Dendrimeric peptide – montmorillonite intercalation compound

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Abstract: The solution intercalation method was used for immobilization of low-molecular-mass lysine-based peptide dendrimers (Lys-D, hydrochloride form) on montmorillonite (MMT). The intercalation was confirmed by X-ray diffraction and elemental analysis of the products. Analysis of Brunauer-Emmett-Teller (BET) nitrogen adsorption–desorption isotherms indicates that both interlayer and external clay surfaces are involved in the binding of dendrimeric peptides. Depending on the structure of Lys-D, including the location of protonated amino groups in peptide branches, the intercalation involves ion exchange between sodium montmorillonite and peptide ionic groups or proceeds leaving the intact peptide counterions. Thermogravimetric analysis (TGA) studies of MMT-Lys-D compounds indicate that the major mass-loss associated with the decomposition of peptide component is shifted to higher temperatures compared to the peptide itself.

Keywords: dendrimeric peptides, montmorillonite, intercalation, X-ray diffraction, BET analysis.

Montmorylonit interkalowany dendrymerami peptydowymi

Streszczenie: Metodą interkalacji z roztworu przeprowadzono immobilizację niskocząsteczkowych dendrymerów L-lizyny (Lys-D, w formie chlorowodorku) na montmorylonicie (MMT). Wyniki analizy metodą dyfrakcji rentgenowskiej (XRD) oraz analizy elementarnej (C, H, N, Cl) potwierdziły interkalację peptydu w MMT. Na podstawie analizy izoterm adsorpcji–desorpcji azotu Brunauera-Emmetta-Tellera (BET) stwierdzono, że związanie peptydu następuje zarówno w przestrzeni międzywarstwowej, jak i na zewnętrznej powierzchni MMT. W zależności od struktury peptydu Lys-D i położenia protonowanych grup aminowych interkalacja połączona jest z wymianą jonową pomiędzy montmorylonitem sodowym i peptydem lub przebiega z zachowaniem wyjściowych przeciwjonów chlorkowych Lys-D. Analiza TGA związków MMT-Lys-D wskazuje, że główny ubytek masy związany z rozkładem składnika peptydowego zachodzi w wyższym zakresie temperatury niż w przypadku peptydu niezwiązanego.

Słowa kluczowe: dendrymery peptydowe, montmorylonit, interkalacja, dyfrakcja rentgenowska, analiza BET.

The interaction between aluminosilicate clays and peptides has been a subject of investigations in various research fields including drug delivery, soil ecosystems or even early life evolution [1]. Among clay minerals, particular attention has been paid to the smectite group, with a 2:1 layered structure consisting of two tetrahedral sheets of silica fused to octahedral sheet of alumina. The most common smectite mineral is montmorillonite (MMT), which forms plate-shaped crystals composed of 1-nm-thick layers. Part of Al^{3+} cations in MMT octahedra are substituted by Mg^{2+}, generating a negative charge of the layers, which is counterbalanced by the cations present in the interlayer space, so-called gallery. Consequently MMT has cation-exchange properties, moreover it can absorb high quantities of water, which leads to expansion of the interlayer spacing. As a result MMT has a large, both external and internal surface able to adsorb various molecules by ionic, electrostatic or other physical interactions.

Gougeon and collaborators studied adsorption of two homopolypeptides, poly(D-lysine) and poly(D-glutamic acid) on a synthetic MMT clay in acidic medium [2]. CP-MAS NMR (cross polarization magic angle spinning nuclear magnetic resonance) analysis indicated changes in the peptide conformation, with unfolding and adoption of more extended random coil structure. Also, XRD and N_2-adsorption BET experiments showed that intercalation of polypeptide fragments within the clay interlayer

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takes places, although polypeptide backbone do not enter the interlayer space. These polypeptide-clay compounds can be heated up to 110 °C without any degradation of the peptide component. The effect of pH on the adsorption of lysozyme, ovalbumin and ovotransferrin on the particles of synthetic smectite in aqueous solutions was investigated by Kolman and coworkers [3]. The incorporation of small oligopeptides derived from soil organic matter into montmorillonite and thermal stability of the obtained organoclay complexes were studied by Block *et al*. [4].

