Materials Science and Engineering C 42 (2014) 466–473

Contents lists available at ScienceDirect

Materials Science and Engineering C

journal homepage: www.elsevier.com/locate/msec

Antibacterial efficiency of vermiculite/chlorhexidine nanocomposites and results of the in vivo test of harmlessness of vermiculite

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ARTICLE INFO

Article history:
Received 2 December 2013
Received in revised form 26 May 2014
Accepted 29 May 2014
Available online 4 June 2014

Keywords:
Organovermiculites
Antibacterial activity
In vivo tests
Histological examination

ABSTRACT

Clay minerals have been proposed as very useful materials for modulating drug delivery. These are the commonly used materials in pharmaceutical production both as inorganic carriers or active agents. We focused on the development of suitable long-acting material for local treatment of oral infection where clay minerals act as inorganic drug carriers. Organovermiculites with antibacterial activity were prepared by ion exchange reactions using different concentrations of chlorhexidine diacetate. The samples were characterized by X-ray diffraction (XRD), Fourier transform infrared spectroscopy (FTIR) and thermal analysis (TGA). The antibacterial activity was evaluated by finding the minimum inhibitory concentration (MIC). All studied organoclays possessed good antibacterial activity after 24 h exposure against *Escherichia coli*, *Enterococcus faecalis* and particularly against *Staphylococcus aureus*. *Pseudomonas aeruginosa* however proved very resistant as only the sample with the highest concentration of CA that successfully inhibited bacterial growth. Furthermore, clay mineral vermiculite was subjected to in vivo toxicological analysis and its influence on gastrointestinal tract during its oral application was investigated. Tissue samples from buccal mucosa, tongue, esophagus, stomach, terminal duodenum, small intestine, caecum, distal colon and liver were subjected to histological examination, both macroscopically and microscopically. Neither systemic nor local reactions were observed. Therefore the toxicity of vermiculite to a mammal model organism can be excluded.

1. Introduction

The development of suitable antibacterial materials that could be utilized in the future for medical purposes still rates among the most current topics of medical research. Of particular interest are substances that can act as carriers of antibacterial or antimycotic agents for local treatment (e.g. of oral cavity infections) and thus to avoid treatment on the level of the entire organism in the first phase of the therapy. Recently, increased attention is paid to so-called inorganic carriers based on clay minerals when literature reports their action in modified release systems [1–3]. Some of these are even already used for treatment (e.g. Smecta — diosmectite is used for therapy of diarrhea) [4–6]. Clay minerals are hydrated aluminium phyllosilicates with layered structure [7,8], moreover they are naturally occurring inorganic cation exchangers, so they may undergo ion exchange with functional molecules and/or particles via intercalation process, especially with basic drugs. The layered 2:1 clay mineral vermiculite (abbreviated as Ver), due to its greater layer charge than the most commonly used montmorillonite (abbreviated as Mt), becomes a promising carrier in the area of antibacterial materials.

Antibacterial agents are generally divided into two basic types, i.e., inorganic and organic antibacterial materials. Most frequently used antibacterial inorganic cations for modifying Mt were heavy metals such as Ag^+ [9–12], Cu^{2+} [13–16] and Zn^{2+} [17]. One of the few available papers describes antibacterial activity of cation exchanged Ver with silver and/or copper [18,19]. Hundáková et al. [18] prepared Ag^+Ver, Cu^{2+}Ver or combined Ag^+/Cu^{2+}Ver and found that these samples were more effective against Gram-negative bacteria than Gram-positive. Moreover, combined Ag^+/Cu^{2+}Ver stopped the bacterial growth at lower MIC value. Among the organic cations with antibacterial activity, alkyl trimethylammonium cations, especially quaternary amines, were a commonly used group of agents [20–24]. Antibacterial activity of cetyltrimethylammonium (CTA) and cetylpyridinium (CP) exchanged

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http://dx.doi.org/10.1016/j.msec.2014.05.054
0928-4931/

