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Solid lipid and chitosan particulate systems for delivery of siRNA

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Due to the recent advances in molecular biology, there are promising gene therapy studies for prevention and treatment of cancer, genetic and infectious diseases. Many technologies in molecular biology and biotechnology were developed, and among those technologies, 'antisense technology' has become prominent in recent years. In this study, non-viral gene delivery systems such as solid lipid and chitosan nanoparticles were developed for improving intercellular delivery of siRNA. Commercially available Bcl-2 siRNA which is specific for Bcl-2 mRNA was used as a genetic material. Particle size, zeta potential, siRNA binding abilities and cytotoxic properties of the systems were evaluated and transfection assay was performed on among the prepared formulations. When the results of those studies were compared with Lipofectamine® 2000, prepared formulations were found to show usable results. A novel method was developed in this study for producing solid lipid nanoparticles (SLNs) with highly efficient siRNA encapsulation. The results of this study showed that the genetic materials can be encapsulated in SLNs and SLNs have the potential to be used as a transfection agents.

1. Introduction

Antisense technology is a well-equipped technique for functional genomic studies, along with its feasibility in the discovery of human, animal and plant diseases and specific treatments for disease. There is a post-transcriptional gene silencing mechanism in this method, i.e., RNA interference (RNAi) performs a gene silencing process with double-stranded RNA that is connected to the messenger RNA, causing destruction following the passage from the nucleus to the cytoplasm of messenger RNA. Thus, inhibition of protein production,or transcription and translation, is maintained. Approximately 21-23 nucleotide-long small interference RNA (siRNA) or small silencer RNA playa major role in this technology (Zhang et al. 2007). Among the various methods for siRNA transfer into mammalian cells, electroporation, injection of naked siRNA to the cell nucleus or cytoplasm (Li et al. 2006), or transfection of plasmids and viruses can produce siRNA (Takabatake et al. 2007).

Solid lipid nanoparticles (SLNs) were introduced in the early 1990's as an alternative to traditional delivery systems such as emulsions, liposomes, and polymeric systems (Souto et al. 2004; Chime and Onyishi 2013). SLNs are nano-sized particles prepared by using lipids which are solid at room and body temperatures and can be stabilized with surfactants. Triglycerides (tristearin, etc), fatty acids (stearic acid, etc), steroids (cholesterol, etc), and waxes (cetyl palmitate, etc) are examples of the solid lipids used (Saupe and Rade 2006). SLNs can be prepared by high pressure homogenization (hot or cold), microemulsion, solvent emulsification-evaporation, emulsification-diffusion and high speed stirring, and ultrasonication methods (Chaturvedi and Kumar 2012).

Chito-oligosaccharide, the product of partial hydrolysis of chitosan, has antimicrobial, antioxidant, anti-inflammatory, and anti-tumor activities (Park and Kim 2010). The high cationic character of chitosan results in the capability of electrostatic interaction with negatively charged molecules such as DNA and RNA (Alves and Mano 2008).

The objective of this study was to formulate low toxic, biodegradable, and biocompatible lipid and polymeric delivery systems to protect genetic material from enzymatic degradation and also can easily pass into the cell. According to our results, cSLNs have the appropriate particle size and zeta potential for transfection assay. Results of transfection studies showed that cSLN's are a suitable material for efficient delivery of siRNA.

2. Investigations, results and discussion

siRNA delivery systems, which can be applied both systemically and locally in different diseases, have gained significant importance (Oh and Park 2009). Issues such as particle size, zeta potential, stability of siRNA-loaded system, ability to transport genetic material to cell cytoplasm, as well as release in the cytoplasm for interaction of RISC and siRNA, should be taken into consideration in the development of siRNA delivery systems (Aigner 2007; Howard 2009).

2.1. Characterization of SLNs

2.1.1. Particle size distribution and zeta potential

It is known that SLNs can deliver genetic material but there is only a limited number of studies on SLN and siRNA (Kim et al. 2008). Preparation of SLN using sonication method is

Table 1: Ratios of formulations (n = 3)

