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Preparation of protein loaded chitosan microparticles by combined precipitation and spherical agglomeration

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ABSTRACT

Combined precipitation and spherical agglomeration was carried out in the non-miscible region of ethyl acetate–ethanol–water ternary solvent system. At first, w/o type quasi emulsion was prepared by sequential introduction of aqueous solutions of human serum albumin (HSA), chitosan (CS), and poly(4-styrenesulfonate) (PSS) into an ethyl acetate–ethanol solvent mixture. HSA was used to model a protein type drug, while CS and PSS served as matrix material in the obtained composite particles. PSS also served as chemical precipitation agent for both of the HSA and CS. The solubility of all these substances was reduced by introduction of additional amounts of ethyl acetate–ethanol mixture and/or ethanol as poor solvents. Due to the counter-diffusion of the good and poor solvents between the water rich droplets and the ethyl acetate–ethanol rich continuous phase, the aqueous phase gradually disappeared and partial agglomeration of the precipitated solids and their transfer to the continuous organic phase took place. The paper gives a report on the effect of several process variables on the quality of the obtained microparticles, such as their shape and stability against disintegration. The effects of the composition of the ternary solvent mixture, the route of its variation, the feeding method and composition of the added poor solvents, the stirring rate and the duration of agitation were studied.

1. Introduction

Polymeric microparticles containing various active ingredients are important drug delivery systems. There are a wide variety of methods available for production of such type of particles. One of them is the polymeric spherical crystallization which was developed from the classical spherical crystallization technique by Kawashima et al. [1–3] where crystallization and agglomeration take place simultaneously.

Spherical agglomeration may dramatically improve the micromeritic properties of drugs, such as flowability, packability, solubility and compaction behaviour compared to the original products prepared e.g., by the usual crystallization processes [4,5]. Carrying out agglomeration simultaneously with crystallization is called spherical crystallization [6]. Particles obtained by this method typically have mean sizes of 300–500 μm and more or less spherical shape. The composite structure of drug–polymer agglomerates helps to achieve suitable pharmacokinetic profile useful for controlled drug release. The spherical agglomeration technique is used more frequently to improve the solubility and/or the dissolution rate of a solid dosage form especially for water-insoluble drugs. According to Cui et al. [7] and Tapas et al. [8] after incorporating hydrophilic polymers into the microparticles by spherical agglomeration the drug solubility was enhanced significantly.

Spherical crystallization is carried out by various techniques that are categorized into two major groups. In a *typical spherical crystallization* process (*i*) the material to be crystallized is dissolved in its good solvent. Crystallization is induced by adding a poor solvent, which is miscible with the good solvent but diminishes the solubility of the dissolved material in the mixture. Adding a small amount of binding liquid e.g., a wetting solvent that is immiscible with the former two solvents and adheres to the surface of the newly formed primary crystals and/or partially dissolves them, the primary particles stick together to form agglomerates. Collisions and shear during stirring lead to a dynamic equilibrium between the agglomeration and disintegration resulting in a balanced mean particle size and near spherical shape. If crystallization is forced by other methods, e.g., cooling, salting-out or chemical precipitation, the so called *non-typical spherical agglomeration* (*ii*) takes place as was described by Szabó-Révész et al. [9].

Kawashima describes two basic mechanisms in spherical crystallization, namely the spherical agglomeration (SA) and emulsion solvent diffusion (ESD) processes [10,11]. In an SA process the precipitated crystals are transferred from a good solvent into a poor solvent where the crystals can simultaneously agglomerate, due to a small amount of bridging liquid. The general guidelines for the process were summarized by Chow and Leung [5].

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In a typical ESD process the substance to be separated in form of spherical agglomerates is dissolved in its good solvent. This solution is dispersed in a poor solvent, where counter diffusion of the good and poor solvents takes place through the boundary of the formed droplets. The increase of the concentration of poor solvent in the droplets reduces the solubility and crystallization is induced in the droplets. A small amount of good solvent remaining at the surface of crystals serves as bridging liquid that can bind them together. If a polymer material is also present it can be co-precipitated together with the crystals, acting as matrix material in the composite particle [11]. The primary agglomerates formed within the droplets are transferred to the continuous phase, where they further grow to larger agglomerates.

Kawashima et al. [1] established a process for direct spherical agglomeration of salicylic acid crystals carried out simultaneously with their formation in a mixture of water, ethanol and chloroform. Ikegami et al. [12] proposed a spherical agglomeration (SA) method to produce dry inhalation form of a steroid drug and studied the kinetics of crystal growth during the process. In this process, the active ingredient dissolved in acetone was poured into water under stirring which induced crystallization in the organic phase. Then, a small amount of ethyl acetate bridging liquid was added to produce spherically agglomerated particles of 200–300 μm median diameters.

Espitalier et al. studied the formation mechanism of spherical agglomerates in a quasi-emulsion crystallization process [13]. In a two-solvent system acetone was applied as good solvent of the studied drug, and water with polyvinyl alcohol emulsifier served as poor solvent. A model was established to study the effects of droplet size and the initial temperatures of phases, but no further agglomeration of the particles originated from different droplets was considered. Chow and Leung supposed that agglomeration is the result of partial dissolution of the particles, and re-crystallization at the contact points between them serving as solid bridges [5].

Spherical agglomeration can be a competitive method due to its simplicity, relatively low costs and easy scaling up to commercial level [14]. Spray drying e.g. needs considerable energy to evaporate the solvent from the droplets of the solution of an active ingredient, but the latter generally may be heat sensitive which does not allow such a treatment. The formed particles obtained by spray drying have compact structure thus making difficult to obtain a tunable drug-carrier composite structure for proper drug release kinetics. Supercritical techniques have also growing importance in particle formation, however the obtained particle size is generally too small and requires secondary treatment (e.g. granulation with a carrier material) to get suitable size and form for controlled drug release.

Polymer-drug composites have great potential in controlled drug release. The most promising biodegradable and biocompatible matrix materials are of natural origin such as chitosan, alginate, or polymers made of building blocks occurring in living organisms, such as DL-lactic-acid polymer (PLA), or DL-lactide glycolide copolymer (PLGA). Certain biologically tolerable synthetic polymers such as polyacrylate copolymers (Eudragit), polycaprolactones, polyaspartamides, or polyamidoamines are also eligible.

Perumal [15] studied the effect of formulation parameters and the technical design of reaction vessel on the properties of microspheres prepared from the ibuprofen model drug and a methacrylic polymer Eudragit® RS 100. Applying side baffles along the circumference of the vessel considerably improved the particle size attributes (lower mean size, narrower size distribution and more regular shape) and the yield of microspheres. Increasing the rate of agitation had also beneficial effect, in agreement with Kawashima et al. [16].

