Design and preparation of a new multi-targeted drug delivery system using multifunctional nanoparticles for co-delivery of siRNA and paclitaxel

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Abstract

Drug resistance is a great challenge in cancer therapy using chemotherapeutic agents. Administration of these drugs with siRNA is an efficacious strategy in this battle. Here, it was tried to incorporate siRNA and paclitaxel simultaneously into a novel nanocarrier. The selectivity of carrier to target cancer tissues was optimized through conjugation of folic acid (FA) and glucose onto its surface. The structure of nanocarrier formed from ternary magnetic copolymers based on FeCo-polyethylenimine (FeCo-PEI) nanoparticles and polylactic acid-polyethylene glycol (PLA-PEG) gene delivery system. Biocompatibility of FeCo-PEI-PLA-PEG-FA(NPsA), FeCo-PEI-PLA-PEG-Glu (NPsB) and FeCo-PEI-PLA-PEG-FA/Glu (NPsAB) nanoparticles and also influence of PTX-loaded nanoparticles on in vitro cytotoxicity were examined using MTT assay. Besides, siRNA-FAM internalization was investigated by fluorescence microscopy. The results showed the blank nanoparticles were significantly less cytotoxic at various concentrations. Meanwhile, siRNA-FAM/PTX encapsulated nanoparticles exhibited greater cytotoxicity on MCF-7 cells. NPsAB/siRNA/PTX nanoparticles showed greater effect on MCF-7 cell viability than NPsA/siRNA/PTX and NPsB/siRNA/PTX. Also, they induced significantly higher cytotoxic effects on cancer cells compared with NPsA/siRNA/PTX and NPsB/siRNA/PTX due to their multi-targeted properties using folic acid and glucose. We concluded that NPsAB nanoparticles have great potential for co-delivery of both drugs and genes for use in gene therapy and chemotherapy.

Introduction

Cancer has been identified as the second main cause of mortality worldwide , after heart diseases (Sant, Iyer, Gaharwar, Patel, & Khademhosseini, 2013). Chemotherapy is one of the most common treatment procedures for cancer therapy which is used after surgery and in a combination with radiotherapy (Cao et al., 2011; Lv et al., 2014; Zou et al., 2012). Although the chemotherapy has been proved to be useful in many cases, nonetheless, the severity of its side effects and also the drug resistance to these drugs lead to reducing the efficacy of chemotherapeutic agents (Eggenberger et al., 2010; Zintchenko, Philipp, Dehshahri, & Wagner, 2008). During the previous few decades, nanoparticles were employed to optimize the effect of drugs and genes, reduce their side effects and deliver the medicines in a targeted manner for efficient therapy (Hami, Amini, Ghazi-Khansari, Rezayat, & Gilani, 2014a). Folic acid (FA) is an essential nutrient for the human body which can enter cells through folate receptors (FRs) (Zwicke, Mansoori, & Jeffery, 2012). In the human body, folic acid is used in the synthesis of the DNA. Therefore, the expression level of folate receptor in cancer cells is higher compared to normal cells, due to theirs need to high level of folic acid (Alibolandi et al., 2016; Mojica Pisciotti et al., 2014). Glucose is another important nutrient in the human body which provides the energy that is needed in many metabolic functions and synthetic processes of different compounds in cells, serum and tissues (Chatterjee, 2013; Talpur, Echard, Ingram, Bagchi, &

Preuss, 2005). So, glucose transporters (Gluts) are over-expressed on the cell membrane of many malignant cells (Smith, Volkert, & Hoffman, 2005; Qiang Wang et al., 2010). In 1927, Warburg et al. reported that glucose uptake was significantly enhanced in cancer tissues compared to those in normal tissues because of their high metabolic activity (Warburg, Wind, & Negelein, 1927). After that, glucose has been used to target cancer cells for different diagnostic and therapeutic applications (Li, Ma, Dang, Liang, & Chen, 2014; Mamaeva et al., 2016).

It is well known that the targeted endocytosis pathway of nanoparticles is based on the interaction between cell surface receptors and ligands (S. Wang et al., 2013; Xia et al., 2013). Therefore, the efficiency of drug delivery was limited up to saturation of cell surface receptors by specific ligands of nanoparticles. Therefore, it seems that specific targeting of tumor tissues could be improved through simultaneous incorporation of several ligands such as glucose and folic acid inside the structure of nanoparticles. So, in this study it was aimed to optimize the surface of nanoparticles with co-addition of folic acid and glucose.