Formulations	Composition (% w/w) SLN Gelucire/Span + Tween/Oct	Chitosan	High Speed Homogenisation (HSH) or Sonication (1min)	Filtration/ Dialysis	Particle size (nm) ± SE	PI ± SE	Zeta potential (mV) ± SE
G0	4/4/-	—	HSH	+/-	52.04 ± 1.58	0.28 ± 0.05	−20.60 ± 0.90
G1	4/4/0.0125	—	HSH	+/-	55.16 ± 3.95	0.32 ± 0.10	−6.15 ± 0.31
G2	**4/4/0.125**	**—**	HSH	**+/-**	**117.43 ± 0.87**	**0.26 ± 0.02**	**11.10 ± 0.28**
G3	4/4/0.25	—	HSH	+/-	224.80 ± 3.79	0.45 ± 0.03	16.43 ± 0.30
G4	4/4/0.5	—	HSH	+/-	267.20 ± 4.52	0.30 ± 0.04	16.37 ± 0.24
G5	4/4/1.25	—	HSH	+/-	388.90 ± 4.64	0.44 ± 0.07	17.19 ± 0.27
S1	2/4/-	—	Son.	+/+	179.60 ± 2.12	0.30 ± 0.02	−18.6 ± 0.10
S2	2/4/0.250	—	Son.	+/+	224.30 ± 3.54	0.32 ± 0.03	12.40 ± 0.21
S3	4/4/-	—	Son.	+/+	150.20 ± 1.02	0.29 ± 0.04	−21.67 ± 0.41
S4	4/4/0.125	—	Son.	+/+	208.10 ± 1.23	0.33 ± 0.04	10.60 ± 0.24
S5	**4/4/0.250**	**—**	Son.	**+/+**	**137.03 ± 2.24**	**0.31 ± 0.03**	**25.93 ± 0.49**
K1	—	4 (1 kDa)	—	—	159.23 ± 0.59	0.7 ± 0.028	7.90 ± 0.17
K2	—	4 (1-3 kDa)	—	—	88.07 ± 1.43	0.65 ± 0.07	11.27 ± 0.14
K3	—	4 (3-5 kDa)	—	—	70.35 ± 2.30	0.47 ± 0.07	9.60 ± 0.29
K4	**—**	**4 (10 kDa)**	**—**	**—**	**207.07 ± 2.79**	**0.25 ± 0.09**	**42.07 ± 1.28**

widely used. It was shown that an increase in sonication time decreases particle size. In a study carried out by Zhu et al. (2011), particle sizes of about 300-350 nm were obtained at the end of the 1.5-min sonication period applied to DNA-loaded SLN/PLGA nanoparticles. However, it was further reported that long sonication time applied to decrease particle size and polydispersity index causes metal contamination (Mehnert and Mäder 2001). Based on the results of previous studies, it was decided in this study that sonication time should be 1 min at 20 % amplitude for an efficient encapsulation process in which the genetic material is not damaged. Increase in lipid concentration increases particle size (Olbrich and Müller 1999). Addition of octadecylamine to Gelucire® 50/13 formulations prepared in this study increased particle sizes of all formulations except the S5 formulation (Table 1). One and 2 min-sonication were applied for S1, S3 and S4 formulations, and similar particle sized dispersions were obtained. In addition, a particle size increase was observed for S2, while there was a decrease for S5 formulation after 2 min sonication (data not shown). As a result, it was determined that the sonication time does not have a significant impact on particle size.

SLN dispersions which do not include a cationic agent were found to be negatively charged (Padhye and Nagarsenker 2013). An increase in octadecylamine amount of the formulations prepared resulted in an increase of zeta potential value. This increase was observed for all formulations (Table 1). Zeta potentials of formulations containing the same amounts of cationic agent and prepared using two different methods were found to be very close to each other. However, when G3 formulation containing the same amounts of lipid and octadecylamine were compared with S5, it was observed that S5 had a higher zeta potential. It was hypothesized that reduction in particles leads to an increase in zeta potential.

A variety of solid lipidic compounds have been used in the preparation of SLNs, including stearic acide, monostearin, Compritol 888 ATO, cetylpalmitate, Precirol ATO 5, glyceryl trioleate, cholesterol, soya lecithin, and glyceryl monostearate. The choice of the lipid is very important for the gene delivery properties of the SLN formulation. Moreover, the choice of the suitable method to prepare SLNs should be also considered. Issues such as size and stability of drugs during the production process may be affected by composition. In a study carried out by Montana et al. (2007) the cationic SLNs produced by microemulsion using Compritol ATO 888 as matrix lipid and dimethyldioctadecylammonium bromide (DDAB) as cationic lipid, as *Paracentrotus liVidus bep3* RNA carriers. In another study (Kim et al. 2008), SLNs composed of cholesteryl ester, triglyceride, cholesterol, dioleoylphosphatidylethanolamine (DOPE), and 3-beta-[N-(N′,N′-dimethylaminoethane) carbamoyl]-cholesterol (DC-chol) was prepared using a modified solvent-emulsification method and was provided interaction electrostatically siRNA conjugated polyethylene glycol (PEG). A similar study was done by He et al. (2013). They prepared solid lipid nanoparticles by solvent diffusion method as DNA delivery system containing monostearin/octadecylamine/protamine and DNA adsorption was achieved. Although the method of preparation and amounts of excipients were different, the results obtained were close to our study for particle size, zeta potential, and cytotoxicity.

2.2. Characterization of chitosan particles

2.2.1. Particle size distribution and zeta potential

It was determined in studies carried out with chitosan particles that the molecular weight of chitosan, degree of acetylation, preparation method, and nucleic acid/chitosan molar ratio affect particle size (DeMartimprey et al. 2009). It was found that particle size increases with the increase in molecular weight of chitosan. Particle size of formulations prepared using different molecular weight chitosan (1 kDa, 1-3 kDa, 3-5 kDa and 10 kDa) varied between 70 nm and 207 nm (Table 1). It is well known that particle size should be in the nanometer range in order to increase the efficiency of drug delivery systems (Park and Kim 2010) and cellular uptake *via* endocytosis (Choi et al. 2006). Particle sizes of all formulations prepared using chitosan in this study seem to be appropriate for efficient delivery.

