

Effect of Fluorination Modification on Transfection Efficiency of Non-Viral Gene Carrier Systems Based on Chitosan

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Keywords

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Gene therapy

Abstract: The aim of this study was to examine the transfection efficiency of the fluorination modification without the addition of any cationic charge on the chitosan (Chi) molecule. The fluorination reaction on Chi (ChiF) was carried out with using 1H,1H,2H,2H-Perfluorooctyltriethoxysilane (SiF). The characterization of ChiF was realized by Fourier transform infrared (FTIR) analysis and its molecular weight (Mw) and polydispersity index (PDI_{Mw}) were determined using GPC-SEC system. The physical properties of nanoparticles (nChiF) obtained by ionic gelation method were determined. The gel electrophoresis analysis was applied to the nanoparticles for determine the gene complexing capacity. The cytotoxicity of ChiF onto Human Embryonic Kidney (HEK293) cells was determined via MTT colorimetric assay. The cell confluency (after transfection) and transfection efficiency of nChiF on HEK293 cells were evaluated. The results showed that the nChiF2:pEGFN1 complex (ratio of 35:1) with a particle size of 98.1±2.2 nm and zeta potential of 34.7±6.5 mV, is more superior agent for transfection efficiency in HEK293 cells due to its high transfection effect and higher cell confluency. As a result, it has been showed that the fluorination reaction onto Chi without any cationic charge modification enhance the transfection efficiency for HEK293 cell lines.

Kitosan Esaslı Viral Olmayan Gen Taşıyıcı Sistemlerin Transfeksiyon Verimliliği Üzerine Florlama Modifikasyonunun Etkisi

Anahtar Kelimeler

Kitosan,
Florlama,
Transfeksiyon etkinliği,
Gen terapi

Özet: Bu çalışmanın amacı, kitosan (Chi) molekülü üzerine herhangi bir katyonik yük eklenmeden florinasyon modifikasyonunun transfeksiyon etkinliğini incelemektir. Chi üzerindeki florlama reaksiyonu (ChiF), 1H, 1H, 2H, 2H-Perfluorooktiltrioksasilan (SiF) kullanılarak gerçekleştirildi. ChiF'in karakterizasyonu, Fourier Transform Infrared Spektroskopisi (FTIR) analizi ile gerçekleştirilmiş ve molekül ağırlığı (Mw) ve polidispersite indeksi (PDI_{Mw}), GPC-SEC sistemi kullanılarak belirlenmiştir. İyonik jelleşme yöntemiyle elde edilen nanopartiküllerin (nChiF) fiziksel özellikleri belirlenmiştir. Gen kompleksleme kapasitesini belirlemek için nanoparçacıklara jel elektroforez analizi uygulandı. ChiF'nin İnsan Embriyonik Böbrek (HEK293) hücreleri üzerindeki sitotoksitesi, MTT kolorimetrik deneyi ile belirlendi. HEK293 hücreleri üzerine nChiF'nin transfeksiyon etkinliği ve hücre yayılımı (transfeksiyon sonrası) sonuçları değerlendirildi. Sonuçlar, 98.1 ± 2.2 nm partikül büyüklüğüne ve 34.7 ± 6.5 mV zeta potansiyeline sahip nChiF2: pEGFN1 kompleksinin (35: 1 oranı), yüksek transfeksiyon etkisi ve daha yüksek hücre yayılımı gösterdiğinden dolayı HEK293 hücrelerinde transfeksiyon etkinliği için daha üstün bir ajan olduğunu göstermiştir. Sonuç olarak, Chi üzerine herhangi bir katyonik yük modifikasyonu olmadan florinasyon reaksiyonunun, HEK293 hücre hatları için transfeksiyon verimliliğini arttırdığı görülmektedir.

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1. Introduction

Gene therapy has a great potential therapeutic method for the treatment of various diseases such as cancer and genetic disorders [1, 2]. The main purpose is to ensure that the relevant disease-therapeutic gene is directed appropriately to the target cell nucleus. There are several application methods for this purpose in the literature [3]. In particular, the gene delivery systems with viral and non-viral carriers are the preferable methods recently [4, 5].

