

# Preparation and characterization of PLA-PEG/Chitosan-FA/DNA for gene transfer to MCF-7 cells

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## Research Article

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# Abstract

The ability of Chitosan-Folic acid and PLA-PEG polymers for gene delivery into MCF-7 cells was evaluated. The PLA-PEG copolymer is one of the most appealing nanoparticles for gene transfer to human cells because of its biocompatibility and high blood circulation. However, one of the barriers to the use of these polymers in gene therapy has always been the low effectiveness of these nanoparticles. In this study, Chitosan and folic acid were utilized to improve gene transfer efficiency and encapsulation of PLA-PEG/Chitosan-FA/DNA nanoparticles. PLA-PEG/Chitosan-FA/DNA nanoparticles with different concentrations of Chitosan-FA were prepared using double emulsion-solvent evaporation technique. The biological properties (i.e., biocompatibility, DNA release, and gene transfer ability to MCF-7 cells in vitro) as well as the physical and chemical properties (i.e., particle size, zeta potential, and the morphology of the resultant nanoparticles) of PLA-PEG/Chitosan-FA/DNA nanoparticles were investigated. The results showed that increasing the Chitosan-FA concentration in the PLA-PEG/Chitosan-FA/DNA nanoparticles resulted in an increase in zeta potential, DNA release, encapsulation, and gene delivery efficiency. The MTT assay showed PLA-PEG/Chitosan-FA/DNA nanoparticles exhibit low cytotoxicity and good compatibility. On the other hand, fluorescence microscopy and flow cytometry were used to test the ability of PLA-PEG/Chitosan-FA/DNA nanoparticles with varied concentrations of chitosan-folic acid to transfer the gene to MCF-7 cells. Flow cytometry and fluorescence microscopy analysis showed that increasing the concentration of chitosan-folic acid in PLA-PEG/Chitosan-FA/DNA nanoparticles improved gene transfer efficiency to MCF-7 cells.

# Introduction

Gene therapy and the use of cationic nanocarriers with high ability to transfer genes to eukaryotic cells have helped us focus on treating genetic disorders and cancers (Wang et al. 2016; Nasab et al. 2020). The usage of cationic transporters has become important due to current gene therapy methods and attention to factors, such as biocompatibility, DNA loading percentage, and the ability of these nanoparticles (cationic nanocarriers) to transfer genes to cancer cells. In fact, cationic nanocarriers are important because of their ability to interact with and neutralize negative DNA charges, as well as compress and bundle DNA into appropriate structures. Furthermore, many of these nanocarriers have the ability to protect DNA against enzyme digestion in the plasma medium (Xing et al. 2020).

Polyethylene glycol (PEG) is one of the most commonly utilized polyether polymers in pharmacy. The molecular weights (MW) of PEGs range from 200 to 35000 kDa. PEGs with a low MW (under 400) are usually clear viscous liquids, whereas those with a high MW (more than 1000) are usually opaque solids or powders. Hydrophilic polymers such as polyethylene glycol (PEG) and macrogols are commonly employed in pharmaceuticals. Due to their water-soluble qualities, they are utilized in medical (e.g., wound dressings and hydrogels), food (e.g., preservatives in food supplements), and cosmetic products (e.g., emollients and emulsifiers) (Massadeh 2021; Sellaturay et al. 2021). Other common polymers in gene transfer include biodegradable polymers, such as polylactic acid (PLA). Because of its great biocompatibility and ability to continually manage medication, it received great interest. On the other

hand, the negative interactions of polylactic acid with proteins in the plasma increase the size of these nanoparticles, which the immune system detects and excretes (Zhang et al. 2020). When compared to PLA nanoparticles alone, surface modification of hydrophobic polymers with the hydrophilic polyethylene glycol and manufacturing of the amphiphilic polymer PLA-PEG polymer can reduce the size of the resultant particles (due to increased hydrophilicity), as well as increase DNA encapsulation and blood circulation length of time (Farboudi et al. 2020). Because of their ease of synthesis, high drug loading, and possibility for sustained drug release, several groups throughout the world have looked into PEG-PLA-based nanoparticles and micelles as drug carriers for hydrophobic medicines. However, due to the limited partitioning of more hydrophilic pharmaceuticals into the micelles' hydrophobic core, these polymeric micelles have exhibited a relatively low drug-loading potential in the case of hydrophilic medications (Danafar et al. 2017).

Researchers have attempted to improve the solubility and bioavailability of vincristine in recent years by encapsulating it in biodegradable polymeric nanoparticles. Chitosan is a glucan derivative made up of repeated chitin units derived from crab skin. This polysaccharide is totally biocompatible, because of the presence of amine groups on its surface and it has a positive charge. In reality, chitosan's ability to neutralize negative charges and transfer genes has been demonstrated in various investigations (Garg et al. 2019; Mujtaba et al. 2020). In other words, studies have shown that Chitosan nanoparticles, which are stable and less toxic, are commonly utilized to deliver hydrophobic medicines, vitamins, proteins, minerals, and phenolics into biological systems (Ruman et al. 2021).

Today, nanocarriers have been produced that can detect and carry medications to specific tissues, such as cancerous tissues, due to breakthroughs in materials science and a better understanding of cell biology. Due to the high rate of division in cancer cells, the need for folic acid has increased in these cells due to the utilization of this molecule in the process of DNA creation, and as a result, cancer cells have more folic acid receptors than other cells (Teo et al. 2015; Salim et al. 2020). In reality, folic acid (FA) is the completely oxidised form of vitamin B9 essential for purine and pyrimidine synthesis, amino acid metabolism, methylation activities, and formation. In recent years, folic acid has been used as a target molecule in various nanocarriers for targeted drug delivery to cancer cells and tissues (Farahnak Roudsari et al. 2021; Pinto et al. 2021). Also, Biodegradable polymers coupled with folic acid (FA) have been investigated as effective gene and medication delivery methods (Chen et al. 2017).

