BIOPHARMACEUTICAL UNDERSTANDING OF FORMULATION PREPARATION VARIABILITY OF PLGA NANOPARTICLES LOADED WITH ERYSIMUM EXTRACT

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Polymeric nanoparticles (PNPs) are a nanotechnology-based system fabricated for pharmaceutical purposes [2,3]. In recent years interest towards polymer nanoparticles has been especially increased due to their favorable characteristics in terms of simple elaboration and good biocompatibility. PNPs have a marked role since they can bring therapeutic agents in the human body with excellent efficiency [4,5]. In addition, they transport active ingredients to intended position at the specified concentration and impart stability and longer duration. Thus, PNPs are considered one of the ideal candidates for drug delivery systems [6]. The design of a PNP delivery system requires efficient control of quality characteristics. Moreover, the development of an unstable nanocomposition results in the uncontrolled and unpredictable behavior of nanoparticles in a complex biological environment [4]. That is why it is critically important to develop nanoparticles with stable, reproducible properties. The properties of polymeric nanoparticles depends on various factors, such as polymer nature, physical-chemical properties of active substance and target characteristics of nanoparticles. Accordingly, understanding process and formulation variables influencing the nanoparticles properties is very important. Though, most of the nanoparticles preparation methods need to be developed and optimized [7,8]. Various process variables influence the characteristics of nanoparticles prepared, which needs to be determined and strictly adjusted during nanoparticle fabrication process. The purpose of this study was to evaluate the effect of process and formulation variables on the preparation of biodegradable polymeric nanoparticles. Poly-lactide-co-glycolide (PLGA) was selected as the most widely used biodegradable polymers [2], which protects active pharmaceutical ingredient from human defence system. Also, PLGA as a nanocarrier, is good candidate to insure sustained release of active ingredient. PLGA based nanoparticles were prepared by modified emulsification method [4,7]. During experiment we studied impact of various biopharmaceutical factors on colloidal characteristics of nanoparticles.

Material and methods. Biodegradable polymer PLGA, Poly(D,L-Lactide-co-Glycolide, (LA:GA 50:50, MW 7000-17000),Surfactants: polysorbates-Tween 80, tween 20, Sorbitan monooleate (MW 1310), polyvinyl alcohol (PVA, Mowiol 8-88, MW 67,000), Kolliphor P188 (Poloxamer 188) were purchased from Sigma-Aldrich (Germany). Organic solvents: acetone, chloroform, 1,2-dichloroethane were provided by Tbilisi State Medical University. Freeze-dried crude extract of Erysimum contractum Somm. Et Levier was obtained from Neopharmi LTD, Tbilisi, Goergia. Crude extract of Erysimum contractum Somm. etLevier is rich with of flavanoidsand indole, pyridine alkaloids. Cytotoxic activity of crude extract of Erysimum contractum Somm. etLevier is also evaluated by Dr. Dali Beridze.

Preparation of the NPs (General Procedure). The polymer NPs were prepared according to the modified emulsification-solvent evaporation method. All experiments of NPs formulation were performed at room temperature. In a typical procedure, a definite amount of PLGA was dissolved in an organic solvent. The organic phase was added to the aqueous phase containing surfactant and stirred on the magnetic stirrer at 2500 rpm. Evaporation of organic phase takes place at room temperature. Particles are washed three times with 20 ml distilled water. Nanoparticles are washed and collected by centrifuging (15 000 g, 15 min). The influence of the different factors such as organic solvents, surfactants, as well as a polymer concentration in the organic phase, surfactant concentration in the aqueous phase, the organic/water phase ratio on the NPs fabrication process was studied.

NPs Size and size distribution. The mean particle diameter of NPs was characterized by size and size distribution (Polydispersity Index, PDI). Measurement was performed by dynamic light scattering (DLS) using a Zetasizer Nano ZS (Malvern Instruments, U.K.) at 25°C. The MPD and PDI are presented as an average of three individual measurements± standard deviation (SD).