The group of Torres described the compounds of MMT and nisin, polycyclic peptide with a molar mass around 3500 Da, consisting of 34 amino acids, used as a food preservative [5]. On the basis of XRD and transmission electron microscopy (TEM) analyses they suggested that the cationic portions of nisin were intercalated into the interlayers located near the edges of the clay, similarly to the "frustrated intercalation" observed in organoclays based on dendritic surfactants [6]. On the other hand, adsorption of some small protein molecules in the cationic forms can proceed *via* complete intercalation. Johnston *et al.* [7] reported the successful introduction of hen egg white lysozyme (HEWL) into the interlayer of saponite (trioctahedral mineral of the smectite group). XRD analysis showed that the interlayer distance in the HEWL-saponite complex increased to a value of 4.4 nm and was consistent with the crystallographic dimensions of HEWL [7].

The objective of this study was to investigate the compounds of low-molecular-mass, lysine-based peptide dendrimers (Lys-D) with montmorillonite clay prepared by solution intercalation method. The preparation and properties of Lys-D dendrimeric peptides were described in [8, 9]. The immobilization of peptide dendrimers on aluminosilicate clays can be advantageous for applications where peptide stabilization or controlled release is needed.

EXPERIMENTAL PART

Materials

Sodium montmorillonite Nanomer PGW (cation exchange capacity 145 meq/100 g) was supplied by Nanocor (USA). Two dendrimeric peptides, presented in Table 1, were synthesized according to the procedure described in [9]. All other used chemicals were of analytical grade quality.

Preparation of montmorillonite clay – dendrimeric peptide compounds

10 g MMT was dispersed in 200 cm^3 water by ultrasonication for 2 hours using a probe sonicator (400 W, Dr. Hielscher UP400 S). The resulting suspension was mixed with 200 cm^3 of dendrimeric peptide (DP) solution in methanol (containing 1 or 2 g DP) and ultrasonicated for 3 hours with cooling in an ice bath. Then, the suspension was diluted with methanol and the solid product separated by centrifugation at 10 000 rpm for 30 minutes. The product was suspended in 200 cm^3 methanol and centrifuged to wash out the unbound peptide. The same procedure was repeated twice using water to remove the salt by-product. The final product was dried under vacuum at 50 °C.

Methods of testing

Elemental analysis was carried out using a Series II CHNS/O Perkin-Elmer analyser. X-ray diffraction patterns of powdered samples were recorded using Bruker-AXS D8 Advance Series 2 diffractometer working with Co-K$_\alpha$ radiation (λ = 0.179 nm); 2 θ angle was in the range of 2–60°. Specific surface area and volume of pores were determined from nitrogen adsorption–desorption isotherms measured at -196 °C using Micromeritics TriStar II 3020 analyzer. Before the measurement, the samples were degassed at 50 °C under vacuum (10.67 kPa) for 12 h. Thermogravimetric analysis was carried out using a Mettler Toledo TGA SDTA 851 analyzer at a heating rate of 20 °C/min.

