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PRODUCTION OF 3′,5′-CYCLIC DIADENOSINE MONOPHOSPHATE COMPLEX WITH MG,AL-LAYERED DOUBLE HYDROXIDE

Abstract. The possibility of using Mg,Al-layered double hydroxide (LDH) for production of nanoparticles comprising molecules of pharmaceutically valuable 3′,5′-cyclic diadenosine monophosphate (cyclic di-AMP) was demonstrated. Experimental conditions were optimized to produce LDH complexes with cyclic di-AMP (average size of 300 nm), which loading capacity in regard to the target compound reached 60 mass %. The fact of cyclic di-AMP release from LDH complex in pH-dependent mode (proceeding slightly slower at pH 7.4 than at pH 4.5) was stated. The obtained results testify in favor of applying the studied nanocomplexes for delayed delivery of cyclic di-AMP to the target immune cells.

Keywords: 3′,5′-cyclic diadenosine monophosphate, Mg,Al-layered double hydroxide, nanoparticle, adjuvant, dinucleotide Mg,Al-layered double hydroxide complex, delayed delivery of drug to target cells

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ПОЛУЧЕНИЕ КОМПЛЕКСОВ 3′,5′-ЦИКЛИЧЕСКОГО ДИАДЕНОЗИНМОНОФОСФАТА С MG,AL-СЛОИСТЫМ ДВОЙНЫМ ГИДРОКСИДОМ

Аннотация. Показана возможность использования Mg,Al-слоистого двойного гидроксида (СДГ) для получения наночастиц, включающих молекулы фармацевтически важного 3′,5′-циклического диаденозинмонофосфата (цикло-ди-АМФ). Подобраны экспериментальные условия для получения комплексов СДГ с цикло-ди-АМФ (размером порядка 300 нм), емкость которых в отношении цикло-ди-АМФ достигает 60 мас.%. Установлено, что при pH среды 4,5 цикло-ди-АМФ высвобождается из его комплекса с СДГ более активно, чем при pH 7,4. Полученные результаты свидетельствуют о возможности применения изученных нанокомплексов для пролонгированной доставки цикло-ди-АМФ в иммунные клетки-мишени.

Ключевые слова: 3′,5′-циклический диаденозинмонофосфат, Mg,Al-слоистый двойной гидроксид, наночастица, адъювант, комплекс динуклеотида с Mg,Al-слоистым двойным гидроксидом, пролонгированная доставка лекарства в клетки-мишени

Для цитирования: Получение комплексов 3′,5′-циклического диаденозинмонофосфата с Mg,Al-слоистым двойным гидроксидом / М. А. Винтер [и др.] // Вес. Нац. акад. навук Беларусі. Сер. біял. навук. – 2024. – Т. 69, № 3. – С. 249–253. https://doi.org/10.29235/1029-8940-2024-69-3-249-253

Introduction. The likelihood of imminent pandemics caused by respiratory viruses dictates the need to seek new antiviral agents [1].

We have previously proposed [2] to use pharmacologically promising 3′,5′-cyclic diadenosine monophosphate (cyclic di-AMP) (Fig. 1) recently discovered in bacteria and archaea [3], as a universal antiviral agent. The compound is involved in the control of many complex physiological processes and acts as an inducer of endogenous interferon [4, 5], which protects human and animal bodies from various infectious agents.

A grave challenge for pharmacological application of this compound is its low stability in the blood stream. In addition, the cyclic di-AMP molecule is negatively charged and, hence can hardly penetrate into immune cells [6]. Various nanoparticle carriers have been proposed to overcome this barrier. Among them, nanoscale inorganic layered double hydroxides (LDH) (Fig. 2) are regarded as the most promising carrier.

LDHs, also called "anionic clays", represent a family of materials that have attracted a growing interest in recent years due to their technological benefits in catalysis, separation technology, optics, medical science and nanocomposite materials engineering [8].

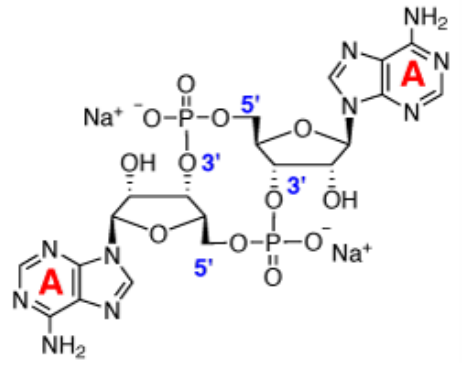

Fig. 1. Structure of cyclic di-AMP (sodium salt)

