MATERIALS SELECTION FOR PRODUCTION OF MICROSPHERES PROTECTING LACTOFERRIN UNDER ACID CONDITIONS

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Abstract: Lactoferrin is a multifunctional protein of the transferrin family, which can be found in the human and other mammals milk. On the basis of many biological functions of lactoferrin, researchers have considered various possibilities of its application in health care, in the prevention and treatment of infectious and inflammatory diseases. However, lactoferrin is exposed to pepsin degradation in the gastrointestinal tract, decreasing its bioactivity after oral administration. In this regard, appropriate delivery system of lactoferrin, which ensures its delivery intact to the receptors in the small intestine requires. In this study, the different compositions of the capsules in combination with tannic acid, formed mainly by hydrogen and hydrophobic interactions were showed. Its stability toward acidic conditions (0.1 M HCl) was investigated. Complexes with polyelectrolytes (and its pairs): PSS/PAH, Parg as the first adsorbed layer on $CaCO_3$ particles formed by electrostatic interactions were presented. As the results, the adsorption of these polyelectrolytes led to greater stability of the obtained capsules. Bovine serum albumin and tannic acid, in particular with the use of poly-L-arginine as the first layer, increasing the stability of the obtained microspheres were selected as the most promising materials for the microcapsules synthesis. The changes in the morphology of [BSA/TA] and Parg [BSA/TA] capsules depending on the various number of bilayers (from 3 to 6) were analyzes. The thickness of capsule was increased on 1–2 nm by applying each subsequent layer. It was noted BSA/TA capsules looked thinner than Parg [BSA/TA] capsules with the same number of bilayers.

Keywords: Lactoferrin, microspheres, acid conditions, resistance, materials

INTRODUCTION

Lactoferrin is a polyfunctional protein of the transferring family, which has anti-bacterial, anti-viral, anticancer, antifungal, antiparasitic, antioxidant and regenerative properties. Lactoferrin can be found in the human and other mammals milk [1, 2]. On the basis of the many biological activities of hLF, researchers have considered a wide variety of possible applications in human health care, such as prophylaxis and treatment of infectious and inflammatory diseases [3].

One of the most important lactoferrin receptors are receptors on the intestinal mucosa and the GALT – related cells. However, in order to have any effect in the small intestine, it is necessary to survive in the upper gastrointestinal tract [3]. Lactoferrin is exposed to pepsin degradation in the gastrointestinal tract, decreasing its bioactivity, and do not easily get access to the target sites of the intestine due the high viscosity of the mucous layer. Therefore, the protection of lactoferrin from the action of enzymes of the gastrointestinal tract at oral administration of lactoferrin is important task [4]. In this regard, the appropriate delivery system of lactoferrin with the ability to overcome these obstacles is required.

Special systems of lactoferrin delivery: liposomes (Trif, M. et al 2001; Ishikado, A et al 2005; Ogue, S et al 2006) [5–7], the PEG-conjugates (Nojima, Y et al 2008; Bailon, P. et al 2009; Foster, GR. 2003) [8-10], enteral formulations (Takeuchi et al 2006), microparticles (Onishi et al 2007) [11], polyelectrolyte complexes (Qing-Xi Wu and others 2009, Onishi et al 2010, Koyama, K et al 2009) were developed [12]. However, many used microencapsulation methods have disadvantages such as: use of organic solvents and hard conditions of encapsulation that in the case of lactoferrin may to result to significant loss of activity.

Currently the technology of layer-by-layer (Layer-by-Layer (LBL)) of oppositely charged polyelectrolytes (PE) adsorption onto colloidal inorganic microparticles is very promising technology[13].

LBL method is simple and universal approach which allows to choice of polyelectrolytes and the range of substances which can be encapsulated. This technology allows to obtain microcapsules of certain shape and size depending on the used cores. The shell

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of the microcapsules provides the required catalytic or affine properties, stability, permeability, compatibility, and regulation of the release of the inner material of the capsule. No time-consuming methods of polymer chemistry is require for obtaining of microcapsules with the help of this technology. In addition, the use of this method allows to perform microencapsulation under physiological conditions (temperature, pH), which is especially important at encapsulation of labile bioactive compounds [14].

LBL assembly method provides a unique opportunity to include various functional and responsive components of the layers together. In the case of lactoferrin, the following functions are important: a) protection from enzyme (pepsin); b) protection from low pH (~ 1.4); and b) targeted delivery to the mucosa of the small intestine or the gut – associated lymphoid tissue [15].

PSS/PAH coating is the most studied pair of polyelectrolytes used in the production of delivery systems. Despite the successful examples of the use of this LBL, cytotoxicity limits of this approach. In general, the toxicity of the PSS/PAH LBL assembly is due to positive charge of cationic polyelectrolytes, which form pores in cell membranes with subsequent cell damage and death [16, 17].

The aim of this study is investigation of different capsules (based mainly by hydrophobic and hydrogen bonds) in regard to the resistance in acid conditions.

LBL assembly, proposed in the present study, mainly based on hydrophobic and hydrogen bonds. Non-toxic, non-ionic and biocompatible the reagents are used as main components. Synthetic polyelectrolytes have been used to increase the stability and improve the adsorption of subsequent layers.

We prepared LBL capsules with tannic acid and various polymers (Fig. 1), including polyelectrolytes (poly(styrene sulfonate), PSS; poly (allylamine hydrochloride) PAH; poly-L-arginine hydrochloride (Parg), bovine serum albumin (BSA), polyethylenimine (PEI), tannic acid (TA). Stability of these LBL assembly under acidic conditions of the stomach is studied.

Tannic acid (TA) is a natural high molecular weight polyphenols, which exhibit a unique set of properties for biomedical, pharmaceutical, food additives and other applications. The technique of LBL is used for the production of composite films containing TA and various polymers [18]. This strategy was based on electrostatic and/or hydrogen bonds of TA with cationic or electrically neutral polymers.

The chemical structure of TA (Fig. 1) includes several gallic groups, which contribute to the formation of electrostatic, hydrogen and hydrophobic bonds [18, 19]. It belongs to the group also known as hydrolyzable tannins and contains a central carbohydrate (glucose) core esterified by phenolics (gallic acid). Tannins are well-known for their astringent properties; they precipitate proteins such as collagen, gelatin, albumin, some polysaccharides, and alkaloids from solution [20]. TA is among the most intriguing building blocks in nanotechnology due to the unique chemical properties of this material, which allow interactions with various metals, minerals, metal oxides, carbon nanotubes and graphene [19–21].

