Specificity of release from biocompatible microcapsules with ovomucoid integrated into the shell

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Abstract—The possibility of control of model substance release from microcapsules made of bovine serum albumin (BSA) and tannic acid (TA) by introducing ovomucoid into their shell was investigated. Since tannic acid, bovine serum albumin and ovomucoid are natural compounds, the systems should be non-toxic, biodegragable and biocompatible. Polymer microcapsules are often used for drug and biological molecules delivery (e.g., DNA, peptides, and polysaccharides). It was obtained that stable complexes consisting of tannic acid, BSA and ovomucoid formed and structures of these complexes were investigated. This system can be used in future for oral delivery of different proteins and peptides.

Keywords—layer-by-layer; tannic acid; release; ovomucoid; scanning electron microscope

I. INTRODUCTION

More than 10 billion injections of peptides and proteins are performed worldwide every year [1]. It means that injections still remain the most common administration route. In one hand, injections are the quickest route. In the other hand, this route is limited by serious side-effects and little half-life of drugs. These limitations are the main reason for new protein and peptide drug delivery systems discovery.

Since 2016, the most promising directions in drug delivery systems development can be selected [1 - 6]:

- 1. Therapeutical drugs based on microcapsules
- 2. Anti-cancer drugs based on nanoparticles
- 3. Transdermal drug delivery systems
- 4. Implants
- 5. Antibody-protein conjugates

The concept of protein and peptide drug delivery based on microcapsules was to manage the delivery of drugs into the body and their prolonged and sustained release reducing the number of injections.

Inclusion of proteins and peptides into microcapsules obtained by layer-by-layer adsorption of oppositely charged natural polyelectrolytes is one of the promising methods. These delivery systems generally consist of natural and/or synthetic polymers strictly selected for delivery (protection, prolongation, binding to other objects, etc.) and more than others suitable for oral administration. Release of a drug from polymeric microcapsules is usually determined by dissolution of the protective coating caused by pH changes of the medium. Thus, a pair of polyelectrolytes capable to protect drugs from the acidic environment of patient's stomach and to ensure its controlled release in intestinal environment is selected. In a number of studies, different inhibitory agents (for example, aminobenzamidine-n) and various modified amino acids are widely used to protect proteins in microcapsules from enzymes action [7-9].

To form a shell of microcapsules biocompatible natural compounds - protein bovine serum albumin (BSA) and polyphenol tannic acid were selected.

Due to the large number of hydroxyl groups of tannic acid (TA) strong connection with complex hydrocarbons and various proteins forms. These complexes can be either soluble or insoluble and are usually formed by hydrophilic interactions.

Due to the natural stability of tannic acid to enzyme action it can be expected that microcapsules will effectively protect included substances when it pass gastro-intestinal tract. However, it is known [10], that trypsin destroys tannic acid and BSA complex.

Therefore, to protect microcapsules from trypsin and to slow content release additional layers of trypsin inhibitor (ovomucoid) were applied. Ovomucoid (OM) is a glycoprotein with molecular weight of 30 kDa. Amino groups of trypsin react with acidic functional groups of ovomucoid forming complex with it and inhibiting activity of trypsin. Since ovomucoid is a protein, it is also capable (as BSA) to form complex with tannic acid.

The aim was to demonstrate the ability to control the release of model substance poly(allyl)amine hydrochloride (polymer, that is safe, non-toxic, soluble in water, stable to hydrolysis and not sensitive to pH changes), from microcapsules, made of bovine serum albumin (BSA) and tannic acid (TA), by introducing ovomucoid into their shell.

II. MATERIALS AND METHODS

A. Materials

The following chemical reagents: sodium carbonate (Na₂CO₃), calcium chloride (CaCl₂_2H₂O), sodium chloride (NaCl), tannic acid, ovomucoid, bovine serum albumin labelled with fluorescein isothiocyanate, rhodamine B, poly(allyl)amine hydrochloride were used as received from Sigma-Aldrich (puriss. p.a.)

