

Research Article

Study on Apoptosis of Prostate Cancer Cells Induced by $I\kappa B\alpha$ Overexpression Synergistic with Poly (Lactic-Co-Glycolic Acid)-Curcumin Nanoparticles

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Objective. To investigate the synergistic effects of $I\kappa B\alpha$ overexpression and poly (lactic-co-glycolic acid)-curcumin nanoparticles (PLGA-Cur-NPs) on prostate cancer (PC) and reveal the underlying mechanisms of cooperative sensitization induced by curcumin. **Methods.** Proliferation and apoptosis rate in $I\kappa B\alpha$ overexpressed and PLGA-Cur-NPs-treated PC cells were detected by MTT and flow cytometry assay. The expression levels of $I\kappa B\alpha$, apoptosis-related, and signaling proteins were measured by western blotting assay. **Results.** PC cell proliferation was significantly inhibited by the overexpression of $I\kappa B\alpha$. The apoptosis rate of PC cells was significantly increased at a high concentration of curcumin exposure, while the activation of NF- κB pathway was obviously inhibited. In addition, PLGA-Cur-NPs treatment synergistic with $I\kappa B\alpha$ overexpression enhanced the apoptosis of PC cells by suppressing the NF- κB pathway activation. **Conclusion.** $I\kappa B\alpha$ overexpression synergistic with PLGA-Cur-NPs could obviously inhibit proliferation, induce apoptosis, and suppress the activation of NF- κB pathway in PC cells. These findings may provide an experimental basis to establish the tumor gene therapy combined with traditional Chinese medicine treatment, thus promoting the clinical application of both tumor gene therapy and anti-tumor Chinese medicine.

1. Introduction

Up to now, the incidence of prostate cancer (PC) has been gradually increasing. It is worth noting that the clinical treatment of advanced PC faces the challenge of androgen resistance in PC cells, which will lead to the loss of anti-androgen therapy. Therefore, it is extremely urgent to develop new treatments [1–3]. Cationic poly lactic-co-glycolic acid nanoparticles (PLGA-NPs) are a new particle coated with a cationic surfactant and charge to reconcile agent gene nanoparticles. Multiple studies showed the advantages of cationic PLGA-NP carrier in aspects of genetic, nucleic acid protection, transfection efficiency, and target gene expression. As a new type of drug carrier, PLGA-NPs could improve the water solubility of drugs and control the drug release rate by encapsulating drugs, increasing drugs' bioavailability, and reducing adverse drug reactions [4–8]. PLGA-NPs have been approved as carriers to carry drugs for human experimental research due to their biodegradable and nontoxicity.

Nuclear transcription factor (nuclear factor kappa B predominate, NF- κB) has been proved to play a vital role in occurrence and development in many human tumors, including PC, and participate in the process of PC cells' antiapoptotic [9, 10]. Looking for an approach to restrain the NF- κB activation may be effective for the treatment of PC. In most of the cells, NF- κB specifically binds to the family members of its inhibiting protein $I\kappa B\alpha$ (inhibitor of the NF- κB) and mainly exists in the cytoplasm as an inactive complex [11]. Given that the front 36 amino acids of the N terminal of $I\kappa B\alpha$ only involved in mediating the $I\kappa B\alpha$ degradation rather than functionality required area [12], the loss of 36 N-terminal amino acids in $I\kappa B\alpha$ still retains the function of inhibiting the NF- κB without degradation. Therefore, the overexpression of $I\kappa B\alpha$ might be used to target the inhibition of NF- κB .

The Chinese medicine therapy of curcumin (Cur) in tumor occurrence and development of various stages (initial, promotion, and evolution) has drawn attention.

Previous studies have shown that Cur could directly kill PC cells and be nontoxic to normal cells. However, insoluble into water, susceptible to oxidizing and metabolizing, frequent administration, and low bioavailability in vivo limit the clinical application of Cur. Thus, in this study, we constructed the PLGA-Cur-NPs and validated its own effects or synergistic effects with $\text{I}\kappa\text{B}\alpha$ overexpression in PC cells in vitro, which would reveal possible therapeutic strategy for PC treatment.

