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SCIENTIFIC PAPER

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DESIGN OF PECTIN BEADS FOR ORAL PROTEIN DELIVERY

Protein and peptide drugs are becoming a very important class of therapeutic agents owing to recent and remarkable advances in the area of biotechnology. However, most of them are not therapeutically active after oral administration due to severe physical and enzymatic barriers of the gastrointestinal tract. A combination of colonic targeting using pectin–based systems and microencapsulation methods was used to increase the oral bioavailability of proteins. The purpose of this investigation was, therefore, to study under gastrointestinal the release conditions of bovine serum albumin from calcium pectinate beads prepared by extrusion/external gelation. Matrix reinforcement with dextran sulfate and different types of coatings was investigated.

Key words: Pectin beads, Protein delivery, Oral delivery, Extrusion, External gelation.

Recent advances in the field of biotechnology have been increasing the use of protein and peptides for pharmaceutical purposes and have permitted their production in a large scale. Due to their low stability, low permeability, rapid proteolysis and short circulatory life, these drugs are currently delivered by parenteral administration and frequent injections are required. Unsurprisingly, the local discomfort and inconvenience caused by the need of multiple administrations affect patient compliance.

The oral route is the easiest and most acceptable route of administration. However, although protein and peptide drugs are highly potent and specific in their physiological functions, most of them are generally not therapeutically active after oral administration. Once taken, the acidic fluid in the stomach may cause their denaturation. Moreover, the upper gastrointestinal (GI) tract has a high intensity of enzymatic activity and, consequently, the environment to which the proteins or peptides are exposed to is extremely aggressive and leads to the loss of biological activity. Amongst the several approaches that have been proposed to overcome those problems, the delivery of protein drugs to the colon has many potential benefits and represents a promising alternative. The colonic region has a slow transit, contains a lower level of peptidases and a number of polymeric carriers are degraded by bacteria located essentially in the colon, providing controlled release of the drug.

To achieve the delivery of a model protein to the colon, an alternative is its entrapment in pectin beads since this polysaccharide is degraded by colonic bacteria and is not digested by gastric or intestinal enzymes. Also, an *in vivo* study showed that beads based on amidated pectin exhibited rapid gastric emptying, passed through the small intestine intact, while there was evidence of slow degradation in the colon [1]. In addition, the advantages of multiunit dosage forms are the suppression of drug loss prior to entry in the colon, which occurs with single unit preparations [2], the conception of a more predictable and the reproducible system [3], improvement of the bioavailability of the embedded drug, less localized GI disturbances and greater product safety [4].

Pectin is a heterogeneous anionic polysaccharide found in the cell wall of most plants and extracted for commercial purposes from citrus peels or apple pomaces. It mainly consists of D–galacturonic acid residues joined together by α–(1$\rightarrow$4) linkages. The carboxylic acid groups present in the uronic units can react with calcium ions to form an insoluble and strong network, possibly similar to that known as the "egg–box" structure proposed for calcium alginate [5].

The ionotropic gelation of pectin is a simple, gentle and inexpensive immobilization technique. For this reason, calcium pectinate beads prepared by extrusion/external gelation have been used to encapsulate a number of drugs [6–11], proteins [12–17], cells [18,19], dyes [4] and radiolabeled compounds [1]. Besides, various advantages over calcium alginate beads, such as a higher stability constant and less sensitivity to damaging ions and chemical agents, have been recognized [19].

As mentioned, pectin beads have been evaluated as a carrier for the oral delivery of protein specifically to the colon [12–15,17]. However, nearly all the reported release studies were conducted only in alkaline buffer solutions, without simulating the passage through the stomach. In this study we performed protein release studies using the Bio–Dis III extended release tester. This is an advanced instrument designed for the dissolution testing of a variety of sustained release preparations the properties of which call for the use of the USP XXII Apparatus 3. Its standard configuration consists of six rows of seven 300 mL media vessels and, thus, it is possible to concomitantly run a control with six

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samples to be tested. Flexibility is also provided in allowing the operator the choice of as many as six different media. This permits the establishment of a gradient or a change from gastric to intestinal fluid without intervention by the operator. All the tubes are maintained at a set temperature by immersion in a thermostated water bath. The inner tubes, that contain the dosage form being tested, dip into the outer tubes and are moved up and down at a programmed rate. The amplitude of this movement and the depth of immersion are such that the dosage form remains completely immersed in the dissolution medium throughout the test. When the medium is to be changed, the inner tubes are lifted out to drain and shifted to the next row for a new cycle.