RESULTS AND DISCUSSION

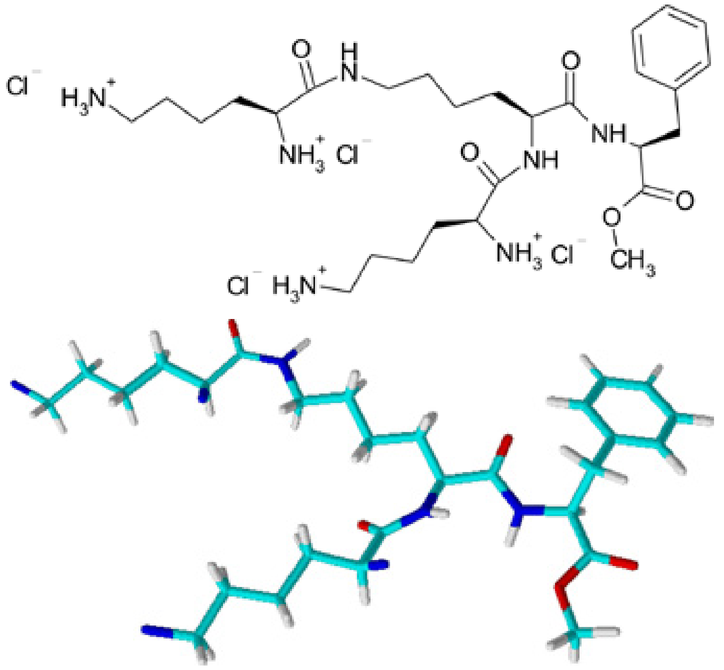
In order to prepare intercalation compounds, methanolic solutions of dendrimeric peptides Lys-D1 and Lys-D2 (with the chemical and spatial structures shown in Schemes A and B, respectively) were mixed ultrasonically with an aqueous solution of sodium montmorillonite.

The resulting product was isolated by centrifugation and purified by washing with methanol and water to remove the excess of free peptide and the sodium chloride formed in the ion exchange process. The elemental analysis shows that using 10 weight parts of Lys-D1 peptide by 100 weight parts of clay practically quantitative

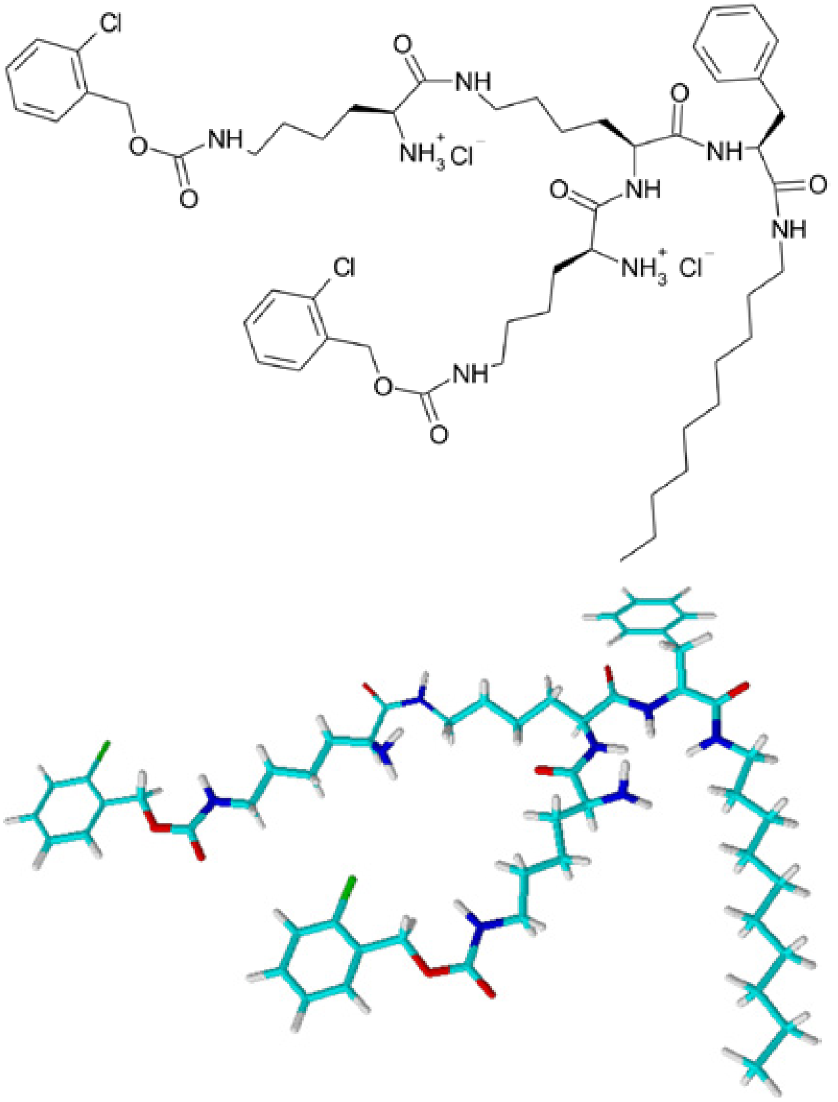
T a b l e 1. Characteristics of dendrimeric peptides used in the study