The objectives of this research were to: 1) design and encapsulate DNA into PLA-PEG/Chitosan-FA/DNA nanoparticles for gene transfer and improved medication delivery to MCF-7 cells and 2) develop a method to protect nucleic acid from enzyme digestion damage and increase its transfer in plasma. In this research, new nanoparticles were created by combining PEG, PLA, chitosan, and folic acid. The study evaluated the physical and chemical properties of nanoparticles, and the encapsulation efficiency and the pathway pattern of DNA drug from PLA-PEG/Chitosan-FA/DNA nanoparticles. The biocompatibility of PLA-PEG/Chitosan-FA/DNA nanoparticles was also investigated, as well as gene transfer to MCF-7 cells using PLA-PEG/Chitosan-FA nanoparticles DNA.

# Materials And Methods

## Synthesis of chitosan-folic acid (Chitosan-FA)

One mM of folic acid was dissolved in 10 mM of Tris-HCL (pH=7.4) solution, and 50  $\mu$ M of chitosan was dissolved in 10 mM, Tris-HCL buffer (pH = 5.6). Each solution was then centrifuged at 15000 rpm for 30 min at 4°C. The supernatants were removed and, the chitosan-folic acid was washed several times with phosphate buffer. Finally, it was dried by lyophilization and stored in -20°C. To evaluate and confirm the synthesis of chitosan-folic acid, FTIR, UV spectroscopy and TEM were used.

## Investigating the ability of PLA-PEG/Chitosan-FA nanoparticles to interact and neutralize negative DNA charges

Thirty  $\mu$ l of TE buffer and 1 mg of PLA-PEG/Chitosan-FA/DNA nanoparticles were dissolved and was electrophoresed for 2 h at 80 volts on 0.8% agar gel.

## Investigating the toxicity of PLA-PEG/Chitosan-FA/DNA nanoparticles

To evaluate the toxicity of the copolymer, MCF-7 cells were transferred to 96-well plates at a density of 7000 cells per vial. 5% CO<sub>2</sub> was then held for 24 h at 37°C. 250, 500, and 1000 ml of PLA-PEG/Chitosan-FA nanoparticles were applied to each well separately and kept for another 24 h. After that, cell viability was determined using the MTT test. Each treatment in this test was replicated three times.

### MTT test

MTT is a yellow salt that can be used to determine the viability of cells. To perform the MTT test, the supernatants were removed and the cells were washed several times with phosphate buffer, then 100  $\mu$ l of new culture medium containing 10% MTT was added to each well and incubated at 37°C for 4 to 5 h in the dark. Formazan crystals were then dissolved in DMSO (100  $\mu$ l per each well). The absorbance this solution was measured at 570 nm by an ELISA reader (Wang et al. 2020). Finally, the % viability was calculated using the following formula:

$$\text{Viability\%} = \frac{(\text{absorbance of treated cells})}{(\text{absorbance of control cells})} \times 100$$

Formula 1

## Preparation and characterization of PLA-PEG/Chitosan-FA/DNA nanoparticles

PLA-PEG/Chitosan-FA/DNA nanoparticles were measured by the method developed by (Perez et al. 2001). Thirty mg of PLA-PEG copolymer, 500 g of plasmid pEGFP-N1, and different amounts of chitosan-folic acid (3, 6, 15 and 30 mg) (ratios of 2:30, 6:30, 15: 30 and 30:30 of PLA-PEG to chitosan-folic acid) were under sonication at 0°C for 30 s. The resulting solution was emulsified in 1.5 ml of 1.5% polyvinyl alcohol (PVA) solution under sonication at 0°C for 60 s. After that, 25 ml of 3% PVA solution was added to the samples and underwent vigorous stirring at room temperature for 3 h. Then, the resulting

nanoparticles collected were centrifuged at 15000 rpm for 1 h at 4°C and washed several times with deionized water. Finally, the nanoparticles were dried by lyophilization and stored in a -20° C.

### **Investigating the DNA release pattern from PLA-PEG/Chitosan-FA nanoparticles**

One mg of PLA-PEG/Chitosan-FA/DNA nanoparticles was dissolved in 10 ml of PBS buffer (pH = 7.4). Then, it was placed on a shaker at 45 rpm and 37°C. The nanoparticles were then separated for 3, 7, 14 and 28 dby centrifugation at 15000 rpm for 1 h at 4°C. Finally, the supernatant was measured by a 260 nm spectrophotometer.

### **MCF-7 cells transformation using PLA-PEG/Chitosan-FA/DNA nanoparticles**

MCF-7 cells with a density of 20000 cells per ml of RPMI-1640 culture medium containing 10% FBS were transferred to each of the 24-well plate wells. Then they were stored at 37°C and 5% CO<sub>2</sub> in the dark. One ml of each PLA-PEG/Chitosan-FA/DNA nanoparticle was added to each well independently in three replications. Cells treated with PLA-PEG/Chitosan-FA/DNA nanoparticles, and were stored at 37° C and 5% CO<sub>2</sub> for 7 h. The cell supernatant was then replaced with a new RPMI-1640 medium containing 10% FBS and kept for 48 h. To evaluate the transfection efficiency of PLA-PEG/Chitosan-FA/DNA nanoparticles, a fluorescence microscope and fluorescence device were used.

### **Statistical analysis**

All quantitative traits were performed in three replications. Mean comparison was performed using Duncan's multi-range test at a 5% probability level. The statistical analysis was carried out using SPSS 16 statistical package and Excel application software. The standard deviation of means was then calculated from the average of each treatment.

## **Results And Discussions**

### **Synthesis of chitosan-folic acid (Chitosan-FA)**

The results of chitosan as well as chitosan attached to folic acid are shown in Figure. 1 (image A and B). The results showed that there was a significant difference in the morphology of free chitosan and chitosan attached to folic acid. The results show that free chitosan has a spindle-shaped and a relatively uniform distribution. The size of nanoparticles obtained from 100 kDa chitosan was about 20-40 nm. The morphology of chitosan-derived nanoparticles and chitosan-folic acid nanoparticles were significantly different as well. The nanoparticles made from chitosan-folic acid exhibited high self-aggregation and cluster formation.