Zeta potential values of all chitosan formulations prepared were determined to be positive due to the natural cationic property of chitosan (Alves and Mano 2008). It was determined that K4 formulation showed the highest zeta potential, while K1 formulation had the lowest value (Table 1).

2.3. pH Value of dispersions

Studies have noted that the transfection efficiency at pH 6.9 is greater than that of pH 7.5 when they investigated transfection efficiency using varying pH values (6.9-7.5) in A549 cells (Sato et al. 2001). In our study, it was determined that pH values of SLN formulations prepared were very close to each other and

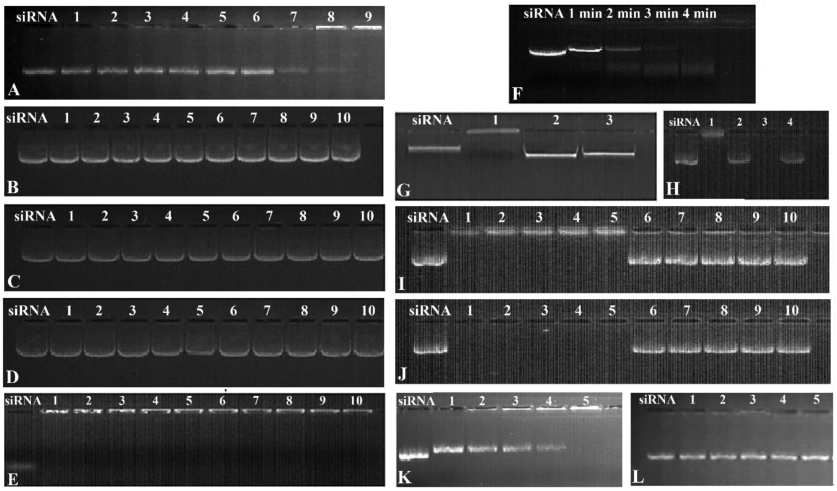

Fig. 1: Agarose gel electrophoresis results of all formulations and siRNA**, **A;** siRNA binding ratios of G2 formulation (siRNA/G2) 1:1, 1:2, 1:3, 1:4, 1:5, 1:7.5, 1:10, 1:15, 1:20 respectively (1-9), **B;** siRNA binding ratios of K1 formulation (siRNA/K1) 1:1, 1:2, 1:3, 1:4, 1:5, 1:7.5, 1:10, 1:15, 1:20 and 1:25 respectively (1-10), **C;** siRNA binding ratios of K2 formulation (siRNA/K2) 1:1, 1:2, 1:3, 1:4, 1:5, 1:7.5, 1:10, 1:15, 1:20 and 1:25 respectively (1-10), **D;** siRNA binding ratios of K3 formulation (siRNA/K3) 1:1, 1:2, 1:3, 1:4, 1:5, 1:7.5, 1:10, 1:15, 1:20 and 1:25 respectively (1-10), **E;** siRNA binding ratios of K4 formulation (siRNA/K4) 1:1, 1:2, 1:3, 1:4, 1:5, 1:7.5, 1:10, 1:15, 1:20 and 1:25 respectively, **F;** Extracted naked siRNA in distilled water and organic solvent after sonication times respectively, 1 min, 2 min, 3 min, 4 min., **G;** (1) G2 formulation, (2) siRNA after extraction of siG2* formulation, (3) extraction process applied on naked siRNA, **H;** (1) K4 formulation and (2) siRNA after extraction of siK4* formulation, (3) S5 formulation and (4) siRNA after extraction of siS5* formulation, **I;** Serum stability of siRNA before extraction from siK4* formulations (1-5) after extraction from siK4* formulations (6-10) for 1 hour, 4 hours, 10 hours, 24 hours and 48 hours respectively with 10 % FBS containing DMEM, **J;** Serum stability of siRNA before extraction from siS5* formulations (1-5) after extraction from siS5* formulations (1-6) for 1 hour, 4 hours, 10 hours, 24 hours and 48 hours respectively with 10 % FBS containing DMEM, **K;** Serum stability of siRNA before extraction from siG2* formulations (5-1) for 1 hour, 4 hours, 10 hours, 24 hours and 48 hours respectively with 10 % FBS containing DMEM, **L;** After extraction from siG2* formulations (1-5) for 1 hour, 4 hours, 10 hours, 24 hours and 48 hours respectively with 10 % FBS containing DMEM, **3 μg naked siRNA was applied into the first well as a control and the formulations which includes about 3 μg was used.

the average pH value was 6.1. The average pH was determined to be 5.5 for chitosan formulations.

