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Original Research

A comparison between PLGA-PEG and NIPAAm-MAA nanocarriers in curcumin delivery for hTERT silencing in lung cancer cell line

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Abstract: Lung cancer is the most common cancer among men. Since the main reason of cancer cells immortality is telomerase activity, targeting of such enzyme can be a promising approach in cancer therapy. Curcumin is a safe and efficient anticancer agent in this context, but its applications in cancer therapy are limited because of its hydrophobic structure and low solubility in water. Today, using nanocarriers for delivery of such anticancer agents is a well performed method. Here, we developed and compared the efficiency of two nanocarriers (PLGA-PEG and NIPAAm-MAA) in delivery of curcumin and also in levels of hTERT silencing in lung cancer cell line (calu-6). Scanning electron microscopy, MTT assays and real-time PCR were used for imaging, cytotoxicity testing and measuring the expression levels of hTERT after treatment of cells with different concentrations of free curcumin and curcumin loaded nanocarriers. The MTT results demonstrated that the IC_{50} values of curcumin loaded nanocarriers were in lower concentrations than free curcumin. The hTERT expression levels were decreased by curcumin loaded PLGA-PEG more than curcumin loaded NIPAAm-MAA and free curcumin. Our results showed that the curcumin loaded PLGA-PEG can be a useful nano based carrier for delivery of anti-cancer agents such as curcumin to fight lung cancer.

Key words: Lung cancer, curcumin, hTERT, calu-6 cell line, PLGA-PEG, NIPAAm-MAA.

Introduction

Cancer is a major cause of death in many developed and developing countries. Smoking, poor diet and physical inactivity are behaviors which increase the risks for cancer. In 2014, lung cancer accounted for 28% of diagnosed cancers in men (1). Additionally, it was a leading reason of cancer death in males (2, 3). Many studies demonstrated telomerase overexpression in almost 85% of cancer cells. This overexpression leads to immortality in many cancerous cells (4). Human telomerase is composed of two major subunits: a reverse transcriptase (hTERT) and an RNA template (hTRC) (5). Since, many studies showed the high levels of hTERT expression in cancerous cells selectively, the role of telomerase in human carcinogenesis is clear. Therefore, the hTERT suppression can be an effective way in cancer treatment (6, 7). Following cancer diagnosis, a variety of therapeutic approaches such as surgery, radiotherapy and chemotherapy can be administrated. These therapeutic procedures could be prescribed alone or in combination with the other methods.

Chemotherapy is the most common method to treat metastatic cancers. In some approaches synthetic anticancer agents can be used; on the other hand, natural products can be of interest candidate (8-11). Besides antioxidant and immunomodulatory effects of natural products such as herbal metabolites, they assumed to be less toxic and have a lower side effects in cancer treatment (12-15). Curcumin is a yellow herbal powder obtained from the herb *Curcuma longa* Linn which is called turmeric (16). In many researches, it was found that curcumin has antioxidant, anti-inflammatory, chemo preventive and anti-tumor properties (17). Moreover, it can induce apoptosis and prevents proliferation in many cancerous cells. Due to the low solubility of curcumin in aqueous environment and its rapid degradation and consequently its low bioavailability, there are some limitations in using of this compound in animals and humans (16).

Nowadays, various types of nano-vehicles are prepared which can overcome this problem. There are many polymeric biodegradable nanoparticles for safe and targeted drug delivery (18-20). Drug delivery using biodegradable polymers is one of the favorite research lines in cancer therapy (8). Poly (lactic-co-glycolic acid) or PLGA or is a biodegradable polymer which hydrolyzes into lactic acid and glycolic acid in body (21). These metabolites then consumed in Krebs cycle. Because of its safe metabolites, PLGA was approved by US FDA and European Medicine Agency (EMA) as a drug delivery system in human. After in vivo administration, these nanoparticles can be uptaken by cells via pinocytosis and clathrin-mediated endocytosis. Followed by

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cellular entrance, these nanoparticles can escape from endosomal compartments, enter and release their cargo into cytosol (22-25).

