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Development and Evaluation of Solid Witepsol Nanoparticles for Gene Delivery

Gen Aktarımı Amacıyla Katı Witepsol Nanopartiküllerin Geliştirilmesi ve Değerlendirilmesi

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ÖZ
GİRİŞ ve AMAÇ: Gen terapisi, etkili bir geleneksel tedavi yaklaşımı bulunmayan hastalıklarda kullanımı nedeniyle tıbbi, farmasötik ve biyoteknolojik sektörlerde dikkat çekmektedir. Katyonik katı lipit nanopartiküller kullanılarak yapılan viral olmayan uygulama, hedef hücrelere büyük nükleik asitlerin aktarımı için yararlı bir stratejiyi temsil etmektedir. Başarılı bir gen aktarım sistemi elde etmek için bileşenlerin ve miktarlarının dikkatli bir şekilde seçilmesi çok önemlidir. Mevcut çalışmada, katı Witepsol nanopartiküller formüle edilmiş, karakterize edilmiş ve gen aktarımı amacıyla in vitro olarak değerlendirilmiştir.
YÖNTEM ve GEREÇLER: Katı Witepsol nanopartikülleri, iki farklı sınıf Witepsol ve üç farklı yüzey aktif madde (Cremephor RH40, Kolliphor HS15 ve Peceol) kullanılarak mikroemülsiyon-dilüsyon tekniği ile formüle edilmiştir. DDAB sisteme katyonik lipit olarak dahil edilmiştir. Böylece, toplamda on iki formülasyon hazırlanmıştır. Elde edilen nanopartiküller parçacık boyutları, zeta potansiyelleri, DNA bağlanma ve koruma kabiliyetleri ile sitotoksisite ve transfeksiyon açısından değerlendirilmiştir.
BULGULAR: Hazırlanan katyonik katı lipit nanopartiküllerin (cSLN'ler) parçacık boyutlarının 13.43 ± 0.06 ile 68.80 ± 0.78 nm arasında olduğu bulunmuştur. DNA bağlanması için de önemli bir parameter olan zeta potansiyelleri +40 mV'den daha yüksek olarak belirlenmiştir. Jel geciktirme deneyi, cSLN'lerin yeşil floresan protein kodlayıcı plazmid DNA (pDNA) ile kompleksleşme yeteneğine sahip olduğunu ve bu kompleksin pDNA'yı DNaz-I aracılı parçalanmadan koruyabildiğini ortaya koymuştur. Nanopartiküllerin sitotoksisite değerlendirmesi L929 Hücre Hattı üzerinde gerçekleştirilmiştir. Ayrıca, in vitro transfeksiyon verileri, katı Witepsol nanopartiküllerinin fibroblast hücre hücrelerini in vitro olarak etkili bir şekilde transfekte edilebildiğini göstermiştir.
TARTIŞMA ve SONUÇ: Bu çalışma, mikroemülsiyon-dilüsyon tekniği ile hazırlanan katı witepsol nanopartiküllerin viral olmayan gen terapisi için umut verici sistemler olduğunu göstermektedir.

Anahtar Kelimeler: gen aktarımı, Witepsol, lipit nanopartikül, transfeksiyon, pDNA

ABSTRACT

INTRODUCTION: Gene therapy approaches continues increase its attraction in the medical, pharmaceutical and biotechnology industries due to its application for the treatment of diseases for which there no effective conventional therapy. Non-viral delivery using cationic solid lipid nanoparticles represents a useful strategy to introduce large nucleic acids to target cells. A careful selection of components and their amounts is critical to obtain a successful delivery system. In the present work, solid Witepsol nanoparticles were formulated, characterized and evaluated in vitro for gene delivery purpose.

METHODS: Solid Witepsol nanoparticles were formulated by microemulsion-dilution technique using two different Witepsol grades and three different surfactants namely, Cremephor RH40, Kolliphor HS15, and Peceol. DDAB was incorporated into the system as a cationic lipid. Twelve different combinations of these ingredients were formulated. The obtained nanoparticles were then evaluated for particle sizes, zeta potentials, DNA binding and protection ability, cytotoxicity and transfection.

RESULTS: The particle sizes of the prepared cationic solid lipid particles (cSLNs) were found to be between 13.43 ± 0.06 and 68.80 ± 0.78 nm. Their zeta potential, which is important for DNA binding efficiency, were determined higher than +40 mV. Gel retardation assay revealed that obtained cSLNs have ability to form a compaq complex with green fluorescent protein encoding plasmid DNA (pDNA) and this complex is able to protect pDNA from DNase-I mediated degradation. The cytotoxicity evaluation of nanoparticles performed on L929 cell line. Furthermore, in vitro transfection data showed that solid Witepsol nanoparticles could be effectively transfect the fibroblast cells.

DISCUSSION AND CONCLUSION: This study indicates that solid Witepsol nanoparticles prepared by microemulsion-dilution technique are promising non-viral delivery systems for gene therapy.