Entrapment efficiency. The amount of entrapped active pharmaceutical ingredient (crude extract of Erysimum contractum Somm. etLevier) was calculated by dissolving the nanoparticle sediment into DMSO (dimethyl sulfoxide) and entrapment efficiency was calculated according to the equation: EE%= amount of extract determined in sediment x100%/applied amount of extract. The absorbance was measured at 425 nm.

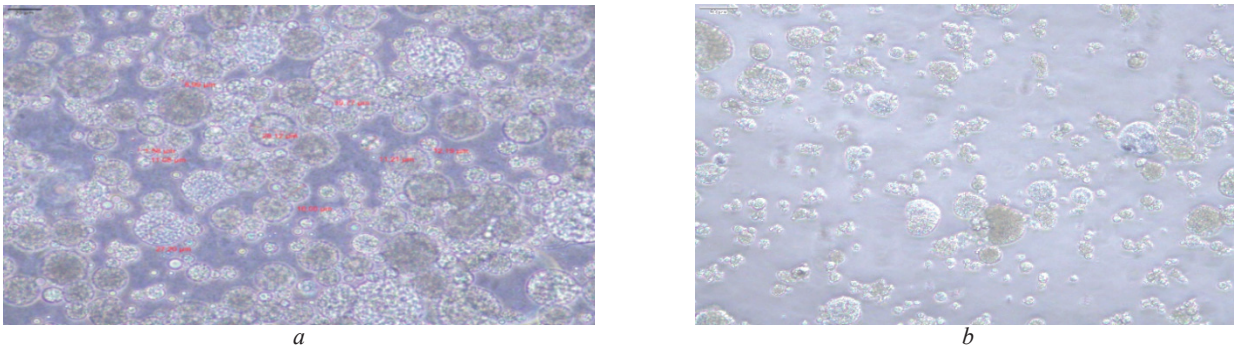
Results and discussion. *Influence of surface active substances on particle size and size distribution.* Surface active substances (surfactant) are used to reduce the surface tension and stabilize the droplet phase during emulsification process. The nature and concentration of the surfactant influence on particle size, polydispersity index and their colloidal stability [5].

Low concentration of the surfactant results in colloidal instability of the nanocomposites. While at high concentration, as surfactant acts as a solubilizing agent, the active substance diffuse in aqueous medium and is dissolved in the form of micelles, thus entrapmentefficiency is decreased. This phenomenon also reduces surfactant tconcentration in aqueous medium, which results in colloidal instability of system [9]. Thus, determination of optimal concentration of the surfactant is one of the critical parameters during nanoparticle fabrication. Different non-ionic surfactants were used in nanoparticle preparation process:poloxamer 188, sorbitan monooleate (Span 80), polysorbate 20 (tween 20), polysorbate 80 (tween 80), polyvinyl alcohol (PVA). The NPs were prepared according to the general procedure described above. The parameters of the NPs fabrication process are given below: concentration of the polymer in organic phase 50 mg/ml, organic solvent - 1,2 dichloroethane, organic phase/ water phase ratio – 1:20. Fifteen composition was developed to study influence of surfactants.

Empirical screening of the prepared nanocomposites was implemented by visual evaluation of nanoemulsion. Exclusion criteria was phase separation and formation large aggregates. In nanocomposites where no large aggregates are visually observed, biological microscope and dynamic light scattering (DLS) are used to characterize size distribution of nanoparticles. List of the formulations, as well as their visual evaluation results are given in the Table 1.

