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Optimization of Lipofectamine-2000/siRNALipoplexLoaded PLGANanoparticles for Efficient EGFR Gene Silencing: An *in Vitro* Study

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In this study, a novel small interfering RNA (siRNA) delivery system based on encapsulation of lipolexes was introduced. A Lipofectamine-2000–siRNA complex was encapsulated in particles of poly (D,L-lactic-co-glycolic acid; PLGA) by double micro-emulsion. Parameters such as surfactant concentration, the volume of the inner water phase and the outer water phase were evaluated to achieve high loading efficiency, small particle size and low polydispersity. The ratio of the internal to the external phase has a significant effect on the particle size and encapsulation efficiency. The various concentration of surfactant has a different effect on the particle size. In order to achieve optimum conditions for siRNA delivery, the luciferase siRNA was used as a reporter gene. The prepared formulations have a particle sizes in the range of 222 ± 5.2 nm to 900 ± 20 nm and loading efficiency in the range of 4% to 29%. lipoplex loaded PLGA particles (LPPs) had a zeta potential values ranging from −23±2.5 to −29±1.5 mV. S1 and S3 formulations showed greater efficiency compared to the lipoplexes. The gene silencing pattern of LPPs was different from lipoplex. The cytotoxicity of lipoplex loaded PLGA particles (LPPs) was lower than lipoplexes in H1299 cell line. LPPs showed better stability and higher level transfection in the presence of heparin than lipoplexes. The EGFR silencing of S1 formulation was greater than other formulation in A431 cell line. All together these properties suggest that lipoplex loaded PLGA particles have strong potential as a gene carrier for *in vivo* silencing angiogenesis and treatment of cancer.

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Introduction

Cancer is a global disease that occurs due to the abnormal growth of cells that can invade or spread to other parts of the body. The number of new cancer cases diagnosed in the United States increased from about 1.4 million in 2007 to 1.7 million in 2016 [1, 2]. Metastasis of cancer is a complex process that can be divided into five major steps: invasion, intravasation, extravasation, dissemination and colonization [3]. The different methods used for treating cancer are: surgery, radiation, chemotherapy and hormone therapy. however, these methods are not very efficient in treating metastatic cancer [4, 5]. The different types of cancer sometimes occur due to the overexpression of various proteins. The inhibition of protein biosynthesis by gene silencing, especially the synthesis of proteins that are over expressed in cancer cells, and are essential for their growth and division, has recently attracted attention as an effective therapy that has low levels of adverse effects. Therefore, the silencing of a target gene may be less toxic than nonspecific chemotherapy of normal cells [6].

Gene silencing is a novel mechanism that shows promise in cancer therapy. RNA interference (RNAi) is a powerful gene-silencing process that limits the transcript level by suppressing transcription or activating the RNA degradation process [7, 8].

Tyrosine kinase, an epidermal growth factor receptor (EGFR), is overexpressed in many cancers and is known to play a significant role in cell proliferation, tumor growth, invasion, metastasis and inhibition of apoptosis [7]. The inhibition of EGFR signaling is an established strategy for treating various types of cancer including lung cancer, colorectal cancer, and head and neck squamous cell carcinomas. A number of approaches have been used to block EGFR including monoclonal antibodies and tyrosine kinase inhibitors. Monoclonal antibodies bind with extracellular domain EGFR, thereby preventing ligand binding. The tyrosine kinase inhibitor binds with the tyrosine kinase portion of the EGFR.The limitations of the single-agent EGFR tyrosine kinase inhibitor has led to adding other therapeutic strategies, including enhanced targeting of the EGFR. Currently, many studies focus on the inhibition of EGFR by siRNA [9-12].

Although siRNA therapy showed promising results in the *in vitro* studies, a few of the main drawbacks of siRNA for *in vivo* therapies are its safe delivery and efficiency. At present, most of the siRNA delivery systems use either viral or non-viral vectors. Although viral systems are efficient *in vivo*, their major drawbacks are toxicity, immunogenicity and the potential of causing inflammation which may lead to the degeneration of the tissue. Non-viral systems based on biocompatible lipids and polymers are preferred because of their safety, stability, large scale production and characterization, cost effectiveness, size of transgenic DNA and immunogenicity [13]. The siRNA therapy is limited by its ineffective delivery to the target systems. The delivery of siRNA to its cellular site of action requires overcoming extracellular and intracellular obstacle such as intravascular degradation, tissue penetration, stability of the siRNA delivery system in the presence of extracellular glycosaminoglycans, uptake across extracellular barriers, endosomal escape, intracellular trafficking and release of siRNA into the cytosol [14-16]. UN modify siRNAs are highly sensitive to nucleases and serum .unmodifieldsiRNA digested quickly in serum [17].