Cationic polymeric vectors, among the non-viral delivery systems, have recently been selected for their promising properties, such as low toxicity, easy synthesis and desirable modifications to enhance transfection efficiency. Among these cationic vectors, chitosan (Chi) is most widely used because of its low toxicity, biocompatible and biodegradable structure but it does not show high transfection efficiency alone. Therefore, the various modifications which are graft polymerization, nitration, phosphorylation, amination, reducible reaction, were applied on Chi [6-8].

All of these modifications are applied not only to increase the cationic charge density on Chi but also to increase cell membrane interaction, provide endosomal escape and dissociation of gene-Chi nanoparticle. As one of these modifications, the fluorination reaction which provides both lipophilic and hydrophobic properties onto the cationic gene carrier structure, is ensured high affinity with the cell membrane, while also achieve endolysosomal escape [9-11]. Wu et al. synthesized fluorinated PEG-polyprotein polyplex micelles to better serum-resistance using as gene delivery systems [12]. Similarly, Belabassi et al. have studied onto the targeted nanoparticles based on PEGylated-fluorinated Chi using in drug delivery systems [13].

In this study, transfection efficiency after fluorination without any cationic load modification on chitosan was investigated. For this purpose, chitosan was fluorinated using SiF on methylol groups (ChiF) and characterized. The nanoparticles of ChiF (nChiF) were formed by using ionic gelation method and specified their z-average particle size (nm), polydispersity index (PDI_n), zeta potential (mV). For determining the complex forming ability, gel electrophoresis analysis was realized. The cytotoxicity of ChiF was determined by MTT assay. The transfection efficiency of the nChiF was realized on HEK293 cells.

2. Material and Method

Chi (Low molecular weight, 75-85% deacetylated, viscosity average $M_w=50.000-190.000$ Da, SKU: 448869) and SiF (SKU: 667420) using for the fluorination modification on Chi (ChiF) synthesis, were purchased from Sigma-Aldrich (USA). Sodium

hydroxide (NaOH), isopropanol, acetone, glacial acetic acid were obtained from Sigma-Aldrich (USA). Sodium tripolyphosphate (TPP) was obtained from Sigma-Aldrich (USA) for using as a crosslinker in ionic gelation method.

Dulbecco modified eagle medium (DMEM), agarose, EDTA and Tris base were obtained from Sigma-Aldrich (USA). Opti-MEM were obtained from ThermoFisher Scientific (USA) for gene transfection tests. 3-[4,5-dimethylthiazol-2-yl]-2,5 diphenyl tetrazolium bromide (MTT) and dimethyl sulfoxide (DMSO) were purchased from Sigma-Aldrich (USA) for cytotoxicity studies.

2.1. Synthesis of ChiF and characterization

In order to synthesize the fluorinated of Chi (ChiF), the mixture of Chi and NaOH (1:5 w/w) were prepared in isopropanol and water (4:1 v/v). Chi is kept at 60 °C for one hour for swelling and alkalinized. Afterward, fluorination reagent (SiF) was added slowly onto this solution as 1 mole glucosamine unit to 1 mole SiF and incubated for 48 h at 60 °C. For purification, the reaction mixture was dialyzed against deionized water with dialyze tubes (MWCO-12-14000 Daltons, Medicell International Ltd.) at 25±1°C twice for two days. Then ChiF was lyophilized at -50±1°C at 0.01 mbar pressure (Lyovac GT2E, Steris, Germany) and after lyophilization, ChiF was stored at +4 °C. The characterization of ChiF was carried out using Cary 630 FTIR spectrometer (Agilent; USA). At the same time, the gel permeation chromatography-size exclusion chromatography (GPC-SEC) system (Tosoh Bioscience; Japan) was used to determine the M_w and $PDIM_w$ of ChiF with using TSK-gel GMPWXL column. The refractive index increment (dn/dc) was 0.142 mL/g and the flow rate was 1 mL/min. The mini-DAWN multi-angle light scattering (MALS) detector and the differential refractive index (dRI) detector (Wyatt, USA) were used to calculate the molar mass distribution and specific dn/dc , respectively. The ASTRA software was used to collect and analyze all of the data. The ChiF : acetic acid solution (2 mg/mL) was diluted to two times with sodium acetate (0.2 mol/L). Then this solution was filtered through 0.22 µm membrane and transferred to vials [14].