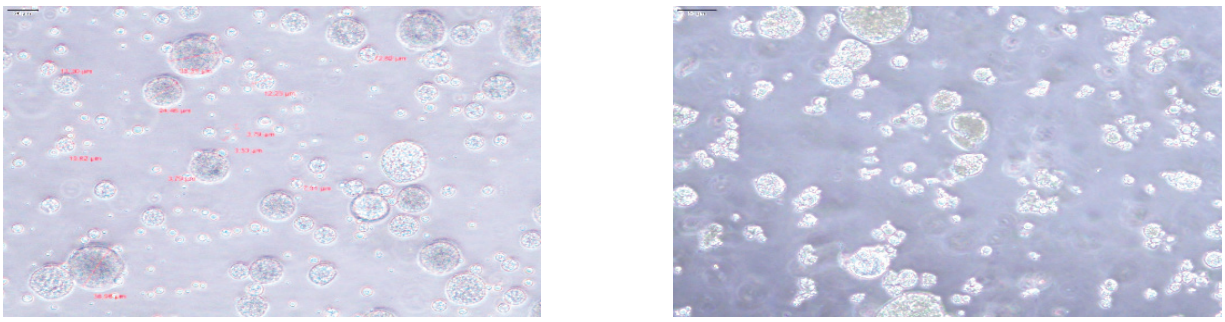
Table 1. The influence of surfactant concentration on PNPs formation

Formulation №	Type of Surfactant	Surfactant concentration, %	Visual evaluation
1	Poloxamer 188	0.1	Complete aggregation
2	Poloxamer 188	0.5	Complete aggregation
3	Poloxamer 188	1,0	Complete aggregation
4	Poloxamer 188	2.5	Aggregation after washing step, difficult resuspendability
5	Poloxamer 188	5	Aggregation after washing step, difficult resuspendability
6	Tween 20	0.5	Complete aggregation
7	Tween 80	0.5	Complete aggregation
8	Span 80	0.5	Complete aggregation
9	Tween 80	2.5	Coalescence, flocculation
10	Tween20	2.5	NPs sedimentation problem
11	Tween 80	2.5	NPs sedimentation problem
12	Polyvinyl alcohol	5	gelatinization of aqueous phase
131	Polyvinyl alcohol	2,5	No aggregation, easily resuspendable
14	Polyvinyl alcohol	1,0	Separate aggregates after resuspension
15	Polyvinyl alcohol	0,5	Separate aggregates after resuspension



a *b*

Fig. 1. Microscopic images of nanocompositions prepared with poloxamer 188 (2.5%)



a *b*

Fig. 2. Microscopic images of nanocompositions prepared with 2.8 % tween 80 (a), 2.5% tween 20 (b)

Considering the surfactant structure, we tried to assess the mechanism of their influence on the properties of nanoparticles. Investigations carried out by us proved that in compositions with low poloxamer 188 concentration (<1%), complete aggregation of polymer occurred instantly (composition 1,2,3, Table 2). While at high concentration of poloxamer 188 (2-5%) composite loses colloidal stability only at purification stage. Evaluation by microscope figures (Fig. 1a,b) clearly shows the colloidal instability mechanisms of the emulsion systems, such as coalescence and flocculation.

The above stated phenomenon can be explained by chemical structure of poloxamers. Chemical structure of poloxamers contain hydrophilic polyethylene oxide blocks (PEO) and hydrophobic polypropylene oxide blocks (PPO), all poloxamers have similar structure, but they differ by molecular weight and PEO/PPO ratio. Hydrophobic (PPO) blocks in poloxamer 188 is less, which results in weak interaction between the hydrophobic polymer (PLGA) particles and the poloxamer. This is the reason why most of the surfactant is removed at washing step and aaggregates are formed. The same mechanism explains the colloidal instability of nanocomposites prepared with low concentration (0.5%) of polysorbates (Tween 20, Tween 80) and sorbitan monooleate (span 80), formulations № 6,7,8 respectively (Table 1). Low concentration of the above stated surfac-

