NUTRITION ADDITIVE OF E322 AS PHOSPHATIDYLCHOLINE SOURCE FOR ENVELOPE IN NANOSCALE FORMULATION

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Abstract. Microporous nanomaterials can provide interesting tools for different goals from bioimaging to the delivery of bioactive molecules. In this study, the procedure based on cryoapproaches was designed to formulate the nanoparticles of clinoptilolite from Zeolites mineral family. Applying scanning electron microscopy, clinoptilolite particles were imaged. Retting the nanoparticles in the ethanol solution of phosphatidylcholine (lecithin), the particle was encapsulated in lecitin envelope. Registering the diminution of optical density (OD 235) for ethanol/lecitin solution, the sorption of lecitin by clinoptilolite nanoparticles was studied with UV spectrometry. The kinetics of lecitin/clinoptilolite complex development was shown to exhibit intricate behavior, when the adsorption of lecitin was followed with its gradual desorption resulting in the increase of lecitin content in ethanol solution back toward the original level. The distribution of final size for lecitin/clinoptilolite complex was determined with dynamic light scattering technique. To our knowledge, there are no reports of natural zeolite nanoparticles that were used as the platform for nanosize capsule with phospholipids shell.

***Key words**: phosphatidylcholine (lecithin), UV spectrometry, scanning electron microscopy, nanocapsule.*

The most natural way of introducing biologically active molecules into the body is through the gastrointestinal tract. This approach is especially effective if it is necessary to directly deliver substances to the gastrointestinal epithelium [1]. This situation occurs during the treatment of a number of complications due to gastrointestinal upset. However, the effect of the drug can be significantly reduced by enzymes (peptidases, proteases, amylases, lipases, nucleases) and acidity (pH ~ 2–8). In order to maintain specific activity in an aggressive environment of the stomach and intestinal lumen, the introduced substances are converted into a colloid or suspension of nanoparticles [2-6].

Inorganic substances are considered as promising materials for creating the micro and nanocontainers [7-9]. Zeolite is one of the most abundant natural mineral, widely distributed throughout the world and used in food production and agricultural technologies [10-13]. The microporous structure significantly increases the surface area of zeolite particles available for adsorption. Among the naturally occurring Zeolites family, clinoptilolite is the most widespread and studied for biomedical purposes as the antioxidant [14] and anti-inflammatory agents [15], and excellent detoxifying [16]. A method has been developed to obtain synthetic zeolite nanoparticles in laboratory conditions [17-22]. Even though there are several synthetic species of zeolites, their production is limited by small volumes, the cost of final products and the need to purify it from the ingredients involved in the synthesis. An alternative to synthetic nanoparticles may be natural zeolite, which is mined on an industrial scale [12, 23].

In the case of mucous epithelium, the affinity for the nanoparticle is with the mucous layer, which contributes to its adhesion on the surface of the layer and further diffusion to the apical membrane of enteric cells. The coating of particle with shell allows the biocompatibility of water-insoluble [24-26] or solid materials [27, 28]. For this purpose, the surface of nanoparticles can be modified with phospholipids [29, 30] the main components of cellular membranes. The phospholipids shell also provides prolonged release of the active substance and protects it from the aggressive environment of gastrointestinal tract. Therefore, a structure of zeolite (core) and phospholipid (shell) is considered a promising composition for producing a nanoplatform.

An example of the successful use of aluminosilicate for the transport of biomolecules is a study in which a synthetic zeolite-L nanocrystal was employed as a container for the delivery of nucleic acids and organic molecules [24]. In this work, a poly-L-lysine coating allows the transport of the nanoparticle into the HeLa cell to be more efficient. To our knowledge, there are no reports of natural zeolite nanoparticles with phospholipids shell. In addition, there is no data on how a phospholipid will behave when interacting with negatively charged clinoptilolite nanoparticles. Thus, the aim of this work was to: (1) develop a method for producing nanoscale particles of natural clinoptilolite, (2) study the sorption of phosphatidylcholine by the particles obtained.

