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Original Article

Increased production of soluble vascular endothelial growth factors receptor-1 in CHO-cell line by using new combination of chitosan-protein lipid nanoparticles

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Abstract: The soluble vascular endothelial growth factor receptor-1 (VEGFR1) or sFLT-1 has important role in antiangiogenesis. In this study, the increase expression and production of sFLT-1 fragment by newly designed ChPL-NPs nanoparticles (chitosan-protein lipid) using Chinese hamster ovary cell line (CHO) was evaluated. The assessment and purification of sFLT-1 were carried out by western blotting and fast protein liquid chromatography (FPLC). Thereafter, the angiostatic effect of gene transfer of sFLT-1 in Human umbilical vein endothelial cell line (HUVEC) was evaluated. Our results showed a significance rate of transfection with ChPL-NPs (80-85%) in comparison to standard lipofectamine^{2000} (65-70%) ($P < 0.05$). The anti-angiogenic action of sFLT-1 was observed by in-*vitro* culture of recombinant protein (sFLT-1; 50 ng/ml) with HUVEC cell lines (5×10^6). The ChPL-NPs nanoparticles can consider a potential carrier system for large scale production of sFLT-1, which ultimately may be use as therapeutic agent in targeting solid tumor tissues.

Keywords: VEGFR1, sFLT-1, ChPL-NPs, nanoparticles, antiangiogenesis

Introduction

Angiogenesis is the formation of new capillary blood vessels from a preexisting vascular network [1]. When irregular, it contributes to development of vessel overgrowth (e.g. cancer) or vessel insufficiency (e.g. cardiovascular diseases) [2]. Already studies showed a positive correlation between angiogenesis with degree of metastasis, tumor recurrence and shorter survival rates [3-5]. These up regulations were usually monitored by angiogenic cytokines and growth factors such as vascular endothelial growth factor (VEGF) [2, 5-7]. VEGF is a 45 KDA glycoprotein, homodimeric that act as an autocrine as well as paracrine stimulation of tumoral angiogenesis [5]. Therefore, VEGF considered as a privileged target for control of angiogenesis in an anti-tumoral goal [2]. Recently, the regulatory action of VEGF receptor (also called sFlt-1) as anti-VEGF factors has been extensively studied [8, 9]. The sFlt-1 is a protein or peptide that binds to VEGF with high affinity and reduces the *in-vitro* and *in-vivo*-effect of VEGF [5-10]. Due to its important role, several research based studies tried to modify the sFlt-1 production using viral or non-viral vector [11-13]. But in most of performed studies the limitation of recombinant sFLT-1 production such as serious toxicity, lack of glycosylation and in correct folding of protein was not fully resolved [14-17]. Thereby, production of recombinant sFLT-1 in biological format with therapeutic effect, which does not alter immune response, is still under investigation. We recently, constructed sFLT-1 molecule that contained extracellular domains I-III from N-terminal of the sFLT receptor [18]. This model proposed to increase the anti-angiogenic efficacy when administrated as a recombinant protein. Here, we aim to