2.4. Gel retardation of SLN and chitosan particles

Sonication intensity and duration may cause breakage in genetic material (Gvili et al. 2006). For this purpose, the sonication process was applied on the organic solvent containing genetic material without solid lipid and surface active material as well as on genetic material in distilled water. When the integrity of siRNA was examined, based on experiments conducted challenging the time at 20% amplitude, damage of siRNA was determined after the 2nd min (Fig. 1F). As a result, it was decided to use 1 min. and 20 % amplitude for preparing the formulations. Encapsulation of all siRNA can be possible, however, there is also a possibility of existence of free siRNA in aqueous phase (non-encapsulated) which can be adsorbed by positively charged particles. The fact that there was no smear in the siRNA band extracted from siS5* showed that the genetic material was not damaged during preparation of formulation. Meanwhile, siS5* formulation, which was applied directly to the well, remained constant (Fig. 1H).

It was reported that small particle size increases stability and decreases toxicity (Heydenreich et al. 2003). Among the SLN formulations prepared, G2 was selected to load the genetic material due to its lowest particle size and positive zeta potential. siRNA binding ratios of G2 formulation were determined using gel documentation. siRNA and G2 were observed on the gel after leaving it for incubation at room temperature for 30 min in order to attain electrostatic interaction. It was observed that the binding ratio increased gradually as the formulation ratio increased, starting from 1:10 ratio (siRNA-G2 μg/μL) (Figure 1A). Various systems were prepared in studies using solid lipids such as DOTAP, cholesterol, lesitin, and stearic acid in order to bind siRNA. Increasing ratios of those systems to siRNA amount showed that siRNA can bind to a higher extent in the nanoparticulate complex (Yuan et al. 2008; Jacobson et al. 2010). The results obtained for G2 in this study are in accordance with those in the literature.

Since a ratio of 1:15 was used in transfection studies, the extraction process was applied keeping this value in mind. In addition, the same process was carried out on naked siRNA in order to determine the effect of extraction process on siRNA. It was determined that intact siRNA in the third well, which contained the same ratio of siRNA, was not affected from the extraction process, and siRNA extracted from siG2* in the third well was obtained without a certain loss (Fig. 1G).

It was stated that the genetic material binding ratios of low molecular weight chitosan decreased due to their low number of free amino groups (Choi et al. 2006). Molecular weight of chitosan used in K4 is higher than other chitosan. When siRNA binding was tested for K1, K2, K3, and K4 (Fig. 1B, C and D), it was observed that siRNA was bound effectively only in K4 formulation (Figure 1F). Those ratios increased from 1:50 to 1:300 for K1, K2 and K3 and siRNA could be bound completely only at 1:300 ratio of K3 (data not shown). Formulation applied to cells would increase in cell culture studies and therefore, K1, K2 and K3 formulations were not evaluated; only K4 formulation was selected for cell culture studies.

Fernandes et al. (2012) have shown that the siRNAs could not be kept with low MW chitosan particles. Unlike this result, our Bcl-2 siRNA was successfully held according to our gel retardation study.

2.5. Stability in serum

Various lipases, nucleases, and high density lipoproteins inside the serum can interact with the delivery systems. These substances may lead to release of lipid content in the lipidic delivery systems (Rao 2010). It was determined that lipidic siS5* protected the genetic material throughout the study (Fig. 1I), and the genetic material adsorbed by siG2* and siK4*

were not damaged in the existence of the serum (Fig. 1 J,K and L). However, this was not valid for siG2* since it was observed that siG2* started releasing the genetic material at the end of the 4th hour (Fig. 1K). Comparing the stability of intact siRNA and encapsulated siRNA, it was reported that encapsulated siRNA is more resistant to serum degradation (Gary et al. 2007). Similar results were obtained for siRNA encapsulated in siS5* with complete protection.

2.6. Particle size and zeta potentials of siRNA-loaded formulations

Lipidic formulations are generally uptaken by endocytosis to enter the cells. The literature also states that while complexes with particle sizes less than 300 nm were uptaken by clathrin-mediated endocytosis, complexes with sizes above 500 nm are uptaken by caveolar endocytosis (Rejman et al. 2004). Accordingly, particle size of the genetic material/formulation to be used for transfection is important. Gradual increase in particle sizes of siG2* and siK4* formulations were observed by keeping the siRNA ratio constant but increasing the formulation amount (Table 2). This may be due to siRNA adsorbed on the particle surface holding other particles together, as well as adsorbing on the surface of other particles and thus leading to the movement of several particles together. A significant increase was not observed in particle size, owing to siRNA encapsulated in siS5* and no possibility of contact with other particles as in siG2* and siK4*. Thus, the obtained particle size has been found to be suitable for transfection.