MATERIALS AND METHODS

Pretreatment of clinoptilolite. We used the commercial product Litovit M (Russia), which was manufactured from a natural zeolite originated from Kholinsky deposit, Russia. Cascade mechanical activation allowed the industrial method to purify natural zeolite from impurities and to obtain the powder of 100% content for active component - clinoptilolite. The microsize particles were achieved after clinoptilolite powder is triturated in a porcelain mortar at room temperature.

Scanning electron microscopy. The investigated **s**amples were visualized by scanning electron microscopy. The suspension of clinoptilolite particles in distilled water (30 mg/100 ml) was pre-treated with ultrasound for 20 seconds.

The suspension droplet of ~ 2 μl was placed on the surface of the sample holder for the electron microscope. After the water was evaporated at room temperature in the stream of clean air, the specimen was coated with approximately 10 nm thick platinum layer in a JFC-1600 (JEOL, Japan) unit. A Pt film significantly enhances the signal of secondary electrons, removes the electrostatic charge and protects the sample from heating, which causes its mechanical destruction. The fine structure of the object's relief was studied in a scanning electron microscope JSM-6390A (JEOL, Japan), at an accelerating voltage of 25 kV in the secondary electron mode.

Extraction of phosphatidylcholine. Phosphatidylcholine was extracted from nutrition additive of E322 (Cargill Lecigran 1000P, Germany), which is a mixture of polar phospholipids. A portion of 600 mg was thoroughly mixed in 6 ml of 100% Ethyl Alcohol (ETON) until a homogeneous suspension. The extraction of ETON soluble phosphalidylcholine (lecithin) was carried out at room temperature for 24 hours. The undissolved component was separated by centrifugation at 600g for 10 minutes. The uterine extract obtained in this way was taken into a clean glass sealed tube, which was stored at 4 ° C in the dark. For the experiments, a working solution was used, which was obtained by diluting the mother liquor 30 times with ethanol.

Application of phosphatidylcholine to the particle surface. A portion of 20 mg of clinoptilolite particles was incubated at room temperature for a different time interval (1 / 4h, 1 / 2h, 1h, 1.5h, 2h, 3h, 4h, 6h) in 4 ml of a working solution of lecithin stirring with horizontal shaking. The resulting suspension was centrifuged at 600 g for 10 minutes. Then, 3 ml of supernatant was taken into a quartz cuvette. The lecithin solution sample thus obtained was investigated by UV spectrometry.

UV spectrometry. In the ultraviolet region of the absorption spectrum of phosphatidylcholine, three maxima are recorded: 200-210 nm, 235 nm and 280 nm. For measurements, we used the absorption peak of carbonyl groups at a wavelength of 235 nm. The choice was due to the instability of the signal at a wavelength of 200-210 nm, as well as a relatively low peak level at 280 nm. The optical density of the initial working solution of lecitin at a characteristic wavelength (235 nm) is 0.76 units, which corresponds to optimal conditions for analytical measurements on a Specord M40 spectrophotometer (Carl Zeiss, Germany).

RESULTS AND DISCUSSION

Obtaining nanoscale particles of natural clinoptilolite. After grinding in a planetary mill and a porcelain mortar, clinoptilolite powder was ground at -35 ° C in an agate mortar. The powder obtained at low temperature was transferred