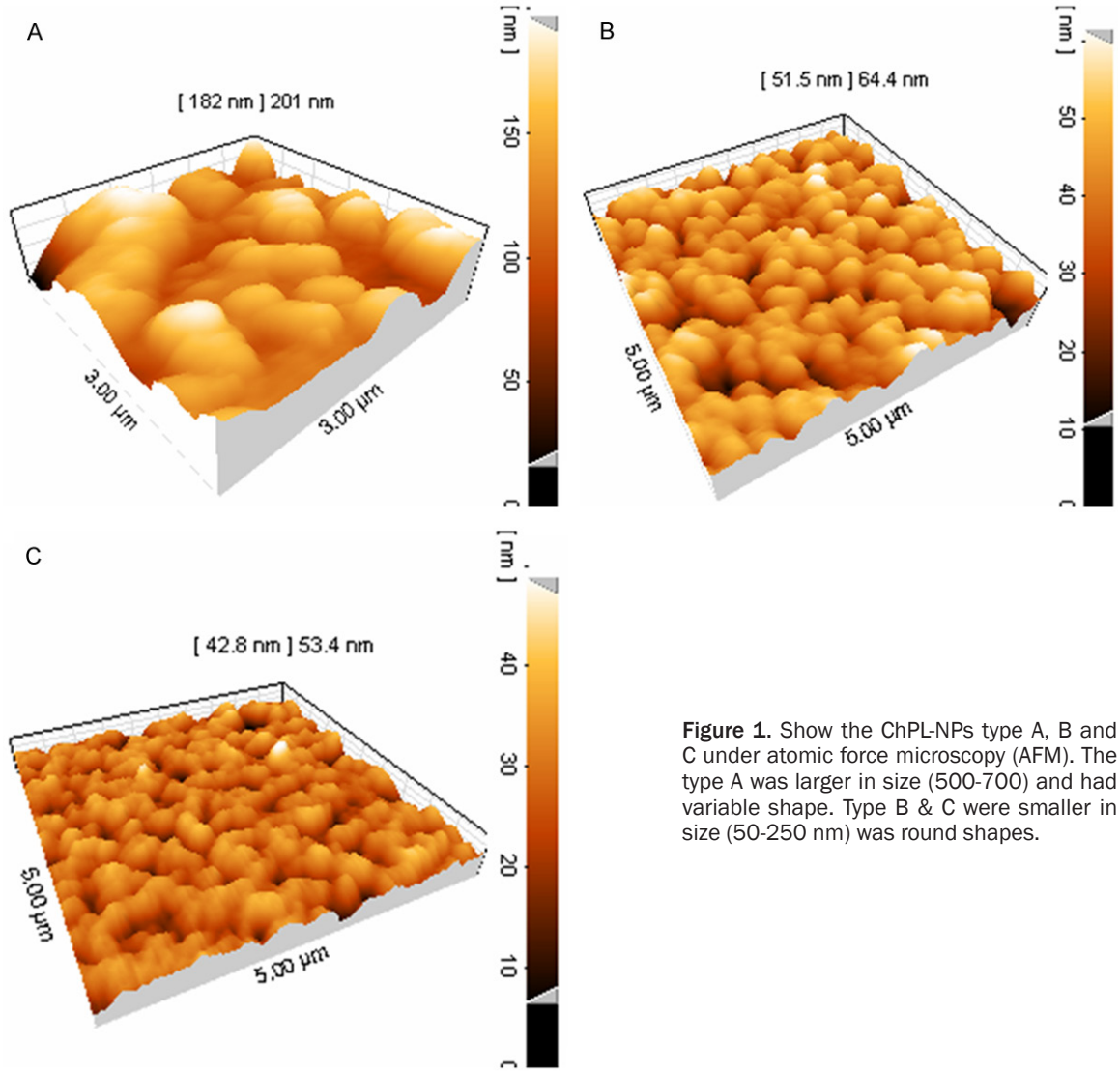

Figure 1. Show the ChPL-NPs type A, B and C under atomic force microscopy (AFM). The type A was larger in size (500-700) and had variable shape. Type B & C were smaller in size (50-250 nm) was round shapes.

improve sFLT-1 production by nanoparticles. For this reason, a nanoparticles with new biological or chemical composition i.e., chitosan, protein and lipid (ChPL-NPs) was designed. Then, the rate of transfection in CHO cell line by nanoparticles, in comparison to commercial liposome i.e., lipofectamine^{2000} was analyzed. Thereafter, the effectiveness of produced sFLT-1 in inhibiting the growth of endothelial cell was investigated. The designed nanoparticles propose to act as *in-vivo* bioactive delivery agent, which may need further investigation.

