EXPERIMENTAL ARTICLES

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SYNTHESIS AND CHARACTERIZATION OF POLY(D,L-LACTIC-CO-GLYCOLIC)ACID MICROPARTICLES LOADED BY DIPHTHERIA TOXOID

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The aim of the study was to synthesize poly(D,L-lactide-co-glycolide) particles — PLGA and obtain their complexes with a non-toxic diphtheria toxin recombinant fragment subunit B fused with enhanced green fluorescent protein EGFP-SubB; to characterize the main physical and chemical properties of the resulting complexes.

Two types of the diptheria toxoid loaded PLGA microparticles were obtained: particles with immobilized diphtheria toxoid (PLGA 1) and particles with encapsulated diphtheria toxoid (PLGA 2). Micropartcles obtaines were characterized for antigen loading, particle size, polydispersity index and in vitro antigen release.

Key words: diphtheria toxoid, poly (lactic-co-glycolic-acid) microparticles.

Diphtheria toxin (DT) is one of the most important pathogenic factors of diphtheria bacilli *Corynebacterium diphtheriae* [1–4]. It consists of single polypeptide chain, which can be broken into two subunits A and B. Subunit A possess catalytic activity, and subunits B is responsible for binding to diphtheria toxin receptor HB-EGF (heparin binding EGF-like grows factor) on cell surface [5].

Diphtheria is an infectious disease that affects only humans. Highly infectious disease can be transmitted from human to human airborne. This disease was very dangerous and killed amount of humans until vaccination era. Vaccination by diphtheria toxoid is one of the precautions that can be taken against this disease [6].

Vaccination with the diphtheria toxoid was discovered by Ramon Gaston during the 1920s has been used in medical practice. Currently, vaccination against this disease depends on cultivation of *C. diphtheriae* bacilli in bio-secure conditions and inactivation of toxin by chemical methods. New generation more effective vaccines need to be developed. In the last few decades the studies to develop a recombinant vaccine against diphtheria focused on obtaining inactive mutants of DT-toxoids. For this reason, using adjuvants in order to obtain a more robust immune response is aimed.

Particle carriers are effective delivery systems for antigens and increase the antigen uptake on cellular level. They are effective on controlled antigen release and can protect the antigen integrity from degradation [7].

Fortunately, modern nanotechnology provides new scientific means for mucosal immune response regulation. Biodegradable and biocompatible polymers, like poly(D,L lactide-co-glycolide) (PLGA), are widely used for the design of mucosal immunizing tools. PLGA, which one of the most widely used co-polymer, is a biopolymer approved by FDA and has several advantages such has inert properties in physical environments, degradability in biological environments, biocompatible properties and being able

to be degraded into non-toxic products [8]. Importantly, that the way of particle preparation plays an important role in PLGA biodegradation and antigen release.

It is believed, that strong induction of antitoxic immunity in mucosal tissues can protect the body against the infection. All current diphtheria vaccines have parenteral route of administration. Such vaccines results in formation of high serum antitoxin levels in the blood, predominantly IgG class. However, most important role in the mucosal surfaces protection is playing secretory IgA antibodies [7]. Undoubtedly, oral administration of antigens would be the most patient-friendly way of immunization. However, the efficacy of free antigens oral administration is limited by their degradation in the gastrointestinal tract and poor absorption by M-cells (microfold cells) in the follicle-associated epithelium of the Peyer's patches, which play the major role in stimulating mucosal immunity [8].

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In this study, diphtheria toxin subunit B, genetically fused to EGFP, encapsulated PLGA micro-particles were synthesized for using vaccine models. Synthesized microparticles were characterized by scanning electron microscopy (SEM), loading yields and antigen release under acidic pH were measured.