Keywords: gene delivery, Witepsol, solid lipid nanoparticle, transfection, pDNA

Introduction

Genetic-based treatment approaches show promise for numerous diseases from cancer to inherited illnesses. The use of naked nucleic acids is not therapeutically effective due to their hydrophilic character, anionic charges, and high molecular weights which hinder them from crossing cell membranes in the target areas [1,2]. To overcome these challenges, viral or non-viral gene delivery systems are used. Production of non-viral delivery systems that have a capacity for carrying high amounts of genetic material, low toxicity, low risk for immune responses, and easy production is one of the research areas of pharmaceutical sciences [3,4]. In recent years, lipid-based carrier systems such as liposomes, emulsions, nanostructured lipid carriers, and solid lipid nanoparticles (SLNs) gained interest as therapeutic gene carrying systems. Among these systems, SLNs come to the forefront as promising systems in the field of non-viral gene delivery by owing to the various advantages [5]. Specifically, SLNs are appropriate for large-scale production and show long-term stability. They are usually developed by using nontoxic, biodegradable lipids [6,7]. SLNs could be produced in nanosize and with cationic properties which allow them to bind nucleic acids and enhance cellular influx and/or intracellular transition due to electrostatic interactions [8]. Furthermore, modifying the size and charge of SLNs is possible by changing production strategies in accordance with the production purpose [9].

Essential components for SLN formulations are lipid, emulsifier, and aqueous phase. The melting point of lipids is usually higher than the room and body temperature. An example for lipids could be used in SLN formulation is Witepsol, mainly used in pharmaceutical research and development as excipients. A range of Witepsol grades that comprise different proportions of triglycerides, diglycerides, and monoglycerides are available to meet requirements in pharmaceutical technology, production, and also in biopharmacy. To allow a better survey of this huge variety the Witepsol grades classified in different categories such as H, W, S and E. For example, Witepsol H series lipids consist mostly of triglycerides with a proportion of, at most, 15% of diglycerides and not more than 1% of monoglycerides. So as, they are characterized by hydroxyl values up to 15 and have small gap between the melting and solidification temperatures. On the other hand, Witepsol W series lipids consist of a mixture of triglycerides (65–80%), diglycerides (10–35%), and monoglycerides (1–5%). Their hydroxyl values are between 20–50 and have a bigger difference between melting and solidification points [10,11]. As a result of the diversity in their composition, these Witepsol grades show different characteristics, thus the selection during formulation developments conduct according to these properties [11,12].

The main surfactants used in this study are Kolliphor HS15 (Macrogol 15 hydroxystearate) and Cremephor RH40 (Macrogolglycerol hydroxystearate). They are non-ionic oil-in-water solubilizer and emulsifying agents with hydrophilic-lipophilic balance (HLB) values 14–16, making it suitable for o/w emulsion formulation [11,13]. However, they exhibit different emulsifying capacity depends on the used formulation components.

In this respect, the purpose of this research was to develop stable solid Witepsol nanoparticles appropriate for gene delivery. In accordance with this aim, the microemulsion-dilution technique was employed and various formulation parameters were investigated to achieve non-toxic, biocompatible, cationic charged SLNs with optimal size and surface properties as to be used in gene delivery. Obtained SLNs were characterized and then complexed with pDNA. Protection potential against *DNase* I enzyme, cytotoxicity properties, and transfection ability was investigated. According to our findings, this study is one of the first research about the development of solid Witepsol nanoparticles by microemulsion-dilution technique for gene delivery.

MATERIALS AND METHODS

Materials

Witepsol H35 (WH35; a mixture of triglycerides (65–80%), diglycerides (10–35%), and monoglycerides (1–5%)) and Witepsol W35 (WW35; a mixture of triglycerides with a portion of, at most, 15% of diglycerides and not more than 1% of monoglycerides) were obtained from IOI Oleo GmbH (Germany). Cremophor RH40 (CRH40) and Kolliphor HS15 (KHS15) were kindly donated by BASF (Germany). Peceol (Pec) was gifted from Gattefosse (France). Dimethyldioctadecylammonium bromide (DDAB) was provided from Sigma-Aldrich Co. (USA). Ethanol was obtained from Merck-Co. (Germany). L929 murine skin fibroblast cell line and Cos-7 African green monkey fibroblast cell line were obtained from American Type Culture Collection (USA). Alamar Blue cell proliferation assay kit was provided from Thermo Fisher Scientific (USA). Agarose was obtained from Sigma-Aldrich Co. (USA). pEGFP-C1 plasmid DNA and maxiprep plasmid DNA purification kit were purchased from Invitrogen (USA). DNase I was purchased from Fermentas, Thermo Fischer Scientific (USA). Ultrapure water (UPH_2O) was used in all stages needed as water.

Plasmid DNA

The model plasmid pEGFP-C1 from Invitrogen, USA, encoding green fluorescent protein (GFP) under CMV promoter (pDNA) was used and amplified in *Escherichia coli DH5α* strain. Maxiprep plasmid DNA purification kit was performed to purify plasmid from

Invitrogen (USA). Following purification, the plasmid unity was investigated by restriction enzyme digestion and visualized by a horizontal electrophoresis system. Furthermore, the purity and the concentration of the plasmid was evaluated by measuring its absorbance at 260/280 nm wavelengths. Finally, the purified plasmid DNA was diluted to 100 µg/mL, aliquoted, and stored at -20ºC until use.