In recent years, drug resistance was emerged as one of the biggest therapeutic challenges in cancer therapy and so it attracted many research attentions (Bentley-Goode, Newton, & Thompson, 2017; Marques et al., 2014). Simultaneous co-targeting of cancer cells using two or more drugs seems to be a novel and effective approach to overcome the problem of drug resistance (Camirand, Lu, & Pollak, 2002; Q. He, Liu, Sun, & Zhang, 2004; Mancini et al., 2014).

The use of siRNA to suppress expression of oncogenes has been considered as a promising solution for cancer treatment in recent years (Kumar, Yigit, Dai, Moore, & Medarova, 2010; Patil & Panyam, 2009). Although, this method can be helpful lonely, however, due to the complexity of human cancer, other complementary therapies may be needed to improve the efficacy of cancer therapy by siRNA (Peng, Hsu, Lin, Cheng, & Hsu, 2017; X. Z. Yang et al., 2011). Combination therapy of siRNA and chemotherapeutics such as paclitaxel has been considered as a useful strategy for increasing the effectiveness of cancer therapy (Kapse-Mistry, Govender, Srivastava, & Yergeri, 2014).

Various carriers were used to deliver genetic agents. Cationic polymers such as polyethyleneimine (PEI) are of the most common ones and have the ability to interact with RNA and DNA and form complexes with high transfection efficiency. However, studies showed that application of PEI is associated with critical cytotoxicity effects (Abebe et al., 2015b; Moret et al., 2001). In addition, hydrophilic polymers such as PEI have low capacity to use as a nano-vector for delivering hydrophobic and neutral drugs such as PTX into cancer cells.

Several methods have been developed to transfer different compounds using nanoparticles to cancer cells (Amani, Zare, Asadi, & Asghari-Zakaria, 2018; Kircheis et al., 1999). Synthesis of copolymers by binding of hydrophobic polymers such as PLA to cationic hydrophilic polymers allows simultaneous co-delivery of siRNA and PTX to cancer cells.

Biodegradable polymers such as polylactide (PLA), poly(D,L-lactide-co-glycolide) (PLGA), and polyglycolide (PGA) have great application potential as drug carriers due to their good biocompatibility and biodegradability (Lu et al., 2014; Perez et al., 2001a; J. Wang, Xu, Liu, Sun, & Yang, 2016).

Researchers have developed tri-block copolymers using PLA, PEI, and PEG such as tri-block PEI-PLA-PEG copolymers, as potential gene delivery nano-vectors (Abebe et al., 2015a, 2015b; Sim et al., 2017). The hydrophobic PLA segment affords a degree of biodegradability to nanoparticles. It also increases the stability of nano-carrier through charge shielding effects. In recent decades, much study has been done on multifunctional nanoparticle. Multifunctional nanoparticles are attractive for cancer treatment due to high potential to overcome the barriers in various extracellular and intracellular conditions (Chen et al., 2015; Liu et al., 2016; Yan, Li, Li, Zhu, Shen, Yi, Wu, Yeung, Xu, Xu, & Chu, 2014). This study presents multifunctional biodegradable magnetic nanoparticles including PLA, PEG, PEI, FeCo nanoparticles, folic acid and glucose for co-delivery and targeting of siRNA and PTX.

Materials and methods

2.1. Materials

2-Azidoethyl β-D-galactopyranoside was purchased from Synthose Inc. (Canada), polyethyleneimine (Mw 1800 Da) was purchased from Polysciences, Inc. (Germany), polyethyleneimine (PEI), 1.8 kDa, branched was obtained from polyscience (USA), stannous octoate, tetraethyl orthosilicate (TEOS), 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide (EDC), polyvinyl alcohol (PVA), dichloromethane (DCM), MTT, RPMI 1640, DMSO, penicillin/streptomycin solution, N-hydroxylsulfosuccinimide (NHS), fetal bovine serum (FBS) were purchased from Sigma-Aldrich (USA). HO-PEG-COOH was obtained from Rapp Polymere GmbH (Germany). N- hydroxysuccinimide, Folic acid and dicyclohexylcarbodiimide were purchased from Acros (Germany). Agarose gel, PVP, MgCL2, Tris-HCl, and EDTA were purchased from Merck (Germany).