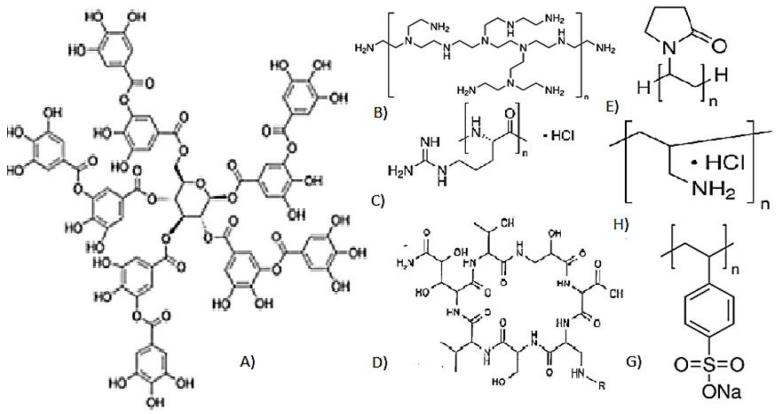


Fig. 1. Chemical structure of compounds involved in the formation of LBL assemblies of this study with tannic acid (tannic acid) (A): polyethylene imina (PEI) (B) poly-L-arginine (Parg) (C), dextran (dextran) (D), poly(N-vinylpyrro-lidone) (PVP) (E), Poly(allylamine hydrochloride) (PAH) (H), Poly(4-styrenesulfonic acid) (PSS) (G).

OBJECTS AND METHODS OF STUDY

Reagents. The sources of chemicals were as follows: poly (styrene sulfonate, sodium salt) (PSS, Mw 70,000) and poly (allylamine hydrochloride) (PAH, Mw 70,000), dextran sulfate, sodium salt (Dex, MW > 500 000), poly-L-arginine hydrochloride (PARG, MW > 70 000), bovine serum albumin (BSA), polyethylene imine (branched, average MW ~ 25.000) (PEI), tannic acid (TA), hydrochloric acid, calcium chloride dihydrate, anhydrous sodium carbonate, ethylenediaminetetraacetic acid trisodium salt (EDTA). All reagents were from "Sigma" (USA).

The water used in all experiments was prepared in a three-stage Millipore Milli-Q Plus 185 purification system and had a resistivity higher than 18:2M X cm.

$CaCO_3$ template synthesis. $CaCO_3$ particles were synthesized by mixing 1 ml of 1 M $CaCl_2$ and 1 M Na_2CO_3 on the magnetic stirrer for 1 min. After mixing, the resulted solution was centrifuged and washed thoroughly (3 times) with water Milli-Q.

Capsule preparation. The method of layer-by-layer adsorption of polyelectrolytes was used to prepare capsules. Adsorption of polyelectrolytes (at a concentration 2 mg/ml in 0.5 M NaCl) was performed by adding 1.5 ml of the respective polyelectrolyte to precipitate of $CaCO_3$ particles. The solution was incubated with constant mixing for 15 min. The resulted solution was centrifuged. The precipitate was washed (3 times) in water. After adsorption of the desired number of layers, the capsules were treated in HCl solution, pH = 1.1, to decompose Ca_2CO_3 cores. The obtained capsules were washed with water to neutral pH. The capsules were analyzed using confocal laser scanning and scanning electron microscopy.

Confocal Laser Scanning Microscopy (CLSM). Confocal micrographs were taken with an inverted confocal laser scanning microscope from Leica, equipped with a 100 x oil immersion objective. The samples were mounted with nail lacquer. This prevented the evaporation of water.

Scanning Electron Microscopy (SEM). SEM analysis was performed using a FEI Quanta FEG microscope at 5–10 kV. Samples were prepared by depositing a drop of a particle or capsule suspension on a silicon wafer and allowing it to dry at room temperature. Before imaging, dried specimens were sputter-coated with approximately 5 nm silver film using a Denton sputter-coater.

Measurement of ζ-potential. Sequential deposition of layers onto particles of calcium carbonate and the measurement of ζ-potential at pH ¼ 6 and pH ¼ 9 were carried out on Zetasizer Nano-ZS equipment (Malvern). The pH of the capsules solution were adjusted using 0.01 M HCl or NaOH. Each value was obtained by averaging three independent measurements.

The determination of capsules stability in 0.1 M HCl solution. The obtained capsules were incubated in 0.1 M HCl for 2 hours. pH was controlled during 2 hours. After incubation, the capsules were washed with water (3 times), and analyzed by scanning electron microscope, as described above.

Statistical processing of data. All of the experiments were done in triplicate. Results were expressed as the mean ± SD. Student's t-test was applied to check significant differences. Differences were reported statistically significant for $P < 0.05$.

RESULTS AND DISCUSSION

The characteristics of the microcapsules

$CaCO_3$ particles, which in contrast to the widely used melamine formaldehyde cores are non-toxic and have good ability to elimination from capsules were synthesized as a basis.

Particles were obtained by mixing solutions 0.1 M $CaCl_2$ and 0.1 M of Na_2CO_3. In the results, quite homogeneous spherical particles with size 2.0 ± 0.5 μm were obtained.

$CaCO_3$ particles, before its coating with layers of polymer are presented in Fig. 2.

Further, the following shells: $[BSA/TA]_3$, Parg $[BSA/TA]_3$, $[TA/PEI]_3$, PSS/PAH $[Ta/PVP]_2$, $[TA/PVP]_3$ were formed by layer-by-layer method.

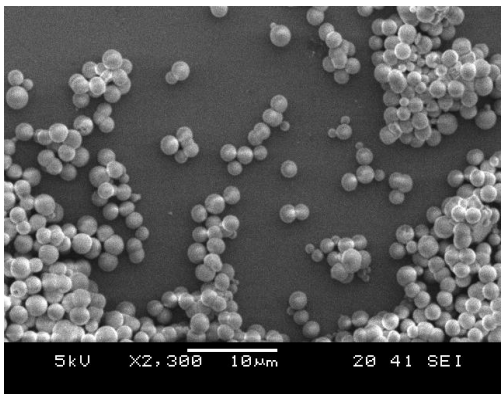


Fig. 2. $CaCO_3$ particles.