Solutions of salts for obtaining CaCO₃ cores were prepared using deionized water with a conductivity of 0.056 mS/cm (Vital Diagnostics, St. Petersburg, Russia).

B. Microcapsule formation

The cores consisting of CaCO₃ were formed during precipitation across the reaction between calcium chloride and sodium carbonate. The certain volume of 0.33 mol/L water solution of CaCl2 (3 mL) was rapidly added into water solution of Na2CO₃ of the same volume and concentration of 2 mg/ml during mixing with a magnetic stirrer (600 rpm). The mixture was stirred for 30 s and then the obtained suspension was left for 15 min. Then the mixture was centrifuged (3000 rpm) with Centrifuge 5418 (Eppendorf, Germany) and supernatant was collected. The cores were washed with ethanol and then were dried in FD 115 (Binder, Germany) convection oven at 50° C.

After that polyphenol-protein multilayer assembly were formed on the cores by alternating deposition of tannic acid, bovine serum albumin and ovomucoid as described on Fig. 1 and elsewhere [11-13].



Fig. 1. Scheme of microcapsule formation.

C. Encapsulation efficiency

The amount of protein loaded into cores (I) was calculated by means of the following equation:

$$I(\%) = \frac{\left(C \times V\right)_{s} - \left(\sum_{i} \left(C_{i} \times V_{i}\right)\right)}{\left(C \times V\right)_{s}} \times 100\%$$
(1)

where V_s is the volume of the starting solution; C_s is the concentration of the starting solution; V_i is the volumes of filtrate and the liquid used for washing filters (in the co-precipitation method) or the volume of the supernatant measured after reaching equilibrium (in the adsorption method) and C_i is the concentration of filtrate and the liquid used for washing filters or the

concentration of the supernatant measured after reaching equilibrium (in the corresponding methods).

The efficiency of PAH inclusion into carbonate cores was defined by PAH load (L) i.e. the amount of the included substance per unit of the core weight:

$$\hat{L} = I \bullet (P_{pr} / P_{templ}), \tag{2}$$

where P_{pr} is the initial weight of substance and P_{templ} is the weight of cores synthesized in co-precipitation experiments or used in adsorption experiments.

D. Preparation of trypsin solution

Trypsin solution was prepared as follows: 10 mg of trypsin was dissolved in 10 ml of 0.1 mM HCl. The resulting solution was then diluted with 0.2 M Tris (hydroxymethyl) aminomethane (TRIS) until pH of 7.5-8.5 was obtained.

E. Release study

Suspensions with microcapsules were incubated in 2 ml of 0.2 M TRIS solution with trypsin (prepared as described above) constantly stirring with a MixMate vortex shaker (Eppendorf, Germany) at 100 rpm and 37° C. At certain intervals of time aliquots (0.1 ml) of the suspension were pipetted (Eppendorf, Germany) and an equivalent volume of trypsin buffer was added. Then aliquots were centrifuged at 5000 rpm for 5 minutes, supernatant was separated and concentration of PAH labeled with rhodamine В was determined with spectrophotometer. The release efficacy of PAH was evaluated by the ratio of its content in the supernatant and in the starting suspension.

F. Scanning Electron Microscopy (SEM)

Photographs of microcapsules were obtained using a Supra 40 VP scanning electron microscope (Carl Zeiss, Germany). The microscope is equipped with a computer control system of scanning with electron beam. It also has signal and image digital registration, x-ray microanalyzer INCA and INCA WAVE X-MAX. The samples were attached to the Si plates and then coated with gold. Microparticles were assessed using SEM).

G. Spectrophotometry

Optical measurements were carried out using a SF-2000 spectrophotometer (LOMO, Russia). For microcapsules and their contents visualization fluorescent labels to bovine serum albumin (BSA) and poly (allyl) amine hydrochloride (PAH) were attached (Fig. 2). Concentrations of labeled PAH were calculated from the calibration curve at $\lambda_{max} = 560$ nm (the maximum in the UV region of rhodamine B absorption spectra).