Synthesis of Azido-Functionalized Folate

Preparation of PLA-PEG-FA copolymer was started with the synthesis of OH-PLA-PEG-COOH according to the method described by Hami et al (Hami, Amini, Ghazi-Khansari, Rezayat, & Gilani, 2014b). Briefly, definite amounts of lactide monomers was added to HO-PEG-COOH under dry argon, then the reaction was continued at 140 °C for 24 h in the presence of stannous octoate (Sn(Oct)2) as catalyst. The mixture was dissolved in chloroform and precipitated by a mixture of ether and methanol (v/v= 1:1). Then, the HO-PLA-PEG-COOH obtained as precipitate was dried. This obtained polymer was dissolved in DCM with DCC (1.2 mmol) and NHS (0.6 mmol). The product was stirred at 0 °C and 25°C for 1 h and 24 h respectively. Triethylamine (TEA) and propargylamine (0.6 mmol) were added into the above product. Next, the mixture was stirred at 25°C for another 24 h. The product (HO-PLA-PEG-alkyne copolymer) was precipitated in cold ether, filtered and dried at 30 °C under vacuum. Then, the HO-PLA-PEG-alkyne copolymer (0.1 mmol) was conjugated with acryloyl chloride (C3H3ClO) (0.2 mmol) in dry toluene containing triethylamine (C6H15N) (0.2 mmol). The sample was stirred for 10 h at 80 °C. After that, the product was cooled to 25°C, filtered and precipitated in n-hexane then dried in vacuum oven to produce acrylate-PLA-PEG-alkyne copolymer. Acrylate-PLA-PEG-Folic acid and Acrylate-PLA-PEG-Glucose were synthesized stepwise by the addition of Acrylate-PLA-PEG-alkyne copolymer (0.1 mmol) and Azido-functionalized folate and Azido-functionalized glucose in 30 mL aq. NH4HCO3 (10 mM) separately. Then, sodium ascorbate and CuSO4 solutions were added to acrylate-PLA-PEG-alkyne copolymer and stirred with a rotator at 25 °C for 24 h. Next, the product was filtered with a 0.22 μm membrane filter. The reaction solutions were diluted with sufficient NaCl aqueous solution, and then extracted several times by dichloromethane. The resulting products were concentrated under vacuum and precipitated in cold ether. Finally, acrylate-PLA-PEG-FA copolymer and acrylate-PLA-PEG-Glu copolymers were dried under vacuum.

Synthesis of FeCo nanoparticles

First, FeO(OH) and Co3O4 in appropriate amount were dissolved in Trioctylamine (TOA), then the mixture was loaded into a flask and dehydrated at 160 °C for 2 h under a flow of argon. Afterward, the mixture was quickly heated to 370 °C while vigorous stirring is maintained using a mechanical stirrer. Next, the product was cooled to room temperature and collected by centrifugation at 10,000 rpm. Finally, the product washed with hexane and ethanol, and dried under vacuum to obtain FeCo nanoparticles (FeCoNPs).

Synthesis of FeCo@SiO2-SS-PEI nanoparticles

To synthesize the FeCo@SiO2-SH, 200 mg of FeCo NPs dispersed in ethanol by ultrasound, followed by sequential addition of 1 mL NH3.H2O, 20 mL TEOS and 1 mL MPTMS in the ultrasound for 3 hours (Zhang, 2014).

Then, the nanoparticles were collected using magnet, and washed with ethanol and water for 5 times and then the magnetic nanoparticles were dried under vacuum at 25 ºC. To synthesize FeCo@SiO2-SS-COOH nanoparticles, 200 mg of Sulfhydrylated FeCo nanoparticles was dispersed in 12 mL methanol, and then, the 2- carboxyethyl 2-pyridyl disulfide (200 mg) was added and reaction performed for 36 hours, the obtained nanoparticles were washed and then dried as mentioned above. Finally, to synthesize FeCo@SiO2-SS-PEI nanoparticles, 200 mg of FeCo@SiO2-SS-COOH nanoparticles was dispersed in 16 mL PBS buffer (pH =

7.4) and then, the activation of the carboxyl groups was performed with addition of 200 mg of EDC and 100 mg NHS at 25 C^{o} for half an hour. After that, 4 mL PBS buffer containing 200 mg PEI (1800 Da) was added and stirred at 25 C^{o} for 2 days. The resulting nanoparticles were washed with distilled water and ethanol, then the nanoparticles were lyophilized (Lifilizator Alpha model 1-2 LD plus, Christ, Germany).

