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GASTRORETENTIVE MONTMORILLONITE-TETRACYCLINE NANOCLAY FOR THE TREATMENT OF *HELICOBACTER PYLORI* INFECTION

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Abstract

The paper aims to explore the potential benefits provided by an organically modified montmorillonite (nanoclay) in the problematic management of the *Helicobacter pylori* gastric infection that is one of the most prevalent infectious diseases worldwide. Two nanoclay samples were produced by the intercalation of tetracycline (TC) into the interlayer of montmorillonite (MM) under two different pH reaction conditions (pH 3.0 and 8.7). MM/TC nanoclays were characterized by EDX, XRD, FTIR, DSC, drug

adsorption extent, *in vitro* mucoadhesiveness and desorption in simulated gastric media. The reaction between MM and TC led to a complete MM cation (Na^+ and Ca^{2+}) exchange process, an increase of MM characteristic interlayer spacing as well as an involvement of NHR_3^+ group of TC, regardless of the reaction pH value. However, MM/TC nanoclay obtained under alkaline conditions provided a lower TC adsorption as well as a drug fraction weakly linked to MM in comparison with the nanoclay obtained in acidic conditions. Both the nanoclays exhibited good mucoadhesion properties to porcine mucin and TC desorption occurring mainly *via* a cation exchange process by H^+ ions. Based on the results obtained, TC intercalation into MM nanoplatelets could represent a potential advantageous approach allowing the antibiotic to distribute homogeneously on the gastric mucosa, diffuse through the gastric mucus layer and achieve the microorganism localization.

Keywords: Helicobacter pylori, Montmorillonite, Tetracycline, Nanoclay, Mucoadhesion

Chemical compounds studied in this article

Magnesium Aluminum Silicate (PubChem CID: 3084116); tetracycline hydrochloride (PubChem CID: 54704426); Cesium chloride (PubChem CID: 24293); Chitosan (PubChem CID: 71853).

1. Introduction

Helicobacter pylori (Hp) gastric infection is one of the most prevalent infectious diseases worldwide with an estimation of more than 50% of the world population. Hp infection is associated with several gastroduodenal pathologies such as chronic gastritis, gastric and duodenal ulcers. Furthermore, oxidative and nitrosative stresses in combination with inflammation observed in Hp-infected patients play an important role in gastric carcinogenesis, being Hp estimated to be responsible for approximately two-thirds of gastric lymphomas (Gisbert and Calvet, 2013). For these reasons, the Food and Drug Administration (FDA) has recently added Hp to the list of qualifying pathogens that have the potential to pose a serious threat to public health (FDA, 2014).

In accordance with FDA and European Maastricht guidelines (Malfertheiner et al., 2012), the recommended therapy involves different regimens based on the oral administration of two or more antibiotics combined with proton pump inhibitors or antacids twice a day for up to 14 days, and in fed conditions to increase the gastric residence time of the drug. However, this latter condition is relevantly affected by inter-individual differences in gastric emptying as well as by the amount and variety of the ingested food. Even with a right selection of drugs, bacterial eradication may fail in up 20-40% of patients owing to antibiotic resistance, insufficient antibiotic concentration reaching the site of infection (under the gastric mucus gel layer), uneven drug distribution in the gastric lumen, short contact time between drug formulation and gastric mucosa, and poor patient compliance due to the complex therapeutic regimen which also produces side effects (Gasparetto et al., 2012;