Lipoplexes that is complexes of negatively charged nucleic acids with cationic liposomes are widely used as a non-viral carrier for nucleic acid delivery. Although liposome vesicles are produced from lipids, they may protect the nucleic acids from degradation and also target them to the site of action. However, they have shown relatively poor storage stability, cytotoxicity, dissociation in the presence of glycosaminoglycans or following freeze-drying and rapid clearance from the blood [18, 19].

Recently, combinations of polymers and lipids have attracted a lot of attention to overcome the mentioned limitations [20]. Since the United States Food and Drug Administration approved of poly (D,L-lactide-co-glycolic acid (PLGA), it has been widely used for the engineering of both micro- and nano-sized particles loaded with a variety of drugs. PLGA nanoparticles are of particular interest for siRNA delivery because their size

allows efficient tissue penetration and cellular uptake [21]. The encapsulation of siRNA inside the PLGA micro- and nanoparticles is a common strategy to improve the stability of particles in the presence of glycosaminoglycans, but this method suffers from low transfection efficacy [22, 23]. To overcome this obstacle, PLGA has been incorporated with cationic excipients like dioleoyltrimethylammoniumpropane (DOTAP), polyethyleneimine (PEI) and chitosan to increase the gene silencing efficiency of siRNA[24-27].In terms of formulation development, it is challenging to efficiently encapsulate high amounts of hydrophilic macromolecular compounds like siRNA into PLGA particles. This is mainly because of the hydrophobic nature of PLGA and the absence of electrostatic interactions between siRNA and PLGA [28]. Encapsulation of polyplexes and lipoplexes into PLGA can not only improve the properties of these complexes, as mentioned earlier, but also improve the stability of particles whose *in vivo* transfection of lipoplexes suffers from low stability. In this study, we have producedlipofectamine-2000/siRNA lipoplex loaded PLGA particles and evaluated their gene silencing efficacy.

Materials and Methods

Materials

Anti-luciferase siRNA (sense5′-pGGUUCCUGGAACAAUUGCUUUUAca-3′, antisense 5′-UGUAAAAGCAAUUGUUCCAGGAACCAG-′3) was purchased from Santa Cruz Biotechnology, USA. The epidermal growth factor receptor (EGFR) siRNA was gifted by Professor Gert Storm from Utrech University. The sequence of EGFR siRNA was 5′-GUU-UGC-CAA-GGC-ACG-AGU-AdTdT-3′; 3′-dTdTC-AAA-CGG-UUC-CGU-GCU-CAU-5′.Polyvinylalcohol (PVA) 88 kDa with % 80.0 degree of hydrolysis was provided by Merck, Germany. Lipofectamine-2000 was purchased from Invitrogen, Breda-Netherland. Luciferase assay reagent was obtained from Promega, USA. A431 which is a human epidermoid carcinoma cell line over expressed EGFR was obtained from the Pasteur Institute in Iran. The human lung cancer cell line H1299 which expresses firefly luciferase was gifted Professor Gert Storm from Utrech University. Cell culture medium, fetal bovine serum, penicillin-streptomycinand trypsin-EDTA were purchased from Ato-cell, Austria.

Methods

Preparation of lipoplex loaded PLGA particles

Lipoplex loaded PLGA particles were prepared by double emulsion-solvent evaporation. Lipoplexes were prepared according to the manufacturer's protocol (20pL of siRNA was complexed with 1 µL of Lipofectamine). Then, Lipoplexes(referred to as the inner phase) with different volume of Lipoplexes were mixed with 250 µL dichloromethane (DCM) containing PLGA with concentration of 0.006 mg/mL (oil phase), and the mixture was emulsified by sonication for 1.5 min using an ultrasonic processor (Bandelinsonopuls, Germany).The resulting first emulsion (W/O) was injected and emulsified into outer water phase with varying concentrations of PVA by sonication for 1 min at power of 50 W over ice bath to produce a double W/O/W emulsion. DCM was evaporated by stirring magnetically for 100 min at room temperature, and particles were collected by centrifuging at 21400 g for 30 min at 4 °C. The precipitate was washed twice with 1 ml of RNase-DNase free water, and re-suspended in RNAse-DNAse free water [29]. The volume of inner phase, outer phase and PVA concentrations in the outer water phase for the preparation of the different formulations (S1-S5) are shown in Table 1.