Mt studied by Praus et al. [21] showed that CP exchanged Mt exhibited an antibacterial activity against *E. coli* higher by six orders of magnitude compared to CTA exchanged Mt, within 24 h. Chlorhexidine diacetate (CA), substituted biguanidine with strong basic character, has a very wide range of antibacterial activity, being effective against both Gram-positive and Gram-negative bacteria and it is widely used in dental applications such as mouthwash as an active ingredient reducing dental plaque and oral bacteria. Previous studies describe antibacterial effects of CA intercalated into montmorillonite [25–27]. Zhou et al. [26] investigated the release of CA from Mt. In vitro release study showed that CA continuously released from clay matrix over 72 h, therefore they suggested Mt as advanced drug delivery carrier with controlled release characteristics. Our team [28] prepared CA/Na^+Ver and studied their bacteriostatic and bactericidal effects. Antibacterial test showed that higher content of CA (concentrations 2.0, 3.0 and 4.0 × CEC) at organovermiculite samples led to bactericidal effect. As far as treatment of oral infections is concerned, the current market lacks any curative form for a local long-acting application that would enable therapy without the need to use systemic treatment. A solution might be offered by application of mucoadhesive films with incorporated inorganic clay molecules carrying antibacterial or antimycotic agents that would gradually be released directly into the target area. For these inorganic carriers to be used in clinical practice, evaluation of their interactions with living organisms (toxicological analysis) as well as of their antibacterial and antimycotic activities (bacteriological analysis) must be performed. So far, no information is available in literature on acute or chronic toxic reaction to Ver in any experimental animal model. The situation is similar as far as in vitro cytotoxicity and genotoxicity of Ver are concerned. So far, only one study was published describing experimental intrapleural application of Ver (25 mg in 0.2 cm^3 solvent) in 21-days old rats and assessing possible carcinogenic effects. The animals were exposed to amorphous particles sized <2 nm and <5 nm. The conclusion was that even small particles did not cause any neoplasm, not even after 104 days after application [29].

In our study, organovermiculites using different mass ratio of chlorhexidine diacetate were successfully prepared by ion exchanged reactions. The resultant organovermiculites were characterized using X-ray diffraction (XRD), Fourier transform infrared spectroscopy (FTIR) and thermal analysis (TGA). The antibacterial activity was evaluated by finding the minimum inhibition concentrations (MIC) against Gram-positive *Staphylococcus aureus* and *Enterococcus faecalis* and Gram-negative *Escherichia coli* and *Pseudomonas aeruginosa*. Since there is no information about toxicity of clay mineral Ver, we also focused on in vivo tests of pure Ver on experimental animals (rats in our instance), and its influence on gastrointestinal tract during its oral application. The main objective of our research is to develop a long-acting material for treatment of oral infection in the form of mucoadhesive films. Therefore, this work is a pilot study which brings us important results about bacteriological and toxicological properties of antibacterial nanocomposites based on natural Ver.

2. Materials and methods

2.1. Materials

Clay mineral Mg^{2+}-vermiculite (abbreviated as Mg^{2+}Ver) from Letovice (Czech Republic) was utilized for experiment. The Mg^{2+}Ver obtained from a weathered zone of the ultrabasic body of metamorphosed basalts in the Letovice complex, in the eastern part of the Bohemia Massif (Czech Republic) was milled in a planetary mill, sieved, and the fraction <45 μm was used for experiment. This sample didn't contain other mineral phases identifiable by X-ray diffraction. Its crystallochemical formula calculated from the results of the elemental chemical analysis was $(Si_{3.13}Al_{0.86}Ti_{0.02})(Mg_{2.53}Fe_{0.45}Al_{0.02})O_{10}(OH)_2(Mg_{0.19}K_{0.01}Ca_{0.02})$ per $O_{10}(OH)_2$ with the cation exchange capacity (CEC) of 140 cmol(+)/kg. The chemicals used for the sample preparation were chlorhexidine diacetate (CA, $C_{22}H_{30}N_{10}Cl_2 \cdot 2C_2H_4O_2$, Sigma Aldrich) and ethanol as a solvent.