Dendrimeric peptide designation	Molecular mass g/mol *)		Elements wt %				
			C	H	N	Cl	Cl/N ratio
Lys-D1	709.58	calc.	47.40	7.53	13.82	19.99	1.45
		exp.	47.13	7.48	11.83	14.36	1.21
Lys-D2	1099.02	calc.	57.92	7.34	10.20	12.90	1.26
		exp.	56.21	7.45	9.71	13.22	1.36

*) hydrochloride form.



Scheme A



Scheme B

Table 2. Characteristics of montmorillonite – dendrimeric peptide compounds

MMT-dendrimeric peptide compound	Peptide weight parts by 100 weight parts MMT	MMT interlayer spacing d_{001} nm	Elements wt %				
			C	H	N	Cl	Cl/N ratio
MMT-Lys-D1-10	10	1.76 3.09	4.29	2.55	1.34	0.40	0.30
MMT-Lys-D1-20	20	1.48	6.25	2.04	1.84	0.60	0.33
MMT-Lys-D2-10	10	1.52 3.08	7.49	2.77	1.46	2.05	1.40

binding of peptide with MMT was obtained. As it is expressed by chlorine/nitrogen ratio the product, denoted as MMT-Lys-D1-10, contains some amount of chlorine in water-insoluble form corresponding to 20 % of the chlorine present in the starting Lys-D1 (Table 2).

This indicates that most of the amino groups of peptide took part in the ion exchange reaction with anionic centres of MMT interlayer. Also, the peptide intercalation is confirmed by XRD pattern, which shows an intensive d_{001} reflection corresponding to an interlayer spacing of 1.76 nm, increased in comparison to that of sodium montmorillonite. The interlayer spacing of the latter changes from 0.95 nm for a dry material to about 1.2 nm for the clay in the form of monolayer hydrate [10]. A second, broadened diffraction peak (3.1 nm) in the XRD pattern of MMT-Lys-D1-10 may indicate the presence of more expanded arrangements of the peptide molecule in MMT interlayer.

The twofold increase of the Lys-D1 concentration in the reaction mixture results in only 50 % increase in the carbon and nitrogen content in the product MMT-Lys-D1-20. Further changes in peptide arrangement in the clay gallery are revealed in the XRD pattern (Fig. 1).

The maximum of the most intensive d_{001} reflection shifts to 1.48 nm. For comparison, montmorillonite intercalated with L-lysine in hydrochloride form (pH = 4) has interlayer distance of 1.36 nm [11], while the compound of MMT with high molecular weight L-lysine homopolymer is characterized by d_{001} spacing between 1.37 and 1.64 nm, depending on the water content [2].