Table 1. Effect of different parameters on particle size, polydispersity and loading efficiency

Code	Volume of Inner phase (µl)	Volume of outer phase (µl)	Concentration of PVA٪(w/v)	Size (nm)	PDI	Zeta (mV)	Load (%)
S1	100	700	0.0005	513 ± 32.25	0.5 ± 0.05	-25 ± 3	29 ± 1
S2	100	700	0.001	222 ± 5.2	0.18 ± 0.02	-25.2 ± 2	4 ± 1
S3	100	700	0.002	254 ± 22.25	0.22 ± 0.03	-25.3 ± 2	16 ± 1.5
S4	50	700	0.001	900 ± 20	0.4 ± 0.1	-23 ± 2.5	20 ± 2
S5	50	1250	0.001	617 ± 20	0.57 ± 0.3	-29 ± 1.5	10 ± 2

Particle size and zeta potential

NPs were re-dispersed in RNase-DNase free water by sonication in a bath sonicator for 1 min. The mean particle diameter (Z-average) and polydispersity index (PDI) of NPs were determined by dynamic light scattering using the photon correlation spectroscopy (PCS) technique, and the zeta potential of NPs was measured by the laser Doppler electrophoresis technique. The measurements were performed on undiluted samples (n= 3) at 25 °C using a Malvern Nano ZS (Malvern Instruments Ltd., Worcestershire, UK) equipped with a 633nm laser and 173°detection optics. The voltage used for the zeta potential measurements was selected automatically based on the measured conductivity of the sample.

Morphology

The surface morphology was examined using a scanning electron microscopy (SEM,HIT-4160-02, Hitachi, Japan) and atomic force microscopy (AFM) (DME Dualscope TM C- 26).
Prior to examination for , the samples were lyophilized, fixed on a brass stub, and coated with a gold-palladium layer under argon atmosphere using a gold sputter module in a high vacuum evaporator then samples for atomic force microscopy were prepared by depositing a 10µl aliquot of nanoparticles on freshly cleaved mica surfaces and allowed to air dry.

siRNA loading

Glycosaminoglycans and freeze-drying can disassemble lipoplexes and release of siRNA. To measure loading efficacy, formulations were exposed to heparin (26 µg/µL) for 1h(30), and after that, they were frozen for 8 day at -70 °C and freeze-dried. Frozen powders were re-dispersed into RNase and DNase free water [20]. Particles were collected by centrifuging at 21400 g for 30 min at 4 °C, concentration of siRNA in supernatant was measured by a Nano-drop (Thermo scientific, USA) at 260 nm, and loading efficacy (EE) was calculated as follow:

$$\boldsymbol{siRNA\ Loading\ Efficacy} = \frac{(\boldsymbol{amount\ of\ total\ siRNA}}{} \quad (1)$$

Cell viability assay

H1299 cells were seeded in a 96-well plate at a density of 2×10^4 cells/well and grown for 24 h at 37 °C. The cells were then incubated for 3 h in antibiotic and serum free media

containing either lipoplexes or LPPs[31]. Then medium was replaced with complete medium containing 10% FBS and incubated for further 48 h. To measure cell viability, 20 µL of MTT was added to each well, and plate was incubated for 3 h. Then, medium was removed by aspiration, and the formazan crystals were dissolved in 150µL/well DMSO and absorption was measured in a micro plate reader (Hybrid SynergyH1, Biotec,USA) with a test wavelength of 540 nm and a reference wavelength of 630 nm to obtain sample signal (OD540-OD630). The cell viability was calculated as a follow:

$$\textbf{Cell viability}\ (\%) = \frac{\textbf{OD of sample}}{\textbf{OD of control}} \times \textbf{100} \quad (2)$$ [32]

In vitro gene silencing

Luciferase gene silencing

H1299 cells were seeded into 96-well plate at a density of 2×10^4 per well in 200 µLof RPMI containing antibiotics and 10% FBS, 24 h prior to transfection. At the time of transfection, the medium in each well was replaced with serum and antibiotic free medium, and the cells were transfected with a desired formulation. After 3 h of transfection, the medium was removed and replaced with complete medium. After 48 and 96 h, cells were washed with PBS twice and lysed with 20µL of cell lysis buffer (Promega, USA). The luciferase activity in cell extracts was assayed by a luciferase assay kit (Promega, USA) and using a plate reader (Hybrid Synergy, USA). The relative light units (RLU) were normalized against protein concentration in the cell extracts using Bradford assay. The percent of gene silencing was measured by following formula[31]:

$$\text{The gene silencing}\ (\%) = \frac{\text{the normalized RLU of transfected wells}}{\text{the normalized RLU of blank wells}} \times 100 \quad (3)$$