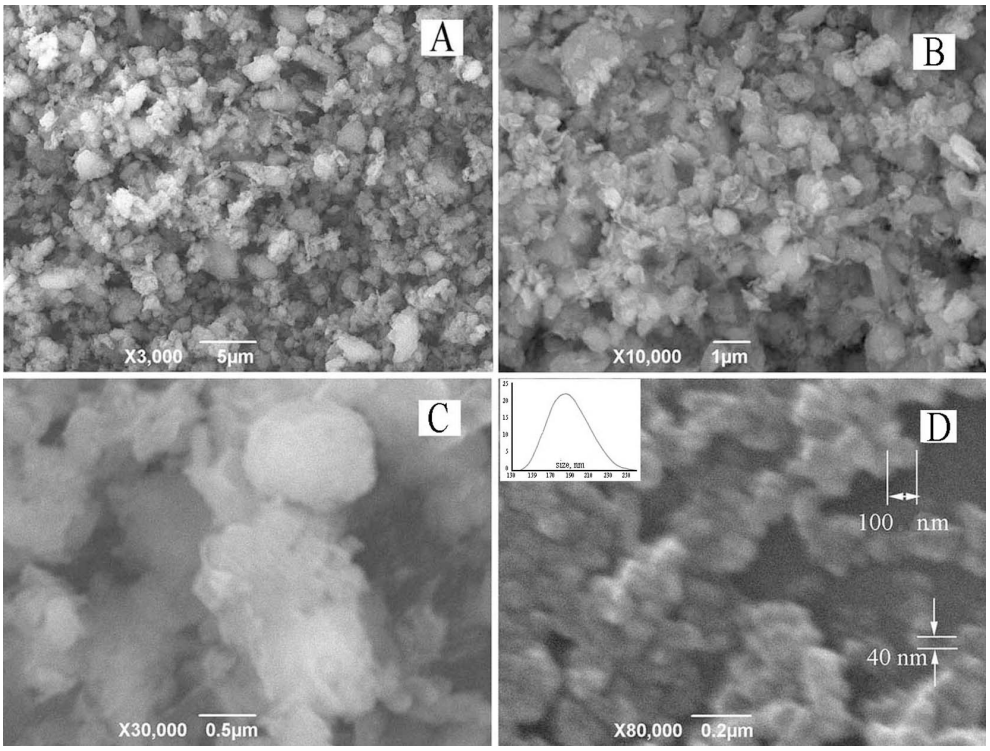

Figure 1. Microphotographs of clinoptilolite particles: (A) the original sample of "Litovit M"; (B) the powder after grinding "Litovit M" in a planetary ball mill; (C) the powder obtained after grinding the powder in a porcelain mortar at room temperature; (D) nanosize particles obtained after additional grinding of powder in an agate mortar at a low temperature of -35 ° C followed by low-temperature dehydration in vacuum. Insertion: the size distribution of nanoscale particles of clinoptilolite with a lecithin shell

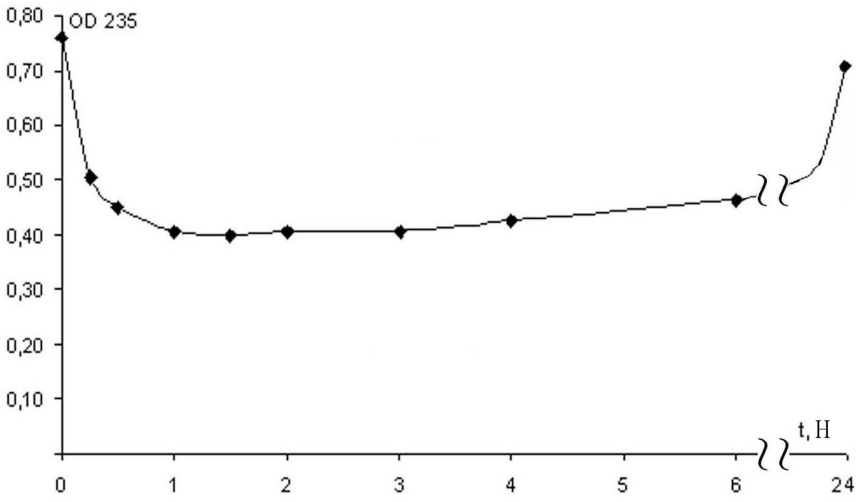

Figure 2. The change in optical density (OD 235) at a wavelength of 235 nm in the absorption spectrum of the working solution of alcohol-soluble lecitin depending on the incubation time of clinoptilolite nanoparticles in it

