded solid (**S0**) was isolated by vacuum filtration, washed with 300 mL of water adjusted to pH 2, and dried at room temperature for 24h. This loading process was optimized in further assays (*vide infra in 2.5*).

To obtain **S1**, 100 mg of **S0** were suspended in 4 mL of acetonitrile and an excess of N3 (0.43 mL, 0.015 mmol) was added. The final mixture was stirred for 5.5 h at room temperature. The loaded and functionalized solid (**S1**) was isolated by vacuum filtration, washed with 300 mL of water adjusted to pH 2, and dried at room temperature for 24h.

2.5 *Folic acid release studies*

To determine the effect of pH in FA release from the non-gated (**S0**) and amine-gated (**S1**) mesoporous silica particles, 10 mg of the corresponding solids (**S0** or **S1**) were placed in 25 mL of water at pH 2 and pH 7.5. At a certain times aliquot were separated, the suspension filtered and the solution analysed by HPLC.

2.6 *Folic acid loading optimization*

With the aim of optimize the amount of FA loaded inside the MSP, two different loading methods were tested: immersion (A) and impregnation (B). Table 1 summarizes the 8 loading conditions assayed. Solids were synthetized by triplicate.

For the immersion method (A), 100 mg of MCM-41 were immersed into a PBS solution containing 4 different amounts of FA, stirred for 24 h, filtered and washed. Then, loaded solids were functionalized with 0.43 mL of N3 in acetonitrile following the procedure described in the synthesis of **S1**. Using this method, 4 different loaded and functionalized solids were obtained (**A1-4**).

For the impregnation method (B), FA dissolved in PBS (10mg/mL) was added to 100 mg of MCM-41 employing 4 different FA amounts and cycles of addition (see Table 1). After each addition cycle solids were dried at 30ºC to eliminate water content. Then, each of the loaded solids (**B1-4**) were functionalized with 0.43 mL of N3 using different media; i.e. water at pH 2 (solids **BW#**), acetate buffer at pH 2 (**BB#** solids) or acetonitrile (**BA#** solids). The loaded and functionalized solids were isolated by vacuum filtration, washed with 300 mL of water adjusted to pH 2 with HCl, and dried at room temperature for 24h. Finally, 12 solids were obtained: 4 functionalized in water at pH 2 with HCl (**BW1-4**), 4 functionalized in acetate buffer at pH2 (**BB1-4**) and 4 functionalized in acetonitrile (**BA1-4**).

Table 1. Conditions employed in loading optimization assays: immersion method (A) and impregnation method (B).

Loading mechanism	*Solid*	*MCM (mg)*	*Folic (mg)*	*PBS (mL)*	C_{folic} *(mg/mL)*	*Cycles (amount of solution per cycle)*
Immersion	A1	100	35	7	5	
	A2	100	35	3.5	10	
	A3	100	70	7	10	
	A4	100	70	3.5	20	
Impregnation	B1	100	10	1	10	1 (1 mL)
	B2	100	10	1	10	2 (0.5 mL)
	B3	100	15	1.5	10	3 (0.5 mL)
	B4	100	20	2	10	4 (0.5 mL)

2.7 Loading efficiency evaluation

Loading efficiency of each of the 16 obtained solids was determined by quantification FA delivered in PBS after 5h by HPLC. The "relative loading efficiency" was calculated according to the following equation:

Relative loading efficiency (%) = FA_D/FA_L x 100

where FA_D are the mg of folic delivered per 1mg of loaded solid and FA_L are the mg of folic acid employed for the loading of 1 mg of MCM-41.

2.8 Determination of *in vitro* folic acid bioaccessibility

FA bioaccessibility (FA delivery from the prepared solids) was determined by simulating a human digestion in the stomach and small intestine adapting the procedure described by Versantvoort et al. [36] (Fig. 1). The large intestinal track was not taken into account since in vivo folic acid absorption occurs throughout the jejunum [1]. In a typical experiment, 10 mg of the corresponding solid were suspended in 12 mL of gastric juice and incubated for 2h at 37ºC. Finally, 12 mL of duodenal juice, 6 mL of bile, and 2 mL of bicarbonate solution (1M) were added simultaneously. After the addition, the mixture was maintained under stirring at 37ºC for 2h. All

digestive juices were heated to 37ºC before being mixed. During this period aliquots were taken, filtered and analysed by HPLC.

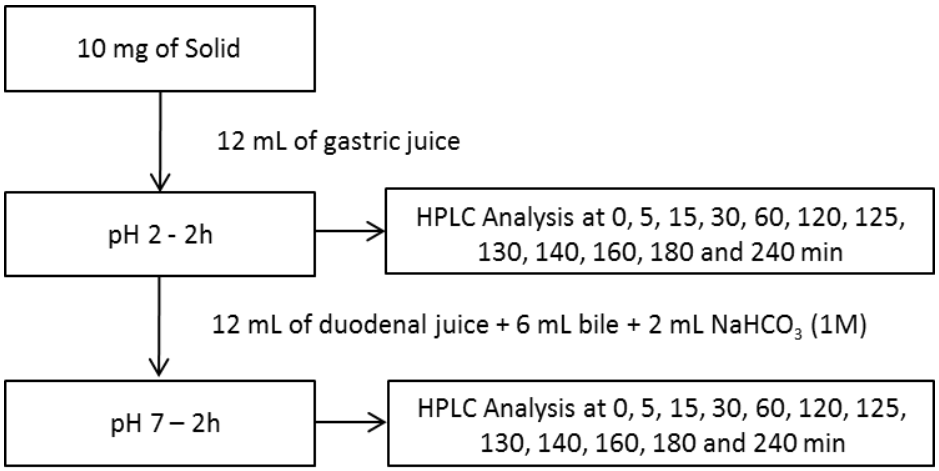

Figure 1. Schematic representation of the *in vitro* digestion model.