For improving the in vivo half-life and bioavailability of the hydrophobic nanocarriers such as PLGA, many studies decorated the surface of nanocarriers with hydrophilic molecules such as Polyethylene glycol (PEG) (26). Polyethylene glycol (PEG) is a best known hydrophilic and non-ionic polymer which is used for surface modification(26). Many reports show that PEG increases blood-circulation, half-life and biocompatibility of PLGA. Positively-charged nanoparticles easily interact with the cell membrane and can be up taken by cell in higher rates (27). Other nano-polymeric models are temperature-sensitive hydrogels, a group of well-studied polymers which are suitable for targeted drug delivery. Temperature-sensitive polymers have many hydrophilic groups such as methyl and propyl groups (28). Poly (N-isopropylacrylamide) or NIPAAm is a well-characterized temperature and pH-sensitive polymeric nanoparticle in drug delivery context. Because of the high temperature and acidic environment of cancerous cells, NIPAAm can release its cargo in these cells gradually (4). So far, many researches have been done for cancer treatment. However, a safe procedure without any side effects has not been discovered, yet. In present study, we developed and compared the efficiency of two co-polymeric nanoparticles used in delivery of the anticancer herbal metabolite "curcumin" to suppress hTERT expression in lung cancer cell line.

Materials and Methods

Materials

Glycolide and (d,l-lactide), PEG (6000), dichloromethane (DCM), stannous octoate (Sn(Oct)2) (stannous 2-ethylhexanoate), curcumin, [3-(4,5-Dimethylthiazol-2-yl)-2,5-Diphenyltetrazolium Bromide] (MTT), and poly(vinyl alcohol) (PVA) were purchased from Sigma-Aldrich (USA). RPMI 1640, fetal bovine serum (FBS), EDTA-Trypsin, TRIzol (TRI reagent), and antibiotics were purchased from Invitrogen (Germany) and Gibco. SYBR Green real-time PCR master mix kit was purchased from Roche (Germany). Primers were purchased from Takapouzist. RNX-Plus kit for extraction of total RNA was purchased from CinnaGen (Iran) and first strand cDNA synthesis kit was obtained from Thermo Scientific. Calu-6 lung cancer cell line was purchased from Pasteur Institute cell bank of Iran.

PLGA-PEG Copolymer Preparation

D,l-lactide (Sigma-Aldrich, USA) and 0.285 g glycolide (Sigma-Aldrich, USA) With a molar ratio of 3:1, 1.441 g were combined. After that, PEG (PEG 6000 (45% w/w)) was added and the mixture was heated (140 °C) and was melted in a bottleneck flask connecting to a nitrogen gas source. As a catalyst, 0.05% (w/w) stannous octoate (Sn(Oct)2) (stannous 2-ethylhexanoate) (Sigma-Aldrich, USA) was added to the mixture. The temperature was increased to 180 °C. The mixture was retained for 3 hours in 180 °C. A vacuum was used to perform the polymerization. The synthetized copolymer was dissolved in dichloromethane. For precipitation of the copolymers, ice-cold diethyl ether (Sigma-Aldrich, USA) was used (6).

NIPAAm-MAA copolymer preparation

N-Isopropylacrylamide (NIPAAM) (Sigma-Aldrich, USA) and methacrylic acid (MAA) (Sigma-Aldrich, USA) are water soluble monomers and were mixed in 8.5: 0.3 molar ratios. 2-(Dimethylamino) ethyl methacrylate (DMAEMA) (Sigma-Aldrich, USA) was used as a cross linker. After that 0. 17 gr of banzoeilperoxide (BPO) was added to the mixture as initiator. Using free radical polymerization method under N_2 atmosphere at 70 °C, monomers in 1, 4-dioxane were polymerized. Polymer was precipitated in cold n-Hexan and afterward solvent was evaporated (4).

Characterization of prepared nanoparticles

Scanning electron microscopy (SEM) (KYKY, EM3200, Beijing, China) was used to determine the size and shape of NIPAAm-MAA, NIPAAm-MAA-curcumin, PLGA-PEG and PLGA-PEG-curcumin.

Drug loading capacity assessment

To determine curcumin encapsulation efficiency, 120 mg of each nanoparticle (PLGA-PEG and NIPAAm-MAA) were dispersed in 10 mL dichloromethane containing polyvinyl alcohol solution. After that, whole solution was stirred for 2 minutes and rotatory evaporator (Heidolph Instruments, Hei-VAP series, Schwabach, Germany) was used to evaporate the dichloromethane. After 25 minutes centrifugation in 9000 rpm, the supernatants were isolated and compared to curcumin contained solution (20 mg curcumin in 3 mL methanol). An ultraviolet 2550 spectrophotometer (Shimiadzu, Tokyo, Japan) was used to measure the amounts of free curcumin (λmax = 420 nm). Using the following formula, where (OD1) and (OD2) present the total amount of curcumin and remained curcumin in supernatant, curcumin encapsulation efficiency of nanoparticles was measured. Encapsulation efficiency % = [(OD1 – OD2) / (OD1)] ×100%.

