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Stabilizing liposomes loaded with proteins, amino acids, vitamins, and microelements

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Abstract:
Many protein-containing drugs have limited application in the prevention and treatment of diseases due to their instability in the gastrointestinal tract. Therefore, there is a need for complex liposomal drugs with stabilizing components that can enhance their therapeutic effect.

Our objects of study included soy lecithin, egg albumin, immunoglobulin, insulin, chitosan, amino acids, tocopherol, ascorbic acid, riboflavin, zinc sulfate, and iron (III) chloride. The concentrations of nutrients were determined by the colorimetric and titrimetric methods. We also used the peroxide value and the dynamic light scattering method.

Liposomes obtained by the injection method had a diameter of 4.7 ± 0.2 μm, which makes them suitable for oral drug administration. Protein incorporation at 98, 95, and 83% was achieved by 1.0 mg/mL insulin, 1.6 mg/mL globulin, and 30 mg/mL albumin, respectively. The most optimal concentration of albumin in liposomes was 30 mg/mL. The highest degrees of incorporation of amino acids and their mixtures were 94–98 and 90%, respectively. Stabilizing liposomes with vitamins B_2 and C, as well as zinc and iron, increased the liposomal incorporation of amino acid mixtures and ensured their release in the model gastrointestinal tract.

The protein corona increased the release of target components in the small intestine and improved liposome stability during storage. Modifying the surface of liposomes with chitosan decreased the release of albumin in the oral cavity, stomach, and intestine.

Complex liposomes proved to have better stability in the model gastrointestinal tract and during storage. The results obtained can be used to create complex nutriceuticals.

Keywords: Liposomes, soy lecithin, protein therapy, albumin, globulin, insulin, amino acids, vitamins, microelements

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INTRODUCTION

Protein components are the most common functional elements involved in the life processes of the human body. They act as enzymes (biocatalysts of biochemical reactions), immunoglobulins regulating defense mechanisms against antigens, building blocks of muscle and connective tissues (collagen, keratin, elastin, fibrinogens, and thrombins), signal molecules (hormones, cytokines, and growth factors), as well as carriers of oxygen and carbon dioxide from tissues to the lungs (hemoglobin) [1].

Protein therapy has recently revolutionized the treatment of many diseases. Its main advantages are high specificity and lack of side effects. For quite a long time, the clinical rationale for the use of many therapeutic proteins was undermined by undesirable immune responses, which limited their effectiveness and affected their safety profile.

Currently, the protein-based pharmaceutical market is thriving, with sales exceeding $380 billion in 2023 and expected to rise to over $650 billion by 2030 [2].

Therapeutic proteins can be classified into four groups. The first group includes proteins with enzymatic or regulatory activity. They are mainly applied to replace deficient proteins in people with hereditary genetic diseases or mutations. The second group comprises proteins with targeting activity such as monoclonal antibodies that act as diagnostic tools for tumor lesions and other diseases. The third group includes vaccines based on protein or glycoprotein components of a pathogen,

capable of causing an immune response. Therapeutic proteins of the fourth group are recombinant proteins that are mainly used in diagnostics [3].

A disadvantage of protein-based pharmaceuticals is their limited stability in physiological fluids containing proteolytic enzymes (pepsin, trypsin, chymotrypsin). Another disadvantage is their low pH values in the stomach [4].

To overcome these drawbacks, protein-based drugs can be encapsulated in liposomes or hydrogels. The encapsulation material must be biocompatible, biodegradable, and stable under the conditions of prolonged release. In addition to the type of biomaterial, the choice of a way to introduce protein-based drugs is also important. For example, they can be introduced into a hydrogel either by forming a gel in the presence of a protein or by soaking the gel in a protein solution. The former technique can lead to denaturation of protein molecules due to impurities or by-products of gel formation. With the latter technique, proteins are usually displaced from the gel networks due to their heterogeneous size. Because of this limitation, only less than 0.1% can be incorporated when using this method [5].

Lipid-based delivery systems, or liposomes, are effective due to their ability to deliver antigens (peptides, proteins, and nucleic acid systems) to cells stimulating protective immune responses. Modern engineering of nanomedicines varying in composition, particle size, and surface charge can help in spatial and temporal drug delivery [6, 7].

In addition to high-molecular compounds, liposomes contain various low-molecular substances, such as amino acids, vitamins, microelements, carotenoids, etc.

The presence of various functional groups in amino acids makes it possible to synthesize a wide range of molecules to form liposomal aggregates with different morphologies [8–12]. In addition, natural L-amino acids can promote the biotransformation of other molecules [13, 14].

Denieva *et al.* [15] produced mixed liposomes based on natural amino acids (L-alanine, L-serine, and L-ornithine) and cationic lipids. They found that L-serine-based liposomes were capable of accumulating in the endoplasmic reticulum of cells for 1 h. Also, their transfection activity significantly exceeded that the commercial drug Lipofectamine-2000. The system proposed by the authors had a slight toxic effect (IC_{50} – 0.75 mg/mL, with a safe working concentration of 0.24 mg/mL).

Tosato *et al.* [16] formulated a dietary supplement based on 1.66 g of L-arginine and 500 mg of liposomal vitamin C. The authors showed its efficiency in restoring physical performance and reducing fatigue during the recovery from COVID-19.

Microelements and vitamins are also introduced into liposomal nanocontainers to increase their bioavailability, ensure prolonged release, and protect them from biodegradation in the digestive system [17]. For example, Liu *et al.* [18] applied liposomal co-encapsulation to extend the shelf life and improve the bioavailability of vitamins and a wide range of hydrophilic and hydrophobic molecules, such as peptides, proteins, and nucleic acid-based drugs, which are used for pharmaceutical, cosmetic, biochemical, and nutraceutical purposes.

Liposomal forms of vitamins have advantages over their free forms. In free form, vitamins have low absorption due to their passing through cell membranes and oxidation in the digestive system [19]. Liposomes loaded with vitamins are embedded in damaged areas of cell membranes, transferring the active compound into the cells. Another benefit of using liposomes as a transport carrier is that they are of the same nature as fat-soluble vitamins.

Since vitamin C is an acid, it can irritate the gastric mucosa, especially in people with peptic ulcers. This irritation can be avoided by enclosing the vitamin in liposomes. Nechaev *et al.* [20] developed liposomes loaded with vitamins C and E, which enhance each other's effects. The liposomes had stable quality, antioxidant effect, and good sensory indicators during 90 days of storage at 4 ± 2°C.

Lipovitam-Beta is an antioxidant drug of the new generation. The liposomal encapsulation of its active substances (beta-carotene, vitamins E and C) ensures their high bioavailability (over 90%, compared to 10–30% in traditional drugs) [21].

Vitamin B_2 (riboflavin) is involved in dehydrogenase reactions in the form of prosthetic groups FAD and FMN. Gupta *et al.* [22] studied the effect of liposomes on the photostability of riboflavin in an aqueous composition under fluorescent lighting. They found that the photostability of riboflavin was higher in the presence of neutral and negatively charged liposomes but lower in the presence of positively charged liposomes. In addition, higher concentrations of dimyristoylphosphatidylcholine in the liposomes increased the photostability of riboflavin.

Iron (II) and (III) ions are included in liposomes for a number of reasons. Firstly, in high doses, iron (II) and (III) cations have a negative effect on the intestinal mucosa, causing irritation. This effect can be avoided by taking iron (II) and (III) salts with food. However, taking iron with food reduces its bioavailability, with only 2–10% of absorption for non-heme iron (III) [23]. Secondly, the walls of the small intestine and duodenum mainly absorb the Fe^{2+} cation, but iron (II) ions are unstable in solutions and are oxidized to iron (III) ions. As a result, Fe^{2+}-based drugs cannot be used in liquid forms. Moreover, liquid forms of drugs based on Fe^{2+} and Fe^{3+} tend to stain tooth enamel, cause an unpleasant metallic taste, and pass through the gastrointestinal tract undigested [24].

The liposomal form of Fe^{3+} is an innovative method of delivering iron ions into the human body. It is a trivalent iron pyrophosphate enclosed in a double-layer lipid shell that has a similar structure to that of a cell membrane. When entering the lumen of the small intestine, liposomes containing iron (III) ions penetrate enterocytes by endocytosis and are transported into the

lymph. With the lymph, the liposomes enter mainly the liver, where iron (III) ions are released from the liposomal shell into active metabolism. The liposomal iron does not cause the typical side effects that traditional preparations of iron (II) and (III) salts do. The liposomal form of Fe^{3+} is highly efficient in patients with iron deficiency anemia associated with chronic and acute blood loss, deficiency of iron (II) or (III) ions in the diet, as well as anemia of chronic disease. Treating anemia of chronic disease with standard drugs based on Fe^{2+} and Fe^{3+} ions is ineffective due to changes in their absorption in the intestine and regulation of hepcidin. In inflammatory bowel diseases, iron-based drugs are usually administered intravenously, since their oral administration is ineffective. However, the liposomal form of iron (II) or (III) ions administered orally showed high efficiency in patients with Crohn's disease [25].