2.9 Folic acid determinations

FA was determined by reversed-phase gradient HPLC method according to the method described by Póo-Prieto et al. [37] with minor modifications. The HPLC instrument consisted of a Hitachi LaChrom Elite liquid chromatograph (Hitachi Ltd., Tokyo, Japan) equipped with an auto-sampler and UV detector (model L-2400). A Kromaphase 100 C18 (250 mm x 4.6 mm i.d., 5 μm particle size analytical column) (Scharlab, Barcelona, Spain) was used for the separations. Mobile phase consisted of (A) 0.125 mM of NaH_2PO_4, 0.875 mM of Na_2HPO_4 and 0.4mM of TBAHS in water and (B) acetonitrile-phase A 65:35 (v/v). The flow rate employed is described in table 2. The wavelength of UV detector was set at 280 nm. Solutions for preparation of calibration standards were made at 1, 5, 10, 25, 50, 75, 100 μg FA/mL in PBS.

Table 2. Elution program for HPLC analysis.

Time (min)	Flow (mL/min)	Mobile phase A (%)	Mobile phase B (%)
0	1.0	90	10
5	1.0	90	10
15	1.0	64	36
30	1.0	40	60
35	1.0	90	10
40	1.0	90	10

A: NaH_2PO_4 (0.125 mM), Na_2HPO_4 (0.875 mM) and TBAHS (0.4mM) in water.

B: Acetonitrile-Phase A 65:35 (v/v)

2.10 Solids characterization

X-ray diffraction (XRD), transmission electron microscopy (TEM), N_2 adsorption-desorption isothermes and thermogravimetric analyses (TGA) were employed to characterize the synthesized materials. XRD were performed on a BrukerD8 Advance diffractometer using CuKα radiation. TEM images were obtained with a JEOL JEM-1010. The N_2 adsorption-desorption isotherms were recorded by a Micrometrics ASAP2010 automated sorption analyser. Samples were degassed at 90ºC in vacuum, overnight. The specific surface areas were calculated from the adsorption data within the low pressure range using the BET model. Pore size was determined following the BJH method. Thermogravimetric analyses were carried out on a TGA/SDTA 851e Mettler Toledo balance, using an oxidant atmosphere (air, 80 mL/min) with a heating program consisting of a heating ramp of 10 °C per minute from 393 to 1273 K and an isothermal heating step at this temperature for 30 min.

2.11 Data analysis

The results of the FA delivery from the different solids prepared were statistically processed using Statgraphics Centuriun XV (Manugistics Inc., Rockville, MD, USA). Statistical analysis on FA concentrations was made using an analysis of variance (One-Way ANOVA).

3. Results and discussion

3.1 *FA molecular modelling and geometrical dimensions*

Molecular dynamics calculations on FA were carried out. The molecular structure of FA is shown in Figure 2. As it can be seen, FA (pteroyl monoglutamic acid) consists of a pteridin ring linked to the para-amino benzoic acid (PABA) and a molecule of glutamic acid. In the larger dimension, FA exhibits a length of 1.45 nm, whereas the calculated volume is 1.16 nm^3. These calculations were used to determine the total theoretical amount of vitamin that could be encapsulated in the silica mesoporous matric (vide infra).

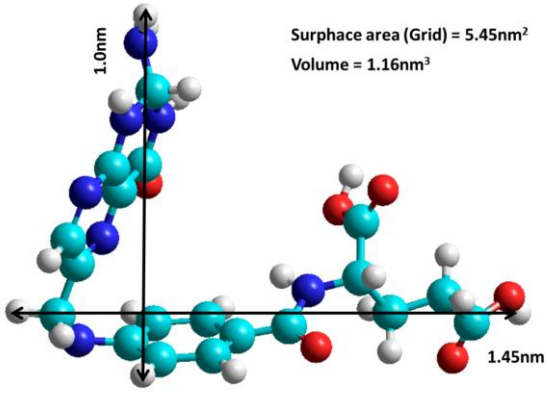

Figure 2. 3D *FA molecular structure and geometrical dimensions*

3.2 Design, synthesis and characterization of the gated particles

For the design of the proposed pH-controlled and sustained release system described above MCM-41 mesoporous silica microparticles were selected as an inorganic support due to its high loading capacity, homogeneous porosity in the 2–3 nm range, high inertness, and ease of functionalization. This starting support was loaded with FA to obtain solid **S0**. Moreover, this FA-loaded material was further capped with a pH-responsive ensemble (i.e. 3-[2-(2-aminoethylamino)ethylamino]propyltrimethoxysilane, N3) in order to obtain the gated polyamine-functionalised material **S1**. In this work pH was chosen as a suitable digestive stimulus for the modulation of FA release from the inner of the MCM-41 voids. In stomach, pH is very acid (pH 1-2) to help the degradation of proteins and to provide a non-specific immunity, retarding or eliminating various pathogens. In the small intestine, the duodenum provides critical pH balancing to activate digestive enzymes. The liver secretes bile into the duodenum to neutralise (pH 7-7.5) the acidic conditions from the stomach. Also the pancreatic duct empties into the duodenum, adding bicarbonate to neutralize the acidic chyme, thus creating a neutral environment. FA is known to be mainly absorbed in the small intestine (jejunum; pH 7.5) from where it is distributed to the tissues through the bloodstream and stored in the liver.