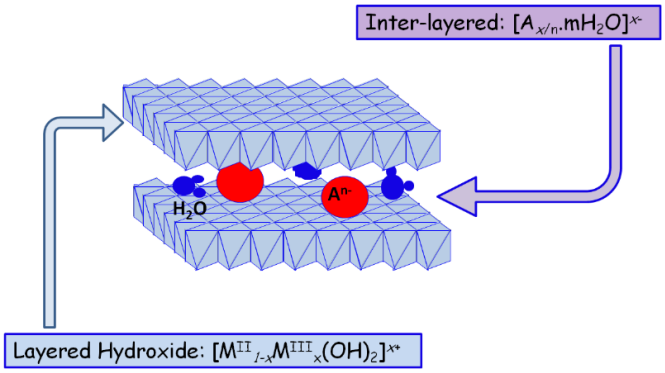

Fig. 2. Structure of LDH [7]

LDHs consist of positively charged metal hydroxide layers, in which the anions (along with water) are stabilized in order to compensate the positive layer charges. The general chemical formula of LDH clays is expressed as $[M^{II}_{1-x}M^{III}_{x}(OH)_2]^{x+}(A^{n-})_{x/n}\cdot yH_2O$, where M^{II} is a divalent metal ion, such as Mg^{2+}, Ca^{2+}, Zn^{2+}, etc., M^{III} is a trivalent metal ion, such as Al^{3+}, Cr^{3+}, Fe^{3+}, Co^{3+}, etc. and A^{n-} is an anion of any type with charge n, such as Cl^-, CO_3^{2-}, NO_3^-, etc. [9, 10].

The electrostatic interactions, hydrogen bonds between layers and contents of the gallery cement the architecture, shaping a three-dimensional structure. Diverse combinations of divalent and trivalent cations can form LDHs. For these ions, the only limitation is that their radii are not strikingly different from those of Mg^{2+} and Al^{3+}. The anions occupy the interlayer region of layered crystalline materials.

LDHs are increasingly explored as drug and gene carriers, since they are characterized by low toxicity, enhanced cell penetration capacity and the ability to release transported compounds at reduced pH values, corresponding to pH inside endosomes/lysosomes [11].

Several methods have been described for synthesis of LDHs. Co-precipitation in aqueous solution is the most common procedure for LDH production [12]. The benefit of this technique is that several synthesis parameters can be controlled independently during precipitation process (e. g. temperature and pH of the reaction medium, concentration of the metallic salts and alkaline solution).

In the present study, we prepared artificially a novel bio-inorganic nanohybrid of Mg,Al-LDH and biomolecule (pharmaceutically valuable cyclic di-AMP) as well as assessed application prospects of intercalated LDH as a drug delivery system.

Materials and research methods. Cyclic di-AMP sample was enzymatically synthesized from ATP as previously described [13]. Milli-Q water was used in all experiments.

The nanocomplex of cyclic di-AMP with Mg,Al-LDH was produced by co-precipitation method according to [14], with subsequent crystallization at 75 °C in the presence of ammonium hydroxide. Briefly, 10 ml of salt solution containing 20 mM $Mg(NO_3)_2$ and 10 mM $Al(NO_3)_3$ was added drop-wise with vigorous stirring at room temperature to 10 ml of 10 mM NH_4OH solution comprising 0.25, 0.5, 1 or 2 mM cyclic di-AMP. The reaction slurry was aged at 75 °C for 12 h. The resulting white precipitate was collected by centrifugation (20 000 g for 10 min) and washed with water until no cyclic di-AMP was detected in the supernatant. All the samples were air-dried at 60 °C for 5–6 h. To obtain LDH nanoparticles without cyclic di-AMP the above procedure was performed without addition of cyclic di-AMP to solution of NH_4OH.

Amount of cyclic di-AMP incorporated in nanohybrides was estimated by the following method. A known amount of the nanocomposites was placed in a 10 ml volumetric flask and LDH layers were dissolved with 50 mM HCl solution. Concentration of cyclic di-AMP in the solution was determined by monitoring the absorbance at λ = 259 nm (ε = 27 000 $M^{-1}\cdot cm^{-1}$) with Solar PB2201 spectrophotometer (CJSC Spectroscopy, Optics and Lasers – Modern Developments, Belarus).

The binding capacity of LDH in regard to cyclic di-AMP was calculated according to the following formula:

$$A = \frac{m_1}{m_2}\cdot 100,$$

where A is the binding capacity, %; m_1 is the amount of cyclic di-AMP intercalated into the LDH nanoparticles; m_2 is the total amount of cyclic di-AMP added into reaction mixture.