in vapor of liquid nitrogen to a vacuum chamber, where the sample was heated to room temperature. This technique eliminates the condensation of water vapor from the atmosphere to the surface of a cold sample, which causes aggregation of particles. The obtained clinoptilolite sample was visualized by scanning electron microscopy. Figure 1 shows the set of microphotographs demonstrating changes in the size of clinoptilolite particles at different stages of the proposed technology.

It is seen that after the planetary mill from the initial preparation (Fig. 1A) we obtain micron-sized particles (Fig. 1B). As a result of subsequent grinding of the sample in a porcelain mortar at room temperature, spherical submicron clusters are formed (Fig. 1C). After the final stage of low-temperature processing, nanosized particles were obtained (Fig. 1D), the magnitude of which varies. The inset in the figure shows a histogram of the size distribution of the clinoptilolite / lecithin complex in the range of 140 nm - 260 nm and a maximum in the region of 190 nm. Data obtained by dynamic light scattering.

Clinoptilolite/lecithin complex. Sorption of the obtained zeolite particles of lecithin from the working solution was carried out in accordance with the described procedure. The presence of an effect was determined by a decrease in the content of phosphatidylcholine, measuring the optical density of the solution at a wavelength of 235 nm (OD 235). Figure 2 shows the value of this parameter at different time intervals for the incubation of nanoparticles.

It is seen (Fig. 2) that the kinetics of sorption of phosphatidylcholine is complex. A decrease in optical density means a decrease in the concentration of lecithin in solution as a result of the formation of a clinoptilolite / lecithin complex. This process is realized through adsorption, since the size (~ 0.2 nm) of the pores of the zeolite does not allow phospholipid molecules to penetrate into the particles. Thus, a nanoscale platform is formed with a clinoptilolite core and a layer of lecithin on the surface.

Within two hours, the lecithin content in the solution reaches a minimum value (~ 0.4 OD), but the adsorption process is reversible. Over time, the optical density of the solution is restored to the initial level (Fig. 2), which means the presence of desorption of phosphatidylcholine. This experimental fact allows us to apply the method of direct identification of the presence of clinoptilolite / lecithin complex. For this purpose, the precipitate, which was obtained by incubating 20 mg of zeolite particles in 4 ml of a working solution of lecithin, was transferred to 15 ml of distilled water to remove unbound phosphatidylcholine. After centrifugation (600 g) of the suspension, water was carefully taken and 3 ml of ethanol was added to the precipitate. Further desorption of lecithin was carried out for 24 hours at room temperature in the dark. The effect was evaluated by increasing the optical density (OD 235nm) of the alcohol solution (Fig. 3).

The diagram (Fig. 3) shows that relative to the beginning of the experiment (Fig. 3-1), as a result of phospholipid adsorption on clinoptilolite particles, the concentration of lecithin in the working solution decreases (Fig. 3-2). After adding ethanol to the sediment of the clinoptilolite/lecithin complex (Fig. 3-3), desorption of phosphatidylcholine is observed. As a result, an increase in the optical density of the solution is recorded (Fig. 3-4), which indicates the presence of a lecithin shell in the zeolite particle. The combination of nanoscale and phospholipid coating is a prerequisite for the successful diffusion of particles through the barrier layer of mucus of the enteric epithelium [31, 32]. We met these criteria when creating a nanoplatform from a natural clinoptilolite with a lecithin shell. The possibility of transferring biomolecules by a zeolite nanoparticle is shown by the example of nucleic acids [24]. However, one should take into account not only the carrier properties of the zeolite nanoparticles, but other possible uses including excellent detoxifying [16] and as the antioxidant [14] or anti-inflammatory agents [15].