The bridging liquids should have good wettability to the precipitated solids, immiscible or only partially miscible in the poor solvent. For selection of a suitable bridging liquid Amaro-Gonzales and Biscans [17] developed a method based on the capillary rise of liquid in packed column filled with the relevant powder. The quality of spherical agglomerates is influenced by the amount of bridging liquid. The use of surfactants improves the wetting of the primary crystals, but often lowers the interfacial tensions and weakens the strength of the bonds. Crosslinkable polymers may contribute to the gelation and also can improve the mechanical strength of the bond [18]. Wetting agents such as PEG, or polymers having electrostatic interaction with the precipitated materials enhance the bridging force before solidification of liquid bridges. Ribardiere et al. [19] studied the effect of several polymer additives on the structure of microspheres produced in acetone/water system.

The polymeric spherical crystallization technique was applied by Cui et al. [3] to develop acrylic polymer microspheres for controlled drug release. A water soluble model drug and the polymer were dissolved in acetone–ethanol mixture serving as good solvent containing a small amount of water. This solution was poured under stirring into cyclohexane as dispersing medium also served as poor solvent which resulted in a quasi w/o emulsion. The droplets were solidified by the precipitation of drug crystals and simultaneous coacervation of the polymer. High ethanol ratio reduced the available water for bridging, resulting in dispersed drug and polymer powder, while too high water ratio led to not solidified viscous droplets. Similar observation was done by Amaro-Gonzales and Biscans [17], i.e. when the amount of wetting agent was too small, no significant agglomeration took place. But increasing it excessively, the agglomerates became soft and pasty.

Usha et al. [20] applied a three solvent system for spherical crystallization of aceclofenac drug, using acetone as good solvent, dichloromethane as bridging liquid and water as poor solvent. Crystallization was induced by pouring the drug–acetone–dichloromethane solution into a larger volume of water under continuous stirring by a controlled speed mixer. The amount of bridging liquid and stirring rate both were critical parameters in this respect. Not enough bridging liquid led to improper wetting of the crystals therefore irregular shaped and smaller agglomerates were obtained, while too much dichloromethane resulted in large and irregular agglomerates. Bausch and Leuenberger [21] proposed a spherical agglomeration technique to form agglomerates from fine powder containing BSA as model drug, recombinant bovine alpha-interferon as active ingredient, and mannitol as excipient. The obtained agglomerates were nearly spherical with high yield of the size fraction between 250 and 1000 μm. However, their method was quite different from the spherical crystallization because the primary particles were prepared in a previous step.

Recently, several studies were carried out to prepare chitosan drug carrier particles by various methods including precipitation and complex coacervation of recombinant human interleukin-2 containing chitosan microspheres [22]. Sinha et al. [23] published a detailed review on the advantages, preparation methods and applications of chitosan microspheres as potential drug carriers. However, only one paper was referred that dealt with spherical agglomeration of chitosan to prepare drug carrier microparticles [24].

Chitosan is a linear polysaccharide of natural origin composed of randomly distributed β-(1–4)-linked D-glucosamine and N-acetyl-D-glucosamine units. It is obtained commercially by the deacetylation of chitin, which occurs in great quantities in the nature as structural element of exoskeleton of crustaceans (crabs, shrimp, etc.). The degree of deacetylation (%DD) in commercially available chitosans is in the range 60–100% with molecular weight between 3,800 to 20,000 Daltons depending on its production process. Chitosan has useful properties for pharmaceutical applications: it is a biodegradable, non-toxic, and biocompatible natural polymer with high charge density. It also has hypoallergenic and natural antibacterial feature. It shows mucoadhesive behaviour [25], therefore drug carrier particles composed of or coated with chitosan are especially advantageous when prolonged action should be achieved after oral or pulmonary administration of poorly absorbable ingredients, especially peptid drugs [26] or other protein type medicines.

One of the available methods to prepare chitosan microparticles is complex coacervation based on interionic interaction between oppositely charged polymers such as with sodium alginate, sodium carboxymethylcellulose, sodium polyacrylic acid. Emulsion solvent

diffusion methods are also applicable to prepare chitosan microparticles for controlled drug release [27]. Because in these works considerable initial burst of model drugs was observed, efforts should be done to prepare chitosan microparticles that have suitable inner structure to achieve appropriate release profile. Spherical agglomeration can be a promising technique for this purpose. Therefore, in this work, spherical agglomeration technique was selected to prepare chitosan microparticles because of its suitability to produce particles for direct tableting. Since agglomeration takes place here simultaneously with crystallization, precipitation or phase separation, this process can be realized in a low cost apparatus, reaching relatively high encapsulation efficiency and drug loading. Our aim was to develop a method to obtain chitosan based composite microspheres applicable for controlled release of protein type drugs. The process was carried out in ethyl acetate–ethanol–water ternary solvent system using human serum albumin as a model ingredient.

2. Methods and materials

2.1. Materials

Chitosan (CS) with degree of deacetylation DD ≥ 85% originated from crab shells was purchased from Sigma. Sodium poly(4-styrenesulfonate) (PSS), M_w = 70,000 was obtained from Aldrich, and human serum albumin (HSA) was kindly presented by Trigon Ltd, Hungary. Ethanol (EtOH) was provided by Reanal Ltd, ethyl acetate (EtOAc) and acetic acid were bought from Spektrum-3D Ltd, Hungary. All chemicals used in this work were of analytical grade. Water used for the aqueous solutions and solvent systems was freshly prepared by double distillation.

Stock solutions of chitosan (CS) and PSS were prepared by dissolving their powder in 4.0% (w/w) aqueous acetic acid to get 1.0% (w/w) CS and 2.0% (w/w) PSS concentrations, respectively. HSA was used in 3.8% and 7.28% (w/v) solutions in phosphate buffered saline (PBS).

2.2. Methods

2.2.1. Phase equilibrium data

Phase equilibrium data for the partially miscible region of the EtOAc-EtOH-H_2O system were taken from the literature [28,29] (data points x on Fig. 2). However, some lacking data had to be determined by own measurements, carried out in a test tube at 25 °C. First 5 mL solvent mixture with selected global composition was pipetted into the test tube, and was mixed by a vibration shaker for a few seconds. Then several minutes rest was allowed for reaching the equilibrium. Starting from various solvent mixtures where, in equilibrium, two phases (i.e. separated aqueous and organic phases) were present, stepwise increasing amounts of ethanol were added till getting a global composition where the mixture became homogeneous, i.e. no turbidity could be observed. The resulted compositions were accepted as the measured points of the phase equilibrium curve (data points ● on Fig. 2).

2.2.2. Particle preparation

Particle preparation was carried out by a combined precipitation–spherical agglomeration process as described in the next section of the paper in details.