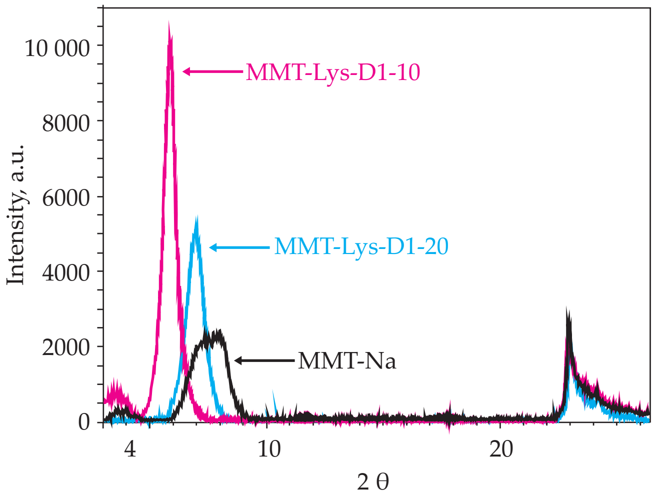


Fig. 1. XRD pattern of sodium montmorillonite and dendrimeric peptide Lys-D1 intercalation compounds

In contrary to the former product, the compound of MMT with the peptide Lys-D2 (denoted as MMT-Lys-D2-10) is characterized by nearly the same chlorine: nitrogen ratio than Lys-D2 itself. The 2 % content of chloride remains unchanged even after the prolonged washing with water. This indicates that the binding process does not involve the ion exchange of sodium cations in MMT gallery for ammonium cations of the peptide dendrimer. This difference can be explained by the fact that the protonated amino groups in Lys-D2 dendrimer are located only in the internal part of the molecule and they are surrounded by bulky chlorobenzyl groups. In effect, the process of intercalative ion exchange can be hindered. Nevertheless, an intercalation of Lys-D2 molecules into MMT interlayers is confirmed by XRD pattern with two d_{001} reflections corresponding to about 1.5 and 3 nm spacing.

The surface properties of MMT-dendrimeric peptide compounds were analyzed using Brunauer-Emmett-Teller (BET) method based on isothermal adsorption and desorption of nitrogen (Table 3). The obtained isotherms (demonstrated in Fig. 2) display hysteresis loops typical for MMT clays, usually classified as type H3 or H4 according to IUPAC recommendations (12–14).

They are characteristic for plate-like particles possessing slit-shaped pores. Specific surface area of sodium MMT determined from the BET measurements (29.7 m^2/g) correspond to the sum of external surface area (18.2 m^2/g) and micropore area (11.5 m^2/g) calculated from t-plot analysis (based on the comparison between the experimentally determined volume of adsorbed N_2 and its standard thickness on the nonporous material as a function of the relative gas pressure). For MMT-dendrimeric peptide compounds a significant reduction in BET surface area (by 70–77 %) was observed, including a decrease in the external area from 48 % (MMT-Lys-D1-20) to 62 % (MMT-Lys-D2-10). Density functional theory (DFT) calculations show that the intercalation of peptide gives 91–95 % decrease in the total area of pores greater than 2.5 nm. These results indicate that, besides an intercalation of dendrimer molecules or their fragments into MMT interlayer (which in general is not accessible to nitrogen), a considerable amount of peptide is bound to the external clay surface blocking the pores and reducing the surface area determined by BET method. Simi-

T a b l e 3. BET surface area analysis of sodium montmorillonite and dendrimeric peptide intercalation compounds

Symbol of sample	BET surface area m^2/g	The t-plot external surface area m^2/g	Total area in pores greater than 2.5 nm m^2/g (DFT)
MMT-Na	29.67	18.17	18.02
MMT-Lys-D1-10	9.06	10.13	1.28
MMT-Lys-D1-20	8.78	9.56	1.24
MMT-Lys-D2-10	6.77	6.85	0.96