Cell culture

A calu-6 lung cancer cell line (Pasteur Institute, Iran) was cultured in an RPMI-1640 (Gibco, Invitrogen, USA) which supplemented with 10% heat inactivated fetal bovine serum (FBS) (Gibco, Invitrogen, USA), 80 mg/mL Penicillin G (Merck, Germany), 50 mg/mL Streptomycin (Merck, Germany), and 2 g/mL sodium bicarbonate at 37 °C air containing 5% CO_2.

To assess the drug release behaviors of nanocarriers, 3 mg of curcumin loaded nanoparticles were dispersed in 3 ml of solution containing 3 mL phosphate buffered (pH:7.4) and acetate buffer (pH: 5.8). Incubation was done at two different temperatures (37 °C and 40 °C). At definite time intervals, 3 mL of sample was removed and replaced by same amounts of acetate and phosphate-buffered solutions. After that, curcumin release was determined by UV spectrophotometry.

MTT assay

Cytotoxicity effects of PLGA-PEG- curcumin and NIPAAm-MAA-curcumin on cancerous cells were assessed by MTT assay. After seeding the cells and applying 24 h incubation (37 °C, 5% CO_2), treatment

of the cells with different concentrations (10, 20, 30, 40, 50, 60, and 70 µM) of PLGA-PEG- curcumin and NIPAAm-MAA-curcumin was performed for 24, 48, and 72 h. Cells with 200 µL culture medium containing FBS served as control. After incubation and changing the medium of wells with a fresh one, cells were left in incubator for 24 h. Next steps include removing the medium of all wells, adding 50 µL of 2 mg/ml MTT dissolved in PBS and incubation for 4 h were performed. Content of the wells was removed and for dissolving blue formazan precipitate, 200 µL pure dimethylsulfoxide (DMSO) was added to each well. Afterwards, 25 µL Sorensen's glycine buffer was added. Lastly, after shaking the plates, ELISA plate reader with reference wavelength of 630 nm was used for reading the absorbance of each well in 570 nm.

Total RNA extraction, cDNA synthesis and real-Time PCR

Cells were treated with different concentrations of PLGA-PEG- curcumin and NIPAAm-MAA-curcumin for 72 h and Total RNA was extracted. For determination of their purity and concentrations and also RNAs integrity, nanodrop and electrophoresis were used. First strand cDNA synthesis kit was used for synthesis of complementary DNA (cDNA). Reaction samples were prepared and each of them contained 1 µg/µL total RNA, 1 µL of olgo dT primer, 4 µL of 5X Reaction Buffer, 2 µL of dNTP Mix, 1 µL RiboLock RNase inhibitor, 1 µL of RevertAid M-MuLV Reverse Transcriptase and 10 µL of nuclease-free water. Afterwards, incubations in 37 °C for 15 minutes (Reverse Transcription); 85 °C for 5 sec (in order to inactivate of reverse transcriptase) and 4 °C were performed. To determine the expression level of the hTERT, Real time PCR was done. 2 µL of forward and reverse hTERT primers (5' GTCTGGAGCAAGTTGCAAAG-3', 5' TGACCTCTGCTTCCGACA-3'), β-actin (5'- TCCCTGGAGAAGAGCTACG-3', 5'-GTAGTTTCGTGGATGCCACA-3'), 2 µL of cDNA sample, 6.5 µL of Real-Time PCR master mix and 2 µL of deionized water were used for Real time PCR reaction.168 bp and 181 bp, were the amplicon sizes of hTERT and β-actin. Equal volumes of each RNA sample were prepared, and the amplification of hTERT and β-actin were done by Real-Time PCR in triplicate. The following conditions were used for incubation of reaction mixture: 95 °C for 5 minutes, 1 cycle (Holding step); 95 °C for 15 seconds, 40 cycles (Denaturation); 60 °C for 15 seconds, 40 cycles (Annealing); 72 °C for 30 seconds, 40 cycles (Extension), 72-95 °C, 1 cycle (Melting). To record infrared spectra in real–time, a Perkin Elmer series FTIR (Fourier Transform Infrared) was used.