2.2.3. Size analysis and morphology

The size distribution of the obtained spherical agglomerates was determined by Malvern-2600 type particle size analyzer. The particles were characterized by the volume mean diameter and the relative width (span, SP) of the distribution. Span data were calculated from the 10%, 50% and 90% quantiles of volume distribution as

$$SP = \frac{D[v,0.1] - D[v,0.9]}{D[v,0.5]} \quad (1)$$

Size measurements were carried in suspension after the samples of dry agglomerates were re-suspended in mother liquor of same composition from they were separated. The stability of the agglomerates against disintegration in suspension was evaluated by observing the change of size distribution under stirring at 400 rpm in the suspension after 5–20 min. The shape of the agglomerates was observed under an optical microscope attached to a computer. The surface morphology of the agglomerates was examined by Philips XL30 ESEM scanning electron microscope. The crystals were splutter coated with gold before scanning. The photomicrographs were taken at 20.0 kV and magnification at 25X and 300X.

2.2.4. FTIR

The spectra were recorded with Varian Scimitar FTS2000 spectrometer (128 scans, 4 cm^{-1} resolution) equipped with liquid nitrogen cooled MCT detector and Pike GladiATR (with diamond micro-ATR element) accessory.

2.2.5. Efficiency of protein encapsulation

The amount of the HSA model protein incorporated into the formed composite particles from the total amount introduced to the ternary solvent system was determined in several experiments from its concentration remaining in the mother liquor after filtering out the obtained spherical agglomerates. The encapsulation efficiency of protein was calculated both from the FTIR spectra by linear combination of the spectra of the solid components and from the UV spectrum of the remained protein in the filtrate. The latter was determined by colorimetric technique, similar to the original Bradford method [30], by measuring the absorbance at 590 nm and comparing it to a calibration series.

3. Experiments

Composite CS–HSA–PSS microparticles were prepared in ethyl acetate–ethanol–water ternary solvent system by combined chemical and antisolvent precipitation, accompanied with spherical agglomeration. The process, shown schematically in Fig. 1, was started in w/o type disperse phase system (in a quasi emulsion). For this, a closed glass reaction vessel of 100 mL inner volume and stirred by a paddle mixer was applied. The temperature during the whole process was kept at 25 °C by a heat transfer liquid circulated in the jacket of the vessel through a Julabo thermostat. To avoid the loss of solvents a glass condenser was set at the top of the vessel.

3.1. Determination of the initial solvent composition range suitable for spherical agglomeration

The behaviour of chitosan and PSS separately, and also that of the chitosan and HSA together were investigated in the non miscible region of the EtOAc–EtOH–H_2O ternary solvent system represented by the ternary phase diagram shown in Fig. 2. The solubility or phase equilibrium data of the aqueous and organic phases were taken from the literatures (data points indicated by **x** on the phase equilibrium curve *c*), or were determined by own measurements (data point indicated by ● on the equilibrium curve).

As for the behaviour of chitosan in solvent mixtures of different compositions, its precipitation was observed at higher EtOAc concentrations. More exactly, at every solution composition where the concentration of EtOH is 25% (v/v) and the concentration of EtOAc ≥ 40% (v/v), the chitosan has become precipitated. At concentrations of the EtOH = 15% and 20% (v/v), and at those solution compositions where two phases exist, there was no precipitation, but the chitosan accumulated at the interface of the two liquid phases as a desolvated and stiffened polymer film. Along the phase equilibrium curve (i.e. the conode) separating the heterogenous two-phase and the homogeneous one phase regions of the ternary phase diagram, and above this curve (in the one phase

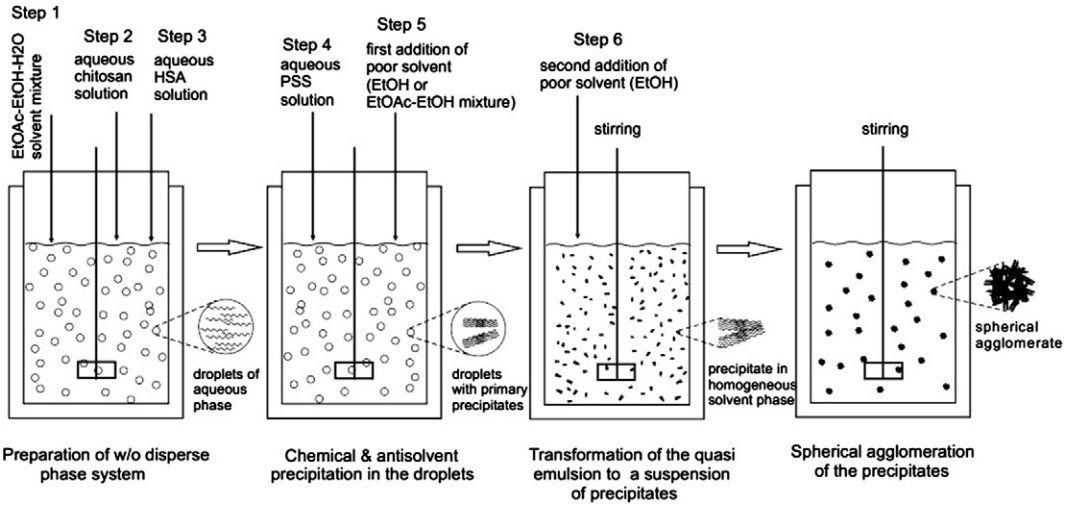

Fig. 1. Schematic diagram of the studied spherical agglomeration process.

region) there precipitation was observed. PSS alone acted similarly in this respect as the chitosan.

The behaviour of the chitosan and HSA together was very important to select the suitable starting composition of the solvent mixture: the polymer and the HSA should not be precipitated at these compositions and the aqueous phase (whose compositions are shown by the left hand side of the conode) should easily be dispersed in the continuous organic phase (whose compositions are shown by the right hand side of the conode) to be able to generate a w/o (water in oil) type quasi-emulsion system. These conditions could be realized when the concentration of EtOAc was higher than 50% (v/v). The solvent compositions inside the shaded zone (see on Fig. 2.) proved to be suitable for the experiments as a starting point: w/o quasi emulsion were generated, and no spontaneous crystallization took place.

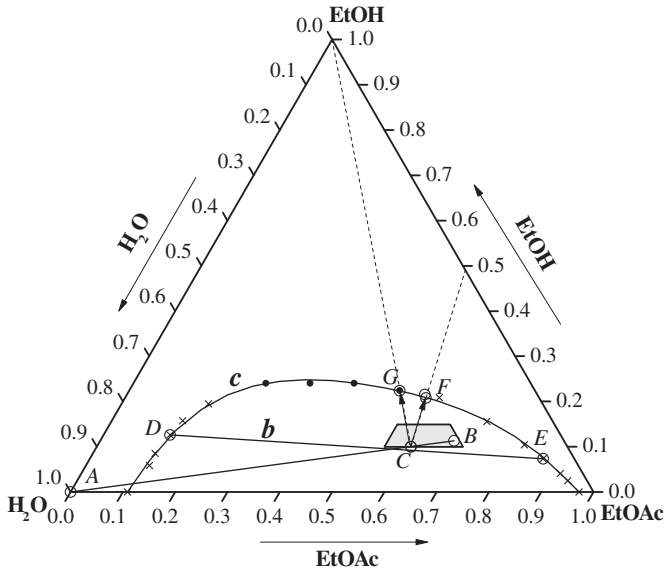

Fig. 2. Principle of the applied spherical agglomeration method. c – phase equilibrium curve (bimodal curve or conode), b – the straight line (binode) connecting the equilibrium compositions of the aqueous and organic phases, A – pure H_2O, the solvent of the initial HSA, CS and PSS solutions, B and C – compositions of the EtOAc-EtOH-H_2O solvent mixture before and after the addition of the HSA, CS and PSS solutions, respectively. D and E – compositions of the aqueous and organic phases in equilibrium. The shaded zone represents the composition range where there was no precipitation in the initial mixture.