Synthesis of FeCo@SiO2-SS-PEI- PLA -PEG-FA and FeCo@SiO2-SS-PEI-PLA-PEG-Glu and FeCo@SiO2-SS-PEI-PLA-PEG-Glu-FA

FeCo@SiO2-SS-PEI, acrylate-PLA-PEG-FA and acrylate-PLA- PEG-Glu were dissolved in 6ml of chloroform, separately. The chloroform solution of acrylate-PLA-PEG-FA and acrylate-PLA-PEG-Glu was added dropwise into FeCo@SiO2-SS-PEI solution separately. Then the mixture was stirred for 24 h at 45 °C and stirred for 24 h. The resulting nanoparticles were washed with distilled water and ethanol, then the dispersion was centrifuged at 11068 ×g for 30 minutes and the supernatant was removed. The nanoparticles were lyophilized. To synthesize FeCo@SiO2-SS-PEI-PLA-PEG-Glu-FA nanoparticles, the same amounts of acrylate-PLA-PEG-FA and acrylate-PLA-PEG-Glu were added into FeCo@SiO2-SS-PEI solution. Then the FeCo@SiO2-SS-PEI-PLA-PEG-Glu-FA nanoparticles were synthesized as protocol mentioned above.

Encapsulation of siRNA-FAM and PTX into NPsA, NPsB and NPsAB

The drug-loaded nanoparticles NPsA/siRNA/PTX, NPsB/siRNA/PTX and NPsAB/siRNA/PTX NPsAB/siRNA/PTX were prepared based on the previous report with minor modification (Perez et al., 2001a).

Briefly, to synthesis of NPsA/siRNA/PTX, 0.5 ml water solution containing siRNA-FAM (250µg) was added dropwise into 4 ml of aceton/DCM solution containing NPsA (30 mg) and PTX (5 mg)The reaction mixture was sonicated on ice for 30 seconds (Sonicator® XL, Misonix, NY), then slowly added to 6 mL of PVA solution (1% w/v) and sonicated again for two minutesNext, the reaction mixture was poured dropwise into a stirred PVA solution (30ml, 0.3% w/v).

After 5 min, the solvent (DCM and acetone) from the nanoparticles fraction was evaporated using a rotary vacuum evaporator. Then, the NPsA/siRNA/PTX were collected were collected from the solution by centrifugation at 15000 rpm (16602 ×g) for 30 min (Sigma 1-14K), and after washed several times with deionized water to remove the unloaded drug, the the resultant were filtered using a 0.1 and 0.45 micrometer membrane filter to remove large scattering particles and free NPsA, and finally, the product was lyophilized. The NPsB/siRNA/PTX and NPsAB/siRNA/PTX were also prepared as described above.

The encapsulation efficiency of siRNA-FAM and PTX in nanoparticles was determined by measuring the amount of siRNA-FAM and PTX that was not encapsulated in nanoparticles. Therefore, the amount of siRNA-FAM and PTX in the supernatant of the nanoparticle were measured using spectrophotometer at 490 (siRNA-FAM) and 230 (PTX) nm respectively. Then, the amount of siRNA-FAM and PTX in the supernatant was compared with the total amount of siRNA-FAM and PTX used in the encapsulation process (Son & Kim, 2010). encapsulation efficiency (EE) of siRNA-FAM and PTX was determined as follow Eq. (1):

$$(\%\text{EE}) = \frac{Amount\ of\ used\ drug - Amount\ of\ drug\ in\ supernatant}{\text{Amount of used drug}} \times 100 \quad (1)$$

The PTX and siRNA release kinetics from the nanoparticles was assessed in PBS buffer (pH 7.4). The nanoparticles were treated separately with 5 ml of PBS buffer (1 mg/ml) then, collected via centrifugation (16602×g for 30 minutes) at predetermined time intervals (i.e. 30 minutes - 30 days after inoculation). After each collection, the supernatants were employed to determine the amount of siRNA and PTX released from the nanoparticles. Afterwards, nanoparticles were re-suspended in 5 ml of fresh PBS buffer (pH 7.4), and incubated at 37 °C until the next scheduled time interval. The total DNA release ratio for each sample was calculated using the following Eq. (2):

$$\text{Cumulative Release } (\%) = \frac{\text{Total drug content in supernatant}}{\text{The amount of encapsulated drug}} \times 100 \quad (2)$$

Characterization of nanoparticles

The morphology of NPsA/siRNA/PTX, NPsB/siRNA/PTX and NPsAB/siRNA/PTX nanoparticles were examined using transmission electron microscopy (TEM, JEM-2100).