### **Confirmation of chitosan-folic acid synthesis using TGA**

Thermal Gravimetry Analysis (TGA) is a method that records the weight changes of materials as a function of time or temperature in a controlled environment. In this method, heat is applied to the material

at a specific temperature and in one environment. This method is used to identify and determine the amount of volatiles. In the range of 300 to 400° C, the TGA diagram generated from chitosan showed only one weight loss phase, as shown in Figure. 3, image (C). It shows that chitosan destruction is straightforward and just requires one step. In addition, the TGA device was used to record the weight variations of folic acid in a constant environment and at different temperatures. The results showed that weight loss was detected in folic acid at temperatures lower 200° C, which is likely resulted from loss of water molecules in folic acid. At temperatures over 200° C, folic acid degradation was more severe, with about 50% of its weight being reduced between 250 and 350° C. At temperatures ranging from 350 to 600° C, the third stage of weight loss was observed. In general, the process of folic acid weight loss was observed in three stages of 100 to 200°C, 250 to 350°C, and 350 to 600°C with increasing temperature at the same atmospheric pressure (Figure. 1, image C).

Unlike chitosan, the weight loss trend in chitosan-folic acid nanoparticles included 3 to 4 stages of weight reduction. At temperatures ranging from 120 to 250° C., about 5% of the weight was reduced. In addition, in the temperature range of 250 to 350° C, the second weight loss phase was observed, in which more than 50% of the weight was reduced. In the temperature range above 400° C, another weight loss was observed. Considering that the weight loss process in chitosan-folic acid nanoparticles is somewhat in the middle of the weight loss process of chitosan and folic acid, it can be concluded that the synthesis of chitosan-folic acid nanoparticles has been done correctly (Figure. 1, image C).

### **Confirmation of chitosan-folic acid synthesis**

Because the binding of folic acid to chitosan causes many changes in the amount of chitosan absorption, UV spectroscopy of chitosan-folic acid nanoparticles was Figure 1, image D shows that the binding of folic acid to chitosan increases absorption in the 290 nm range, whereas, chitosan uptake was very low in this range.

### **Confirmation of chitosan-folic acid nanoparticle synthesis using FTIR spectroscopy**

The FT-IR spectra of the synthesized chitosan-folic acid nanoparticles are shown in Figure. 1, image E. The peaks observed in the range of 3320 are associated with the O-H and N-H functional groups in chitosan. Furthermore, the peaks in the 2830-2900 range are linked to the C-H functional groups in chitosan. Also, the peak in the 1621 range is related to the amide I in chitosan, whereas the peak in the 1530 range demonstrates N-H binding of the amide groups and primary amine group's acrylates in chitosan. Furthermore, the O-H and C-H bonds of the chitosan circular structure are visible in the 1386 and 1322 ranges, respectively, and 1010 range indicates C-O binding in chitosan.

The bands observed in pure folic acid in the range of 3421 to 3600 pertain to the hydroxyl (OH) group of glutamic acid and the NH-group of the pteridine ring. Also, The C=O bond in folic acid is related to the strong band observed in the 1730 range, and the NH-bond in folic acid is related to the band observed in the 1607 range. The intensity of bonds related to C=O binding related to the amide I group in the range of 1010, as well as bands related to C-N bonds with N-H related to amide II in chitosan, decreased after folic

acid binding. The hydrophilic interaction between C = O, C-N, and N-H in amide I and amide II causes the strength of peaks related to folic acid and chitosan bonds to decrease. The FTIR spectra of chitosan showed no bands in the range of 2000 to 2200 (Figure 1, image E). ). However, after binding of chitosan to folic acid in this range, bands corresponding to folic acid and chitosan binding were seen. The above results are in agreement with the findings of (Pinto et al. 2021).

### **Evaluating the ability of PLA-PEG/Chitosan-FA and DNA nanoparticles to neutralize negative DNA charges**

The results of the ability of PLA-PEG/Chitosan-FA nanoparticles to neutralize DNA negative charge showed that the speed of DNA movement in an agarose gel was gradually lowered by increasing the chitosan-folic acid ratio in PLA-PEG/Chitosan-FA/DNA nanoparticles compared to control DNA (uncoated DNA). DNA fully ceased moving at a PLA-PEG to chitosan ratio of 30 to 15, indicating that PLA-PEG/Chitosan-FA/DNA nanoparticles may neutralize DNA's negative charge (Figure 2).

### **Investigating the size and zeta potential characteristics of PLA-PEG/Chitosan-FA/DNA nanoparticles by DLS device**

Increasing the amount of chitosan-folic acid in PLA-PEG/Chitosan-FA/DNA nanoparticles increased the amount of zeta-positive potential. Also, the results showed that the PLA-PEG/Chitosan-FA/DNA nanoparticles with the lowest percentage of chitosan-folic acid had the lowest zeta potential, whereas PLA-PEG/Chitosan-FA/DNA nanoparticles with the highest percentage of chitosan-folic acid had the highest zeta potential. On the other hand, the effect of increasing the percentage of chitosan-folic acid in PLA-PEG/Chitosan-FA/DNA nanoparticles on the size of nanoparticles was due to other factors studied by DLS. The results showed that despite the zeta potential, there was no link between increasing the percentage of chitosan-folic acid in PLA-PEG/Chitosan-FA/DNA nanoparticles and particle size. In addition, PLA-PEG/Chitosan-FA/DNA nanoparticles with the lowest percentage of chitosan-folic acid had the highest particle size. On the other hand, increasing the percentage of chitosan-folic acid in PLA-PEG/Chitosan-FA/DNA nanoparticles did not result in the same direct reduction in nanoparticle size. The size of PLA-PEG/Chitosan-FA/DNA nanoparticles increased again when the ratio of chitosan-folic acid to PLA-PEG was increased from 30:6 to 30:30 (ratio is not %) (Figure 3, B).