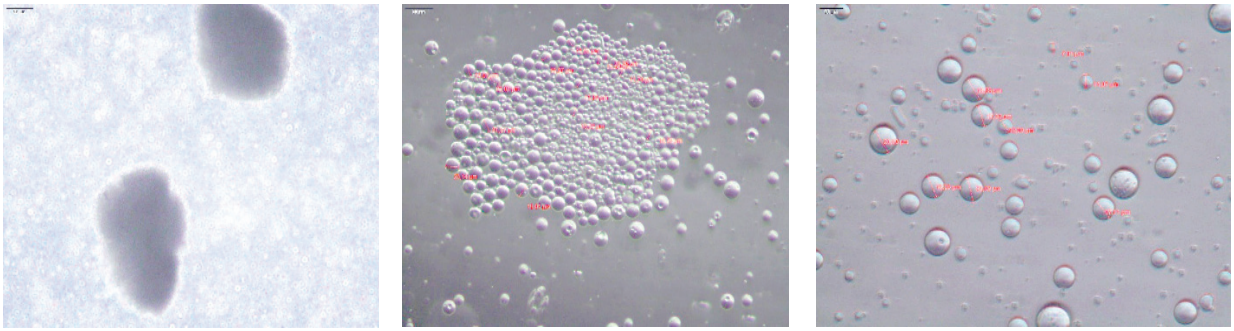


Fig. 3a - PVA 2.5% Fig. 3b. - PVA 0.5% Fig. 3c. PVA 0.1%

tants (Tween 20, Tween 80, span 80) are not sufficient to ensure colloidal stability of composite, visible aggregates are formed instantly. The reason is excess of hydrophilic blocks in their structure, which prevents from strong interaction of surfactant and polymer particle surface. But, in case high concentration (≤2.5%) no visually detectable aggregates are formed, although microscopic images prove colloidal instability, such as coalescence, flocculation (Fig. 2a,b).

When high concentration of sorbitan monooleate (span 80) was used nanoparticle sedimentation problem occurred independence of centrifugation speed and duration. The reason of this problem was also in the structure of surfactant. The so-called netlike positioning of hydrophobic blocks of sorbitan monooleate (SPAN 80) in water phase prevents sedimentation of nanoparticles (especially of small size particles).

During our experiments polyvinyl alcohol was used as a surfactant. There are partially hydrolyzed and fully hydrolyzed polyvinyl alcohols. Amount of residual acetyl group in fully hydrolyzed polyvinyl alcohol is approximately 1.5% which provides weak interconnection between the surfactant and PLGA particle surface. Partially hydrolyzed polyvinyl alcohol contains more hydrophobic acetyl groups (10%), that provides stronger interation of polyvinyl alcohol to the surface of PLGA particles. That is why partially hydrolyzed polyvinyl alcohol is preferred for nanoparticle fabrication.

The above stated hypothesis was proved by the experiments too, in the formulation (№12, 13, 14, 15), where partially hydrolyzed polyvinyl alcohol was used no aggregation of particles was detected. Influence of polyvinyl alcohol concentration is also interesting. In particular, in the experiment we used various concentrations of the surfactant (Table 2). In case of 5% concentration (formulation-12) increased viscosity of water phase prevented further separation of phases and sedimentation of NPs. Microscopic images (Fig. 3a,b,c) clearly show that the particle size is not homogeneous and they are of micro scale size (within 10-40 micrometer).

Re-suspension of nanocomposites prepared with low concentration of PVA (0.1-0.5%) doesn't occur completely. At the stage of washing, because of removal of a surfactant, the composite loses its stability and collected particles are hardly re-suspended. This is proved microscopically by the presence of single aggregates (Fig. 3a,b,c). Thus, application of 2.5% partially hydrolyzed polyvinyl alcohol provided formation of relatively stable nanocomposites (Fig. 3a). Nanoparticles are easily re-suspendable, particles are of micro-size, with non-homogeneous distribution, although microscopic images do not prove presence of single aggregates. Thereafter, size and poly-dispersion degree of formulation №12 were evaluated by DLS, the size and posidispersity index were 731.5±71.02 and 0.786±0.022, respectively. Results demonstrate that particles at nanoscale are obtained with broad size distribution. At the next stage, influence of other formulation variables was studied to obtain nanoparticles with narrow size distribution.

