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ORIGINAL ARTICLE / ÖZGÜN MAKALE

PREPARATION AND *IN VITRO* CHARACTERIZATION OF SOLID LIPID MICROPARTICLES FOR PROTEIN DELIVERY

PROTEİN VERİLİŞİ İÇİN KATI LİPİT MİKROPARTİKÜLLERİN HAZIRLANMASI VE İN VİTRO KARAKTERİZASYONU

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ABSTRACT

Objective: *The aim of this research was to assess the effect of the process and formulation parameters during the preparation of solid lipid microparticles. Solid lipid microparticles (SLMs) have evident advantages such as biocompatibility, simplicity of production and characterization, prolonged release, and especially high protein loading capacity, despite being less investigated than lipid nanoparticles.*

Material and Method: *SLMs were prepared via emulsion solvent diffusion technique using glyceryl tridecanoate (GTD) as a biocompatible and biodegradable lipid. The optimum formulation conditions for producing homogenous spherical microparticles were found and represented by a triangle phase diagram area. After optimizing the particle size and encapsulation efficiency by changing the formulation parameters, the microparticles were characterized by in vitro release, morphological analysis, thermal analysis and electrophoretic analysis on the selected formulations.*

Result and Discussion: *The maximum drug loading efficiency was achieved by combining 100 mg of lipid, 60% triacetin and 3% emulsifier. The average microparticle size was observed as 8.9 µm. The in vitro drug release were analyzed in pH 7.4 phosphate buffer and were mainly completed at 8th hour.*

Keywords: *Bovine serum albumin, glyceryl tridecanoate, lipid microparticle, triacetin, triangular phase diagram*

ÖZ

Amaç: *Bu araştırmanın amacı, katı lipid mikropartiküllerinin hazırlanması sırasında proses ve formülasyon parametrelerinin etkisini değerlendirmektir. Katı lipid mikropartiküller (SLM'ler), lipid nanopartiküllerinden daha az araştırılmış olmalarına rağmen biyouyumluluk, üretim ve karakterizasyon kolaylığı, uzun süreli salım ve özellikle yüksek protein yükleme kapasitesi gibi belirgin avantajlara sahiptir.*

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Gereç ve Yöntem: *SLM'ler, biyouyumlu ve biyolojik olarak parçalanabilen bir lipid olarak gliseril tridekanoat (GTD) kullanılarak emülsiyon çözücü difüzyon tekniği ile hazırlanmıştır. Homojen küresel mikropartiküller üretmek için en iyi formülasyon koşulları belirlenmiş ve bir üçgen faz diyagram alanı ile temsil edilmiştir. Mikropartiküller, formülasyon parametreleri değiştirilerek partikül boyutu ve enkapsülasyon etkinliği optimize edildikten sonra, seçilen formülasyonlar in vitro salım, morfolojik analizler, termal analiz ve elektroforetik analiz ile karakterize edilmiştir.*

Sonuç ve Tartışma: *En yüksek etken madde yükleme etkinliği 100 mg lipid, %60 triasetin ve %3 emülgatör kullanılarak elde edilmiştir. Ortalama mikropartikül boyutu 8.9 µm olarak gözlenmiştir. İn vitro etken madde salımı pH 7.4 fosfat tampon çözeltisinde değerlendirilmiş ve 8. saatte tamamlanmıştır.*

Anahtar Kelimeler: *Gliseril tridekanoat, lipid mikropartikül, sığır serum albümini, triasetin, üçgen faz diyagramı*

INTRODUCTION

In recent years, protein delivery has emerged as a particularly interesting subject of research due to the selectivity, efficacy, and relative maturity of recombinant protein expression techniques. Solid lipid particles have shown excellent potentials against proteolysis of integrated proteins among protein delivery methods designed for parenteral administration. These particles also provide steric barriers that can contribute in the controlled release of drugs. [1]. The lipid matrix of SLM is composed of physiological lipids, which lowers the risk of both acute and long-term toxicity [2,3]. On the other hand, SLMs provide high biocompatibility and stability, and can encapsulate different protein therapeutic agents effectively [4].