The loading capacity of LDH in regard to cyclic di-AMP was calculated according to the formula:

$$E = \frac{m_1}{m_3} \cdot 100,$$

where E is the loading capacity, %; m_1 is the amount of cyclic di-AMP intercalated into the LDH nanoparticles; m_3 is the amount of LDH/cyclic di-AMP nanohybrid.

The size of LDH nanoparticles was measured in collaboration with colleagues from Institute of Bioorganic Chemistry, NAS of Belarus, by method of dynamic light scattering using DynaPro NanoStar (Wyatt Technology, USA) in compliance with the manufacturer's guidelines.

To measure the amount of cyclic di-AMP released from the nanohybrids, the *in vitro* drug release tests were performed at room temperature by stirring a certain amount of powdered LDH/cyclic di-AMP nanohybrids in 10 mM phosphate-citrate buffer solution (pH 4.5 or 7.4, respectively) [15]. 1 ml aliquots of the suspension were sampled at desired time intervals, centrifuged and the cyclic di-AMP contents in supernatant were determined by reading UV absorbance at λ = 259 nm to calculate the amount of dinucleotide released from the nanohybrids. The percentage released at each time point was expressed as a fraction of the total amount of the dinucleotide.

Experimental data obtained in this research represent confidence range of arithmetic means for 95 % probability level.

Results and its discussion. The initial investigation stage was devoted to synthesis of LDH nanoparticles loaded with cyclic di-AMP. Mixing of aqueous solutions containing Mg^{2+}, Al^{3+} and cyclic di-AMP according to the procedure described in the section "Materials and research methods" resulted in the transparent, slightly opalescent solution. The loading and binding capacity of nanoparticles in regard to the molecules of ligand (cyclic di-AMP) and their size were chosen as the main parameters characterizing the nano products. Results of the experiment illustrated by Fig. 3 testify that increasing the concentration of cyclic di-AMP from 0.25 to 2 mM in the reaction mixture is likely to promote the holding capacity up to 600 mg of cyclic di-AMP/g nanocomplex, reducing concomitantly the loading percentage from 90 to 40.

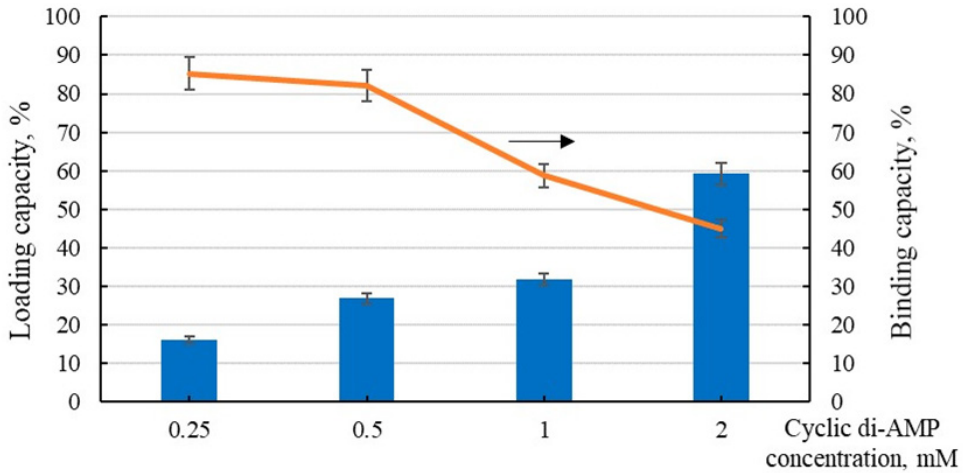

Fig. 3. Binding of cyclic di-AMP to LDH

In order to investigate the prospects of using intercalated LDH nanohybrid as a drug delivery system, deintercalation of cyclic di-AMP was examined under different pH values (7.4 and 4.5). These pH parameters were chosen in line with the data that blood in the vessels is circulating at pH about 7.4, and pH inside the lysosomes is lying in the range from 4 to 5. Sodium citrate-phosphate buffer was applied to provide stable pH values in the appropriate range. Typical kinetic curves of cyclic di-AMP release from the nanocomposites at different pH values are shown in Fig. 4.

The elution curves indicate fast release of cyclic di-AMP into supernatants in all samples, except one incubated in mQ water, regardless of pH value of the solutions.