2.2. Preparation of nChiF

As seen in Table 1, the 150 mg of ChiF was dissolved in the 50 mL of 1% acetic acid solution. At the same time, the aqueous solution of TPP (0.1% m/v) was added dropwise at 250 rpm for one h onto the ChiF solution which was diluted with deionized water for preparing in different concentrations (final total volume: 5 mL). The resulting nanoparticles were stored at +4 °C for using in future analysis and were determined their size (nm), zeta potential (mV) and PDI_n values, at 25 °C (Zetasizer Nano Series Malvern instrument; England).

Table 1. The amounts for preparation of the nChiF.

Nanoparticle	ChiF (%)	ChiF Solution (mL)	ChiF:TPP	Amount of TPP (mL)
nChiF ₁	0.05	0.833	3:1	0.833
nChiF ₂			4:1	0.625
nChiF ₃			5:1	0.500
nChiF ₄			6:1	0.417
nChiF ₅			7:1	0.357
nChiF ₆			8:1	0.313

2.3. Gel electrophoresis assay

The ability to bind green fluorescent protein circular plasmid DNA (pEGFN1) to different amounts (w/w) of nanoparticle was investigated by gel electrophoresis (Cleaver Scientific Ltd., England). Briefly, Agarose gel (1%, w/v) containing 0.5 µg/mL ethidium bromide (EtBr) was prepared in Tris-acetate-EDTA (TAE) buffer (pH 8.0). Then the mixture of pEGFN1-ChiF complexes (gnChiF) with loading dye was loaded onto the agarose gel and subjected to electrophoresis for one hour at 100 V. The images were visualized by UV transilluminator (UVITEC; Cleaver Scientific; England).

2.4. Cytotoxicity assay

The cytotoxicity study of ChiF was carried out via MTT colorimetric assay. Briefly HEK293 cells were seeded at 1×10^4 cells/well in 96-well plates. Then they were cultured in 100 µL of DMEM at 5% CO₂, 37 °C in an incubator for one day. After incubation, the medium was drained, and different ChiF concentrations from 50 to 500 µg/mL in 100 µL of FCS free DMEM were added in triplicate wells and incubated for four h. The supernatant was discarded, and 100 µL of MTT solution (1 mg/mL in FCS free DMEM) were added to each well and allowed to react for four h at 37 °C. After the reaction time, the supernatant solution on cells was discarded, and these cells were washed twice each with phosphate buffered saline (PBS). Finally, the formed formazan crystals were dissolved with 100 µL of DMSO. The microplate reader (ELx800, Biotek Instruments, USA) was used to record the optical densities (OD) of the wells at 550 nm [15, 16]. The following equation was used to calculate the cell viability;

$$\text{Cell viability (\%)} = \frac{C_{\text{ChiF}}}{C_{\text{DMEM}}} \times 100 \quad (1)$$

where C_{ChiF} and C_{DMEM} are the average absorbance value of wells incubated with ChiF and FCS free DMEM, respectively.

2.5. In vitro transfection efficiency

The transfection efficiency of gnChiF was determined in the HEK293 cell line. Briefly, the cells were seeded

at 3.5×10^4 cells/well in 24-well plates. Then they were cultured in 100 µL of DMEM at 5% CO₂, 37 °C in an incubator for one day. After one day, the microscope (Olympus, Japan) was used to control the proliferation of the cells. Then, DMEM on cells was removed, and the various gnChiF concentrations with Opti-MEM cell culture medium (Gibco-BRL, Rockville, Maryland) were added. After four h incubation, the supernatant was discarded and DMEM was added again. The cells were incubated for three days in 5% CO₂ at 37°C. The inverted fluorescence microscope (Olympus IX-71, Japan) under 460-480 nm fluorescence light was used to visualize the cell confluency and EGFP-expressing cells.

