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# Organo-modified bentonite for gentamicin topical application: Interlayer structure and in vivo skin permeation

Valentina Iannuccelli, Eleonora Maretti, Alessia Bellini, Daniele Malferrari, Guido Ori, Monia Montorsi, Moreno Bondi, Eleonora Truzzi, Eliana Leo

## Highlights

An organo-modified raw bentonite was developed as novel antibacterial material.

Thermal reactions could support drug intercalation occurrence.

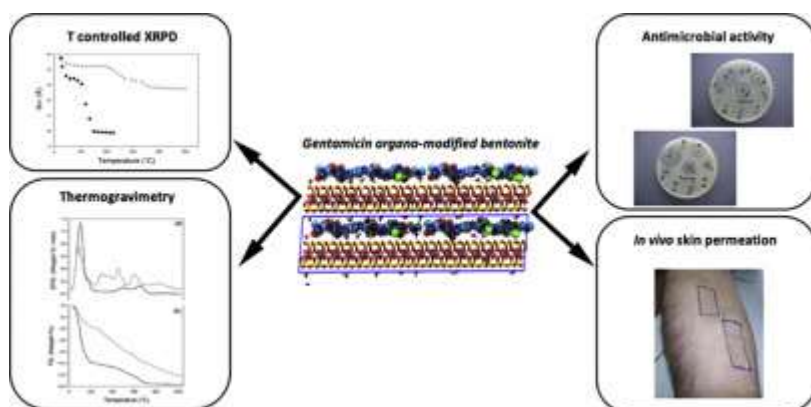
MD simulations showed gentamicin monolayer arrangement within Mnt interlayer.

*Trans*-epidermal route was favored by drug intercalation as arisen from *in vivo* data.

## Abstract

Recent [biomedical applications](#) of clay materials have included organically modified clays or clay minerals with the purpose of modifying and improving drug biological activity. The present research aims to explore the potential benefits provided by a raw [bentonite](#) (Bt) modified by gentamicin (GM) adsorbed within [montmorillonite interlayers](#) in the management of cutaneous infectious diseases. Information arisen from controlled X-ray powder diffraction, [thermogravimetry](#) coupled with evolved gas [mass spectrometry](#), and molecular dynamics simulations pointed out GM monolayer arrangement within montmorillonite framework without producing substantial effects on the layer periodicity. Concerning skin biomedical application, unlike the pure antibiotic permeating along the trans-follicular pathway across *stratum corneum*, the organo-modified Bt/GM would favor the trans-epidermal route along inter-cluster [corneocyte](#) region, as *in vivo* skin penetration studies by means of tape stripping test indicated. Based on the results obtained, GM [intercalation](#) could represent a potential advantageous approach allowing a long-term Bt/GM reservoir for sustained antibacterial activity.

## Graphical abstract



## Keywords

Bentonite, Gentamicin, Intercalation, Thermal analyses, Molecular dynamics, *In vivo* skin penetration

## 1. Introduction

There is a strong demand to identify new strategies in order to set optimal drug delivery systems for antibiotic treatments. [Intercalation](#) of organic molecules into layered inorganic solids provides a useful and convenient approach to prepare hybrids that show properties of both the inorganic host and organic guest in a single material ([Aguzzi et al., 2007](#); [Rodrigues et al., 2013](#)). In the last five decades the ability of both raw and synthetic [smectites](#) to exchange cations with several organic compounds has been exploited in many application fields. An archetypical example of such [versatility](#) is represented by the polymeric [nanocomposites](#) employing organo-modified [bentonites](#) ([Benelli et al., 2017](#); [Franchini et al., 2008](#); [2011](#); [Morgan and Wilkie, 2007](#)).

More recently, smectites have been proposed as materials for modulating drug delivery or improving dissolution of poorly water-soluble drugs ([Aguzzi et al., 2005](#); [Iannuccelli et al., 2015](#); [Joshi et al., 2009](#)). Among smectites, the 2:1 layered montmorillonite is probably the most investigated clay mineral. The reasons that drive this interest mainly arise from its high specific surface area, swelling and [adsorptive capacity](#), high cation exchange capacity (CEC), specific rheological properties, drug-carrying capability and ability to modulate drug release ([World Health Organization, 2005](#)). Montmorillonite is mainly used as auxiliary material in the [pharmaceutical industry](#) for oral or topical dosage forms, recorded in the United States, European, and British Pharmacopeias. Montmorillonite, following to its high [swelling behavior](#), can intercalate therapeutic compounds between the layers generating a host for oral or topical drug delivery ([Aguzzi et al., 2005](#); [Bello et al., 2015](#); [Bonina et al., 2007](#), p. 200; [de Paiva et al., 2008](#); [Forni et al., 1987](#); [Iannuccelli et al., 2015](#); [Iliescu et al., 2011](#); [Joshi et al., 2009](#); [Kant and Datta, 2016](#); [Katti et al., 2010](#); [Kim et al., 2016](#); [Mohamed et al., 2014](#); [Rapacz-Kmita et al., 2015](#)). Concerning topical use, montmorillonite has beneficial effects in dermatological and cosmetic applications (geotherapy, paleotherapy) ([Carretero, 2002](#); [López-Galindo et al., 2007](#)).

The present work focuses on the assessment of a raw bentonite (Bt), a montmorillonite rich clay recently characterized in a previous work ([Iannuccelli et al., 2016](#)), for the development of a novel gentamicin/clay hybrid material for the topical use. Gentamicin (GM) is an aminoglycoside antibiotic widely used in the treatment of severe infections, caused by many Gram-negative and Gram-positive bacteria, such as meningitis, nephritis, and post-operative infections. Although it presents a very broad spectrum of action, its use is limited to serious infections caused by Gram-negative bacteria because of its high toxicity. Gentamicin is commonly administered as injections, topical and ophthalmic dosage forms because of poor absorption following the oral administration. The well-known poor gastrointestinal membrane permeability and the consequent low [bioavailability](#) (class III of the biopharmaceutical classification system) are likely connected to the high polarity of this cationic compound. Various approaches have been investigated in order to increase GM oral bioavailability, including the co-administration of absorption-enhancing agents such as surfactants ([Hu et al., 2001](#); [Ito et al., 2005](#)), bile salts and glucosteroids ([Axelrod et al., 1998](#)), and liposaccharides ([Ross et al., 2004](#)). Although good gastrointestinal absorption enhancing effects were demonstrated, cytotoxicity and damage to the mucosa have been reported ([Aungst, 2000](#); [Ross et al., 2004](#); [Swenson et al., 1994](#)). Another strategy aiming to promote GM oral bioavailability could involve the use of microparticulate carriers to be taken up by the intestinal lymphoid tissue ([des Rieux et al., 2007](#); [Hussain et al., 2001](#); [Iannuccelli et al., 2011](#); [McClean et al., 1998](#); [Moyes et al., 2007](#)) or to be implanted for bone infection treatment also exploiting drug interaction with anionic polymers ([Iannuccelli et al., 1996, 2011](#)). Gentamicin is extensively used topically against severe microbial infections especially in burns and wounds ([Chang et al., 2006](#)), but also in the treatment of impetigo, infected bed sores, nasal staphylococcal carrier state, pyoderma, infections of the external eye, and adnexa ([Nishijima and Kurokawa, 2002](#)). Gentamicin applied to the skin has only a low systemic absorption due to the difficult penetration through the deep layers of the skin, related, probably, to its cationic nature; for this reason, its use is limited to the local effect that involves mainly the most superficial skin layers. Despite its benefits, GM short-life, bacterial

barriers and adverse effects such as nephrotoxicity, ototoxicity, and neurotoxicity upon prolonged use limit GM daily dosage (Roberts, 2007). In fact, many clinicians are reluctant to use it, even for a short term (Drusano, 2007). Efforts have been made to reduce toxicity associated with prolonged use by means of liposomes, micellar systems, hydrogels, microgels, or nanospheres (Ahangari et al., 2013; Ayhan and Ozkan, 2007; Changez et al., 2003; Eljarrat-Binstock et al., 2004; Jia et al., 2008; Nnamani et al., 2013; Sökmen et al., 2008; Umeyor et al., 2012). Local delivery of GM can solve the major disadvantages of the systemic administration by maintaining a high local antibiotic concentration for an extended time (Zalavras et al., 2004). Particularly, drug delivery systems exhibiting high initial release rate followed by a sustained release at an effective antibiotic concentration may allow local control of infection while minimizing side effects and preventing bacterial resistance (Aviv et al., 2007; Persson et al., 2006).