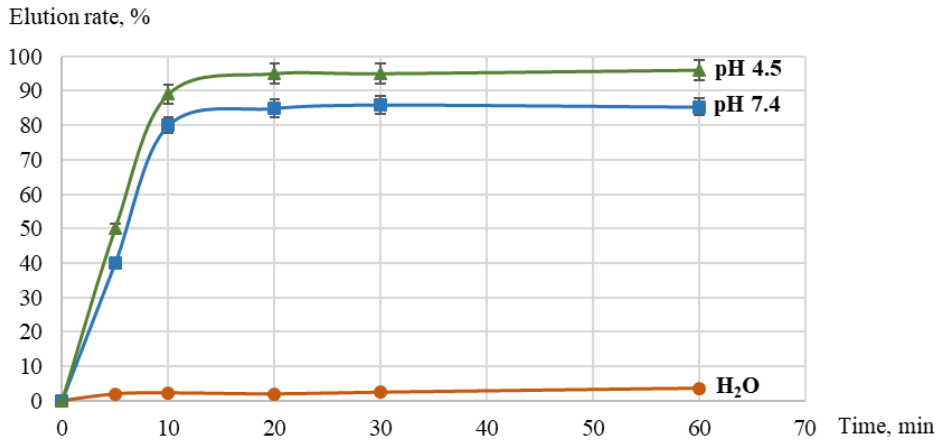

Fig. 4. The release of cyclic di-AMP from complex with LDH in water, in buffer at pH 7.4 and pH 4.5

The obtained results match the literature data stating that the exchange ability of supplied anions tends to augment with increasing charge and decreasing ionic radius. The order of intercalation is as follows: $CO_3^{2-} > HPO_4^{2-} > SO_4^{2-}$ for divalent anions [16]. It seems logical therefore that phosphate ions show high affinity for interlayer space and can readily displace cyclic di-AMP molecules resulting in their fast elution rate even around pH 7.4.

The experimental results show that the nanoparticles of all formed complexes differ in size (Fig. 5).

The proven fact of cyclic di-AMP liberation from LDH complexes evidences in favor of engaging the studied system for delayed delivery of active dinucleotide to the target cells.

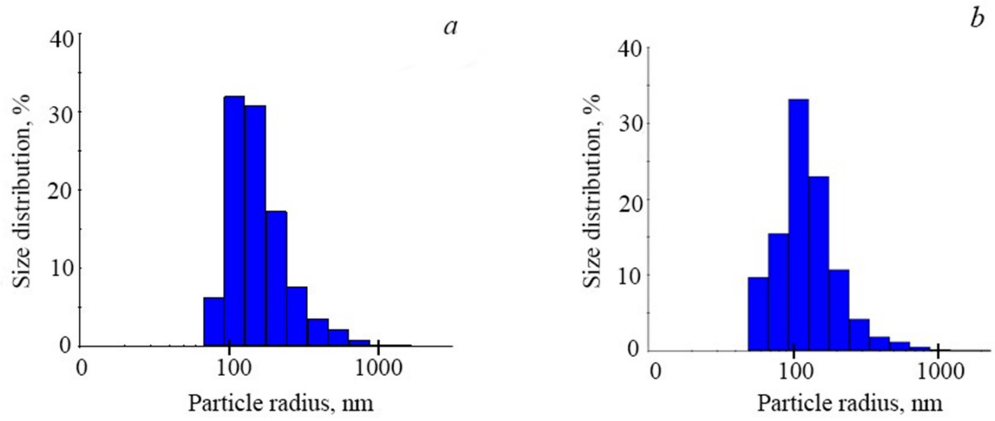

Fig. 5. The size of obtained particles: empty LDH (*a*); cyclic di-AMP/LDH complex (*b*)

Conclusion. Earlier we proposed to use the inducer of endogenic interferon cyclic di-AMP as the universal antiviral agent. The advantage of this compound over the other numerous interferon inducers is that cyclic di-AMP represents an element of naturally evolved mechanism ensuring universal protection of vertebrata from attack of multiple viral pathogens. Unfortunately, a molecule of this dinucleotide carries two negative charges presumably complicating its penetration within virus-infected and malignant cells also charged negatively on the surface.

In literature the problem is solved by immobilizing active compounds on positively charged supports, including LDH carriers.

The present study revealed the fact of delayed cyclic di-AMP release from LDH complex in mildly acidic media and indicated the principal possibility of applying the studied nanocomplexes for prolonged delivery of cyclic di-AMP to target immune cells.

References

1. Pagani I., Ghezzi S., Alberti S., Poli G., Vicenzi E. Origin and evolution of SARS-CoV-2. *European Physical Journal Plus*, 2023, vol. 138, no. 2, art. 157. https://doi.org/10.1140/epjp/s13360-023-03719-6

2. Zinchenko A., Vinter M., Kazlovskii I. Interferons and their inducers as the elements to control COVID-19. *Nauka i innovatsii* [Science and innovations], 2023, no. 2, pp. 24–29 (in Russian).