The DNA encapsulation efficiency is shown in Figure 3 (A). According to the results, the PLA-PEG/Chitosan-FA/DNA nanoparticles with the highest chitosan-folic acid ratio (63.82%) had the maximum DNA encapsulation effectiveness. In general, increasing the chitosan-folic acid ratio and DNA loading by PLA-PEG/Chitosan-FA nanoparticles had a direct and significant association. As a result, increasing the chitosan-folic acid ratio in PLA-PEG/Chitosan-FA nanoparticles increased the percentage of DNA loading. The neutralization of DNA negative charge by interaction with amino groups in chitosan could be one of the reasons for the increase in DNA encapsulation efficiency at high chitosan-folic acid ratios because of the negative charge in the DNA phosphate group as well as the carboxyl group in the PLA polymer.

## **Toxicity of PLA-PEG/Chitosan-FA/DNA nanoparticles**

The cytotoxicity of some nanocarriers is a major obstacle to gene delivery systems. Therefore, determining the nanocarriers cytotoxicity is critical (Amani et al. 2019). The comparison of the mean results of the toxicity test of PLA-PEG/Chitosan-FA/DNA nanoparticles by the MTT test showed that the nanoparticles used in this study had very low toxicity (Figure 3, C). So, after treatment with PLA-PEG/Chitosan/DNA nanoparticles at a concentration of 1 mg/ml and PLA-PEG/Chitosan-FA/DNA nanoparticles containing a w/w ratio of 30: 30, (PLA-PEG: Chitosan-FA), MCF-7 cells showed the lowest percentage of viability (87.64%). As a result, increasing the chitosan-folic acid to PLA-PEG ratio in PLA-PEG/Chitosan-FA/DNA nanoparticles had no effect on MCF-7 cell mortality.

## **DNA release pattern from PLA-PEG/Chitosan-FA/ DNA nanoparticles**

The DNA release pattern from PLA-PEG/Chitosan-FA/DNA nanoparticles is shown in Figure 3 (D). The results showed that the DNA release pattern from PLA-PEG/Chitosan-FA/DNA nanoparticles was explosive at first, then gradually decreased. More than 60% of the total amount of DNA released during the 28 days was released in the first three days. With increasing the amount of chitosan-folic acid in PLA-PEG/Chitosan-FA/DNA nanoparticles, DNA release increases significantly. The release rate of DNA was 63.74% after three days of incubation of PLA-PEG/Chitosan-FA/DNA nanoparticles in PBS buffer containing a 1: 30 ratio of PLA-PEG to chitosan-folic acid. However, after three days of incubation in PBS buffer, the amount of DNA released from PLA-PEG/Chitosan-FA/DNA nanoparticles containing equal ratios of PLA-PEG to chitosan-folic acid was only 82.16%. The results of DNA release from different nanoparticles used in this study showed that more than 60% of the total DNA released was released explosively in the first three days after nanoparticles were treated with PBS buffer.

## **MCF-7 cells transformation using PLA-PEG/Chitosan-FA/DNA nanoparticles**

Fluorescence microscope image results showed that in some MCF-7 cells treated with PLA-PEG/Chitosan-FA/DNA nanoparticles, green fluorescence was observed (Figure 4, A, B, C and D). This demonstrated the ability of PLA-PEG/Chitosan-FA/DNA nanoparticles to transmit and release DNA within MCF-7 cells. However, the control DNA, was unable to transfer to MCF-7 cells. An electrostatic repulsion is formed between DNA and the cell membrane due to the negative charge in DNA and the cell membrane due to the presence of phosphorus groups in the structure of DNA, as well as the phospholipid membrane of cells, which inhibits DNA from penetrating into the cell. In addition, the appropriate structure and large size of DNA are other factors in the inability of control DNA to transmit to the cell. The results also indicated that increasing the ratio of chitosan-folic acid to PLA-PEG in PLA-PEG/Chitosan-FA/DNA nanoparticles increased gene transfer efficiency in a linear and significant manner. In a way, the efficacy of GFP gene transfer to MCF-7 cells was improved by increasing the ratio of chitosan-folic acid to PLA-PEG in PLA-PEG/Chitosan-FA/DNA nanoparticles. Cells treated with PLA-PEG/Chitosan-FA/DNA nanoparticles comprising equal proportions of PLA-PEG and chitosan-folic acid had the highest gene expression (38.22%). According to the results, the PLA-PEG/Chitosan-FA/DNA nanoparticles with the lowest chitosan-folic acid to PLA-PEG ratio (30: 2 PLA-PEG: Chitosan-FA) had the lowest gene transfer

efficiency (34/18%). Gene expression efficiencies were 23.2% and 36.5% in PLA-PEG/Chitosan-FA/DNA nanoparticles having 30:6 and 30:15 (PLA-PEG: Chitosan-FA) ratios, respectively (Figure 4, E).