The preparation of a GM-based organo-modified bentonite (Bt/GM) may therefore represent a valuable alternative to assure safer and more effective utilization of GM for topical treatment. Based on these premises, the present research includes a thorough characterization of Bt/GM by means of several instrumental analyses as well as the comparison of the experimental results with Molecular Dynamics simulations (MD modeling) to provide a more detailed understanding about the interlayer arrangement and interactions promoted by the organic guest molecules confined in the montmorillonite framework. Moreover, GM antimicrobial activity, *in vitro* desorption, and *in vivo* skin permeation on human beings were assessed in the perspective of contribution to a novel antibiotic material.

## 2. Experimental part

### 2.1. Materials

A bentonite (Bt) of volcanic origin from Iglesias (Sardinia, Italy) deposit (average mineralogical composition from the producer's datasheet: montmorillonite 80%, quartz 13%, illite-kaolinite 5%, plagioclase 2%) was donated by Eurit srl (Colorobbia Group, Sovigliana Vinci, Italy). Gentamicin sulfate (GM, Fig. 1), composed of gentamicin C1 ( $C_{21}H_{43}N_5O_7 \cdot H_2SO_4$ , <45%), gentamicin C1a ( $C_{19}H_{39}N_5O_7 \cdot H_2SO_4$ , <35%), and gentamicin C2 ( $C_{20}H_{41}N_5O_7 \cdot H_2SO_4$ , <25%),  $pK_a = 12.55$  in acidic condition; 10.18 in basic condition, was purchased by Polichimica (Bologna, Italy). All the chemicals and reagents were of analytical grade (Sigma-Aldrich, Milan, Italy).

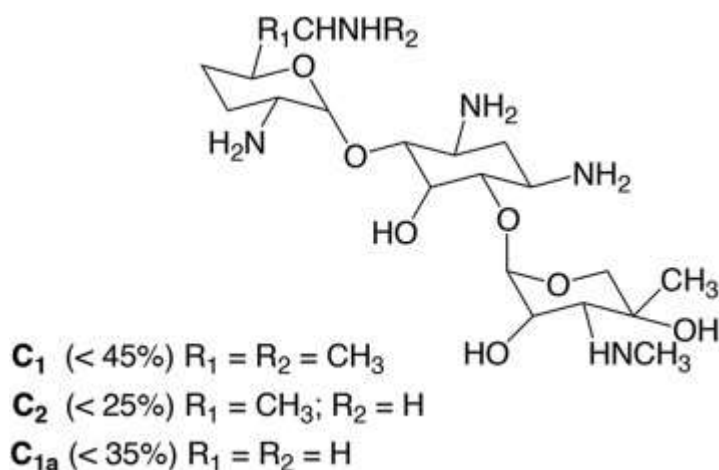


Fig. 1. Molecular structure of gentamicin sulfate.

### 2.2. Bt activation

Bt activation and thus the implementation of its organophilic behavior are provided by the saturation of the montmorillonite interlayers with a homogeneous cationic population through the cation exchange reaction

hereafter detailed. A defined amount of Bt was grinded by a vibratory [ball mill](#) (Fritsch GmbH, Idar-Oberstein, Germany) for 10 h to remove particle aggregates. Batches of dispersions were prepared mixing 1 g of milled Bt and 25 mL of NaCl 0.1 M and were shaken with a [magnetic stirrer](#) at room temperature for 24 h. The supernatant was centrifuged (mod. 4235, 188 ALC International, Milan, Italy) at 2115  $\times g$  for 20 min and the solid was twice subjected to the same treatment. The separated solids were washed several times with 35 mL of distilled water under magnetic stirring at room temperature for 4 h followed by centrifugation at 2115  $\times g$  for 2 h. The solid was dried under vacuum at room temperature and the supernatant analyzed for NaCl absence by [titration](#) with 0.1 M [silver nitrate](#) solution according to [U.S. Pharmacopeia](#). The [activation process](#) was carried out in triplicate.

### 2.3. Bt/GM preparation

Gentamicin was adsorbed onto both activated and non-activated Bt at constant drug concentration corresponding to about two times Bt CEC measured for activated Bt/GM (aBt/GM) and non-activated Bt/GM (Bt/GM), respectively. Glass tubes filled with 20 mL GM water solution (1 mg/mL) and 100 mg of milled Bt were horizontally shaken in the darkness for 24 h, a time suitable to fully saturate the montmorillonite interlayer with GM. The dispersions were centrifuged (2115  $\times g$ , 20 min) and solids washed twice with 35 mL [deionized water](#) under magnetic stirring for 15 min. The obtained organo-modified clays were dried under vacuum at room temperature and stored in the darkness. Three batches were prepared for each sample.

### 2.4. Gentamicin adsorption measurements

In this paper, the term “adsorption” was used to generally refer to the [immobilization](#) of GM onto Bt thus without distinguish between [intercalation](#) in the interlayer of montmorillonite and adsorption on the outer surface of montmorillonite and illite-kaolinite. However, when dealing with each single mineral phase the term adsorption and intercalation will be suitably used.

The amount of GM adsorbed onto Bt in both aBt/GM and Bt/GM was calculated as the difference between the initial GM concentration and that in the supernatants obtained during organo-modified clay preparation. GM was derivatized by reaction with o-phthalaldehyde of an aliquot of 1 mL from the supernatants according to Sampath and Robinson method ([Sampath and Robinson, 1990](#)) and determined spectrophotometrically (Lambda 3B, Perkin-Elmer, Norwalk, CT, USA) at 274 nm wavelength. The GM concentrations were expressed as drug/clay weight percentage as well as yield (actual/theoretical drug) percentage on three determinations from three different batches.

### 2.5. Size, surface charge, and pH value

Bt/GM particle size, [Polydispersity Index](#) (PDI), and Z-potential were determined on 10 mg/mL organo-modified [clay water](#) dispersion by using Photon Correlation [Spectroscopy](#) (PCS) (Zetasizer version 6.12, Malvern Instruments Ltd) equipped with a 4 mW He—Ne laser (633 nm) and a DTS software (Version 5.0) and compared with those of Bt. pH value of 2% Bt/GM water dispersion was measured by [potentiometry](#) immediately after the preparation of the dispersion and after 1 h; obtained data were compared with the value of Bt dispersion, according to U.S. Pharmacopeia monograph for bentonite, and with 0.1% (w/v) GM water solution. The reported values were averaged on three determinations from three different batches.

### 2.6. CHN elemental microanalysis

CHN elemental [microanalysis](#) (Elemental analyzer, mod. 1106, Carlo Erba, Milan, Italy) was performed on Bt/GM in comparison with bulk Bt and GM. The analysis was carried out in triplicate.

## 153 2.7. Elemental composition by EDX analysis

154 Clay elemental composition was determined by Energy Dispersive X-ray (EDX, Oxford INCA-350, FEI  
155 Company-Oxford Instruments, Oregon, USA) analysis coupled with an Environmental Scanning Electron  
156 Microscopy (ESEM, Quanta 200 Fei Company-Oxford Instruments). Elements can be identified qualitatively  
157 and semi-quantitatively in function of the X-ray energy emitted by their electrons transferring from a higher  
158 energy shell to a lower energy one. X-ray emission from  $K\alpha$  or  $K\beta$  levels of the atoms calcium, potassium,  
159 oxygen, sodium, magnesium, aluminum, silicon, and other elements with atomic numbers from 4 were  
160 recorded by the selected area method related to whole clay particles from samples mounted without a  
161 [conductive coating](#) on carbon stubs with the following experimental settings: low vacuum (0.70 Torr),  
162 [accelerating voltage](#) 12 kV, spot size 3, element detection limit  $\sim 0.05$  wt%, spatial resolution  $0.1\ \mu\text{m}$ , total  
163 spectrum counts  $>250,000$ , accuracy within  $\pm 5\%$  relative errors by reference to standards. [EDX spectra](#)  
164 representing the plots of X-ray counts (intensity) vs. energy peak (keV) of each element were acquired and  
165 semi-quantitative compositions, obtained by a standardless method of acquisition and expressed as  
166 relative weight percentage of each element, were calculated. The carbon peak at low energy level, related  
167 to the hydrocarbon contamination growing the carbon stub signal, was not considered ([Rolland et al.,](#)  
168 [2004](#)). The reported data were averaged on three determinations for each sample.

## 169 2.8. FT-IR measurements

170 FT-IR measurements were performed using a Perkin-Elmer FT-IR 1600 (abscissa accuracy of  $0.01\ \text{cm}^{-1}$  using  
171 HeNe laser reference; resolution from 2 to  $16\ \text{cm}^{-1}$ ; lithium [tantalite](#) temperature-stabilized detector) on  
172 bulk GM, bulk Bt, Bt/GM, and Bt/GM physical mixture at GM content corresponding to that of the organo-  
173 modified clay. The samples were maintained in a drier, dispersed in a Nujol mull (typically 2% w/w) and  
174 measured. Spectra were collected in air in the MID Infra-Red (mid-IR) region. The analyses were performed  
175 in triplicate.