3.2. General description of the process

At first, w/o type quasi emulsion was prepared by sequential introduction of aqueous solutions of human serum albumin (HSA), chitosan (CS), and poly(4-styrenesulfonate) (PSS) into an initial water–ethyl acetate–ethanol solvent mixture. HSA was used to model a protein type drug, while CS and PSS served as matrix material in the obtained composite particles. PSS also served as chemical precipitation agent for both of the HSA and CS.

The general steps of the process are shown on the ternary phase diagram in Fig. 2: the solvent composition of the introduced HSA, CS and PSS solutions correspond to the left vertex of the triangle denoting 100% v/v H_2O concentration (point A). The composition of the initial water–ethyl acetate–ethanol solvent mixture is denoted by point B on the phase diagram. After adding the three aqueous solutions to the initial water–ethyl acetate–ethanol mixture, the composition of the resulted solvent mixture corresponds to point C on the line drawn between A and B. Its location in the line A–B depends on the volume ratio of the aqueous solutions to the volume of the initial water–ethyl acetate–ethanol mixture. The higher is the volume ratio of the latter phase the closer will be the point C to point B.

In the equilibrium, the composition of the two liquid phases are indicated by points D (aqueous phase) and E (organic phase) located on the left and right hand legs of the *conode*, respectively. During stirring, the relative volumes of the two phases determine that which phase forms the dispersed and the continuous phases: generally the smaller volume will be dispersed in the larger volume.

During the experiments, the volumes of the aqueous solutions of HSA, CS, and PSS, and the volume of the initial H_2O–EtOAc–EtOH solvent mixture was selected in a way that point C was located in the heterogeneous region of the phase diagram, where $V_{aq}/V_{org} < 1$ to get a w/o disperse system: i.e. the aqueous phase was present in form of droplets in the continuous organic phase. At the same time, the amounts of the dissolved HSA, CS, and PSS were so selected, that initially they were in dissolved state in the aqueous droplets (i.e. no solid precipitation took place at solvent composition D).

Then, the solubility of these substances was reduced by introduction of certain amount of ethyl acetate–ethanol mixture and/or pure ethanol as poor solvents. In the former case the global composition of the heterogeneous solvent mixture moved from point C to point F, or from point C to point G, respectively, close to the transition point from the heterogeneous phase system to the homogeneous solvent mixture (not regarding the possibly precipitated solid phase). At the same time, due to the principles shown above (*conode* and *lever rule*), the composition of the dispersed aqueous phase moved from

point D to point G (on addition of pure EtOH), or from point D to point F (on addition of EtOH–EtOAc mixture). During this process, according to the lever rule, the volume of the aqueous phase also diminished and then became totally dissolved in the continuous organic phase, while the composition of the continuous organic phase moved from point E to points G or F.

The increasing concentration of the poor solvents in the droplets, and the decrease of their volumes can cause supersaturation, resulting in a rapid precipitation of the dissolved solid materials. The rising solid content allows agglomeration of the primary crystals within the droplets, and the collisions of droplets with each other during agitation can lead to their coalescence and thus further agglomeration of their solids content.

3.3. Preparation of spherical agglomerates

Spherical agglomerates were produced by several consecutive steps as follows:

Step 1. A ternary solvent mixture of about 40 mL was prepared by filling 24.4 mL ethyl acetate, 4.0–6.4 mL ethanol and 6.45–7.45 mL double distilled water into the vessel depending on the intended ratios of the components. According to the results of preliminary experiments, the initial volume ratios of ethyl acetate, ethanol, and water in this mixture could be varied within relatively narrow ranges, namely between 65.2 and 69.7, 11.3 and 17.1, and 17.7 and 21.0% (v/v), respectively.

Step 2. Chitosan solution of 1.0% (w/w) concentration in 4% (w/w) aqueous acetic acid solution was added under stirring to the solvent mixture by a pipette. Because, considering the results of our preliminary tests, two considerably differing precipitation routes were studied in these experiments, the introduced volumes in two different series of experiments were 1.15 and 2.1 mL, respectively.

Step 3. Just after the chitosan addition, 0.30–0.31 mL aqueous HSA solution was introduced to the mixture under stirring in two series of experiments with 40 and 72.8 mg/mL concentrations, respectively.

Step 4. Finally, depending on the amounts of CS and HSA added previously in the two series of experiments, 1.15 or 2.10 mL aqueous PSS solution with 2.0% (w/w) concentration was added, respectively.
Due to the negative electric charge of PSS molecules and the positive charge of CS and HSA molecules, the PSS served as precipitation agent for both CS and HSA. Therefore, the applied mass ratio of PSS to the total amount of CS + HSA was kept constant at about 1.0.

Step 5. For complete separation of the introduced CS and HSA, and to harden the gel like particles, pure ethanol or 1:0.96 mixture of ethyl acetate–ethanol was added to the quasi emulsion. By this, the water ratio in the heterogeneous solvent mixture was considerably diminished, and its composition was shifted very close to the *bimodal curve*. During this step, due to the counter diffusion between the droplets and the surrounding continuous organic solvent phase, the concentration of the poor solvents in the dispersed droplet phase was increasing which, together the precipitation effect of the PSS, resulted in the complete precipitation of the introduced CS and HSA. FTIR measurements carried out in the samples taken from mother liquor confirmed that complete separation of CS, HSA and PSS was achieved by this process, i.e. practically no dissolved solids remained in the liquid phase.

Step 6. Further portion of ethanol was added to enhance the agglomeration and to solidify the obtained composite particles. On the effect of agitation agglomeration of the precipitated solids within a given droplet, coalescence of different droplets, and agglomeration of the not fully solidified particles took place.
At the end, the obtained spherical agglomerates were separated by filtering on glass fibre filter, and were washed by 5 mL pure ethanol twice and air-dried.

4. Results and discussion

From the results of the experiments it was seen that the quality of the obtained particles depended on many factors: e.g. too fast or excessive ethanol addition quickly diminishes the water content inside the particles causing rapid phase separation before stable solid binding bridges could be formed. This resulted in not proper agglomeration, bad stability against disintegration, irregular shape, or powder like products. Optimal amount and rate of ethanol addition helped to develop good agglomerates with almost spherical shape and mechanical strength against disintegration. If not enough ethanol is added, too much water remained in the binding bridges between the primary particles, leading to sticky and pasty agglomerates with irregular shape.