2. Methods and Materials

2.1. PLGA-Cur-NPs Synthesis. By the ethanol extraction and petroleum ether skim to silica gel column chromatography, Cur complex [Cur I (0.1%), Cu II (0.1%), and Cur III (99%)] active monomer was extracted and separated. In the following experiments, Cur III active monomer was used to construct PLGA-Cur-NPs. Cur-NPs' liposomes were synthesized by the nanoprecipitation method. In brief, we weighed the prescription amount (10 mg/mL) of soybean lecithin and Cur-NPs accurately ($v_{\text{soybeanlecithin}}: v_{\text{curcumin-nano}} = 10:1$) and dissolved them in methanol as the organic phase. Besides, we weighed the prescription amount (1%) of auxiliary stabilizer (poloxamer 188) accurately and dissolved it in water as the aqueous phase. The volume ratio of the organic phase to the aqueous phase is 1:10. Under the magnetic stirring at a certain speed (1,000 rpm/min), the organic phase was dropped into the aqueous phase of the ice bath (0–2°C) at a constant rate (5 mL/min) by using a microinjection pump. After the organic phase was added, the preparation was transferred to room temperature (25°C) and stirred to volatilize the organic solvent to prepare Cur-NPs liposome. The stability of Cur-NPs liposome was measured by using HPLC. The encapsulation efficiency (%) and loading efficiency (%) of Cur-NPs liposome was $90.36 \pm 1.37\%$ and $3.82 \pm 0.07\%$, respectively. Poly (lactic/glycolic acid) (PLGA, LA:GA = 75:25, 10 kDa) was purchased from Boehringer Ingelheim. The surface charge of the nanoparticles was tested under the Zetasizer NanoZS instrument (Malvern Co. Ltd., UK). PLGA was dissolved, injected in a DSPEmPEG2000 emulsifier, and rigorously emulsified by a high-power sonicator.

2.2. Vector Construction. PC cell line LNCaP was purchased from ATCC (American-type culture collection). The cells were cultured in RPMI-1640 medium (Gibco) containing 15% Fetal Bovine Serum (FBS) at 5% CO_2 in a humidified incubator. Vector DNA with $\text{I}\kappa\text{B}\alpha$ gene was produced by a four-plasmid calcium phosphate transient transfection method as previously [13]. The residual plasmid DNA was discarded after being treated with Benzonase (Novagen, San Diego, CA, USA). After the cells were seeded onto a 6-well plate at a density of 5×10^5 cells/well, the $\text{I}\kappa\text{B}\alpha$ overexpression vector ($\text{I}\kappa\text{B}\alpha$ -OE) or Blank-vector (negative control, NC) with green fluorescent protein (GFP) was transfected into the cell line LNCaP. GFP expression was assessed after 96 h using fluorescence microscopy (Olympus IX71).

2.3. MTT Assay. The cytotoxicity assay of nanocurcumin in different concentrations was examined by MTT assay. 1×10^5 cells per well were cultured in 96-well plates for 24 h at 37 °C. Subsequently, 5 $\mu\text{g/mL}$ of MTT was added to the cells in each well, and these cells were then cultured for 4 h. After that, 100 μL of DMSO (Beijing Solarbio Science and Technology Co., Ltd.) was added. The absorbance of each hole was detected by a microplate bio-reader (Beijing Potenov Technology Co. Ltd.) at 560 nm wavelength.

2.4. Flow Cytometry. Flow cytometry for apoptosis assay was performed on a FC500 MPL system (Beckman Coulter) using the Annexin V-FITC/PI Apoptosis Detection kit (Solarbio, Beijing, China). Cells were inoculated in 12-well plates at the density of 2×10^4 cells per well for 72 h. Cells were centrifuged and resuspended in Annexin V binding buffer, and these cells were stained with Annexin V-PI (Life technologies, Carlsbad, CA, USA) for 15 min at room temperature. After being resuspended with 500 μL of PBS, at least 1×10^4 cells were tested using a flow cytometer. FlowJo software was conducted to analyze data.

2.5. Western Blot. Total proteins were extracted from cells using RIPA protein lysate. Next, protein concentration was detected by the BCA protein assay kit (Beyotime, China). Protein samples (30 μg) were separated by 12% SDS-PAGE and transferred to PVDF membranes. These protein membranes were blocked with 5% skimmed milk powder at room temperature for 1 h and incubated with primary antibodies against $\text{I}\kappa\text{B}\alpha$ (1:1000; ab126605; Abcam), Bcl-xl (1:20000; ab178844; Abcam), Bcl-2 (1:1000; ab32124; Abcam), NF- κB p65 (1:1000; ab32536; Abcam), Lamb1 (1:1000; ab108536; Abcam), GAPDH (1:5000; ab9485; Abcam), and β -actin (1:2000; ab8227; Abcam) at 4°C overnight. Then, membranes were incubated with horseradish peroxidase-conjugated secondary antibodies (1:3000; ab6877; Abcam) at room temperature for 2 h. Blots were washed with TBST 3 times, bands were visualized using an ECL kit (Millipore, Billerica, MA, USA), and the protein gray value was analyzed by Image J software (NIH, MD).