The main purpose of this research was therefore to investigate the effect of process (drying method) and formulation (addition of dextran sulfate and different types of coatings) variables and to compare the release profiles of a model protein under conditions simulating the GI tract.

EXPERIMENTAL

Materials

Amidated low–methoxyl pectin (GRINDSTED pectin LA 410) was purchased from Danisco (Grindsted, Denmark), calcium chloride was acquired from Panreac (Barcelona, Spain) and dextran sulfate sodium salt was obtained from Sigma–Aldrich (Steinhem, Germany). Low molecular weight chitosan (MWč150 kDa), medium molecular weight chitosan (MW~400 kDa) and high molecular weight chitosan (MW~600 kDa) were provided by Fluka Chemie GmbH (Buchs, Switzerland) and polyvinylpyrrolidone (Kolidon 30) was purchased from Vaz Pereira (Lisbon, Portugal). Freeze–dried bovine serum albumin was obtained from Sigma–Aldrich Co. (St. Louis, USA). Other chemicals were reagent grade.

Methods

Preparation of calcium pectinate beads

Bovine serum albumin (BSA) was first dissolved in distilled water and pectin and dextran sulfate (DxS) powders were then dispersed in the BSA solution. After complete homogenization the solution was stored until degassing. Thirty millilitres of pectin/BSA mixture was dropped into 500 mL of calcium chloride through a syringe with a 0.8 mm diameter needle, with gentle agitation and at room temperature. After gelation, the beads were filtered, washed with distilled water and dried at room temperature or lyophilized at 0°C for 48 h (Lyph–lock apparatus, Labconco, USA).

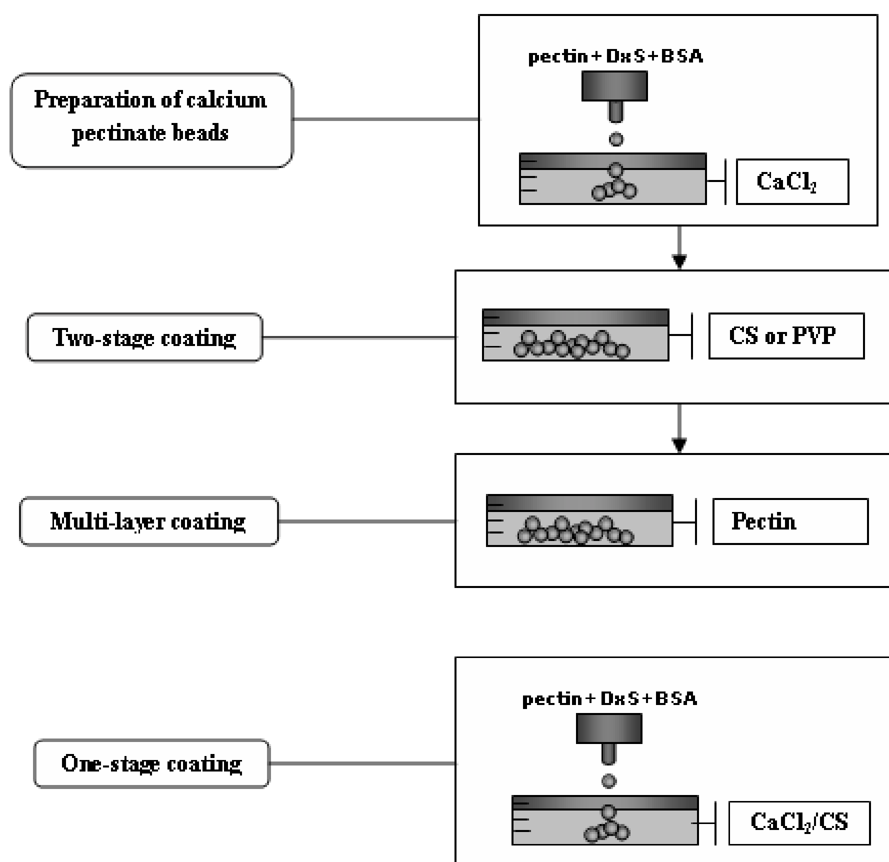


Figure 1. Scheme of preparation of BSA–loaded calcium pectinate beads

Preparation of coated beads

Calcium pectinate beads were coated by one–stage, two–stage and multi–layer methods (Figure 1). The one–stage procedure consisted in extruding the solution containing BSA into a calcium chloride/chitosan solution and the beads were recovered as before. For the two–stage coating, the beads were placed into a chitosan (CS) solution or into a polyvinylpyrrolidone (PVP) aqueous solution and allowed to stand for 10 min, filtering and washing with distilled water. The multi–layer coating was applied by placing the previously prepared calcium pectinate beads into a chitosan solution for 10 min, filtering and washing with distilled water. Then, the beads were immersed in a pectin solution for 10 more min and recovered as before.