Formulation of solid Witepsol nanoparticles

The microemulsion-dilution technique was used to develop SLNs [2]. As a first step, Witepsol was used as an internal oil phase to obtain an oil in water (o/w) microemulsion system. CRH 40 / KHS15 and Pec (2:1, w:w) was used as surfactants (S), and ethanol as co-surfactant (CoS), respectively. Cationic lipid DDAB was added into the solid lipid phase to achieve cationic microemulsion [14]. SLNs prepared with the DDAB were abbreviated as "cSLN". Compositions of the designed SLNs are listed in the Table 1. All components were weighted as the total solid lipid amount is 50 mg per formulation and heated to 50 °C (10 °C above the Witepsol melting temperature). Previously warmed ultrapure water at the equivalent temperature was then added onto the lipid and S:CoS (1:1, w:w) mixture with nearly 2% of total weight increase per addition. Transparent regions were indicated on the phase diagram to determine o/w microemulsion formation area. Hereby, a microemulsion consisting of 4 % oil, 36 % S:CoS, and 60% water was selected for further studies for all developed SLNs. The acquired hot o/w microemulsion was then rapidly dispersed in 8 mL ultrapure distilled water (0−4 °C) stirring at 1000 rpm at a ratio of 1:5 (v/v). Once hot microemulsion droplets applied to cold water, SLNs were occurred. The concentration for the final SLNs was calculated to be 7.5 mg/mL for all formulations, with respect to the amounts of solid lipids.

Table 1. The investigated formulation parameter and particle characterization results of Witepsol nanoparticles.

Characterization of solid Witepsol nanoparticles

The particle size, polydispersity index (PDI), and zeta potential values of SLNs, cSLNs and cSLN:pDNA complexes with various formulation parameters were measured by Dynamic Light Scattering (DLS, Zeta sizer Nano ZS, Malvern Insturements Ltd., UK) [15]. Disposable polystirene microcuvettes were used to measured particle size and PDI for each sample. The zeta potential was measured in standard zeta cuvettes and calculated by the software using Smoluchowski equation. Measurements were repeated at least triplicate for each sample.

Stability of cationic solid Witepsol nanoparticles

The particle size and zeta potential of cSLNs were followed in terms of stability for 3 months. The measurements were performed at various time intervals by DLS (Zeta sizer Nano ZS, Malvern Instruments Ltd., UK). Samples were stored at 4 ºC in this period.

Gel retardation assay

cSLN:pDNA complexes were generated by electrostatic interactions between cationic charged nanoparticles and anionically charged pDNA [16]. The complex formation ability of pDNA with cSLN determined by 1% (w/v) agarose gel electrophoresis. The purity and concentration of the pDNA were spectrophotometrically assayed at 260 and 280 nm by Nanovette (Beckman Coulter, USA). The ratio of absorbance at 260/280 nm was measured as 1.752 (~1.8), indicating the purity of pDNA [17]. A stock solution of pDNA (100 µg/mL) was prepared in nuclease-free UPH_2O. cSLN:pDNA complexes were prepared by incubating pDNA (100 µg/mL) with cSLNs for 30 minutes at 25ºC on a benchtop shaker for increasing cSLN:pDNA ratios (0.5:1, 1:1, 2:1, 3:1 (v/v)) to provide the binding of pDNA onto cSLNs electrostatically.

Glycerol (2%) was added to each sample and electrophoresis was carried out at 100 V for 60 min. Then, ethidium bromide solution (in 500 ng/mL) was used to stain the gel. The stained pDNA bands were then monitorized under the UV transilluminator (Vilber Lourmat, France) to determine the optimal pDNA:cSLN ratio. Naked pDNA was used as a control.

***In vitro DNase* I protection assay**

The protection of pDNA via cSLNs was evaluated using *in vitro* DNase I protection study. For this purpose, *DNase* I was added to freshly prepared cSLN:pDNA systems to get 1 IU *DNase* I/2.5 µg pDNA as the final concentration and incubated at 37º C for 30 min. To terminate the enzymatic reactions and to obtain the release of pDNA, SDS (1%, v/v/) was added when the incubation time is over. The integrity of pDNA was visualized by horizontal gel electrophoresis [18].

Cytotoxicity analysis

The cytotoxicity evaluation of formulations was examined on L929 cell lines. Dulbecco's Modified Eagle's Medium supplemented with 10% fetal bovine serum (FBS) and 2 mM L-glutamine was used as culture medium. Penicillin–streptomycin (100 UI/ml penicillin, 100 µg/mL streptomycin) was added to prevent bacterial contamination.

Briefly, cells were plated in 96-well plates at a density of 5×10^3 cells per well in 100 µL medium and incubated for 24 h at 37ºC in a humidified 5% CO_2 atmosphere. Then, the medium was renewed and the cells were treated with cSLNs at increasing concentrations (225, 450, 675, 900, 1125 µg/mL, with respect to solid lipids). Following 24 h incubation period, the cells washed twice with PBS (pH=7.4) and Alamar Blue cell viability assay (Thermo Fisher Scientific, USA) was used to evaluate the proportion of viable cells. Cell viability was determined by normalizing the fluorescence of media from treated cells to untreated cells [19].

Transfection Studies

Transfection ability of developed formulations is particularly important to transport therapeutic genes into the cells. For this study, Cos-7 cells were cultured in 6-well plates at a density of 5×10^4 cells/mL and incubated until 70% confluency. The medium was then removed. Following the washing process with PBS (pH 7.4) fresh growth medium (500 µl) was instantly added so as to avoid cells from desiccation. Determined doses of cSLN:pDNA complexes (2:1, v/v) which contains 2.5 µg of pDNA were applied to the cells in accordance to cytotoxicity test results and incubated for 4 h at 37 °C in a humidified (5% CO_2) atmosphere. Following the incubation period, the medium containing SLN suspension in the wells was replaced with fresh growth medium, and incubation time was prolonged 48 h more to grow cells which would be able to provide GFP gene expression properly. Transfection capacity was observed under a fluorescence microscope (Olympus, Japan).