The data obtained allow us to make the following conclusions. This work describes a very first example of the use of nanoparticles from zeolite of natural origin as cores to create the capsule with phospholipide shell. Clinoptilolite nanoscale particles were successfully produced using ordinary laboratory equipments and the commercial product containing clinoptilolite (100%). The mode size of ~100 nm was achieved after its grinding at low temperature of -35ºC

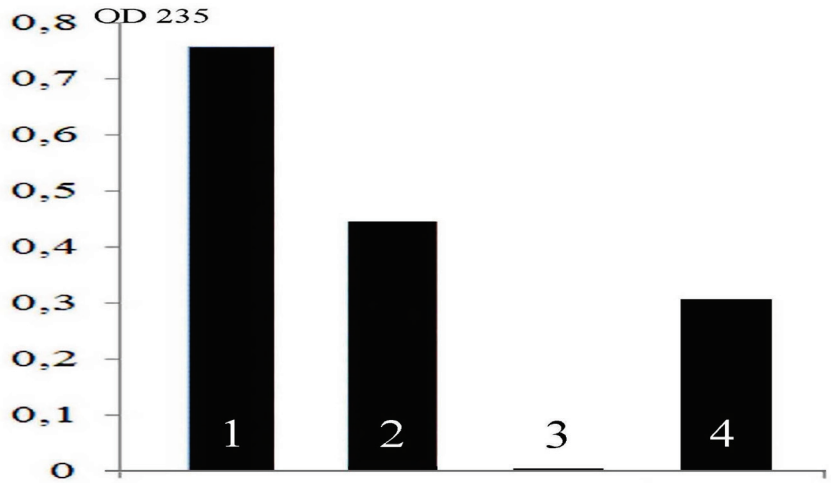

Figure 3. Changes of optical density (OD 235) at a wavelength of 235 nm in the absorption spectrum of lecitin/ethanol solution: (1) the solution of lecithin in ethanol, (2) after incubation (2 hours) of clinoptilolite nanoparticles in lecitin/ethanol solution, (3) ethanol, (4) after incubation (24 hours) of clinoptilolite/lecitin complex in ethanol

followed with freeze-drying. These results are very promising from the point of view of micro- and nanogrinding of tailored products. Using a solution of phosphatidylcholine in ethanol, a method for creating a nanoplatform based on a clinoptilolite core with a phospholipid shell has been developed. It was shown that the formation of lecitin/clinoptilolite complex has complex kinetics when the adsorption of lecithin with its subsequent desorption into a ethanol is recorded in the initial phase.

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References:

1. Simonoska-Crcarevska M., Glavas-Dodov M., Goracinova K. Chitosan coated Ca–alginate microparticles loaded with budesonide for delivery to the inflamed colonic mucosa. *Eur. J. Pharmaceutics and Biopharmaceutics*, 2008, vol. 68, pp. 565-578.

2. Render D., Samuel T., King H., Vig M., Jeelani S., Babu R.J., Rangari V. Biomaterial-Derived Calcium Carbonate Nanoparticles for Enteric Drug Delivery. *J. Nanomaterials*, 2016, vol. 2016, pp. 1-8.

3. Allemann E., Leroux J.-C., Gurny R. Polymeric nano- and microparticles for the oral delivery of peptides and peptidomimetics. *Advanced Drug Delivery Rev*., 1998, vol. 34, pp. 171-189.

4. Hu Y., Jiang X., Ding Y., Ge H., Yuan Y., Yang C. Synthesis and characterization of chitosan–poly (acrylic acid) Nanoparticles. *Biomaterials*, 2002, vol. 23, pp. 3193-3201.

5. Jintapattanakit A., Junyaprasert V.B., Maob S., Sitterberg J., Bakowsky U., Kissel T. Peroral delivery of insulin using chitosan derivatives: A comparative study of polyelectrolyte nanocomplexes and nanoparticles. *Int. J. Pharmaceutics*, 2007, vol. 342, pp. 240-249.