Actually, the formation of the particle and the particle shape did not depend directly on water content, because the main influencing factor was the changing rate of the water content in the droplets, which in turn depends on the rate of ethanol feeding. If it takes place too fast, the particles spend too short time in their plastic (i.e. deformable) state, when spherical shape can be formed by multiple collisions with the vessel wall and each other.

Therefore to study the effects of the process conditions, more than 60 experiments in two distinct series (Groups I and II) were carried out with various combinations of parameters, to discover the influence of solvent mixture compositions at various stages of the process, and the effect of the routes of changing these compositions. Namely, the composition of the heterogeneous solvent mixture was varied

- when the precipitation started, i.e. after adding the PSS solution in Step 4, and
- after adding the first portion of poor solvent in Step 5, and
- after adding the final portion of poor solvent in Step 6.

The applied poor solvent in Step 5 was pure ethanol (in Group I) or 1:0.96 ethyl acetate–ethanol mixture (in Group II), and pure ethanol in Step 6 (in both Groups). For the experiments the following conditions were applied:

- the total solid concentration (CS, HSA and PSS together) in the aqueous dispersed phase was about 5.5 mg/mL in Group I, and 6.2–6.4 mg/mL in Group II after Step 4, while after Step 6 it was about 1.2 mg/mL in Group I, and 2.2 mg/mL in Group II, and the CS:HSA:PSS mass ratio was about 0.25:0.25:0.50
- the first portion of poor solvent was generally introduced in feeding rate of 12 mL/min and one shot, but in some experiments feeding rates of about 5.5 mL/min were also tested
- in Step 6 generally 0.55 mL pure ethanol was introduced in one shot, but in some experiments its amount was 0.55–1.0 mL
- generally 400 rpm stirring rate was applied, but in some experiments 200, 300 or 500 rpm were also tested
- generally 15 and 30 min stirring times were applied in Steps 5 and 6 respectively, but in some experiments other combinations of 15, 30 or 45 min time of agitation were also tested.

The obtained composite agglomerates were characterized by their

- volume mean size and size distribution,
- morphology (shape and structure), and
- stability against disintegration.

To evaluate the quality of the obtained agglomerates, the following categories were defined:

- **1A** – Almost spherical shape, relatively smooth surface, good stability
- **1B** – Almost spherical shape, but poor stability

- **2A** – Less spherical shape, coarse surface, good stability
- **2B** – Less spherical shape, coarse surface, poor stability
- **3A** – Merged agglomerates (composed of doublets, triplets or more), good stability
- **4A** – Irregular shape, coarse surface, good stability
- **4B** – Irregular shape, coarse surface, poor stability
- **5B** – Loose lumps and debris, poor stability

Examples for the most characteristic conditions and results are given in Table 1.

4.1. Proposed mechanism of particle formation

In Steps 1–4 a w/o type quasi-emulsion was developed where initially four different kinds of aqueous droplets could be present. In Step 1 water rich aqueous droplets were present without any dissolved solids dispersed in a continuous ethyl acetate rich phase. Then, by adding different aqueous solutions to the mixture in Steps 2, 3 and 4, separate droplets of aqueous CS, HSA and PSS solutions were formed respectively. Then, because of perpetual coalescence and division of droplets under stirring, the composition of different droplets became more and more uniform. Finally all the three substances (CS, HSA and PSS) could be present in each droplet in a ratio which corresponds to their global concentrations. Therefore, due to the precipitating effect of PSS, as was proved by separate experiments in a continuous aqueous phase of identical composition, gelation took place in each droplet.

Adding poor solvent (either ethanol or ethyl acetate–ethanol mixture) to the quasi emulsion in Step 5, the composition of the ternary solvent mixture was shifted towards higher ethanol or ethyl acetate–ethanol ratios. Due to a drastic reduction of solubility of any of the solutes, particle formation and agglomeration took place. Initially, the agglomerates could be sticky and deformable therefore they could grow by collisions with other aggregates. Spherical shape could be raised by multiple collisions between the pre-matured i.e. still somewhat plastic agglomerates, and by interactions with the stirrer blades and vessel wall during their drift.

The plasticity or rigidity of binding bridges and their change during the process should be critical from respect of the quality of the achievable final product.

4.2. Effect of solvent mixture composition

Two series of experiments were carried out to study the influence of the global composition of solvent mixture. In the first series (Group I) after adding the PSS solution (Step 4) the mixture contained 23.0% water, 16.0% ethanol and 61.0% ethyl acetate. The total CS, HSA and PSS content in these experiments before adding the poor solvent was quite low, about 1.151 mg/mL. In order to increase the volumetric productivity of the process a second series of experiments (Group II) has also been carried out by increasing the solute content to about 2.146 mg/mL. For this, the composition of ternary mixture had to be shifted toward more water ratios: in Step 4 the global water content was increased to about 30.0%, the ethanol ratio was decreased to 10.0–10.5%, while the ethyl acetate was varied between 60.1 and 63.2%. The volume fraction of the dispersed aqueous phase (droplets) containing all the solutes was thus increased from 21.0 to 34.5%.

After adding the total amount of poor solvents (Step 6) in the first series of experiments (Group I) the water, ethanol and ethyl acetate in the solvent mixture were 21.2–21.4%, 21.8–22.6%, and 56.2–56.8%, correspondingly. In Group II these ratios were in that order 18.6–21.7%, 20.7–22.3%, and 56.6–59.6%. The studied range of the mixture compositions were rather narrow because, according to our previous experiences, the process was rather sensible to that ratios, and seemingly no appropriate microparticles could be obtained outside of these ranges.

The composition of solvent mixtures after Steps 4, 5 and 6, and the quality of the resulted agglomerates are listed in Table 1. From these it seems that in Step 4 mixture composition in the studied range had no significant influence on the quality of agglomerates. As for the mixture compositions after Step 6, it looks that the most stable and spherical agglomerates were produced at relatively higher water ratio around 21.4% in both Groups I and II, and the worst quality of particles was obtained at somewhat less water ratio, mainly between 18.6 and 20.1%. However, the effect of this variable alone is not fully unambiguous and, probably due to other effects, less appropriate agglomerates also occurred at water ratio of 21.0–21.4%.

In addition to the water content, the ethanol ratio in the final step of the process also should have certain effect, because it can change the extent and rate of water distraction from the not fully solidified agglomerates, i.e. the development of binding bridges between the primary particles. However, because of the complexity of this process the effect of this variable could not be cleared up separately.

Table 1
Conditions and result of some representative experiments ranked according to product quality categories.