Materials and Methods

Materials

In the work there were used: acrylamide, N,N′-methylenebisacrylamide from AppliChem GmbH (Germany); 2-mercaptoethanol from Helicon (Russia); plastic Petri dishes from Greiner BioOne (Austria); kanamycin, glucose from "Kyivmedpreparat", Arterium Co. (Ukraine); poly(lactic-co-glycolic acid) — PLGA, polyvinyl alcohol (PVA), dichloromethane (DCM), fetal bovine serum (FBS), LB medium, NaN_3, RPMI-1640 medium with L-glutamine, sodium dodecyl sulphate (SDS), stock solution of amphotericin B, penicillin G and streptomycin for cell culture, Trisma base, Triton X-100, urea from Sigma (USA); imidazole from Shanghai Synnad (China); KCl, NaCl, Na_2HPO_4, NaOH, KH_2PO_4 from Miranda-C (Ukraine); bovine serum albumin (BSA), isopropyl β-D-1-thiogalactopyranoside (IPTG), nickel-nitrilotriacetic acid-agarose (Ni-NTA agarose), prestained protein markers for gel electrophoresis from Thermo Fisher Scientific (USA).

All chemical reagents were of analytical grade. Ultra-pure water was acquired from Adrona Water Purification system.

Antigen preparation

The recombinant proteins used in this study: EGFP, EGFP-SubB, were obtained as previously reported [11]. Briefly, bacterial culture of Escherichia coli BL 21 (DE3) Rosetta (Novagen, Reno, NV USA) transformed by corresponding genetic constructions was grown at 37 °C under intensive stirring (250 rpm) up to extinction A600 — 0.5–0.7. Expression of the proteins was triggered via incubation with 1 mM of isopropyl β-D-1-thiogalactopyranoside (IPTG) up to 3 hours at 30 °C under intensive stirring (250 rpm). Recombinant protein was purified by the Ni^{2+}-NTA-agarose column chromatography with imidazole elution gradient and stored in PBS, pH = 7.2.

Electrophoretic separation of proteins

Electrophoresis in polyacrylamide gel with sodium dodecyl sulfate was performed following the modified method by H. Schagger [12].

Preparation of Microparticles

Two types of PLGA particles were prepared and characterized: particles with immobilized diphtheria toxoid (PLGA 1) and particles with encapsulated diphtheria toxoid (PLGA 2).

(1) The PLGA particles of the first type (PLGA 1) were prepared by solvent displacement method [13]. Briefly, 3 ml of 0.5% w/v PLGA (lactide:glycolide — 65:35) (Sigma-Aldrich Co., St. Louis, USA) in acetone were added drop-wise to 30 ml of pure distilled water under magnetic stirring. Acetone was evaporated overnight at room temperature.

Afterwards particles suspension was filtered through 10 μm filter. PLGA 1 particles were collected by centrifugation at 12,000 g for 3 min and resuspended in PBS contained protein (1% w/w PLGA). The emulsion was stirred overnight on magnetic stirrer at 4 °C. Afterwards, the particles were collected by centrifugation for 20 min at 12 000 rpm the pellet was resuspended in 1 ml of PBS and stored at 4 °C.

(2) The PLGA particles of the second type (PLGA 2) were prepared by previously reported method [14] with some modifications (double emulsification using solvent evaporation method). Briefly, 2 ml of 0.5% w/v PLGA (lactide:glycolide — 50:50) in methylene chloride were sonicated with 2 ml of protein solution (1 mg/ml) in 0.5% PVA by ultrasound homogenizer LabsonicM (Sartorius, Germany) for 2 min at an amplitude of 90%, and a duty cycle of 0.9 s. Afterward, the water-in-oil emulsion was added to 8 ml of 3% PVA solution and sonicated in ice bath for 10 min. Obtained emulsion was stirred overnight at room temperature on a magnetic stirrer for evaporation of the organic solvents. Afterwards, the particles were collected by centrifugation for 20 min at 12 000 rpm the pellet was resuspended in 1 ml of PBS and stored at 4 °C.

Characterization of Microparticles

In this study, obtained microparticles were detailed analyzed by following parameters: encapsulation efficiency (EE), drug loading (DL), particle size (Z-ave) and polydispersity index (PDI).

Antigen immobilization measurement

The electrophoretic separation of the obtained particles was carried out in a polyacrylamide gel with sodium dodecyl sulfate using the modified Schagger H. technique [12]. The effectiveness of antigen immobilization on the particles was determined by electrophoregram with densitometric method using TotalLab TL120 software. The effectiveness of antigen immobilization on the particles was determined by electrophoregram by densitometric method using Origin 8.0 (OriginLab Corporation, USA) and Fiji (Open Source software project) [15]. The effectiveness of immobilization was calculated based on the ratio of protein concentrations in the solution before and after conjugation with the particles.