Influence of organic solvent on NPs sizes and size distribution. Selection criteria of organic phase for nanoparticle preparation was solubility of PLGA polymer. As usual, selection of organic solvent is made empirically. Mechanism of organic solvent influence on particle size is not clearly known. According to one of the hypotheses, organic solvent diffusion coefficient in water can be used as an indicator of particle sizes and distribution degree. Organic solvent with high diffusion factor provides relatively small size and monodisperse particles, while organic solvent with low diffusion factor works on the contrary. The following organic solvents were used in study: chloroform, 1,2 dichloroethane and acetone, formulations are given in Table 2.

Particle size and polydispersity index in composites were evaluated by DLS. Results are given in Table 3.

Table 2. Compositions with different organic phase

Formulation №	Organic phase				Aqueous Phase	
	PLGA, mg	1,2 dichloroethane, ml	Chloroform, ml	Acetone, ml	Polyvinyl alcohol, %	Water, ml
13	50	1	-	-	2,5	20
16	50	-	1	-	2,5	20
17	50	-	-	1	2,5	20

Table 3. Influence of organic solvent on NPs size and size distribution

Formulation №	Z-Ave (nm)	Polydispersity index (PDI)
13	731± 71	0.786± 0.022
16	1299± 308	0.663± 0.114
17	571± 53	0.337± 0.12

Table 4. Impact of aqueous and organic phase ratio on the particle size and entrapment efficiency

Composition № F	Z-Ave (nm)	Polydispersity index (PDI)	Entrapment efficiency (%)
17 (1:20)	571±53	0.337±0.12	25
18 (1:10)	27 ±5	0.228±0.012	48
19 (1:5)	232±3.25	0.18±0.004	73

The obtained results demonstrates (Table 3) that organic solvent greatly influence on NPs size and size distribution. The obtained results proved the hypothesis regarding organic solvent mentioned above. Improved size distribution was obtained in the formulation prepared with water miscible organic solvent, acetone (formulation F17). According to the literature data, nature of organic solvents can influence on interaction of surfactant with polymeric nanoparticles, which itself impactson thecolloidal stability. Apparently, in case water miscible organic solvent is applied, number of surfactants associated with (formulation-17). Therefore, acetone was selected as an organic solvent.

Influence of polymer concentration on nanoparticle sizes and size distribution. To determine impact of polymer concentration on particle size we used 50 and 100 mg PLGA. The condition of the NPs fabrication process is: polyvinyl alcohol – 2.5%, organic solvent – acetone, organic phase/ water phase ratio – 1:20. Variable parameters: polymer concentration- 50 mg/ml, 100 mg/ml. By increasing polymer concentration from 50 mg to 100 ml NPs size was increased from 571 nm to 882 nm, respectively. Polydispersity index was also increased from 0.33 to 0.68. By increasing polymer concentration, the viscosity of the organic phase is rised. This increases viscosity force and decreases homogeneous distribution of emulsion drops, which contributes to formation of big size particles. Thus, 50 mg PLGA was considered as an optimal concentration of polymer.

Influence of aqueous and organic phase ratio on the entrapment efficiency. Ratio of aqueous and organic phase mainly influences on the entrapment efficiency of active substances in the nanoparticles, especially when organic solvent (acetone) is water miscible.

Alongside with it, ratio of aqueous and organic phase influence on the particles meophology as well. Increase of aqueous phase ensures rapid solidification of nanoparticles, their surface is smoother with a smaller number of pores. By decreasing ratio of aqueous and organic phase the number of pores increases, which in its turn results in high speed of drug release.

Therefore, to achieve high entrapment efficiency of active substance we studied the impact of aqueous and organic phase ratio. The condition of the NPs fabrication process is: polyvinyl alcohol – 2.5%, organic solvent – acetone, amount of PLGA- 50 mg. Variable parameters: organic phase/ water phase ratio- 1:20, 1:10, 1:5.