The average size, zeta potential and magnetic properties of nanoparticles were investigated using dynamic light scattering (DLS, Malvern Instruments, Westborough, MA, USA) and vibrating sample magnetometer (VSM, a Standard 7403 Series, Lakeshore) respectively.

In vitro cytotoxicity and transfection assay

The in vitro cytotoxicity assay for blank nanoparticles (NPsA, NPsB and NPsAB) and siRNA-FAM/PTX encapsulated nanoparticles (NPsA/siRNA/PTX, NPsB/siRNA/PTX and NPsAB/siRNA/PTX) was evaluated on MCF-7 cell lines. The cells were seeded in 96-well plates at a density of approximately 7000 cells per well, in 100 µL of complete growth medium (RPMI 1640 culture medium supplemented with 1% Penicillin/Streptomycin and 10% FBS) and were incubated at 37°C in 5% CO2 for 24 hours. Then the cells were exposed to various concentrations of the blank nanoparticles (100, 200, 400, 600, 800 and 1000 µg/ml) for 48 hours at 37°C. Next, each well was supplemented with 20 µL of MTT solution (5 µg/mL in culture medium) for 5 hours at 37°C. The supernatant was removed and 200 µl of dimethyl sulfoxide (DMSO) was added per well to dissolve the Formazan crystals. Following this step, the plates were incubated at 37°C for 30 minutes. Next, the absorbance level at 570 nm was recorded by a multiwall plate reader (BioTek Instruments; Winooski, VT, USA) (Qian Wang et al., 2017). Finally, the percentage for the cell viability was determined according to the following Eq. (3):

$$\text{Cell viability } (\%) = \frac{OD \text{ of each sample at } 570 \text{ nm}}{OD \text{ of negative control at } 570 \text{ nm}} \text{x} 100 \quad (3)$$

The in vitro cytotoxicity assay for the nanoparticles encapsulated with siRNA-FAM/PTX (NPsA/siRNA/PTX, NPsB/siRNA/PTX and NPsAB/siRNA/PTX) was evaluated with equal amount of PTX (equivalent PTX concentration of 40 nM for each sample) in each sample on the MCF-7 cell lines as described above. To investigate the transfection ability of nanoparticles, the cells were incubated with NPsA/siRNA/PTX, NPsB/siRNA/PTX and NPsAB/siRNA/PTX, then were washed twice using PBS, and the siRNA-FAM transfection efficiency was determined using an inverted-fluorescent microscope (Nikon TE200).

Results and discussion

Synthesis of PLA-PEG-FA and PLA-PEG-Glu copolymers

The chemical structure of the PLA-PEG-FA and PLA-PEG-Glu copolymers were examined with proton nuclear magnetic resonance spectroscopy (1H NMR). Fig. 1 showed the 1H-NMR spectrum of PEI-PLA-PEG-FA copolymer, where the presence of CH2 protons of PEG in PEI-PLA-PEG-FA copolymer was observed around 3.6 ppm (-CH2CH2O-). Moreover, the appearance of signals at 5.1 and 1.6 ppm are attributed to the methine (CH) protons and methyl protons – (CH3) of PLA, respectively (Fig. 1 A) (Heald et al., 2002; Sim et al., 2017).

The appearance of chemical shifts at 6.5-8.6 ppm (aromatic protons associated with FA) and 7.9 ppm (s, weak, 1H, triazoles) provided evidence for successfully obtaining PEI-PLA-PEG-FA copolymer (S.-J. Yang et al., 2010)(Fig. 1 A).

Also in the Fig. 1 B The multiple sharp peaks at 3.6-3.8 ppm and weak peak at 5.4 ppm which were assigned to methylene protons in PEG and aromatic proton in glucose respectively, appeared on the spectrum after the reaction of PLA-PEI with PEG-Glu (Fig. 1 B)(Sadeghi et al., 2015).

The structures of PLA-PEG-FA and PLA-PEG-Glu were ascertained by the FTIR spectrum (Fig. 2). The sharp peak appearing at 1767 cm-1 was assigned to the carbonyl (C=O) group in the PLA-PEG copolymer (Xiong et al., 2011).

The peaks that appeared in the regions of 1193 cm-1 and 1460 cm-1 are related to stretching C-O and the bending of -CH2- groups in PLA-PEG copolymer, respectively (Amani, Kabiri, Shafiee, & Hamidi, 2019;

Danafar, Rostamizadeh, Davaran, & Hamidi, 2017).