## Discussions

According to the results of the present study, it was found that free chitosan has a spindle-shaped and relatively uniform distribution. Also, the size of nanoparticles obtained from 100 kDa chitosan was about 20–40 nm. These results are in agreement with the findings of (Wang et al. 2011). Also, binding of folic acid to chitosan increased the uptake in the range of 290 nm, while chitosan uptake was very low in this range. These results are in agreement with the findings of (İnce et al. 2020). The results in Fig. 3 (B) showed that increasing the amount of chitosan-folic acid in PLA-PEG/Chitosan-FA/DNA nanoparticles increased the amount of zeta-positive potential. In fact, the increase in zeta potential in PLA-PEG/Chitosan-FA/DNA nanoparticles with a high percentage of chitosan-folic acid was not unexpected and was consistent with the expected results due to the presence of the amine group in chitosan. In fact, Surface charge and nanoparticle size are two key parameters that influence gene transfer efficiency and nanoparticle stability. Therefore, by electrostatic interaction of particles with cell membranes, increasing the positive potential of particles, in addition to causing repulsion between co-charged particles and preventing their accumulation, increases the efficiency of gene transfer (Shim et al. 2013). Lack of complete DNA compression at low Chitosan-FA ratios as well as free Chitosan-FA accumulation in solution around PLA-PEG/Chitosan-FA/DNA nanoparticles can be considered as two size enhancers at low and high ratios of Chitosan-FA (Dufès et al. 2005). Many researchers have been drawn to biodegradable polymers like PLA because of their biocompatibility and biodegradability. They stated that the hydrophobicity of PLA polymers causes the accumulation of proteins in the plasma around the nanoparticles, resulting in an increase in the size of the nanoparticles. Finally, the ability of the immune system to detect and remove these nanoparticles will increase. On the other hand, the low efficiency of PLA polymers in DNA encapsulation is another obstacle to the use of these nanoparticles in gene therapy (Perez et al. 2001; Abebe et al. 2015). In fact, it seems that the presence of a positive charge in chitosan and the neutralization of the negative charge of DNA by it, has reduced the repulsion between DNA and PLA, as a result of which the rate of DNA encapsulation has increased. The above results are in agreement with the findings of (Messai et al. 2005; Bravo-Anaya et al. 2016, 2019; Amani et al. 2019). It is stated that the electrostatic interactions between cationic polymers and the cellular anionic surface are of great importance for the adsorption of DNA-carrying nanoparticles into cells. However, these membrane interactions may cause direct damage to target cells (Chen et al. 2007). Cationic polymers used in gene transfer such as PEG and chitosan are among the pH sensitive polymers. These polymers are protonated in an acidic environment and will have a more positive charge. The presence of amino group charges on the surface of cationic polymers such PEG actually improves their interaction with cell membranes. Also, Chitosan possesses a favorable positive charge for gene transfer to eukaryotic cells due to the presence of amine groups in its structure. Furthermore, no toxicity has been reported due to the biocompatibility and biodegradability of this polysaccharide. As a result, in our study, increasing the ratio of chitosan-folic acid to PLA-PEG in PLA-PEG/Chitosan-FA/DNA nanoparticles had no effect on the lethality of MCF-7

cells. A similar observation had been reported by other researchers (Amani et al. 2019; Baghaei et al. 2021). For example, Amani *et al.* reported that in MCF-7 cells, PLA-PEG-PLA copolymer, (PLAPEG-PLA)-DNA, and PLA-PEG-PLA/PEI/DNA nanoparticles showed almost no cytotoxicity (cell viability > 83%). Also, Baghaei *et al.* reported that the chitosan nanoparticles were not toxic to the cells (the percentage of cell survival was about 90%), which is consistent with the results of the present study. Furthermore, our study indicates that the PLA-PEG/Chitosan-FA/DNA nanoparticles are safe carriers, which can be due to the incorporation of Chitosan-FA into the encapsulated PLA-PEG/Chitosan-FA/DNA nanoparticles. Also, the results showed that more than 60% of the total amount of DNA released during the 28 days was released in the first three days, and with increasing the amount of chitosan-folic acid in PLA-PEG/Chitosan-FA/DNA nanoparticles, DNA release increases significantly. Due to the hydrophilicity of chitosan, the creation of a water channel inside the nucleus of nanoparticles by these nanoparticles has allowed the hydrophobic core of PLA to hydrate more quickly. As a result, the solubility of nanoparticles increased, leading to nanoparticle disintegration and DNA release. The effect of combining hydrophilic polymers with fragment polymers that have both hydrophilic and hydrophobic components on DNA release rate was observed. In other words, the hydrophilic polymers have been shown to accelerate the rate of DNA release by forming water channels within the matrix. Recent studies (Amani et al. 2021; Guo et al. 2021; Mateti et al. 2021) have reported the ability of copolymers containing PLA and PEG components to control drug release, which is in support of the results of this study. The ability to penetrate and transport PLA-PEG/Chitosan-FA/DNA nanoparticles into MCF-7 cells was demonstrated using fluorescent microscopy images (Fig. 4, A, B, C and D). Given that the premise for gene transfer by these nanocarriers is dependent on the phenomena of endocytosis, and that intracellular nucleases destroy more than 99 percent of the DNA entering the cell, as a result, in gene therapy research, protecting DNA from harmful enzymes is critical [33]. Previous studies have demonstrated that polycations constitute a physical barrier to restriction enzyme binding due to their complexation with DNA, therefore shielding DNA from digestion by these enzymes [34]. The ability of PLA-PEG/Chitosan-FA/DNA nanoparticles to penetrate and safely release DNA into MCF-7 cells was demonstrated by fluorescent microscopy images of MCF-7 cells treated with these nanoparticles (Fig. 4, E). The GFP gene was observed to emit green fluorescence inside the cells. It appears to be the proper interaction of PLA-PEG/Chitosan-FA/DNA nanoparticles with cell membranes due to the neutralizing of negative DNA charge by chitosan that is one of the most important reasons for MCF-7 cell transfection by these nanoparticles.

## Conclusions

The objective of this research was to evaluate the multifunctional micelle system for gene transfer that has both the benefits of cationic, biodegradable and dual-friendly polymers. The results of the study showed that folic acid, chitosan, PLA, and PEG protected DNA from enzymatic digestion damage. In addition, they are biocompatible and improved the release of DNA. Furthermore, the results indicated that PLA-PEG/Chitosan-FA/DNA nanoparticles had a high ability to transfer genes to MCF-7 cells.

## Declarations

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## Competing Interests

The authors have no relevant financial or non-financial interests to disclose.