Material and methods

Nanparticle preparations

The ChPL-NPs particles were prepared in three consecutive steps by modification of our previously published and pending reports [US Patent number; 20110167962; US patent pending [45 2014/0370500A1] [19, 20]. Briefly, 1.76 gram of chistosan powder (Cat no; 44.886-9 Sigma-Aldrich, USA) with a molecular mass of 90KD and 80% degree of deacetylation was dissolved in 100 ml of 0.8% acetic-acid. The solution was first incubated at shaker incubator for 16 hours (37°C at 120 rpm), and then centrifuged for 15 minutes at 4000 rpm. The obtained gel in supernatant (PH ≈ 5.5) was taken off and kept at 4°C until uses. In the next step, 0.4 to 0.6 grams of gelatin type A in 100 ml of phosphate buffer solution dissolved and the mixture kept in shaker incubator (120 rpm) for 3 hrs at 40°C. Ten ml of dissolved gelatin was mixed with 70 ml of chitosan and kept in shaker incubator for 30 minutes at 37°C with

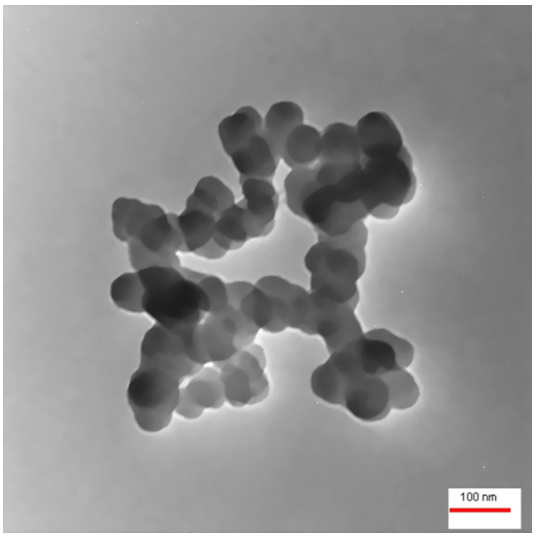

Figure 2. Shows the ChPL-NPs type B and C under transmission electron microscopy (TEM). The ChPL-NPs type B and C had uniform sizes.

140 rpm. In a glass round bottle flask, 0.2 gram lyophilized phosphatidycholine (Cat no; 3556, Sigma-Aldrich, USA) dissolve with 10 ml of chloroform, and after removing the chloroform by rotary vacuum evaporator, phosphatidycholine (lecithin) was precipitated in inner wall of flask and dispersed stably in gelatin-chitosan colloidal solution [20]. To obtain nanoparticles from this colloidal suspension three different methods were applied; 1) vigorous magnetic stirring or self assembling, 2) high-pressure homogenier under 120 mps of nitrogen gas and 3) high speed homogeneizer with 6 stainless Blade up to 20000 rpm [21, 22]. The morphological characteristic of nanoparticles was investigated by transmission electron (TEM) and atomic force microscopy (AFM) [23, 24].

Cell culture for sFLT-1 RNA extraction

Human umbilical vein endothelial like cell line (HUVEC; obtained from Pasture Institute, Tehran, Iran) were cultured in Dulbecco's modified Eagle's medium [DMEM/F-12 (1:1)]. Supplemented with 10% fetal Bovine serum plus Penicillin (100 unit/ml) and streptomycin (100 µg/ml) and incubated at 5% CO_2 at 37°C with 90% humidity. The sub-cultures of these cells were used for RNA-Extraction as previously demonstrated [18].

