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Photophysical and antibacterial properties of complex systems based on smectite, a cationic surfactant and methylene blue

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ABSTRACT

Solid or colloidal materials with embedded photosensitizers are promising agents from the medical or environmental perspective, where the direct use of photoactive solutions appears to be problematic. Colloids based on layered silicates of the saponite (Sap) and montmorillonite (Mon) type, including those modified with dodecylammonium cations (C12) and photosensitizer – methylene blue (MB) were studied. Two representatives of bacteria, namely *Enterobacter cloacae* and *Escherichia coli*, were selected for this work. A spectral study showed that MB solutions and also colloids with Sap including C12 exhibited the highest photoactivities. The antimicrobial properties of the smectite colloids were not directly linked to the photoactivity of the adsorbed MB cations. They were also influenced by other parameters, such as light *vs*. dark conditions, the spectrum, power and duration of the light used for the irradiation; growth phases, and the pre-treatment of microorganisms. Both the photoactivity and antimicrobial properties of the colloids were improved upon pre-modification with C12. Significantly higher antimicrobial properties were observed for the colloids based on Mon with MB in the form of molecular aggregates without significant photoactivities. The MB/Mon colloids, both modified and non-modified with C12 cations, exhibited higher antimicrobial effects than pure MB solution. Besides the direct effect of photosensitization, the surface properties of the silicate particles likely played a crucial role in the interactions with microorganisms.

1. Introduction

Hybrid materials and nanomaterials represent an extensively developed topic of materials chemistry, influencing various branches of natural sciences. In the last few decades, the antimicrobial properties of nanomaterials have been reported and novel hybrid systems exhibiting promising disinfectant and biocidal properties have been developed. Natural nanomaterials have also received a lot of attention because of their active role in the biosphere [1]. Smectites, which are clay minerals and expandable layered silicates, are probably one of the most important inorganic nanomaterials present and formed in nature. Under specific conditions, smectites exhibit a large surface area (about 800 $m^2\ g^{-1}$) and a high capacity to adsorb inorganic and organic cations.

The antimicrobial properties of clay minerals have only been systematically investigated in the last few decades [2,3]. A lot of attention has been paid to hybrid systems based on smectites as carriers of antimicrobially-active substances. These include systems with sulfonamides [4], tetracyclines [5], sulfathiazoles [6], quaternary alkylammonium and alkylphosphonium ions [7,8], or biocidal polymers [9]. General antibacterial and antifungal properties have been observed for colloids based on montmorillonite and the photosensitizer methylene blue (MB) [10]. The role of montmorillonite particles was identified as the delivery of dye molecules onto the microbe cell surface. The photophysical and photochemical properties of MB in colloids are very complex and depend on various parameters [11]. For example, MB molecular aggregates, often formed at interfaces, exhibit significantly altered photophysical properties. H-aggregates, which are structurally sandwich-type molecular assemblies, exhibit reduced photoactivity. The effect of negatively-charged surfaces on the properties of MB has been well documented [12–14]. In order to improve the antimicrobial properties of photoactive compounds, hybrid systems based on nanomaterials as carriers of active photosensitizers

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have also been investigated [15]. However, there has been a lack of information on the antimicrobial properties of more complex systems including clay minerals, photosensitizer and additional bioactive or surface-active substances. The objective of this work was a detailed characterization of the photophysical and antibacterial properties of MB in solution, in the colloids of smectites, including materials modified with the surfactant dodecyltrimethylammonium (C12), to the Gram-negative bacteria *Enterobacter cloacae* and *Escherichia coli*. This study should provide more information about the interactions in such complex systems with focus on the validation of antimicrobial effectiveness.

2. Materials and methods

2.1. Smectites, photosensitizers and chemicals

Cationic dye MB (chloride salt, LOBA, Feinchemie, Austria, M_r = 319.86) was used as a photosensitizer and as a component in the hybrid systems with smectites. A synthetic layered silicate of saponite type, with the product name Sumecton (Sap), was purchased from Kunimine Ind., Japan. It was used as received. Montmorillonite SWy-2 (Mon) was purchased from the depository of the Clay Mineral Society. The Na^+ form was prepared and the fine fraction (<2 μm) obtained by the standard method based on sedimentation. The cationic surfactant C12 (chloride salt, M_r = 263.8) was obtained from Sigma Aldrich (Germany). The stock solution of MB was prepared in distilled water and standardized after an appropriate dilution by visible absorption spectroscopy at 664 nm ($\varepsilon_{664} = 9 \cdot 10^4$ mol^{-1} dm^3 cm^{-1}). The solution was filtered through a membrane filter with a pore size <0.22 μm (TPP, Switzerland). It was storaged at 4 °C and used for at most two weeks.