Zinc is another important microelement responsible for the normal functioning of innate immunity. Zinc exhibits antioxidant properties in relation to active forms of nitrogen and oxygen. It participates in the modulation of cytokine release and induces the proliferation of CD8+ T-cells, as well as maintains the integrity of the skin barrier and mucous membranes. In acquired immunity, zinc plays an important role in the growth of immune cells. It facilitates the intracellular binding of tyrosine kinase to T-cell receptors, promoting the development and activation of T lymphocytes. Zinc deficiency leads to a lower production of lymphocytes (especially T-cells), thymus atrophy, and disruption of cytokine synthesis, causing the development of oxidative stress and inflammatory processes [26].

Zinc enhances the antiviral properties of many mammalian cells by stimulating natural immunity. It is also an important structural component in a number of enzymes. Along with zinc-dependent protein complexes in liposomal compositions, this microelement can effectively fight viral infections by activating individual functional complexes of immune cells in the respiratory tract. Numerous studies have shown that taking zinc in combination with hydroxychloroquine significantly reduced the risk of death during COVID-19 [27].

The main requirements for liposomes are biodegradability, stability, the absence of immune response, a possibility of prolonged action, and non-toxicity. However, liposomes have a number of limitations due to their thermodynamic instability and tendency to aggregate in the human body. Liposomes lose their stability even with minor changes in environmental parameters, such as temperature, pH, or ionic strength. When stored in the dry form, they are destructed due to lipid peroxidation under the influence of oxygen. Therefore, scientists are faced with the task to obtain stabilized liposomal forms of bioactive substances and study the structural and functional properties of the resulting complexes [28].

Liposomes can be stabilized by creating additional shells (e. g., polymer-based) on top of the main bilipid layer. The resulting multilamellar liposomes have a more stable membrane and are much better able to retain the active compound during long-term storage. This is because their outer shells protect their deep bilipid layers from the effects of oxygen. Shells are often based on protein substances or polysaccharides (e. g., chitosan). Chitosan is easy to use for layer-by-layer adsorption of polyelectrolytes or ionotropic gelation and it contributes to effective particles. Even chitosan alone used in the outer shell can already increase the stability of liposomes. However, more significant results can be achieved by using an additional gelling agent such as polyacrylic acid [29]. This can reduce the intensity of liposome peroxidation during storage and improve the preservation of the active compound as it passes through the stomach.

Chitosan is commonly combined with sodium alginate to produce a shell for stabilized multilayer liposomes. The chitosan-alginate complex can effectively bind to the hydrophilic surface of the bilipid layer and release liposomal particles under certain pH values in the small intestine [30].

Also common are liposomal shells based on casein and whey protein in functional nutrition. Such shells are less resistant and stable in the stomach. However, their advantages include high accessibility and lower susceptibility to peroxidation [31, 32].

Liposomes can also be stabilized with α-tocopherol, an antioxidant that prevents lipid peroxidation. In addition, vitamin E has a cholesterol-like effect, ordering unsaturated bilayers and disordering saturated ones. It forms stabilizing complexes by interacting with free fatty acids. Finally, introducing vitamin E into liposomal nutraceuticals can make them effective dietary supplements [33].

Currently, most protein drugs are administered parenterally, which allows for higher bioavailability compared to the oral route. However, this approach has a number of disadvantages that deteriorate the quality of human life. Therefore, the pharmaceutical industry is seeking to develop oral delivery methods for more cost-effective and flexible forms of drugs [34].

In view of the above, we aimed to obtain protein and amino acid preparations to be incorporated into liposomes, as well as to study their properties and ways of stabilization [35, 36].

STUDY OBJECTS AND METHODS

Our study objects included: moslecithin – soy lecithin (65% phospholipid fraction; Ekobio Plus, Russia); egg albumin (99% active ingredient; Roskar Poultry Farm, Russia); Globfel-4 globulin – γ- and β-globulin fractions of horse blood serum hyperimmunized with industrial strains of panleukopenia and infectious rhinotracheitis viruses, calicivirus, and chlamydia in cats (Vetbiokhim, Russia); Actrapid® HM – a short-acting insulin preparation (genetically engineered soluble human insulin, 100 IU/mL; Novo Nordisk, Denmark); chitosan (200 kDa, 85% deacetylation; Sigma-Aldrich, USA); LifeEvit vitamin E in capsules (400 mg alphatocopherol acetate; Saneka Pharmaceuticals, Slovak Republic); amino acids: serine, histidine, proline, alanine, lysine, and arginine (99.5% active ingredient; PanEco,

Russia); ascorbic acid (99.5% active ingredient; Dia-M, Russia); riboflavin (99% active ingredient; Pharmstandard-Ufa Vitamin Plant, Russia); zinc sulfate, chemically pure (Khimmed, Russia); and iron chloride (III), chemically pure (Ruskhim, Russia).

The protein content in the solutions was measured by the microbiuret method. The method was based on the spectrophotometric determination of the optical density of the colored product (biuret complex) resulting from the interaction between copper ions and groups of peptide bond atoms under alkaline conditions at 330 nm. The total amino acid content was determined by a reaction with ninhydrin to form a violet-colored complex [37]. The riboflavin content was measured by the optical density at 445 nm. The ascorbic acid content was determined by the reduction reaction of 2.6-dichlorophenolindophenol, measuring the optical density at 510 nm. The trivalent iron content was determined by a reaction with thiocyanate ion in a strongly acidic medium to form a red-colored complex with maximum absorption at 500 nm. The zinc content was determined by the titrimetric method by a reaction with 0.1 M solution of sodium EDTA salt, 1 mL of which corresponded to 13.63 mg of zinc chloride [38]. The liposomal stability in the gastrointestinal model and during storage was assessed by determining the peroxide value based on the ability of hydroiodic acid to reduce peroxides with the release of molecular iodine. The amount of molecular iodine was determined by titration with sodium thiosulfate in the presence of starch [39]. The size of microparticles and the zeta potential of the suspensions formed by them were determined automatically by the dynamic light scattering method on a Photocor Compact Z particle size analyzer (Photocor, Russia).

The thermal method for preparing liposomes. Samples of lecithin were homogenized in different volumes of distilled water with lipid contents of 0.75, 1.00, 2.00, and 3.00 wt. %. The homogenate was hydrated for 2 h. Then, 3.0 vol. % glycerol was added to the resulting suspension and thermostatted for 30 min at 65–70°C with constant stirring in a magnetic stirrer. The mixture was then kept at the specified temperature for 1 h. The prepared solutions were analyzed for liposome formation using microscopy after 1 week [40].

The injection method for preparing liposomes. The injection method is based on the spontaneous formation of bilayer vesicles when the liposomal base enters the hydrophilic solvent medium due to injection-induced pressure difference. This technique was used as described in [41]. In particular, 50 mg of egg lecithin was dissolved in 1.0 mL of 96% ethanol and added to 15 mL of 0.01 M phosphate buffer (pH 7.5). Previously, the material to be incorporated in the liposomes had been dissolved in the buffer by constant stirring in a magnetic stirrer. The prepared solution was allowed to stand overnight at room temperature and then analyzed by microscopy at a magnification of 400×.

The thin film hydration method. The thin-film hydration method is used for loading hydrophilic substances into liposomes. It is based on the rehydration of lipid films in the presence of a buffer containing the substances to be loaded. Phospholipids are dissolved in an organic solvent, usually chloroform, which is then evaporated under reduced pressure until a thin lipid film is obtained. Then, an aqueous phase is added to the film, with the hydrophilic ingredients dissolved, and a liposomal dispersion is formed [42].

Evaluation of liposome stability during storage. One of the main causes of liposome destruction is lipid peroxidation. The degree of destruction of liposomal particles during storage or use depends on their initial oxidation, which is indicated by their peroxide value. Liposomes were incubated for 8 weeks at 6°C in a hermetically sealed container. Their samples were taken throughout the storage period to determine the peroxide value [39].

***In vitro* digestion modeling.** The human gastrointestinal tract was modelled in accordance with the International Standardized Protocol for Digestion of Foods [43, 44]. The conditions for simulating digestion at each stage were as follows:

– the oral cavity: simulated salivary fluids (SSFs), pH 7.0;
– the stomach: simulated gastric fluids (SGFs), pH 3.0, 2 h, pepsin (1:1000 enzyme:substrate ratio by weight);
– the intestine: simulated intestinal fluids (SIFs), pH 7.0, 2 h, trypsin + α-chymotrypsin (1:1000 enzyme:substrate ratio). A mixed solution of trypsin and chymotrypsin had been previously prepared at a 4:1 ratio of their activities, respectively, according to the Protocol.

At all the stages, the solutions were kept in a shaker thermostatted at 37°C and stirred at 100 rpm.

To construct a parallel release profile, 0.3 g of liposomes was placed into a test tube and 2 mL of a simulating solution (SGFs or SIFs) was added to the tube. The liposomes were incubated for a given time, after which they were separated from the solution by centrifugation at 11,000 rpm for 30 min.