One of the methods to produce lipid nanoparticles is the solvent emulsification-diffusion method, in which the lipid and active component are dissolved in an organic solvent miscible with water and dispersed in an external aqueous phase including surfactant. Emulsification is carried out by mixing at high speed. The resulting emulsion is then mixed with the aqueous phase to encourage organic solvent migration from droplets to the aqueous phase, which solidifies the lipid molecule particles [3]. While this technique is reproducible and it has the advantage of not exposing the active substance to high temperature, it has the disadvantage of diffusing especially the water-soluble active substance into the aqueous phase and lowering the encapsulation efficiency.

Several advantages of SLMs have driven numerous studies for the application of SLMs as peptide/protein carrier system for oral [5-7], nasal [8] and particularly for parenteral administrations [9-11]. The physicochemical properties and stability of SLMs also depend on the properties of the encapsulated active substance. In this context, the effect of formulation parameters on the physicochemical characteristics of SLMs should be evaluated to obtain an optimal SLM formulation for each active ingredient. With this objective, glyceryl tridecanoate and triacetin were used as lipids for SLM formulations in this research for bovine serum albumin (BSA) delivery as a model protein. The use of glyceryl tridecanoate as a matrix material for SLM formulations for peptides and proteins has rarely been reported [1, 12, 13]. Triacetin is an oil with many unique features such as non-toxicity, high

drug dissolving capacity, self-emulsifying formulation ability, and plasticizer properties. There are few studies in the literature on the use of triacetin in lipid microspheres produced by solvent diffusion method. It has been stated that the use of triacetin in the microsphere formulation allows the solvent and triacetin to diffuse easily into the polymer matrix and increases the drug retention efficiency [14, 15].

MATERIAL AND METHOD

Materials

GTD, triacetin, BSA, Pluronic F127 and ethanol were obtained from Sigma-Aldrich Co (USA). All other reagents were of analytical grade.

Pseudo-Ternary Diagram

In this research, the first step in formulating emulsion-based microparticles was to create the phase diagram used in the literature to determine the stable emulsion region in the optimization process of microparticle formulations [16]. The pseudo-ternary phase diagram was constructed based on the selected lipids and combination of surfactant at different ratios. The lipophilic phase consisted of a GTD and triacetin solution in ethanol, and Pluronic F127 was used as a surfactant. By maintaining the GTD concentration constant (100 mg) and altering the amounts of the surfactant and oil, it was possible to obtain the stable emulsion formation area in a ternary phase diagram. Appropriate amounts of lipophilic phases were weighed in the beaker and mixed for sufficient time to reach equilibrium, then mixed with the aqueous phase containing Pluronic F127 and homogenized with a high-speed homogenizer (IKA T 18 digital ULTRA-TURRAX®) at 13500 rpm for 5 min. The resulting mixture was first visually examined in terms of formation of emulsion. Afterwards, all mixtures produced in the phase diagram where no separation was observed visually were subjected to particle size analysis to confirm the microparticle formation.

Preparation of solid lipid microparticles

In order to prepare solid lipid microparticles, firstly, Pluronic F127 was mixed in a calculated amount of water and kept in the refrigerator overnight to form a solution. GTD was dissolved in 2 ml of alcohol and triacetin was added and mixed. Afterwards, the lipophilic phase was added to the aqueous phase containing Pluronic F127 and homogenized with a high-speed homogenizer at 13500 rpm for 5 min. Then, for the removal of the ethanol, the formulations were taken into a dialysis bag (Spectra/Por Biotech CE Tubing, 300 kD MWCO) and dialyzed for a total of 9 h by changing every 3 h at 350 rpm against one liter of water. The formulation taken from the dialysis bag was frozen at -40°C and

lyophilized. In order to encapsulate BSA into SLMs, 10 mg of BSA was first dissolved in 1 ml pH 7.4 PBS by keeping it in the refrigerator and added to the lipophilic phase.

Particle size measurements

A sample of 10 µL formulation was diluted with ultrapure water. The particle size and size distribution of microparticles were evaluated at 25°C using Malvern Mastersizer 3000 (Malvern Instruments, UK).