Impact of aqueous and organic phase ratio on the particle size and entrapment efficiency is given in Table 4.

The obtained results (Tables 4) proved the above referred theoretical hypothesis: decrease of aqueous medium increases the entrapment efficiency. This is explained by the fact that, increasing volume of aqueous phase, more active substances migrate from organic phase to aqueous phase. This process is more intensive when water miscible organic solvent (acetone) is used in the formulation. This is the explanation, that in formulations № 17 and 18 entrapment efficiency is low, 25% and 48% respectively. Decrease of aqueous and organic phase ratio positively influences on the particles size distribution as well. This is explained by the fact that mixing in small volume is much more intense and homogenous, than in case of big volume. According to the results optimal ratio of aqueous medium and organic phase is considered to be 1:5. This condition obtained smaller nanoparticles (232 nm) with narrow size distribution (0.18) and 73% of entrapment efficiency.

In summary, based on the performed experiments optimal formulation of nanocomposite is suggested: polyvinyl alcohol – 2.5%, organic solvent – acetone, amount of PLGA- 50 mg, organic phase/ water phase ratio 1:5.

Conclusion. The result of the study demonstrates that the formulation variables could be effectively altered to achieve the desired characteristicsof polymeric nanoparticles. The influence of the various biopharmaceutical factors such as type of organic solvent, surfactant, as well as surfactant concentration in the aqueous phase, polymer concentration in the organic phase, the organicphase/water phase ratio on the NPs size, size distribution and entrapment efficiency were studied. The influence mechanism of different biopharmaceutical factors on the colloidal characteristics of polymer nanoparticles has been theoretically explained and experimentally confirmed. Based on performed study optimal formulation and preparation method of nanocomposite is provided.

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SUMMARY

BIOPHARMACEUTICAL UNDERSTANDING OF FORMULATION PREPARATION VARIABILITY OF PLGA NANOPARTICLES LOADED WITH ERYSIMUM EXTRACT

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The purpose of this study was to evaluate effect of process and formulation variables on the preparation of Erysimum extract loaded PLGA nanoparticles.

The influence of the various biopharmaceutical factors such as type of organic solvent, type and concentration of surfactant, polymer concentration in the organic phase, ratio of organic phase and water phase were studied. Modified emulsification solvent evaporation method was used for preparation of nanoparticles. Based on the performed experiments optimal formulation of nanocomposite is suggested. Nanoparticle size, size distribution and entrapment efficiency were determined. Among five non-ionic surfactants polyvinyl alcohol provided more stable nanocomposite. Influence mechanisms of different surfactants on nanoparticle formation are provided. Water miscible organic solvent, acetone obtained 232 nm nanoparticles with improved size distribution. Entrapment efficiency was increased to 73% by reducing ratio of organic and water phases. Based on experiments nanoparticles with stable, reproducible properties are fabricated.

Keywords: polymeric nanoparticle, PLGA, formulation variables, endemic plant species.

РЕЗЮМЕ

ОЦЕНКА ВЛИЯНИЯ БИОФАРМАЦЕВТИЧЕСКИХ ФАКТОРОВ НА СВОЙСТВА НАНОЧАСТИЦ PLGA, СОДЕРЖАЩИХ ЭКСТРАКТ ERYSIMUM CONTRACTUM SOMM

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Тбилисский государственный медицинский университет, [1]кафедра фармацевтической технологии; [2]направление фармакогнозии и фармацевтической ботаники, Грузия

Целью исследования явилась оценка влияния биофармацевтических факторов на свойства наночастиц PLGA, содержащих экстракт *Erysimum contractum Somm*.