To construct a sequential release profile, 0.6 g of liposomes was placed into a test tube and 3 mL of a solution simulating the oral cavity (SSFs) was added to the tube. The mixture was incubated at 37°C for 15 min, after which the liposomes were separated by centrifugation at 11,000 rpm for 30 min. A solution simulating the stomach (SGFs) was then added to the microparticles and they were incubated for 90 min under the same conditions. The liposomes were separated by centrifugation in the same mode. Finally, a solution simulating the small intestine (SIFs) was added and the liposomes were incubated for 120 min under the same conditions. They were separated again, as described above.

At the end of the modeling, all the supernatants were analyzed for the concentrations of incorporated components by standard methods. Based on the data, we determined the degree of release of the target components under the influence of the gastrointestinal enzymes at each stage [44].

Modification of liposomes with chitosan. A chitosan solution was added dropwise with stirring to an

emulsion of liposomes obtained by the injection method (5 mg/mL) in a potassium phosphate buffer solution (pH 7.4) until a given concentration of chitosan in the emulsion was achieved. After that, the liposome-chitosan complexes were incubated for 1 h with vigorous stirring at room temperature. The modified liposomes were separated by centrifugation at 10,000 rpm for 7 min.

Modification of liposomes with ovalbumin ("protein corona"). Stock solutions of egg albumin with concentrations of 10–100 mg/mL were prepared in 0.01 M potassium phosphate buffer (pH 7.4). Liposomes were obtained by the injection method. The resulting emulsion was added dropwise to the protein solution in a volume ratio of 1:1 and mixed. The mixture was incubated at room temperature for 4 h and then centrifuged at 10,000 rpm for 7 min. To remove unbound protein, the liposomes were washed twice with distilled water and then centrifuged under the same conditions.

RESULTS AND DISCUSSION

Comparative analysis of the injection and thermal methods for obtaining soy lecithin liposomes. There are numerous methods for producing liposomes, but many of them are difficult to scale up for industrial use. Therefore, we aimed to compare the thermal and injection methods since both can be easily adapted for industrial production of liposomal forms of drugs.

To prepare liposomes by the thermal method, soy lecithin was used at concentrations of 0.75, 1.00, 2.00, and 3.00 wt. %. The resulting liposomes were examined microscopically with a magnification of 400×. The most optimal concentrations of soy lecithin were 1.0 and 3.0 wt. %. Therefore, these concentrations were used for further experiments (Fig. 1).

As can be seen in Fig. 1, the obtained liposomes were significantly heterogeneous in size. Therefore, they were homogenized using an IKA T25 digital ULTRA-TURRAX laboratory homogenizer. The select the best process time, solutions with a lipid content of 1.0 and 3.0 wt. % were homogenized for 15–120 s with an interval of 15 s. The homogenized particles were microscopically examined at each stage (Fig. 2). The sample homogenized for 90 s had even, rounded particles of approximately the same size. A further increase in time caused intense foam formation. Thus, 1.5 min was determined as the most optimal time, after which the size and shape of the preparations remained unchanged.

This experiment also showed that the suspensions with a phospholipid content of under 3.0 wt. % were most effectively homogenized, so this concentration was used for further analyses. The resulting liposomes were dried at 50°C.

According to laser light scattering, the size of the thermally obtained liposomes was 4–10 μm, which exceeded the permissible microparticle diameter of 100 nm–5 μm for oral drug delivery [45].

Next, we determined changes in the size and ζ-potential of the liposomes during incubation in various sections of the gastrointestinal tract for a parallel profile. For this, the stages of the gastrointestinal tract (oral cavity, stomach, and intestine) were simulated (Table 1).

We found that over time, the liposomes significantly increased in size in the gastrointestinal model. For example, exposure to the stomach environment for 120 min caused a ten-fold increase in size. At the same time, the ζ-potential of the liposomes decreased. This was due to the destruction of liposomes, as well as changes in the configuration of negatively charged polar groups

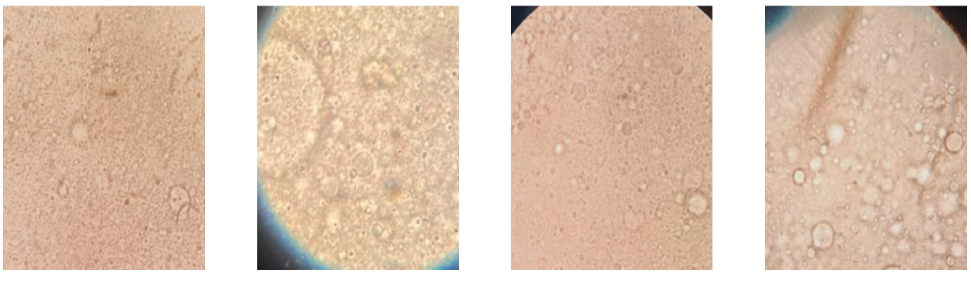

Figure 1 Liposomes prepared by the thermal method with soy lecithin contents of: (a) 0.75%; (b) 1.00%; (c) 2.00%; and (d) 3.00% (400× magnification)

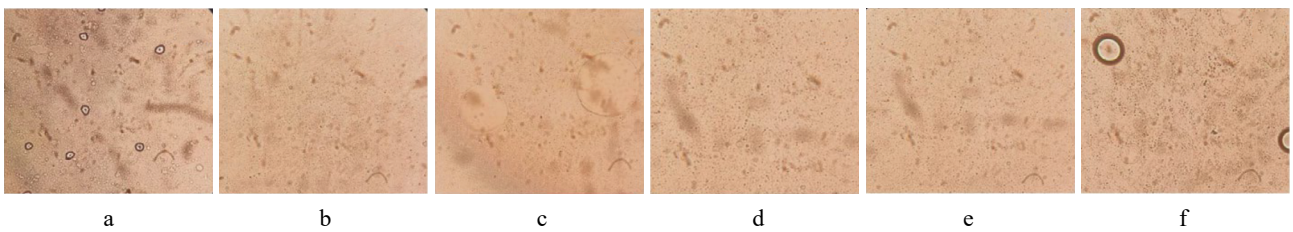

Figure 2 Liposomes prepared by the thermal method with 3% lecithin after homogenization for: (a) 15 s; (b) 45 s; (c) 75 s; (d) 90 s; (e) 105 s; and (f) 120 s (400× magnification)

Table 1 Changes in the size and ζ-potential of thermally obtained liposomes in model sections of the gastrointestinal tract for a parallel profile

Sample	ζ-potential, mV	Size, μm
Initial	36 ± 4	7.0 ± 0.4
Simulated salivary fluid (SSF) (15 min)	25 ± 2	17.5 ± 0.9
Simulated gastric fluid (SGF) (60 min)	25 ± 2	75.3 ± 3.8
Simulated gastric fluid (SGF) (120 min)	8 ± 1	115.5 ± 5.8
Simulated intestinal fluid (SIF) (30 min)	28 ± 3	65.4 ± 3.3
Simulated intestinal fluid (SIF) (60 min)	24 ± 2	43.3 ± 2.2

Table 2 Changes in the size and ζ-potential of thermally obtained liposomes in model sections of the gastrointestinal tract for a sequential profile

Sample	ζ-potential, mV	Size, μm
Simulated salivary fluid (SSF) (15 min)	34 ± 2	17.2 ± 0.9
Simulated gastric fluid (SGF) (120 min)	24 ± 1	68.3 ± 3.4
Simulated intestinal fluid (SIF) (60 min)	32 ± 2	46.7 ± 2.3

Table 3 Changes in the size and ζ-potential of liposomes obtained by injection in model sections of the gastrointestinal tract for a parallel profile

Sample	ζ-potential, mV	Size, μm
Initial	36 ± 4	4.7 ± 0.2
Simulated salivary fluid (SSF) (15 min)	25 ± 2	12.4 ± 0.6
Simulated gastric fluid (SGF) (60 min)	25 ± 2	45.3 ± 2.3
Simulated gastric fluid (SGF) (120 min)	8 ± 1	76.7 ± 3.8
Simulated intestinal fluid (SIF) (30 min)	28 ± 3	55.7 ± 2.8
Simulated intestinal fluid (SIF) (60 min)	24 ± 2	68.4 ± 3.4

Table 4 Changes in the size and ζ-potential of liposomes obtained by injection in model sections of the gastrointestinal tract for a sequential profile

Sample	ζ-potential, mV	Size, μm
Simulated salivary fluid (SSF) (15 min)	33 ± 2	12.4 ± 0.6
Simulated gastric fluid (SGF) (120 min)	25 ± 1	48.2 ± 2.4
Simulated intestinal fluid (SIF) (60 min)	21 ± 1	56.4 ± 2.8

of phospholipid molecules during oxidation. Moreover, the decrease in ζ-potential was caused by changes in the orientation near the surface of the bilayer membrane. As the liposomes passed through each of the gastrointestinal sections, their absolute ζ-potential values dropped to below 30 mV, which indicated liposomal adhesion. The greatest decrease was observed in the acidic environment of the stomach.

Then, we analyzed changes in the size and ζ-potential of the liposomes in the gastrointestinal model for a sequential profile. Samples were collected after the maximum incubation time for each gastrointestinal section – the oral cavity, stomach, and small intestine (Table 2).