Determination of the encapsulation efficiency

To evaluate the encapsulation efficiency (EE) an accurately weighted amount of beads was dissolved in 55 mM sodium citrate for 2 h and the BSA content was assayed by UV spectrophotometry (Shimadzu UV 1603, Kyoto, Japan) at 280 nm.

Protein release studies

Release studies were performed using the USP apparatus 3 (Bio–Dis III extended release tester, VanKel^{TM}, Cary, NC, USA) (Figure 2).

Figure 2. "Bio–Dis III" dissolution apparatus

The simulated gastric fluid was a hydrochloric buffer at pH 1.2 (USPXXVI) (for 2 h) and the simulated intestinal fluid (USPXXVI) (for 6 h) was a phosphate buffer at pH 7.4, both without enzymes, and the experiment was carried out at 37±1°C. Calcium pectinate beads (250 mg) were placed into vessels fitted with 250 mL of medium and a standard dip rate of 10 rpm was used. Sample solutions (3 mL) were collected at specific time intervals and replaced by an equal volume of fresh test medium. The BSA concentration was evaluated spectrophotometrically at 280 nm and expressed as the percentage amount of drug released. Each determination was made in triplicate.

RESULTS AND DISCUSSION

Appearance of the calcium pectinate beads

The beads had good spherical geometry and a smooth surface. For the studied parameters, no significant variation in particle size was observed, the mean diameter being 2.5±0.1 mm. The blank beads were transparent (Figure 3a), but when BSA was encapsulated they acquired some opacity (Figure 3b). Coating with chitosan by one–stage or two–stage procedures gave a more opaque aspect (Figure 3c,d). Upon air–drying all types of beads shrank to mean diameters of 1.0±0.1 mm.

BSA encapsulation efficiency in calcium pectinate beads

Overall, the encapsulation efficiencies were very high (above 70%) considering the protein hydrophilicity. Most of the protein loss occurred during the gelation phase because of its hydrophilic nature.

Table 1 shows how greatly the calcium chloride concentration influenced the BSA encapsulation efficiency.

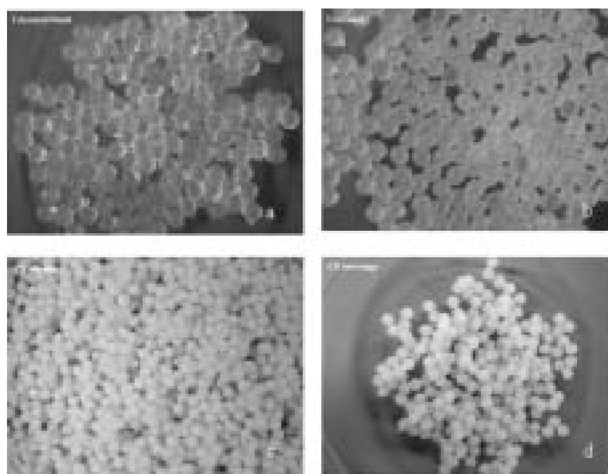


Figure 3. Digital photographs of uncoated and chitosan–coated BSA–loaded calcium pectinate beads. (a) uncoated–blank; (b) uncoated; (c) one–stage chitosan coated; (d) two–stage chitosan coated.

Table 1. Influence of the calcium chloride concentration on the encapsulation efficiency of BSA–loaded calcium pectinate beads

Parameter	EE ± SD (%)
$CaCl_2$ concentration (%m/v)	
0.5	72.07 ± 1.93
1.0	65.16 ± 3.06
2.0	54.23 ± 1.96

Increasing the calcium concentration significantly decreased the amount of protein encapsulated. It appears that the calcium chloride solution favored protein diffusion, probably explained by an increase of the counter ion ionic force. The lowest calcium concentration (0.5%w/v) guaranteed a good gelation and was, therefore, employed throughout the experiment.

When dextran sulfate was incorporated in the calcium pectinate matrix or when different coatings were applied, we did not find additional losses of BSA to either the reticulation media or the coating solution. Actually, we observed small increases in the encapsulation efficiency (Table 2 and Table 3). Because the critical step of protein loss is the gelation phase, we attribute those results to external variations introduced by inter–day conditions.