EGFR gene silencing

A431 cells were cultured and transfected as mentioned in previous sections (2.2.6.1). Total RNA from A431 cells was extracted using high pure isolation kit (Roche, Mannheim, Germany) according to the manufacture instructions. Quality and quantity of total RNA was assessed by spectrophotometer (Nano Drop TM 2000, USA) and samples stored at -80°C until use. The polymerase chain reaction (PCR) primers to detect human EGFR (forward primer sence 5-GACAGCTATGAGATGGAGGAA -3, reversed primer sence: 5'-GAGTCACCCCTAAATGCCA- 3') and human β-actin (forward primer sence: 5'-TCATGAAGTGTGTGACGTGGACATC-3', reversed primer sence: 5'-CAGGAGGAGCAATGATCTTGATCT-3'). The specific primers for EGFR(NM_005228) were designed using NCBI primer design and selected from published studies(33, 34). β-actinhas been frequently considered as constitutive housekeeping genes for RT-PCR and used to normalize changes in specific gene expressions. in many studies , β-actin is used as housekeeping gene for EGFR expression in A431 cell line(35-38).Thermal cycler conditions were 15 min at 50 °C for cDNA synthesis, 10 min at 95 °C followed by 40 cycles of 15 s at 95 °C to denature the DNA, and 45 s at 60 °C to anneal and extend the template. Melting curve analysis was performed to ascertain specificity by continuous acquisition from 65– 95 °C with a temperature transient rate of 0.1 °C/S. All reactions were performed in triplicate in a Corbett system (Australia). The value obtained for the target gene expression were normalized to β-actin and analyzed by the relative gene expression - ΔΔCT method, where –ΔΔCT= (CT target –CT β-actin) Unknown – (CT target – CT β-actin) Calibrator.

Results and discussion

Characterization of lipoplex loaded particles

Various formulations and process parameters were examined for the production of lipoplex-loaded PLGA particles (LPPs) with the desired properties such as small particle size, a low polydispersity index (PDI) and high loading efficiency.

These process parameters included: (1) the emulsifier concentration, (2) the volume of the outer water phase and (3) the volume of the inner water phase. The evaluated formulations, coded as S1–S5, are shown in Table 1.

The mean diameter of lipoplexes was 270±20 nm, which agrees with the Hidetoshia Arima result [39] Other studies also demonstrated that 200–400 nm is the optimal size for lipoplexes [40]. The influence of the concentration of poly (vinyl alcohol) (PVA) on the colloidal properties of LPPs was investigated at the constant volumes of 100 and 700 µL for the inner and outer water phases, respectively. Increasing the concentration of PVA in the outer water phase resulted in a significant decrease in the mean particle size. An increase of PVA concentration from 0.0005% (S1) to 0.001% (S2) reduced the particle size and PDI from 513±32.25 to 222±5.2 nm and 0.5±0.05 to 0.18±0.02, respectively. The results from other studies showed that increasing the PVA concentration led to smaller particle sizes [41].

However, a further increase in PVA concentration had a relatively reverse effect on the particle size. The particle size was increased by enhancing the PVA concentration from 0.001% (S2) to 0.002% (S3).The oil droplet break-up efficiency and release during the second emulsification step was prevented by the higher viscosity of the outer water phase [42].

The particle size of the S2 formulation was 222±5.2 nm, which is smaller than the size of the lipoplexes. The encapsulation of lipoplexes in hydrophobic PLGA particles causes shrinkage of the lipoplexes, thus decreasing the particle size. As shown in Table 1, the particle size and PDI value of the S3 formulation were also statistically lower than the S1 formulation and lipoplexes, indicating that the S3 formulation is more stable than the S1 formulation and the lipoplexes.

The effects of the inner and outer water phase volumes on the particle size and loading efficiency were also investigated at a constant concentration of PLGA and an optimum concentration of PVA (0.001%). It was observed that the mean particle size increased with decreasing volume of the inner water phase (S4). This occurs because the coalescence of droplets is prevented by the presence of a large amount of organic solvent[43] Also, the loading efficiency increased significantly when the volume of the inner water phase was decreased to 50 µL. Decreasing the volume of the inner water phase resulted in the increase of siRNA concentration in the inner phase, which can be a reason for its higher loading efficiency [44].

The ratio of the internal to the external phase has a significant effect on the particle size and encapsulation efficiency. The reduction in the lipoplexes' encapsulation with an increase in the external phase volume could be due to the lower shear stress and tendency of the lipoplexes to diffuse into the external phase [45]. Previously, some factors affecting the characteristics of particles produced by the double-emulsion method had been reviewed. It was reported that the particle size increased with the increase of the continuous phase volume [41].