EE was determined by using of the supernatants obtained after centrifugation for each of microparticles and determined using the formula given below:

$$EE\ (\%) = \frac{AA - AN}{AA} \times 100,$$

where AA — the amount of polypeptide initially added (mg);
AN — the amount of non-encapsulated polypeptides (mg).

The DL of polypeptide was calculated using the formula given below:

$$DL\ (\%) = \frac{AA - AN}{AM} \times 100,$$

where AA — the amount of polypeptide initially added (mg);
AN — wherehe amount of non-encapsulated polypeptides (mg);
AM — Amount of prodused particles.

Particle Size and Polydispersity Index of Microparticles

Determination of particle size was carried out using two different methods: Nanoparticle Tracking Analysis (NTA) using NanoSight NS300 (Malvern Instruments, UK) and dynamic light scattering (DLS) using Zetasizer Nano ZS Analyzers (Malvern Instruments, UK).

SEM Measurements of Microparticles

Particles size and morphology were studied using a scanning electron microscopy method (SEM) performed on a Mira 3 Tescan microscope, with an accelerating voltage of 10 kV, other parameter is shown on the pictures. To avoid sample charging during SEM investigation, samples was previously coated by thin conductive layer using Gatan Pecs, in this work we use 20 nm of Au/Pd.

In vitro antigen release studies

To determine the effect of the medium pH on the antigen release rate, the PLGA particles loaded with the recombinant antigen EGFP-SubB were incubated at pH 1.2 in an environment that simulates the acidity of the gastric juice (solution of hydrochloric acid at pH 1.2) and as a control at pH 7.4 in phosphate buffer solution (PBS). The microparticles suspension was incubated in a shaking incubator (100 rpm) at a stable temperature, close to the temperature of the human body — 37 °C. Samples were removed from solutions at various time intervals: after 15 min, 30 min, 60 min, 120 min and 240 min. Since the particles were loaded with fluorescent

recombinant antigen, the determination of their degree of degradation was carried out using Flow cytometry.

Results and Discussion

Encapsulation Efficiency and Drug Loading of Microparticles

A prerequisite for successful immunization and the development of an effective immune response is the sufficient amount of introduced antigen. In view of this, an important indicator is the effectiveness of the antigen sorption of particles.

We synthesized PLGA-based microparticles and conjugated them with the protein in two different techniques, which allowed obtaining two varieties of PLGA particles: particles with immobilized diphtheria toxoid (PLGA 1) and particles with encapsulated diphtheria toxoid (PLGA 2).

The effectiveness of antigen immobilization on the particles was determined by electrophoregram by comparing the amount of proteins initially added and in the samples containing the non-encapsulated proteins (supernatant after centrifugation). Densitometric analysis of electrophoresis results was performed using Fiji and OriginLab software. The effectiveness of immobilization was calculated based on the ratio of protein concentrations in the solution before and after conjugation with the particles. Obtained results are given in Table.

It can be seen that the EI was higher for particles synthesized by the second method (PLGA 1 — 72%, PLGA 2 — 90%). At the same time, by the flow cytometry it was demonstrated that 99% of the PLGA 1 particles are loaded with EGFP-SbB, while only 92% of PLGA 2 particles are bound to the protein. According to the obtained results, it can be said that the loading is more effective in encapsulating antigens within the particle, but the amount of particles containing the protein in its composition is slightly less in such synthesis technique.

Particle size Analysis of Microparticles

Also, produced microparticles were analyzed for particle size and polydispersity index (PDI) The particle sizes were slightly different according two distinct techniques (NTA — number based, the software tracks individual particles; DLS — scattering intensity weighted), however demonstrate similar patterns. The size distributions of the produced microparticles and PDI values were summarized in Fig. 1 and in Table.

NTA data showed that the particle sizes of PLGA 1 ranges from 50 nm to 510 nm, most of the particles had a size of 81.4 nm. The particle sizes of PLGA 2 ranges from 70 nm to 400 nm, most of the particles had a size of 155.9 nm. The mean particle sizes were 192.8 nm and 183.8 nm for PLGA 1 and PLGA 2, respectively (Fig. 2).