Run	Step 4		Step 5		Step 6		Feeding method of the poor solvent[b]	Mean size, μm	Span	Quality category
	Good solvent volume ratio[a]	EtOH volume ratio in the poor solvent[a]	Good solvent volume ratio	EtOH volume ratio in the poor solvent	Good solvent volume ratio	EtOH volume ratio in the poor solvent				
I-10	0.230	0.208	0.217	0.264	0.214	0.277	F1b	278	1.2	1A*
II-25	0.299	0.143	0.230	0.208	0.214	0.277	F4	286	1.0	1A
II-28	0.281	0.143	0.217	0.264	0.214	0.277	F2	324	0.8	1A
II-29	0.291	0.142	0.217	0.264	0.214	0.277	F2	301	0.9	1A
II-26	0.299	0.143	0.217	0.264	0.214	0.277	F2	309	0.9	1A
II-2	0.299	0.143	0.217	0.264	0.215	0.274	F2	260	1.0	1B
II-21	0.281	0.143	0.217	0.264	0.214	0.277	F3	244	1.0	1B
II-20	0.299	0.143	0.217	0.264	0.214	0.277	F2	261	1.0	2B
I-14	0.230	0.208	0.217	0.264	0.212	0.287	F1b	306	0.8	3A
II-3	0.281	0.143	0.203	0.265	0.201	0.274	F3	234	0.9	4A
II-4	0.263	0.142	0.188	0.265	0.186	0.274	F3	290	0.9	4A
II-23	0.299	0.143	0.203	0.265	0.201	0.274	F3	282	1.0	4A
II-24	0.299	0.143	0.191	0.257	0.189	0.265	F2*	277	1.0	4A
II-27	0.299	0.143	0.214	0.277	0.213	0.281	F3	175	0.8	4A
II-1	0.291	0.142	0.203	0.265	0.208	0.274	F3	243	1.2	4B
I-9	0.230	0.208	0.214	0.279	0.214	0.279	F1a	183	1.5	5B

[a] Good solvent means H_2O; poor solvent means various mixtures of EtOAc and EtOH.
[b] Feeding methods: F1 – ethanol to reach the bimodal curve + EtOH, F2 – EtOH:EtOAc mixture to reach the bimodal curve + EtOH in one or two portions, F3 – EtOH:EtOAc mixture close to, but above bimodal curve + EtOH, F4 – EtOH:EtOAc mixture + EtOH to reach the bimodal curve + EtOH ,*: reach the curve at higher EtOAc concentration, a: one portion, b: two or more portions.

Depending on the route from a given initial solvent mixture composition to its final composition, agglomerates can be obtained either with appropriate quality or not, i.e. no direct correlation was found between the quality of the obtained particles and the solvent composition after the Step 4. In any case, when a solvent composition after adding the first amount of poor solvent was exactly on the bimodal curve at the given composition point F, the formed particles were almost spherical (see the column with title of the feeding method of the poor solvent in Table 1). It was found that at too high ethyl acetate ratio in this stage, particles with irregular shape but good stability were obtained. However, when this composition was somewhat above the bimodal curve, the form of the obtained particles substantially differed from spherical shape. In the next step the composition of the solvent mixture was shifted more above the bimodal curve to solidify the obtained particles.

As for the composed effect of the solvent mixture and the feeding rate of the poor solvent, it was found that in the initial solvent mixture there was no precipitation, and the ratio of the aqueous and the organic phase made possible to form w/o quasi emulsion. Changing the water content by appropriate rate of feeding poor solvent allowed enough time to develop spherical agglomerates. In case of increased water content (and with this the solute content) in the initial solvent mixture, instead of pure ethanol we had to use ethanol-ethyl acetate solvent mixture to get the same final solvent mixture composition. The composition at point F on Fig. 2 shows the solvent mixture where the particles are deformable enough to form spherical particles.

4.3. Effect of feeding method and poor solvent composition

Four typical SEM micrographs obtained in runs I-9, I-10, I-14 and II-2 are shown in Fig. 3*a–d*. The particles shown in Fig. 3a (run I-9) were produced by adding ethanol in one portion only, i.e. the Steps 5 and 6 were merged together in this case. This resulted in unstable, not fully agglomerated lumps and debris with relatively wide size distribution (Span = 1.5). This can be explained by an excessively rapid water distraction from the droplets and fast precipitation of the remaining solids, which did not give enough time to develop suitable bridging bridges and round agglomerates. Therefore the obtained particles were brittle and vulnerable to disintegrate.

The products shown in Fig. 3b and c (runs I-10 and I-14) were obtained by feeding the poor solvent (ethanol in these runs) in two portions, allowing enough time for agglomeration and rounding up. These products proved to be quite stable with almost spherical shape.

The composition of poor solvent also had some effect on product quality: in experiments of Group I pure ethanol was added both in Steps 5 and 6, which produced generally better shape compared to Group II, where 1:0.96 ethyl acetate–ethanol mixture was used in Step 5. This can be the consequence of the fact that ethyl acetate is worse solvent than ethanol therefore the available solutes remained in the liquid bridges at this stage precipitated more quickly, i.e. the binding bridges in Group II solidified too early, resulted in stiff agglomerates before obtaining spherical shape (Fig. 3d).

The tendency for fusion of two or more agglomerates as shown in Fig. 3c was probably caused by the less ethyl acetate content and higher ethanol ratio applied in run I-14, which possibly made the agglomerates softer thus allowing secondary agglomeration.

From Fig. 4a–d it is also seen that the size distribution of products were generally log-normal type, not regarded the lower size ranges where not agglomerated particles or debris were present. This is also seen from Figs. 5a and 6a and b, where the left hand upper parts of the curves were shifted towards smaller particle size regions.

The size distributions of agglomerates also may vary depending on certain process conditions as can be seen on Figs. 4 and 5. Comparing the size distributions of runs I-9 and I-10 plotted together in Fig. 4b, it is obvious that the product obtained in run I-10 had essentially narrower distribution (Span = 1.2) than that of in run I-9 (Span = 1.5), caused by different kind of poor solvent addition.

However, when the poor solvent was added in two steps, no significant relation was found between the width of size distribution and the poor solvent composition: as it is seen in Table 1, i.e. the