RESULTS AND DISCUSSION

Due to rapid progress in molecular biology and genetics, the need to use nanoparticles as applicable vehicles for the drug and gene delivery systems has increased. In this regard, the use of SLNs is of particular importance. During the last decade, SLNs have mostly experienced as drug delivery systems [20–22]. Even cationic SLNs developed by using cationic lipids have made it possible to use as gene carrying system, there is only a few literature concerning their application in gene therapy [23,24,25]. In these previous studies, relatively higher melting point solid lipids such as glyceryl behenate, behenic acid, stearic acid (melting point over 60ºC) were used as solid lipid matrix in the preparation of SLNs [9,14]. However, in this current study, Witepsol -which has a melting point around 37 ºC- has been used. The low melting point of solid lipid matrix brings several advantages. First of all, the energy requirement for large scale production has decreased. This energy conservation reduces the production cost of nanoparticular carriers and provides an advantage to enter the pharmaceutical market [23]. Furthermore, following endocytosis, the genetic material-loaded SLNs presumably encounter consecutive pH drop in the endosome and lysosome by the acidic character of the endosomal media [26]. In this step, the low melting point of Witepsol which is similar to body temperature might facilitate the release of the carried genetic material into the cytoplasm comparing high melting point lipids [2]. In this study, it was aimed to produce SLNs with the stated advantages by using Witepsol in the solid matrix. For this purpose, plotting pseudo triple phase diagrams, which is the initial process of SLN production by microemulsion dilution method, was carried out at the lipid melting temperature [27].

Preparation of pseudo-ternary phase diagrams

For the formation of the o/w microemulsion system, the HLB of the system was adjusted between 10 to 12 with the nontoxic surfactants. One of the surfactants used in these formulations is Peceol. It is a readily dispersible, solubilizing agent consist of a mixture of mono- and diglycerides of oleic acid. Since the HLB value of peceol is quite low (around 3), the HLB value of surfactant mixtures were augmented by using CRH 40 and KHS15 to allow o/w microemulsion formation. The pseudo-ternary phase diagrams constituted by titration with UPH_2O into the oil (Witepsol), S (CRH 40:Pec or KHS15:Pec, 2:1, w:w) and CoS (Ethanol) mixture are presented in Fig. 1. According to the construction of the phase diagram, the final formulations were selected providing microemulsion requirements such as being oil-in-water type and transparent as well as having high proportion of solid lipid and low proportion of S:CoS [28,29]. Since the o/w microemulsion formation areas are similar for all different formulations, the same ratios were selected to compare the effect of solid lipids (WH35/WW35) and S:CoS (CRH 40:Pec / KHS15:Pec). So as, the o/w microemulsions consisting 4 wt% solid lipid, 36 wt% S:CoS, and 60 wt% water were prepared and used for SLN preparation for all formulations.

Figure 1. Pseudo-ternary phase diagrams for different formulations SLN_1, SLN_2, SLN_3, SLN_4. Green area shows o/w microemulsion formation region.

Physicochemical characterization

To examine the particle size and zeta potential of nanoparticles, DLS measurements were performed. As shown in Table 2, the obtained solid Witepsol nanoparticles are in the nanometer size range (from 13.43 to 80.49 nm). Complex formation with pDNA increased the particle size of the system. SLNs without cationic lipids have zeta potential between -2.4 to 13.7 mV. In case of CRH 40 was used as a surfactant, the zeta potential values of nanoparticles were measured as +13.7 and +12.7 for SLN 1 and SLN 3, respectively. On the other hand, when KHS15 was used instead of CRH 40, the measured zeta potentials were decreased to -0.5 and -2.4 for SLN 2 and SLN 4, respectively. That could be attributed to the

glycerol group in the CRH 40's chemical structure. Furthermore, following the DDAB incorporation, the zeta potential of cSLNs was measured at over 40 mV for all formulations. These results were as expected due to the cationic amphiphilic character of DDAB that is commonly used in non-viral gene delivery studies [30]. Then, the final zeta potential of the formulations was decreased by complexing with pDNA but still remained higher than 30 mV for all four formulations. The obtained positive zeta potential of formulations plays an important role to achieve interaction between the negatively charged nucleic acids and cell membranes for transfection [31,32]. The PDI values of the produced SLNs increased after the DDAB was incorporated into the system as a cationic lipid. However, following the complex formation with pDNA, polydispersity of the formulation decreased. The particle sizes of the final cSLN:pDNA vector systems were measured lower than 100 nm that is necessary for i.v. injection. Also, they are small enough to provide the stability of disperse system by having Brownian motion [33].

Table 2. Characterization results of SLN formulations

Physicochemical stability of the developed cSLNs was also followed. For this aim, cSLNs were kept at 4 °C and particle size, and zeta potentials were measured at up to 90 days (Table 3). As can be seen from the measurement results, particle sizes and zeta potentials of cSLNs are stable for a period of 21 days. Particle size of the cSLNs increase dramatically within 90 days. Only the particle size of $cSLN_3$ stayed under 100 nm. Even so, it can be say that the developed formulations can be used for complexation with pDNA at least the following 21 days since their particle size are under 100 nm and zeta potential are over 40 mV [34].

Table 3. The physicochemical stability of the cSLNs at 4 °C.