sFLT1 gene amplification, cloning

The total RNA was isolated using RNeasy Mini kit according to manufactures instruction (Cat no; 74104: QIAGEN, Hilden, Germany). The isolated RNA (1/µl) was subjected to one-step RT-PCR kit (QIAGEN). The forward primer (5'-ATG GTC AGC TAC TGG GAC ACC G-3') and the reverse primer (5'-TAT GCA CTG AGG TGT TAA CAG ATT TG-3') were designed to amplify the coding sequence from 286 to 1268 bp of sFlt-1 regions with 327 aa. The sequence was retrieved from Gen Bank (Accession No; 001159920). The PCR Product was re-amplify by primers (sFlt-F 5'-AAG CTT ATG GTC AGC TAC TGG GAC ACC G-3'; sFlt-R1 5'-GGA TCC TAT GCA CTG AGG TGT TAA CAG ATT TG-3'; sFlt-R2 5'-GGA TCC TTA TAT GCA CTG AGG TGT TAA CAG ATT TG-3') containing Hind III and Bam HI restriction enzymes sites [18, 25]. The product was first ligated in PTZ57R vector and transformed intoE. Coli XL1 Blue competent cells. The PTZ57R plasmid (7 µl) were digested with dual restriction endonucleases enzymes (1 µl Bam HI + 1 µl Hind III + 3 µl buffer, 8 µwater) at 37°C for one hour. The digested product was seen on 0.8% agarose gel. The desired bands of 1 kb were extracted and recovered using gel extraction kit (Cat No; 11732676001, Roche Diagnostic GMbH, Germany).

Sub cloning of sFLT1 gene into pEGFP-N1

The pEGFP-N1 shuttle vector was digested with restriction endonucleases enzymes BamHI and HindIII at 37°C for 1 hour. The digested product was aanalyzed by 0.8% agarose gel. The desired band of 4.7 Kb was extracted and recovered using gelextraction kit. Thereafter, the PCR product (9 µl), pEGFP-N1 (3 µl), T4 DNA ligase (1 µl), 5Xbuffer (6 µl) and Water (11 µl) were added into micrfuge tube. The reaction mixture was overnight incubated at 4°C. The ligation products were transfect to XL1 Blue competent cells and inoculated into LB agar culture medium containing Kanamycin (50 µg/ml). Thereafter, the selected colonies were sub cultures into LB liquid media containing Kanamycin (50 µg/ml). The correct recombinant plasmid pEGFP-N1 identified by using plasmid mini-preparation kit. The isolated recombinant plasmid was confirmed using PCR and restriction analysis [18, 25, 26].

Sequencing of inserted gene segment

Sequencing of inserted gene segment in recombinant plasmid was performed by sending the segment to commercial company (Cinagene; Tehran, Iran) [18].

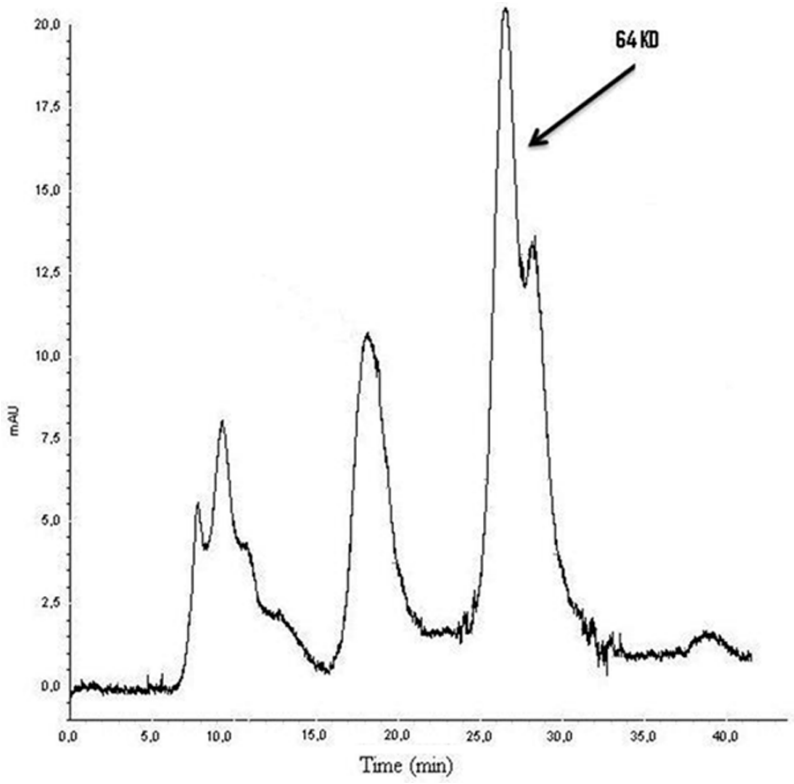

Figure 3. Shows the FPLC graph. Within 30 minutes duration a 64 kd recombinant sFLT-1 were isolated.