DLS data also showed that the mean PLGA 1 particles size was 203.3 nm and the

Microparticles properties including EE, DL, particle size and PDI

Type of particles	EE (%)	DL (%)	Mean Particle Size NTA (nm)	Mean Particle Size DLS (nm)	PDI
PLGA 1	725	7	183.8	203.3	0.143
PLGA 2	90	8	192.8	211.6	0.115

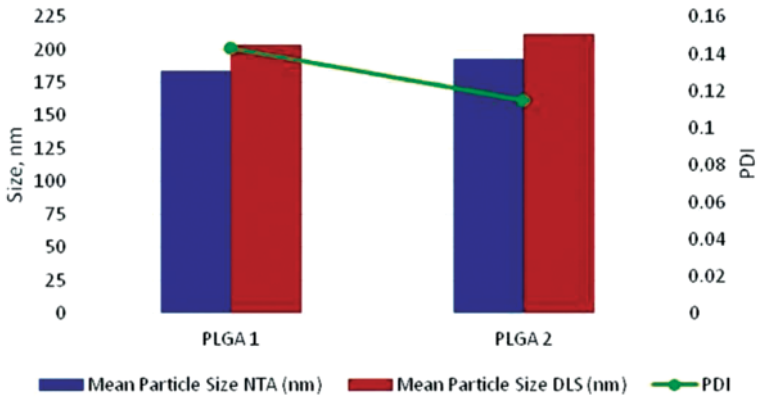


Fig. 1. **Z-ave of PLGA 1 and PLGA 2 particle sizes according two distinct techniques:** NTA and DLS (bars); and PDI (line)

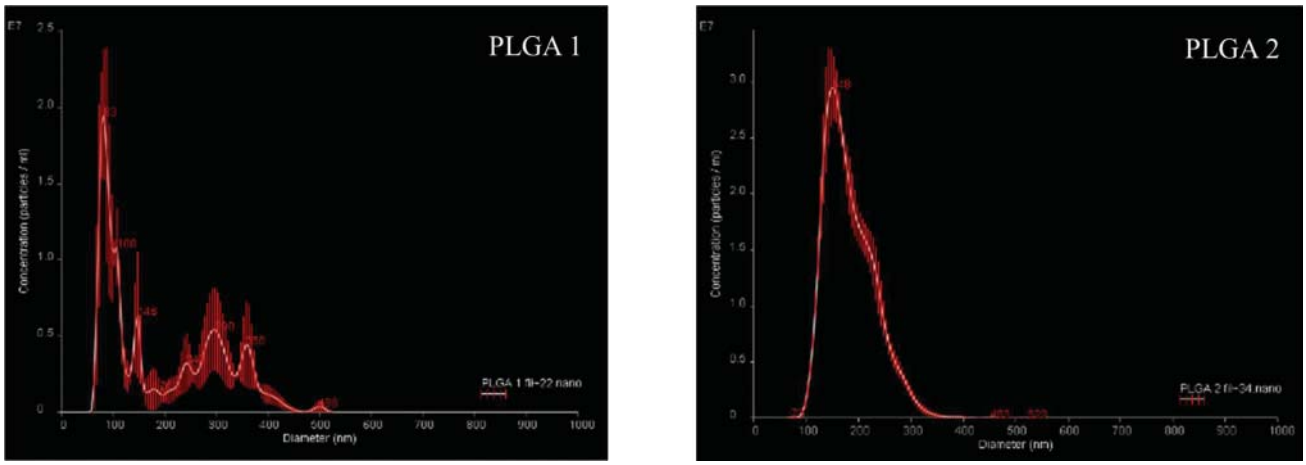


Fig. 2. **Particle size distribution of PLGA 1 and PLGA 2 respectively according NTA technique**