As stated above, pH has been chosen in this paper as triggering stimulus. In this particular system changes in the pH are expected to modulate FA delivery in two different ways. On the one hand, it is known that FA under neutral/basic conditions is about 1000 times more soluble than FA in an acidic environment due to protonation (acid) and deprotonation (neutral/basic) of FA in aqueous environments [38]. On the other hand, it has been reported that MSP functionalised with the polyamine derivative 3-[2-(2-aminoethylamino)ethylamino]propyl-trimethoxysilane (N3) are suitable pH-responsive controlled release systems able to allow or inhibit delivery as a function of pH changes due to the transformation of amines (open gate at neutral/basic pH) to polyammonium groups (closed gate at acidic pH). These two mechanisms of action are illustrated in Figure 3.

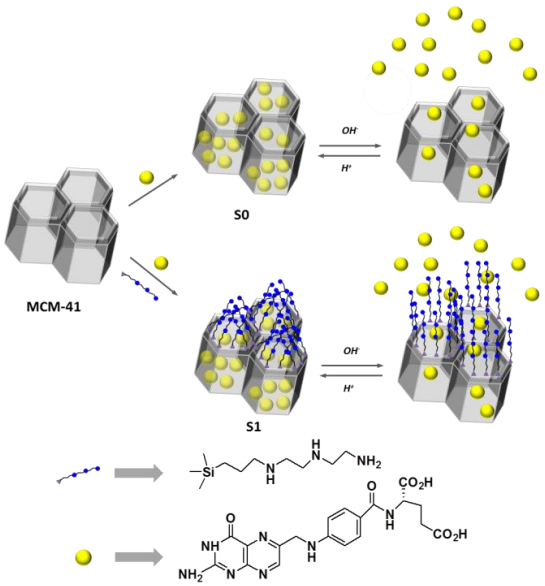

Figure 3. Illustration of the synthetic procedure for the preparation of solids **S0** and **S1**, and the mechanism of FA delivery at neutral or acidic conditions.

The different solids prepared were characterized according to standard techniques. X-ray patterns of the solids MCM-41 as synthesized (*a*), calcined (*b*), loaded with folic acid (**S0**) (*c*) and loaded with folic acid and functionalized with amines (**S1**) (*d*) are shown in Figure 4. Curve *a* shows the expected four peaks of a hexagonal ordered array indexed as (100), (110), (200) and (210) Bragg reflections. A significant shift of the (100) reflection in the XRD powder of the MCM-41 calcined sample is clearly appreciated in the curve *b*, corresponding to a cell contraction related to condensation of silanols during the calcination step. Curves *c* and *d* show that reflections (110), (200) and (210) are lost, most likely due to a reduced contrast that can be attributed to the presence of FA in the pore voids and the anchored N3 molecule. Nevertheless, the existence in all cases of the (100) peak in the XRD patterns indicated that the process of pore loading with FA, and the additional functionalization with the polyamine, did not modify the typical porosity of the mesoporous MCM-41 scaffold.

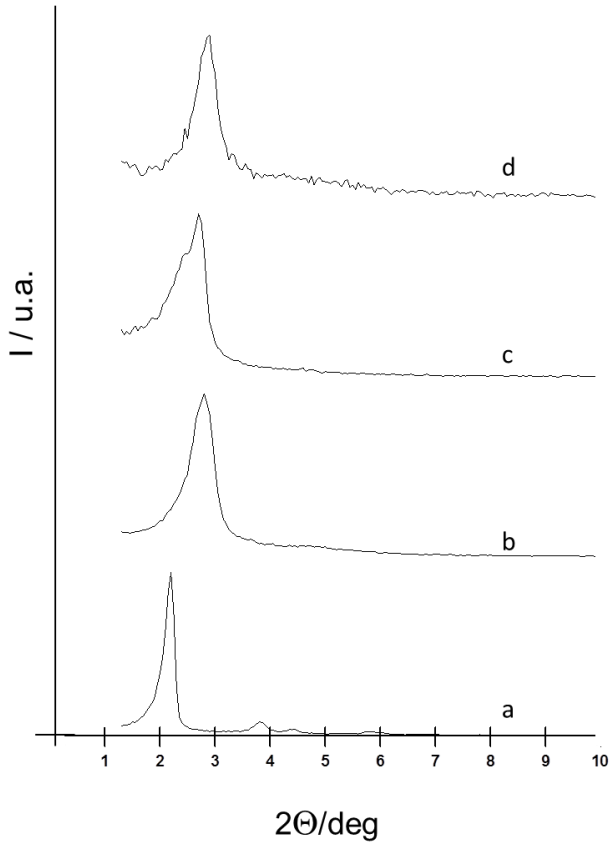

Figure 4. Powder X-ray patterns of the solids (a) MCM-41 as-synthesized, (b) MCM-41calcined, (c) the uncapped solids containing the vitamin B_9 (**S0**) and (d) the capped mesoporous system (**S1**).

The MCM-41 mesostructure mesostructure after loading with FA and functionalization with polyamines was also confirmed by TEM images (see Figure 5).