Transfection of CHO cell lines with pEGFP-N1 containing sFLt-1gene fragment by lipofactamin^{2000} & ChPL-NPs particles

Chinese hamster ovary cell line (CHO; C111-obtained from *Pasture Institute*, Tehran, Iran), were cultured in Dulbecco's modified Eagle's medium (DMEM/F-12 {1:1}) supplemented with 10% fetal Bovine serum plus Pen (100 unit/ml) and streptomycin (100 µg/ml) [18]. They were incubated at 37°C with 90% humidity under 5% CO_2, then cells were passaged with 0.25% trypsin-EDTA and counted [18]. They were further diluted and inoculated into 24-well culture plates containing Opti-MEM (1X) (Gibco; lot No 1161191) at a concentration of 4×10^4 per. Plate [18, 27]. In each experimental set up, two sets of 24-well culture plates was used; one for transfection with lipofactamin^{2000} (Invitrogen; Lot No 11668-027) and the other for ChPL-NPs particles. In the first set, CHO cell lines (4×10^4 per) incubated with equal volume of diluted plasmid (05-1 ug of pEGFP-N1/50 µl Opti-MEM) and lipofactamin^{2000} (50 µl). Whereas, in transfection using ChPL-NPs, 2 µl of 50 µg concentrations of particles types A, B, C was investigated. The transfected cells with pEGFP-N1 plasmid emit a green-light florescence. All the culture plates were investigated under inverted florescent-microscopy after 48 hours of incubation.

Protein analysis by SDS-PAGE and immunobloting

The supernatant of cell culture were visualized using SDS-PAGE electrophoresis. Then Western bloting performed with primary antibody (anti-VEGF receptor 1 antibody, ab32152) with 1: 1000 and secondary antibody (anti-Rabbit Ig (G) AP conjugate, Promeage; 53-73B) with 1:7.500 dilutions were used for staining [12]. It is important to note that since in the sub-cloning of sFLT-1 gene in the pEGFP-N1 shuttle vector, the stop-codon was not included in designed sequences; the expression of hybrid protein was occurred. In another words, the expression of EGFP marked the SFLT-1 expression. The observed molecular weight was 64KD by SDS-PAGE electrophoresis [12, 18].

Anti-Angiongesis properties of sFLT-1

HUVEC cells in 5×10^6 were grown as a monolayer in PureCol® Collagen Coated T-25. After 24 hours, 10 ng/ml basic fibroblast growth factor (bFGF) F8924-25UG Sigma and one percent of Estradiol Valerate (Aburaihan Co. Iran. Batch No: 1021) was added to each flask. Each experiment consists of 2 culture flask; one without recombinant sFLT-1, and one with 50 ng/ml of purified protein. Every 3 days, the culture were look for dendrite like cells formation.

Statistical analysis

All results are expressed as mean ± standard deviation. Differences were analyzed by Student's t test and considered statistically significant at $P \leq 0.05$.

Results

Nanoparticles

The result discussed here is average of observation. Overall, each procedure was repeated for 5-6 times and each time the ChPL-NPs were observed under TEM and AFM microscopes. The morphological characteristic of nanoparticles type A (self assembling), B (high-pressure homogenier) and C (high speed homogeneizer) was shown in **Figure 1**. The ChPL-NPs type B & C had a uniform shape (rounds) and the size (from 50 to 250 nm). Whereas, self assembled Nan particle (type A) was larger in size (400 to 700 nm) (**Figure 2**).