Fig. 2. Structure of fluorescent labels: a - fluorescein isothiocyanate (FITC); b - rhodamine B.

III. RESULTS AND DISCUSSION

A. Synthesis of carbonate cores

Since the core is the basis of microcapsules presented in this study, the size and the shape initially embedded in it will determine morphology of microcapsule itself. Three modifications of calcium carbonate are known [14]: a vaterite (spherical form, hexagonal matrix), calcite (cubic form, rhombohedral matrix) and aragonite (needle-shaped form, orthorhombic matrix). One of these modifications, the vaterite, was chosen because of its better solubility in comparison with calcite or aragonite, highly dispersed porous structure and high specific surface density. The CaCO₃ vaterites are formed by precipitation of CaCl₂ and Na₂CO₃. It has already been shown [15] that in order to obtain calcium carbonate vaterites of various sizes, it is necessary to correct some parameters of their synthesis process, in particular: time and intensity of solution stirring, drying methods of the cores, the presence/absence of ethylene glycol in the initial solutions (CaCl₂ and Na₂CO₃), temperature changes.

Scanning electron microscopy images of microparticles are presented in Fig. 3. The SEM images in Fig. 3 give information about the shape of the vaterite, its size and pore structure. The obtained CaCO₃ cores had a narrow size distribution ranging from 2 to 4 μ m.



Fig. 3. CaCO₃ core structure: a, b – intact cores; c – pores structure of the core. Magnification \times 60 000 (a), \times 30 000 (b), \times 90 000 (c).

Porosity is an important feature of CaCO3 microparticles because it is connected with drug inclusion and capsule formation. By BET (Brunauer, Emmett and Teller) the specific surface area of the cores and its pore size distribution was measured in [16]. The pore size was about 35-40 nm that is also confirmed in [17, 18]. From SEM image in Fig. 3c it is also seen that the pore size is approximately 40 nm.

Zeta-potential of carbonate core is mainly determined by its surface charge. CaCO₃ particles zeta-potential in water (pH 5.5) was measured by means of ZetaSizer Nano ZS (Malvern, UK) and was negative ($-6.8 \div -8.3$ mV).

B. Microcapsule formation

Drug incorporation can be performed before, during, or after microcapsule formation. In one case, a drug can be mixed with a biopolymer solution during a shell of microcapsules formation. Alternatively, carbonate core could be formed first and then a drug could be adsorbed into the pores. Drug-loaded cores also can be obtained by the co-precipitation method. In brief, CaCl₂ solution containing a drug is added to Na₂CO₃ solution under stirring. A drug is captured by growing CaCO₃ cores.

 $CaCO_3$ microparticles with PAH labeled with rhodamine B were coated with: 8 layers of BSA and tannic acid (1); 4 layers of BSA and tannic acid and 4 layers of ovomucoid and tannic acid (2). Scanning electron microscopy images of microparticles are presented in Fig. 4.



Fig. 4. Capsule formation: a – 8 layers of BSA and tannic acid; b – 4 layers of BSA and tannic acid and 4 layers of ovomucoid and tannic acid. Magnification × 30 000.

Ovomucoid like other proteins is binding to tannic acid through hydrogen bonds and is forming a dense shell (Fig. 5). Protein included into the shell of the microcapsules was previously labeled with fluorescein isothiocyanate (FITC). The resulting microcapsules had an average PAH labeled with rhodamine B inclusion 54.7% and a loading of 24.6 μ g / mg. At the same time, the loss of the model substance during the microcapsule formation was 1.3% per layer.



Fig. 5. Capsule formation: $a - CaCO_3$ core with a shell of BSA, tannic acid and ovomucoid; b - final capsule obtained after an hour in 0.2 M EDTA. Magnification \times 40 000.

Passing through the shell pores, ovomucoid interacts not only with tannic acid on the surface of the microcapsules, but also with free tannic acid and BSA groups of earlier layers (Fig. 6). Thus, the application of subsequent layers of ovomucoid significantly increases the microcapsule's thickness (Fig. 6b).