The genetic material/delivery system components should have a net positive charge, to provide interaction with the negatively charged cell membranes (Reynolds 2007). No significant change was observed in the zeta potential values of siG2* and siS5* as a result of adsorption and encapsulation of siRNA. There was a zeta potential decrease of approximately 50 % in 1:1 ratio of siK4* (Table 2). As a result, it was determined that siG2* with 1:15 ratio, siS5*, and siK4* with 1:2 ratio were at appropriate size for transfection and have net positive charges to interact with the cell membrane.

2.7. Cytotoxicity of siRNA-loaded formulations

It is important that the formulations developed have low toxicity on body cells. It was reported that the superior character of SLN is its non-cytotoxic property (Yuan et al. 2008; Subedi et al. 2009). It is known that Bcl-2 gene reduces the death of cells. Among the objectives of this study is the transfection of genetic material for silencing Bcl-2 gene, using the formulations prepared. It is expected that Bcl-2 mRNA is silenced after transfection, and thus the Bcl-2 protein production is reduced and leads to cell death. It is important that the carrier systems are not toxic for the cell. A549 and MCF-7 cancer cells were used in cytotoxicity studies. Cell viabilities of G2 formulation at the lowest concentration at the end of 24 and 48 h were determined to be 82 % (IC_{50}: 11.8 μL/100 μL) and 93 % (IC_{50}: 5.7 μL/100 μL), respectively. S5 formulation demonstrated 98 % cell viability (IC_{50}: 1.20 μL/100 μL) in 24 h at the lowest concentration, while this rate decreased to 83 % (IC_{50}: 0.90 μL/100 μL) at the end of 48 h (Fig. 2). The reason for the difference in cytotoxicity and the decrease in cell viability of G2 and S5 formulations containing the same amounts of solid lipid, cationic agent, and emulsifier may be attributed to the release of octadecylamine following degradation of particles after uptake. Those results suggested the higher cellular intake of S5 formulation than G2. Comparing the cytotoxicity results

Table 2: siRNA/formulations ratios and results of particle size / zeta potential (n = 3)

Ratios	G2	siRNA/G2			S5	siRNA/S5	K4	siRNA/K4		
		1:15	1:20	1:25		62.5 μg/mL		1:1	1:2	1:3
Particle size (nm) ± SE	117.43 ± 0.87	125.90 ± 1.06	189.83 ± 3.68	230.75 ± 6.78	137.03 ± 2.24	158.63 ± 5.50	207.07 ± 2.79	214.70 ± 1.67	260.30 ± 4.30	298.50 ± 8.30
Zeta Potential (mV) ± SE	11.10 ± 0.28	12.13 ± 0.18	12.01 ± 0.49	11.95 ± 0.53	25.93 ± 0.28	24.10 ± 0.09	42.07 ± 1.28	23.30 ± 0.36	40.03 ± 0.78	41.20 ± 2.15

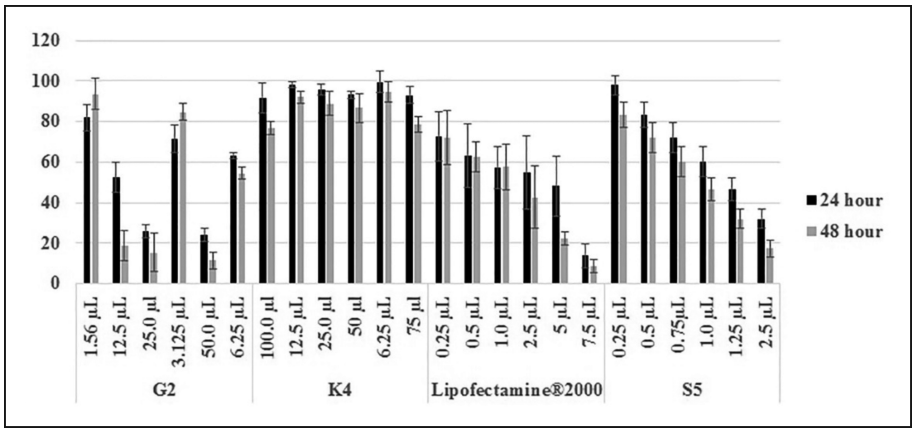

Fig. 2: Effect of Lipofectamin®2000, S5, G2 and K4 formulations of the cell viability on human A549 lung carcinoma cells for the indicated times and doses. Mean (I) SE of triplicate independent experiments.

of S5 and Lipofectamine®2000, S5 showed lower cytotoxicity at low concentrations (0.25, 0.5 and 1 µL/100 µL).
Cell viability of K4 did not decrease below 90 % at the end of 24 h for all concentrations, while it was determined to be 76 % at the highest concentration at the end of 48 h (Fig. 2). The data in the literature reveals the non-toxic character of chitosan (Kumar et al. 2004; Venkatesan et al. 2010). Hence, chitosan formulations are superior to SLNs in terms of cytotoxicity. It was reported that cationic polymers with high zeta potential values have a lithic effect on cells, and were therefore identified as cytotoxic (Jiang et al. 2009).