Siddalingam and Chidambaram, 2014; WGO Global Guidelines, 2010). Among these difficulties connected with Hp cure, failure of therapy is mostly associated to antibiotic resistance and patient non-compliance (Graham and Shiotani, 2008). Indiscriminate use of antibiotics combined with non-adherence to therapy have led to a growing increase in the resistance of bacteria to antibiotics, particularly macrolides, fluoroquinolones and metronidazole (Kim et al., 2001; Malfertheiner et al., 2012; Oleastro et al., 2011; Papastergiou et al., 2014; Siavoshi et al., 2010). Another reason for the failure of the conventional therapy lies in Hp nature. Hp colonization occurs because the microorganism survives in the acidic gastric environment owing to its urease activity producing ammonia and bicarbonate from urea. After that, Hp adheres to the gastric epithelium under the mucus gel layer where H^+ ions are neutralized by HCO_3^- secreted by the epithelial cells (Allen and Garner, 1980; Henriksnäs et al., 2006). Therefore, access for antimicrobial drugs to the infected site is limited by both the acidic gastric lumen affecting drug stability and mucus gel layer which hinders the attainment of sufficient antibiotic concentrations for bactericidal activity (Lopes et al., 2014; Umamaheshwari et al., 2004). Therefore, a new treatment approach that is also simple to encourage patient compliance aiming to achieve higher eradication rates is urgently needed (Gatta et al., 2013; Siddalingam and Chidambaram, 2014; Vakil and Vaira, 2013). On these assumptions, a stomach-site specific drug delivery system would increase the localized concentration and the residence time of the drugs at the site of action making less variable the gastric emptying time, reducing dosage frequency, and increasing patient compliance. For this reason, scientific research has proposed floating or mucoadhesive systems that prolong intragastric residence time, gradually releasing the drug in the gastric region for a longer time than normal gastric emptying, requiring a single dose per day and being scarcely influenced by food

ingested. In addition, the entire dose could be released in the stomach resulting in an increased efficacy of the formulation. Most of these studies involved multiparticulate delivery systems able to be distributed over a wider surface of the gastric mucosa in comparison with monolithic devices (Adebisi and Conway, 2014; Bardonnet et al., 2006; Ishak et al., 2007; Liu et al., 2005; Patel and Patel, 2007; Sahasathian et al., 2010). Concerning the mucoadhesion approach, animal model studies demonstrated that mucoadhesive microparticulate systems can reside in the stomach even after 10h from the administration and that the transport of an antibiotic from the gastric lumen through the mucus layer is more effective than drug absorbed through the basolateral membrane from blood circulation as well as drug administered by means a conventional formulation (Prasanthi et al, 2011; Siddalingam and Chidambaram, 2014; Umamaheshwari et al., 2004).

On the basis of these premises, the purpose of this study was to exploit the use of a clay material from the smectite family in order to produce a mucoadhesive organically modified clay by means of the interaction between the clay and an anti-Hp antibiotic. Smectites are 2:1 (tetrahedral:octahedral sheet) layered silicates, characterized by octahedral and tetrahedral substitutions and high Cation Exchange Capacity (CEC), so they may undergo ion exchange with basic drugs in solution within their interlayer space. Smectite ability to exchange cations with several organic compounds has been used for five decades, and more recently, they have been proposed as materials for modulating drug delivery or improving dissolution of poorly water-soluble drugs (Aguzzi et al., 2005; Joshi et al., 2009). In addition to these properties, smectites such as clays in general possess strong bioadhesive properties, and gastroprotective antacid activity due to their interaction with the mucus glycoproteins neutralizing the gastric acidity (Droy-Lefaix and Tateo, 2006). Among smectites, montmorillonite (MM), the

main component of bentonite, could be considered as the essential clay material, known to have platelet structure with average dimension of 1 nm thick and 70 to 150 nm wide from which nanoclay definition originates (Patel et al., 2006; Uddin, 2008). Montmorillonite is mainly used as auxiliary material in the pharmaceutical industry for oral or topical dosage forms, recorded in the United States Pharmacopoeia, European Pharmacopoeia, and British Pharmacopoeia.

Therefore, an organically modified MM nanoclay was developed by intercalating tetracycline (TC) into MM nanoplatelets. TC was used in the first effective therapies against Hp and is currently used as part of second-line therapy (triple or quadruple regimen) eradicating Hp. Hp isolated from 138 patients resulted sensitive to TC with a MIC $\leq$ 0.38 mg/L and Hp resistance to TC is considered as rare in most countries owing to a stepwise process involving a triple-base-pair substitution within Hp rRNA genes (Gerrits et al., 2003; Samra et al., 2002). Adsorption of tetracyclines onto soils and clay minerals were conducted as early as 1950's even if to a limited extent. To date these studies focused on removal TC as an organic contaminant of water or soils to improve environmental quality (Aristilde et al., 2013; Avisar et al., 2009; Chang et al., 2009; Wang et al., 2010), and on biopharmaceuticals effects due to the co-administration of TC and clays acting as suspending agents (Browne et al., 1980; Porubcan et al., 1977), or topical excipients (Parolo et al., 2010). Conversely, clays have not been hitherto object of study for the development of a gastroretentive TC delivery system.