Gel retardation assay

Gel retardation assay is widely used to examine the nucleic acid binding ability of cationic nanoparticles [16]. cSLNs were evaluated for complex formation ability with pDNA using agarose gel retardation assay to specify the optimal cSLN:pDNA ratio. The migration of naked pDNA and cSLN:pDNA complexes for different Witepsol nanoparticles in agarose gel is shown in Figure 2. Obtained cSLNs showed pDNA binding ability and the migration of pDNA in agarose gel was utterly stopped when the ratio of cSLN:pDNA reached 2:1 (v/v) for all cSLNs.

Figure 2. Agarose gel image of pDNA and cSLN complexes for the constant amounts of pDNA and increasing amounts of $cSLN_1$, $cSLN_2$, $cSLN_3$, or $cSLN_4$. (cSLN:pDNA ratios are 0.5:1, 1:1, 2:1, 3:1, (v/v), respectively.)

Protection ability of nanoparticles against *DNase* I degradation

The agarose gel photograph of cSLN:pDNA complexes incubated with the *DNase* I was shown in Figure 3. Lane 1 is the untreated plasmid (negative control). According to lane 2, naked pDNA was entirely digested by the *DNase* I enzyme. Lanes 3-5, indicate the protection of cSLN formulations against *DNase I* enzyme at three different cSLN:pDNA ratios (1:1, 2:1, 3:1 (v:v)). This evidence confirmed that obtained cSLNs were efficiently protected the pDNA from degradation by *DNase* I [35].

Figure 3. *DNase* I protection of cSLN:pDNA complexes. (1:Control pDNA; 2:Naked pDNA incubated with *DNase* I; 3, 4, 5: pDNA released from cSLN:pDNA complexes following incubation with *DNase* I for the cSLN:pDNA ratio 1:1, 2:1, 3:1 (v:v), respectively. (Lane A1-5 for $cSLN_1$, Lane B1-5 for $cSLN_2$, Lane C1-5 for $cSLN_3$, Lane D1-5 for $cSLN_4$).

Cytotoxicity

The cytotoxicity assay was applied to find the non-toxic doses of obtained nanoparticles before transfection. The results obtained from the Alamar Blue cytotoxicity assay on L929 cell line supported that all excipients used in this study have low cytotoxicity. Significant cytotoxicity was not determined on L929 cells in the concentration range of 225-1125 µg/mL. The applied doses showed over 70% cell viability which is considered as the minimum reference cell viability dose for further transfection studies [33]. Furthermore, concentration dependent cytotoxicity was observed for all formulations (Figure 4).

Figure 4. Viability of L929 cells exposed to cSLN formulations. (Each condition was tested at least in four replicates for 24 hours.)

Cellular uptake studies

Uptake of the Witepsol nanoparticles in Cos-7 cell line was determined using pEGFP-C1 plasmid and visually investigated under a fluorescence microscope (Figure 5). Green color shows the fluorescence signal from GFP expressed in the cells. According to the transfection results, all obtained formulations have transfection ability [36,37]. Since these nanoparticles showed efficient transfection on cell culture, it may be possible that using the developed gene delivery systems *in vivo* for different genetic based-diseases by loading disease specific therapeutic genes.

Figure 5. Images of GFP-positive cells following cSLN:pEGFP-C1 application observed by inverted fluorescent microscopy after 48 h of transfection for $cSLN_1$ (a), $cSLN_2$ (b), $cSLN_3$ (c), $cSLN_4$ (d) pEGFP-C1 complexes.

CONCLUSION

In this study, solid Witepsol nanoparticles were successfully prepared and their characterization studies were carried out. The obtained cationic nanoparticles indicated pDNA binding and protection ability. The characterization of formulations in terms of their particle size, zeta potential, PDI was analyzed and resultant nanoparticles are in the nanometer size range. Cytotoxicity results proved the suitability of nanoparticles for gene delivery. Transfection studies showed that developed nanoparticles are capable of carrying genetic materials into the cells efficiently. Consequently, it can be inferred that solid Witepsol nanoparticles are considered promising delivery vehicles for non-viral gene therapy.

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Table 1. The investigated formulation parameter and particle characterization results of Witepsol nanoparticles.

Code	**Solid Lipid**		**DDAB**	**Surfactant**		**pDNA**
	WH35	WW35		CRH40:Pec (2:1, w:w)	KHS15:Pec (2:1, w:w)	
SLN_1	+	-	-	+	-	-
SLN_2	+	-	-	-	+	-
SLN_3	-	+	-	+	-	-
SLN_4	-	+	-	-	+	-
$cSLN_1$	+	-	+	+	-	-
$cSLN_2$	+	-	+	-	+	-
$cSLN_3$	-	+	+	+	-	-
$cSLN_4$	-	+	+	-	+	-
*$cSLN_1$:pDNA	+	-	+	+	-	+
*$cSLN_2$:pDNA	+	-	+	-	+	+
*$cSLN_3$:pDNA	-	+	+	+	-	+
*$cSLN_4$:pDNA	-	+	+	-	+	+