## 176 2.9. X-ray powder diffraction

177 X-ray powder diffraction (XRD) was employed mainly to detect basal periodicity variation in  
178 montmorillonite before and after treating Bt with GM. The XRD patterns were recorded from (00 $l$ ) oriented  
179 mounts, in the temperature range from 25 to  $500\ ^\circ\text{C}$  with a [heating rate of](#)  $10\ ^\circ\text{C}/\text{min}$  using a PANalytical  
180 X'Pert PRO [diffractometer](#) equipped with X'Celerator detector. Before measurements all the samples were  
181 simultaneously equilibrated at the same environmental conditions. Experimental conditions were: Incident  
182 beam: monochromatic  $\text{Cu}\ K\alpha_1$  radiation ( $1.54060\ \text{\AA}$ ), 40 kV and 40 mA; filter, nickel; Soller slits,  $0.04\ \text{rad}$ ;  
183 anti-scatter mask, 20 mm; anti-scatter slit,  $1/4^\circ$ ; divergence slit,  $1/4^\circ$ . [Diffracted beam](#): X-ray detector,  
184 X'Celerator (Position Sensitive Detector, PSD); anti-scatter mask, 5.0 mm; Soller slits,  $0.04\ \text{rad}$ ; integration  
185 time, 20 s in continuous scanning (PSD length of  $2.12^\circ 2\theta$  corresponding to a step size of  $0.0170^\circ 2\theta$ ).  
186 [Diffraction patterns](#) were recorded from  $3$  to  $75^\circ (2\theta)$  at room temperature, and from  $3$  to  $20^\circ (2\theta)$  when  
187 measuring in non-ambient temperature conditions. NIST corundum was used as calibrating standard.

## 188 2.10. Thermogravimetric measurements coupled with evolved gas mass spectrometry

189 Thermogravimetric analyses were performed with a Seiko SSC 5200 thermal analyzer equipped with a  
190 [quadrupole mass spectrometer](#) (ESS, GeneSys Quadstar 422) to analyze gases evolved during [thermal](#)  
191 [reactions](#) (MSEGA). This device samples gases *via* an inert, fused silicon capillary system, heated to prevent  
192 gas condensation. Analyses of evolved gas phases were carried out in multiple ion detection mode (MID),  
193 which allows the qualitative determination of evolved masses vs. temperature or time. MID analyses were  
194 carried out measuring the  $m/z$  ratios 17 and 18 for  $\text{H}_2\text{O}$ , 28 and 44 for  $\text{CO}_2$ , 30 for NO and  $\text{NO}_2$ , 34 for  $\text{H}_2\text{S}$ ,  
195 46 for  $\text{NO}_2$ , and 48, 64, 66 for  $\text{SO}_2$ , where  $m/z$  is the ratio between the mass number and the charge of an  
196 ion; SEM (Secondary Electron Multiplier) and FARADAY detectors operating at 900 V were employed with  
197 1 s of integration time on each measured mass.



Measurements were performed on Bt, Bt/GM, and bulk GM air-dried samples at the following experimental conditions: heating rate: 10 °C/min for Bt and Bt/GM, and 20 °C/min for pure GM; heating range: 25–1000 °C (25–900 °C for pure GM); data measurement: every 0.5 s; purging gas: ultrapure helium, at a flow rate of 100 µL/min.

### 2.11. Differential scanning calorimetry

Bt/GM was subjected to thermal analysis on a Differential Scanning [Calorimeter](#) (DSC-4, Perkin-Elmer) and compared with GM and Bt/GM physical mixture at GM content corresponding to that of the organo-modified clay. The samples (6–7 mg) were accurately weighed in crimped aluminum pans and heated from 30 °C to 280 °C at a scanning rate of 10 °C/min under dry nitrogen flow (30 mL/min). All the thermograms were obtained in triplicate.

### 2.12. Molecular dynamics simulations

A  $X_{0.75}[Si_{7.75}Al_{0.25}][Al_{3.5}Mg_{0.5}]O_{20}(OH)_4$  (X = monovalent cation) ideal montmorillonite model was used for the present study. This model shows  $-0.75e$  per unit cell, leading to a CEC of 101.9 meq/100 g, a value close to the experimental one. The simulation box consists of one layer made of 64 unit-cells corresponding to a basal surface of 4.14 nm × 7.17 nm. In order to mimic the partial cation exchange process obtained in the experiments, we consider the total negative charge of the layer to be counterbalanced by ~79.2% by C-1(GM) molecules and ~20.8% of sodium ions (see Supporting information for details). A certain amount of water molecules was also inserted in the interlayer (corresponding to the 2% wt.). In order to accommodate the organic cations, the [interlayer distance](#) was pre-expanded to 2.5 nm and the C-1 and water molecules and Na ions were randomly inserted. An initial [energy minimization](#) and MD simulation in the NPT-ensemble of 1 ns (298 K, 1 atm, time step 1 fs) have been used to optimize the interlayer region reaching an equilibrium density. Different initial starting configurations have been tested (in terms of organic, water, and sodium ions placement) and in all cases for a given system they converge to similar final arrangements and layer-to-layer distances. Then, the ones with the lowest energy have been further simulated for 1 ns in the NVT-ensemble. The last 0.5 ns have been used for the data analyses in terms of density profiles, pair correlation functions, ion coordinations. In all the simulations, both the atoms of the montmorillonite layer as well as the atoms confined in the interlayer were allowed to move and the periodic boundary conditions were applied. MD simulations were performed using the Discover module of the Materials Studio package (v. 5.0, Accelrys Inc.).

### 2.13. Antimicrobial activity

A microbiological agar well diffusion method was performed ([Giamarellou et al., 1975](#)) on Bt/GM in comparison with the respective physical mixture as well as pure GM and Bt. Tryptic soy agar (15 mL) and [Staphylococcus aureus](#) (*S. aureus*) strain ATCC 6538 or [Pseudomonas aeruginosa](#) (*P. aeruginosa*) strain ATCC 27853 ( $10^5$  CFU/mL) were used as growth medium and indicator [microorganisms](#), respectively. The wells in [agar plates](#) were filled with the samples in water dispersions (5 µg/mL, 100 µL). The glass plates were incubated at 37 °C overnight and zone inhibition diameters determined and related to GM concentration of standard water solutions. The analyses were made in triplicate.

### 2.14. In vitro gentamicin desorption

Gentamicin dissolution and desorption from the organo-modified clay 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 phosphate buffer solutions (pH 5.4 or 7.4 according to the European Pharmacopeia) at a temperature of  $37.0 \pm 0.5$  °C under a flow rate of 25 mL/min. The dissolved or desorbed concentration of drug was determined spectrophotometrically (Lambda 3B, Perkin-Elmer) following

242 derivatization according to Sampath and Robinson ([Sampath and Robinson, 1990](#)) at fixed time intervals for  
243 3 h. The reported data were averaged on three determinations.

## 244 2.15. *In vivo* skin permeation

245 *In vivo* skin [permeation](#) study was carried out following the application of Bt/GM, Bt, and GM, mechanically  
246 mixed into petroleum jelly immediately before skin application, on both the two shaved volar forearms and  
247 forehead of 3 healthy Caucasian volunteers (2 females and 1 male, aged 20–62), free of any dermatological  
248 disorder after obtaining informed consent for the experimentation following the recommended guidelines  
249 as set out in the Declaration of Helsinki. A delineated area of 2 × 5 cm received 200 mg of each formulation  
250 containing pure GM (4 mg), Bt/GM (65 mg) both corresponding to GM dose of 0.4 mg/cm<sup>2</sup>, or pure Bt  
251 (65 mg). The samples were homogeneously distributed by means of rubber gloves. After an application  
252 time of 60 min, which had been found to have a predictive value for penetration resulting from longer  
253 times of application ([Howes et al., 1996](#)), the *stratum corneum* (SC) was stripped twelve times by using an  
254 [adhesive tape](#) (Scotch Film Tape 600-3M). This number of stripped tapes is considered proper by Food and  
255 Drug Administration bioequivalence guidelines ([Shah et al., 1998](#)). The tapes were applied to the skin with a  
256 constant pressure by a 500 g roller. The first stripped tape was not considered in the penetration study  
257 because it represents unabsorbed materials. Twelve tapes stripped from SC that has received pure  
258 petrolatum jelly as well as from untreated SC were also assayed as controls. The tapes were combined into  
259 2 groups (group 1: tapes 2–6; group 2: tapes 7–12) in order to increase determination sensitivity and  
260 subjected to an extraction procedure by [isopropyl alcohol](#) to determine GM according to the method  
261 described above. Data were expressed in penetrated GM percentage of the applied dose. A further tape-  
262 stripping test was conducted on the volunteers under the same conditions after a resting period of 14 days.  
263 Tapes n. 2, 6, and 12 were assayed by Energy Dispersive X-ray (EDX) analysis coupled with an Environmental  
264 Scanning Electron Microscopy (ESEM) using the selected area method. An area of 1.25 cm<sup>2</sup> of each tape  
265 was cut from the center of the tape, mounted without conductive coating on a carbon stub. X-ray emission  
266 from K $\alpha$  and K $\beta$  levels of the atoms carbon, oxygen, aluminum, silicon, and sulfur were registered under the  
267 experimental setting described above. EDX spectra representing the plots of X-ray counts vs. elements and  
268 semi-quantitative results expressed as relative weight percentage of the elements present in the specimen  
269 were obtained. The reported data were averaged on the results obtained from the volunteers.