In the present work, MM-TC nanoclays were obtained under two different pH reaction conditions and characterized for the interaction occurred between drug and nanoclay, drug adsorption and *in vitro* desorption extents. In particular, a novel application of Energy Dispersive X-ray (EDX) analysis is proposed to determine clay

Cation Exchange Capacity (CEC) giving more information about the elements involved in the intercalation process compared with the current methods. Moreover, the nanoclay samples were evaluated *in vitro* for mucoadhesiveness in a perspective of a gastroretentive formulation able to improve the performance of Hp infection therapy.

2. Materials and methods

2.1. Materials

For the organically modified nanoclay preparation, montmorillonite (MM) [$(NaCa)_{0.33}(AlMg)_2(Si_4O_{10})(OH)_2 \cdot nH_2O$), Veegum R, Magnesium Aluminum Silicate NF, pharmaceutical grade, >90% montmorillonite] from Vanderbilt Minerals, LLC (Norwalk, CT, USA) and tetracycline hydrochloride (TC, MW 480.90) from Fluka Chemie (Buchs, Switzerland) were purchased. For Cation Exchange Capacity (CEC) determination, cesium chloride, CsCl, was purchased from Sigma-Aldrich (Milan, Italy). For the mucoadhesion assay, chitosan (low molecular weight, >75% deacetylated, viscosity 20-300 cps in 1% acetic acid at 25°C) from Fluka Chemie and mucin from porcine stomach, type II from Sigma-Aldrich were purchased. All the other chemicals were of analytical grade.

2.2. Montmorillonite CEC determination

Montmorillonite Cation Exchange Capacity (CEC) was measured by placing 100 mg clay in a 0.1 M solution of CsCl (125 ml) under magnetic stirring for 24h. After centrifugation (3000 rpm for 15 min), the powder was rinsed with water, vacuum

dried, compressed in a hydraulic press (Perkin-Elmer) at 200 kg/cm^2 for 1 min using 12.5 mm diameter punches, mounted on carbon stubs without conductive coating, and assayed by Energy Dispersive X-ray (EDX, Oxford INCA-350, FEI Company-Oxford Instruments, Oregon, USA) analysis coupled with an Environmental Scanning Electron Microscopy (ESEM, Quanta 200 Fei Company-Oxford Instruments) by the selected area method. EDX analysis is a technique usually used to identify the elemental composition of a sample. In this case, it allowed determining clay CEC, exploiting the peculiar affinity of phyllosilicates for some elements, among which cesium. X-ray emission of Cs atoms was evaluated at the intensity characteristic of this element (Lα= 4.2865 keV) and at the following working conditions: acceleration voltage 12 kV, spot size of 3, detection limit <0.3%. Moreover, X-ray emissions from the atoms calcium, potassium, carbon, oxygen, sodium, magnesium, aluminum, silicon, and other elements with atomic number from 4 were identified.

EDX spectra representing the plots of X-ray counts *vs* elements and semi-quantitative results expressed as relative weight percentage of the elements present in the specimen were obtained. The same procedure was applied to the original specimen, which was not submitted to cationic exchange with CsCl solution.

The relative CEC was determined through the weight percentage of Cs, applying the following equation:

$$\text{CEC (mEq/100 g of clay)} = (1000 \text{ weight\% Cs})/(\text{Cs MW})$$

The reported value was averaged on three determinations.

2.3. Tetracycline adsorption onto montmorillonite

Tetracycline adsorption onto MM was performed at constant drug concentration, corresponding to about two times MM CEC value, and at two different pH values. Then, glass tubes were filled with 20 ml of tetracycline (TC) water solution (2 mg/ml) at pH 3.0 for hydrochloric acid or water solution at pH 8.7 for sodium hydroxide. Then, 40 mg MM, previously milled by a vibratory ball mill (FRITSCH GmbH, Idar-Oberstein, Germany) (bulk MM) for 10h, was suspended in each solution and the tubes were horizontally shaken in the darkness for 24h. Preliminary studies showed this time as suitable to achieve the equilibration. The suspensions were centrifuged (2500 rpm, 15 min) and the sediments washed twice with 1000 ml deionized water under magnetic stirring for 15 min. The obtained MM/TC nanoclays, named MM/TC pH 3 and MM/TC pH 8.7, were dried under vacuum at room temperature and stored in the darkness. The amount of TC adsorbed was calculated as the difference between the initial TC concentration and that in the supernatant determined spectrophotometrically (Lambda 3B, Perkin-Elmer, Norwalk, CT, USA) at 274 nm wavelength. The reported data were averaged on three adsorption batches.