2.8. Transfection

Cationic SLNs can be formulated for gene transfer using cationic lipids just like liposomal transfection agents. When structural and performance similarities and differences between solid lipids and liposomes were examined, particle size was found to be smaller in comparison to liposomes, and differences were seen in genetic material binding. Lipofectamine® 2000 is a cationic liposomal agent that provides high levels of *in vitro* transfection in a wide range of mammalian cell types (Dalby et al. 2004). Lipofectamine® 2000 was therefore used as a control in this study to compare the efficiencies of formulations prepared.
Transfection studies were performed on A549 and MCF-7 cells with siS5*, siG2* with 1:15 ratio and siK4* with 1:2 ratio. FITC-conjugated siRNA was replaced by Bcl-2 siRNA at the end of transfection in order to obtain radiation. Radiations in all formulations were not observed using fluorescent microscopy for siG2* with 1:15 ratio. It was determined in the previous serum stability study that siG2* released siRNA after a certain time (Fig. 1K). Since serum added to the medium at the end of 6 h during the transfection study caused a loss in electrostatic interaction between G2 formulation,and siRNA and genetic material was released in the medium, it was decided that transfection did not occur (Fig. 3).
According to the transfection results obtained on A549 cells by counting the cells at the end of 24 and 48 h, transfection indices were found to be 47 % and 45 %, 68 %, and 62 %; and 7 % and 10 %, for siS5*, Lipofectamine® 2000, and siK4* with 1:2 ratio, respectively. When siS5*, Lipofectamine® 2000, and siK4* with 1:2 ratio were evaluated statistically, there was no significant difference between Lipofectamine® 2000 and siS5* ($p > 0.05$) at the 24th hour, while a statistically significant difference was

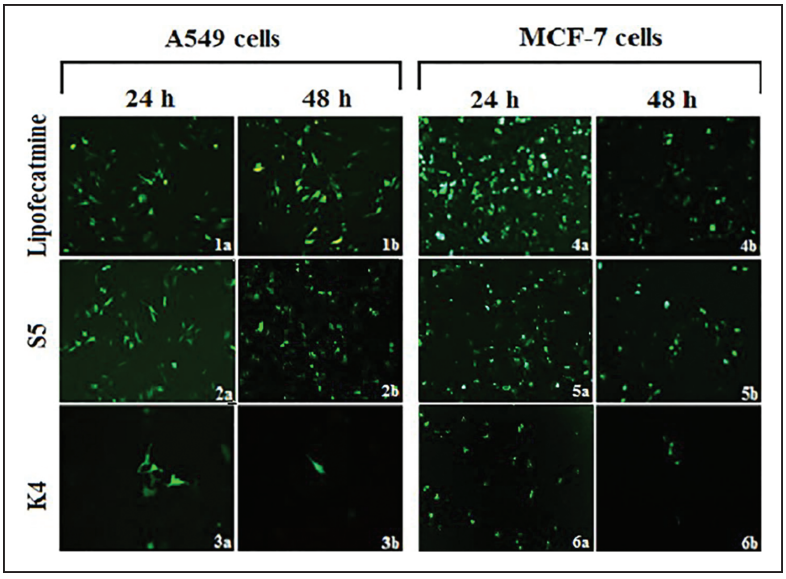

Fig. 3: Fluorescent images demonstrating of siRNA delivery to the A549 and MCF-7 cells with Lipofectamine ®2000(1a-b, 4a-b), siS5* formulation (2a-b, 5a-b) and siK4* formulation (3a-b, 6a-b).

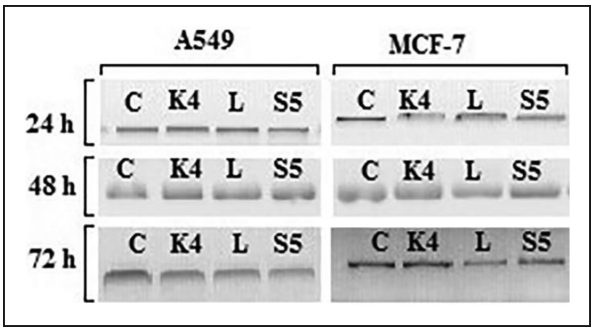

Fig. 4: Western blot analysis of Bcl-2 protein levels of C (Control), L (Lipofectamine® 2000), K4 (siK4* formulation and S5 (siS5* formulation.) were performed on A549 and MCF-7 cells for 24 h, 48 h or 72 h time points.

found between siK4* with 1:2 ratio and Lipofectamine® 2000 and siS5* ($p \leq 0.001$). When the transfection results of the 48th hour were compared, a statistically significant difference was determined between all formulations ($p \leq 0.001$). Counting the MCF-7 cells after 24 and 48 h, transfection indices were determined to be 51 % and 44 % for siS5*, 72 % and 65 % for Lipofectamine® 2000, and 9 % and 5 % for siK4* with 1:2 ratio, respectively. The same statistical results were obtained for A549 cells.