The different parameters evaluated in this study had no significant effect on the zeta potential and no significant differences were observed in the zeta potential. Most of the particles had zeta potential values ranging from −23±2.5 to −29±1.5 mV (Table 1).

The morphology of the S1 particles and lipoplexes was evaluated by scanning electron microscopy (SEM) and Atomic Force Microscope. The particles were spherical with smooth surfaces (Fig. 1A&C) while the SEM images of lipoplexes indicated aggregated particles and no intact liposomes.

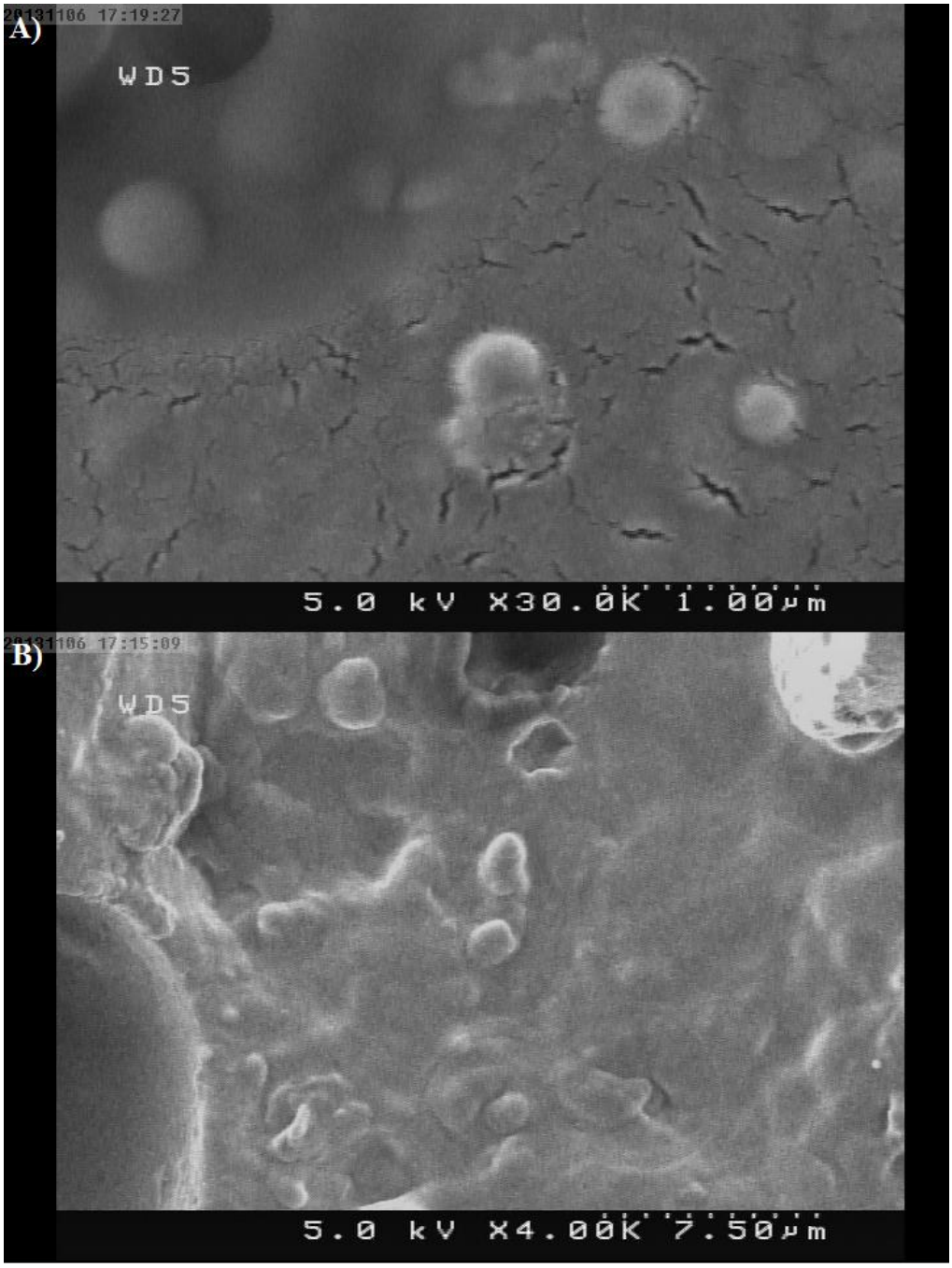

Fig. 1. SEM images of the Lipoplex -loaded PLGA nanoparticles (A) and lipolex (B).