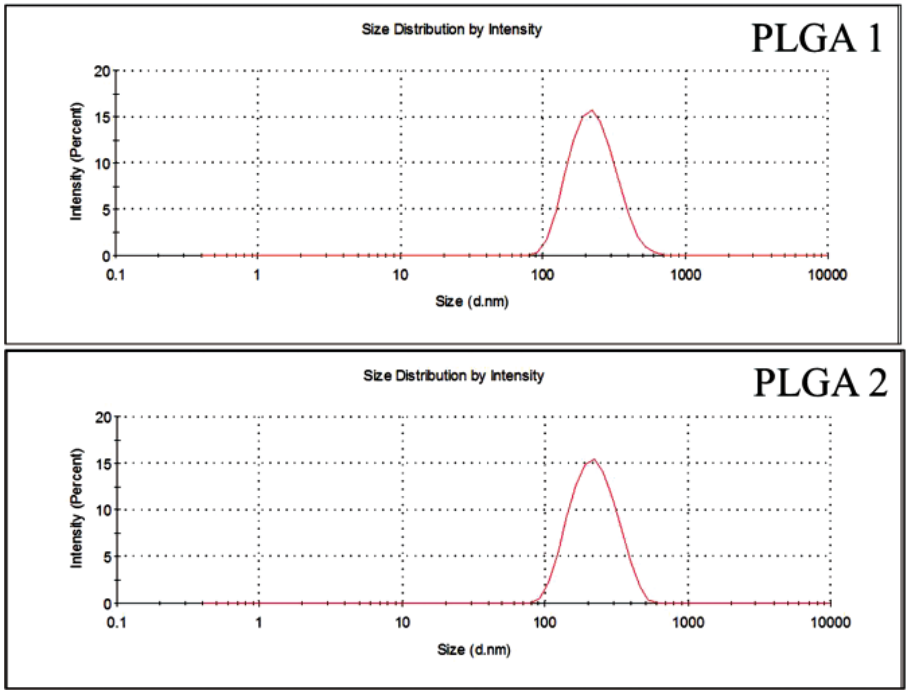


Fig. 3. **Particle size distribution of PLGA 1 and PLGA 2 respectively according DLS technique**

mean PLGA 2 particles size was 211.6 nm (Fig. 3). Moreover, the obtained particles demonstrated similar oval to round shape. The slight difference in particle size estimation is not unexpected as they are two different techniques.

The size distributions are similar for both particles and there is no agglomeration of the particles.

SEM-Analysis of Microparticles

Microparticles synthesized using different methods were analyzed morphologically by scanning electron microscopy (SEM). Obtained SEM images were shown in Fig. 4, *A*, *B*.

The results of electron microscopy of PLGA particles confirmed the results presented above. As can be seen from the pictures (Fig. 4), single particles have similar round correct shape, and their sizes are within the size determined by the data of NTA and DLS.

In vitro Release of Microparticles

To determine the effect of the medium pH on the antigen release rate, the PLGA particles loaded with the recombinant antigen EGFP-SbB were incubated in an environment that simulates the acidity of the gastric juice (solution of hydrochloric acid at pH 1.2) and as a control in a phosphate buffer solution (pH 7.8). Fig. 5 illustrates the dependence of the particle degradation process on incubation time in different environments.

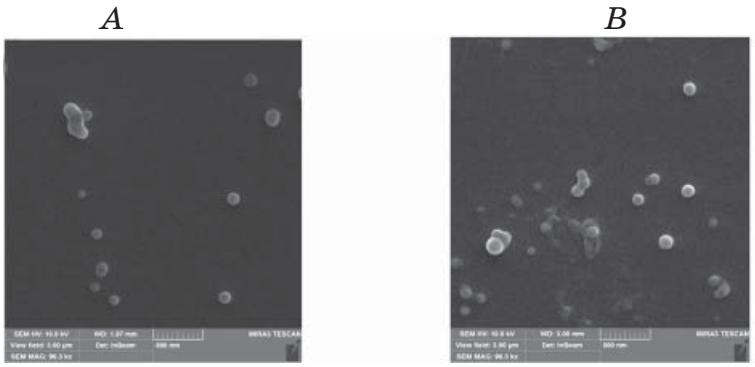
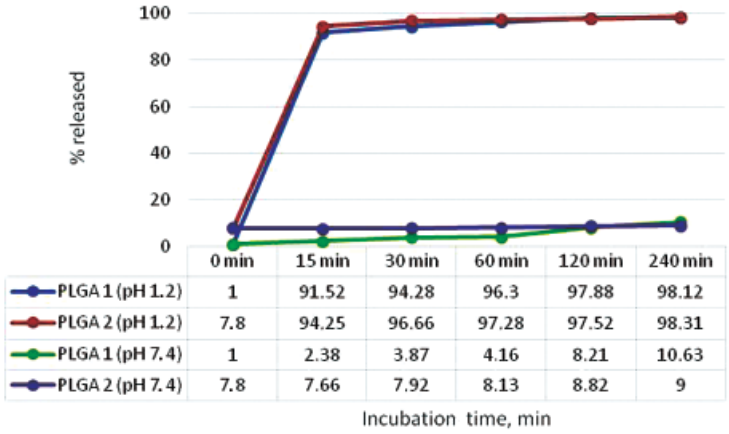