*cSLN:pDNA, cSLN formulations complexed with pDNA

Table 2. Particle size, PDI and Zeta potential values of SLN formulations

Code	**Particle Size (nm ± SD)**	**PDI**	**ZP (mV± SD)**
SLN_1	23.84 ± 0,06	0.08	13.7 ± 0.6
SLN_2	61.90 ± 0,39	0.13	-0.5 ± 0.5
SLN_3	23.42 ± 0,01	0.04	12.7 ± 4.7
SLN_4	47.87 ± 0,28	0.19	-2.4 ± 0.5
$cSLN_1$	13.43 ± 0,06	0.62	43.8 ± 1.8
$cSLN_2$	23.45 ± 1,60	0.91	47.7 ± 0.7
$cSLN_3$	37.57 ± 0,49	1.00	42.2 ± 2.7
$cSLN_4$	68.80 ± 0,78	0.36	41.5 ± 1.7
*$cSLN_1$:pDNA	54.44 ± 0,54	0.60	32.4 ± 2.5
*$cSLN_2$:pDNA	80.49 ± 1,29	0.52	41.8 ± 3.2
*$cSLN_3$:pDNA	37.82 ± 0,09	0.61	34.0 ± 1.6
*$cSLN_4$:pDNA	69.50 ± 0,45	0.57	34.4 ± 8.8

SD, standard deviation. (n=3).

Table 3. The physico-chemical stability of the cSLNs at 4 °C.

Code	**Particle Size (nm ± SD)**				
	Day 0	**Day 7**	**Day 14**	**Day 21**	**Day 90**
$cSLN_1$	13.43 ± 0.06	10.96 ± 0,33	10.02 ± 0.18	12.68 ± 0.12	125.0 ± 44.52
$cSLN_2$	23.45 ± 1.60	22.83 ± 0,25	21.49 ± 1.37	22.34 ± 0.33	109.1 ± 26.94
$cSLN_3$	37.57 ± 0.49	35.49 ± 3.23	39.26 ± 8.31	36.71 ± 2.25	73.58 ± 3.55
$cSLN_4$	68.80 ± 0.78	73.46 ± 9,56	77.30 ± 3.63	70.45 ± 0,39	453.0 ± 43.20
Code	**Zeta Potential (mV± SD)**				
	Day 0	**Day 7**	**Day 14**	**Day 21**	**Day 90**
$cSLN_1$	43.8 ± 1.8	37.0 ± 0.8	37.0 ± 0.7	37.8 ± 4.30	43.9 ± 1.3
$cSLN_2$	47.7 ± 0.7	40.0 ± 10.0	40.0 ± 10.0	43.5 ± 0.49	42.7 ± 1.8
$cSLN_3$	42.2 ± 2.7	40.5 ± 0.3	40.5 ± 0.3	41.0 ± 4.17	42.0 ± 1.5
$cSLN_4$	41.5 ± 1.7	44.6 ± 2.3	44.6 ± 2.3	44.5 ± 2.48	44.7 ±2.4

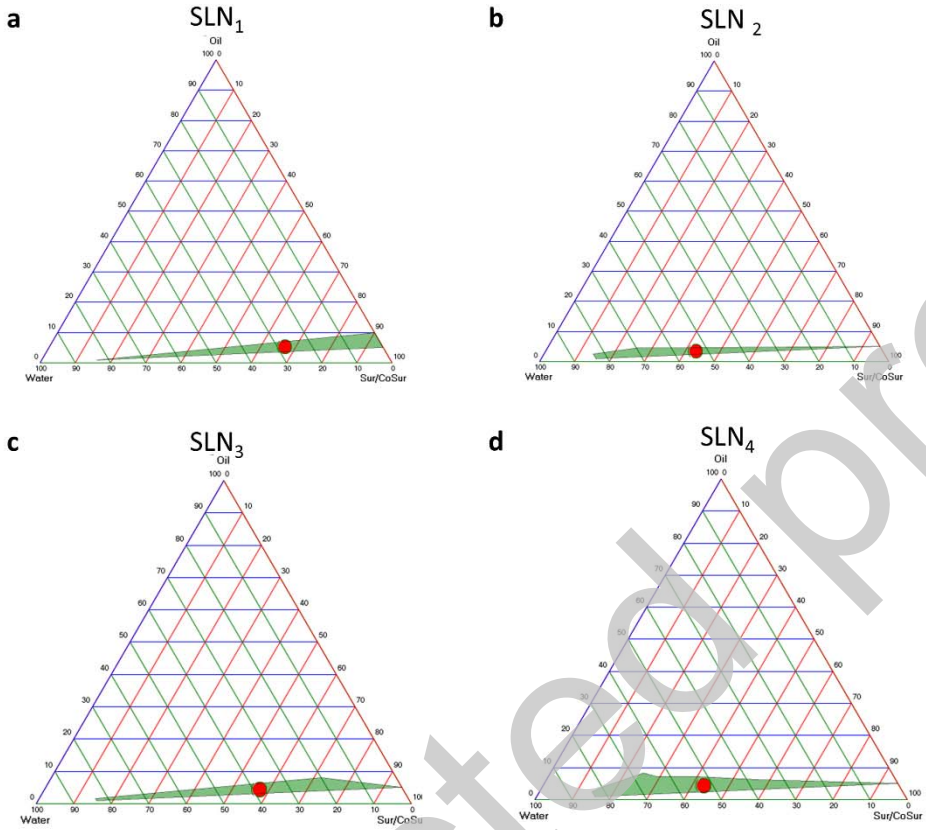

Figure 1. Pseudo-ternary phase diagrams for different formulations SLN_1, SLN_2, SLN_3, SLN_4. Green area represents transparent w/o microemulsion formation region.