The results for the sequential profile were similar to those for the parallel profile (increased particle size and decreased ζ-potential due to liposome destruction). The intestinal environment caused the particles to aggregate, with a decrease in the absolute value of ζ-potential and a slight decrease in size.

Since the preparation of liposomes by the thermal method was time-consuming (about 7 days), we aimed to study the injection method to speed up the process.

As lecithin has low solubility in ethyl alcohol, hexane was used as a solvent and then removed from the mixture by evaporation. We studied changes in the size and ζ-potential of liposomes in the model sections of the gastrointestinal tract for the parallel and sequential profiles (Tables 3 and 4).

We found similar tendencies in the behavior of the liposomes in the gastrointestinal model, regardless of the method of their preparation. However, the liposomes obtained by the injection method had a smaller diameter (4.7 ± 0.2 μm) compared to those obtained by the thermal method. This makes them more promising agents for oral drug delivery. Another advantage of the injection method is its shorter time. Therefore, liposomes were obtained by the injection method for further analyses.

Studying the degree of protein incorporation into liposomes. To determine the effectiveness of liposomes as target protein delivery systems, we studied the conditions that ensured the highest degree of protein incorporation. Albumin, globulin, and insulin were used as model proteins. They were encapsulated by passive diffusion into the pre-prepared liposomes. The liposomes were then separated by centrifugation for 20 min at 8000 rpm. The residual concentration of proteins in the supernatant was determined by the microbiuret method and the degree of their incorporation into liposomes was calculated (Figs. 3 and 4).

The most optimal concentration of albumin for liposomal incorporation was 30 mg/mL, with the incorporation degree of 83% (Fig. 3).

The most optimal concentration of insulin for liposomal encapsulation was 1.0 mg/mL, which was equivalent to a mixture of Actrapid® and 0.1 M phosphate buffer (1:4), pH 7.0. This concentration contributed to 98% of encapsulation (Fig. 4).

For immunoglobulin, the most optimal concentration was 1.6 mg/mL, which was equivalent to a 10-fold

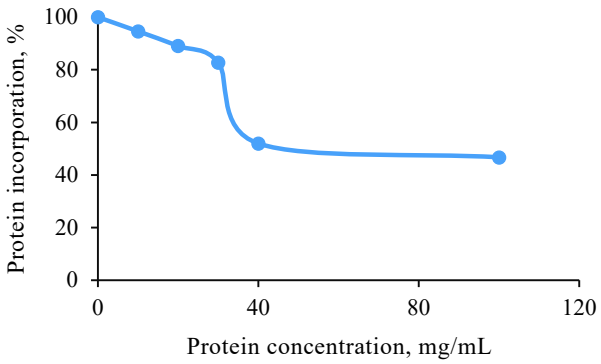

Figure 3 The degree of albumin incorporation into liposomes vs. the initial concentration of albumin in the solution

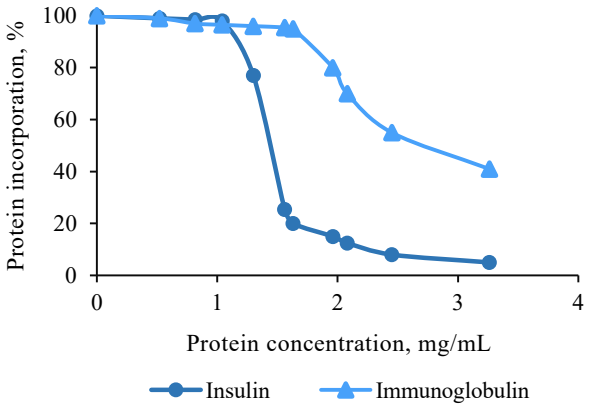

Figure 4 The degree of insulin and immunoglobulin incorporation into liposomes vs. the initial concentration of the proteins in the solution

dilution of Globfel-4 in 1.5 M phosphate buffer, pH 7.0. The degree of immunoglobulin incorporation was 95% (Fig. 4).

Based on the results, the capacity of liposomes was 207.0 mg/g for albumin, 8.5 mg/g for insulin, and 13.0 mg/g for immunoglobulin. The differences between the proteins might be due to their charge at the pH applied, as well as the size of their molecules. In particular, the isoelectric point is near pH 4.9 for albumin, 5.4 for insulin, and 5.2 for immunoglobulin.

The release of target components in the gastrointestinal tract is an important aspect to determine the efficiency of drugs in liposomal form. Therefore, we aimed to study the behavior of protein-loaded liposomes in the model gastrointestinal sections.

Studying protein release from liposomes in the in vitro models of human gastrointestinal tract. An experiment similar to the one for unloaded liposomes was carried out to analyze the release of incorporated proteins in parallel and sequential profiles. Liposomal vesicles were incubated in the respective model sections of the gastrointestinal tract and separated by centrifugation. The residual protein concentrations in the supernatants were measured by the biuret method to calculate the degree of protein release. The results are presented in Fig. 5 for the parallel profile and in Fig. 6 for the sequential profile.

In the parallel profile, the degree of albumin release from the liposomes reached 17% in the oral cavity (with 11% released in the first 5 min of incubation), 38% in the stomach, and 53% in the intestine. Insulin showed the highest degree of release in the stomach (about 50%), 30% in the intestine (28% in the first 30 min), and about 15% in the oral cavity. Similar results were obtained for immunoglobulin: 16% released in the oral cavity, 38% in the stomach, and 50% in the small intestine.

The sequential profile showed the following patterns of protein release. The lowest degree of release was observed in the oral cavity for all the proteins (11, 15, and 17% for albumin, insulin, and immunoglobulin,

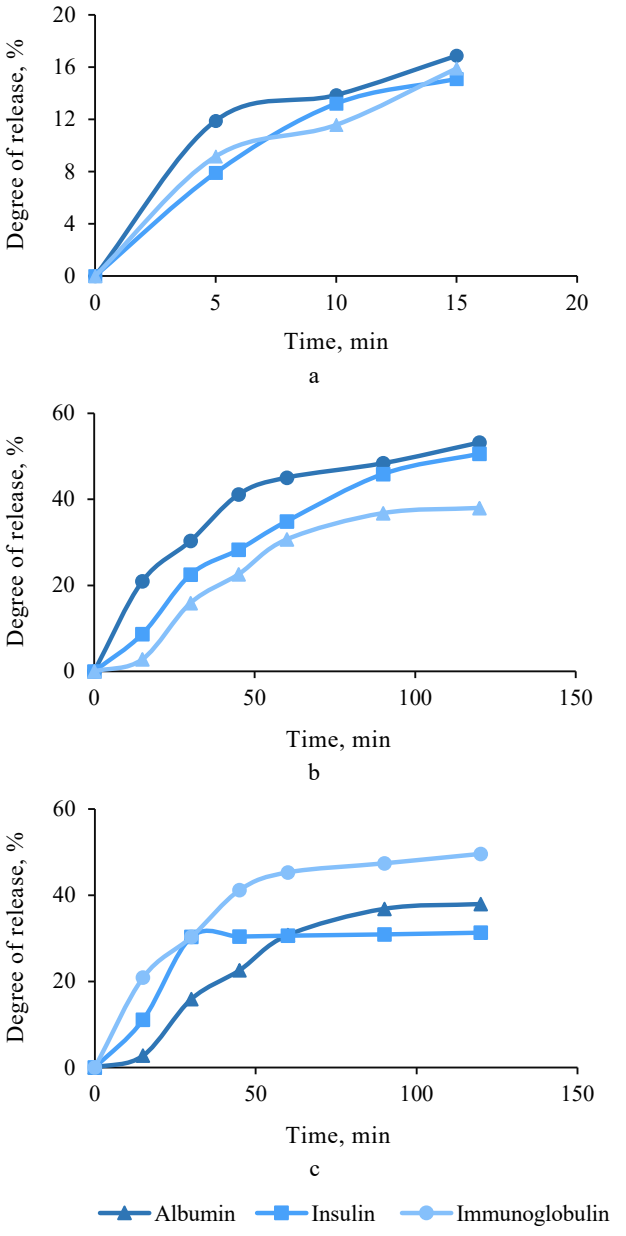

Figure 5 Parallel profile of protein release from liposomes *in vitro* in the models: (a) oral cavity (SSF); (b) stomach (SGF); and (c) intestine (SIF)

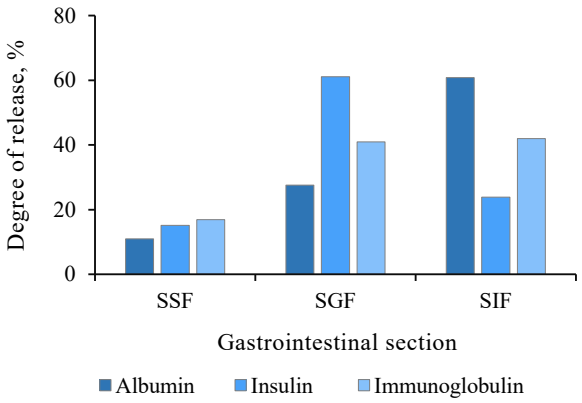

Figure 6 Sequential profile of protein release in the model gastrointestinal sections

respectively). Yet, there were some differences in the subsequent model sections. Albumin and immunoglobulin showed the greatest release (64 and 42%, respectively) in the small intestine, while in the stomach their release reached 28 and 41%, respectively. The maximum amount of insulin (61%) was released in the stomach. The differences between the proteins might be due to their charge at the pH of the model gastrointestinal environment. In the stomach model, albumin was less positively charged compared to the other proteins.