## 270 2.16. Statistical analysis

271 Data obtained were evaluated from a statistical point of view using ANOVA one-way. Differences at *p*-  
272 values < .05 were considered significant.

## 273 3. Results and discussion

274 Adsorption of GM onto [Bt](#) is essentially driven by the CEC of the clay that is related to isomorphous  
275 substitution in octahedral and tetrahedral sheets of [montmorillonite](#). To modulate drug [bioavailability](#)  
276 through interactions with bentonite (Bt), the cationic gentamicin (GM) was selected. Each of the three  
277 major components of GM complex (C1, C1a, C2) contains five basic amino functions exhibiting change in  
278 [protonation](#) state of the amino groups as function of pH. At the acidic pH value of Bt/GM preparation, GM  
279 molecules carry almost fully protonated charges (+5 and +4) ([Lesniak et al., 2003](#)) that are appropriate for  
280 efficacious cation exchange on montmorillonite. The Bt selected to develop Bt/GM organo-modified clay  
281 was previously characterized providing information on mineralogical and physico-chemical features  
282 ([Iannuccelli et al., 2016](#)). Gentamicin capacity to be intercalated in a commercial montmorillonite to be  
283 used as general drug carrier has been demonstrated by [Rapacz-Kmita et al. \(2015, 2017\)](#). In the present  
284 work, more extensive knowledge about the specific interactions between GM and Bt was supplied  
285 providing information on the arrangement of the guest molecule in the montmorillonite [interlayer](#) as well  
286 as the potentiality of a topical application.



### 3.1. Interlayer structure

The first step of the research was to verify the usefulness of Bt activation procedure. Clay activation did not offer advantages in terms of GM adsorption extent (aBt/GM =  $4.49 \pm 0.56\%$ , yield% =  $25.13 \pm 0.62$ ; Bt/GM =  $7.16 \pm 0.91\%$ , yield% =  $35.29 \pm 0.51$ ). Therefore, the study was performed only on the non-activated Bt/GM.

Bentonite treatment with GM generated an increase of clay average particle size ( $p > .05$ ) (Table 1) though remaining in the size range considered proper for several dermatological and cosmetic purposes (Lein and Oussoren, 2015). Both Bt and Bt/GM exhibited negative surface charge with a greater magnitude in Bt compared to Bt/GM ( $p < .05$ ) (Table 1). Bentonite net surface charge is mainly due to montmorillonite pH-independent permanent structural charges accounting for 90–95% of the total charges (Au and Leong, 2016; Pecini and Avena, 2013). Furthermore, montmorillonite is characterized by a surface charge due to the hydrolysis of Si—O and Al—OH bonds on the external surfaces of tetrahedral sheets as well as along the edges. Consequently, the lower Bt/GM Z-potential in absolute value is probably ascribable to GM interactions with the edges of clay particles and/or acidic pH medium of GM water solution (Delgado et al., 1986; Furukawa et al., 2009). Upon contact with water, Bt provided alkaline dispersions (pH of about 9, Table 1), in agreement with the values required by both U.S. and European Pharmacopeias. Alkalinity, that remained unchanged after 1 h, is generated by quick diffusion from the interlayer surfaces of exchangeable  $\text{Na}^+$  ions retained by electrostatic attraction. Conversely, Bt/GM water dispersions exhibited pH values consistent with those of pure GM suggesting the occurred exchange process between montmorillonite interlayer cations and GM.

**Table 1**  
Size, polydispersity index (PDI), surface charge, and pH values of Bt, GM, and Bt/GM. Mean values  $\pm$  SD.

Sample	Z-average ( $\mu\text{m}$ ) $\pm$ SD	PDI	Z-potential (mV) $\pm$ SD	pH value
GM	—	—	—	$4.52 \pm 0.05$
Bt	$3.64 \pm 0.98$	$0.42 \pm 0.15$	$-28.40 \pm 0.98$	$9.43 \pm 0.48$ (time = 0) $9.30 \pm 0.07$ (time = 1 h)
Bt/GM	$6.82 \pm 0.75$	$0.62 \pm 0.12$	$-17.60 \pm 1.30$	$4.66 \pm 0.13$ (time = 0) $4.91 \pm 0.10$ (time = 1 h)

In order to define the arrangement of GM molecules within montmorillonite lattice and elucidate the interaction mechanism occurring in Bt/GM, a suite of analyses was performed.

Elemental CHN analysis carried out on Bt/GM showed a C/N ratio in nice agreement ( $p < .05$ ) with that of GM alone (Table 2) and therefore evidenced that the drug was present in the organo-modified clay.

**Table 2**  
CHN microanalysis and C/N ratios of Bt, GM, and Bt/GM. Mean values  $\pm$  SD.

Sample	C%	H%	N%	C/N
Bt	—	$0.90 \pm 0.21$	—	—
GM	$51.80 \pm 1.05$	$8.91 \pm 0.52$	$15.12 \pm 0.83$	$3.43 \pm 0.11$
Bt/GM	$2.28 \pm 0.20$	$1.26 \pm 0.23$	$0.63 \pm 0.005$	$3.62 \pm 0.30$

The occurrence of a possible exchange process between GM and the exchangeable cations of montmorillonite as a preliminary evidence of GM intercalation was provided by Energy Dispersive X-ray (EDX) analysis carried out on Bt/GM compared with Bt. The identification and relative quantification analysis (Table 3, see Fig. S1) showed the Si and Al elements of tetrahedral and octahedral sheets, respectively, as well as Mg due to Al partial isomorphous substitution. Isomorphous substitutions in the octahedral sheets create an excess of negative structural charge within the lattice that is balanced by inorganic cations (mainly  $\text{Na}^+$  and  $\text{Ca}^{2+}$ ). A significant ( $p < .05$ ) less abundance of  $\text{Ca}^{2+}$  ions in Bt/GM compared with those of Bt suggests the almost complete substitution of  $\text{Ca}^{2+}$  ions by the cationic form of GM and, consequently, a possible drug arrangement within montmorillonite interlayers. The detection of a

minimal content of S in the mixture suggests, however, a non-negligible intercalation of GM without losing the [sulfate](#) group.

**Table 3**

EDX semi-quantitative analysis of the elements present in Bt and Bt/GM samples. All data are expressed as relative percentage of the elements > 0.5%. Mean values  $\pm$  SD.

	Bt	Bt/GM
O	32.25 $\pm$ 3.24	59.48 $\pm$ 0.63
Na	1.13 $\pm$ 0.73	0.90 $\pm$ 0.25
Mg	0.79 $\pm$ 0.59	0.71 $\pm$ 0.20
Al	11.01 $\pm$ 0.76	7.32 $\pm$ 0.23
Si	46.92 $\pm$ 2.42	27.99 $\pm$ 0.45
S	–	0.26 $\pm$ 0.20
K	2.38 $\pm$ 0.60	1.47 $\pm$ 0.17
Ca	2.30 $\pm$ 0.59	0.44 $\pm$ 0.16
Fe	3.22 $\pm$ 0.86	1.43 $\pm$ 0.27

324

Nonetheless, it has to be considered that GM could interact also with additional negative polar sites at the broken edges as well as by exposed hydroxyl end-groups on the terminated planes.