1. **$[BSA/TA]_3$ capsules on $CaCO_3$ particles**

The [BSA/TA] shell (BSA - bovine serum albumin, anionic polyelectrolyte at neutral pH of the aqueous phase; (TA – tannic acid, a polyphenol that reacts with molecules of BSA) was formed by layer-by-layer adsorption.

This shell was formed through the interaction of polyphenols with proteins. Tannins interact with specific sites of proteins, forming strong non-covalent connection with hydrophobic fragments of proteins, such as proline, histidine, and arginine residues. Phenolic groups are excellent hydrogen donor that forms a hydrogen bonds with the carboxyl group of the protein. Interaction of protein and polyphenol can be strong enough to form a stable capsules. Although, electrostatic forces that contribute to the interlayer interaction were not involved in this capsules formation.

Proteases of the gastrointestinal tract can destroy peptide bonds of protein blocks in the capsules formed by the interaction thereby to destroy the complex.

Thus, the capsules formed due to the interaction of protein and polyphenol compounds are expected to have stability in acidic conditions of the stomach and degrade under the action of the small intestine enzymes, thereby gradually release the active ingredient.

Fig. 3 shows the change in surface charge (ζ-potential) at the formation of capsules based on the [BSA/TA].

Unlike LBL assembly of conventional polyelectrolytes, the LBL was formed by hydrogen/ hydrophobic bonds of BSA/TA. The LBL assembly was characterized by overall negative ζ-potential throughout the entire multilayer formation (Fig. 3).

Fig. 4 shows images of scanning electron and confocal microscopes resulted $[BSA/TA]_3$ capsules after dissolving the calcium carbonate core.

The literature presents several possible formulations that mimicking gastric conditions. In our case the behavior of the capsules under the following conditions 0.1M HCl of pH = 1 (for 2 hours) was observed.

Fig. 5 shows images of the capsules after 2-hour incubation in 0.1 M HCl solution.

As Fig. 5 shows, [BSA/TA]3 capsules were not sufficiently stable in 0.1 M HCl. The high concentration of hydrochloric acid resulted to change of the morphology and fracture of the microspheres. The contents of the capsules in the sample were significantly less compared with the control (Fig. 4).

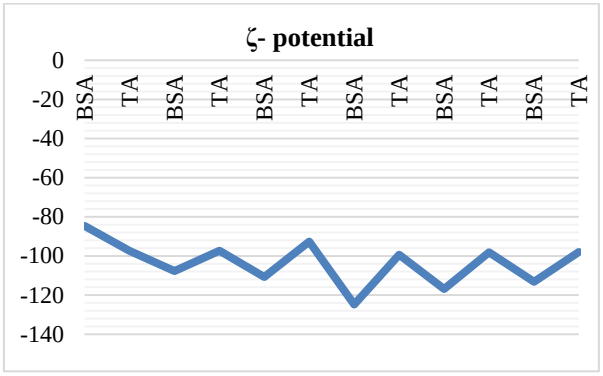
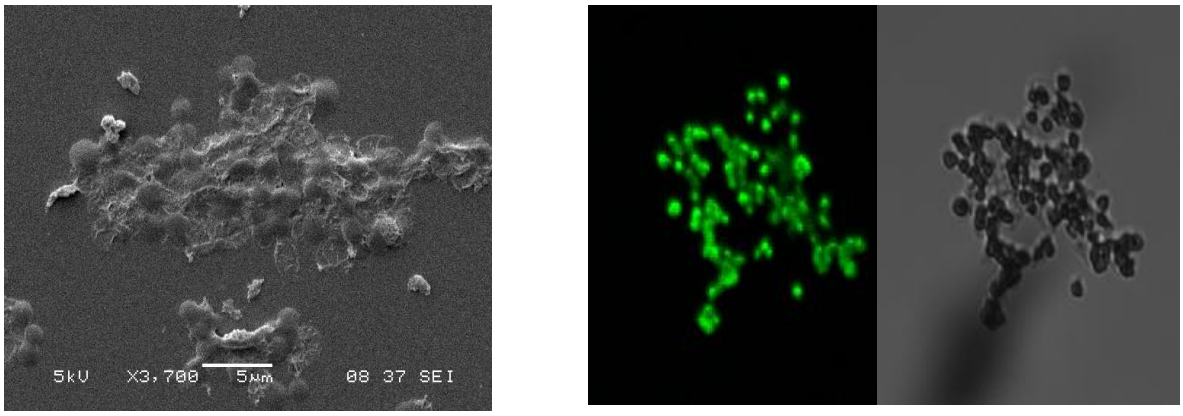
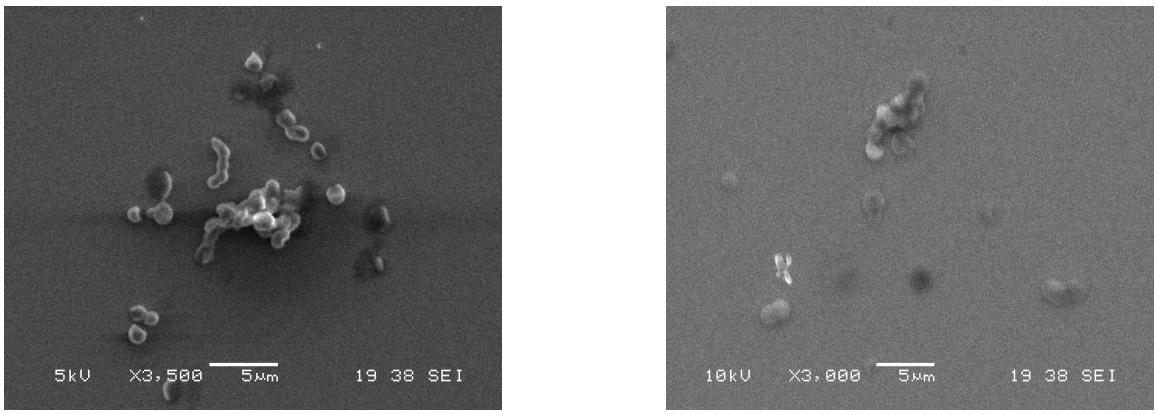


Fig. 3. Change of ζ - potential of the [BSA/TA] capsules at the layers adsorption.