Moreover, the peaks at 1640 and 1095 cm-1 corresponds to the C=O and C-O-C stretching of COOH in the FTIR spectrum of PEG, PLA- PEG-FA and PLA-PEG-Glu suggested that PEG was grafted to the nanoparticles (Ahmed, Arfat, Castro-Aguirre, & Auras, 2016; Rafat et al., 2010).

The FTIR spectra of PLA-PEG-FA copolymer showed that there is also a broad peak in the region between 1580 and 1648 cm-1 belonging to the amine group of FA. Moreover, the peak observed around 2108 and 2116 cm-1 were derived from azido group of FA and Glu respectively. The peak at 3580 cm-1 corresponds to the O-H stretching of OH in the FTIR spectrum of glucose suggested that glucose was grafted to the PLA-PEG.

The FT-IR spectra of FeCo, FeCo-PEI, FeCo-PEI-PLA-PEG-FA, and FeCo-PEI-PLA-PEG-Glu are illustrated in Fig. 2.

After conjugation of PEI with FeCo nanoparticles, the PEI peaks from 1050 cm-1 to 1250 cm-1 were attributed to the amide and amide amino groups , which indicated that the PEI was attached on the surface of FeCo nanoparticles (Fig. 2) (Gultekinoglu et al., 2015).

As also evident in Fig. 2, almost all peaks related to PLA-PEG, FeCo-PEI, glucose and folic acid were observed in the NPsA and NPsB, therefor it is indicated the successful synthesis of these nanoparticles.

Thermogravimetric analysis (TGA) of NPsA and NPsB was performed to find out different chemical compounds in the samples. The TGA curves of PEG, PEI, PLA, PLA-PEG, PLA-PEG-FA and CBP-FA copolymers, are shown in Fig. 3.

TGA analysis of the pure polymers (PEI, PLA and PEG) shows single step degradation while the spectrum of PLA-PEG copolymer exhibits three steps degradation related to the presence of three components in the PLA-PEG copolymer. The initial mass loss in the range of 175 degC to 305 degC, may be due to the loss of adsorbed water molecules from the copolymer (Jadhav et al., 2013).

The second stage of the PLA-PEG degradation occurs between 320 degC and 380 degC, which is related to the thermal degradation reaction of the PLA polymer (Shih & Huang, 2011). The third stage of thermal degradation occurs at temperatures above 380 degC, is associated with degradation of PEG polymer (Kwon & Kim, 2006).

The thermogram of the –FA and –Glu shows several mass losses during the temperature rise from 200 to which proposes that the glucose has higher temperature resistance, compared to other pure PEI, , and folic acid and therefore, temperature resistance of PLA-PEG copolymer increased after binding to glucose.

As it is seen in Fig. 3 the FeCo-PEI nanoparticles has a much better thermal stability than that of PLA-PEG-FA and PLA-PEG-Glu. Therefore, improvement in temperature resistance of PLA-PEG-FA and PLA-PEG-Glu was observed after modification with FeCo nanoparticles, which indicated the successful conjugation of PLA-PEG-FA and PLA-PEG-Glu with FeCo.

Preparation and characterization of nanoparticles

The results of DLS indicated that there was no significant difference between the particle size of NPsA, NPsB and NPsAB (the hydrodynamic diameter of NPsA, NPsB and NPsAB was about 82, 94 and 88 nm respectively). However, the hydrodynamic diameter of the nanoparticles increased after being conjugated with siRNA and PTX (Fig. 4). For example, the particle size of the NPsA increased from 88 nm to 120 nm after being encapsulated with siRNA and PTX. The surface charge of NPsA, NPsB and NPsAB was significantly reduced after being encapsulated with siRNA and PTX. It seems that the negative charge of phosphate backbone of the siRNA leads to decrease in the charge of the nanoparticles. A previous study found that the nanoparticles with a slightly negative surface charge and average particle sizes ranging between 100 and 150 nm have much higher transfer efficiency than larger nanoparticles (Bala, Hariharan, & Kumar, 2004; C. He, Hu, Yin, Tang, & Yin, 2010; Panyam & Labhasetwar, 2003). Although nanoparticles smaller than

100 may have higher transfection efficiency than nanoparticles larger than 100 nm but the high nonspecific uptake of these nanoparticles could increase the side effects on healthy tissue (Puppo et al., 2014).