Another preliminary evidence was provided by mid-IR spectroscopy (see Fig. S2). The most relevant feature of the mid-IR spectra is the band at about 3400  $\text{cm}^{-1}$ , related to the overlapping of asymmetric  $\nu_3$  and symmetric  $\nu_1(\text{H} \cdots \text{O} \cdots \text{H})$  stretching vibrations of water bound by [hydrogen bonds](#) in the interlayer ([Farmer, 1974](#)). Unlike in Bt and Bt/GM physical mixture, the band position in the Bt/GM shift to a lower value, suggesting a decrease of the coordinated water amount induced by the substitution of the exchangeable cations of montmorillonite by GM. This conclusion is supported by the shift of the shoulder at about 3250  $\text{cm}^{-1}$  related to the [overtone](#) ( $2\nu_2$ ) of the bending mode of the solvated water ([Farmer, 1974](#)) and by the positions of the [absorption bands](#) at about 1635  $\text{cm}^{-1}$  attributable to  $\nu_2(\text{H} \cdots \text{O} \cdots \text{H})$  [bending vibration](#). The position of this band is the same in Bt and Bt/GM physical mixture ( $\sim 1637 \text{ cm}^{-1}$ ), it is instead moved to a lower value ( $\sim 1628 \text{ cm}^{-1}$ ) in the Bt/GM suggesting a decrease of the coordinated water amount as demonstrated also by [Madejová et al. \(2002\)](#) in samples where the amount of water has been decreased. Other features of the mid-IR spectra can be related both to montmorillonite and to other mineralogical phases as well ([Iannuccelli et al., 2016](#)). More in detail we refer to the bands at about ([Madejová and Komadel, 2001](#)): i) 3692 and 3620  $\text{cm}^{-1}$  related to OH stretching of structural hydroxyl groups of kaolinite and montmorillonite, respectively; ii) 916  $\text{cm}^{-1}$ , a band related to  $(\text{Al}(\text{OH}))$  bending in montmorillonite; iii) 1100, 795 and 690  $\text{cm}^{-1}$  related to the Si–O vibration of quartz; iv) 1030  $\text{cm}^{-1}$  related to Si–O stretching vibration in montmorillonite. These features, however do not significantly change in Bt, Bt/GM and Bt/GM physical mixture suggesting that they are unaffected by the presence of the antibiotic.

The main technique for detecting structural variations such as changes in the basal periodicity of montmorillonite, is obtained by [XRD](#). The comparison between XRD pattern of Bt and Bt/GM did not highlight considerable differences except for a minor reduction of  $d_{001}$  value in Bt/GM montmorillonite ( $d_{001} = 14.19 \text{ Å}$ ) respect to Bt montmorillonite ( $d_{001} = 14.95 \text{ Å}$ ). Hence, temperature controlled XRD analysis was performed to acquire more information about GM arrangement within montmorillonite framework. In fact, in montmorillonite the thickness of the interlayer depends greatly on the type of occupying molecules (e.g., cations, organic molecules, etc.) and on the amount of solvating water. Conversely, by measuring the variation of the distances between the stacked layers along the  $c$  axis (*i.e.*, the  $d$  values of the (001) reflections) as a function of temperature, information can be acquired on the thermal stability of the molecules occupying the interlayer.

[Fig. 2](#) compares the trends of  $d_{001}$  reflections of montmorillonite in Bt and Bt/GM samples. The most significant data of the comparison is the persistence of the periodicity of Bt/GM montmorillonite despite

the rapid decrease observed for Bt montmorillonite. In fact, at 150 °C Bt montmorillonite interlayer exhibited the typical value of the completely dehydrated interlayer ( $d_{001} = \sim 10 \text{ \AA}$ ), and this value did not change until the occurring of framework collapse at about 600 °C. Conversely, the reduction of the interlayer spacing in Bt/GM montmorillonite only starts after 210 °C. Thermal analyses here after reported, in nice agreement with these finding, will better highlight that at about this temperature begins the [thermal decomposition](#) of the antibiotic.

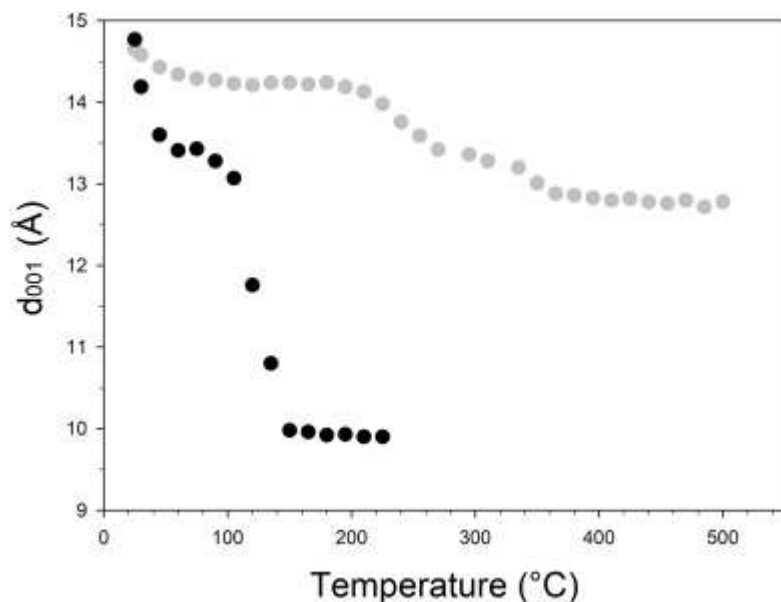
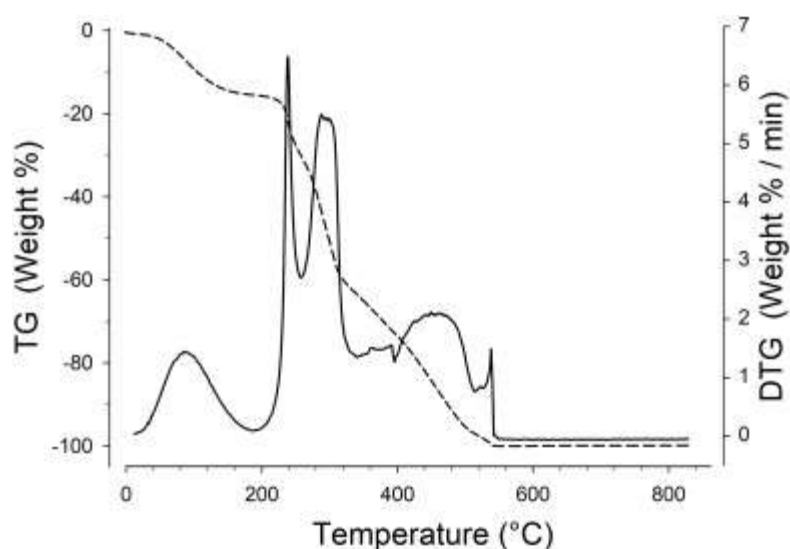


Fig. 2. Plot of  $d_{001}$  values for Bt (black circle) and Bt/GM (gray circle) [montmorillonite](#) as a function of temperature. The position of each (001) peak has been determined at the mid-height of the reflection, through the use of the software X-Pert High Score Plus. The error of the measurement falls within the dimensions of the used symbol.

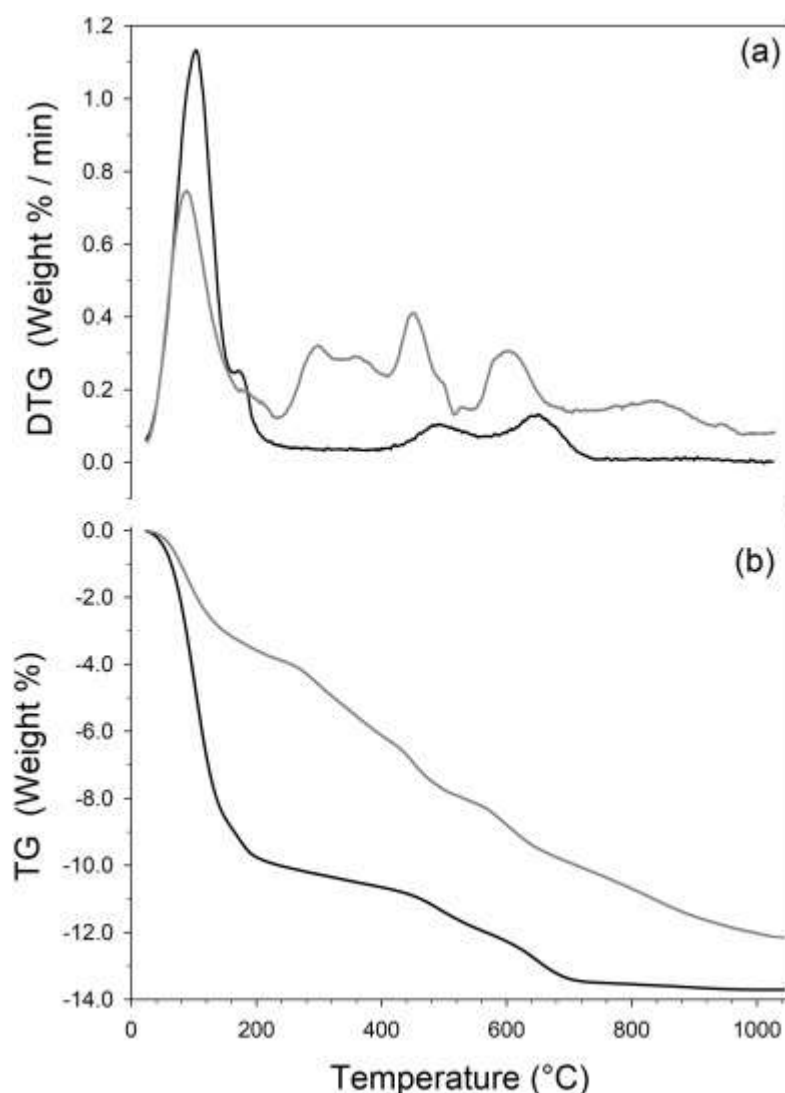
Thermogravimetric (TG) and its first derivative (DTG) curves for GM are shown in [Fig. 3](#). Five main reactions, with maxima at 80, 260, 317, 500, and 582 °C (DTG curve), lead to a nearly complete thermal decomposition of the antibiotic. The weight loss with maximum at 80 °C (mass loss of 14.7%, TG curve) is related to the removal of free water molecules, whereas reactions at higher temperature are related to the thermal decomposition of both the organic fraction and the [sulfate group](#). The asymmetry and/or band-like shape of the DTG peaks, in particular for the thermal event that occurs at  $T > 200 \text{ °C}$ , maybe related to the different forms (C1, C1a, and C2) of GM that thermally decomposed at significantly different temperature values.