The percentage of EGFP-expressing cells were calculated by flow cytometer (Guava-easyCyte; Merck Millipore-France, InCyte software) (n = 3). For this purpose, the medium on cells was drained again. After washing with PBS, the cells were incubated with Trypsin-EDTA 0.25% (250 µL) for 2 min at 37 °C. To stop the trypsin activation and to prevent cell lysis, the cells were washed using 500 µL of DMEM. Then the cells were centrifuged at 1000 rpm for 5 min. The supernatant was removed, the cells were diluted with 300 µL PBS and loaded to 96-well plates.

3. Results and Discussion

3.1. Synthesis and characterization of ChiF

ChiF synthesis was molecularly take placed via the reaction mechanism between methylol groups on the Chi molecule and the ethoxy groups on the SiF molecule (Figure 1).

The formation of ChiF was verified by FTIR spectral analysis. As seen in Figure 2, although the small broad band which was related to the bending vibrations of the free methylol groups were observed at about 1450–1250 cm⁻¹ region (max. at 1426, 1380 and 1320 cm⁻¹) in FTIR spectra of Chi, as seen in FTIR spectra of ChiF, the intensity of this band was decreased at max 1381 cm⁻¹. In addition, new sharp absorption peaks that appeared at 1147 cm⁻¹ and 1240 cm⁻¹ were contributed to the vibrations of CF₂ and CF₃ groups of SiF. Moreover, the small shoulders which were related to the stretching vibrations of the Si-O groups and which were related to the C-H stretching of the CH₂ groups could be observed at about 700-800 cm⁻¹ region (at max. 708 cm⁻¹) and at max. 2925 cm⁻¹, respectively. On the other hand, due to the increased degrees of deacetylation of Chi, the intensity of the broad shoulder which was related to the bending vibrations of the primary NH₂ groups (at max 1596 cm⁻¹) was increased in FTIR spectra of ChiF (at max 1580 cm⁻¹) [17, 18]. At the same time, FTIR technique was used to calculate the N-deacetylation degrees of Chi and ChiF (88.7% of ChiF and 81% of Chi) [19].