In vitro siRNA Delivery in H1299 cell line

At present, the major hurdle hampering the successful clinical translation of RNAi is the lack of effective and safe vehicles for siRNA delivery. In this work, the gene-silencing effect of optimized lipoplex loaded PLGA particles in the H1299 cells was investigated. The luciferase activity of the lysed cells was measured 48 hours after transfection by the luminescent oxidation of luciferin to oxyluciferin (Fig 2). S1 and S3 formulations showed greater efficiency compared to the lipoplexes. The observed knockdown of luciferase by the S1 formulation was 86%±3 while

the knockdown of lipoplexes was 65%±5. Luciferase knockdowns of the S3 and S4 formulations were 80% ±5 and 60% ±3, respectively. The effect of S1 formulation on luciferase expression was greater than the other formulations. The effect of encapsulation into PLGA became remarkable when the cells were incubated over an extended period of time. After 96 hours of incubation, no significant decrease was observed in the activity of S3 and S4 with a gene silencing efficiency of 75%±3.5 and 60%±3, respectively. The gene silencing efficiency of lipoplexes (50%±4) and S1 (32%±5) were significantly decreased after 96 hours (Figure 3). Initially, the lipoplex showed a strong effect while the down-regulation effect became gradually weaker. This suppression pattern was quite different from the LPPs, which was initially weak and then gradually became stronger. These phenomena could be accounted for by the release of sustained siRNA from the LPPs in the cytosol. Our results were in agreement with the previous study by Tahara *et al*. [21]. However, an unexpected result was observed with S1 formulation, wherein its gene-silencing efficiency was reduced during the time while its loading efficacy was the highest among all the formulations.

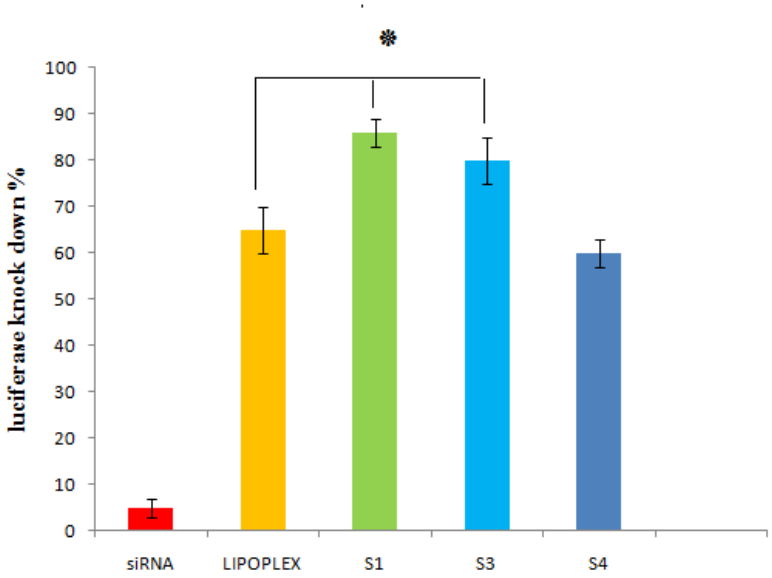

Fig. 2. Release of siRNA from lipoplex and PLGA particles in presence of heparin; Significance was calculated by ANOVA (*$p \leq 0.05$).

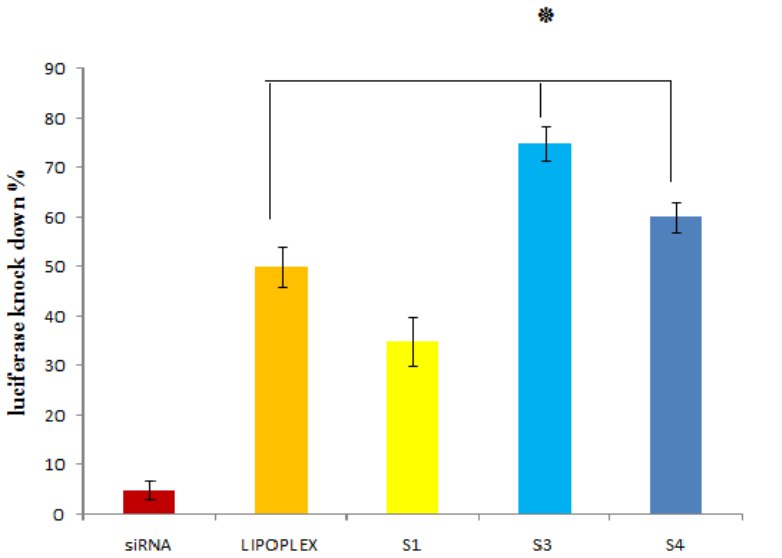

Fig. 3. Effect of heparin on gene silencing in H1299 cell line after 48 h; Significance was calculated by ANOVA (*$p \leq 0.05$).