(a) (b)

Fig. 4. SEM (a) and confocal microscope images (b) $[BSA/TA]_3$ capsules after dissolving the $CaCO_3$ core.



(a) (b)

Fig. 5. SEM images of the $[BSA/TA]_3$ capsules after incubation in HCl solution for 2 hours.

2. Parg $[BSA/TA]_3$ capsules on $CaCO_3$ particles

For improving adsorbtion of first layers of BSA/TA capsules, the cationic polyelectrolyte poly-L-arginine was adsorbed as the first layer, followed adsorption of BSA through electrostatic interaction. The change of ζ-potential during the formation of this shell is presented in Fig. 6.

In the case of Parg [BSA/TA], after the adsorption of positively charged polyalanine ζ-potential of the capsules varies from of 63.14 ± 2.00 mV to -53.02 ± 2.00 mV as a result of coating the negatively charged BSA. Further, the change of ζ-potential occurs only in the negatively charged part of the scale the same as [BSA/TA].

Morphology of the obtained capsules is presented in Fig. 7 (a, b).

Figure 8 shows images of the capsules after 2-hour incubation in 0.1 M HCl solution.

As Fig. 8 shows, Parg$[BSA/TA]_3$ capsules were more resistant to 0.1 M of HCl than $[BSA/TA]_3$. High concentration of hydrochloric acid did not lead to significantly change of the morphology and the destruction of the capsules.

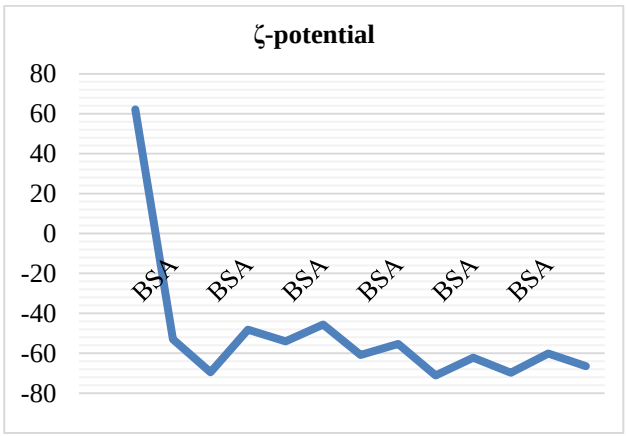
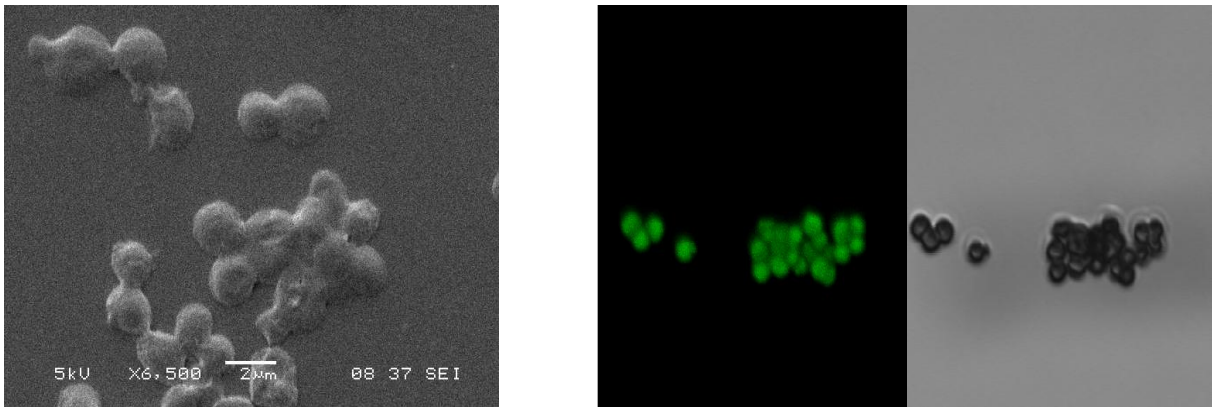
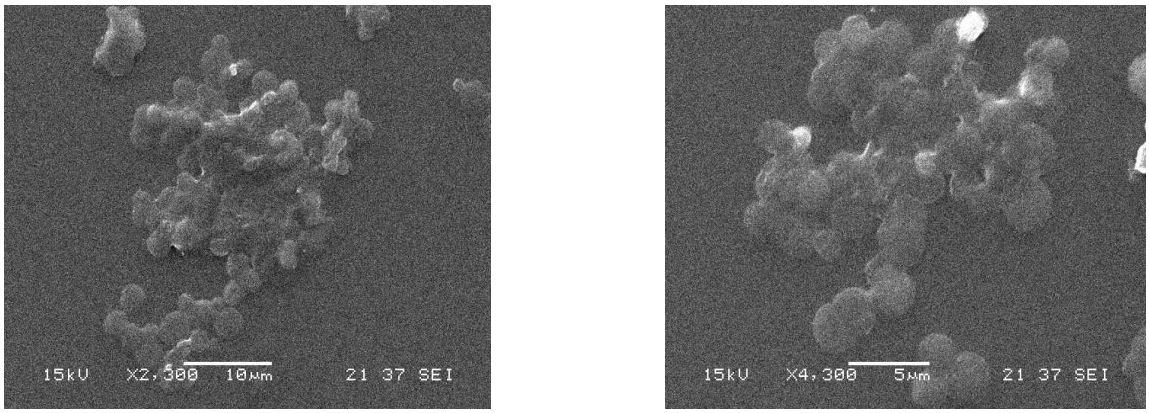


Fig. 6. Change of ζ - potential of the Parg [BSA/TA] capsules at the layers adsorption.



(a) (b)

Fig. 7. SEM (A) and confocal microscope images of Parg[BSA/TA]$_3$ capsules after dissolving the core.



(a) (b)

Fig. 8. SEM image of Parg[BSA/TA]$_3$ capsules after incubation in 0.1 M HCl for 2 hours.