Determination of Encapsulation Efficiency (EE) and Loading Capacity (LC)

To determine the BSA encapsulation efficiency of SLM formulations, 5 mg of SLM was weighed and dissolved in 0.5 ml of ethanol. 4.5 ml of ultrapure water was added and mixed in a shaking water bath for 24 h. Then the mixture was centrifuged at 4000 rpm for 10 min and the amount of BSA in the supernatant was determined by microBCA analysis [17]. Encapsulation efficiency and loading capacity of SLM formulations were calculated with the following equations:

$$EE\ \% = \frac{\text{Total amount of BSA} - \text{Free BSA}}{\text{Total amount of BSA}} * 100 \qquad \text{Eq. (1)}$$

$$LC\% = \frac{\text{Total amount of BSA} - \text{Free BSA}}{\text{Weight of SLM formulation}} * 100 \qquad \text{Eq. (2)}$$

***In vitro* BSA release**

In vitro release of BSA from SLM formulations carried out with slight modification of the method used by Sedyakina et al. [18]. In order to determine the *in vitro* release rate of BSA, the SLM formulation containing 200 µg of BSA was placed in Eppendorf tube and 2 ml of pH 7.4 Phosphate Buffered Saline (PBS) was added. Eppendorf tubes were placed in a shaker incubator at 37°C and were shaken horizontally at 50 rpm. At certain time intervals for 24 h, 0.2 ml of sample was taken and replaced with fresh medium. The amount of BSA in the samples was determined by microBCA analysis and in vitro release graphs were created.

Morphology of the SLMs

The morphology of the SLM formulation was determined by both optical microscopy and AFM. The morphology of the F6 formulation was observed using optical microscopy (BX4, Olympus, Tokyo, Japan) with a 10× objective lens. Images were acquired using a digital camera (Q- Color 3, Olympus, Tokyo, Japan). On the other hand, for AFM analysis, F6 formulation was dispersed in distilled water and then dropped onto mica and dried under nitrogen. Then, the mica surface was scanned, and imaging was performed with the AFM device (ezAFM, Nanomagnetics Inst., UK).

DSC Analysis

The thermal properties of BSA, GTD, triacetin, and BSA loaded SLM formulation (F6) were analyzed with the Shimadzu DSC-60 device. Samples weighing 3-5 mg were placed on an aluminum pan and compressed. Samples were heated linearly at a rate of 10 °C/min between 30 and 300 ° C while being examined using nitrogen gas at a flow rate of 50 ml/min. The blank was an empty pan.

SDS-PAGE Analysis

SDS-PAGE was carried out under reducing conditions. The in vitro release samples of the F6 formulation and various amounts of BSA standards produced in PBS (pH 7.4) were analyzed. The protein solutions (20 µL) were mixed with 5 µL of sample buffer, which was boiled for 5 minutes at 100° C. The sample buffer contains 1.0 M Tris (pH 6.8), 1 % bromophenol blue, 2-mercapto ethanol, and 50% glycerol. The samples of 10 µL were loaded onto 15% SDS-PAGE gel and electrophoretically separated. Coomassie brilliant blue was used to visualize protein bands.

RESULT AND DISCUSSION

Construction of the phase diagram

All batches were made with various concentrations of the oil and surfactant and a constant GTD concentration throughout the optimization procedure. Since the GTD concentration was kept constant in the lipid phase, the pseudoternary diagram was created using the varying percentage of triacetin. Pluronic F127 was only employed below 9 % concentration because gel formation was observed above this rate. The region marked in the phase diagram is the region where the emulsion is stable and microparticle formation is observed (Figure 1). The formulations were prepared according to the ratios in this designated area and the formulation compositions are shown in the Table 1.

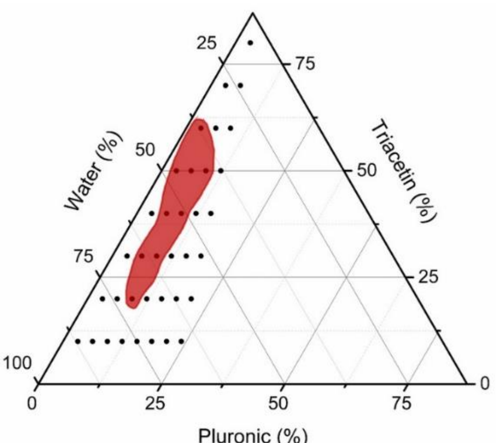

Figure 1. Triangular phase diagram of the water/oil/surfactant system. The stable region is framed by a line and represents formulations yielding spherical microparticles.