Effect of process and formulation parameters on the protein release

Figure 4 shows that, after 2 h in pH 1.2, almost all the protein content was released from the calcium pectinate beads. The high release of BSA in this medium can be partially attributed to a conversion of calcium pectinate to pectinic acid due to the sequestration of calcium ions from the matrix [20], similarly to the case of calcium alginate [21]. The morphology of the beads is not disrupted, but the polymeric network looses its ability to protect the protein from being released. Also, if the encapsulated compound is soluble, the release pattern will be similar to a conventional hydrophilic matrix system, exacerbated by the higher surface area of the multiparticulate formulation [7]. The calcium pectinate beads by themselves cannot be used to control the BSA release rate.

Effect of the addition of dextran sulfate. As mentioned before, although the beads do not dissolve at gastric pH, the protein is still able to escape from the hydrogel matrix. Restriction of release can be achieved by decreasing the swelling degree, erosion or diffusion [7]. An alternative is the incorporation of an additive capable of retaining the protein inside the beads during acid incubation. Following the work of Silva et al. [22], dextran sulfate was therefore added to the pectin/BSA solution.

Table 2. Influence of the matrix composition and different coatings on the BSA encapsulation efficiency

Parameter	EE ± SD (%)
Blending with dextran sulfate (DxS)	
No DxS	72.07 ± 1.93
DxS (0.5%)	83.66 ± 1.89
One–stage coating	
$CaCl_2$ (0.5%)/CS–LMW (0.15%)	81.21 ± 4.07
Multi–layer coating	
CS–LMW (0.15%) → LMA pectin (0.15%)	84.65 ± 1.38

Table 3. Influence of the polymer in the two–stage coating procedure on the BSA encapsulation efficiency

Parameter	EE ± SD (%)
Two–stage coating	
Chitosan molecular weight	
CS–LMW	86.09 ± 1.94
CS–MMW	83.78 ± 2.84
CS–HMW	87.73 ± 0.68
Chitosan–LMW concentration (% m/v)	
0.15	86.09 ± 1.94
0.20	86.09 ± 1.84
0.25	88.53 ± 0.62
Polyvinylpyrrolidone (PVP)	
PVP (0.15%)	99.89 ± 2.48

After incorporating the dextran sulfate in the polymeric matrix, we verified a remarkable change in the BSA release profile (Figure 4). The protein was completely retained in the presence of acidic medium. We believe that BSA preferentially binds to dextran sulfate through its sulfate negatively charged groups, as positively charged groups on a protein ($-NH_3^+$) interact more strongly with $-SO_3^-$ groups than with $-COO^-$ groups [23]. The sulfate groups create a negative microenvironment, which is independent of the pH

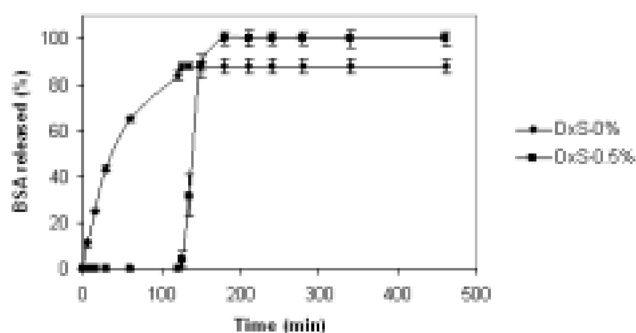


Figure 4. Effect of dextran sulfate (DxS) incorporation into the pectin/BSA solution on BSA release profile

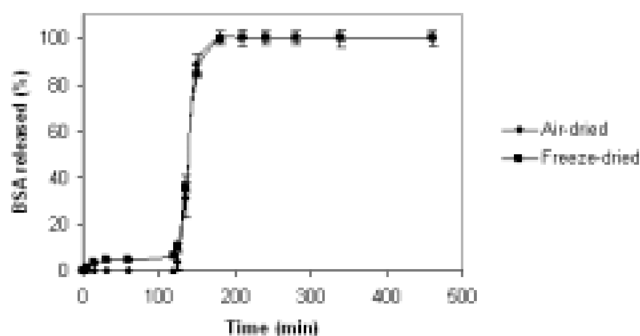


Figure 5. Effect of drying conditions on BSA release profile

interval, holding the protein inside the beads. The same justification can be used to explain the increase in BSA encapsulation efficiency (Table 2).