3. [TA/PVP]$_3$ capsules on $CaCO_3$ particles

Tannic acid showed interesting interaction with polymers such as polyvinylpyrrolidone (PVP). It is known that acquires a negative charge resulting as a result the deprotonation of phenol groups. Polyvinylpyrrolidone is electrically neutral polymer containing hydrogen-host of the carbonyl groups. Polyvinylpyrrolidone interacts with tannic acid through hydrogen bonds involving the carbonyl groups of PVP and phenolic hydroxyl groups of TA. This PVP-TA complex is a very promising basis for capsules.

The change of ζ-potential at the [TA/PVP]$_3$ formation is presented shown in Fig. 9.

As in the case of BSA/TA capsules, the change of ζ-potential at the formation of [TA/PVP]3 shell is characterized by negative ζ-potential throughout the entire multilayer formation.

Morphology of the obtained capsules is presented in Fig. 10. The [TA/PVP]$_3$ assembly forms a rather thin capsules with size of ~2 µm.

For the evaluation of the obtained microspheres to regard to the acidic conditions of the gastrointestinal tract, capsules were incubated in 0.1 M HCl. Images of scanning electron microscope of the obtained samples are presented in Fig. 11.

As Fig. 11 shows, the $[TA/PVP]_3$ capsules were unstable to regard to 0.1 M HCl. The high concentration of hydrochloric acid resulted to almost complete destruction of the obtained microspheres.

4. PSS/PAH $[Ta/PVP]_2$ capsules on $CaCO_3$ particles

The pair of anion-cation PSS/PAH formed through electrostatic bonds was used to increase the stability and improve the layers adsorption, formed via hydrogen interactions in [Ta/PVP]. PSS/PAH is the most commonly used anionic/polycation pair during LBL formation.

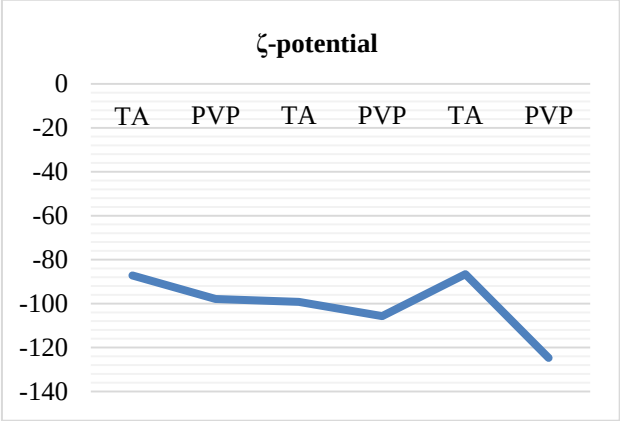
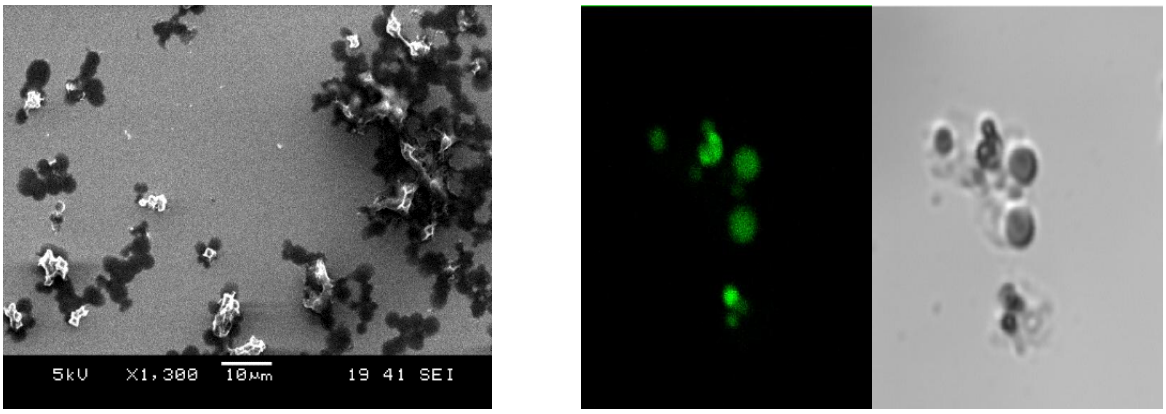
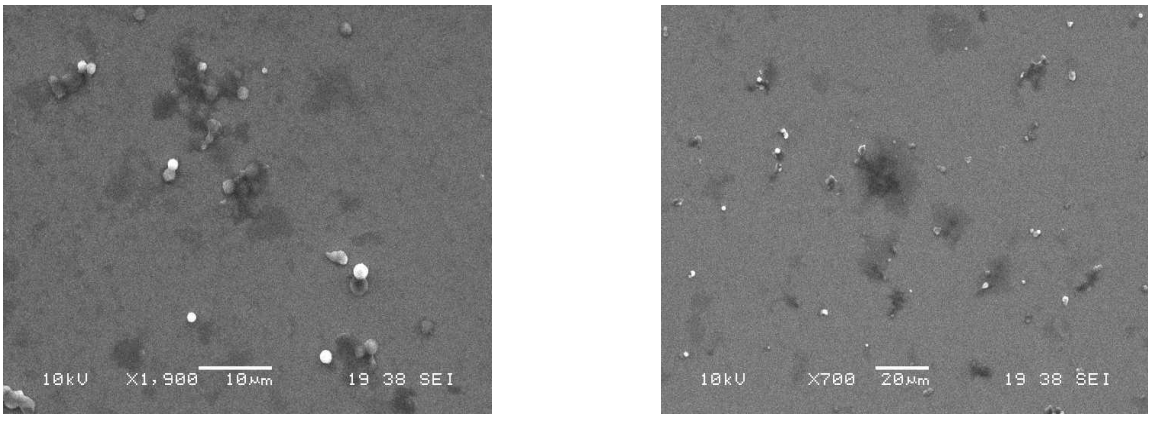


Fig. 9. Change of ζ - potential of the $[TA/PVP]_3$ capsules at layers adsorption.



(a) (b)

Fig. 10. SEM (A) and confocal microscope images of $[TA/PVP]_3$ capsules after dissolving of the core.



(a) (b)

Fig. 11. SEM images of $[TA/PVP]_3$ capsules after incubation in 0.1 M HCl for 2 hours.