376

377 Fig. 3. TG (dashed line) and DTG curves (solid line) of GM.

378 The [thermal behaviors](#) of Bt and Bt/GM are compared in [Fig. 4](#). The DTG curve ([Fig. 4a](#)) of Bt shows four  
 379 [thermal reactions](#) with maxima at 103, 165, 485 and 645 °C. The first two reactions occurred between 25  
 380 and 210 °C and are attributed to the dehydration of montmorillonite with the removal of two water layers  
 381 differently bound to the interlayer cation (maxima at 103 and 165 °C with mass losses of 9.0 and 0.84%,  
 382 respectively - [Fig. 4b](#)). This finding is in agreement with the presence of [divalent cations](#) ( $\text{Ca}^{2+}$ ) as major  
 383 interlayer species ([Iannuccelli et al., 2016](#); [Mackenzie, 1970](#)) and with XRD data ([Fig. 2](#)). The two reactions  
 384 at higher temperature are ascribable to the dehydroxilation of the octahedral sheets of kaolinite (maximum  
 385 at 485 °C, mass loss 1.2%) and montmorillonite (maximum at 645 °C, mass loss 1.9%). Differential thermal  
 386 analyses (not reported) additionally evidenced an [endothermic reaction](#) with maximum at 573 °C related to  
 387 the transition from the trigonal  $\alpha$  to the hexagonal  $\beta$  form of quartz ([Iannuccelli et al., 2016](#)).



388

389 Fig. 4. DTG (a) and TG (b) curves of [Bt](#) (black line) and Bt/GM (gray line).

390 The TG and DTG curves of Bt/GM nearly parallel those of Bt, but with some major differences, arising from  
 391 the presence of GM, that are: i) a drastic reduction of the mass loss related to the removal of hydration  
 392 water (reactions between 25 and 235 °C, mass loss 3.85%); ii) two additional thermal events with maxima  
 393 at 298 and 360 °C (reactions between 220 and 405 °C, mass loss 2.30%); iii) a shift toward lower  
 394 temperature values of the two dehydroxylation reactions; iv) an additional thermal events with maximum at  
 395 about 830 °C.

396 The lower amount of water in Bt/GM compared to Bt may explain the small difference in layer periodicity  
 397 of montmorillonite measured by XRD at room temperature. The two reactions between 220 and 405 °C  
 398 occur nearly in the same temperature of those observed for pure GM, however with a lower decomposition  
 399 rate (DTG curve). As already pointed out, it is not negligible that the reaction at temperature at which starts  
 400 the first of the two reactions (220 °C) is about the same at which begins the reduction of layer periodicity  
 401 ([Fig. 2](#)). This finding may be thus definitively attributed to the beginning of the thermal decomposition of  
 402 the intercalated antibiotic, as moreover suggested by MS-EGA curves.

403 The thermal decomposition of pure GM is complete at about 600 °C ([Fig. 3](#)); however, TG curves indicated  
 404 that the intercalated antibiotic follows a different thermal path. More in detail, MS-EGA curves (see [Fig. S3](#))  
 405 indicated that the two just mentioned reactions lead to the release of H<sub>2</sub>O (m/z = 18), NO (m/z = 30), and  
 406 CO<sub>2</sub> ([Fig. S3](#)), further supporting the intercalation of GM. The detection of a MS-EGA signal for SO<sub>2</sub>

407 highlights, however, the presence of marginal content of GM in its sulfate form, in accordance with the  
408 elemental analysis. XRD measurements showed that the layer periodicity of Bt/GM montmorillonite after  
409 the reaction with maximum at 360 °C was about 13 Å, higher than that in Bt montmorillonite (*i.e.*, 9.9 Å). It  
410 may be concluded that after the two thermal reactions occurring between 220 and 405 °C, a pillar-like  
411 residue, that thermally decomposes at higher temperature, forms in the interlayer of montmorillonite.  
412 However, as above mentioned, all these high temperature reactions (included the modification of the  
413 temperature at which occurred dehydroxylation) have not been taken into account in the present work, as  
414 they are related to the [thermal evolution](#) of the already decomposed antibiotic and mineral. Nevertheless,  
415 they well support the hypothesis of intercalation.

416 Further evidence supporting the intercalation of gentamicin is provided by [DSC](#) measurements that showed  
417 the absence of GM endothermic reaction in Bt/GM. On the opposite, this reaction is visible when DSC  
418 curves are collected for pure GM and physical mixture of Bt and GM.

419 Comparison of experimental measurements with MD simulations provided a more detailed understanding  
420 of the arrangement and bonding promoted by the GM molecules intercalated in montmorillonite. A  
421 computational approach attempted to simulate the interactions in the inter-structure of an ideal model of  
422 montmorillonite ( $X_{0.75}[Si_{7.75}Al_{0.25}][Al_{3.5}Mg_{0.5}]O_{20}(OH)_4$  which corresponds to a CEC of 101.9 meq/100 g),  
423 gentamicin molecules (using C1 as a molecule model), sodium ions and [solvent molecules](#) ( $H_2O$ ).  
424 Gentamicin components present both hydroxyl and amino groups and this affects the total positive charge  
425 (from 1 to 5 possible positive charge) of each molecule depending on the pH of the solution ([Lesniak et al.,](#)  
426 [2003](#)). In this study the system was simulated considering a pH ~ 5 [aqueous environment](#) corresponding to  
427 the preparation pH value at which GM molecules carry almost fully protonated charges (~85% of +5 and  
428 ~15% of +4), taking into account the distribution diagram of the different protonated species of component  
429 C1. This was achieved by inserting into the interlayer ~78.9% of molecules with +5 and ~21.1% molecules  
430 with 4+ charges and a certain content of water molecules (~2 wt%). Plus, in order to mimic the partial  
431 exchange process obtained experimentally a certain amount of sodium ions were also inserted. The  
432 simulated interlayer is made of ~79.2% of GM and ~20.8% of sodium ions with respect to the theoretical  
433 CEC. In this situation, the content of GM molecules useful to balance the negative charge of  
434 montmorillonite corresponds to an organic content of ~7.7% by weight.

435 Modeling results clearly show that Bt/GM is characterized by a monolayer arrangement of the GM (C1)  
436 molecules with a resulting layering periodicity of  $14.2 \pm 0.3$  Å, a value in fair agreement with XRD finding  
437 (14.19 Å). The side and top views of a typical configuration of Bt/GM hybrid system optimized by simulation  
438 are shown in [Fig. 5a](#) and b. The analyses of density profiles show clear peaks for all the intercalated species.  
439 On the basis of [radial distribution functions](#), the interatomic distance between the individual components  
440 of the sub-networks was further assessed. In particular, the ammonium H atoms of the GM molecules  
441 resulted located within the 2.1–2.9 Å range from the oxygen atoms of the montmorillonite surfaces, a  
442 distance slightly larger than those found in alkyl ammonium ions-based [organoclays](#) (1.8–2.5 Å) ([Liu et al.,](#)  
443 [2007](#)). This difference can be ascribed to the larger molecular flexibility of the alkyl [ammonium cation](#) due  
444 to its alkyl chain with respect to the more rigid and sterically hindered nature of the cyclic GM (C1)  
445 molecules. Both Na ions and the ammonium H atoms promote strong interactions with the oxygen atoms  
446 of the tetrahedral sheets ([Fig. 5c](#) and d), being mostly located above the surface [siloxane](#) rings (*i.e.* six-  
447 member rings). Water molecules partially affect such interactions by competing with the oxygen atoms of  
448 the tetrahedral sheets in promoting H-bonding with GM (C1) ammonium H atoms and hydroxyl groups and  
449 solvating the inner- and outer-sphere Na complexes ([Brigatti et al., 2011](#)).