2.6. Caspase-3 Colorimetric Assay. Caspase-3 activity was detected by apoptosis Caspase-3 Colorimetric Assay kit (MBL) in strict accordance with the manufacturer's instructions. Briefly, cells at the logarithmic phase were digested with trypsin, centrifuged at 1,000 g for 5 min, and then resuspended in cell lysates. The supernatant of cells was incubated with DEVD-p-NA substrate (final concentration, 200 μM) at room temperature for 1 h. An automatic microplate reader (molecular devices) measured the absorbance of cells at 400 nm wavelength to reflect Caspase-3 activity.

2.7. Statistical Analysis. All data are represented as mean \pm SD. Data were analyzed by SPSS13.0 statistics software (SPSS Inc., Chicago, IL, USA). One-way analysis of variance (one-way ANOVA) was used to compare the

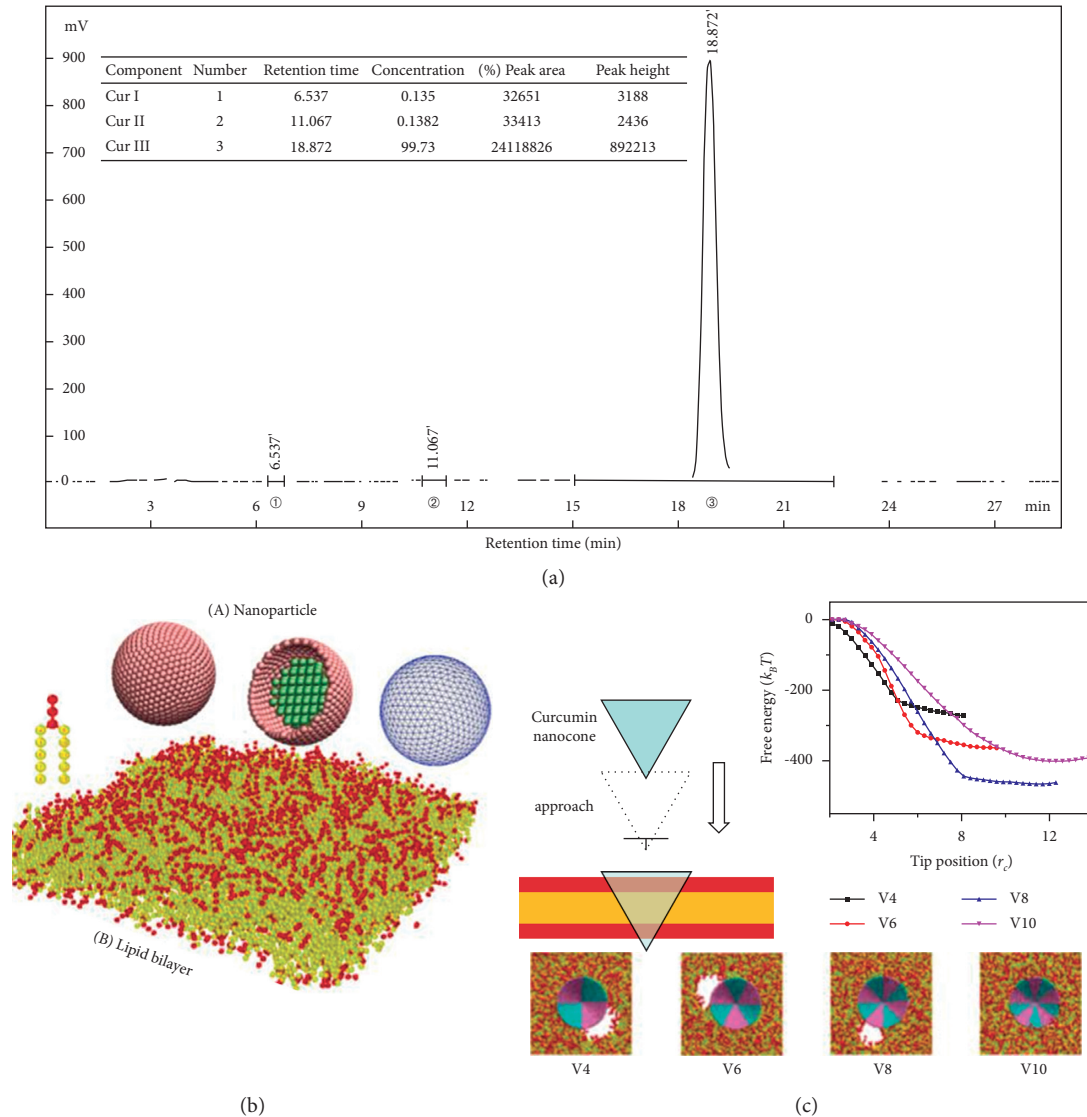


FIGURE 1: PLGA-Cur-NPs preparation and characteristics. (a) Chart of extracted curcumin identification. (b) Coarse granulation model of liposome flexible granules and cell membranes. (c) Free energy and biotoxicity analysis of contact process between conical nanomaterials and cell membranes.

sample means of multiple groups. Student’s *t*-test or paired Student’s *t*-test was performed to compare the sample means of two groups. $P < 0.05$ was considered statistically significant.

3. Results

3.1. PLGA-Cur-NPs Preparation and Vector Validation. In Figure 1(a), HPLC assay showed the extracted and separated active monomer from Cur complex. The characteristics of PLGA-Cur-NPs were shown in Figure 1(b) and 1(c).