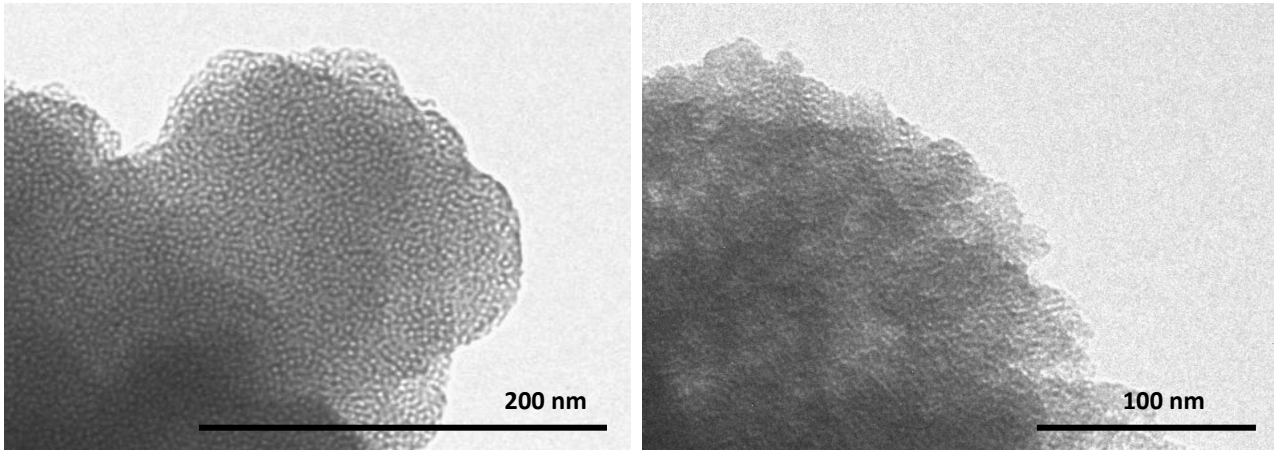

Figure 5. TEM image of (a) MCM-41 calcined and (b) solid **S1** showing the typical porosity of the MCM-41 matrix.

The N_2 adsorption-desorption isotherms of the starting MCM-41 calcined material are shown in Figure 6. The curve shows a well defined and sharp adsorption step at P/P_0 values between 0.1 and 0.3, corresponding to a type IV isotherm, which is typical of mesoporous materials, attributed to nitrogen condensation in the mesopore inlets. With the Barrett-Joyner-Halenda (BJH) model on the adsorption curve of the isotherm, pore diameter and pore volume were calculated to be 2.52 nm and 0.92 cm^3g^{-1}, respectively. The absence of a hysteresis loop in this interval and the narrow BJH pore distribution suggested the existence of uniform cylindrical mesopores. The application of the BET model resulted in a value of 1040 m^2/g for the total specific surface. From the XRD, porosimetry and TEM studies, the a_0 cell parameter (3.98 nm), the pore diameter (2.52 nm), and a value for the wall thickness of 1.69 nm were calculated.

Considering the pore size of the MCM-41 support and the FA structure (see section 3.1), it can be stated that FA can be perfectly encapsulated in pores of 2.52 nm of diameter. Moreover, bearing in mind the volume of the FA molecule (1.16 nm^3), the specific volume of the MCM-41 (0.92 cm3/g) and tentatively assuming that ca. 75% of the pore volume in the mesoporous support can be occupied by FA, it can be roughly stablished that 1 mg of MCM-41 could host as a maximum 436 μg (0.98 mmol) of FA in its porous network.

Table 3 also shows the change on the textural properties of the starting silica after the vitamin adsorption (**S0**) and the functionalization with the N3 molecule (**S1**). The incorporation of the FA leads to a decrease of ca. 37% and 46% for the BET surface area and the BJH mesopore volume, respectively. This evolution indicates that the FA molecules must be incorporated inside the mesopores. Under the pH conditions used for the drug uptake (7.5), the two carboxilate groups must be deprotonated, and consequently, the interaction with the silanol groups at the silica surface must be mediated by H^+ species. Taking into account the presence of partially filled mesopores in the solid **S0** together with the relatively large arm of the N3 molecule, the incorporation of these last species preferentially must occurs in the external surface at the mesopore entrances. As expected, the incorporation of the N3-gates leads to an additional decrease of both the surface area as the volume of 31 and 57%, respectively. In a parallel way,

the size of the mesopores decreases after FA inclusion. A large variety of FA aggregates on the silica are possible which is consistent with the wider peak observed in the pore size distribution curve. An additional mesopore size reduction occurs after functionalization with N3 groups. Hence, solid **S1** shows a wide pore size distribution with a principal peak centred at 1.82 nm and a residual signal at ca. 2.38 nm. The first peak could be associated to a majority of the mesopores well surrounded by N3 molecules and the residual peak must be attributed to a small proportion of mesopores showing some deficiencies respect to an optimum presence of N3 groups.

Table 3. Analytical and textural parameters from TGA and N2 adsorption-desorption isotherms.

	SBET (m2g-1)	Pore volume (cm-3g-1)	Pore size (nm)
MCM-41	1040	0.92	2.52
S0	653	0.49	2.28
S1	451	0.21	1.82 (2.38)