Results

Characterization of nanoparticles

PLGA-PEG, PLGA-PEG-Curcumin, NIPAAm-MAA and NIPAAm-MAA-Curcumin are shown in Figure 1. The size of PLGA-PEG and NIPAMm-MAA was about 55 and 45 nm and there was a significant increase in dispersion of nanoparticles (based on SEM images). The size of nanoparticles after curcumin loading was about 300 and 290 nm for PLGA-PEG and NIPAAm-MAA, respectively.

Drug encapsulation and in vitro releasing

Encapsulation efficiency/drug loading capacity (DL%) of PLGA-PEG and NIPAAm-MAA was 84.5% and 89.6%, respectively. Releasing of curcumin from both PLGA-PEG-curcumin and NIPAMm-MAA-curcumin was time dependent and in comparison to pH: 7.4 and temperature of 37 °C, significantly increased in acidic pH (pH: 5.8) and in 40 °C.

Cell cytotoxicity (MTT assay)

Table 1 shown the results of MTT assays after treatment of calu-6 lung cancer cell line with different concentrations (10 - 70 µM) of PLGA-PEG-Curcumin, NIPAAm-MAA-Curcumin and free Curcumin for 24, 48, and 72h.

Quantitative mRNA analysis

After treatment of cells by different concentrations of NIPAAm-MAA-Curcumin, PLGA-PEG-Curcumin and also pure curcumin, real-time PCR results demonstrated that there was a decrease in hTERT expression levels by increasing in the concentrations of the samples. Changes in gene expression levels of hTERT between

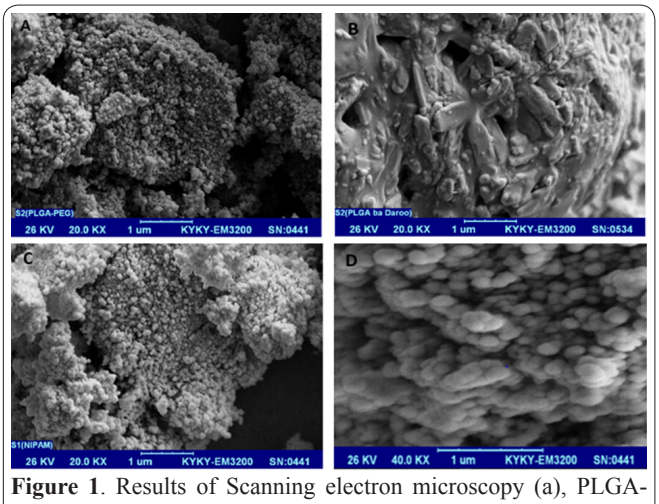

Figure 1. Results of Scanning electron microscopy (a), PLGA-PEG, (b), PLGA-PEG- curcumin, (c), NIPAAm-MAA, and (D) NIPAAm-MAA-Curcumin.

Table 1. IC_{50} values of curcumin, PLGA-PEG-Curcumin and NIPAAm-MAA-Curcumin after treatment of cells and incubation at different times.

	IC_{50} values Mean ± SD		
Incubation time (h)	**Curcumin**	**PLGA-PEG Curcumin (µM)**	**NIPAAm-MAA Curcumin(µM)**
24	27.32 ± 1.03	14.47 ± 1.25	13.25 ± 1.23
48	11.36 ± 1.8	6.78 ± 1.65	5.11 ± 1.12
72	8.13 ± 1.56	5.22 ± 1.36	4.23 ± 1.33