3.2. Vector Validation and the Effects of PLAG-Cur-NPs on Proliferation in PC Cells. As shown in Figure 2(a), GFP was detectable in the transfected PC cells, suggesting the transfected efficiency of vectors. The results of western

blotting assay showed that transfected with $I\kappa B\alpha$ over-expression vector could significantly increase the protein expression of $I\kappa B\alpha$, compared to the NC group (Figure 2(b), $P < 0.05$). In Figure 2(c), overexpression of $I\kappa B\alpha$ significantly inhibited cell proliferation in a time-dependent manner ($P < 0.05$).

3.3. The Effects of PLAG-Cur-NPs on Proliferation and NF- κ B Signaling in PC Cells. Figure 3(a) shows PLAG-Cur-NPs suppressed cell proliferation in a dose-dependent manner ($P < 0.05$). Western blotting results showed that PLAG-Cur-NPs treatments significantly increased the protein levels of $I\kappa B\alpha$ and decreased the levels of P65 (Figure 3(b)).

3.4. Apoptosis and NF- κ B Signaling of PC Cells following PLAG-Cur-NPs Treatment. In Figure 4(a), PLAG-Cur-NPs’

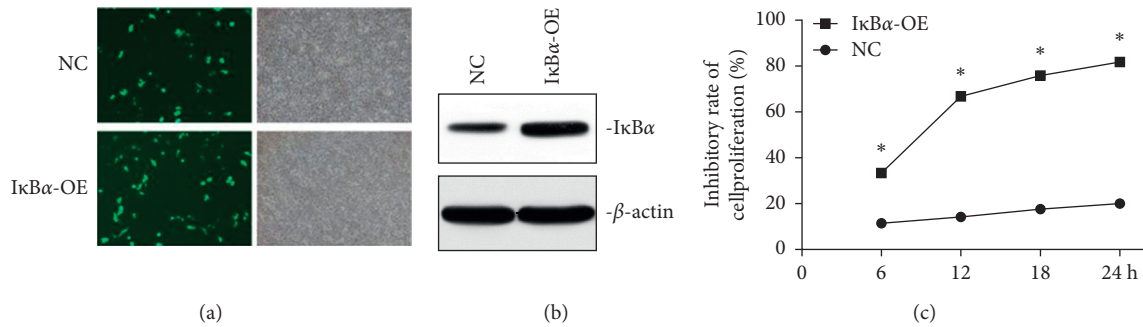


FIGURE 2: Vector validation and the effects of PLAG-Cur-NPs on proliferation in PC cell. (a) Transfected with IκBα-OE vector or Blank-vector (NC), the fluorescence of GFP in PC cells. (b) Western blotting assay was performed to measure the protein change of IκBα in PC cells transfected with IκBα-OE vector. (c) MTT assay was used to detect the proliferation of PC cells transfected with IκBα-OE vector. Compared with the NC group, * $P < 0.05$.