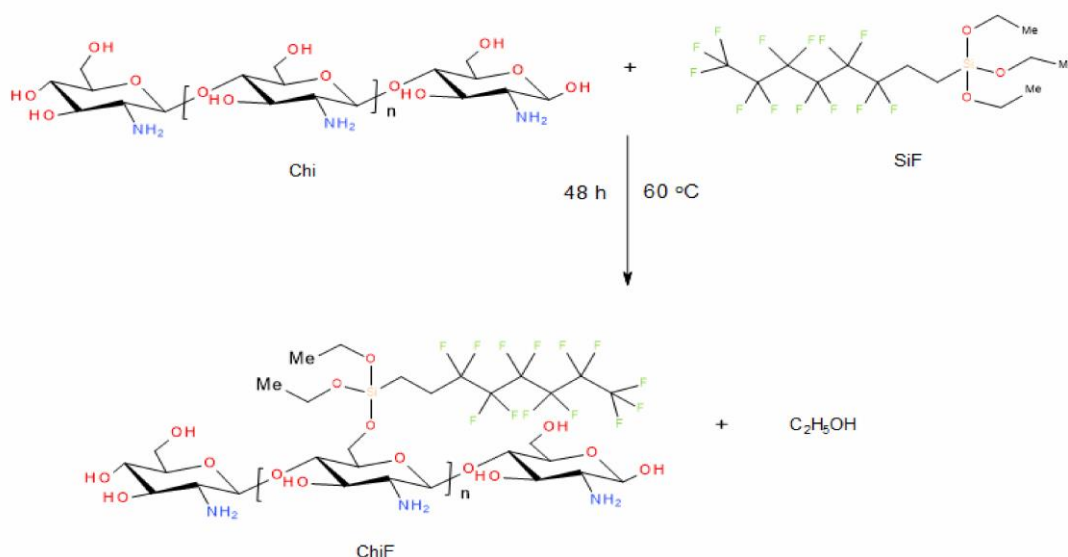


Figure 1. The reaction mechanism of the fluorination reaction

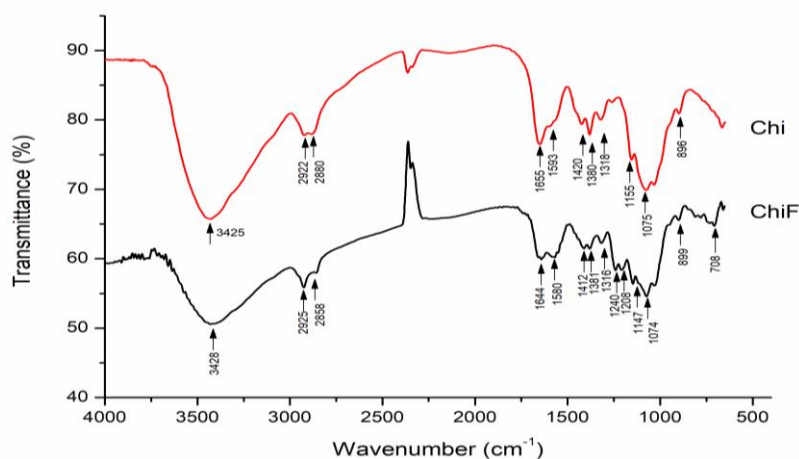


Figure 2. FTIR Spectra of Chi and ChiF

In addition, the M_w and PDI_{M_w} for ChiF were determined to be 29 ± 0.343 kDa and 1.378 ± 0.03 by using GPC-SEC system, respectively. In the ChiF synthesis, the partial degradation of Chi was due to the temperature (60°C) and NaOH effect and when compared to Chi results in my previous study, a monodisperse ChiF structure is obtained from the polydisperse Chi structure [20].

3.2. Characterization of nChiF

Nanoparticles for gene delivery studies mean that nanocore or nanocapsules containing therapeutic genes. The size of the nanoparticles obtained from the materials prepared by various polymerization methods can generally vary from 5-10 nm to 1000 nm, in particular it must be bigger than 10 nm and smaller than 200 nm for gene therapy studies. Moreover, the zeta potential values of these nanoparticulate systems should be more than +30 mV for expressing the stability of the particles [21-22]. As seen in Table 2, z-average particle size, PDI_{nano} and zeta potential results of the nanoparticles, which

were obtained with ionic gelation method, are available. According to the results of $n\text{ChiF}_2$, $n\text{ChiF}_3$, and $n\text{ChiF}_4$, both of them can be used in gene transfer studies. In particular, $n\text{ChiF}_2$ has the most suitable properties with 98.1 nm of particle size, 0.329 of PDI_{nano} and 34.7 ± 6.5 mV of zeta potential values, so it was used in the following experiments.

Table 2. Z-average particle size, PDI_{nano} and Zeta Potential values of the nChiF

Nano Particle*	Particle Size (nm)	PDI_{nano}	Zeta Potential (mV)
$n\text{ChiF}_1$	146.5 ± 4.7	0.531 ± 0.08	25.7 ± 4.1
$n\text{ChiF}_2$	98.1 ± 2.2	0.329 ± 0.04	34.7 ± 6.5
$n\text{ChiF}_3$	100.5 ± 1.8	0.431 ± 0.04	38.6 ± 5.4
$n\text{ChiF}_4$	147.6 ± 5.2	0.397 ± 0.05	43.8 ± 5.2
$n\text{ChiF}_5$	149.6 ± 3.8	0.516 ± 0.06	41.7 ± 8.3
$n\text{ChiF}_6$	152.7 ± 5.6	0.501 ± 0.05	45.2 ± 5.4