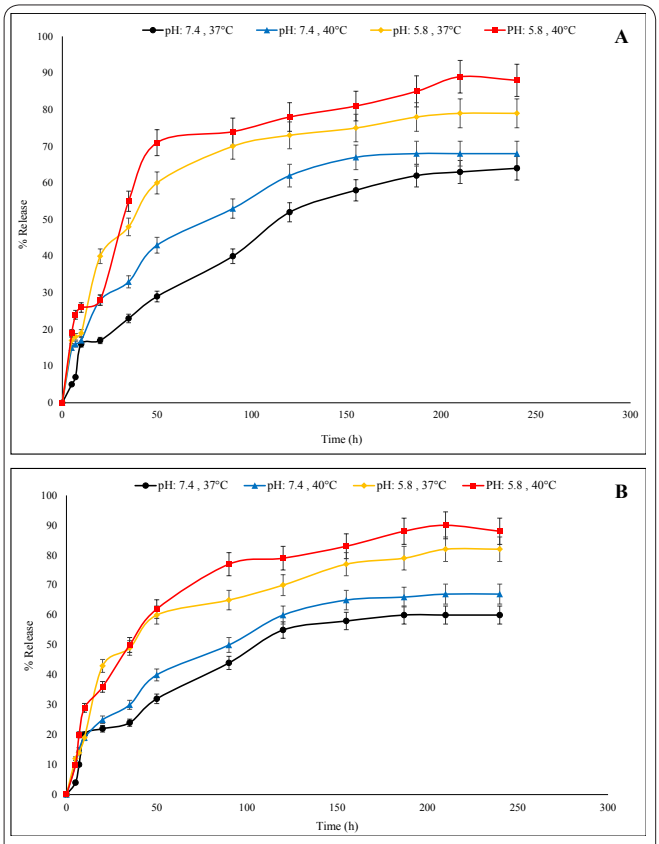

Figure 2. In vitro release of curcumin in different pH and temperatures. NIPAAm-MAA-Curcumin (A); PLGA-PEG-Curcumin (B).

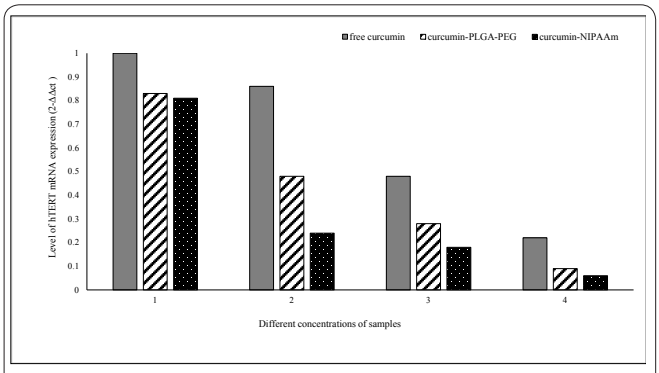

Figure 3. Expression levels of hTERT after treatment of cells by different concentrations of Curcumin- NIPAAm-MAA, Curcumin-PLGA-PEG and free curcumin.

treated calu-6 cells and the control were normalized to housekeeping gene (β-actin) and calculation was performed via the $2^{-\Delta\Delta ct}$ method (Real-time PCR results are shown in Figure 3).

Discussion

Until now, different types of nano-vehicles were developed to deliver various anti-cancer drugs such as doxorubicin (29-31), methotrexate (32, 33), paclitaxel (34, 35), curcumin (36, 37), etc. Besides all of the necessary features of a nano-vehicle, the biodegradability of these nanosystems is in the center of the picture. Lower cytotoxicity, better encapsulation and bioavailability are some main advantages of these nano-vehicles (38).

In this comparative study, PLGA-PEG and NIPAAm-MAA as two biodegradable nano-vehicles were developed for delivery of curcumin in lung cancer cell line (calu-6). Efficiency of these two polymeric nanoparticles in drug encapsulation, drug releasing and suppression of hTERT also were evaluated by calu-6 cell line. Although curcumin is a well-known and safe anticancer agent, its low water solubility led to some limitations in administration of this agent(39, 40). For delivering of curcumin, it was shown that, the problem of low water solubility and low bioavailability could be solved by using some nanocarriers (41).