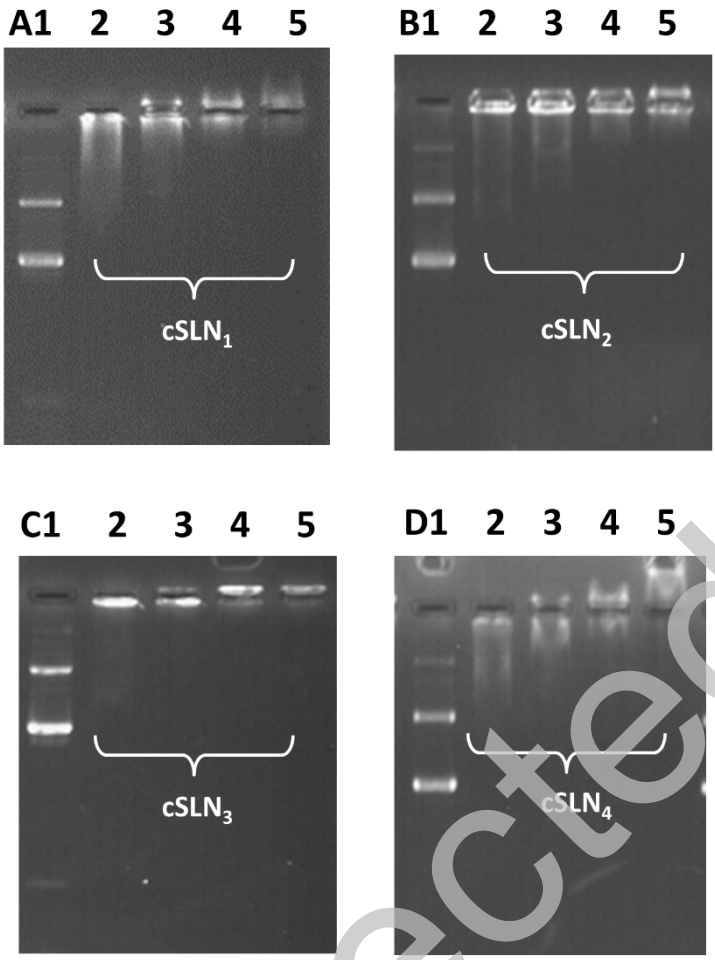

Figure 2. Agarose gel photograph of complexes containing constant amounts of pDNA and increasing amounts of $cSLN_1$, $cSLN_2$, $cSLN_3$, or $cSLN_4$ for ratios of 0.5:1, 1:1, 2:1, 3:1, v/v.