Fig. 6. Microcapsules made of (BSA*FITC + TA)₂, with 1 layer (a) and 2 layers (b) of ovomucoid. Magnification \times 60 000. Microcapsule size 1.8-2 µm.

C. Release study

In general, the release mechanisms from polymer microcapsules can be divided on three: 1 - diffusional-controlled release; 2 - degradation-controlled release; 3 - triggered release.

In case of diffusional-controlled release, the rate of drug diffusion depends on the pore size of microcapsules. If the pore size is larger than the size of a drug, than the diffusion coefficient is high. If the pore size is smaller/equal to the size of a drug, than the coefficient is low due to steric barrier.

The release of a drug from polymer microcapsule as a rule is also induced by degradation of the shell. Degradation can be caused by different factors: pH or ionic strength changes, temperature changes, enzyme's action, etc. Degradation of the microcapsule may also occur due to surface erosion, weakening of the links between compounds that form the shell, chemical changes in compound's structure.

In most cases it is important for drug delivery system to protect an encapsulated object (especially, proteins and peptides) from degradation by acids or enzymes. When a drug delivery system enters stomach, highly acidic gastric fluids (pH 1 to 3), high mineral content (calcium, potassium, etc.) and various types of digestive enzymes (proteases, lipases, etc.) influences on it. After a drug delivery system enters intestinal tract, alkaline intestinal fluids also containing different enzymes influences on it. All these factors (enzymes and pH) should be taken into account when drug delivery systems is developing.

The obtained release profiles of poly (allyl) amine hydrochloride labeled with rhodamine B from the system with 8 layers of BSA and tannic acid showed that presence of trypsin in buffer with alkaline medium increases the release rate (Fig. 7c) after 2 hours of contact (it was about 30%). Release from microcapsules without a modified shell placed in alkaline medium without trypsin after 2 hours was about 22% (Figure 7a). Similar differences in release rate of rhodamine B-labeled PAH from microcapsules into alkaline medium with trypsin (about 20%) and without it (about 15%) were observed for systems with 4 layers of BSA and tannic acid and 4 layers of ovomucoid and tannic acid (Fig. 7b and 7d).



Fig. 7. Release profiles of PAH labelled with rhodamine B: a - medium of 0.2 M TRIS-HCl without trypsin, without ovomucoid in the shell; b - medium of 0.2 M TRIS-HCl with trypsin, with ovomucoid in the shell; c - medium of 0.2 M TRIS-HCl with trypsin, without ovomucoid in the shell; d - medium of 0.2 M TRIS-HCl without trypsin, with ovomucoid in the shell. Incubation temperature 37°C.

Introduction of ovomucoid into the shell of the investigated microcapsules reduced (almost 2.5 times) the release rate of the model substance into alkaline medium without trypsin (Fig. 7a and 7d). Similar results were obtained for microcapsules with ovomucoid in the shell after their contact with alkaline medium with trypsin (Fig. 7b and 7c).

CONCLUSION

In present work, microcapsules obtained by layer-by-layer adsorption of bovine serum albumin and tannic acid and containing a model substance - poly (allyl) amine hydrochloride (PAH) were studied. Spherical porous microparticles (vaterites) of $CaCO_3$ with co-precipitated model substance were used as a core for the microcapsules formation. The results of scanning electron microscopy showed significant differences in microcapsules structure of the first (the shell was without ovomucoid) and the second (the shell with introduced ovomukoid) series of experiments.

The release kinetics of the model substance (PAH) into the medium with trypsin showed that inclusion of two layers of ovomucoid into the shell of microcapsules reduced the rate of their destruction almost half.

Thus, it can be assumed that since all substances in microcapsules are allowed for pharmaceutical use, these microcapsules in future can be used for new oral delivery systems for therapeutic proteins and peptides development.

ACKNOWLEDGMENT

We acknowledge technical and financial support from RASA Center of Peter the Great St. Petersburg Polytechnic University.

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