2.4. MM/TC nanoclay size and surface charge

MM/TC nanoclay particle size, expressed as the diameter of the most represented dimensional class (main class), was determined by laser granulometer (Mastersizer 2000, Malvern Instruments Ltd, Worcs, U.K.) and compared with that of bulk MM.

Z-potential values of MM/TC nanoclays and bulk MM were measured by using Photon Correlation Spectroscopy (PCS) (Zetasizer version 6.12, Malvern Instruments Ltd) equipped with a 4mW He-Ne laser (633 nm) and a DTS software (Version 5.0). The determinations were carried out in triplicate from three sample batches.

2.5. MM/TC nanoclay analyses

The elemental composition of MM/TC nanoclays was determined by means of Energy Dispersive X-ray (EDX) analysis coupled with an Environmental Scanning Electron Microscopy (ESEM), as described previously in section 2.2.

MM/TC nanoclays were subjected to thermal analysis on a Differential Scanning Calorimeter (DSC-4, Perkin-Elmer). The samples (6-7 mg) were accurately weighed in crimped aluminum pans and heated from 30°C to 250°C at a scanning rate of 10°C/min under dry nitrogen flow (30 ml/min). Thermograms of bulk TC and MM/TC physical mixture at 30% TC were also recorded as comparison.

Fourier Transform Infra-Red (FTIR) spectra provided by MM/TC nanoclays, TC, bulk MM, and MM/TC physical mixture at 30% TC, suspended in Nujol mull, were obtained by a 1600 FTIR spectrophotometer (Perkin-Elmer).

Powder X-ray diffraction (XRD) patterns of MM/TC nanoclays were recorded on a X'PertPRO diffractometer (Philips, Eindhoven, The Netherlands) equipped with an X'celerator detector configured for an active length of 2.12° (2θ). The diffractograms were recorded from 3° (2θ) to 75° (2θ). Diffractograms of bulk TC and MM/TC physical mixture at 30% TC were also recorded as comparison.

2.6. Mucoadhesiveness assay

Mucoadhesion properties of MM/TC nanoclays and bulk MM were assayed by using a modified Dognon-Abribat tensiometer (du Nouy method). The samples (0.5 g) were compressed in a hydraulic press (Perkin-Elmer) at 200 kg/cm^2 for 1 min using 12.5 mm diameter punches, wetted with simulated gastric fluid (USP, without pepsin), and placed in contact with the platinum plate previously immersed into 20% porcine mucin suspension in 0.1M HCl. Mucoadhesiveness forces were calculated by applying the following equation:

$$\text{Mucoadhesiveness force} = M \times (g/A)/1000$$

where M is the measured force acting on the plate (dine/cm^2), g is the gravitational acceleration (cm/s^2), and A is the contact area between sample and lamina (cm^2).
To validate the method, chitosan, a known mucoadhesive material, subjected to the same treatment of the samples, was used as the positive control, while a glass slide as the negative control. The reported values are averages of three determinations.

2.7. TC desorption in simulated gastric media

TC dissolution and desorption from the nanoclays were examined under sink conditions using the flow-through cell, USP Apparatus 4 (Dissotest CE-1, Sotax, Basil, Switzerland) on exactly weighed samples in 100 ml of both simulated gastric fluid (pH 1.2, according USP, without pepsin) and pH 3.0 saline solution using HCl and 0.9% NaCl, at a temperature of 37.0 ± 0.5°C under a flow rate of 25 ml/min. The dissolved or desorbed concentration of drug was determined spectrophotometrically (Lambda 3B, Perkin-Elmer) at a wavelength of 274 nm, at fixed time intervals for 5h.

Subsequently, 1 ml HCl 1M was added to the medium to mimic the cation renewal from the gastric acid secretion and TC concentration was determined spectrophotometrically up to 24h. The reported data were averaged on three determinations.

2.8. Statistical analysis

Data obtained were evaluated from a statistical point of view using ANOVA one-way. Differences at P values <0.05 were considered significant.