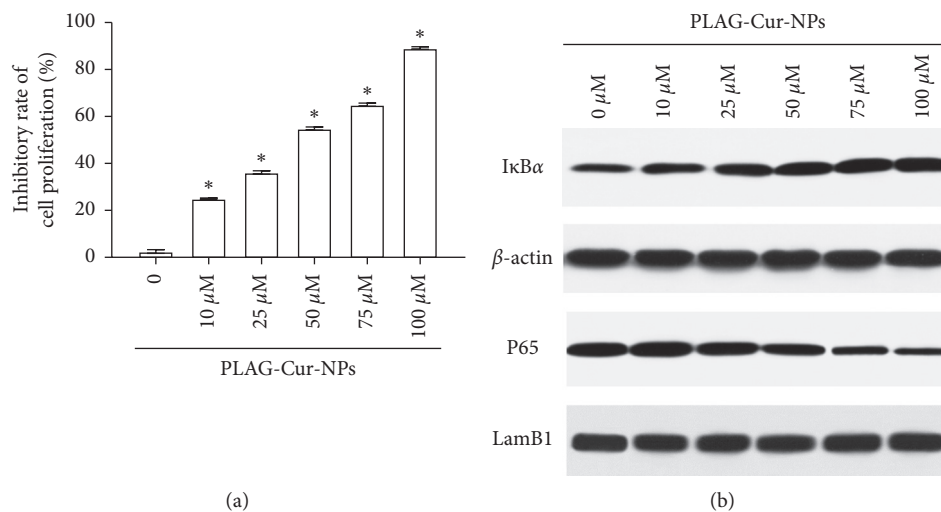


FIGURE 3: The effects of PLAG-Cur-NPs on cell proliferation and NF-κB signaling. (a) The inhibitory effects of PLAG-Cur-NPs at different concentrations (0, 10, 25, 50, 75, and 100 μM) on cell proliferation by MTT assay. (b) Western blotting assay was used to detect the effects of PLAG-Cur-NPs on NF-κB signaling related proteins. Compared with the PLAG-Cur-NPs (0 μM) group, * $P < 0.05$.

treatment significantly induced cell apoptosis ($P < 0.05$). Western blotting results showed that PLAG-Cur-NPs' treatment increased the protein levels of IκBα and decreased the levels of P65, Bcl-xL, and Bcl-2 (Figure 4(b)). Caspase-3 activity in PLAG-Cur-NPs-treated cells was significantly upregulated (Figure 4(c), $P < 0.05$). These results indicated that PLAG-Cur-NPs induced the apoptosis of PC cells.

3.5. The Synergistic Effects of IκBα-OE and PLAG-Cur-NPs. To determine whether IκBα overexpression could induce cell apoptosis synergetically with PLAG-Cur-NPs, PC cells were transfected with IκBα-OE or NC vector. In Figure 5(a), PLAG-Cur-NPs' treatment could significantly inhibit the activation of NF-κB signaling pathway, decreased the levels of the antiapoptotic protein Bcl-2, and increased the apoptotic protein Bcl-xL in PC cells. Besides, when the PC cells overexpressed IκBα at the same time, those effects of PLAG-Cur-NPs treatment were more obvious. MTT assay revealed that the inhibitory rates of proliferation were elevated in the co-treated with IκBα-OE vector and PLAG-Cur-NPs group,

compared to the NC + PLAG-Cur-NPs group (Figure 5(b), $P < 0.05$). In Figure 5(c), the results of apoptosis rates in PC cells were consistent with the changes of Bcl-xL ($P < 0.05$).

4. Discussion

Due to the lack of ideal tumor suppressor genes and transgenic vectors, the effect of tumor suppression on the proliferation of PC cells is not obvious. The transfection efficiency of target genes is unsatisfactory, which makes it unable to introduce the corresponding target genes to tumor cells, thus limiting the efficacy of gene therapy. Cationic PLGA nanoparticles are a new type of gene-carrying nanoparticles, which can improve the gene embedding rate and cell transfection rate through charge attraction and positive modification [7, 8]. Additionally, PLGA-NPs contribute to the drug delivery to the tumor site by small size and excellent release efficiency. Based on above studies, we investigated the effects of PLGA-Cur-NPs in PC cells, which had reliable biotoxicity (the flowchart of our experiment could be seen in Figure 6).