According to our results, the liposomes loaded with albumin showed the greatest loading capacity and degree of release in the gastrointestinal tract. Therefore, they were used for further analyses. However, due to the high degree of albumin release in the stomach, the liposomes need to be stabilized.

Currently, there is a trend towards producing complex liposomal preparations containing several high- and low-molecular components. In this study, we used amino acids, vitamins, and microelements as low-molecular components. In each case, we determined the capacity of liposomes for these components, as well as their release from liposomes in the model gastrointestinal sections in a sequential profile.

Incorporation of amino acids into liposomes. Amino acids such as arginine, alanine, proline, serine, lysine, tryptophan, and histidine are used in medicine to prevent and treat various diseases. Their incorporation into liposomes can enhance the therapeutic effect of other medicinal substances. When used together with proteins, they can improve the amino acid score of the resulting drugs.

Arginine, alanine, proline, and serine are nonessential amino acids. Arginine is one of the key metabolites in nitrogen metabolism processes. It participates in the synthesis of creatine, which is part of muscle tissue, and also strengthens the immune system [46]. Alanine is a source of energy. It promotes the synthesis of carbohydrates, the removal of toxins from the liver, and the absorption of glucose. The intake of these amino acids is important for increased physical activity or stress. Proline and serine are neuromodulators. They are present in significant amounts in the human brain and regulate memory functions and the nervous system as a whole. Their deficiency can lead to disorders of the nervous system, Alzheimer's disease, and encephalopathy [47]. Lysine, tryptophan, and histidine are essential amino acids. Lysine and histidine are the main components of neuronal proteins, tRNA, and nucleoprotein complexes of ribosomes, stimulating RNA synthesis in nerve cells [48]. Their deficiency can lead to anemia, enzyme deficiency, and epileptic seizures. When tryptophan enters the blood, it binds to albumin, facilitating the transfer of amino acids to the central nervous system through the blood-brain barrier. Tryptophan deficiency causes the development of pellagra and dementia.

The amino acids were incorporated into liposomes by the injection method. For each amino acid, we determined a concentration that ensured its complete incorporation into the liposomes. For this, the liposomes were separated by centrifugation and the residual concentration of the amino acid in the supernatant was determined by the ninhydrin method. Based on the data, we calculated the degree of amino acid incorporation and the capacity of the liposomes (Fig. 7, Table 5).

As can be seen, the highest degree of incorporation was observed for tryptophan (94%), histidine (98%), proline (91%), and lysine (95%).

In order to create complex liposomal preparations, the liposomes were loaded with mixtures of two and all seven amino acids. Their ratios were based on the previously established liposomal capacities and the amino acid score (Table 6). The experiment was carried out as described above.

As can be seen, the co-incorporation of amino acids into the liposomes increased the percentages of the target components compared to individual amino acids. The arginine-serine mixture had the highest degree of incorporation (96%).

Next, we studied the release of the amino acids under gastrointestinal tract conditions in a sequential profile. The experiment was carried out as described above for the protein-loaded liposomes. Figure 8 shows the release of individual amino acids, while Fig. 9 shows the release of amino acid mixtures.

According to our results, significant amounts of the amino acids (up to 35%) were released in the oral cavity and the stomach, while their residual amounts (up to 20%) were released in the intestine. The release of amino acid mixtures decreased to 10–12% in the oral cavity, except for the histidine-proline mixture (48%). Their release in the stomach remained high, with 75% for the arginine-serine mixture. The lysine-tryptophan mixture showed maximum release in the intestine. Thus, incorporating amino acids into liposomes without additional components did not have a stabilizing effect in the model gastrointestinal tract.

Incorporation of vitamins into liposomes. Vitamins B_2 and C were chosen as auxiliary components incorporated into liposomes to increase the stability, therapeutic activity, and bioavailability of the target

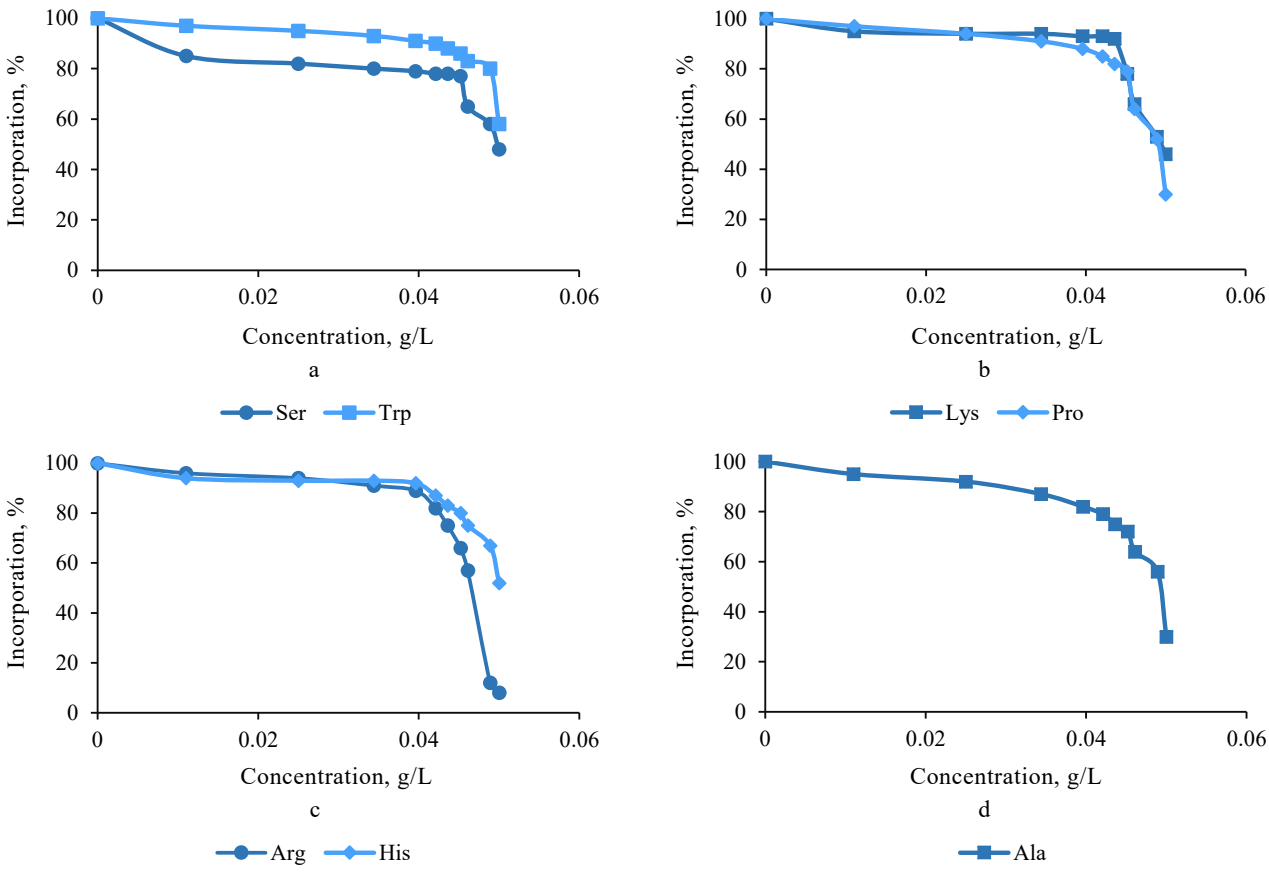

Figure 7 Incorporation of amino acids into liposomes vs. their concentration in the initial emulsion

Table 5 Amino acid incorporation into liposomes

Amino acid	Concentration, mg/L	Incorporation, %	Capacity, mg/mg
Ala	25.0	89	0.122
Ser	38.0	86	0.160
Trp	40.0	94	0.149
His	40.0	98	0.141
Pro	34.4	91	0.141
Arg	40.0	88	0.141
Lys	42.2	95	0.155

Table 6 Incorporation of amino acid mixtures into liposomes

Amino acid mixture	Concentration of amino acids in the mixture, mg/L	Incorporation, %	Specific content of amino acids, mg/g
Arg Ser	8.6 38.4	96	0.167
Lys Ala	42.0 17.6	84	0.211
His Pro	16.3 40.0	89	0.200
Trp Lys	4.0 40.0	79	0.156
Arg Ser Lys Ala His Pro Trp	8.6 2.5 3.6 1.5 1.4 4.2 0.3	90	0.078

preparation. These vitamins participate in the proliferation of B- and T-cells, increase the expression of cytokines, and improve the differentiation of immune cells with the formation of leukocytes [49].