Effect of heparin in gene silencing

Figure 4 shows the results of gene silencing in the presence of heparin. The gene silencing efficiency of lipoplex was 18%±2, which indicates that the lipoplexes have a lower gene-silencing effect in the presence of heparin. Although the loading efficiency of S1 was higher than other formulations, its silencing effect was significantly decreased to 33%±5 in the presence of heparin. S3 and S4 formulations induced 50±5% and 40±3% luciferase knockdowns, respectively.

This confirmed the results of gene silencing after 96 hours wherein S1 did not sustain siRNA release as efficiently as S3 and S4. Faster dissociation of the lipoplexes from S1 may be caused due to the presence of the lipoplexes near the particle surface. S1 had the lowest amount of surfactant and that may have caused the lipoplexes to come to the borderline of particles and play the role of surfactant.

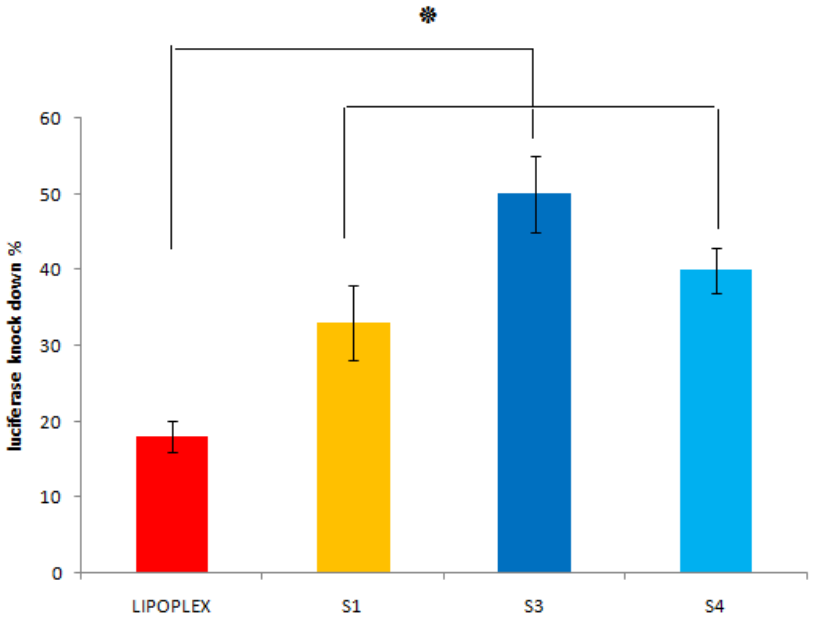

Fig. 4. Relative mean luciferase expression of luc-H1299 cells transfected with lipoplex-loaded PLGA particles after 48 h. Significance was calculated by ANOVA (*$p \leq 0.05$).

Cytotoxicity of Lipoplexes and PLGA particles

The cytotoxicity of the optimum formulations (S1, S3, and S4) and lipoplexes at different concentrations was evaluated by the MTT assay on H1299 cells. A high positive charge on the surface of Lipofectamine leads to the electrostatic interaction with negatively charged components of the cell membrane and causes disruption and damage of cell membrane. A number of studies showed that the cytotoxicity of lipoplex increased with an increase in the concentration of Lipofectamine [30]. PLGA particles have a lower cytotoxicity because they have negative zeta potential, and do not interact with cell membrane. Previous studies reported that the toxicity of PEI complexes was strongly reduced when they were encapsulated in the PLGA particles [46, 47]. The lowest concentrations of S1, S3, and S4 showed a viability of 90%±4, 78%±5 and 77%±4.according to the results, S1 formulation that have a higher loading efficiency showed lower cytotoxicity compare to lipoplex (Fig. 5).