Amplification and identification of sFlt-1 gene

A specific PCR-product with about 981 bp was obtained on 1% agarose gel electrophoresis after one step RT-PCR, from RNA of HUVEC cell lines. The obtained PCR product was 1 kb. Further cloning and digestion of PTZ57R/sFlt-1plasmid using *Bam* HI and *Hind* III restriction enzymes, showed two bonds of approximately 3000bp and 1000 bp on 0.8% agrose gel. The first segment was equal to pTZ57R plasmid and the later one was equal to the size of sFlt-1. Sub cloning of extracted band of 1000bp into pEGFP-N1 shuttle vector and digestion of PCR products results into two bonds of 4700 bp & 1000 bp on 0.8% agarose gel, respectively (**Figure 3**). Sequencing of extracted bond (1000 bp) showed complete consistent of inserted segment with the size and sequence of nucleotide acid of sFlt-1 protein (Gene bank; NP_0011-53392. 1).

Detection of sFlt-1 gene in CHO cell lines using lipofactamin^{2000} & nanoparticle types A, B, and C

After 24 hours of green fluorescence was observed (range 10 to 40%) in the cytoplasm and reaches its maximum after 48, 72 hours with lipofactamin^{2000} and ChPL-NPs type B & C, respectively. The range of transfected cells was 60 to 70% with lipofactamin^{2000}, and 80-85% with ChPL-NPs type B & C ($P < 0.05$). The rate of transfection with nanoparticle type A was lower than lipofactamin^{2000}, ranging from 15-20%. No significant change in proportion of cells expressing PEGFP-N1/sflt-1 plasmid was detected during next 5th days of extending period of culture.

Expression and purification of sFlt-1 protein

The western blot analysis using a Rabbit monoclonal antibody assayed the expression of sFlt-1. It proved that sFlt-1 could be expressed in CHO cell lines. Purified protein was assessed by FPLC using Agilent Bio SEC-3 (250 mm × 20 mm) (**Figure 3**).

Determination of Anti-Angiongesis properties of sFLT-1

Dendrite like cells formed in HUVEC cell culture flask which had no sFLT-1. In contrary, no dendrite like cells formed in culture flask that contains sFLT-1 (50 ng/ml). The HUVEC cell had normal growth with no morphological changes.

Discussion

In this study, the expression and production of sFLT-1 in CHO cell lines by ChPL-NPs (chitosan, protein and lipid) in comparison to standard Lipfectamine^{2000} was analyzed. The sFLT-1 belong to the class III receptor-type tyrosine kinase (RTK) family [5, 7]. Several studies documented a correlation between the enhanced expression of sFLT-1 with diminished endothelial cell-proliferating activity and suppression of angiogenesis [7, 11, 12, 16, 17]. Goldman et al. was among the first who showed restricted growth of transfected tumor cells in sFLT-1 plasmid [14, 18]. Furthermore*, in-vivo* experiments showed suppressed activity of peritoneal metastases after sFLT-1 injection [28]. As a result, the potential use of sFLT-1 as a therapeutic target has gained more scientific attention [13, 25, 26, 27]. Although in most of previous study, the post translation modification of produced sFLT-1 was discussed in bacterial or yeast system. We used CHO cell lines, because it has a good growth rate and can be used as protein production in an industrial scale [29]. On the other side, the growth of virus in CHO cell culture media is very low and even the culture media without a serum can be used for cell-growth [29]. Additionally, they possess especial enzyme for glycosylation and the produced recombinant proteins in this cell lines showed similar functional activity as natural proteins. The main drawbacks of CHO cell line is the low expression of recombinant protein (10-100 folds less than microbial system), that was resolved by signal peptide induction [18], and by using non viral transfection system i.e., ChPL-NPs.