Table 1. Composition of SLM formulations

	GTD (mg)	Triacetin (%)	Pluronic F127 (%)	BSA (mg)
F1	100	30	6	10
F2	100	20	9	10
F3	100	50	3	10
F4	100	40	6	10
F5	100	30	9	10
F6	100	60	3	10
F7	100	50	6	10
F8	100	40	9	10
F9	100	50	9	10

Particle size measurements

The particle size homogeneity of the SLMs has a crucial role in the repeatability of the drug release from formulations. [16]**.** Particle sizes of SLM formulations were found within the range of 1.28–12.4 µm (Table 2). Narrow particle size distributions were obtained in this study. Among the formulations, it was observed that the F4 formulation had the smallest particle size, while the F3 formulation had the smallest particle size distribution.

Encapsulation Efficiency (EE) and Loading Capacity (LC)

Encapsulation efficiencies and loading capacities of SLM formulations were found within the range of 7.6–43.2 % and 0.182-0.996 %, respectively (Table 2). The increase in the amount of triacetin and Pluronic F127 together increased the amount of loaded protein. It is known that triacetin weakens the intermolecular forces between polymer molecules due to its plasticizer function and increases the free volume in the polymer matrix by causing the polymer relaxation [14]. Therefore, the increased encapsulation efficiency in the microspheres with the increase of triacetin concentration can be attributed to the easy diffusion of protein into the polymer matrix. Similarly, in the study of Yüksel et al. [14], the addition of triacetin increased the encapsulation efficiency of indomethacin into microspheres. It was observed that the F6 formulation had the highest encapsulation efficiency and the F3 formulation had the highest loading capacity.

In vitro BSA release

The *in vitro* BSA release of the F4 formulation with the smallest particle size, the F3 formulation with the highest drug loading capacity, and the F6 formulation with the highest encapsulation efficiency were compared. Burst effect was observed in the first hour in all formulations. F6 formulation significantly slowed the release of the active substance compared to other formulations. It was thought that this was due to the higher amount of triacetin in the F6 formulation, and that triacetin increased the encapsulation of BSA into the lipid microparticle structure rather than the surface regions. Similarly, in

the study of Beck et al. [19], the addition of triacetin slowed the release of diclofenac from the nanoparticle-coated inorganic microparticles. In another study triacetin modified the release of indomethacin from the microspheres [14]. F6 was chosen as the optimum formulation in this study because of its high encapsulation efficiency and relatively slow drug release (Figure 2).

Table 2. Characterization of SLM formulations

	Triacetin (%)	Pluronic F127 (%)	Mean size (µm)	Span	Encapsulation efficiency (%)	Loading capacity (%)
F1	30	6	12.4±0.152	3.01	30.0±3.6	0.559±0.067
F2	20	9	3.79±0.009	1.78	11.9±0.7	0.441±0.026
F3	50	3	2.51±0.007	1.44	17.2±0.6	0.996±0.032
F4	40	6	1.28±0.004	2.41	7.6±0.5	0.510±0.036
F5	30	9	4.62±0.009	2.97	29.0±1.3	0.343±0.016
F6	60	3	8.90±0.717	15.4	43.2±2.2	0.260±0.013
F7	50	6	2.03±0.004	1.69	20.7±1.5	0.623±0.045
F8	40	9	5.87±0.046	6.00	38.8±2.5	0.380±0.025
F9	50	9	9.95±0.462	3.77	33.9±5.4	0.182±0.029