## Author Contributions

All authors contributed to the study conception and design. Material preparation, data collection and analysis were performed by Mehdi Afrouz, Farnaz Ahmadi-Nouraldinvand, Yahia yaghooti ajirlu, Fateme Arabnejad, Sabry G Elias, Hadi Eskanlou and HashemYaghoubi. The first draft of the manuscript was written by Mehdi Afrouz and all authors commented on previous versions of the manuscript. All authors read and approved the final manuscript.

## Data Availability

The datasets generated during and/or analysed during the current study are available from the corresponding author on reasonable request.

## Ethics approval

This is an observational study. The University of Mohaghegh Ardabili Research Ethics Committee has confirmed that no ethical approval is required.

## Consent to participate

We had no case for this research

## Declaration of interests

The authors declare no conflicts of interest.

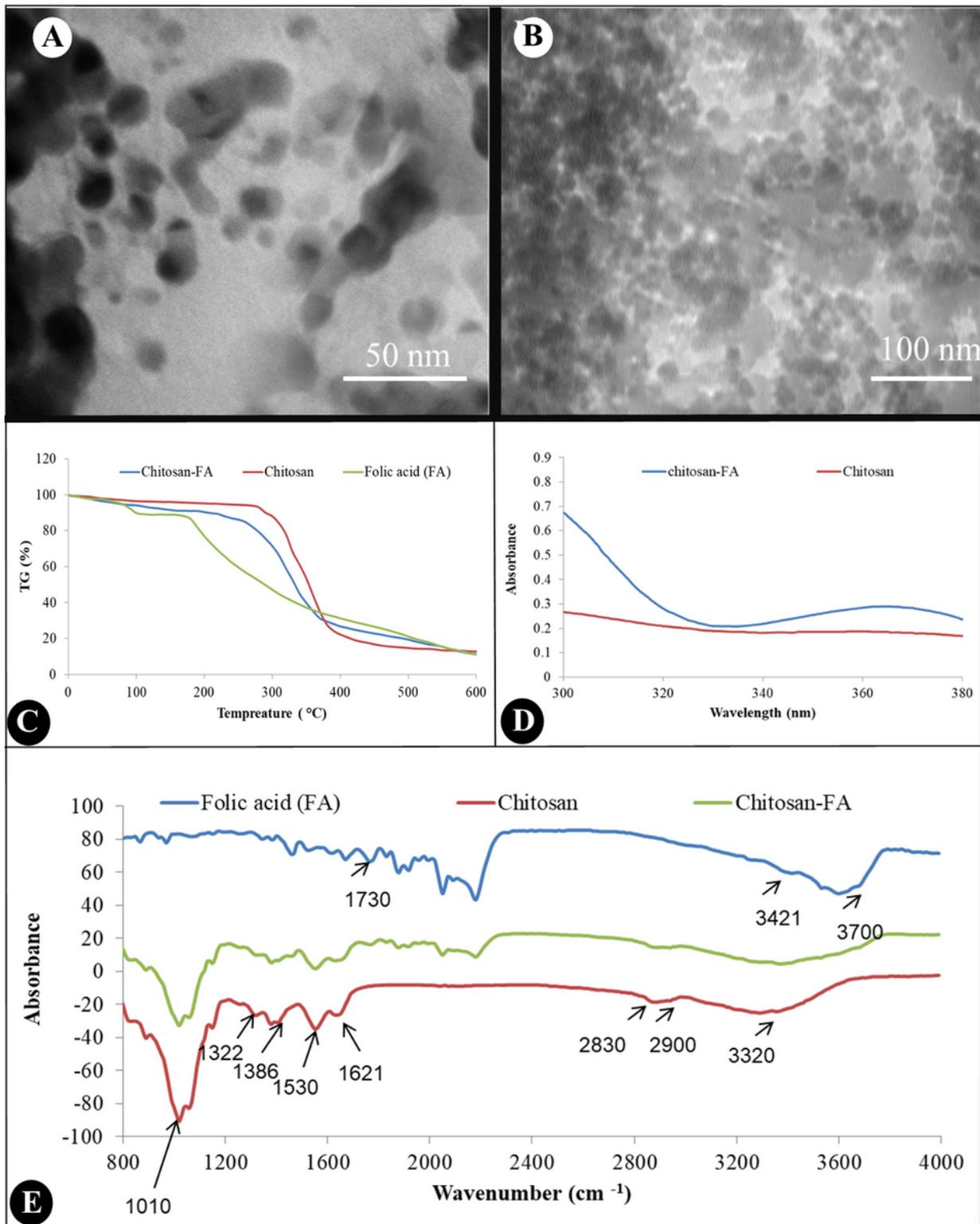
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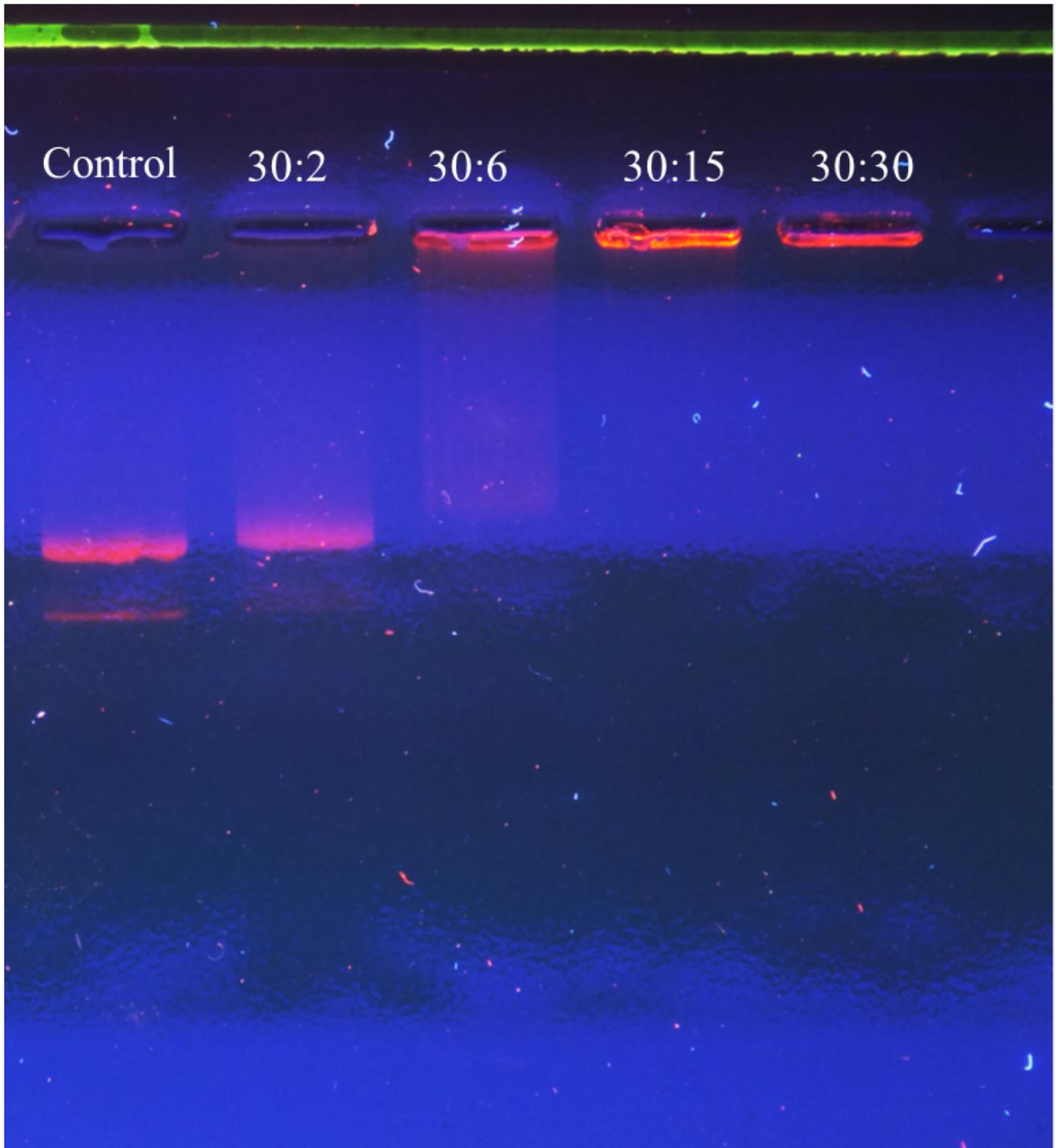
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## Figures