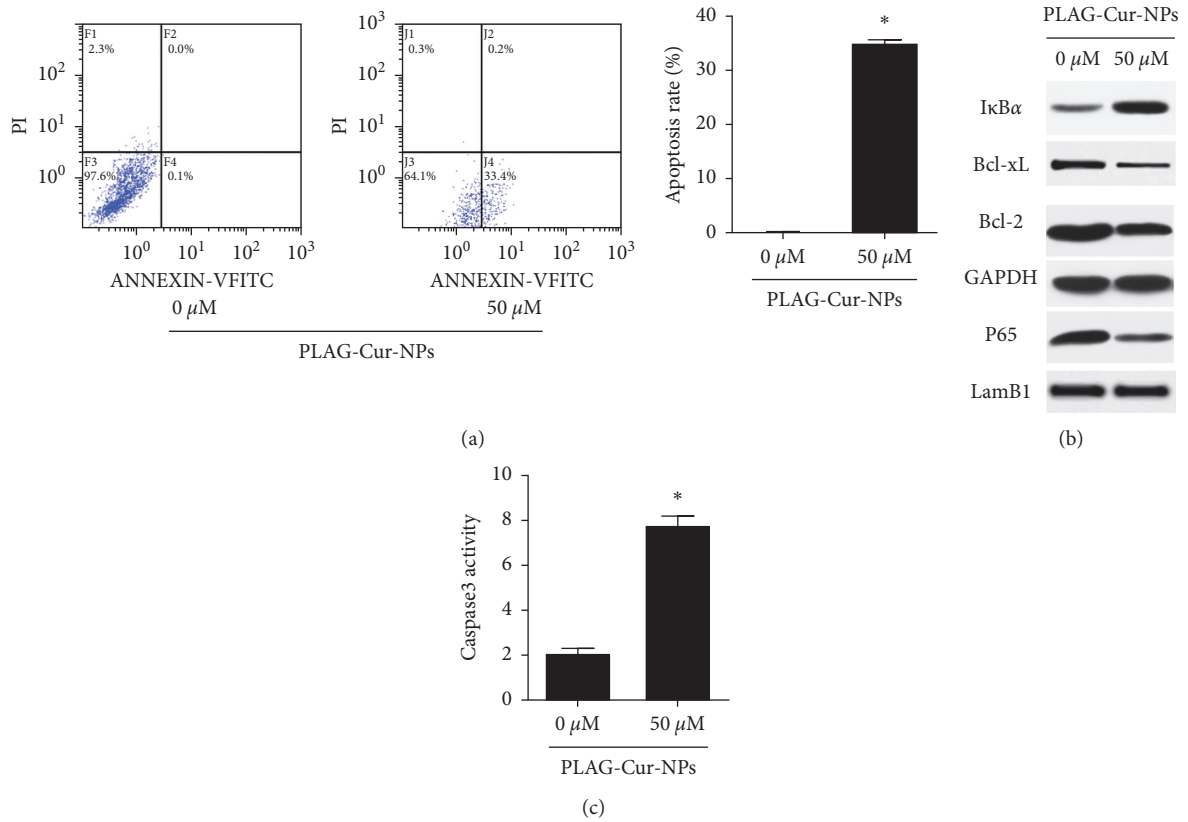


FIGURE 4: Apoptosis and NF- κ B signaling of PC cells following PLAG-Cur-NPs' treatment. (a) Cell apoptosis after PLAG-Cur-NPs' treatment for 12 h was measured by flow cytometry assay. (b) The effects of PLAG-Cur-NPs' treatment on the expression of Bcl-xL, Bcl-2, I κ B α , and p65 protein in PC cells by western blotting assay. (c) Caspase-3 activity assay kit was used to detect Caspase-3 activity after curcumin treatment of PC cells. Compared with the PLAG-Cur-NPs (0 μ M) group, * $P < 0.05$.