3. Results and discussion

The strategy considered in this study for Hp eradication therapy has been directed to the use of a pharmaceutical grade montmorillonite (MM) that can accommodate in its interlayer organic molecules as well as exert mucoadhesive and gastroprotective properties. Tetracycline (TC) was selected as the drug because it is effective against Hp, has also low resistance so from being included in quadruple therapy in case of clarithromycin resistance as both first- and second-line therapy, and represents a lower-cost option (WGO Global Guidelines, 2010). With regard to TC biological activity, its adsorption onto MM without compromising the bactericidal activity was demonstrated (Parolo et al., 2010). Moreover, considering TC degradation in aqueous solutions (Kang et al., 2012; Loftin et al., 2008; Pena et al., 1998) although the high half-life estimated by other Authors in more than 300h (Sah, 2006), a greater chemical stabilization might be provided by the adsorption process onto MM (Chang et al., 2009; Sah, 2006).

TC presents three functional groups that can undergo protonation-deprotonation reactions according to the pH value of the medium (Leeson and Weidenheimer, 2006). It exists predominantly as a cation at pH <3.3, zwitterion at a pH between 3.3 and 7.7, and anion at pH >7.7 (Browne et al., 1980; Porubcan et al., 1977; Wang et al., 2010). Surface interactions (cation bridging, complexation, hydrogen bondings, van der Waals interactions), and/or cation exchange upon contact with MM were described in the literature, even if more research is needed to better understand the reaction process (Parolo et al., 2010, 2008; Wang et al., 2010).

Therefore, the present study involved two MM/TC nanoclay samples obtained by TC adsorption onto MM at two different reaction pH values, pH 3 and pH 8.7, which were selected to exploit TC predominant monocationic species owing to dimethylamino group protonation (pKa_3 9.5), and TC predominant anionic state owing to deprotonation of phenolic diketone (pKa_2 7.7) as well as tricarbonyl methane (pKa_1 3.3) groups, respectively. TC concentration was about two times MM Cation Exchange Concentration (CEC) value, one of the most common concentrations used to obtain organically modified nanoclays.

The CEC is an important characteristic of clays since it represents the tendency of a clay to exchange the inorganic cations located in the interlayer space with chemical moieties on which it comes in contact. CEC arises from isomorphous substitution in MM layer producing Na^+ or Ca^{2+} adsorption as charge compensation, and it is affected by clay chemical composition varying from one original clay deposit to another (He et al., 2010).

The CEC of MM was assayed by using Cs^+ as the exchange cation and by evaluating the modification of the exchangeable elements Na^+ or Ca^{2+} with respect to those exhibited by the bulk MM through EDX analysis. The element identification and

quantification revealed the almost complete Na^+ and Ca^{2+} cation substitution by Cs^+ (Table 1) giving a MM CEC value of 84.81 ± 3.45 mEq/100 g of clay that is in good agreement with typical smectite CEC values (Donahue et al., 1977).

MM/TC nanoclays were examined for physico-chemical features and TC adsorption extent. Moreover, their suitability as gastroretentive dosage forms was exploited by evaluating TC mucoadhesiveness and desorption in simulated gastric media.

3.1. MM/TC nanoclay physico-chemical properties

TC adsorption onto MM generated an increase of MM average particle size and a quasi-monomodal size distribution (>90% population), regardless of the reaction pH value ($P > 0.05$). This finding could be related to the clay sheet expansion occurred during TC adsorption process determining a TC adsorption extent of 29.02 ± 3.56% (w/w) at pH 3.0 and 39.71 ± 5.58% (w/w) at pH 8.7, being the difference between these values significant ($P < 0.05$). Conversely, MM negative surface charge was not modified significantly by TC adsorption process, regardless of the reaction pH value ($P > 0.05$) (Table 1). The negative charge arises from the substitution of a part of aluminum in the aluminum-oxygen octahedron by divalent magnesium atoms and it is considered as a permanent net charge, i.e. not affected by environmental factors such as the pH of the medium or electrolyte concentration (Zhang and Zao, 1997).