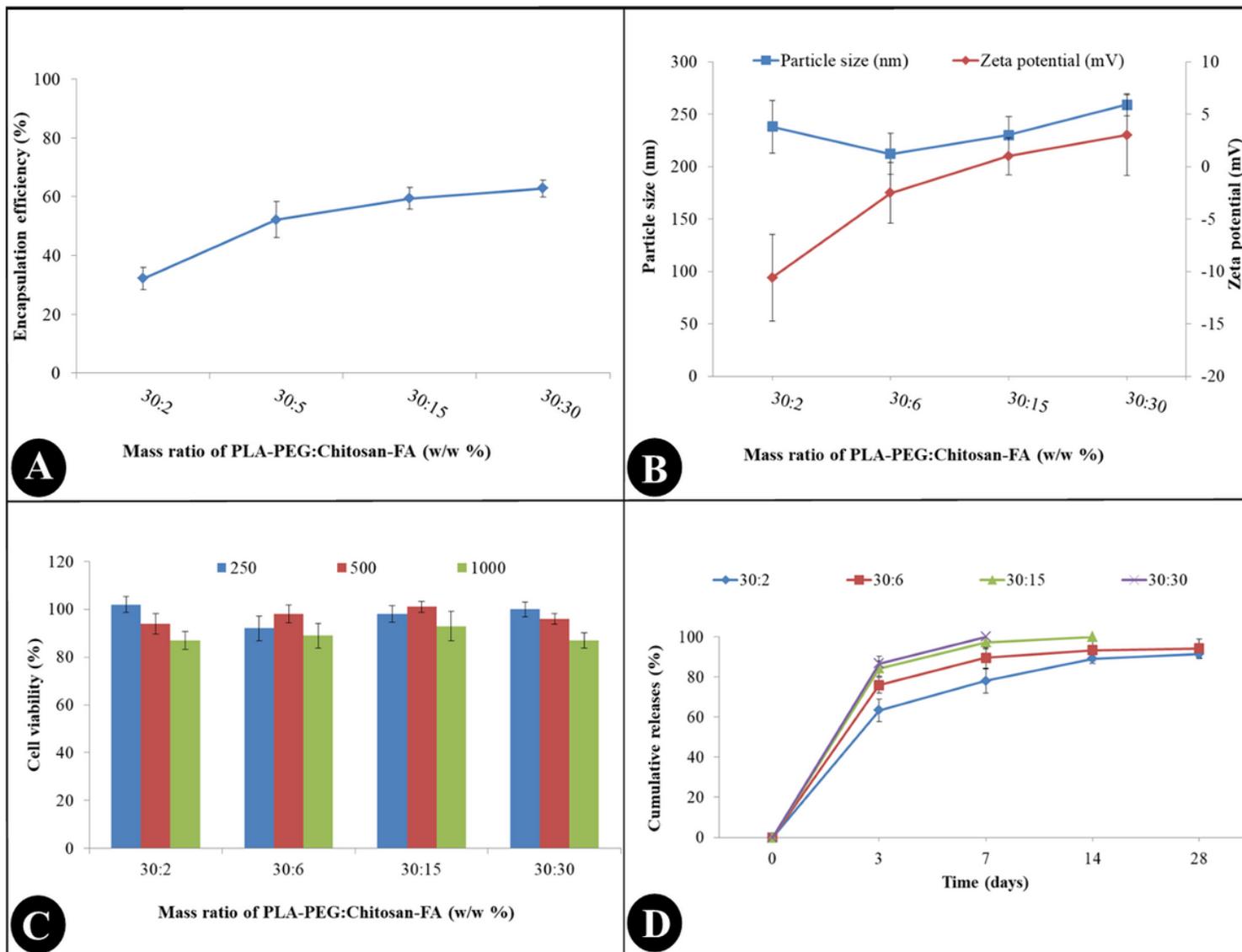
**Figure 1**

TEM images (A and B), UV spectroscopy (Figure D), TGA diagram (Figure C) and FTIR spectroscopy of chitosan-folic acid, chitosan and folic acid nanoparticles (Figure E).