* Due to the wide range of molecular weight distribution of commercially available Chi, the PDI_{nano} values of nChiF are quite high

3.3. Preparation of gene-nChiF complex (gnChiF) and gel electrophoresis assay

The effect of the presence of fluorine and silica groups on the Chi molecule after fluorination modification was investigated on the degree of binding between nChiF₂ and pEGFN1 (gnChiF₂) at different weight ratios (w/w). As seen in Figure 3, the complexation ratio of nChiF₂ with all pEGFN1 is 2:1 (w/w) when the complexation ratio of nChi with all pEGFN1 is 4:1 (w/w). According to these results, the reason for the increase of the binding capacity compared to nChi is the increase in the presence of protonable primary amino groups by increasing the degree of deacetylation of Chi due to the temperature and NaOH effect present in the fluorination modification medium [23].

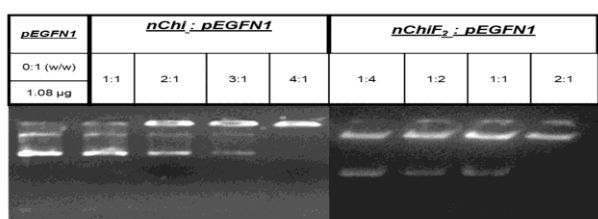


Figure 3. Gel Electrophoresis Analysis of the nChiF (w/w)

3.4. Cytotoxicity of nChiF

In order to understand the interactions between cell and nanoparticles that cause toxic effects, it is necessary to determine the percentage of cell viability. In particular, the structural differences which are molecular weight, cationic charge density, the degree of branching, etc. increase the toxic effect [24].

The toxicity level of nChiF was determined in the HEK293 cells by MTT colorimetric assay, and FCE free DMEM was used as a cell development control. The cell viability values which were calculated with Eq (1), are 84.08%, 72.36% and 71.97% at the nChiF concentration of 50 µg/mL, 125 µg/mL and 250 µg/mL, respectively. As seen in Figure 4, there is no toxic effect at all concentrations, except for 500 µg/mL which is used for determining the toxic level

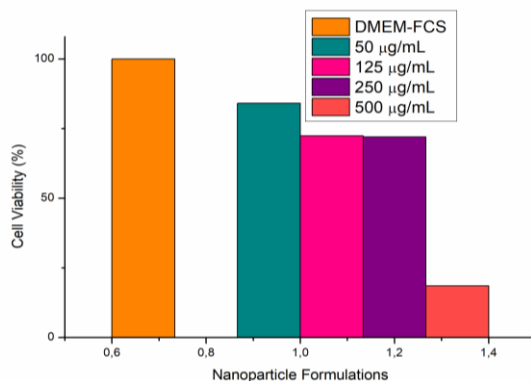


Figure 4. The cytotoxicity of ChiF on the HEK293 cells

of nChiF (18.55% cell viability). According to these results, the applied doses of nChiF for transfection studies show good biocompatibility with the HEK293 cell line.

3.5. Transfection results and cell confluency of gnChiF

The transfection efficiency and cell confluency results of gnChiF₂ using HEK 293 cell line for the amount of plasmid of 1 µg are given in Figure 5A-B. Chi was used as a control group. As seen in Figure 5A, the gene-nChiF₂ complex which nChiF₂:pEGFN1 ratio is 40:1 (w/w), show the highest transfection efficiency results (%21.14±2.14). However, the complex with a ratio of 35:1 nChiF₂:pEGFN1 shows almost the same highest transfection efficiency result as the complex with a ratio of 40:1 nChiF₂:pEGFN1 (%20.88±1.56). On the other hand, as expected, increasing the amount of the complex decrease the cell confluency and hence the complex with a ratio of 40:1 nChiF₂:pEGFN1 shows the lowest cell confluency result (%58.33±2.89). However, for stable transfection, all of the cell confluency results after transfection were obtained in accordance with the literature [25].