In order to elucidate the interaction mechanism between MM and TC according to the reaction pH value, MM/TC pH 3 and MM/TC pH 8.7 nanoclays were investigated by means of EDX, DSC, FTIR and XRD analyses. Variations in elemental compositions originated by TC adsorption onto MM were detected by EDX analysis. The EDX spectra combined with the respective ESEM images from bulk MM and MM/TC

nanoclays are shown in Figure 1 and the relative weight percentage of their elements carbon, Na^+ and Ca^{2+} are listed in Table 1. The bulk MM sample emitted the characteristic peaks of calcium, oxygen, sodium, magnesium, aluminum, and silicon confirming the partial isomorphic substitution of aluminum with magnesium compensated by the exchangeable cations Na^+ and Ca^{2+}. Contrary to MM, both MM/TC nanoclays revealed the almost total absence of the exchangeable cations, and the presence of carbon atoms belonging to TC molecules. In agreement with this latter deduction, carbon weight percentage was higher in MM/TC pH 8.7 in comparison with MM/TC pH 3 ($P < 0.05$), according to TC adsorption extent found higher in MM/TC pH 8.7 than in MM/TC pH 3. EDX findings suggest that a cation exchange between MM and TC has occurred within clay interlayers, regardless of the reaction pH value. Evidently, MM cation exchange by TC might occur not only at pH values rather low at which TC fully protonated form prevails but also at alkaline pH values, at which, despite TC negative form is predominant, positive species could be still present to generate the exchange with MM cations (Parolo et al., 2010; Porubcan et al., 1977).

The thermal behavior of MM/TC nanoclays in comparison with bulk TC, and MM/TC physical mixture is shown in Figure 2. TC exhibited the typical sharp endothermic event at 236°C followed by an exothermic event suggesting melting with thermal decomposition (Fernandes et al., 1999). The peak is still visible in both MM/TC physical mixture and MM/TC pH 8.7 nanoclay even if less pronounced due to the dilution effect whereas it is absent in MM/TC pH 3 nanoclay. Therefore, it can be deduced that only at the acidic pH value TC predominant cationic species have led to a complete drug molecular dispersion, while at the alkaline pH value TC is also present in crystalline form.

Further information on the interactions between MM and TC as a function of the reaction pH value were obtained by subjecting MM/TC nanoclay samples to FTIR analysis in comparison with the bulk materials and their physical mixture (Fig. 3). The bands obtained in the presence of TC correspond to TC adsorbed or interacted with MM that is free from IR absorption except for a strong water bending vibration at approximately 1650 cm^{-1} primarily due to water directly coordinated to the exchangeable cations of the clay. Its absence is already a good indication of the replacement of these cations with TC (Porubcan et al., 1977). The region between 1700 cm^{-1} and 1500 cm^{-1} (Fig. 3a) showed TC characteristic bands at 1615 cm^{-1} and 1581 cm^{-1} associated with amid-NH vibration and C=O stretching vibration at ring C or, alternatively, partly to bending mode of OH10, 12, respectively (Chang et al., 2009; Leypold et al., 2003; Parolo et al., 2010). Unlike the physical mixture, both MM/TC nanoclays showed the band at 1615 cm^{-1} shifted by 10 cm^{-1} to higher frequencies with respect to bulk TC accordingly to previous finding (Chang et al., 2009) but also in contrast with another study indicating a band shift to lower frequencies (Porubcan et al., 1977). The shift to higher frequencies might indicate a strong TC interaction within MM nanoplatelets involving TC amidic group, regardless of the reaction pH value. The absorption band at 1581 cm^{-1} was not changed in relation to the spectra of both MM/TC pH 3 nanoclay and physical mixture, whereas significantly decreased in intensity in the spectrum of MM/TC pH 8.7. In fact, at this alkaline pH value one of the two hydroxyl groups was deprotonated or electrostatically interacted with a counterion (Chang et al., 2009). Other authors explained this result by means of calcium ion bridge between negatively charged TC and negative sites on the clay surface (Chang et al., 2009). Both interactions with Na^{+} or Ca^{2+} counterions and Ca^{2+} bridge mechanism can be

excluded in MM/TC pH 8.7 nanoclay, being these elements absent as highlighted by EDX analysis.

The infrared region between 2800 cm^{-1} and 2400 cm^{-1} (Fig. 3b) exhibited the band at 2660 cm^{-1} associated to $–NHR_3^+$ group (Parolo et al., 2010). Unlike the physical mixture, the absorption band at 2660 cm^{-1} was absent in both MM/TC nanoclays indicating that TC $–NHR_3^+$ group was involved in the interaction, regardless of the reaction pH value. Therefore, TC positively charged groups are present also at the alkaline pH value of 8.7 demonstrating that the cation exchange mechanism may still occur and supporting the results obtained by EDX analysis.