2.9. SDS and western blot

Bcl-2 is strongly expressed in many tumors such as lung, non-Hodgkin lymphoma, and prostate cancer. Strong antitumor effect of the cationic liposome containing siRNA was seen against Bcl-2 in rat models with liver metastasis (Yano et al. 2004). Feng-Fu et al. (2005) showed that Bcl-2 siRNA complex with Lipofectamine® 2000 inhibited Bcl-2 protein at a ratio of 66.5 % on human HelaB2 and BGC-823 cells and rat liver tumors. It was determined in a study with siRNA of the Bcl-2 gene that Bcl-2 siRNA displays high inhibition on gastric cancer cells, depending on both mRNA and protein level, based on time and dose (Hao et al. 2007).

According to the western blot results, siS5* displayed a similar effectiveness to Lipofectamine® 2000 in protein decrease at the 48 and 72-h bands. It was determined that siK4* with 1:2 ratio was not effective in protein decrease, and on the contrary, the results were similar to the control protein amount at the end of the 72nd hour (Fig. 4).

Since the beginning of the 1990 s, the cationic solid lipid nanoparticles (cSLN) have been studied various research groups as an alternative to polymeric nanoparticles. Despite the use of solid lipids as a matrix material for DNA delivery is most common, for the siRNA it is not. Despite similarities, delivery of siRNA also faces distinct challenges due to apparent differences in size, stability, location and mechanism of action. For example, attempts to use commercial cationic lipids with high *in vitro* transfection efficiency such as Lipofectamine® 2000 failed *in vivo* because of the cytotoxic effect due to high zeta potential and the stability problem in serum because of the removal of the genetic material from the system due to enzyme degradation and a decrease in electrostatic interaction (Sato et al. 2001). When encapsulation of the delivery systems was tested, aiming to solve those problems, other problems such as stability and variation in the encapsulated genetic material were generated. In this study, positively charged solid lipid and chitosan systems were shown to have low toxicity and to be stable and reproducible at large quantities. Incorporation of siRNA was accomplished in this lipidic system. Encapsulation of siRNA in SLNs is suggested as a new approach for antisense technology.

3. Experimental

3.1. Materials

Water-soluble chitosans 1K, 1K-3K, 3K-5K and 10K were used as polymer and were purchased from Kitto Life (Seoul, Korean), Gelucire® 50/13 from Gattefosse (Cedex, France) was used as a solid lipid. Span® 85 and Tween® 80, used as surfactants, were obtained from Fluka (Steinheim, Germany) and Merck (Darmstadt, Germany), respectively. siRNA Bcl-2 and Bcl-2 antibody, used for transfection and Western blot studies, respectively, were provided by Santa Cruz Biotechnology (Heidelberg, Germany). The Pierce® Fast Western blot kit was purchased from Thermo Scientific (USA). Lipofectamine® 2000, used as the reference transfection agent, was supplied by Invitrogen (Paisley, UK).

3.2. Preparation of SLNs

3.2.1. Sonication

SLN's were prepared by solvent emulsification/evaporation (del Pozo-Rodríguez et al. 2009) methods, with some modifications. Briefly, Gelucire® 50/13 was dissolved in dichloromethane (DCM) and the surfactants (Tween 80® and Span 85®) were separately dissolved in distilled water. Aqueous phase was added to theoilyphase and sonicated at 20 % amplitude with a Vibra-Cell Sonicator for 1-2 min (Sonics & Materials Inc, USA). Dispersions were evaporatedto remove the organic solvent with rotavapor (Büchi, Switzerland). Formulations were then filtered through a 0.22 μm filter. Prepared formulations have been named as a S1, S2, S3, S4, and S5. Ratios of formulations prepared are shown in Table 1.

3.2.2. High speed homogenization

SLN's were prepared by high-speed homogenization methods, with some modifications (Olbrich et al. 2001). In this technique, solid lipid (Gelucire® 50/13) was heated to the temperature of 10°C above the melting point of solid lipid (~60 °C), and the molten lipid was dispersed in the hot surfactant solution of the same temperature by high- speed stirring at 23.000 rpm using an Ultra Turrax (IKA T-25, USA), and further homogenized for 5 min. The hot dispersion was filtered through a 0.22 μm non-pyrogenic filter. Prepared formulations have been named as a G0, G1, G2, G3, G4, and G5. Ratios of formulations prepared are shown in Table 1.

3.2.3. Preparation of chitosan particles

Different molecular-weighted chitosans (1 kDa, 1-3 kDa, 3-5 kDa and 10 kDa) were used to formulate chitosan nanoparticles. Chitosans were dissolved in distilled water using a magnetic stirrer at 500 rpm for 30 min and filtered through a 0.22 μm filter. Prepared formulations have been named K1, K2, K3, and K4. Ratios of formulations prepared are shown in Table 1.