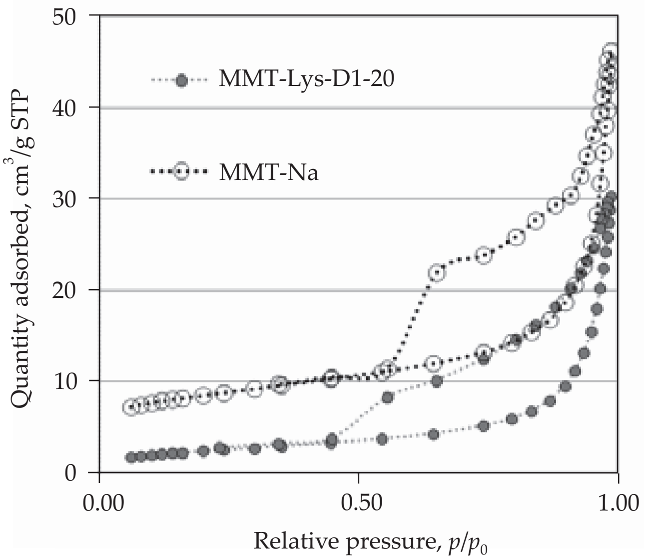


Fig. 2. Nitrogen adsorption–desorption isotherms of sodium montmorillonite and dendrimeric peptide Lys-D1 intercalation compound

lar kind of interaction was reported for montmorillonite MMT and high-molecular-weight poly-D-lysine, where side chain fragments of the peptide penetrate the clay interlayer space while the polymer backbone is adsorbed externally at the MMT surface [2].

The intercalation compound of MMT with peptide Lys-D1 was also characterized by thermogravimetric analysis (Table 4).

The mass loss in the range 30–100 °C may be ascribed to the weakly bound water from the MMT interlayer and interparticle pores [15]. As it is usually observed for the clays intercalated with other organic compounds, the water content (8.5 wt % in sodium montmorillonite) decreases gradually with an increase in dendrimeric peptide content down to 3.1 wt %. It results from a less space available for water molecules.

The degradation of amino acids and their polymers goes through several substages which are difficult for separation. The maximum decomposition rate was observed in the range 330–350 °C for lysine oligomers and polymers [16, 17]. Lysine itself (in hydrochloride form) starts its decomposition at 270 °C (just above the melting point) [18]. The mass loss of Lys-D1 dendrimer in the range of 237–370 °C (58.2 %) is close to that reported for lysine hydrochloride in the range 220–346 °C (55.2 %). Between 237 and 370 °C montmorillonite-Lys-D1 compounds exhibit significantly lower mass losses than those expected from the peptide content determined with elemental analysis. In contrary, in the range 370–500 °C the mass loss of peptide-clay compounds is two times higher than calculated from the composition of intercalation product. This may suggest that the main decomposition stage is shifted to the higher temperatures in the case of peptide dendrimer incorporated in MMT interlayer. However, further studies are needed, including the analysis of deintercalation process, to get a more detailed view on the thermal and structural stability of dendrimeric peptides within the montmorillonite interlayer space.

CONCLUSIONS

Two dendrimeric peptides based on L-lysine unit were successfully intercalated into the montmorillonite interlayers. As it was shown by elemental analysis, the intercalation of dendrimer Lys-D1 containing protonated amino groups at the end of its branches takes place through ion exchange reaction involving most of the chloride counteranions. In contrary, the dendrimer Lys-D2, in which protonated amino groups are surrounded by bulky aromatic substituents, interacts with montmorillonite leading to an expansion of its interlayer spacing, however no evidence of ion exchange was found. BET analysis indicates that both interlayer and external clay surfaces are involved in the adsorption of dendrimeric peptides. TGA studies show that the main decomposition stage of the peptide component bound to montmorillonite is shifted to higher temperatures compared to the peptide itself.

T a b l e 4. TGA analysis of dendrimeric peptide Lys-D1 and montmorillonite-Lys-D1 intercalation compounds

Temperature range °C	Mass loss, wt %				Total mass loss to 500 °C wt %
	30–100	100–237	237–370	370–500	
MMT-Na	8.50	0.26	0.12	0.38	9.26
Lys-D1	4.56	12.78	58.22	18.95	94.51
MMT-Lys-D1-10	5.94	1.84	1.66	3.50	12.94
MMT-Lys-D1-20	3.14	1.94	3.03	5.70	13.78

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