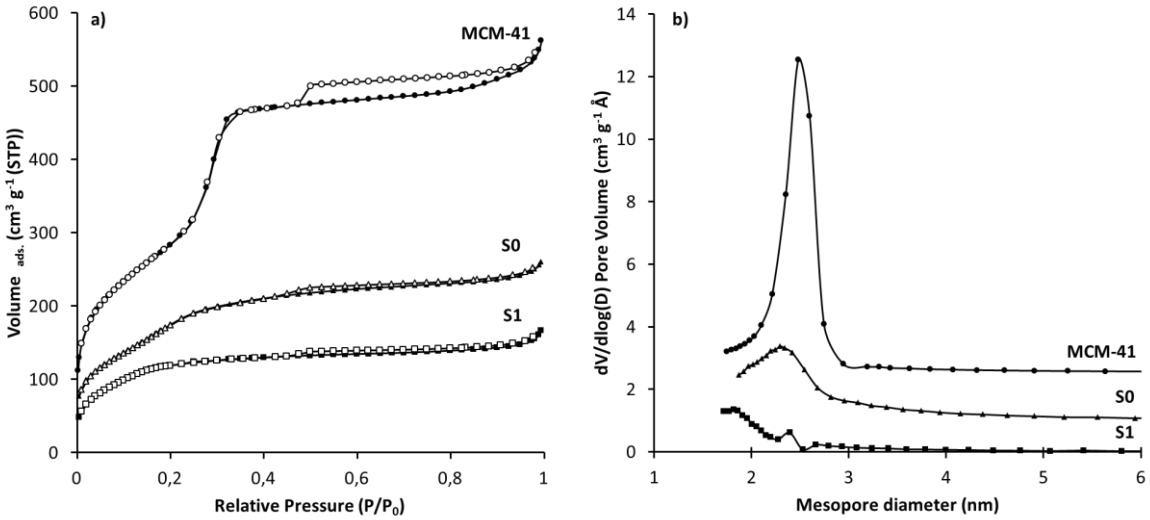

Figure 6. Nitrogen adsorption-desorption isotherms (a) and pore size distribution (b) for MCM-41 mesoporous material, S0, and S1 materials.

3.3 pH gate-like mechanism confirmation

FA delivery from the uncapped solid **S0** in acid and neutral conditions was studied. In a typical experiment 5 mg of **S0** were suspended in 25 mL of two different aqueous solutions (pH 2 and pH 7.5) in an attempt to reproduce the pH of gastric or intestinal fluids. The release profile of **S0** is shown in Figure 7.

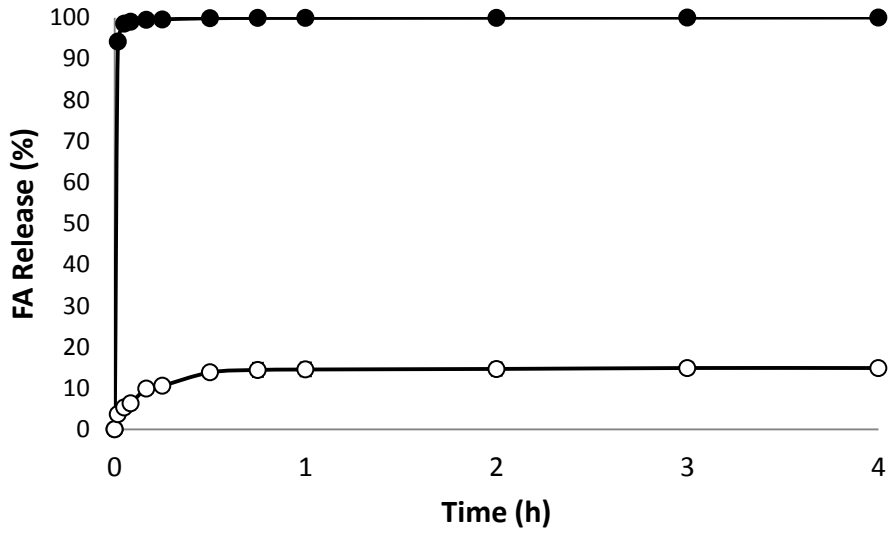

Figure 7. Release profiles of folic acid from the pores of solid **S0** in water at pH 2 (unfilled marker points) and pH 7.5 (filled marker points). Values are Means±SD, n=3.

As observed in the Figure 7, the asymptote of the release curve was achieved in the first few minutes of the delivery at pH 7.5. According to TGA experiments, the maximum delivery achieved (indicated in the figure as 100%) corresponds to 32% of the FA content in the solid. This indicated that, under the experimental conditions, not all the FA loaded was able to be released. In contrast, only a 14.9±1.2% of the maximum delivery capacity was achieved at pH 2 (which corresponds to ca. 5% of the total FA content determined by TGA). Considering that the increase of FA concentration in the water phase is proportional to the delivery of the vitamin from the pores and that FA release is not inhibited by the presence of functional molecular groups on the surface of the MCM-41, the effect of solubility in FA bioacessibity (release) is confirmed. At pH 2, FA is in the form of acid with low solubility, while at pH 7.5 folic is in the form of salt, increasing its solubility, and enhancing the delivery [39] from the pore voids of **S0** to the solution. However, as it can be seen in figure 7 this pH-induced "solubilisation mechanism" is insufficient to modulate a sustained release of FA, a fact that would not allow a proper absorption of FA in the jejunum.

In a second study, delivery of FA from **S1** was tested using similar delivery conditions; i.e. 5 mg of **S1** were suspended in 25 mL of two different aqueous solutions at pH 2 and pH 7.5. As shown in Figure 8, FA delivery after 4h at pH 2 achieved values 0.22±0.03% of the maximum delivery capacity of **S1** (which corresponds to ca. 0.09% of the total FA content determined by TGA). In contrast, at pH 7.5, a progressive delivery of the vitamin was observed, achieving ca. 100% of the release capacity in the performed conditions after 1h (which corresponds to 40% of FA content determined by TGA). In this case, the pH-dependent release behaviour can be explained by considering the different FA solubility as a function of the pH and the presence of the gate-like ensemble based in polyamines. To explain this latter effect, it has to be taken into account that at acidic conditions (pH 2), amines anchored to the surface of the pores are fully protonated. This

fact favours Coulombic repulsions between closely located polyammonium groups, so that tethered polyamines tend to adopt a rigid-like conformation that blocks the pores and practically no release occurs. At pH 7.5, a lower proportion of polyamines are expected to be protonated, favouring hydrogen bond interactions between the different amine chains. As a consequence, pores unblock and vitamin release is produced. Moreover, in amine-based gated ensembles a second cooperative anion-dependent effect occurs. In general polyamines are well-known pH-responsive molecules that can additionally complex anions via electrostatic forces and by formation of hydrogen-bonding interactions in a wide pH range. Additionally, the relative amine/ammonium ratio can control the interaction with anionic species. If electrostatic forces are taken into account (which in general are stronger than hydrogen bonding interactions) the presence of a large percentage of ammonium groups (acidic pH) will favour the interactions of the "gate" with anions in the solution resulting in an additional pore blockage that is not observed at neutral or basic pH [40].