3.3. Encapsulation and adsorption of siRNA

3.3.1. Encapsulation of siRNA in SLN

Sonication technique was used for the encapsulation of siRNA in SLNs (prepared by sonication). 62.5 μg of siRNA was added to 1 mL surfactant solution and sonicated with 20 % amplitude for 1 min. Following evaporation of the organic solvent, formulations were filtered. siRNAloaded formulationswasalso marked with* in the text.

3.3.2. Adsorption of siRNA

Specific amounts of siRNAwere mixed with cationic SLN (prepared by high speed homogenization) and chitosan dispersions. Those complexes were then incubated at 37 °C for 20 min to maintain adsorption of negatively charged siRNA on positively charged particles. siRNAloaded formulationswasalso marked with* in the text.

3.4. Characterization studies on formulations

Mean diameter of the bulk population and the particle distribution *via* the polydispersity index (PI) was analyzed by a Zetasizer NanoZS (Malvern Instruments, UK). Zeta potential was determined using the same equipment. Distilled water whose conductivity value of 50 μS/cm was adjusted using sodium chloride at pH 7.4 was used in zeta potential analyses. Electrostatic mobility was converted to zeta potential using Helmholtz-Smolochowski equation.

3.5. Extraction of siRNA from formulations

For extraction of siRNA from SLN formulations, dichloromethane was added to the formulations, gently mixed and centrifuged for 30 min at 1200 rpm. For extraction of siRNA from chitosan particles, siRNA-adsorbed

chitosan formulations were incubated at 50 °C for 1 night prior to mixing with phenol-chloroform and centrifugation. After centrifugation, supernatant of all formulations were discarded and siRNA was precipitated with 99 % alcohol. Following evaporation of alcohol at room temperature, siRNA was loaded on gel.

3.6. Gel Retardation

Gel retardation studies were used to determine siRNA binding ratio of formulations, to evaluate the possible siRNA degredation in serum and to investigate the effect of sonication and extraction methods on siRNA. Samples were subjected to electrophoresis on 1.5% agarose gel for 2 h at 50 V with Tris/Borate/EDTA (TBE) buffer. Images were obtained using a gel documentation system (Kodak Image Station 440 CF, USA).

3.7. Cytotoxicity

Cell culture studies were performed using S5, G2, and K4 formulations for the determination of cytotoxic effects. Lipofectamine® 2000, known to be a transfection agent, was also tested for toxicity for comparison. Colorimetric MTT method was used for the quantitative determination of cell cytotoxicity (Gencer et al. 2010). A549 and MCF-7 cells were used in the method. Absorbance of the plate was measured at 570 nm using a spectrometric microplate reader (Perkin Elmer Victor X5, England).

3.8. Stability in serum

Aiming for the maximum efficiency in cells, formulations prepared must protect siRNA against the serum components. Therefore, behaviors of siRNA-loaded selected formulations (siS5*, siG2* and siK4*) and also only siRNA in the loaded formulations were examined in the culture medium containing serum components. siRNA complexes at the same ratios with those used in transfection studies were incubated at 37 °C for 1, 4, 10, 24 and 48 h with Dulbecco's Modified Eagle Medium (DMEM) which contained 10 % Fetal Bovine Serum (FBS). Images of samples were taken using a gel documentation system at the end of the period.

3.9. Transfection

For the transfection study, cells in a 96-well plate were seeded with antibioticfree medium and incubated at 37 °C for 24 h, with 5% CO_2 until the cell intensity became 60-70 %. Selected formulations were loaded with FITC-conjugated siRNA, mixed with antibiotic/FBS-free medium, and added drop-wise to the cells. After 24- and 48-hour incubations, plates were examined under a fluorescent microscope. Activity of FITC-conjugated siRNA and transfection efficacy of formulations were compared with effects of Lipofectamine® 2000 using two separate plates. A total of about 10000 cells (transfected and non-transfected) at each 20 different area in the wells were counted. Transfection index was calculated using the following equation; Transfection index: (number of transfected cell/number of transfected cell + number of non-transfected cell)*100.

3.10. Western blot analysis

Following cellular transfection of Bcl-2 siRNA loaded siS5* and siK4* with 1:2 ratio formulations, total protein isolation was performed. Absorbance values of total protein extract were measured with Micro BCA kit according to manufacturer protocol. Western Blot study was performed following SDS to determine the effects of Lipofectamine® 2000, siS5*, and siK4* with 1:2 ratio on Bcl-2 protein in the total protein mixture acquired from the A549 and MCF-7 cells after 24, 48, and 72-hour incubations. G2 was not included in these studies since there was no visible fluorescence in the transfection studies for G2. The proteins were transferred to the PVDF membrane (Millipore Immobilon-P^{SQ} PVDF; polyvinylidene difluoride). Antibody, HRP (Horseradish peroxidase) reagent and detection reagent working solutions were prepared prepared according to manufacturer's protocol. Membrane was illustrated with a CCD camera.

3.11. Statistical analysis

Statistical evaluation of transfection efficiency of the formulations was achieved by a paired t-test and an independent samples t-test.

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