The experiments were carried out as described above. The vitamins were incorporated in the concentrations corresponding to their average daily requirement (0.2 μg/mL for riboflavin and 3.4 μg/mL for ascorbic acid).

Table 7 shows the degree of incorporation of vitamins B_2 and C into the liposomes loaded with individual amino acids and their mixtures. As can be seen, vitamin B_2 had the highest degree of incorporation with serine (95%), tryptophan (91%), histidine (92%), and lysine (94%). The incorporation of vitamin C exceeded 90% with all the amino acids under study.

The co-incorporation of both vitamins (B_2 and C) into the liposomes loaded with amino acid mixtures was 88%. This might be due to a partial loss of vitamin activity during the preparation and storage of the liposomal mixtures, as well as the biodegradation of the liposomal form. As a result, these preparations could not provide the average daily requirements for vitamins B_2 and C.

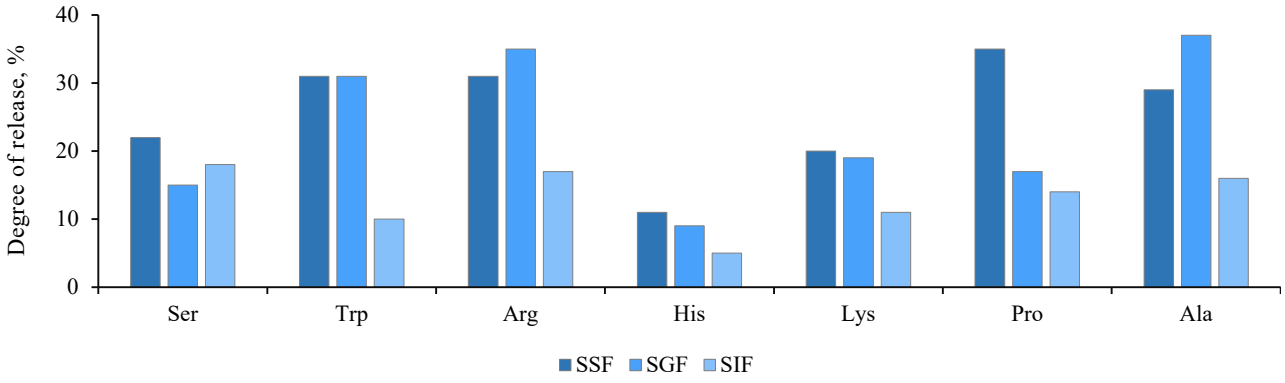

Figure 8 Release of amino acids from liposomes in model gastrointestinal tract in a sequential profile

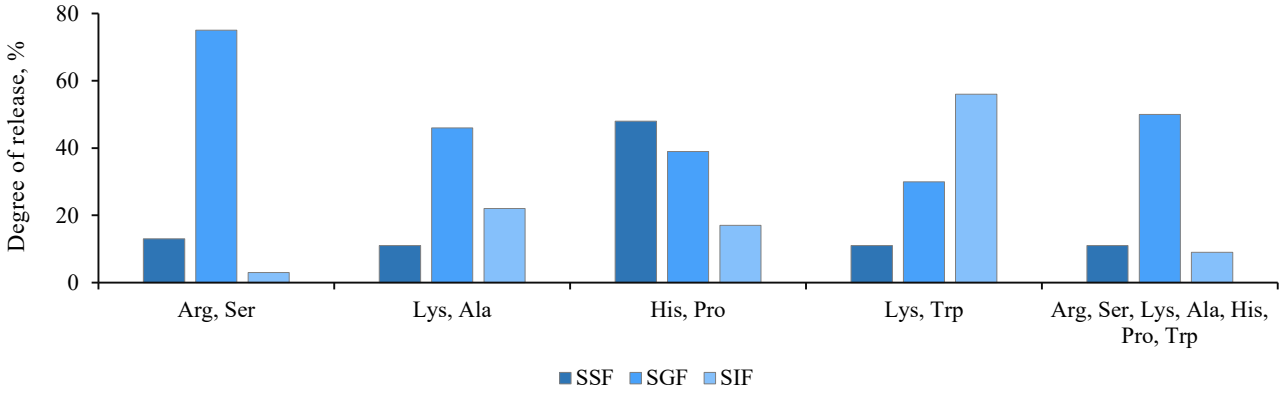

Figure 9 Release of amino acid mixtures from liposomes in model gastrointestinal tract in a sequential profile

Table 7 Incorporation of vitamins B_2 and C into liposomes loaded with amino acids

Amino acid	Incorporation, %		Liposome capacity, μg/mg	
	Vitamin B_2	Vitamin C	Vitamin B_2	Vitamin C
Arg	84	99	0.596	11.94
Ala	88	95	0.625	11.46
Ser	95	98	0.674	11.82
Trp	91	97	0.646	11.70
His	92	98	0.653	11.82
Pro	86	91	0.610	10.98
Lys	94	98	0.667	11.82

Incorporation of microelements into liposomes. Zinc and iron (III) were used as soluble salts to study the effect of microelements on the stability of liposomes. Their bioavailability for the human body is quite low (3–5% for iron and 48% for zinc). The problem of iron absorption is associated with the fact that excess iron ions bind to ferroportin, causing its degradation and storing Fe^{3+} in the form of ferritin. Therefore, the bioavailability of iron (III) ions can be improved by their co-incorporation into liposomes together with B vitamins. Zinc should be combined with vitamin C to improve the absorption of both components in the lymphoid system of the small intestine, without causing any side effects for the other organs [50].

The liposomes were obtained as described above. The microelements were added in the concentrations corresponding to their daily requirements (4.67 μg/mL for Fe^{3+} and 3.00 μg/mL for Zn^{2+}).

According to the results, the degrees of incorporation of iron (III) and zinc ions into the liposomes loaded with amino acids were 98 and 94%, respectively. Noteworthily, the incorporation of amino acids was at least 94%, which might be due to their binding to the metal ions.

Modeling the digestion of liposomes loaded with amino acids and a vitamin-and-mineral complex. We analyzed the release of amino acids from the liposomes loaded with albumin, vitamins B_2 and C, as well as Fe^{3+} and Zn^{2+} ions, in the model gastrointestinal tract (Fig. 10).

The total degree of amino acid release was 90%. Noteworthily, the incorporation of vitamins and microelements led to their stabilization in the oral cavity and stomach, with release degrees of 15 and 27%, respectively. This improved their release and bioassimilation in the small intestine.

Formation of the protein corona. According to literature, the interaction of proteins with lipid vesicles leads not only to liposomal encapsulation, but also to the formation of a protein corona on the surface. The term "protein corona" was proposed in 2007 to describe the spontaneous aggregation and coating of proteins around the surface of nanoparticles [51]. Further studies have

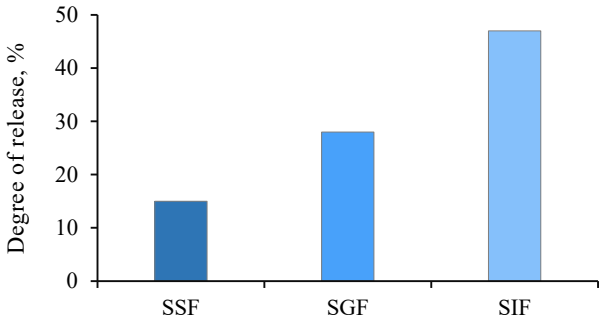

Figure 10 Amino acid release from liposomes with a vitamin-and-mineral complex under *in vitro* digestion conditions

Table 8 Changes in the size and ζ-potential of liposomes depending on the concentration of the albumin solution used for surface modification

Egg albumin solution, mg/mL	Size, μm	ζ-potential, mV
0	4.7 ± 0.2	37 ± 2
10	8.0 ± 0.4	36 ± 2
20	10.6 ± 0.5	34 ± 2
30	12.8 ± 0.6	33 ± 2
40	15.2 ± 0.8	31 ± 3
100	19.0 ± 1.0	28 ± 2

shown that protein adsorption plays an important role in many biological processes [52]. Fundamentally, a protein corona consists of an internal and an external layers. The internal layer ("hard corona") includes tightly bound protein molecules, while the external layer ("soft corona") is represented by proteins that are in dynamic equilibrium with the solution [53].

Protein coronas, which form on the surface of microparticles by changing the surface charge, can help improve the permeability of the intestinal epithelium. This can facilitate the oral administration of drugs and their absorption *in vivo* [54].

Egg albumin was used for a protein corona to form. It has a negative charge at neutral pH and can be adsorbed on the surface of a cationic carrier due to electrostatic attraction. This changes the positive charge of the surface and forms electrically neutral particles.

As we studied the formation of a protein corona on the surface of liposomes, we determined changes in the size and ζ-potential of the particles depending on the concentration of the albumin solution (Table 8).

As can be seen, higher concentrations of egg albumin in the solution for surface modification caused an increase in the liposome size and a decrease in the absolute value of ζ-potential, which indicated the formation of a protein corona. No particles had a charge closer to neutral. This might be due to the heterogeneous albumin coating of the liposomes.