To strengthen the above thesis, TC position within MM nanoplatelets was investigated by recording X-ray diffraction patterns from MM/TC nanoclays in comparison with those from bulk materials and their physical mixture (Fig. 4, Table 2). In MM diffractogram a broad primary 001 reflection was observed at 6.82° 2θ corresponding to a basal spacing *d* of 12.95 Å (Fig. 4a, Table 2) indicating a water monolayer in MM interlayer space (Parolo et al., 2008). Bulk TC diffractogram in the range between 3° and 10° 2θ showed a reflection at 8.80° 2θ with *d* of 10.10 Å (Fig. 4b, Table 2). MM basal spacing was unchanged in MM/TC physical mixture XRD pattern showing also a reflection at 8.75° 2θ with *d* of 10.09 Å attributable to crystalline TC (Fig. 4c, Table 2). Upon contact with TC water solution, MM primary reflection shifted to 4.74° 2θ and 4.60° 2θ, corresponding to increased basal spacing up to 18.64 Å and 19.23 Å, at pH 3 and pH 8.7, respectively (Fig. 4d and 4e, Table 2). This finding gives evidence of TC molecules intercalated within MM interlayer spaces, regardless of the reaction pH value. In addition, MM/TC pH 8.7 diffractogram showed a reflection at 8.05° 2θ (*d* = 10.98 Å) fairly attributable to the presence of a TC crystalline fraction.

The obtained results converge on the following mechanism of interaction. TC intercalation within MM interlayer space occurs *via* cationic exchange as the main reaction whether TC cationic species (pH 3) or those anionic (pH 8.7) predominate. In this latter case, the overall TC adsorption extent is lower and the drug also interacts partially by means of both physically deposition and weakly linkage onto MM surface as minor mechanism.

3.2. MM/TC nanoclay properties for a gastroretentive formulation

Since longer residence times of drugs in the stomach compared with normal gastric emptying times can be achieved by mucoadhesion properties provided by the dosage form, MM/TC nanoclays were examined for their mucoadhesiveness by studying their interaction with porcine mucin. The data obtained are listed in Table 3 in comparison with those obtained from chitosan as the positive control and glass as the negative control. No interaction was provided by the glass. Chitosan, known for its mucoadhesiveness, exhibited an excellent interaction, in agreement with literature data (Hassan and Gallo, 1990). In comparison, bulk MM and both MM/TC nanoclays generated weaker interaction forces, without significant differences among the samples ($P > 0.05$), but adequate to consider the samples mucoadhesive. Mucoadhesion of smectites was observed in a few previous studies (Dong and Feng, 2005), and it could be arisen from weak interactions such as van der Waals bonds on MM surface (Aguzzi et al., 2005; Baek et al., 2012).

TC desorption behavior from MM/TC nanoclays was investigated in simulated gastric media at two different pH values, pH 1.2 and pH 3.0 used as average pH values of the gastric lumen reproducing fasting and fed conditions, respectively. The pH values of

the gastric lumen characterize also the luminal side of the gastric mucus layer on which MM/TC nanoclays would play their function. In fact, the mucus gel layer exhibits a pH gradient from a low value on the luminal side to a pH approaching neutrality on the mucosal side (Allen and Garner, 1980). TC desorption profiles from MM/TC nanoclays are shown in Figure 5 compared with TC dissolution. TC dissolution is fast reaching 100% drug dissolved in a few minutes. Contrary to this, drug desorption from both the nanoclays involved a first burst phase followed by a sustained phase reaching a plateau within approximately 1h, regardless of the medium pH value. Higher TC percentage amounts were desorbed from MM/TC pH 8.7 nanoclay (about 40% in pH 1.2 medium and about 30% in pH 3.0 medium), respect to MM/TC pH 3 (about 25% in pH 1.2 medium and about 10% in pH 3.0 medium). This behavior could be related to the drug fraction physically deposited or weakly linked to MM surface in MM/TC pH 8.7 nanoclay. Moreover, higher TC percentage amounts were desorbed into pH 1.2 medium with respect to pH 3.0 medium from both the nanoclays. Such a difference should reasonably arise from the process of cationic exchange between the intercalated TC and H^+ ions present in the assay media, given the lack of influence from TC solubility difference in function of pH (Wu and Fassihi, 2005). In any case, TC located in MM interlayer cannot be exchanged completely probably due to the ion-exchange reaction reaching its equilibrium according to the available H^+ ions (Joshi et al., 2009). In support of this assumption, the addition of further H^+ ions, reproducing H^+ ions renewal occurring from the physiological gastric secretion, induced the desorption of an additional TC fraction.