6. Zhu J., Hayward R.C. Hierarchically Structured Microparticles Formed by Interfacial Instabilities of Emulsion Droplets Containing Amphiphilic Block Copolymers. *Angew Chem. Int. Ed*., 2008, vol. 47, pp. 2113-2116.

7. Yu X., Khalil A., Dang P.N., Alsberg E., Murphy W.L. Multilayered Inorganic Microparticles for Tunable Dual Growth Factor Delivery. *Adv. Funct. Mater*., 2014, vol. 24, pp. 3082-3093.

8. Kar S. An Overview of Recent Advances in Application of Some Inorganic Materials-Biological and Technological Perspectives *J. Biotechnol. Biomater.*, 2016, vol. 6, pp. 565-578.

9. Stefanache A., Ignat M., Peptu C.A., Diaconu A., Stoleriu I., Ochiuz L. Development of a Prolonged-Release Drug Delivery System with Magnolol Loaded in Amino-Functionalized Mesoporous Silica *Appl. Sci.*, 2017, vol. 7, pp. 237-250.

10. Kojića D., Pajevića S., Jovanović-Galovića A., Puraća, J.E., Škondrićb S., Milovaca S., Popovića Ž., Grubor-Lajšić G. Efficacy of natural aluminosilicates in moderating drought effects on the morphological and physiological parameters of maize plants (*Zea mays* l.). *J. Soil Sci. and Plant Nutrition*, 2012, vol. 12, pp. 113-123.

11. Yada R.Y., Buck N., Canady R., DeMerlis C., Duncan T., Janer G., Juneja L., Lin M., McClements D.J., Noonan G., Oxley J., Sabliov Cr., Tsytsikova L., Vázquez-Campos S., Yourick J., Zhong Q., Thurmond S. Engineered Nanoscale Food Ingredients: Evaluation of Current Knowledge on Material Characteristics Relevant to Uptake from the Gastrointestinal Tract. *Food Science and Food Safety*, 2014, vol. 13, pp. 730-744.

12. Bohacs K., Faitli J., Bokanyi L., Mucsi G. Control of Natural Zeolite Properties by Mechanical Activation in Stirred Media Mill. *Arch. Metall. Mater*., 2017, vol. 62, (2B), pp. 1399-1406.

13. Hazra D.K. Nano-formulations: High Definition Liquid Engineering of Pesticides for Advanced Crop Protection in Agriculture. *Advances in Plants & Agriculture Research*, 2017, vol. 6, pp. 00211-00212.

14. Pellegrino P., Mallet B., Delliaux S., Jammes Y., Guieu R., Schaf O. Zeolites are effective ROS-scavengers in vitro. *Biochemical and Biophysical Research Communications*, 2011, vol. 410, pp. 478-483.

15. Bacakova L., Vandrovcova M., Kopova I., Jirka I. Applications of zeolites in biotechnology and medicine – a review. *Biomater. Sci*., 2018, vol. 6, pp. 974-989.

16. Selim M.M., El-Mekkawi D.M., Aboelenin R.M.M., Sohair A.S.A., Ghada M.M. Preparation and characterization of Na-A zeolite from aluminum scrub and commercial sodium silicate for the removal of Cd^{2+} from water. *J. Association of Arab Universities for Basic and Applied Sciences*, 2017, vol. 24, pp. 19-25.

17. Johnson E.B.G., Arshad S.E. Hydrothermally synthesized zeolites based on kaolinite: A review. *Appl. Clay Sci*., 2014, vol. 98, pp. 215-221.

18. Severance M.A. N*anocrystalline Zeolites: Synthesis, Mechanism, and Applications*. Dissertation for the Degree Doctor of Philosophy in the Graduate School of The Ohio State University, 2014.