In the case of PSS/PAH[Ta/PVP]$_2$ capsules, after adsorption of the negatively charged polyanion the ζ-potential of the capsules was changed to -168.17 ± 2.00 mV. The next bilayer of PSS-PAH is formed via electrostatic interactions. After adsorption of PAH layer the ζ-potential was changed to +70.13 mV. Further, the change of ζ-potential occurs only in the positively charged part of the scale range from +12.45 to +88.23 mV.

The morphology of PSS/PAH[Ta/PVP]$_2$ capsules, obtained after dissolution of the $CaCO_3$ particles is displayed in Fig. 10 (a, b). The obtained fine spherical capsules were with a size of 2 μm. The figure can be noted the complete dissolution of the core.

For the evaluation of the obtained microspheres to regard to the acidic conditions of the gastrointestinal tract, the capsules were incubated in 0.1 M HCl. Images of scanning electron microscope of the obtained samples are presented in Fig. 14.

As Fig. 14 shows, PSS/PAH [Ta/PVP]$_2$ capsules were more resistant to 0.1 M HCl than [Ta/PVP]$_2$ capsules, the morphology of the obtained capsules was not changed. However, the high concentration of hydrochloric acid led to partial destruction of the capsules. Only a little number of capsules was found after exposure for 2 hours in 0.1 M HCl.

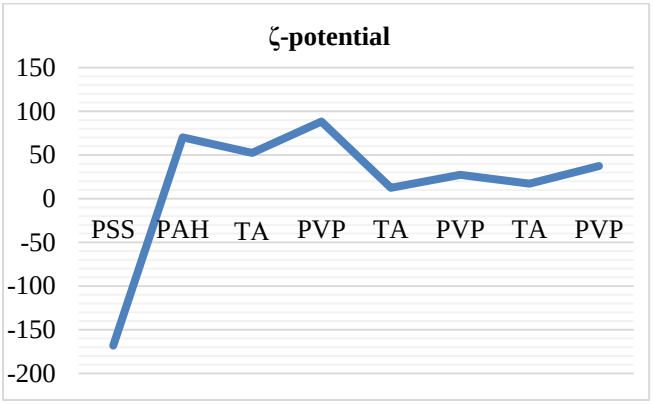
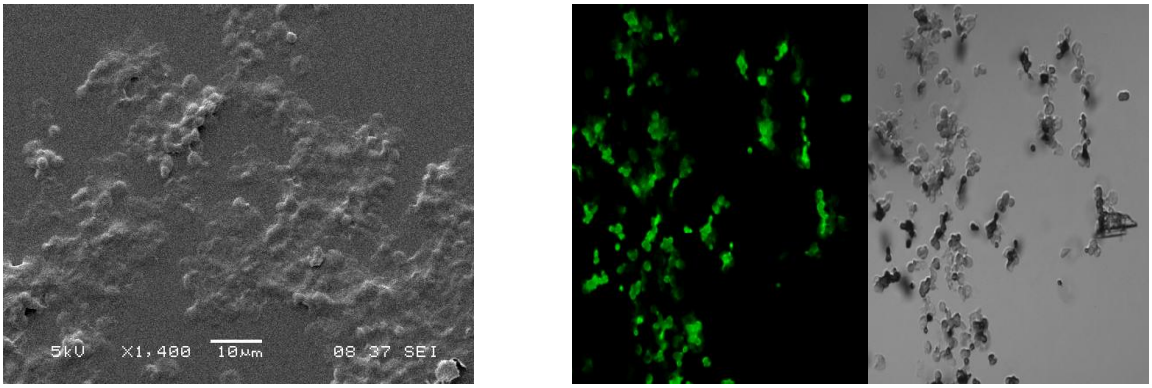
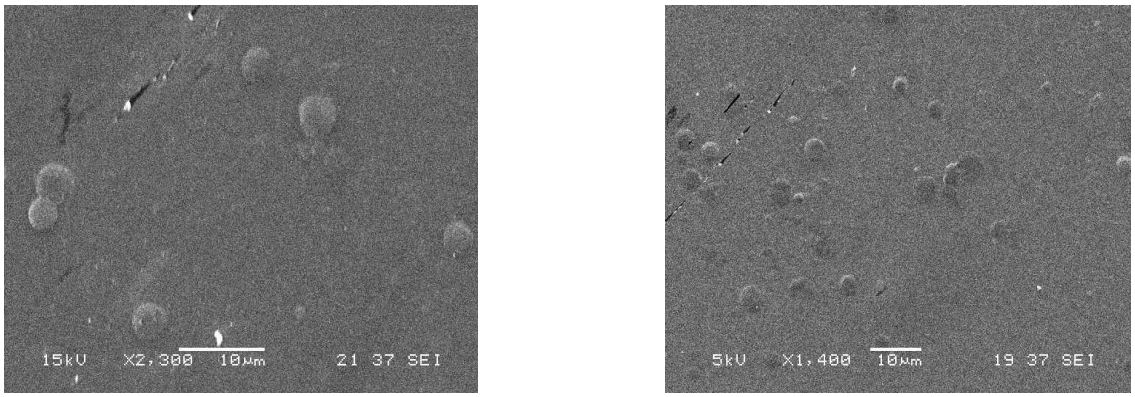


Fig. 12. Change of ζ - potential of the PSS/PAH [Ta/PVP]$_2$ capsules at layers adsorption.



(a) (b)

Fig. 13. SEM (A) and confocal microscope images (B) of PSS/PAH[Ta/PVP]$_2$ capsules after dissolving the core.



(a) (b)

Fig. 14. SEM images of the PSS/PAH [Ta/PVP]$_2$ capsules after incubation in 0.1 M HCl for 2 hours.

5. $[TA/PEI]_3$ capsules on $CaCO_3$ particles

Characteristics of the shell formed due to the interaction of cationic polyelectrolyte PEI and tannic acid were considered. PEI is a cationic polyelectrolyte, the most commonly used in the transfer of genes and oligonucleotides to animal cells.

In the case of $[TA/PEI]_3$ capsules the change of ζ-potential occurs only in the positively charged part of the scale range from +12.45 to +23.25 mV.

Morphology of $[TA/PEI]_3$ capsules, obtained after dissolution of the $CaCO_3$ particles is presented in Fig. 16 (a, b). The obtained spherical capsules were with a size of ~2 μm. The figure can be noted the complete dissolution of the cores.