Here, two developed nanocarriers PLGA-PEG and NIPAAm-MAA were used as suitable models to increase solubility and bioavailability of curcumin in aqueous environment of cancer cells. The body's reticulo-endothelial system recognizes and eliminates hydrophobic particles and prevents drug delivery process by these nanoparticles. To overcome the mentioned problem, one simple method is surface decoration of the nanoparticles with hydrophilic molecules. The main goal of PEGylation of the PLGA nanoparticles in this study was to increase the hydrophobicity of the nanocarrier. The main reason of methacrylate acid (MAA) addition to NIPAAm was to introduce a pH responsive phase transition behavior which is resulted from ionized groups in this molecule(42). After preparation of two nanocarriers, curcumin was encapsulated in nanoparticles via double emulsion method (w/o/w). Scanning electron microscope (SEM) was used for imaging of curcumin loaded nanocarriers. Figure 1 depicts the PLGA-PEG and NIPAAm-MAA before and after curcumin loading. The results showed that, the size of nanoparticles were altered by loading of curcumin. The size of PLGA-PEG and NIPAAm-MAA nanoparticles was changed from 55 nm to 300 nm and 45 nm to 290, respectively. The results indicated the high degree of encapsulation efficiency for two developed nanoparticles (84.5% for PLGA-PEG and 89.6% for NIPAAm-MAA). Releasing of curcumin as expected was in a time, temperature and pH dependent manner. Curcumin releasing from both PLGA-PEG and NIPAAm-MAA was higher in cancerous conditions (pH 5.8 and temperature of 40 °C), compared to normal physiological conditions (pH: 7.4 and temperature of 37 °C). These results highlighted the potential applications of developed nano systems for cancer therapy. After treatment of calu-6 cells with different concentrations of nanocarriers at different time intervals, it was shown that both of the curcumin loaded nanocarriers had a clear cytotoxicity against calu-6 cells. Because of dissimilar IC_{50} values at different times of treatment, it was found that the effects of both curcumin loaded nanocarriers and free curcumin are time dependent. Furthermore, IC_{50} values of the PLGA-PEG-curcumin and NIPAAm-MAA-curcumin were less than free curcumin, since their effects on cancerous cells were more than free curcumin.

These results indicated the enhanced cellular uptake of both curcumin loaded nanocarriers in comparison with free curcumin. No significant differences between the IC_{50} values of curcumin loaded PLGA-PEG and NIPAAm-MAA, demonstrating the potential applications of these two developed nanocarriers in curcumin delivery. Our results were in agreement with other studies which demonstrated the curcumin loaded PLGA (43, 44) and NIPAAm-MAA (45, 46) had a cytotoxic effects on cancerous cells. The results also demonstrated that the nanocarriers retain the anti-tumor activity of curcumin. Induction of apoptosis and disruption of some

processes in cellular growth are some of the well-known properties of curcumin (47).

Activity of telomerase enzyme was seen in lung (48) and more than 80% of all other types of cancers (49). Therefore, targeting and silencing of this enzyme can be considered as a good approach in cancer treatment (50). Although many compounds can be used for silencing the telomerase activity, the side effects of these compounds are undesirable (51). Thus, applying a natural agent to make the side effect as low as possible is necessary. Previous studies stated that, curcumin can inhibit human telomerase activity and down regulates the mRNA of hTERT in leukemia (HL 60), liver cancer (Bel7402), and gastric cancer (SGC7901) cell lines (52). Hence, applying this natural agent might be a useful tool for targeting of telomerase activity and controlling the cell proliferation in lung cancer cells (53).

In the present study, the effect of different concentrations of curcumin loaded PLGA-PEG, NIPAAm-MAA and free curcumin on hTERT mRNA expression was evaluated separately by real-time PCR. Real-time PCR data analysis revealed that by increasing the concentrations of PLGA-PEG-curcumin, NIPAAm-MAA-curcumin and free curcumin, there was a decreasing trend in hTERT expression in calu-6 cells. In comparison with free curcumin, it was observed that both curcumin loaded nanocarriers resulted in a lower hTERT mRNA levels. It was found that the cellular uptakes and efficiency of curcumin loaded nanocarriers were more than free curcumin. These results were in agreement with other research conducted by Kazemi-Lomedasht *et al*. at which the curcumin loaded nanocarriers led to a lower hTERT mRNA levels compared to free curcumin (50). Moreover, in the same concentrations, it was found that the hTERT mRNA levels were more reduced by PLGA-PEG-curcumin nanoparticles compared with NIPAAm-MAA-curcumin nanocarriers. This finding might suggest that the PLGA-PEG nanocarrier is more efficient than NIPAAm-MAA in curcumin delivery and hTERT targeting.

In conclusion, our results introduced two suitable nanocarriers PLGA-PEG and NIPAAm-MAA for delivery of natural agent curcumin in lung cancer cell line calu-6. We suggest that the main problem of curcumin (low solubility in water) can be solved by using nanoparticles as delivery vehicles. The uptake of curcumin loaded nanocarriers was more than free curcumin and both of nanocarriers were able to release their cargo in cancerous cell conditions (pH: 5.8 and 40 °C). The activity of curcumin in lowering the hTERT expression levels was maintained by both of nanocarriers but the PLGA-PEG could be a better candidate in curcumin delivery and telomerase targeting in lung cancer.

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