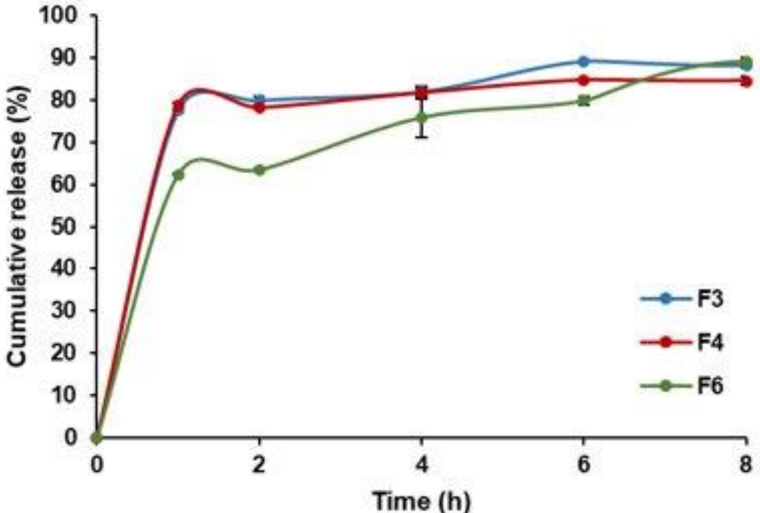

Figure 2. *In vitro* BSA release

Morphology of the SLMs

After the optimization of the process parameters in the previous section, the morphological characteristics of the F6 formulation were investigated. As shown in Figure 3, SLMs were found to be spherical and monodisperse. As a result of the AFM analysis, it was observed that the SLMs were spherical in size and the particle size was similar to the size measured by laser diffraction.

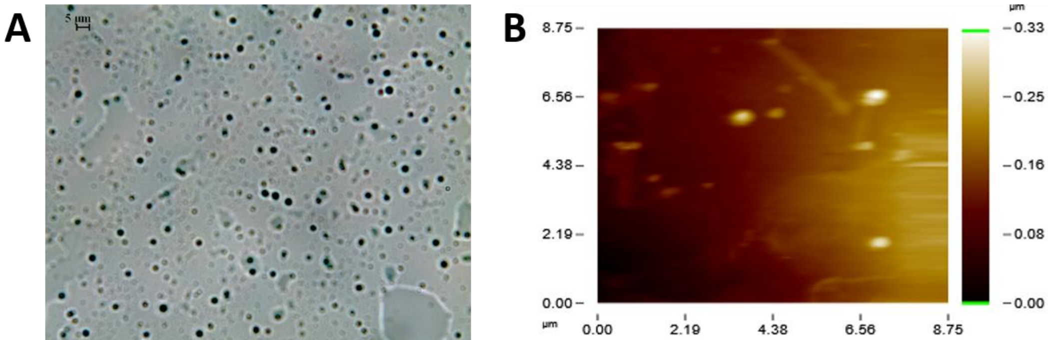

Figure 3. Optical microscope (A) and AFM (B) images of the F6 SLM formulation

DSC Analysis

As a result of DSC analysis, GTD created an endothermic peak at 36°C and is compatible with the literature [1]. Triacetin formed a broad peak at 187°C, corresponding to the flash point [14]. Characteristic peaks of BSA were observed at 89°C and 224°C [20]. DSC thermogram of F6 SLM formulation exhibited only a peak of lipid at 31°C. The absence of characteristic peaks of BSA in the DSC thermogram confirmed that BSA was encapsulated in the lipid microparticle structure (Figure 4).