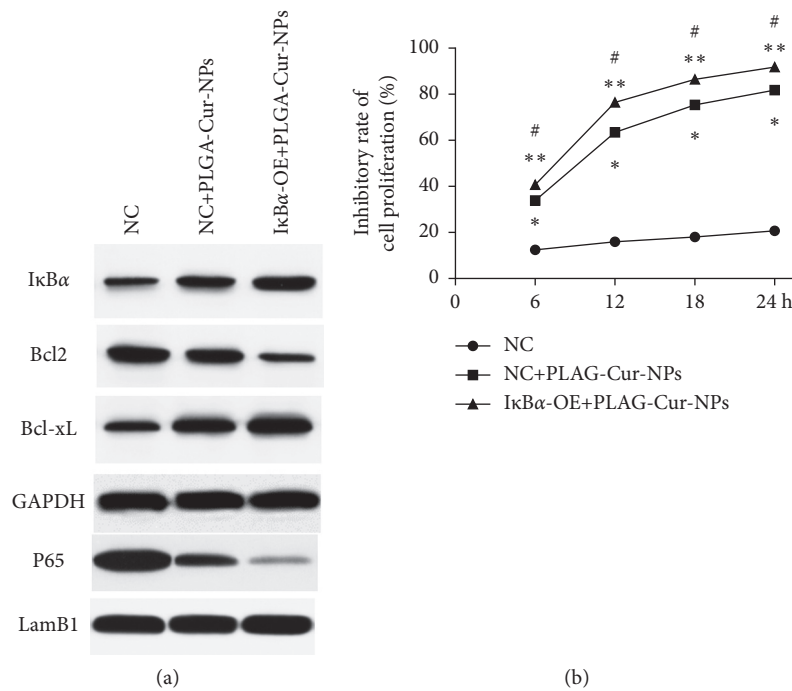
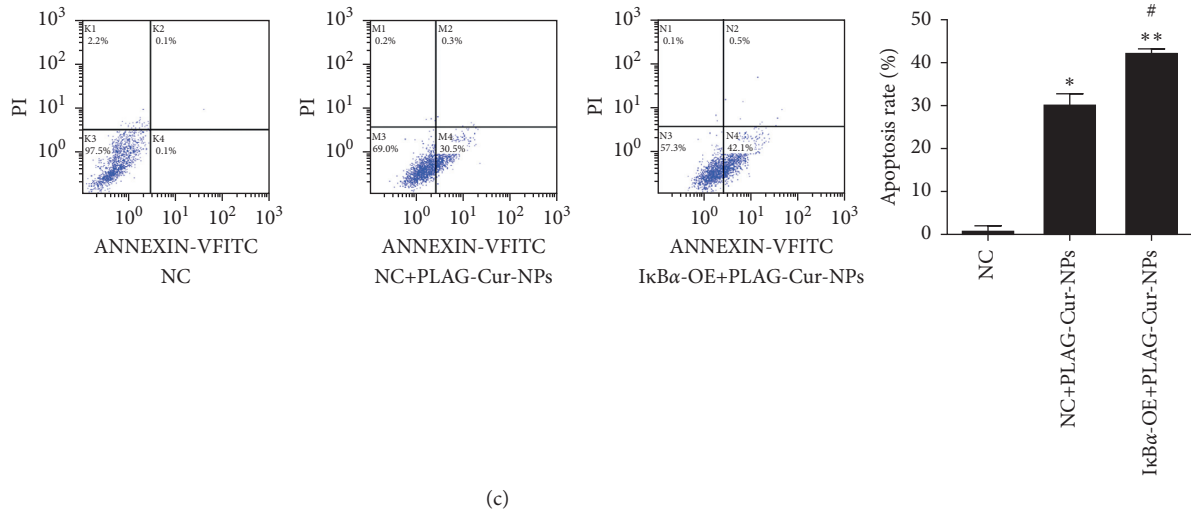


FIGURE 5: Continued.



(c)

FIGURE 5: The apoptosis of PLAG-Cur-NPs-treated PC cells with or without $I\kappa B\alpha$ -OE. (a) Western blotting assay was used to detect the protein expression of Bcl-2, Bcl-xL, and NF- κ B signaling in PC cells treated with PLAG-Cur-NPs in the presence or absence of $I\kappa B\alpha$ -OE. (b) MTT assay was used to detect the inhibitory effects of PLAG-Cur-NPs synergistic $I\kappa B\alpha$ -OE on the proliferation of PC cells. (c) The apoptosis rate of PC cells was detected by flow cytometry. Compared with the NC group, * $P < 0.05$ and ** $P < 0.01$. Compared with the NC + PLAG-Cur-NPs' group, # $P < 0.05$.