Изучено влияние различных биофармацевтических факторов: органический растворитель, тип и концентрация поверхностно-активного вещества, концентрация полимера в органической фазе, соотношение водной и органической фаз. Исследовано влияние пяти неионных поверхностно-активных веществ на свойства наночастиц. Теоретически обоснован и экспериментально подтвержден механизм влияния различных биофармацевтических факторов на коллоидные характеристики полимерных наночастиц. Экспериментально установлено, что полугидролизованный поливиниловый спирт обеспечивает коллоидную стабильность нанокомпозиции.

Для приготовления наночастиц использовали модифицированный эмульсионной метод. Предложено оптимальное содержание нанокомпозиции: поливиниловый спирт - 2,5%, соотношение водной и органической фаз - 1:5, полимер (PLGA) - 50 мг, органический растворитель - ацетон, активное вещество - 5 мг. Определены размер частиц, индекс полидисперсности и инкапсулирование активного вещества. Использование смешиваемых с водой органических растворителей обеспечивает образование наночастиц 232 нм и значительно улучшает степень диспергирования частиц. Уменьшение соотношения водной и органической фаз обеспечило увеличение степени инкапсулирования активного вещества до 73%. Результаты исследования показали, что, изменяя параметры, можно получить наночастицы с желаемыми характеристиками.

რეზიუმე

ბიოფარმაცევტული ფაქტორების გავლენის შეფასება *Erysimum contractum Somm*-ის შემცველი პოლიმერული ნანონაწილაკების მახასიათებლებზე

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თბილისის სახელმწიფო სამედიცინო უნივერსიტეტი, [1]ფარმაციის ფაკულტეტი, ფარმაცევტული ტექნოლოგიის დეპარტამენტი; [2]ფარმაკოგნოზიისა და ფარმაცევტული ბოტანიკის მიმართულება, საქართველო

კვლევის მიზანს წარმოადგენდა ბიოფარმაცევტული ფაქტორების გავლენის შეფასება შეჯგუფული ერიზიმუმის ექსტრაქტის შემცველი PLGA ნანონაწილაკების მახასიათებლებზე.

შესწავლილია სხვადასხვა ბიოფარმაცევტული ფაქტორების გავლენა,როგორიცაა ორგანული გამხსნელი, სურფაქტანტის ტიპი და კონცენტრაცია, პოლიმერის კონცენტრაცია ორგანულ ფაზაში, ორგანულ ფაზისა და წყლის ფაზის თანაფარდობა.

ნანონაწილაკების მოსამზადებლად გამოყენებული იყო მოდიფიცირებული ემულგირება - გამხსნელის აორთქლების მეთოდი. ჩატარებული ექსპერიმენტების საფუძველზე შემოთავაზებულია ნანოკომპოზიციის ოპტიმალური რეცეპტურა. განსაზღვრულია ნანონაწილაკების ზომა, ზომის განაწილება და ჩართულობის ხარისხი. შესწავლილია ხუთი არაიონური სურფაქტანტის გავლენა ნანონაწილაკების მახასიათებლებზე. ექსპერიმენტულად დადგენილია, რომ ნახევრადჰიდროლიზებული პოლივინილის სპირტი უზრუნველყოფს ნანოკომპოზიციის კოლოიდურ სტაბილურობას. ასევე მოწოდებულია სხვადასხვა ზედაპირულად აქტიური ნივთიერებების გავლენის თეორიული საფუძვლები ნანონაწილაკების მახასიათებლებზე. წყალთან შერევადი ორგანული გამხსნელის გამოყენება უზრუნველყოფს 232 ნმ ზომის ნანონაწილაკების ფორმირებას და მკვეთრად აუმჯობესებს ნაწილაკების განაწილების ხარისხს. წყლიანი და ორგანული ფაზის თანაფარდობის შემცირებამ უზრუნველყო მოქმედი ნივთიერების ჩართულობის ხარისხის 73%-მდე გაზრდა. კვლევის შედეგები ცხადყოფს, რომ სხვადასხვა პარამეტრის ცვლილებით შესაძლებელია სასურველი მახასიათებლების მქონე კომპოზიციის მიღება.