For the evaluation of the obtained microspheres to regard to the acidic conditions of the gastrointestinal tract, capsules were incubated in 0.1 M HCl. Images of scanning electron microscope of the obtained samples are presented in Fig. 17.

As Fig. 17 shows, $[TA/PEI]_3$ capsules were quite resistant to 0.1 M HCl, but high concentrations of hydrochloric acid led to changes of the morphology of the capsules and a little distruction. $[TA/PEI]_3$ is one of the most stable compounds presented in the study. But, PEI is to non-destroying compounds in physiological fluids, which does not allow to talk about the prospects of its application in our future research

Thus, researches and analysis of characteristics, presented above, showed that the most promising materials for LBL assembly are BSA/TA (in particular, Parg[BSA/TA]), which will be considered in further studies.

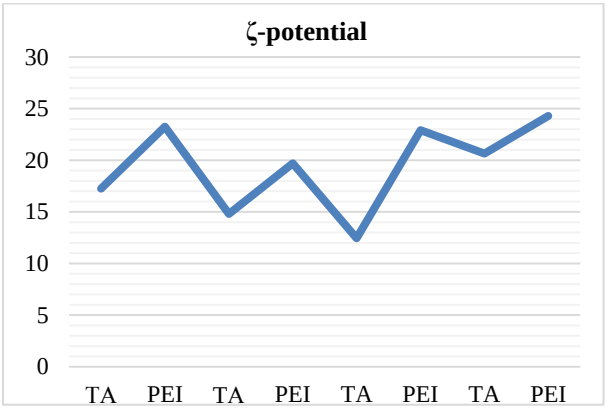
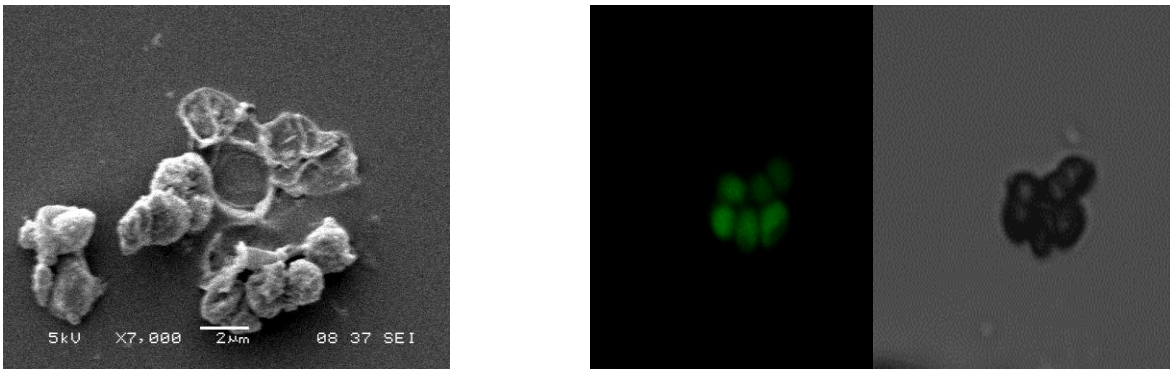
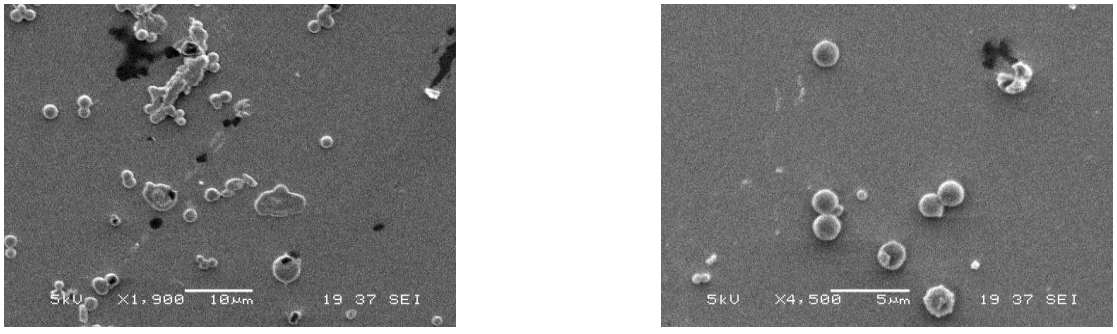


Fig. 15. Change of ζ - potential of the $[TA/PEI]_3$ capsules at layers adsorption.



(a) (b)

Fig. 16. SEM (A) and confocal microscope images of $[TA/PEI]_3$ capsules after dissolving the $CaCO_3$ core.



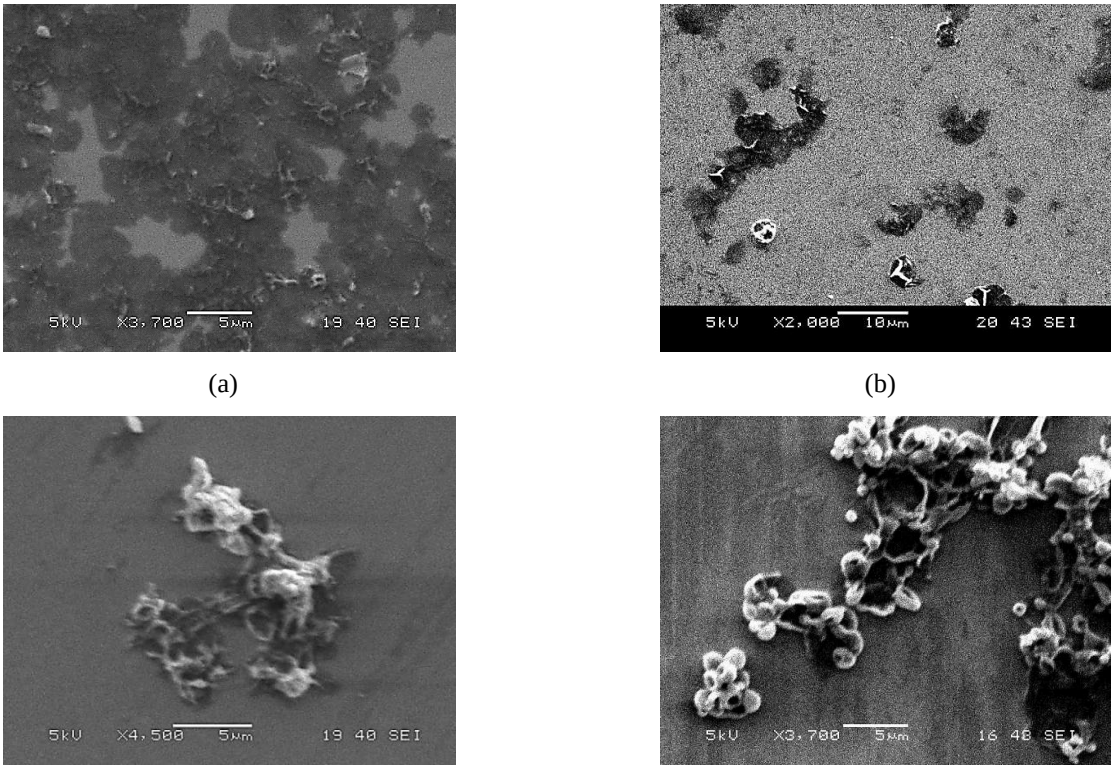
(a) (b)