**Figure 2**

The first well of control DNA. The second to fifth wells of PLA-PEG/Chitosan-FA/DNA nanoparticles in different ratios of PLA-PEG to chitosan-folic acid (PLA-PEG ratio is 30, while the ratio of chitosan-folic acid is variable and is between 2 to 30).



**Figure 3**

(A) The effect of different ratios of chitosan-folic acid on PLA-PEG/Chitosan-FA nanoparticles on DNA encapsulation efficiency ( (B) The effect of different ratios of PLA-PEG and chitosan-folic acid on the size and potential of PLA-PEG/Chitosan-FA/DNA nanoparticles ( (C) Comparison of the effect of increasing chitosan-folic acid ratio in PLA-PEG/Chitosan-FA/DNA nanoparticles on MCF-7 cell toxicity ( (D) DNA release pattern from PLA-PEG / Chitosan-FA / DNA nanoparticles containing different ratios of PLA-PEG to chitosan-folic acid at different times.

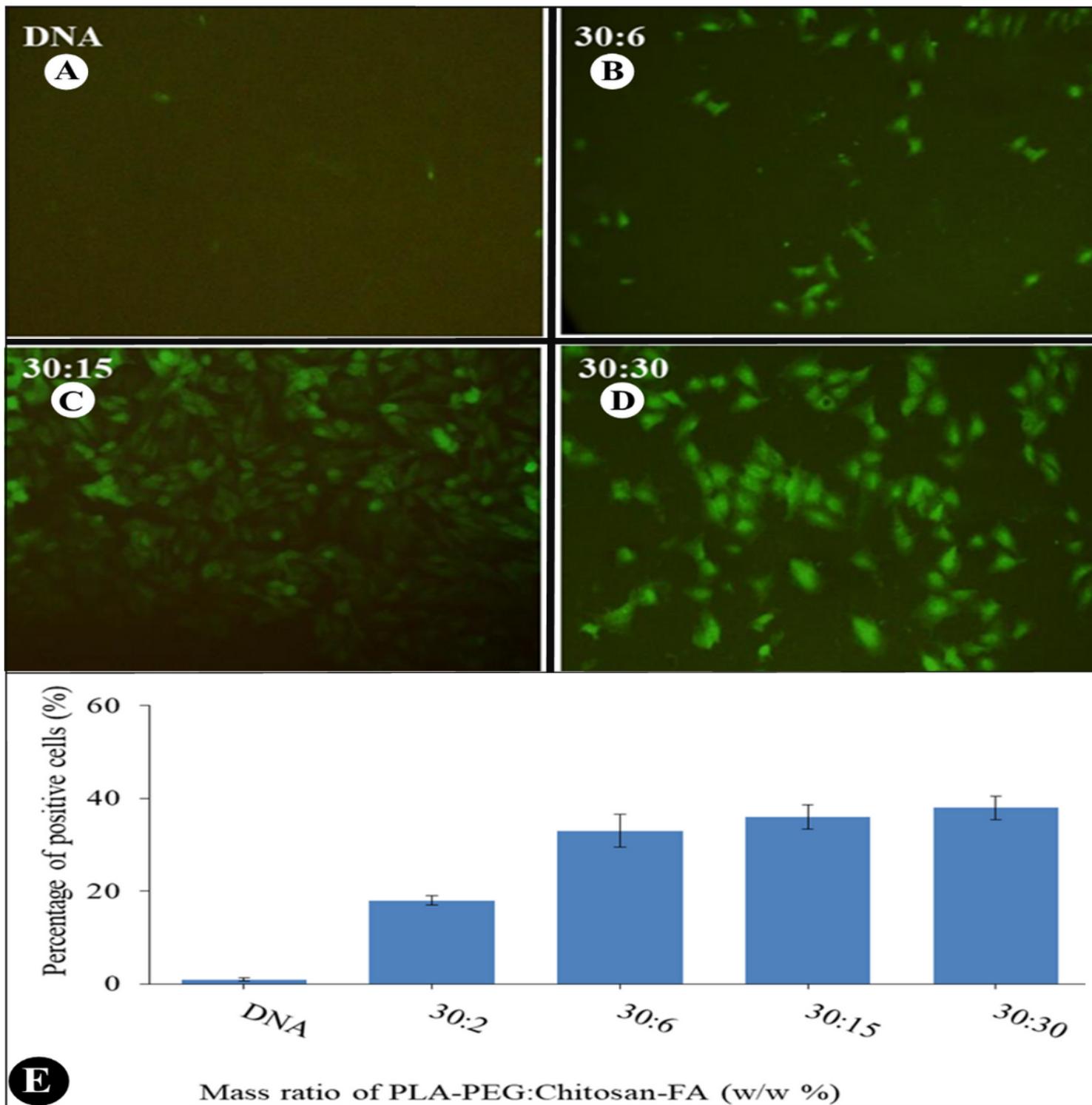


Figure 4

The upper four images (A, B, C, and D) show GFP gene transfer and expression of MCF-7 cells by PLA-PEG/Chitosan-FA/DNA nanoparticles containing different ratios of chitosan-folic acid. The lower graph shows comparison of mean expression of GFP gene in MCF-7 cells by PLA-PEG/ Chitosan-FA/DNA nanoparticles containing different ratios of chitosan-folic acid.

## Supplementary Files

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- [floatimage1.png](#)