Comparing the delivery profiles at pH 2 and 7.5 of the uncapped (**S0**) and capped (**S1**) materials it is apparent that **S0** is not able to completely inhibit FA delivery at acidic pH being the delivery very fast at neutral pH. In contrast, **S1** is tightly capped at pH 2 and displays a sustained delivery (in 1h) when the pH is switch to 7.5. It can be concluded that the gated support **S1** might be a suitable prototype for the development of orally applicable FA delivery systems designed to block cargo delivery in the acidic conditions of the stomach (acid pH, gate closed) yet be able to display a sustained FA release at the intestine (basic pH, gate open).

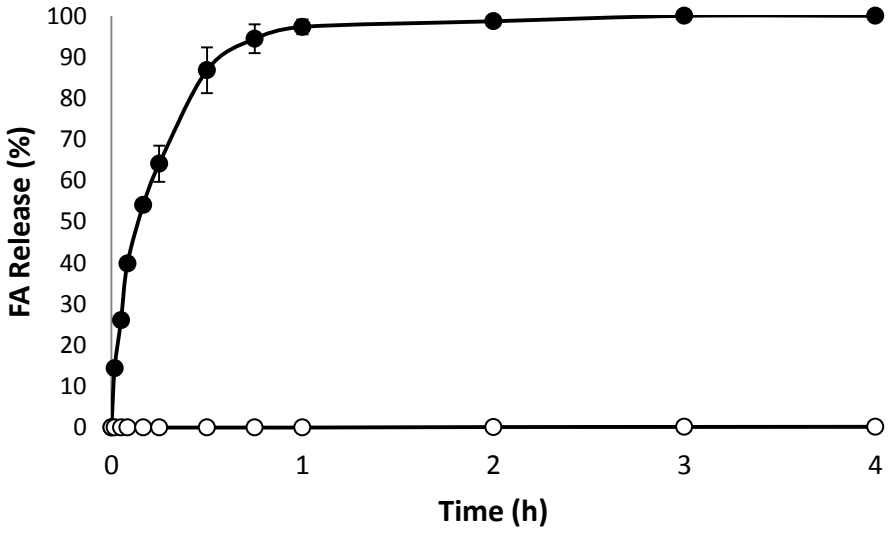

Figure 8. Release profiles of folic acid from the pores of solid **S1** in water at pH 2 (unfilled marker points) and pH 7.5 (filled marker points). Values are Means±SD, n=3

3.3 Loading/releasing optimization process

After studying the delivery of FA from the polyamine-functionalised (**S1**) and unfunctionalised (**S0**) materials, this section deals with a detailed study of FA loading optimization for the preparation of functionalised solids (**S1**-like supports). For this purpose, 16 solids differing in the loading (immersion and impregnation) and the functionalization media used to anchor the polyamine N3 (water at pH 2, acetate buffer at pH 2 or acetonitrile) were prepared. Delivery effectiveness of each solid was evaluated by the determination of FA delivered after 5h at pH 7.5. Moreover, the loading/delivery performance of the solids was evaluated using the "relative loading efficiency" via the determination of the ratio between the amount of FA delivered (after 5h at pH 7.5) per mg of loaded solid and the mg of FA used for loading 1 mg of MCM-41 (see Experimental Section for details).

The first block of bars of Figure 9 shows values of FA delivered per mg of **A#** solids, solids obtained by using a traditional immersion procedure. Among them, solid **A1** was able to deliver 3.7±0.2 μg of FA/mg of solid. By increasing the amount of FA present in the loading solution to 70mg (0.016mmol) (**A3**) the amount delivered increased to 21.2±0.3 μg of FA/mg of solid. However, when the same amount of folic was loaded in the half of solvent, the release capacity decreased (**A4**) to 6.2±0.2 μg of FA/mg of solid. To understand this behaviour the maximum FA amount able to be solubilized in PBS was determined, finding that above of 10mg of FA/mL PBS, FA remains insolubilized, and thus, cannot participate in the loading process.

The same figure shows values of FA delivered by solids loaded by impregnation and functionalized in water at pH 2 (**BW#**), acetate buffer at pH 2 (**BB#**) or acetonitrile (**BA#**). As it can be seen, **BW#** solids exhibited the lowest loading capacity. In fact the 4 solids prepared using these conditions were white, strongly suggesting the presence of a very low amount of FA (pale yellow) in the pores. To understand the cause of this behaviour, it was found that pH reached values of 10 upon the addition of the amine N3 during the synthesis of the solid. At this pH FA is highly soluble and most likely leaked from the pore voids during the synthesis of the materials. In contrast, **B** solids functionalized with N3 in aqueous acetate buffer (**BB#** solids) or acetonitrile (**BA#** solids), showed a remarkable larger FA delivery in water. In this case, the poor solubility of FA in the acetate buffer or acetonitrile during the N3 functionalization avoided the FA leakage during this step of the synthesis. When both **BA#** and **BB#** solids are compared in terms of delivery, it can be stated the **BB#** series display larger delivery ability. In particular, **BB#** solids exhibited a remarkable delivery capacity ($p<0.005$), being solid **BB4** able to release 127±5 μg of FA/mg of solid (see Figure 9). As a conclusion, the choice of the solvent employed for the surface functionalization step is as important as the loading procedure in terms of loading efficiency. Nevertheless, the maximum loading capacity calculated in section 3.2 has not been achieved in any of the designed solids.