4. Conclusions

Montmorillonite was modified by a cation exchange process with tetracycline producing intercalated nanoclay irrespective of the production pH value, i.e. of the fact that the antibiotic cationic form is the prevailing. The obtained nanoclays exhibited good mucoadhesion properties and drug desorption processes based on a cation exchange reaction occurring upon contact with acidic media. Therefore, they could represent an effective approach in a perspective of a potential Hp infection therapy implemented by a wide distribution of the drug adhering on the gastric mucosa capable of promoting antibiotic transport through the gastric mucus layer towards Hp colonization site.

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Table 1 Particle size, z-potential, and elemental composition of bulk montmorillonite (MM), before and after cation exchange by CsCl, MM/TC pH 3, and MM/TC pH 8.7 nanoclay samples. Mean values ± SD.

	MM	MM/TC pH 3	MM/TC pH 8.7
Mean diameter of the main class (μm)	1.06 ± 0.31	6.01 ± 1.50	6.33 ± 1.81
z-potential (mV)	-17.75 ± 1.20	-17.01 75 ± 0.91	-16.88 ± 1.30
Sodium weight (%)	1.27 ± 0.10	0.10 ± 0.03	0.03 ± 0.05
Calcium weight (%)	1.37 ± 0.08	0.14 ± 0.02	0.07 ± 0.01

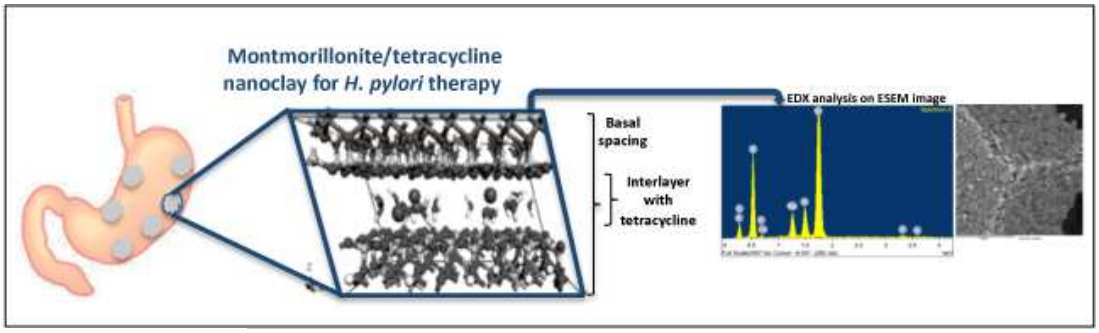
Carbon weight (%)	-	32.43 ± 0.90	40.50 ± 14.01
Cesium weight (%) following CsCl exchange	11.28 ± 0.40	-	-
Sodium weight (%) following CsCl exchange	0.00	-	-
Calcium weight (%) following CsCl exchange	0.78 ± 0.12	-	-

Table 2 Reflections and basal spacings (001) obtained by XRD analysis from MM/TC nanoclays, bulk materials and their physical mixture.

	2θ (°)	d (Å)
bulk MM	6.82	12.95
bulk TC	8.80	10.10
MM/TC physical mixture	6.95 8.75	12.71 10.09
MM/TC pH 3	4.74	18.64
MM/TC pH 8.7	4.60 8.05	19.23 10.98

Table 3 Adhesion forces of bulk MM, MM/TC nanoclays, chitosan (positive control), and glass plate (negative control) on porcine mucin. Mean values ± SD.

	Mucoadhesiveness (dine/cm^2)
bulk MM	2486 ± 170
MM/TC pH 3	2602 ± 112
MM/TC pH 8.7	2746± 65
Chitosan	6389 ± 388
Glass plate	0



Graphical abstract .