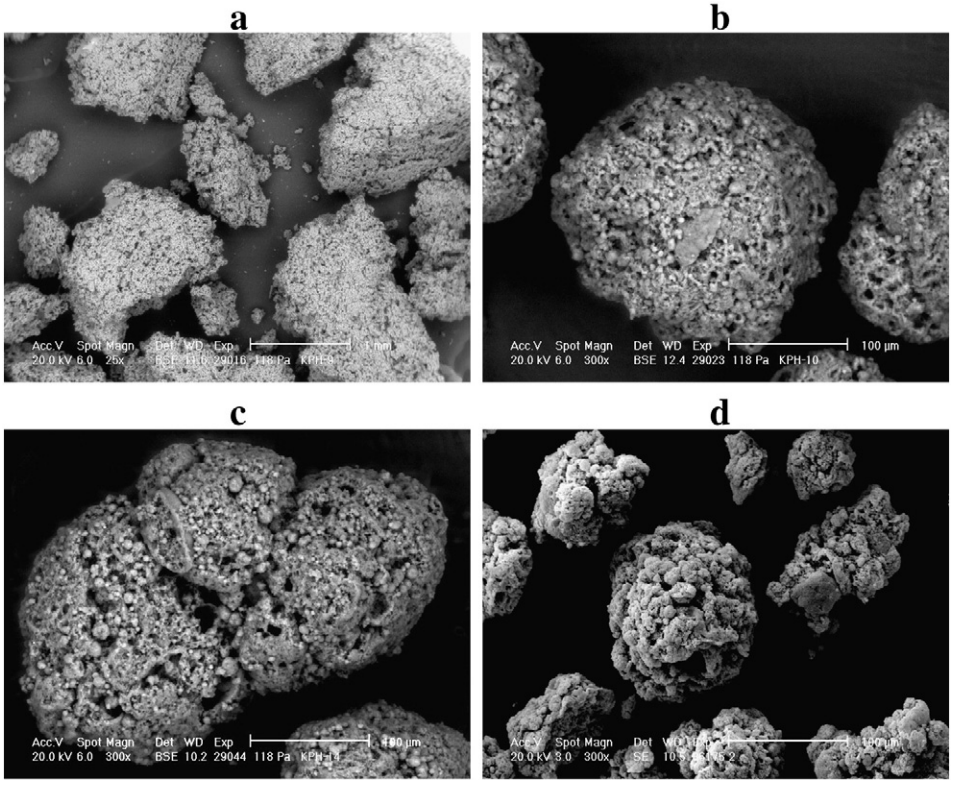

Fig. 3. Typical SEM pictures of agglomerates obtained in various experiments, a – run I-9, b – run I-10, c – run I-14, d – run II-2.

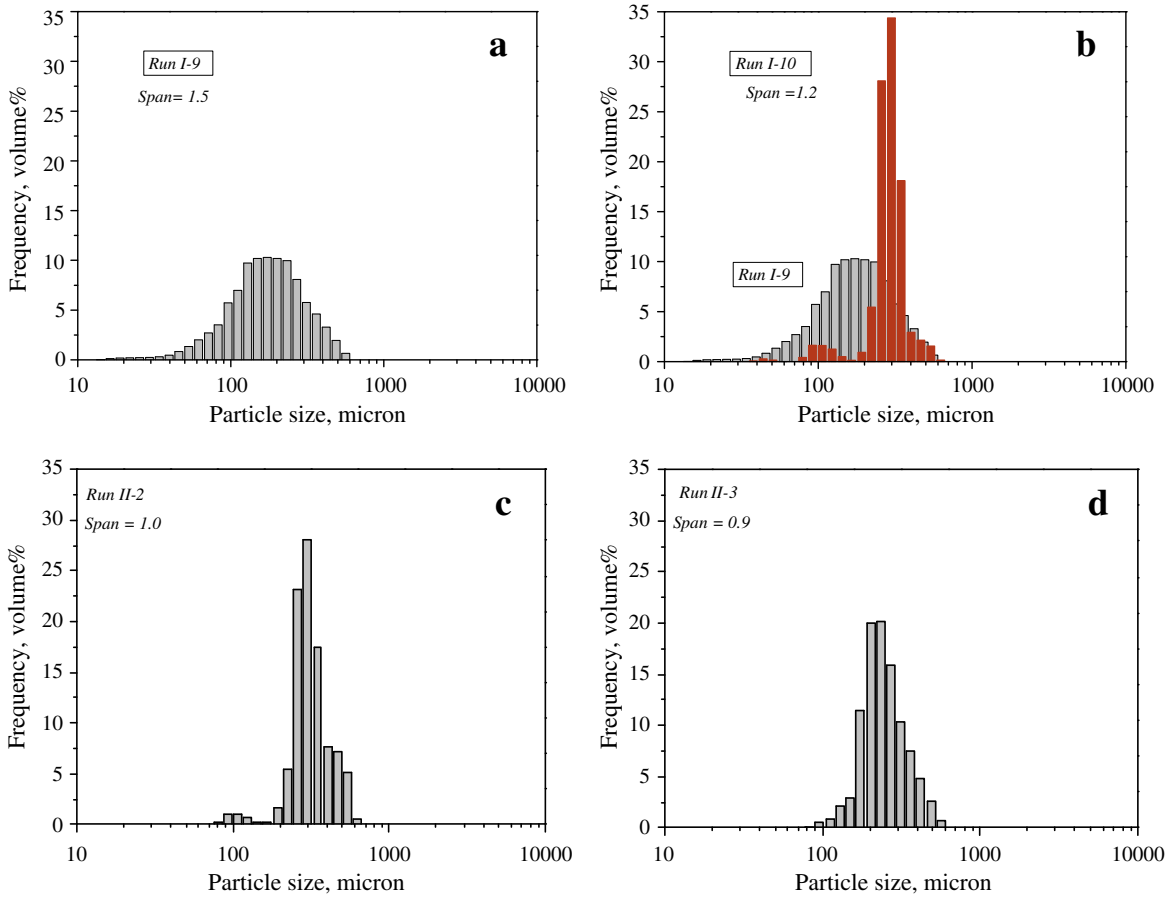

Fig. 4. Typical frequency distribution of the formed spherical agglomerates, a – run I-9, b – runs I-9 and I-10, c – run II-2, d – run II-3.

Span values were scattered within a relatively narrow range between 0.8 and 1.2.

4.4. Effect of the feeding rate of poor solvent

Limited number of experiments was carried out to examine the effect of feeding rates of poor solvent. In addition to the runs where the poor solvent was added in one shot, feeding rates of about 5.5 and 12 mL/min were also tested at different stirring rates. The results listed in Table 2 and shown in Fig. 5a suggest that at high rpm the rate of poor solvent addition had no significant effect, but at low rpm the mean particle size were larger at higher feeding rate. This can be explained by the inhomogeneous spatial concentration distribution at low stirring rates just after the addition of poor solvent. It can therefore be supposed that in certain regions of the mixture the high local concentration of the poor solvent led to stiffer agglomerates, which could serve as nuclei during the next period, because the softer agglomerates formed in the region where the poor solvent had lower concentration could attach to the stiff nuclei resulting in further growth.

4.5. Effect of stirring rate

In the majority of experiments 400 rpm stirring rate was applied. However, to check the effect of this parameter on the obtained size distribution and volume mean size, the stirring rate in several experiments was varied between 200 and 600 rpm. Typical results are plotted in Fig. 5a and b. It is seen that due to the increasing shear force acting on the freshly formed agglomerates their mean size could be diminished to less than the half in the studied rpm range, also depending on the feeding rate of poor solvent. The size distribution of the product was gradually also shifted to smaller size intervals as is seen in Fig. 5b.

4.6. Effect of stirring time

In three series of additional experiments 5–5 different combinations of 15, 30 and 45 min stirring times were applied in Steps 5 and 6 to study their effect on the size distribution of the product. The other conditions were set to the values applied in runs I-10, II-1 and II-3, respectively. Some typical results are plotted in Fig. 6a and b as examples. Surprisingly, it was found that, on the contrary to the effect of stirring intensity, no significant change took place. This can be explained by a supposition that the pre-agglomerates and their equilibrium size distribution could fully developed within 15 min in Steps 5 and 6.