3. Yin W., Cai X., Ma H., Zhu L., Zhang Y., Chou S. H., Galperin M. Y., He J. A decade of research on the second messenger c-di-AMP. *FEMS Microbiology Reviews*, 2020, vol. 44, no. 6, pp. 701–724. https://doi.org/10.1093/femsre/fuaa019

4. Deng L., Liang H., Xu M., Yang X., Burnette B., Arina A. [et al.]. STING-dependent cytosolic DNA sensing promotes radiation-induced type I interferon-dependent antitumor immunity in immunogenic tumors. *Immunity*, 2014, vol. 41, no. 5, pp. 843–852. https://doi.org/10.1016/j.immuni.2014.10.019

5. Ebensen T., Arntz A., Schulze K., Hanefeld A., Guzman C. A., Scherließ R. Pulmonary application of novel antigen-loaded chitosan nanoparticles co-administered with the mucosal adjuvant c-di-AMP resulted in enhanced immune stimulation and dose sparing capacity. *Pharmaceutics*, 2023, vol. 15, no. 4, art. 1238. https://doi.org/10.3390/pharmaceutics15041238

6. Wehbe M., Wang-Bishop L., Becker K. W., Shae D., Baljon J. J., He X., Christov P., Boyd K. L., Balko J. M., Wilson J. T. Nanoparticle delivery improves the pharmacokinetic properties of cyclic dinucleotide STING agonists to open a therapeutic window for intravenous administration. *Journal of Controlled Release*, 2021, vol. 330, pp. 1118−1129. https://doi.org/10.1016/j.jconrel.2020.11.017

7. Yang Y., Zhao X., Zhu Y., Zhang F. Transformation mechanism of magnesium and aluminum precursor solution into crystallites of layered double hydroxide. *Chemistry of Materials*, 2012, vol. 24, no. 1, pp. 81–87. https://doi.org/10.1021/cm201936b

8. Matusik J. Special issue: layered double hydroxides (LDH) and LDH-based hybrid composites. *Materials*, 2021, vol. 14, no. 10, art. 2582. https://doi.org/10.3390/ma14102582

9. Cavani F., Trifiro F., Vaccari A. Hydrotalcite-type anionic clays: preparation, properties and applications. *Catalysis Today*, 1991, vol. 11, pp. 173–301.

10. Kumari S., Sharma A., Kumar S., Thakur A., Thakur R., Bhatia S. K., Sharma A. K. Multifaceted potential applicability of hydrotalcite-type anionic clays from green chemistry to environmental sustainability. *Chemosphere*, 2022, vol. 306, art. 135464. https://doi.org/10.1016/j.chemosphere.2022.135464

11. Choi S. J., Oh J. M., Choy J. H. Anticancer drug-layered hydroxide nanohybrids as potent cancer chemotherapy agents. *Journal of Physics and Chemistry of Solids*, 2008, vol. 69, no. 5–6, pp. 1528–1532. https://doi.org/10.1016/j.jpcs.2007.10.140

12. Olfs H.-W., Torres-Dorante L. O., Eckelt R., Kosslick H. Comparison of different synthesis routes for Mg–Al layered double hydroxides (LDH): Characterization of the structural phases and anion exchange properties. *Applied Clay Science*, 2009, vol. 43, no. 3–4, pp. 459–464. https://doi.org/10.1016/j.clay.2008.10.009

13. Vinter M. A., Kazlouski I. S., Zinchenko A. I. Construction of bacterial strain forming inclusion bodies, exhibiting diadenylate cyclase activity. *Molekulyarnaya i prikladnaya genetika* [Molecular and applied genetics], 2022, vol. 33, pp. 105–109. https://doi.org/10.47612/1999-9127-2022-33-76-82

14. Olanrewaju J., Newalkar B. L., Mancino C., Komarneni S. Simplified synthesis of nitrate form of layered double hydroxide. *Materials Letters*, 2000, vol. 45, no. 6, pp. 307–310. https://doi.org/10.1016/S0167-577X(00)00123-3

15. Doson R., Elliot D., Elliot U., Dzhons K. *Biochemist's Handbook*. Moscow, Mir Publ., 1991. 543 p. (in Russian).

16. Ibrahimova K. A. The synthesis methods and applications of layered double hydroxides – a brief review. *Vestnik Natsional'nogo yadernogo tsentra Respubliki Kazakhstan* [Bulletin of the National Nuclear Center of the Republic of Kazakhstan], 2022, no. 4, pp. 16–29. https://doi.org/10.52676/1729-7885-2022-4-17-29

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