The ChPL NPs consisted of phosphoglycerides (5-10%), chitosan (60-70%) and gelatin (10-20%) combinations [20]. Our results showed that the transfection of sFLT-1 with ChPL NPs were 82-85% of cells, whereas the cell positivity rate with standard Lipfectamine^{2000} was about 60-70%. To our knowledge, the ChPL-NPs complex for transfection of sFLT-1 gene in CHO-cell lines was highlighted for the first time. The CHO cell line showed the maximum tranfection with Lipfectamine^{2000} in 48 hours, but with ChPL NPs the maximum transfection observed within 72 hours. In both experimental set-up, the green light emission was stable till 5 days of post-transfection. Basically, the intensity of the surface charges in nanoparticle is important as it determine their interaction with bioactive compound. In ChPL NPs complex; phosphoglyceriode had negative charges, whereas chitosan and gelatin had positive charges. Consequently, the electrostatic attraction between appositive charges was formed. This electrostatic interaction between oppositely charged ions would ultimately cause an effective system for gene transfer and drug delivery. The applications of nanoparticles as a non-viral vector that facilitates induction of impermeable molecules into the cells has been already discussed by other researchers [31, 32]. Generally, non viral vector is divided into two groups of cationic lipids and cationic polymers [30]. The cationic lipid is not very efficient as they are inactivated in the presence of serum, unstable upon storage, and exhibit some cytotoxicity both *in-vitro* and *in-vivo* [32, 33]. A few studies, uses anionic liposomal DNA delivery vectors as an alternative to cationic liposomes [31]. Although, anionic lipid by themselves, cannot transfect the mammalian cells owing to the lack of interaction between similarly charged DNA and lipid [34]. Among the most important natural polymer for drug delivery system the chitosan and gelatin were highlighted recently [31, 32]. Chitosan belong to a family of cationic polymers of β-1-4 N-acetyl-glucosamine and D-glucosamine residues [32]. It has been used to deliver DNA both *in vitro* and *in vivo* with low efficiency and low specificity [25]. In this regards, Malakootypoor et al. showed the lower rate of transfection of chitosan plasmid DNA nanoparticle than Lipfectamine^{2000} in human bone marrow-derived mesenchymal stem cells (MSCs) [34]. Though, progress made to increase the rate of transfection of chitosan with conjugation of folic acid (FA), but the efficiency was still low [35]. Other investigators underline the potential use of gelatin nanoparticle; gelatin is a partial derivative of collagen which has a low immunogenicity [32]. Zwioreck and his group compared DNA delivery system of gelatin nanoparticles with polyethyleneimine (PEI) using B16F10 cell lines. They also found out a lower transfection rate for gelatin nanoparticles then PEI [32]. In present study, we tried to overcome the problem by using combination of phosphoglycerides, chitosan and gelatin. In our model, the phosphoglyceride with chitosan and gelatin undergoes ionic gelation. Also, as phosphoglycerides are amphipathic molecules i.e., having both polar “head” and nonpolar “tail” the membrane flexibility for attachment and release of nanoparticles into cell improved. Hence, the transfection rate with ChPL-NPs was significantly higher than standard Lipofectamine^{2000}. Based on obtained results, we may propose the ChPL-NPs as a suitable carrier for lipoplic and hydrophilic drugs and genes delivery systems, which needs further clarifications.

We also showed a direct co-relation between qualities of obtained nanoparticles with the performed procedures. The ChPL-NPs type B & C that produced by high speed homogenizer or high pressure homogenizer (size 50-250 nm) were more suitable for transfection of sFLT-1 gene in CHO cell lines. In contrast, the ChPL-NPs type A was larger in size (400-700 nm) and the transfection rate was lower than Lipfectamine^{2000}. Recently Ghorab M et al. [37] showed that the size of solid lipid nanoparticles not only affected and controlled by variation of process, but also with lipid matrix structure. In fact, differences in the chemical nature of lipid matrix could influence the overall hydrophobicity which in turn influences the particle size distribution [37]. Here, we kept the concentrations of lipid (phosphoglycerides), chitosan, gelatin and solvents similar in different procedures and we showed the obtained size of nanoparticles (50-250 nm) was desirable for effective drug delivers, as suggested by others [37, 38].

In conclusions, this study shows that simple, inexpensive and reproducible nanoparticles could successfully transfect a CHO-cell lines to produce a larger scale recombinant protein like sFLT-1. The produced sFLT-1 can act as a safe

and effective vehicle against solid tumors, which need further investigations.

Disclosure of conflict of interest

None.

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