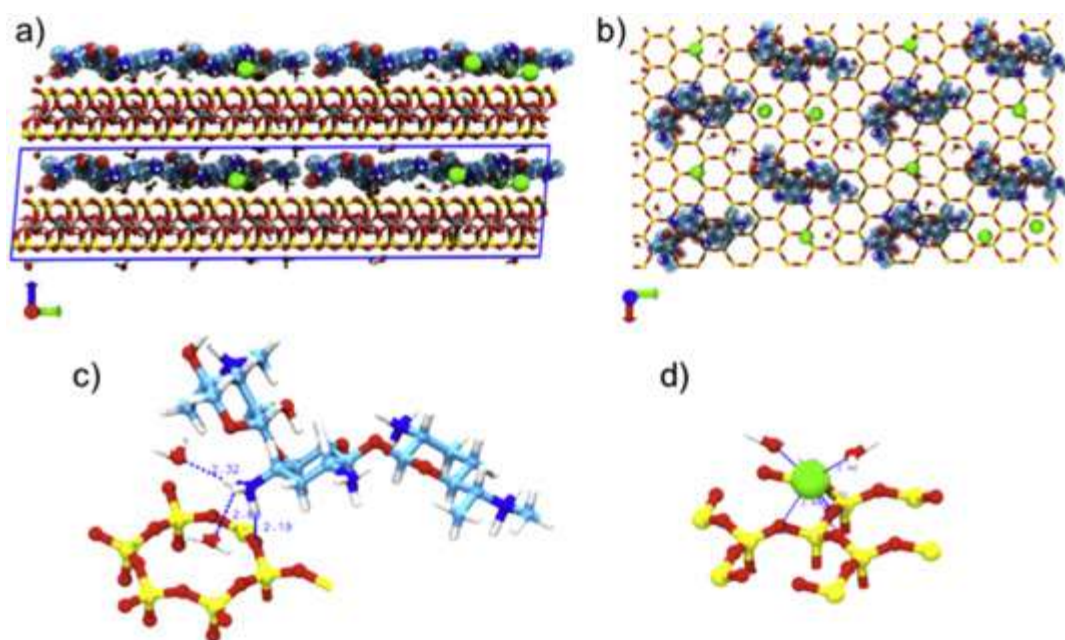


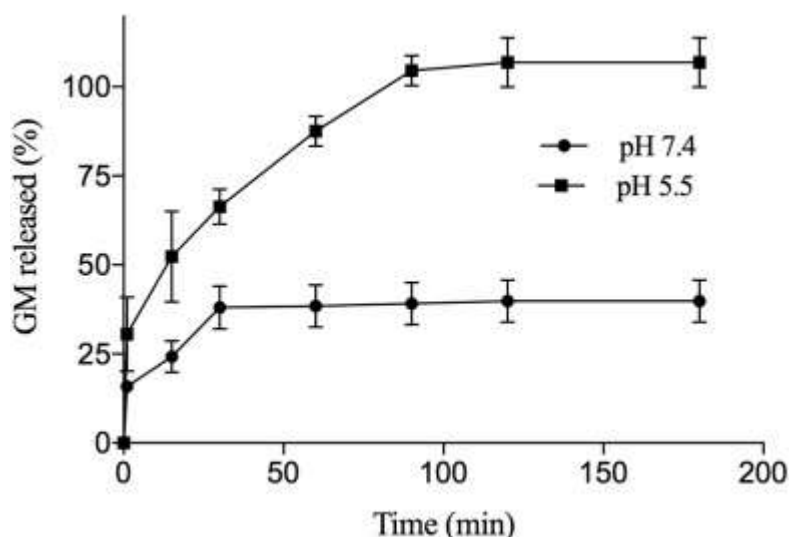
Fig. 5. a) Side view of [montmorillonite](#) in the Bt/GM hybrid system optimized by MD modeling. The simulated [supercell](#) is shown within the blue line and it is replicated twice along the z direction for clearness. b) Top view of the local arrangement of GM and water molecules and Na ions within the montmorillonite [interlayer](#). c) Local environment around the GM ammonium group where the interaction between its [hydrogen atoms](#) with the oxygen of the tetrahedral sheets and the oxygen of water molecules are highlighted with a blue dashed line. d) Local coordination environment of a Na ion located on top of tetrahedral Al (Si substitution, charged site). For clearness, only the bottom [siloxane](#) sheet surface is shown. Color legend: Si atoms, yellow; O atoms, red; H atoms, white; C atoms, cyan; N atoms, blue; Na ions, green. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

### 3.2. Antibacterial activity and *in vivo* skin permeation

In a perspective of Bt/GM use as cutaneous drug delivery system for local or systemic control of infections, the study involved the evaluation of GM biological activity within the organo-modified Bt, *in vitro* desorption, and *in vivo* skin [permeation](#) on human beings. The biological activity of GM within Bt was assessed to point out a possible synergistic clay action. In fact, clay minerals, in particular smectites, have [antimicrobial activity](#) due to their high absorbing/adsorbing power with respect to water forming unfavorable hydrophobic conditions for the growth of [microorganisms](#) (Kim et al., 2016; Williams and Haydel, 2010). Antimicrobial activity of Bt/GM was determined on *S. aureus* or *P. aeruginosa* and compared with that of pure GM, pure Bt and the physical mixture of the antibiotic with the clay in the same ratio as Bt/GM.

The results obtained highlighted the absence of antimicrobial activity of the pure Bt and an almost complete activity of the physical mixture compared with pure GM ( $96.59 \pm 5.68\%$  against *S. aureus*,  $88.01 \pm 2.90\%$  against *P. aeruginosa*). On the other hand, Bt/GM exhibited an activity against both the strains less than those obtained for GM solutions having the same antibiotic concentration ( $50.37 \pm 1.59\%$  against *S. aureus*,  $50.07 \pm 1.88\%$  against *P. aeruginosa*). It is reasonable therefore to suppose that GM, though maintaining antibacterial activity against both Gram-positive and Gram-negative bacteria, desorbs from Bt/GM within the culture media of the [agar plate](#) incompletely indicating that drug intercalation between montmorillonite interlayer reduces drug availability (Ambrogi et al., 2017).

480 Nevertheless, complete GM desorption from Bt/GM was obtained at pH 5.5 buffer solution mimicking the  
 481 acidic environment of skin surface (Fig. 6). Unlike the pure GM dissolving within 1 min regardless of the pH  
 482 value, drug desorption profile involved a burst phase corresponding to about 40% of GM adsorbed onto Bt  
 483 followed by a sustained phase reaching 100% drug delivered in about 2 h. Conversely, following a burst  
 484 phase in which about 15% GM diffused into the medium, drug desorption at pH 7.4 was incomplete  
 485 reaching a plateau corresponding to about 50% of GM payload after about 30 min. Burst phases could be  
 486 related to GM fraction deposited or weakly linked to Bt particle surface whereas the subsequent phases to  
 487 an exchange process between the intercalated GM and the cations present in the media reaching  
 488 equilibrium according to the available cations (Joshi et al., 2009).

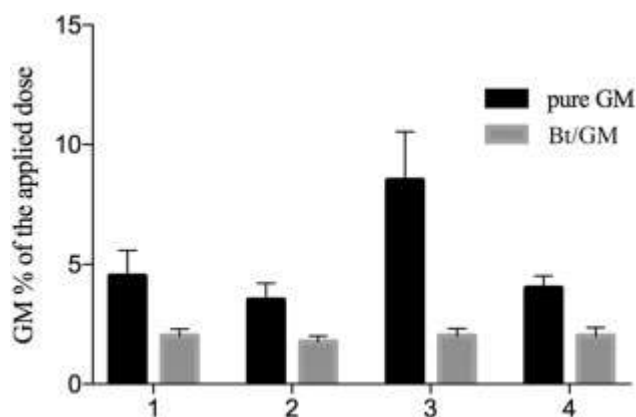


489  
 490 Fig. 6. Profiles of gentamicin desorption from Bt/GM in pH 5.5 and pH 7.4 media.