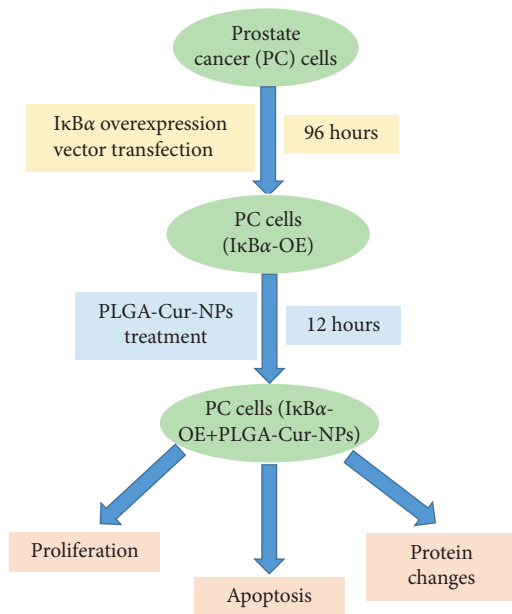


FIGURE 6: Flowchart of the study. Note: prostate cancer (PC); $I\kappa B\alpha$ overexpression ($I\kappa B\alpha$ -OE); poly (lactic-co-glycolic acid)-curcumin nanoparticles (PLGA-Cur-NPs).

Nuclear transcription factor (nuclear factor kappa B predominate, NF- κ B) plays an important role in process of PC, participating in PC cells' antiapoptotic effect. NF- κ B has been regarded as the target of tumor therapy, binding to $I\kappa B\alpha$ would enhance the release of NF- κ B from the DNA site [14–18]. Thus, $I\kappa B\alpha$ could be the hallmark of NF- κ B pathway by controlling the retention of NF- κ B [19]. Previous studies have shown that the activation of NF- κ B could stimulate the division and proliferation of PC cells [20]. Additionally, Ginzburg et al. reported that inhibition of NF- κ B activity reduced

the invasive ability of PC cells [21]. Therefore, we selected $I\kappa B\alpha$, an inhibitor of NF- κ B, as the research target of regulating the progression of PC. In recent years, traditional Chinese medicine has been widely used in treating various tumors, including PC, due to its mild efficacy and fewer adverse reactions. Yallapu et al. have developed a novel Cur drug delivery system [22] and demonstrated that PLGA-Cur-NPs exhibited superior anticancer activity in PC [23]. Thus, we suspected that Cur could inhibit the proliferation of PC cells, promote the expression of $I\kappa B\alpha$, and induce apoptosis in PC cells. In our study, we found that PLGA-Cur-NPs treatments could inhibit PC cell proliferation in a dose-dependent manner. Besides, $I\kappa B\alpha$ -OE could also inhibit the proliferation of PC cells. When the PC cells were co-treated with $I\kappa B\alpha$ -OE and PLGA-Cur-NPs, cell proliferation was suppressed more obvious. The synergistic effects of $I\kappa B\alpha$ -OE and PLGA-Cur-NPs on cell apoptosis were in consistent with cell proliferation.

It was known that NF- κ B/ $I\kappa B\alpha$ was activated in oxidative stress response [24]. A recent study showed that $I\kappa B\alpha$ OE facilitates the susceptibility to cisplatin in nonsmall cell lung cancer therapy [25]. In our study, we found that PLGA-Cur-NPs treatments or combined with $I\kappa B\alpha$ -OE could inhibit the activation of NF- κ B signaling pathway by decreasing the protein levels of P53 and increasing the levels of $I\kappa B\alpha$, which suggests that $I\kappa B\alpha$ -OE or PLGA-Cur-NPs' treatments might facilitate the susceptibility to drugs in PC.

In summary, overexpression of $I\kappa B\alpha$ could inhibit proliferation and induce apoptosis in PC cells. Nanometer carrier is a new gene carrier system because of its anti-tumorigenicity, high gene transfer efficiency, extended time, and slower degradation. The NPs have incomparable advantages with other carrier systems and are expected to provide a new delivery system for gene therapy. Given this, this study suggested PLGA-Cur-NPs' synergistic with $I\kappa B\alpha$ -OE in PC cells is hoping to be a clinical application of tumor

therapy in the future. However, the further studies are still needed in the future.

Data Availability

The data used to support the findings of this study are included within the article.

Conflicts of Interest

The authors declare that they have no conflicts of interest to declare.

Acknowledgments

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