2.2. Modification of clay mineral

Five solutions of CA in ethanol were prepared in different concentrations of CA in accordance with the CEC of Mg^{2+}Ver: 0.2, 1.0, 2.0, 3.0 and 4.0, and then stirred and heated with Mg^{2+}Ver suspended in water. After centrifugation, solid products were dried and samples for the experiment were named Mg^{2+}OVer1, Mg^{2+}OVer2, Mg^{2+}OVer3, Mg^{2+}OVer4 and Mg^{2+}OVer5, respectively.

2.3. Sample characterization

The XRD patterns were measured using the X-ray diffractometer INEL equipped with a curved position-sensitive detector CPSD 120 (Ge-monochromator, $CuK_{\alpha 1}$ radiation) with a 2θ resolution of 0.03° that allows getting complete diffraction patterns in the 2θ range of 0–120°. The measurements were taken in ambient atmosphere (25 °C, 43% of humidity) under constant conditions (35 kV, 20 mA). The samples were fixed in a flat rotation holder and measurement was carried out for 2000 s.

The IR spectra were obtained by the KBr method using a NEXUS 470 Fourier transform (FTIR) spectrometer (Thermo Nicolet, USA). The spectrometer was equipped with a Globar IR source, KBr beam splitter, and DTGS detector. For each spectrum, 128 scans were obtained with a resolution of 4 cm^{-1}. The range of measurements was 400–4000 cm^{-1}.

The TGA analysis was performed on a NETZSCH STA 409 EP instrument at a heating rate of 10 °C/min up to 1000 °C in air atmosphere. Samples of size about 50 mg were placed in aluminium crucibles.

2.4. Antibacterial tests

The minimum inhibitory concentration (MIC) of the prepared samples was determined by their lowest concentration that would completely inhibit bacterial growth. The dilution and cultivation were performed on 96-well microtitration plates. The highest applied concentration was 10% (w/v) over water dispersion. These dispersions were further diluted by a threefold diluting method in glucose stock in such a manner that the second to seventh set of hollows contained sample dispersed in concentrations of 3.33%, 1.11%, 0.37%, 0.12%, 0.04% and 0.01%. The eighth set of wells contained pure glucose stock as a control test. A volume of 1 μl of glucose suspensions of *S. aureus* CCM 3953 (1.1×10^9 cfu ml^{-1}), *E. faecalis* CCM 4224 (1.1×10^9 cfu ml^{-1}), *E. coli* CCM 3954 (1.3×10^9 cfu ml^{-1}) and *P. aeruginosa* CCM 1960 (1.2×10^9 cfu ml^{-1}), provided by the Czech collection of microorganisms (CCM), was applied into the hollows. 1 μl of bacterial suspensions was transferred (after 30, 60, 90, 120, 180, 240, 300 min and then in 24 h intervals for 5 days) from each well into 100 μl of the fresh glucose stock and incubated in a thermostat at 37 °C for 24 and 48 h. Antibacterial activity was evaluated by turbidity, which is a display of bacterial growth [30].

2.5. In vivo tests

During the in vivo experiment, 35 male Wistar rats were used (supplier: MU Brno, 30048/2007-10001, valid until 10th October 2010, registration number: CZ-62760157). Animals were kept in a room with temperature ranging 20–24 °C, humidity 40–60%, and 12:12 L:D cycle (12 h of light, 12 h of darkness). They were fed with a commercially supplied mixture M1 for small laboratory animals, water ad lib. Experimental protocol was approved and monitored by the Ethics Committee of the University of Veterinary and Pharmaceutical Sciences in Brno under reference number 91/2010. Animals were divided into three experimental groups of 10 animals each after a week acclimatization period. The last 5 animals were used as control. Experiment duration was 30 days.