At pH 7.4 the beads rapidly dissolved and the BSA was released. This result could be explained by the excess of hydroxyl ions, at high pH, that increase the electrostatic repulsion between pectin chains, solubilizing the gel [8].

Effect of the drying conditions. Although blended with dextran sulfate, the freeze–dried beads released some protein during incubation at pH 1.2 (Figure 5). The fraction of BSA released was expected to be higher because the lyophilization process confers high porosity to the beads. However, we observed that only 6% of the BSA was released. This result is probably explained by the fact that the freeze–dried beads floated in the release medium, which made contact between the beads and the medium difficult and thus avoided its infiltration. When the beads were changed into pH 7.4, they completely dissolved within 1 h and all the BSA content was released.

Effect of the chitosan molecular weight. Systems composed only of calcium pectinate might be acceptable for less soluble compounds, but another approach to increase protection is needed for soluble drugs. The properties of the calcium pectinate beads may be changed by the formation of a polyelectrolyte complex membrane around the bead using cationic polymers such as chitosan, in a similar way to that used with alginate beads [24]. The complex formation results from the electrostatic interactions between the $-COO^-$ groups of pectin and the $-NH_3$ groups present in chitosan. Moreover, the pectinolytic enzymes can still degrade the beads despite the presence of chitosan [7]. Calcium pectinate beads thus reinforced with chitosan should erode more slowly in phosphate buffer and lead to a suppression of the initial protein release.

Drug loss from calcium pectinate beads under conditions mimicking the stomach and small intestine is reported to be sustained by applying a chitosan coating [7,15,17,25].

But at pH 7.4, after acid incubation, we observed that coating the beads with chitosans of different molecular weights just slightly retained the BSA (Figure 6 – left). After disintegration of the beads, an insoluble complex formed between pectin and chitosan was seen by the naked eye, but this polyelectrolyte complex did not provide any significant protection. Only the low molecular weight chitosan (CS–LMW) somewhat delayed the BSA release.

Effect of the chitosan concentration. Since low molecular weight chitosan gave the best results, it was selected to study the influence of concentration. Contrary to expectations, as the concentrations increased from 0.15 to 0.25% (w/v), the protein release profile was not significantly affected (Figure 6 – right).

Changing the release medium from hydrochloric buffer pH 1.2 to phosphate buffer pH 7.4 also led to a rapid release of BSA. At increasing pH, the deprotonation of chitosan increased and weakened the extent of the interactions inside the microsphere [26] and this is probably why the chitosan coatings did not provide any further protection.

Effect of coating strategy. At pH 7.4, the two–stage coating procedure decreased and retarded the BSA release more than the one–stage or multi–layer coatings (Figure 7 – left).

In the one–stage procedure, a competition between the calcium ions and chitosan for gelation with pectin could have occurred, leading to a disturbance of the polymeric matrix. At pH 1.2 the dextran sulfate retains the BSA inside the beads, but at pH 7.4 the

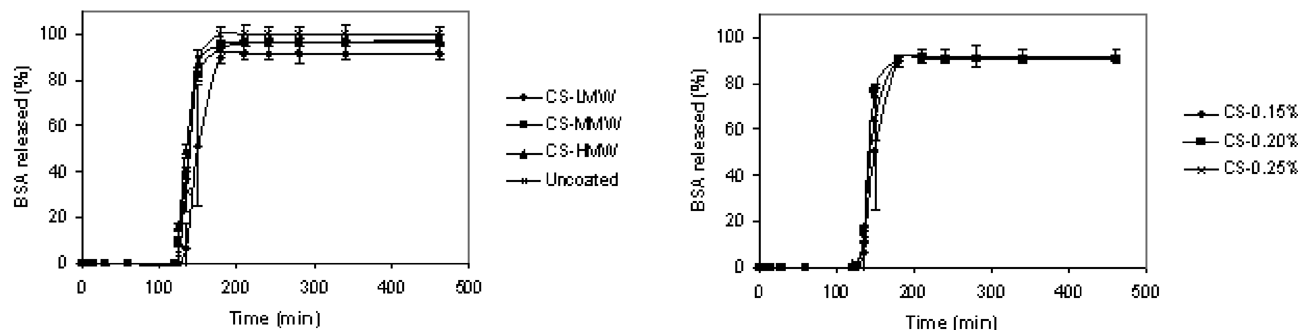
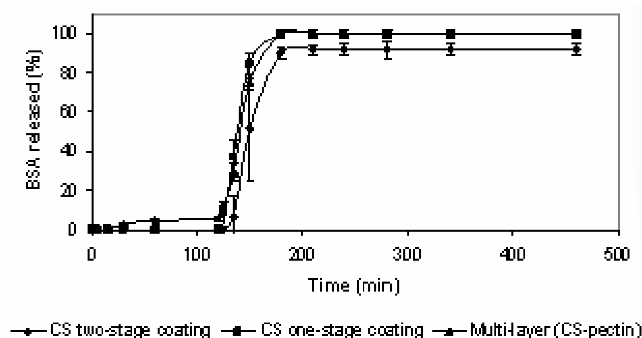