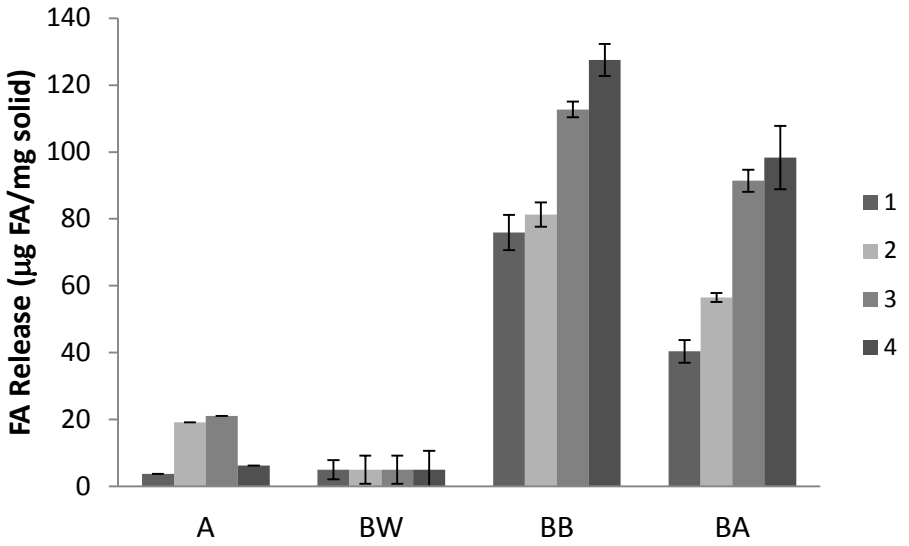

Figure 9. Maximum FA delivery (μg FA/mg solid) for different solids loaded by immersion in PBS and functionalized with N3 in acetonitrile (A# solids) or loaded by impregnation and functionalized with N3 in different solvents: water adjusted to pH 2 (BW), acetate buffer at pH 2 (BB) or acetonitrile (BA). Numbers 1-4 refers to different FA loading conditions described in Table 1. Values are Means±SD, n=3.

From the release ability displayed by the different solids prepared and taking into account the amount of FA employed for the loading of each solid, the relative loading efficiency (RLE) was calculated. Figure 10 shows RLE values for the 16 prepared solids. As expected, solids loaded by immersion (**A#**) were the less efficient in terms of delivery with RLE values of around 1-3%. Moreover RLE values of ca. 2.5-5%, 40-60% and 63-75% were observed for **BW#**, **BA#** and **BB#** supports. In addition, for solids **BA#** and **BB#** RLE increased significantly ($p<0.005$) from 10 mg in 1 cycle (**#1** solids) to 15 mg in 3 cycles (**#3** solids). However, the addition of 1 extra cycle of addition (**#4** solids) did not increase RLE values indicating that, in this case, not all the FA added was loaded inside the pore voids of the mesoporous materials. According to these results, the conditions employed for preparing solid **BB3** should be considered as optimal, in terms of RLE values, for the preparation of MSP loaded with folic acid and gated with N3 moieties.

All these prepared solids were characterized using standard techniques. In all cases XRD patterns and TEM images (data not shown) were equivalent to those obtained for solid **S1** (section 3.1), confirming that the mesoporous structure of the MCM-41 scaffold was maintained in spite of the different loading and functionalization process.

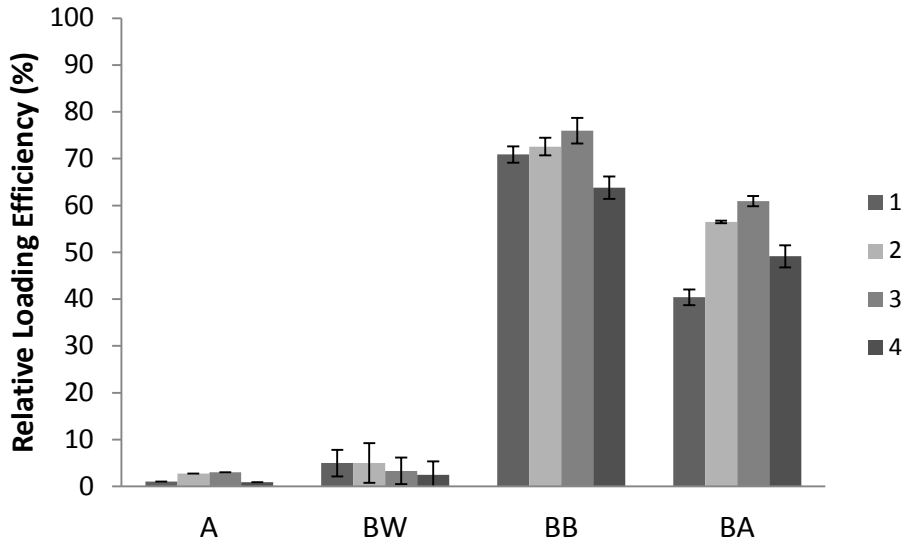

Figure 10. Relative Loading Efficiency for different solids loaded by immersion in PBS and functionalized with N3 in acetonitrile (A# solids) or loaded by impregnation and functionalized with N3 in different solvents: water adjusted to pH 2 (BW# solids), acetate buffer at pH 2 (BB# solids) or acetonitrile (BA# solids). Numbers 1-4 refers to different FA loading conditions described in Table 1. Values are Means±SD, n=3.