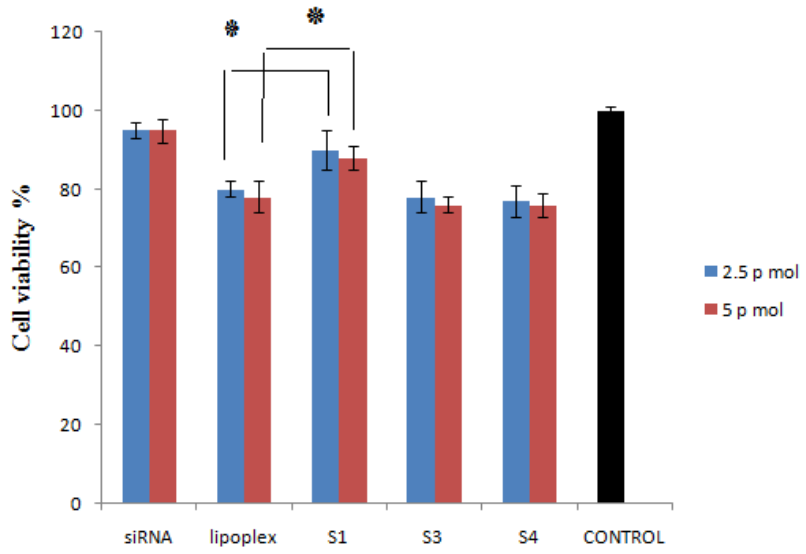

Fig. 5. Relative mean luciferase expression of luc-H1299 cells transfected with lipoplex-loaded PLGA particles after 96 h. Significance was calculated by ANOVA (*$p \leq 0.05$).

Gene silencing on A431 cell line

We performed gene-silencing experiments on the A431 cell line using siRNA against EGFR. The previously studies reported knockdown of EGFR expression (60%) for A431 cells treated with lipoplexes(48). The observed knockdown of EGFR by the S1, S3, and S4 formulations was found to be 80%±5, 72%±2, and 65%±5, respectively (Figure 6). The EGFR is known to be overexpressed in many tumors (e.g. lung, head and neck, colorectal, prostate, and ovarian carcinoma) and involved in cellular proliferation, angiogenesis, and the inhibition of apoptosis. Most recently, RNA interference (RNAi), in which double-stranded RNA (dsRNA) induces sequence specific degradation of the targeted messenger RNA (mRNA), has been extensively developed and studied. RNAi is able to silence the targeted gene expression more efficiently and specifically [49].

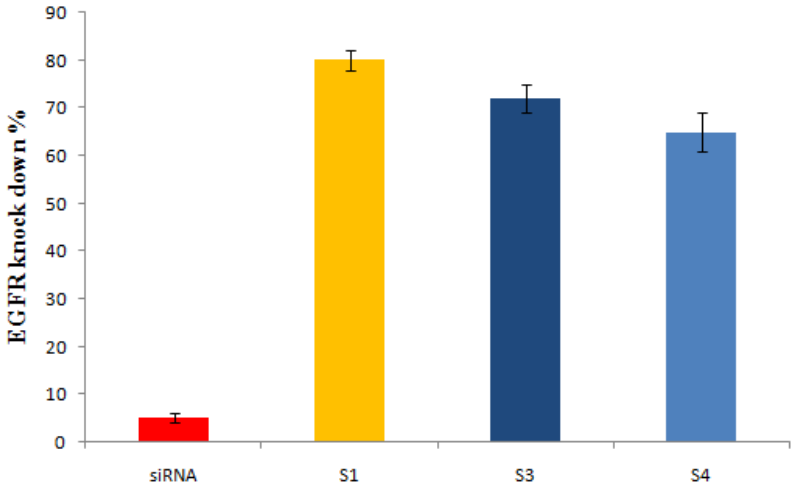

Fig. 6. Cell viability after application of PLGA formulations in H 1299 cell. Values are the mean average ± SD of five wells applied with the same formulation. Significance was calculated by ANOVA (*$p \leq 0.05$).

Conclusion

We haved scribed how to prepare lipoplex-loaded PLGA particles to achieve a siRNA carrier with high loading efficiency, sustained release, stabilityin the presence of glycosaminoglycans such as heparin and high gene-silencing ability. Lipoplexes were encapsulated in PLGA particles with a loading efficiency of16%–29% in optimum conditions. The results showed that the the emulsifier concentration, the volume of the outer water phase and the volume of the inner water phase have a significant effect on particle size and percentage of encapsulated lipoplexes but they had no significant effect on the zeta potential. Encapsulation of lipoplexes into PLGA particles have a sigificant effect on stability of particles against heparin and have asustained gene silencing effect lasting for a longer period of time. The gene silencing efficiency of the optimum formulation (S3) was approximately 15% higher than the lipoplexes at 48 hours and 25% higher than the lipoplexes after 96 hours. The formulations that had higher gene silencing efficiency showed lower cytotoxicity than lipoplexes in the H1299 cell lines and were more biocompatible. EGFR silencing indicated that gene silencing of optimized formulations was significantly higher than that caused by lipoplexes. In conclusion, PLGA combined with lipofectamine have a strong potential as a gene carrier for siRNA.

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Conflict of interest

Authors certify that there is no actual or potential conflict of interest in relation to this article.

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