491 The higher GM percentage amounts desorbed at pH 5.5 with respect to pH 7.4 may reasonably arise from a  
 492 competition by H<sup>+</sup> ions present in the [assay medium](#) on the same interaction sites of GM ([Iannuccelli et al., 2015](#);  
 493 [Joshi et al., 2009](#)). In biological fluids, physiological counter-ions can displace differently the drug  
 494 from the substrate and deliver it into the body ([Aguzzi et al., 2007](#)).

495 The understanding of the mechanism by which insoluble particles can cross the *stratum corneum* (SC) is  
 496 relevant to both prevent any possible local side effects or systemic exposure and properly exploit their  
 497 potential benefits such as the reservoir role inside the [hair follicles](#) ([Wosicka and Cal, 2010](#)) or the drug  
 498 transport modulation ([Scalia et al., 2013](#)). Since the passive transport through intact skin is considered  
 499 highly unlikely ([SCCP, 2007](#)), particle penetration is most likely along the intercellular route following the  
 500 lipid channels between the [corneocytes](#) and the appendage route along the hair follicles. Unlike the open  
 501 question concerning [nanoparticles](#), there is agreement that [microparticles](#) up to 10 µm can enter into the  
 502 follicle orifices that can act as an efficient long-term drug reservoir ([Lademann et al., 2007](#)) from which  
 503 soluble compounds could also diffuse into the viable epidermis ([Borm et al., 2006](#)). In relation to this, the  
 504 density and size of hair follicles as well as the lipophilicity of the material applied on the skin have been  
 505 assumed to contribute to differences in penetration rates ([Feldmann and Maibach, 1967](#); [Knorr et al., 2009](#);  
 506 [Otberg et al., 2004](#)). To assess the mechanism by which the organo-modified bentonite enters the SC, the  
 507 present study has considered the *in vivo* skin [penetration profile](#) of Bt/GM in comparison with that of pure  
 508 GM and pure Bt applying the samples, incorporated in petroleum jelly, on human skin regions having two  
 509 different hair follicle densities, the volar forearm (18 follicles/cm<sup>2</sup> corresponding to 0.09% skin surface,  
 510 78 µm diameter of hair follicle orifice) and the forehead (292 follicles/cm<sup>2</sup> corresponding to 1.28% skin  
 511 surface, 66 µm diameter of hair follicle orifice) ([Otberg et al., 2004](#)).

512 The investigation was performed using EDX analysis on twelve repetitive stripped tapes containing the  
513 outermost layers of SC, generally the *stratum disjunctum* from the 2nd to the 5th tape, the *stratum*  
514 *compactum* from the 6th to the 12th tape ([Jacobi et al., 2005](#)) by both GM extraction from combined tapes  
515 and Bt detection on each tape. Skin exposure to pure GM at the level of the forehead region provided drug  
516 concentrations higher than those obtained on the volar forearm region ( $p < .05$  concerning the first tape  
517 group) suggesting the involvement of the trans-follicular route pathway across SC. This assumption is  
518 consistent with the results from other Authors arguing that skin appendage route gained renewed interest  
519 for [hydrophilic](#) drugs representing a significant access also for gentamicin ([Barry, 2002](#); [Fadli et al., 2015](#);  
520 [Ogiso et al., 2002](#)). Moreover, GM levels are inclined to decrease with the increase of SC depth. The  
521 interaction of GM with Bt decreased antibiotic permeation extent compared with pure GM permeation  
522 ( $p < .05$ ) leading to a constant GM concentration (about 2% of the applied dose), regardless of SC depth  
523 ([Fig. 7](#)).



524

525

526 Fig. 7. GM permeated dose (% of the applied dose) across SC from pure GM and Bt/GM: (1) volar forearm,  
527 tape group 1 (tapes 2–6); (2) volar forearm, tape group 2 (tapes 7–12); (3) forehead, tape group 1 (tapes 2–  
528 6); (4) forehead, tape group 2 (tapes 7–12).

529 It follows a hampering effect of GM permeation provided by its reaction with Bt and the irrelevance of the  
530 application region, *i.e.* follicle density indicating presumably a different pathway. To monitor the possible  
531 translocation and distribution of Bt/GM across SC, each stripped tape (tapes 2, 6, and 12) was assayed by  
532 EDX analysis. All [EDX spectra](#) obtained from both pure Bt and Bt/GM skin exposure exhibited peaks from  
533 carbon, oxygen, aluminum, silicon, and sulfur atoms. The presence of sulfur in all tapes removed from  
534 untreated skin, attributable to the emission from SC [keratin](#), prevented assessing the permeation of GM in  
535 its sulfate form. The elements Si and Al that are not naturally occurring elements in SC entail the presence  
536 of Bt ([Cullander et al., 2000](#); [Moretto et al., 1999](#)). Accordingly, the presence of S atoms and the absence of  
537 Si and Al atoms were pointed out in all the control tapes (untreated skin and skin treated with only  
538 petroleum jelly). By measuring the intensity of characteristic X-rays spectra, the relative weight fraction of  
539 Si and Al can be calculated ([Table 4](#)).

**Table 4**  
EDX analysis: relative element weight percentage detected on tapes 2, 6, and 12 following skin exposure to pure Bt and Bt/GM. Mean values  $\pm$  SD.

Sample	Volar forearm		Forehead	
	Relative silicon weight	Relative aluminum weight	Relative silicon weight	Relative aluminum weight
pure Bt tape 2	29.3 $\pm$ 0.7	7.3 $\pm$ 0.3	13.7 $\pm$ 0.6	4.1 $\pm$ 0.5
pure Bt tape 6	3.9 $\pm$ 0.6	1.8 $\pm$ 0.6	5.5 $\pm$ 0.3	1.6 $\pm$ 0.3
pure Bt tape 12	5.3 $\pm$ 0.4	1.6 $\pm$ 0.4	5.3 $\pm$ 0.2	2.3 $\pm$ 0.2
Bt/GM tape 2	28.9 $\pm$ 0.4	7.0 $\pm$ 0.2	51.4 $\pm$ 0.9	10.8 $\pm$ 0.3
Bt/GM tape 6	17.6 $\pm$ 0.4	4.3 $\pm$ 0.3	18.0 $\pm$ 0.5	4.9 $\pm$ 0.3
Bt/GM tape 12	7.8 $\pm$ 0.5	2.4 $\pm$ 0.4	1.0 $\pm$ 0.5	1.6 $\pm$ 0.5

540

541 The detection of Si and Al atoms in all the tapes reveals the ability of clay particles to translocate across SC  
542 until the *stratum compactum* though Si and Al extents decreased with the increase of SC depth. Differences  
543 in quantitative distribution of Si and Al atoms between volar forearm and forehead application of both Bt  
544 and Bt/GM among the tapes were considered pointless to be argued. The limited influence of the sample  
545 exposure region is consistent with the results obtained by GM extraction from the tapes as the evidence of  
546 drug/clay association at the time of selecting SC pathway, plausibly different from the trans-follicular one.  
547 Besides skin appendages, SC is interrupted by inter-cluster corneocyte regions up to 100 µm in width, made  
548 of unsteady lipid packing generating openings having a low resistance to hydrophilic compounds ([Cevc and](#)  
549 [Vierl, 2010](#); [Dayan, 2005](#); [Iannuccelli et al., 2013](#)). Taking into account the size together with the [hydrophilic](#)  
550 [nature](#) of clayey samples, their motion along this trans-epidermal pathway could be considered as the  
551 favorite pathway. Moreover, Bt could increase SC hydration by means of the occlusive effect decreasing  
552 corneocyte packing and improving the clay transport. Such a route could also bypass the hindering effect  
553 on hydrophilic drug permeation provided by sebum along the trans-follicular route ([Verma et al., 2016](#)).  
554 Even if restricted to the superficial SC layers, it follows that a long-term Bt/GM reservoir for gradual GM  
555 release may be expected to perform leading to sustained antibacterial activity and minimized drug side  
556 effects.

#### 557 4. Conclusions

558 The approach consisting of new carriers redeveloping already-approved drugs and excipients was  
559 addressed to modulate gentamicin release and skin [permeation](#) by exploiting drug arrangement in the  
560 [interlayer](#) of [montmorillonite](#). Comprehensive examination of the organo-modified clay combined with a  
561 computational approach elucidated the mechanism of drug interaction with montmorillonite  
562 demonstrating the occurred [intercalation](#). From that, gentamicin sustained desorption and the possible  
563 pathway across inter-cluster [corneocyte](#) regions of the *stratum corneum* may be ensued in the perspective  
564 of contribution to a novel antibiotic material offering a potential more effective anti-infective therapy.

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