2.2. Illumination system

Two light sources were used for photodynamic inactivation (PDI): 1. Fluorescent lamps with white light (7.404 W m^{-2}, 6 × 36 W, type 33 luminescent tubes of, 4300 K) were placed a distance of 0.4 m over inoculated plates. They were used in some preliminary experiments. 2. Monochromatic LED red light (homemade device, 16.7 W m^{-2}, λ_{max} = 650 nm) was positioned just under transparent inoculated 24-well polystyrene plates separated with a transparent glass sheet of 0.6 cm thickness.

2.3. Preparation of smectite colloids

Sap or Mon powder was added to distilled water and stirred at room temperature (RT) for 24 h. Colloids were sterilized at 120 kPa for 20 min. The colloids containing organically-modified smectites (C12/Sap and C12/Mon) were prepared from smectite colloids and C12 solution. The C12/smectite ratio was always 10^{-3} mol g^{-1}. The mixture was then stirred at RT for 3 h, centrifuged and washed with a mixture of water and ethanol (1:1, v/v) several times in order to remove the excess C12 solution. The final loadings of C12 cations in C12/Sap and C12/Mon colloids determined from carbon analysis were 0.63 and 0.71 mmol g^{-1}, respectively. Finally, the colloids of C12/smectite were repeatedly centrifuged and washed with distilled water to remove the excess ethanol. A sedimented suspension of C12/smectite was overlaid with sterile distilled water to obtain the final volume. The complexes of MB with smectites and C12/smectites were prepared by mixing the dye solution with the respective colloids. The mixtures were stirred for 3 h at RT. The concentrations of the components were varied (see details below) to get a final MB concentration and MB/smectite loading.

2.4. Spectrophotometric and chemometric characterization of the systems

Some systems used in microbiological experiments were characterized by spectroscopy methods. Solutions and colloids were treated under identical conditions to those used for the microbiology experiments before the measurements were performed. UV–VIS absorption spectroscopy and steady-state fluorescence spectroscopy were used for the characterizations. Absorption spectroscopy was done in a Cary 100 UV/VIS Spectrometer (Varian), with water as the reference substance. Steady-state fluorescence measurements were performed using Fluorolog-3 (Horiba Jobin-Yvon) in front-face mode to avoid the interference of light re-absorption and scattering upon the excitation at 570 nm. Spectral data were analysed using principal component analysis (PCA) and multivariate curve resolution – alternating least square (MCR) methods. The range 520–730 nm was selected for the absorption spectrum. PCA was performed using a NIPALS algorithm. MCR was calculated using non-negativity constraints for both the spectral and concentration parameters. More details on the chemometric methods have been described elsewhere [16].

2.5. Bacterial strains and cultivation conditions

Reference strains of Gram-negative bacteria *E. cloacae* CCM 1903 and *E. coli* CCM 3954 (Czech Collection of Microorganisms, Brno, Czech Republic) were used in the experiments. The bacteria were maintained in Skim-Milk Medium (himedia, India) at –20 °C. Before the experiments, the microorganisms were inoculated in Mueller Hinton Broth (MHB, himedia, India) and cultivated aerobically in an incubator (Multitron S-000115690, Switzerland) (150 RPM) at 37 °C for 18 h.

2.6. Assays of microbiological experiments

Prior to the major study, some preliminary experiments were performed. They tested different experimental conditions, like periods of irradiation, light sources, pre-incubation of samples with MB, and inocula prepared from the *lag* and exponential (*exp*) phase of growth, respectively. Two concentrations of MB solution ($2.6 \cdot 10^{-5}$ and $2.6 \cdot 10^{-6}$ mol L^{-1}) were tested in all experiments and are denoted as higher (*H*) and lower (*L*) in the following text. The conditions used for the preliminary experiments testing the antimicrobial properties of MB solutions are summarized in supplementary data SD1. The major study tested microorganisms grown in MHB at 37 °C for 18 h that were directly used for the preparation of bacterial inocula in fresh MHB in order to bring bacteria to the *exp* phase of growth ($OD_{600} \sim 0.5$). The growth phase was determined on the basis of the growth curves of both microorganisms tested (UV–VIS spectrophotometer, Jenway 6305, UK). Additionally, the experiment with inocula from the *exp* growth phase was modified using a 1 h pre-incubation of the bacterial suspension with MB at 37 °C in the dark.

Experiments testing MB solutions or colloids (Tables 1 and 2) were carried out in sterile 24-well polystyrene microtitre plates (Greiner Bio-one, Austria). A 1700 μL volume of the cell suspension in the *exp* phase of growth was applied to polystyrene plates. An additional 200 μL of either aqueous MB solution (*H* and *L* concentration) or smectite colloids or mixtures of them and 100 μL of MHB was then added. The compositions of the second components were adjusted to obtain the final MB concentrations and MB/smectite ratios shown in Tables 1 and 2 or mentioned in the text below. The final volume of the mixtures was 2 mL in each well. Both *H* and *L* concentrations of MB were used in the mixtures. The symbols for the colloids with Sap or Mon denote the presence of active substances: MB/Sap, MB/Mon, MB/C12/Sap and