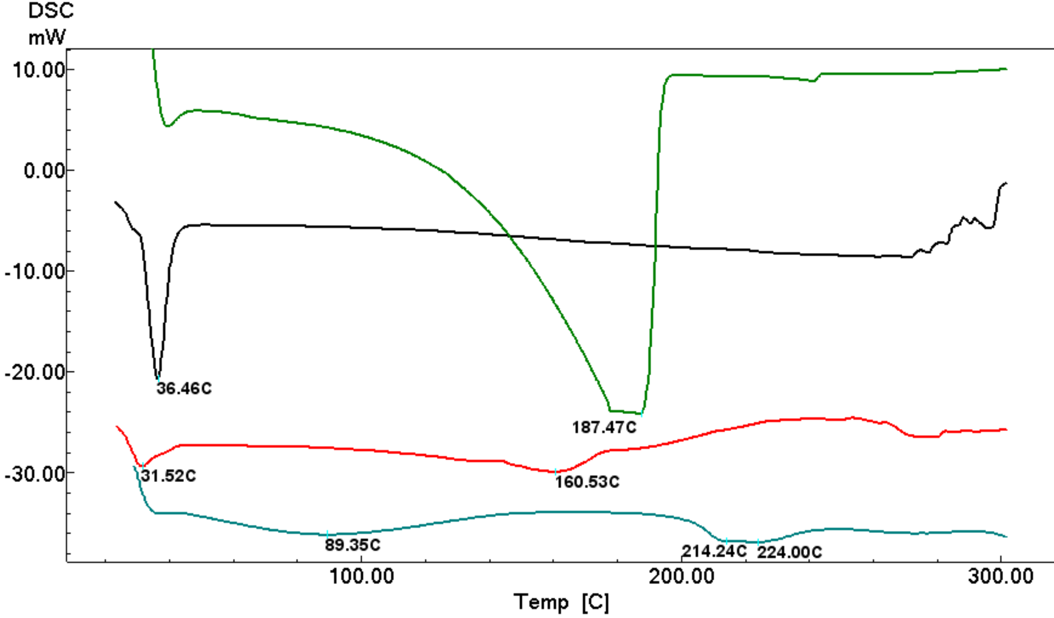

Figure 4. DSC thermograms of triacetin (green), GTD (black), F6 formulation (red) and BSA (blue).

SDS_PAGE Analysis

In the current study, BSA was loaded on SLMs and the molecular weight integrity of BSA released from the NPs was measured by SDS-PAGE whether the structure of the protein was affected during the SLM preparation procedure. As a result of SDS-PAGE analysis, *in vitro* release samples from formulation F6 and BSA standards emerged as a single chain aligned (Figure 5). Apart from these, no

additional bands were observed. This result showed that the protein in the formulation remained stable during the formulation preparation stage and during the 8-hour *in vitro* release period.

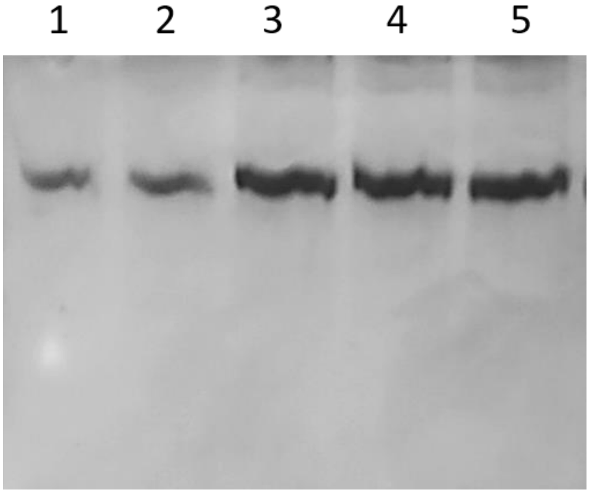

Figure 5. Results of SDS-PAGE analysis. Lane 1: BSA standard 25 µg/ml, Lane 2: BSA standard 50 µg/ml, Lane 3: 2nd hour *in vitro* release sample, Lane 4: 4th hour *in vitro* release sample, Lane 5: 8th hour *in vitro* release sample

In this research, the preparation of SLM formulation as well as its potential for application as a protein carrier system was studied. The triangle phase diagram is used to optimize the effect of emulsion components employed in the emulsion solvent diffusion method on SLM production. The formulations were characterized in detail after loading BSA into the SLM formulations in the region defined by the triangle phase diagram. The optimized F6 formulation was shown to provide BSA release for up to 8 hours and to have acceptable particle size and encapsulation efficiency. Based on these findings, SLM formulations are recommended as an effective protein delivery system.

AUTHOR CONTRIBUTIONS

Concept: *B.K., U.C.Ö., A.B.*; Design: *B.K., U.C.Ö., A.B.*; Control: *B.K., U.C.Ö.*; Sources: *B.K., U.C.Ö., A.B.*; Materials: *B.K., U.C.Ö., A.B.*; Data Collection and/or processing: *B.K., U.C.Ö., A.B.*; Analysis and/or interpretation: *B.K., U.C.Ö.*; Literature review: *B.K., U.C.Ö.*; Manuscript writing: *B.K., U.C.Ö.*; Critical review: *B.K., U.C.Ö.*, A.B.; Other: -

CONFLICT OF INTEREST

The authors declare no conflict of interest.

ETHICS COMMITTEE APPROVAL

The authors declare that this article does not require the ethical committee's approval.

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