Figure 6. Effect of the chitosan molecular weight (left) and chitosan concentration (right) on BSA release profile



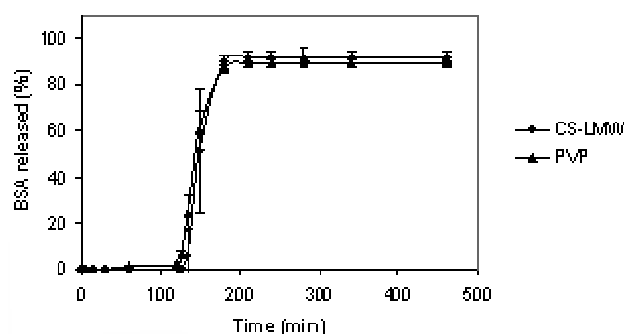


Figure 7. Effect of different types of coating on BSA release profile

protein is released more quickly than in the case of the two-stage coating.

The multi-layer approach exhibited a 6% release at pH 1.2 and accelerated the release at pH 7.4 (Figure 7 – left). In this case, the chitosan layer should interact with both the pectin in the matrix and the pectin in the layer. We suppose that when the pectin layer interacts with chitosan, it somehow introduces some degree of disorder on the surface of the beads thereby changing the behavior.

Effect of coating with other polymers. Polymeric interactions should occur not only between chitosan and pectin but also between pectin and other polymers.

Hydrogels based on hydroxypropylcellulose and PVP have been studied and it was found that the gels richer in PVP were more solid and self-supporting [27]. Furthermore, it seems that PVP is capable of controlling the degree of porosity [28] and exhibit an inhibition of the proteolytic action of pepsin [29]. The unbounded electron pairs on the amine or carbonyl groups located on the five-member ring are expected to confer a high polarity and, consequently, the polymer has a tendency to form complexes with hydrogen donors as well as anionic compounds. Consequently, PVP was used as an alternative to chitosan. This resulted in a small (about 2%) release of protein at gastric pH and at pH 7.4 no significant difference was observed (Figure 7 – right).

CONCLUSIONS

BSA-loaded pectin beads were prepared by extrusion/external gelation with high encapsulation efficiencies. Blending pectin and dextran sulfate provided an increase of the encapsulation efficiency and prevented release in acidic medium. In the presence of alkaline medium, after simulating the gastric passage, the calcium pectinate beads rapidly dissolved and the BSA was released within the first hour. Moreover, although the formation of a chitosan/pectin polyelectrolyte complex or interactions between pectin and polyvinylpyrrolidone have been reported, no additional protein retention was observed by applying different coatings.

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IZVOD

DIZAJNIRANJE PEKTINSKIH ČESTICA ZA ORALNU PRIMENU PROTEINA

(Naučni rad)

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Proteini i peptidi kao lekovi postaju veoma važna kategorija terapeutika zahvaljujuci nedavnim važnim dostignućima u oblasti biotehnologije. Međutim, najveći broj ovih preparata nije terapijski aktivan nakon oralne primene usled ozbiljnih fizičkih i enzimskih barijera u gastrointestinalnom traktu. Kombinacijom pektinskih nosača i mikroinkapsulacionih tehnika omogućena je zaštita i adekvatna raspoloživost proteina nakon *per os* unosa do mesta resorpcije (debelo crevo). Cilj ovog rada bio je proučavanje stepena otpuštanja albumina govedjeg seruma iz kalcijum pektinatnih čestica pripremljenih kombinacijom metoda ekstruzije i spoljašnje želatinizacije. Takođe je ispitivan efekat ojačavanja matriksa čestica primenom dekstran sulfata i različitih načina oblaganja.

Ključne reči: Pektinske čestice, Primena proteina, Oralna primena, Ekstruzija, Spoljašnja želatinizacija.