19. Tian Y. *Synthesis and Characterization of Crystalline Microporous Materials: Investigation of New Synthetic Routes*. A Thesis for the Degree of PhD at the University of St Andrews, 2014.

20. Mintova S., Grand J., Valtchev V. Nanosized zeolites: Quo Vadis? *C. R. Chimie*, 2016, vol. 19, pp. 183-191.

21. Tiburu E.K., Salifu A., Aidoo E.O., Fleischer H.N.A., Manu G., Yaya A., Zhou H., Efavi J.K. Formation of Chitosan Nanoparticles Using Deacetylated Chitin Isolated from Freshwater Algae and Locally Synthesized Zeolite A and their Influence on Cancer Cell Growth. *J. Nano Research*, 2016, vol. 48, pp. 156-170.

22. Tosheva L., Belkhair S., Gackowski M., Malic S., Al-Shanti N., Verran J. Rapid screening of the antimicrobial efficacy of Ag zeolites. *Colloids and Surfaces B: Biointerfaces*, 2017, vol. 157, pp. 254-260.

23. Elhassan A.M. Determination of the Natural Zeolites Characteristics in Sudan Country. *Int. J. Current Advanced Res*., 2016, vol. 5, pp. 1304-1310.

24. Bertucci A., Lülf H., Septiadi D., Manicardi A., Corradini R., De Cola L. Intracellular Delivery of Peptide Nucleic Acid and Organic Molecules Using Zeolite-L Nanocrystals. *Adv. Healthcare Mater*, 2014, vol. 3, pp. 1812-1817.

25. Li Le-Le, Zhang R., Yin L., Zheng K., Qin W., Selvin P.R., Lu Y. Biomimetic Surface Engineering of Lanthanide-Doped Upconversion Nanoparticles as Versatile Bioprobes. *Angew Chem. Int. Ed. Engl*., 2012, vol. 51, pp. 6121-6125.

26. Euliss L.E., DuPont J.A., Gratton S., DeSimone J. Imparting size, shape, and composition control of materials for nanomedicine. *Chem. Soc. Rev.*, 2006, vol. 35, pp. 1095-1104.

27. Grenha A., Remunan-Lopez C., Carvalho E.L.S., Seijo B. Microspheres containing lipid/chitosan nanoparticles complexes for pulmonary delivery of therapeutic proteins. *Eur. J. Pharmaceutics and Biopharmaceutics*, 2008, vol. 69, pp. 83-93.

28. Shchukina E.M., Shchukin D.G. Layer-by-layer coated emulsion microparticles as storage and delivery tool. *Current Opinion in Colloid & Interface Science*, 2012, vol. 17, pp. 281-289.

29. Fricker G., Kromp T., Wendel A., Blume A., Zirkel J., Rebmann H., Setzer C., Quinkert R., Martin F., Müller-Goyman C. Phospholipids and Lipid-Based Formulations in Oral Drug Delivery. *Pharm. Res.*, 2010, vol. 27, pp. 1469-1486.

30. Baldassarre F., Allegretti C., Tessaro D., Carata E., Citti C., Vergaro V., Nobile C., Cannazza G., D'Arrigo P., Mele A., Dini L., Ciccarella G. Biocatalytic Synthesis of Phospholipids and Their Application as Coating Agents for CaCO3 Nano–crystals: Characterization and Intracellular Localization Analysis. *ChemistrySelect*, 2016, vol. 1, pp. 6507-6514.

31. Ensign L.M., Cone R., Hanes J. Oral drug delivery with polymeric nanoparticles: The gastrointestinal mucus barriers. *Advanced Drug Delivery Reviews*, 2012, vol. 64, pp. 557-570.

32. Kotta S., Wadood Khan A., Pramod K., Ansari S.H., Sharma R.K., Ali J. Exploring oral nanoemulsions for bioavailability enhancement of poorly water-soluble drugs. *Expert Opin. Drug Deliv*., 2012, vol. 9, pp. 585-598.