3.4 In vitro Folic Acid bioaccessibility and nutritional implications

After the loading optimization procedure described above, this section deals with the use of solid **BB3**, which exhibited the maximum RLE value, for delivery studies in a more realistic media. In particular, the aim of this part was to study FA release, and therefore FA bioaccessibility, during a simulated pass of the solid through the gastrointestinal tract. To evaluate this, a variation of the dynamic *in vitro* digestion protocol reported by Versantvoort et al. [36] was performed. Briefly **BB3** was suspended in simulated gastric juice and incubated for 2h at 37ºC and then duodenal juice, bile and bicarbonate solution were added to obtain a simulated intestinal fluid (see Experimental Section for details). Note that in this study the pass of the solid through large intestinal track was not taken into account since in vivo folic acid absorption occurs throughout the jejunum [1]. At certain times aliquots were taken, filtered and analysed by HPLC. Results of FA delivery from **BB3** are shown in Figure 11. During the first two hours of simulated digestion, where **BB3** solid was in contact with a simulated gastric fluid, only 10.2±1.4 μg of FA/mg of solid were delivered. After the addition of the simulated intestinal fluid, FA delivered increased progressively to reach a maximum value of 94±9 μg of FA/mg of solid at 2h.

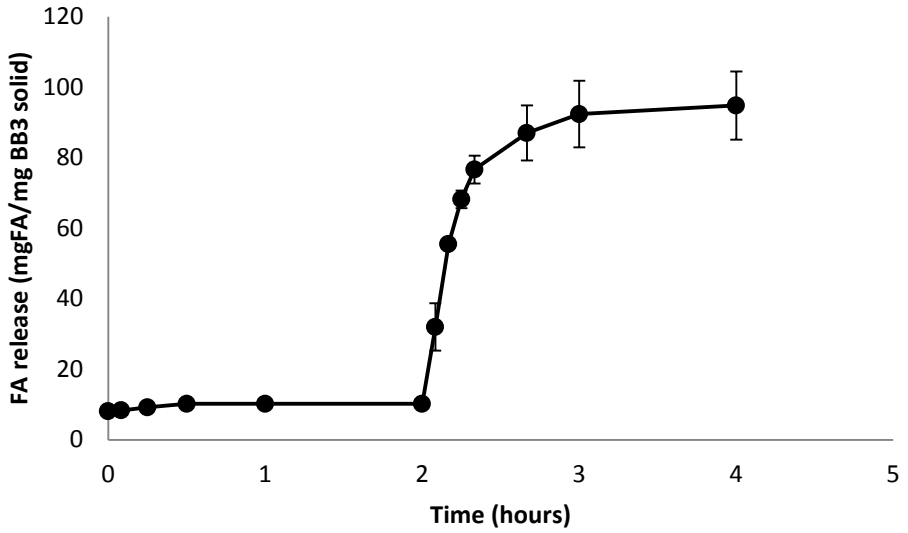

Figure 11. Release profile of FA from solid BB3 along the simulated gastrointestinal digestion process. Time 0-2h correspond to the simulation of stomach conditions and time from 2-4h correspond with the simulation of intestinal conditions. Values are Means±SD, n=3.

Thus, as it can be observed in Figure 9, **BB3** solid is tightly capped in simulated gastric fluid yet opens and deliver the cargo when in contact with simulated intestinal fluid. Moreover, the most important thing is that 1 mg of solid **BB3** is able to release ca. 95 μg of FA during the whole simulated digestion process. This implicates that the higher recommended dietary intakes in human nutrition, established for pregnant woman in 600 μg per day of folates or 360 μg of synthetic PGA (1 μg of dietary folate equivalent = 0.6 μg of folic acid) [9, 41], could be reached by an oral administration of only ca. 4 mg of **BB3,** which is a remarkable low amount. Moreover, based in the oral toxicological evaluation of other mesoporous silica particles carried out by Kupferschmidt et al. [42] -that found that even high amounts of MSP up to 1200 mg of MSP/kg of rat administrated orally were not toxic - our less than 7 mg are very far of a toxicological effect, signifying that the optimized solid **BB3** could be safe for oral administration.

4. Conclusions

Mesoporous silica particles (MSP) have been recently proposed as smart delivery devices able to load large amounts of cargo and release the same using different triggering stimuli. In this study FA has been successfully encapsulated in mesoporous silica particles capped with 3-[2-(2-aminoethylamino)ethylamino]propyl groups. A detailed study of the loading process allowed us to obtain solids with relative encapsulation efficiencies of the 75%. The simulation of an *in vitro* digestion of selected optimized gated support allowed to conclude that the developed MSP capped with amines were not only able to hinder the release of the vitamin in the presence of a simulated gastric fluid, but also were able to deliver progressively the vitamin along the time in presence of a simulated intestinal fluid, offering a mechanism to modulate the bioaccessibility of

the FA vitamin during the pass across the intestine. In this study it was found that 1 mg of the optimized solid was able to release ca. 95 μg of FA indicating that maximum levels of higher recommended dietary intakes in human nutrition could be reached using only 4 mg of the optimized solid. Bearing additionally into account that mesoporous silica particles are non-toxic at low levels for humans, it can be stated that pH-dependent capped SMP are suitable candidates for the design of orally applicable delivery systems designed to protect FA from the acidic conditions of the stomach (acid pH, gate closed) but release the vitamin at the intestine (basic pH, gate open).

Acknowledgements

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