Although transfection efficiency was lower than other studies in the literature, it should be known that the main purpose of the study was not to increase the cationic charge density of Chi, and it was to evaluate the transfection efficiency *in vitro* after fluorination modification on Chi. Wang et al. reported that dendrimers containing fluorine groups were achieved a higher transfection efficiency than commercial products such as Lipofectamine 2000, SuperFect [26]. Similarly, Gong et al. reported that nanoparticles obtained from poly (beta aminoester) containing fluorine groups have high transfection efficiency and low toxicity in HEK-293 and HeLa cells [9]. In this study, As a result of the fluorination reaction to Chi, transfection efficiency was increased compared to Chi. The most important reason for this can be explained by the increased cell membrane interaction with hydrophobic and lipophobic groups after fluorine modification of Chi [27].

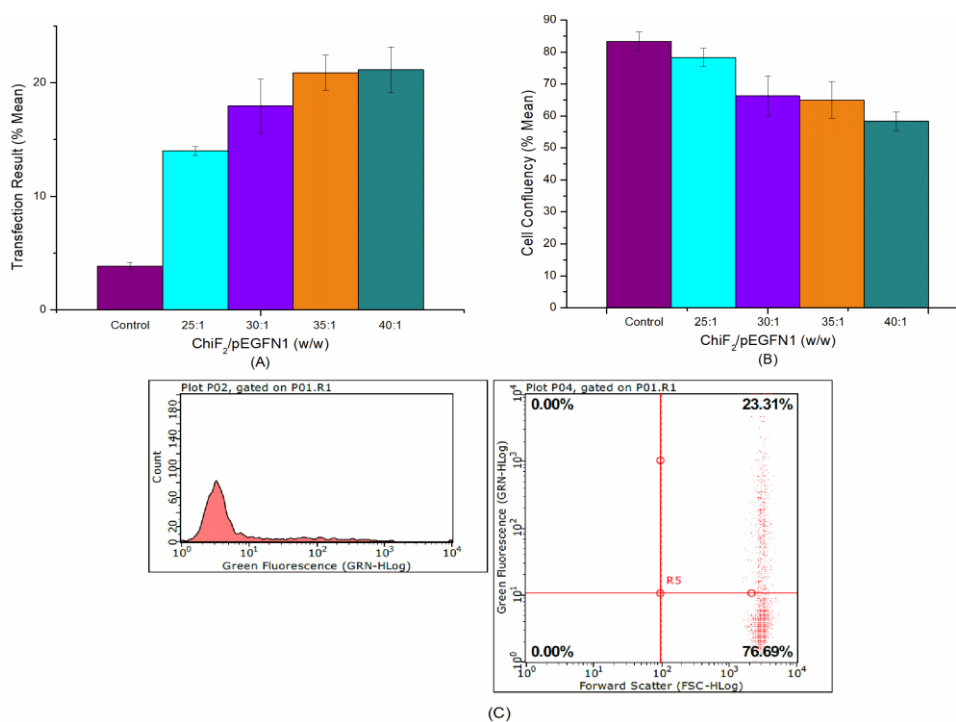


Figure 5. The transfection efficiency and cell confluency results of gnChiF₂ on the HEK293 cell line; A-) Transfection results (% mean) of nChiF₂:gene ratio from 25:1 to 40:1 (w/w), B-) Cell confluency results (% mean) of nChi-F₂:gene ratio from 25:1 to 40:1 (w/w) C-) One of the FACS transfection efficiency results of nChiF₂:gene ratio 40:1 (w/w)

4. Conclusion

In this study, the fluorinated Chi (ChiF) was synthesized, characterized and the nChiF nanoparticles from ChiF were prepared for use in transfection studies. According to the data of this study, the complexing ability with pEGFN1 increased only by the increase in the degree of deacetylation without depending on the fluorination modification. ChiF has low toxicity at the appropriate concentrations. Similar to many other studies in the literature, gnChiF₂ showed lower transfection efficiency compared to studies in the literature, as modifications were not made to increase the cationic charge density on chitosan. However, due to fluorination modification, gnChiF₂ showed about 6 times more transfection efficiency than gnChi.

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