The transmission electron microscopy (TEM) of the nanoparticles showed that the NPsA/siRNA/PTX, NPsB/siRNA/PTX and NPsAB/siRNA/PTX nanoparticles have spherical shape with uniform size distribution and smooth surface (Fig. 5). There was no significant difference between the morphology of the nanoparticles. The results also showed that the average size of the nanoparticles was about 100 nm. These results were consistent with DLS data (Fig. 4).

Magnetic property determination

Saturation magnetization of nanoparticles ranged from 70.6 to 165.2 emu/g. The FeCo nanoparticles showed high saturation magnetization (165.2 emu/g), however these quantities decreased after coating the FeCo with PEI. The values for saturation magnetization of FeCo and FeCo-PEI were determined as 165.2 and 131.4 emu/g respectively (Figure 6 A).

The saturation magnetization values for NPsA, NPsB, and NPsAB were lower than the FeCo and FeCo-PEI nanoparticles (99.41, 107.84 and 94.8 emu/g for NPsA, NPsB, and NPsAB nanoparticles respectively) (Fig. 6 A). Such attenuation pattern of saturation magnetization is often seen in nanoparticles after coating the nanoparticles with non-magnetic compounds.(Pouponneau, Leroux, & Martel, 2009). In general, the saturation magnetization of nanoparticles was reduced with encapsulation of siRNA and PTX into NPsA, NPsB and NPsAB nanoparticles (Fig. 6 A). However, there was no significant difference between the magnetic properties of the nanoparticles after PTX and siRNA encapsulation. Magnetite properties of nanoparticles is an important factor in imaging and targeting using magnetic nanoparticles. Previous studies showed that nanoparticles with magnetite properties up to 9 emu/g are suitable for use in imaging and targeting nanoparticles (Chorny et al., 2010; Yan, Li, Li, Zhu, Shen, Yi, Wu, Yeung, Xu, Xu, & others, 2014). Therefore, it seems that the nanoparticles synthesized in this experiment appear to be suitable for such purposes.

The results of siRNA-FAM and PTX encapsulation efficiency in NPsA, NPsB and NPsAB are shown in Fig. 7. Generally, the encapsulation efficiency of siRNA-FAM was significantly higher than that of PTX. The encapsulation efficiency of siRNA-FAM in various nanoparticles was in the range of 78 to 85% while, in the case of PTX, it was in the range of 38 to 42%. There is no significant difference between encapsulation efficiency of the nanoparticles in each group (Fig. 7). The maximum difference between the high and low encapsulation efficiency values of siRNA-FAM and PTX loaded samples was 7 and 4% respectively (Fig. 6 B). It seems that electrostatic interactions between the negative charge of siRNA-FAM molecules and the positive charge of polyethyleneimine in the nanoparticles is responsible for the increased values of siRNA-FAM encapsulation efficiency compared to PTX.

There was no significant difference between release behavior of PTX and also siRNA from NPsA/siRNA/PTX, NPsB/siRNA/PTX and NPsAB/siRNA/PTX nanoparticles. However, the cumulative release percentage of siRNA from nanoparticles were significantly lower than PTX after 30 days. The cumulative release percentage of siRNA was found to be significantly lower than that of PTX after 30 days incubation at 37 degC in PBS buffer (Fig. 7). It seems that the electrostatic interaction between siRNA and PEI of the nanoparticles may be responsible to this phenomenon. Therefore, it is plausible that electrostatic interactions between the phosphate groups of siRNA and the NH2 groups of the nanoparticles resulted in a reduction in the release rate of siRNA from the nanoparticles.

The PTX and siRNA release from the nanoparticles was observed in two different stages. In the first stage, more than 50% of the PTX-loaded into the nanoparticles was released in the first three days. It was found that the rate of PTX release decreased significantly with increasing the incubation time more than three days. A similar release pattern was observed for siRNA. These results are in agreement with previous studies (Abebe et al., 2015b; Perez et al., 2001b). Those reports have attributed the sustained release property of the nanoparticles to the PLA segment in the structure of the nanoparticles. This property of nanoparticles can be used for intracellular controlled drug release in tumor cells.