Comparing the smallest particle size regions shown in Fig. 6a and b, interesting difference can be observed: under the conditions applied in runs plotted in Fig. 6a significant amount of small fraction corresponding not fully agglomerated particles was obtained at each stirring times. However, in the series plotted in Fig. 6b, such small particle fraction is not present except the product obtained with 45 min stirring in Step 6. It can therefore be supposed that under the applied conditions efficient agglomeration took place during 15 min agitation, but applying too long stirring in Step 6 can lead to considerable disintegration.

By determining the unloaded protein in certain experiments from the UV spectra in the Bradford method, it was found that the amount of encapsulated protein had no significant dependence on the studied process conditions. Encapsulation efficiency was very high, e.g. in sample I-10 it

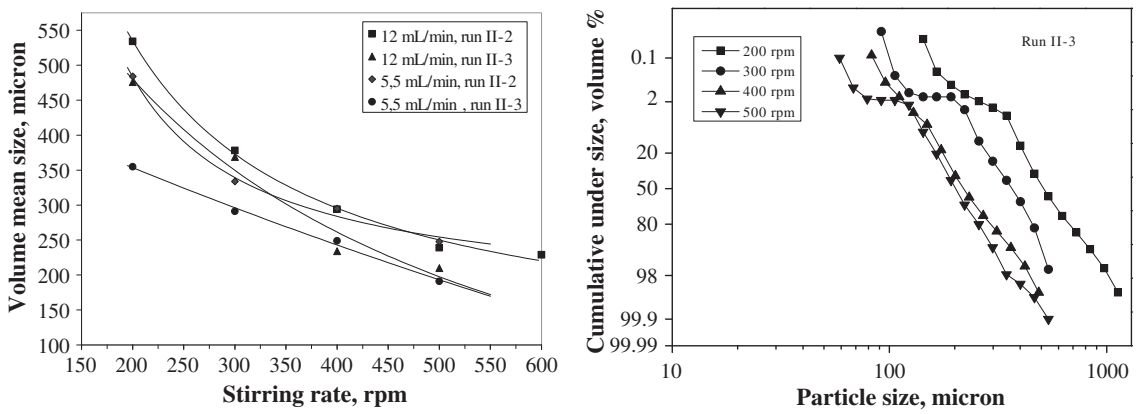

Fig. 5. Effect of stirring rate: a – volume mean size as a function of stirring intensity and the rate of poor solvent addition, b – size distributions obtained by different stirring rates of 12 mL/min. Other parameters: like Runs II-2 and II-3.

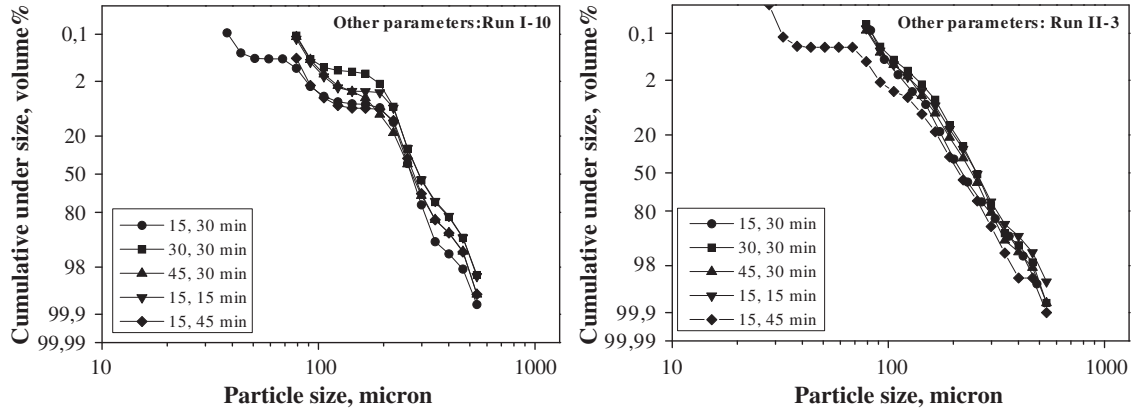

Fig. 6. Effect of stirring times applied in Steps 5 and 6 on the size distribution of the final product. The figures separated by comma in the legends denote the stirring times in minute in Steps 5 and 6, respectively.

was 98% ± 1.5%. From this, the protein loading in the particles was estimated as high as 27% (w/w). This was in good agreement with the FTIR measurement carried out in some runs, which showed that the filtrate after the Step 6 practically did not contain any solutes, meaning that the total amount of the introduced protein has been incorporated into the obtained spherical agglomerates.

5. Conclusions

Investigation was carried out to find out suitable conditions for spherical agglomeration in ethyl acetate–ethanol–water ternary solvent mixtures to produce chitosan–human serum albumin–PSS composite microparticles. The process was realized in w/o type quasi emulsion formed by sequential introduction of aqueous solutions of human serum albumin, chitosan and sodium poly(4-styrenesulfonate) (PSS) under stirring into the solvent mixture. The applied organic solvents are only partially miscible with water, and CS, HSA, PSS alone or together, are practically not soluble in them. Co-precipitation of CS and HSA in the water rich droplets was achieved first by the PSS, and also by the reduction of the water concentration and volume ratio of the droplets. For this purpose only pure ethanol, or ethyl acetate–ethanol mixture and ethanol were added generally in two distinct steps to the mixture under stirring. Under given conditions nearly spherical shape and suitable stability of the formed agglomerates against disintegration was achieved. The paper gives a report on these conditions, describing the effects of the most important process variables such as the compositions of the applied ternary solvent mixture, the feeding method and composition of poor solvent introduced in the last stages of the process, as well as the applied stirring rate and time. The paper proposes a probable mechanism which can lead to proper formation of the protein loaded composite microparticles.

Symbols

D[v, 0.1]	10% of the cumulative volume distribution is below this diameter, μm
D[v, 0.5]	50% of the cumulative volume distribution is below, and 50% above this diameter, μm
D[v, 0.9]	90% of the cumulative volume distribution is below this diameter, μm
SP	span of the granule size distribution (volume basis, Eq. (1)), dimensionless
V	volume, mL

Indices

aq	aqueous phase
org	organic phase
EtOAc	ethyl acetate
EtOH	ethanol
H_2O	water
PSS	poly(sodium 4-styrene-sulfonate)
CS	chitosan
HSA	human serum albumin

Table 2
The effect of the poor solvent addition rate at various stirring rates in runs II-2 and II-3.

Stirring rate, rpm	Other parameters: like in Run II-2		Other parameters: like in Run II-3	
	Poor solvent: 5.5 mL/min	Poor solvent: 12 mL/min	Poor solvent: 5.5 mL/min	Poor solvent: 12 mL/min
200	484	534	355	476
300	334	378	291	369
400	295	294	249	234
500	248	239	191	210

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