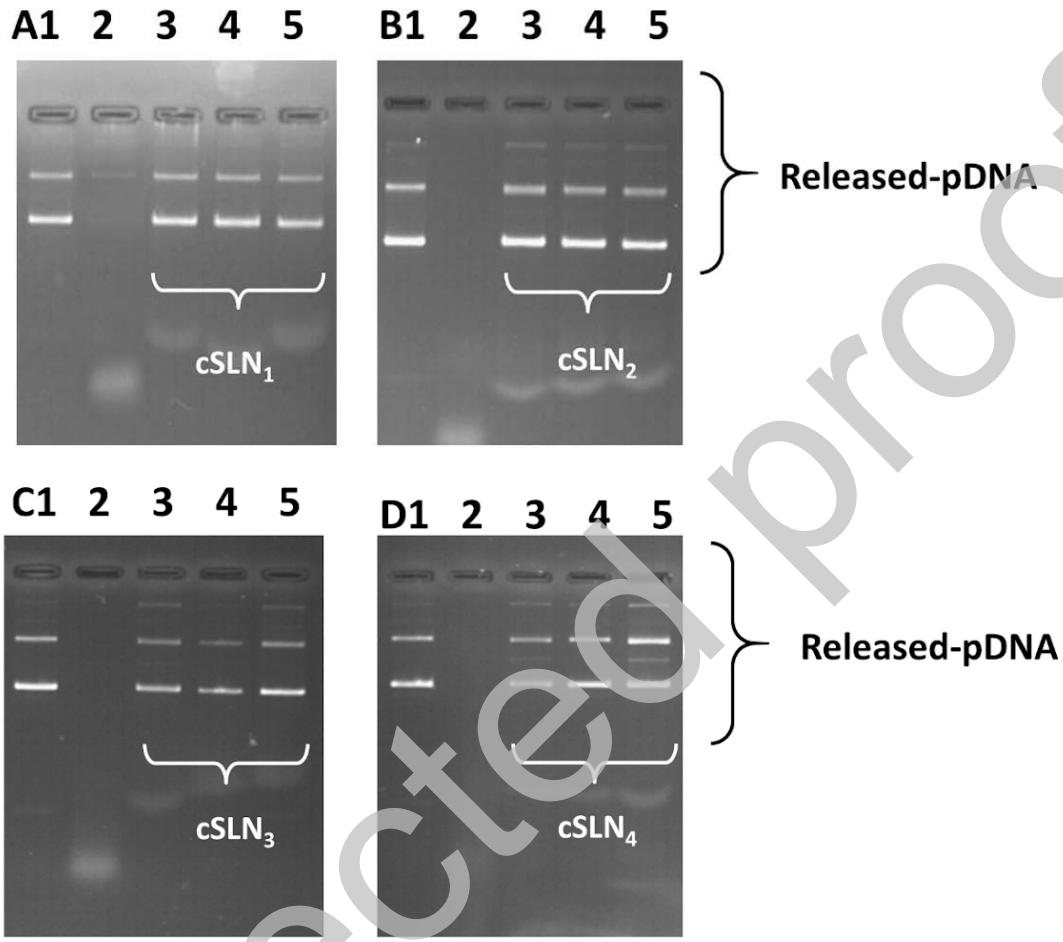

Figure 3. *DNase* I protection of cSLN:pDNA complexes. (1:Control pDNA; 2:Naked pDNA incubated with *DNase* I; 3, 4, 5: pDNA released from cSLN:pDNA complexes following incubation with *DNase* I for the cSLN:pDNA ratio 1:1, 2:1, 3:1 (v:v), respectively. (Lane A1-5 for $cSLN_1$, Lane B1-5 for $cSLN_2$, Lane C1-5 for $cSLN_3$, Lane D1-5 for $cSLN_4$).

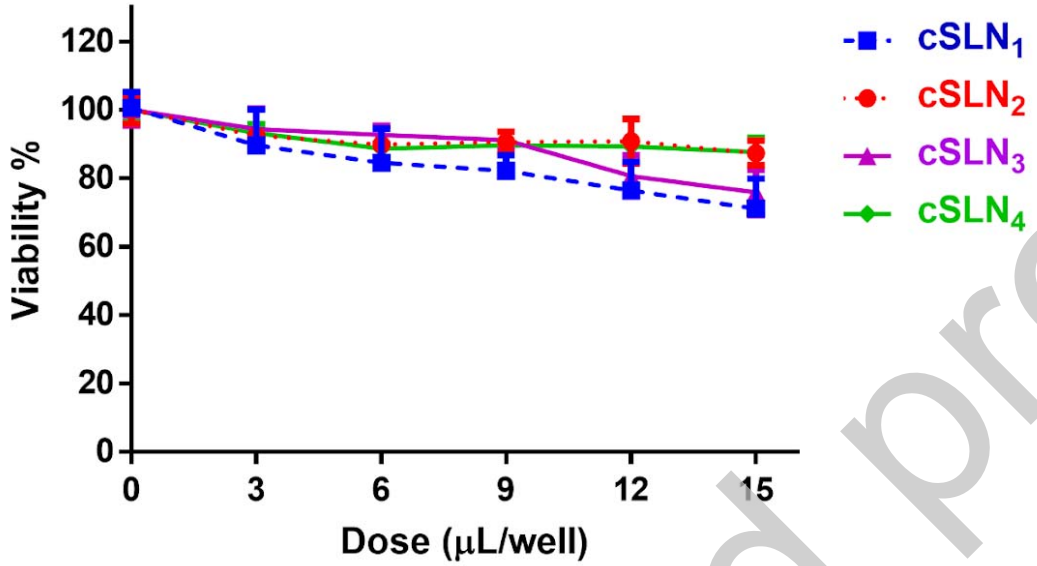

Figure 4. Viability of L929 cells exposed to cSLN formulations. (Each condition was tested at least in four replicates for 24 hours.)

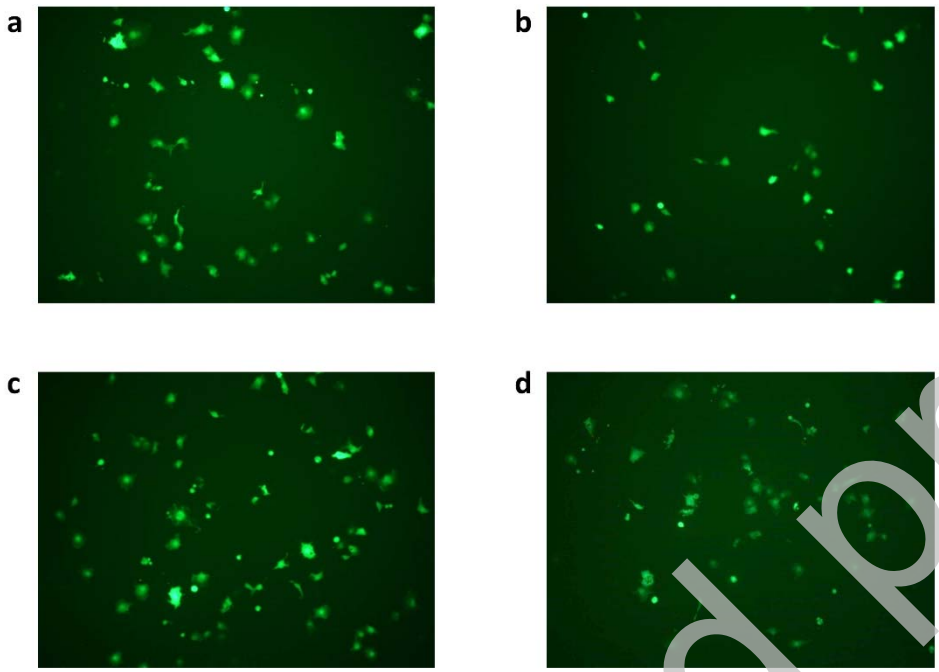

Figure 5. Images of GFP-positive cells following cSLN.pEGFP-C1 application observed by inverted fluorescent microscopy after 48 h of transfection for $cSLN_1$ (a), $cSLN_2$ (b), $cSLN_3$ (c), $cSLN_4$ (d) pEGFP-C1 complexes.