In vitro cytotoxicity and transfection assay

In vitro cytotoxicity of blank nanoparticles and siRNA-FAM/PTX encapsulated nanoparticles were compared after 48 h. As it was seen in Fig. 8A, the siRNA/PTX-free nanoparticles (NPsA, NPsB, and NPsAB) showed good biocompatibility, and no remarkable cytotoxicity was observed after 48 h of incubation with MCF-7 cells. However, its toxicity significantly increased after encapsulation of siRNA/PTX in the nanoparticles (NPsA/siRNA/PTX, NPsB/siRNA/PTX, and NPsAB/siRNA/PTX). As it can be seen in Fig. 8B, siRNA has no toxicity effect on MCF-7 cells. Therefore, it can be concluded that the decrease in cell viability might be due to the effect of PTX on MCF-7 cells. The IC50 value of NPsAB/siRNA/PTX was significantly lower than NPsA/siRNA/PTX and NPsB/siRNA/PTX after 48 h of incubation. Several studies have demonstrated the effect of folic acid and glucose conjugated nanoparticles to increase drug transfer efficiency (Jain, Rathi, Jain, Das, & Godugu, 2012; Macheda, Rogers, & Best, 2005; Zhang et al., 2013).

However, this improvement could be continuing until the saturation of folic acid receptors on the surface of cancer cells by nanoparticles. In other word, after saturating the folic acid receptors by nanoparticles, nonspecific uptake of nanoparticles into healthy tissues increases. The higher cytotoxicity of free PTX may be due to unrestricted and rapid diffusion of PTX into MCF-7 cells, while the nanoparticles are internalized into cancer cells via the receptor-mediated endocytosis pathway (S. Wang et al., 2013). In addition, sustained release of PTX from these nanoparticles due to the presence of polylactic acid in their structure may be another reason for low cytotoxicity of the nanoparticles compared to free PTX.

Fluorescence microscopy was used to evaluate the ability of the nanoparticles for co-delivery of nucleic acid and drug into MCF-7 cells.

Fig. 8 C-E shows fluorescence images of MCF-7 cells after 48 h of incubation with NPsA/siRNA/PTX, NPsB/siRNA/PTX and NPsB/siRNA/PTX nanoparticles. The fluorescence emission observed within the cells indicated the ability of the nanoparticles to transfer siRNA-FAM into MCF-7 cells. Moreover, the circular morphology of MCF-7 cells after incubation with the nanoparticles may be happened since the induction of apoptosis in MCF-7 cells byNPsA/siRNA/PTX, NPsB/siRNA/PTX and NPsB/siRNA/PTX nanoparticles.

Conclusions

In this study, conjugated copolymers of NPAB/siRNA/PTX were successfully synthesized for targeted gene delivery to breast cancer cells. Their chemical structure was confirmed through NMR and FTIR analysis and magnetic properties determination. The results obtained from TEM and DLS revealed that the nanoparticles have spherical morphology, with the hydrodynamic diameter in the range between 100-150 nm, which is suitable for intravenous injection and *in vivo* applications. Invitro drug release showed a sustained release manner of PTX and siRNA, however the cumulative release of PTX is more than siRNA. The cytotoxicity assay demonstrated no significant toxicity of unloaded nanoparticles on MCF-7 cells, however PTX/siRNA loaded nanoparticles caused significant toxicity. In addition, the ability NPAB to improve the transfection efficiency was confirmed through increased uptake of siRNA-FAM in MCF-7 cells via fluorescence microscopy imaging.

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Fig. 1. 1H NMR spectra of PLA-PEG-FA (A) and PLA-PEG-Glu (B) in D2O solvent

Fig. 2. FT-IR spectrum of various copolymers and magnetic nanoparticles

Fig. 3. TGA thermograms of magnetic nanoparticles and different copolymers

Fig. 4. the particle size and zeta potential of the both PTX/siRNA encapsulated and blank nanoparticles

Fig. 5. TEM image of NPsA/siRNA/PTX, NPsB/siRNA/PTX and NPsAB/siRNA/PTX nanoparticles

Fig. 6. Magnetic behavior (A) and encapsulation efficiency (B) of the nanoparticles

Fig. 7. DNA release profiles of the nanoparticles in PBS buffer (pH=7.4) (Mean ± standard deviation) (n = 3).

Fig. 8. Biocompatibility of nanoparticles (A) and the effect of PTX and siRNA loaded into the nanoparticles on MCF-7 cells (B) Fluorescence microscopy images of MCF-7 cells were treated with NPsA/PTX/siRNA, NPsB/PTX/siRNA and NPsAB/PTX/siRNA after 48 hours incubation.

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