Fig. 4. ***A*** **— SEM image of PLGA 1 microparticles;** ***B*** **— SEM image of PLGA 2 microparticles**



	0 min	15 min	30 min	60 min	120 min	240 min
PLGA 1 (pH 1.2)	1	91.52	94.28	96.3	97.88	98.12
PLGA 2 (pH 1.2)	7.8	94.25	96.66	97.28	97.52	98.31
PLGA 1 (pH 7.4)	1	2.38	3.87	4.16	8.21	10.63
PLGA 2 (pH 7.4)	7.8	7.66	7.92	8.13	8.82	9

Fig. 5. ***In vitro*** **release of EGFP-SubB antigen from PLGA particles in solution of hydrochloric acid (pH 1.2) and phosphate buffer (pH 7.4).** Significant compared to control results pH 7.4 ($P \leq 0.05$)

It has been demonstrated that at the beginning of the experiment, 99% of the PLGA 2 particles were loaded with EGFP-SbB protein. There were not observed significant changes in the groups where the incubation was carried out in a buffer environment at pH 7.8. Striking effects were observed after the incubation of PLGA 2 particles in a medium with high acidity. Similar, but even more critical situation was with PLGA 1 particles.

Demonstrated differences in the properties of synthesized particles may have an influence on the immunogenicity of the antigen in their use for oral immunization. The sizes of both particles resemble bacteria size that could increase probability of particles interaction with M-cells. Obtained results can be used for developing a new tool for per os immunization against diphtheria. It can be also expected that the results of our study may be useful for the development of new delivery systems for other means, such as nucleic acids, drugs, vitamins, antigens etc. to the entire body.

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СИНТЕЗ І ХАРАКТЕРИСТИКА МІКРОЧАСТИНОК НА ОСНОВІ ПОЛІ(ЛАКТИД-КО-ГЛІКОЛІДУ), НАВАНТАЖЕНИХ ДИФТЕРІЙНИМ ТОКСОЇДОМ

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Мета роботи — синтезувати частинки на основі полі(лактид-ко-гліколіду) — PLGA й одержати їх комплекси з нетоксичним фрагментом дифтерійного токсину, міченим зеленим флуоресцентним протеїном (EGFP-SubB); охарактеризувати певні фізико-хімічні властивості отриманих кон'югатів і дослідити вплив модифікації поверхні.

Було синтезовано два типи частинок PLGA: з іммобілізованим антигеном на поверхні частинок — PLGA 1 та з інкапсульованим всередині частинок — PLGA 2. Отримані мікрочастинки характеризували за навантаженням антигену, розміром частинок, індексом полідисперсності та вивільненням антигену *in vitro*.

Ключові слова: дифтерійний токсоїд, мікрочастинки полі(лактид-ко-гліколевої) кислоти.

СИНТЕЗ И ХАРАКТЕРИСТИКА МИКРОЧАСТИЦ НА ОСНОВЕ ПОЛИ(ЛАКТИД-КО-ГЛИКОЛИДА), НАГРУЖЕННЫХ ДИФТЕРИЙНЫМ ТОКСОИДОМ

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Цель работы — синтезировать частицы на основе поли(лактид-ко-гликолида) — PLGA и получить их комплексы с нетоксичным фрагментом дифтерийного токсина, меченным зеленым флуоресцентным протеином (EGFP-SubB), охарактеризовать определенные физико-химические свойства полученных конъюгатов и исследовать влияние модификации поверхности.

Были синтезированы два типа PLGA частиц: с иммобилизованным антигеном на поверхности частиц — PLGA 1 и с инкапсулированным внутри частиц — PLGA 2. Полученные микрочастицы характеризовали по нагрузке антигена, размеру частиц, полидисперсному индексу и высвобождению антигена *in vitro*.

Ключевые слова: дифтерийный токсоид, микрочастицы поли(лактид-ко-гликолевой) кислоты.