The size distribution of the resulting liposomes is shown in Fig. 11. We found that high concentrations of albumin increased the number of large liposomes. For example, the sample with an albumin concentration of 100 mg/mL had only 10% of liposomes with a diameter of less than 15 μm.

The formation of a protein corona on the surface of the liposomes can improve the bioavailability of orally administered drugs. Since liposomes under 5 μm in size are used for oral administration, the concentration of albumin should not exceed 10 mg/L for a protein corona to form.

Effect of surface modification on the properties of liposomes. The liposome surface can be modified with chitosan to improve prolonged release of protein and, therefore, its bioavailability. In this experiment, aliquots of the liposomal emulsion were mixed with equal volumes of chitosan solutions at various concentrations. Egg albumin was used as a model protein.

We analyzed changes in the size of liposomes depending on the concentration of chitosan used for modification (Table 9). As can be seen, higher concentrations of chitosan increased the average size of liposomal particles. These findings were confirmed by the distribution of liposomes by size (Fig. 12).

A 0.1% chitosan solution was chosen for further analyses since this concentration provided a significant proportion of liposomes up to 6 μm in size, which is important for oral drug administration.

Effect of antioxidants on the stability of liposomes. Storage of liposomes is challenging due to their instability and high degradability. The stability of liposomes depends on many factors, such as the composition of lipids, the size and shape of particles, the density of packaging, as well as temperature and humidity.

Lipid peroxidation is one of the main causes of liposome destruction [55]. Antioxidants such as vitamin E can be used to reduce the risk of lipid peroxidation and improve the stability of liposomes.

For this experiment, liposomes were loaded with albumin. During their preparation by the injection method, alpha-tocopherol acetate was added to the liposomal mixture at concentrations of 5, 10, and 20 mg per 1 g of lecithin. We analyzed changes in the size and ζ-potential of the liposomes depending on the content of vitamin E, as well as their size distribution (Table 10, Fig. 13).

According to the data, the liposomal emulsions without tocopherol and those with 5 and 10 mg/g of tocopherol were stable and not prone to aggregation. Increasing the content of vitamin E to 20 mg/g caused the liposomes to grow in size (from 4.7 to 18.5 μm).

Next, we studied the behavior of the liposomes modified with a protein corona, chitosan, and vitamin E in the model gastrointestinal tract in a sequential profile (Fig. 14). As can be seen, the liposomes stabilized with vitamin E showed maximum release in the small intestine. This can improve the availability of the drug.

Finally, we analyzed changes in the peroxide value to determine the stability and degree of destruction of liposomes during long-term storage (Fig. 15). According to the results, 10 mg vitamin E/g liposomal base was most effective in reducing the degree of liposome degradation during storage.

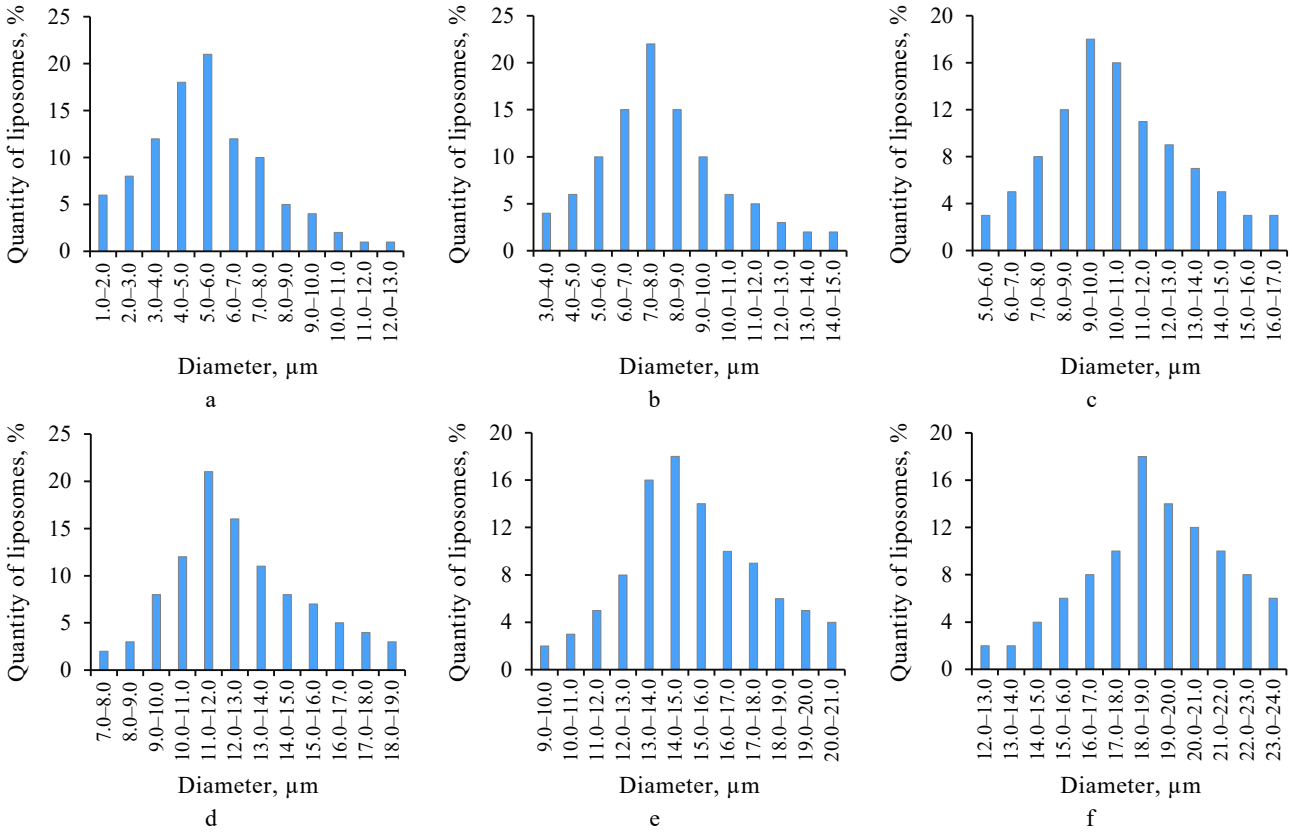

Figure 11 Liposome size distribution after surface modification with albumin at various concentrations, mg/mL: (a) 0; (b) 10; (c) 20; (d) 30; (e) 40; and (f) 100

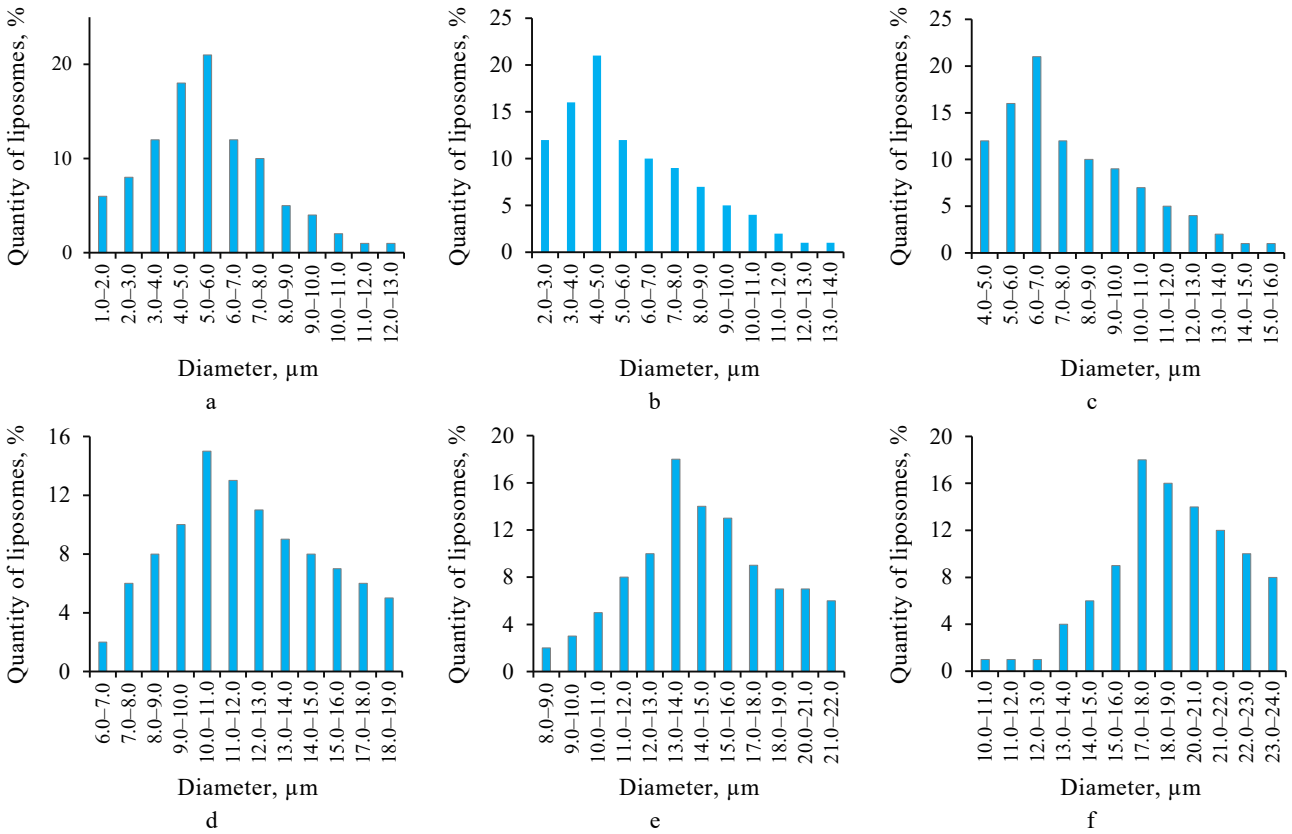

Figure 12 Distribution of liposomes by size depending on chitosan concentration, %: (a) 0; (b) 0.10; (c) 0.15; (d) 0.30; (e) 0.40; and (f) 0.50