Fig. 17. SEM image of the $[TA/PEI]_3$ capsules after incubation in 0,1 M HCl for 2 hours.

Characterization of BSA/TA and Parg[BSA/TA] microcapsules depending on the number of layers

Fig. 18 and 19 allow to note changes of the capsules thickness during the adsorption of each additional layer.

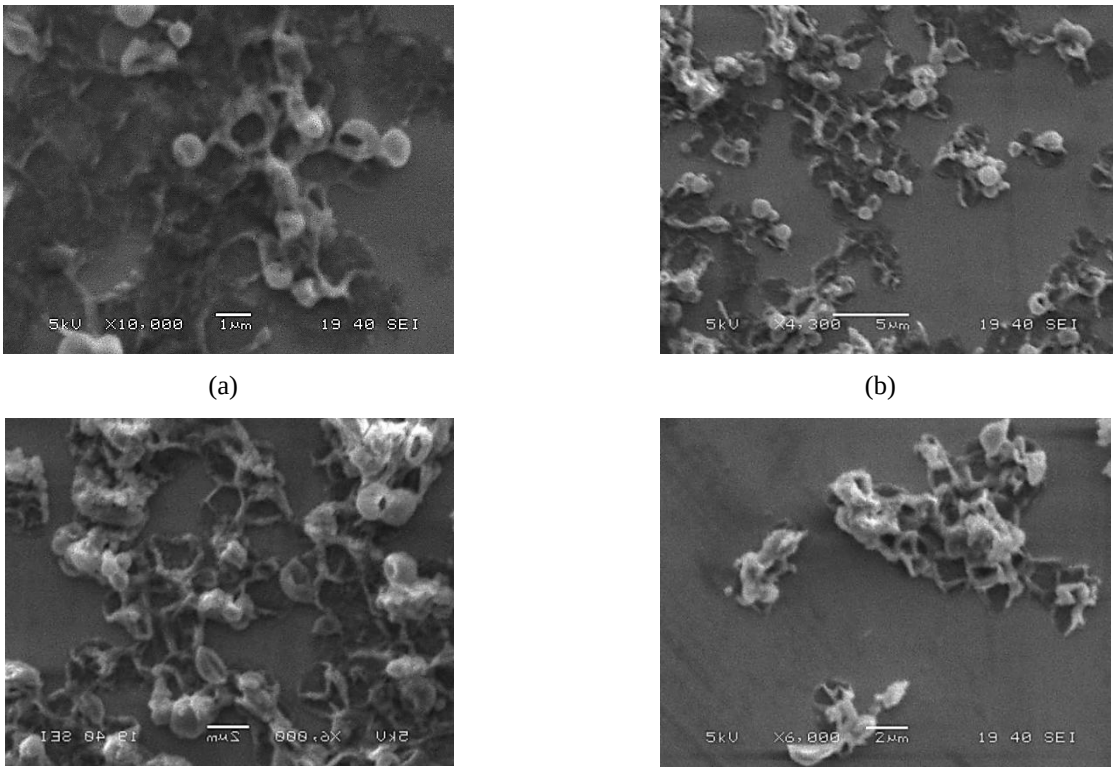
As Fig. 18 and 19 show, BSA/TA capsules looked thinner than Parg [BSA/TA] capsules with the same number of bilayers. The thickness of capsules was increased on 1–2 nm by adsorption each subsequent layer.



(a) (b)

(c) (d)

Fig. 18. SEM images of [BSA/TA] capsules: (a) 3, (b) 4, (c) 5, and (d) 6 bilayers.



(a) (b)

(c) (d)

Fig. 19. SEM images of Parg [BSA/TA] capsules: (a) 3, (b) 4, (c) 5, and (d) 6 bilayers.

CONCLUSION

Thus, in this study the different compositions of the capsules in combination with tannic acid, formed mainly by hydrogen and hydrophobic interactions were showed. Its stability toward acidic conditions (0.1 M HCl) was investigated. Complexes with polyelectrolytes (and its pairs): PSS/PAH, Parg as the first adsorbed layer on CaCO3 particles formed by electrostatic interactions were presented. As the results, the adsorption of these polyelectrolytes led to greater stability of the obtained capsules. Bovine serum albumin and tannic acid, in particular with the use of poly-L-arginine as the first layer, increasing the stability of the obtained microspheres were selected as the most promising materials for the microcapsules synthesis. The changes in the morphology of [BSA/TA] and Parg [BSA/TA] capsules depending on the various number of bilayers (from 3 to 6) were analyzes. The thickness of capsule was increased on 1–2 nm by applying each subsequent layer. It was noted BSA/TA capsules looked thinner than Parg [BSA/TA] capsules with the same number of bilayers.

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List of abbreviations

LTF – hltf lactoferrin is human lactoferrin; EDTA – ethylenediaminetetraacetic acid; TA – tannins (tannic) acid; PEI – polyethylenimine; PVPON or PVP – polivinilpirrolidon; Parg – polyalanine (poly-L-arginine); BSA – bovine serum albumin; PSS – poly-styrenesulfonate, sodium salt; PAH – poly-allylaminehydrochloride; SEM – scanning electron microscope.

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