Table 9 Changes in the liposome size depending on the chitosan concentration used for modification

Chitosan, %	Size, μm
0	4.7 ± 0.2
0.10	5.8 ± 0.3
0.15	7.8 ± 0.4
0.30	12.1 ± 0.6
0.40	14.9 ± 0.7
0.50	18.8 ± 0.9

Table 10 Changes in the size and ζ-potential of liposomes depending on the content of vitamin E

Vitamin E, mg/g	ζ-potential, mV	Size, μm
0	37 ± 2	4.7 ± 0.2
5	32 ± 2	5.2 ± 0.3
10	28 ± 2	12.8 ± 0.6
20	25 ± 1	18.5 ± 0.9

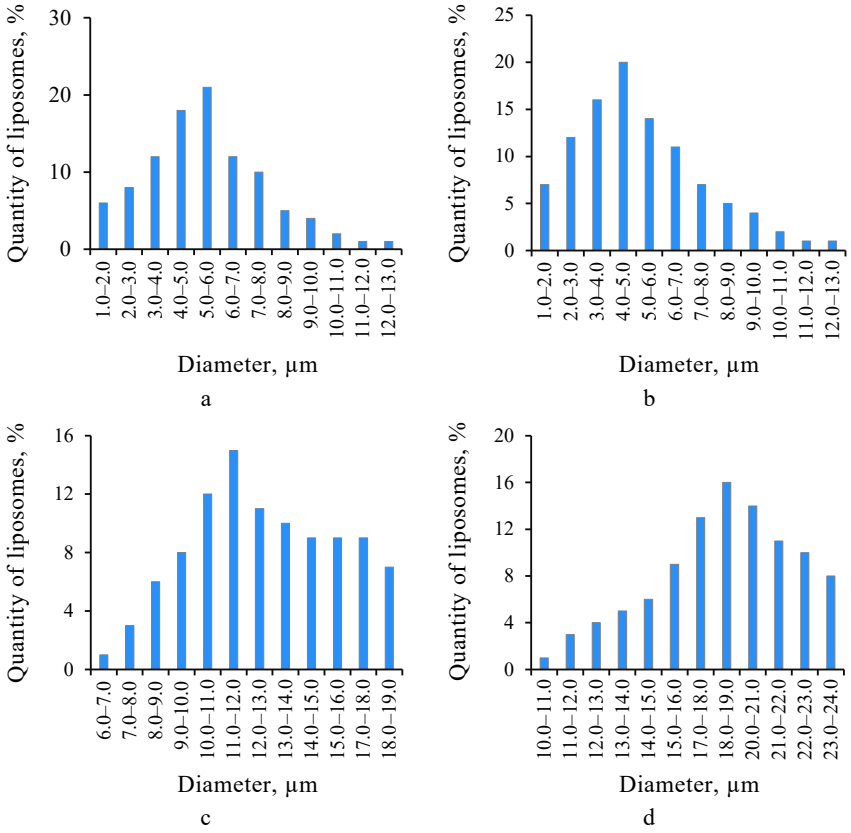

Figure 13 Liposome size distribution with vitamin E modification at concentrations, mg/g: (a) 0; (b) 5; (c) 10; and (d) 20

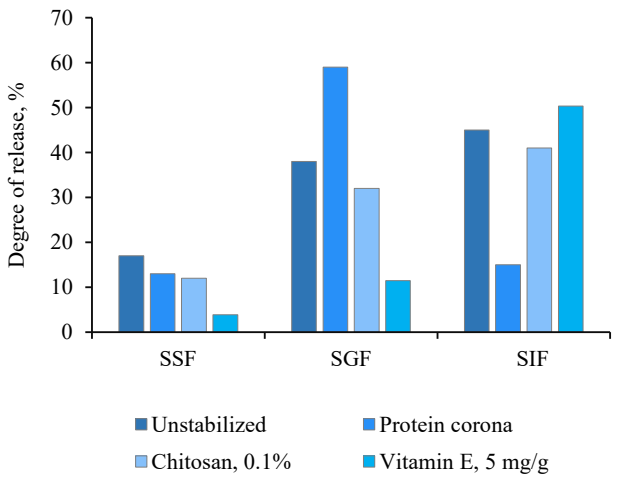

Figure 14 Release of egg albumin from liposomes stabilized in different waysna

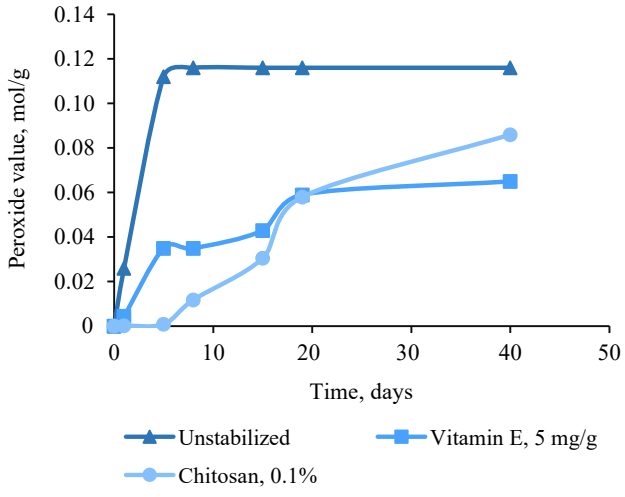

Figure 15 Changes in peroxide value during long-term storage of liposomes

CONCLUSION

Our study showed that the liposomes obtained by the thermal and injection methods had similar properties. However, the injection method produced liposomes with a smaller diameter (4.7 ± 0.2 μm), which made them more promising agents for oral drug delivery. Other advantages of the injection method over the thermal method are ease of use and a shorter duration.

Protein therapy is a promising tool for the future of medicine. Therefore, ways need to be found to prevent the destruction of protein molecules in the gastrointestinal tract. We analyzed the degrees of protein incorporation into liposomes using albumin, insulin, and globulin as model proteins. The most optimal concentrations of albumin, insulin, and globulin were 30, 1.0, and 1.6 mg/mL, respectively. These concentrations ensured their incorporation at 83, 98, and 95%, respectively.

We determined the concentrations of amino acids (arginine, alanine, proline, serine, lysine, tryptophan, and histidine) and their mixtures that ensured their maximum incorporation into liposomes. Among individual amino acids, the highest degrees of incorporation were 94% for tryptophan, 98% for histidine, 91% for proline, and 95% for lysine. Among the mixtures, maximum incorporation was observed for the arginine-serine mixture (96%) and for the mixture of all the amino acids (90%).

The liposomes were stabilized by incorporating vitamins B_2 and C, as well as microelements (zinc and iron (III) ions) in the form of easily digestible salts. We found that a vitamin and mineral complex increased the degree of incorporation of amino acid mixtures into liposomes to 94%.

We studied the release of amino acids from the liposomes loaded with amino acids, albumin, vitamins B_2 and C, and microelements (iron (III) and zinc ions) in the model gastrointestinal tract. The degree of amino acid release was 99%, with 54% released in the small intestine, which indicated a significant degree of drug stabilization and accessibility.

Our results showed a positive effect of the protein corona on the bioavailability of liposomes for oral drug administration. In particular, the protein corona changed the charge of nanoparticles, thereby facilitating drug absorption *in vivo*.

We analyzed the release of albumin, insulin, and globulin under simulated gastrointestinal conditions in both parallel and sequential profiles. Encapsulated albumin had the highest release (64%) in the small intestine, which indicated its efficiency for oral drug delivery and protection in the acidic environment of the stomach. For insulin and globulin, the release degrees were 28 and 50%, respectively.

Antioxidants can be incorporated to improve the stability of liposomes during storage. According to our results, 10 mg of vitamin E per 1 g of liposomal base was the most effective concentration for reducing the degree of liposome degradation during storage.

Modifying the surface of liposomes with chitosan decreased the degree of albumin release in the oral cavity, the stomach, and the intestine by 30, 16, and 11%, respectively.

CONTRIBUTION

A.A. Krasnoshtanova supervised the study, managed the data, and edited the manuscript. I.A. Tsepeleva designed the concept, developed the methodology, conducted the study, and wrote a draft of the manuscript. A.N. Yudina edited the manuscript.

CONFLICT OF INTEREST

The authors declared no conflict of interest regarding the publication of this article.

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