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Codelivery of Antitumor Drug and Gene by a pH-Sensitive Charge-Conversion System

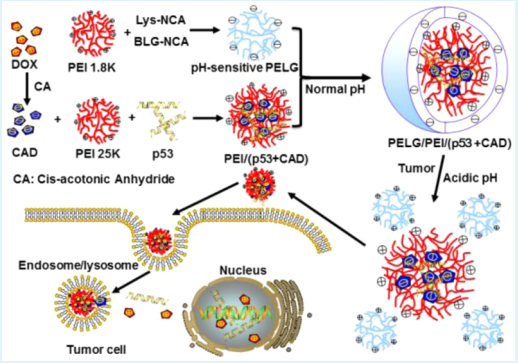
Xiuwen Guan,[†,§] Yanhui Li,*[,‡] Zixue Jiao,[†] Lin Lin,[†] Jie Chen,[†] Zhaopei Guo,[†] Huayu Tian,*[,†] and Xuesi Chen[†]

[†]Key Laboratory of Polymer Ecomaterials, Changchun Institute of Applied Chemistry, Chinese Academy of Sciences, Changchun 130022, P. R. China

[‡]Changchun University of Science and Technology, Changchun 130022, P. R. China

[§]University of Chinese Academy of Sciences, Beijing 100039, P. R. China

ABSTRACT: In the present study, a gene and drug codelivery system was developed by electrostatic binding of polyethylenimine-poly(L-lysine)-poly(L-glutamic acid) (PELG), polyethylenimine (PEI), *cis*-aconityl-doxorubicin (CAD), and DNA. Zeta potential and drug release analysis confirmed the pH-responsive charge conversion and acid-sensitive drug release functional properties of the PELG/PEI/(DNA+CAD) system. Gel retardation assay and transfection experiment showed the codelivery system had effective DNA binding ability and good transfection efficiency on HepG2 cells. The therapeutic gene p53 was further employed to study its combinational effects with CAD. Cytotoxicity assay showed the half inhibitory concentration (IC_{50}) of the PELG/PEI/(p53+CAD) codelivery system was lower than that of the gene or the drug delivery system. Confocal laser scanning microscopy (CLSM) showed that the drug and gene could be delivered into the cells simultaneously. A significant increase of p53 gene expression was achieved after HepG2 cells treated by PELG/PEI/(p53+CAD) codelivery system. The apoptosis experiment indicated clearly that the codelivery system could lead an effective apoptosis on tumor cells, which was beneficial for the treatment of cancer. The biodistribution and tumor accumulation of the codelivery system was explored via in vivo imaging in subcutaneous xenograft and in situ tumor models. The tumor and some major organs were excised and imaged, and the results showed that the codelivery system can accumulate efficiently in tumor for both tumor models. It can be suggested from the above results that the PELG/PEI/(DNA+CAD) codelivery system will have great potential applications in cancer therapy.



KEYWORDS: *codelivery, p53, doxorubicin, pH-sensitive, charge conversion, cancer therapy*

1. INTRODUCTION

Although many researchers in the world have spared no effort to search for the effective ways for antitumor therapy, it is still very hard to fundamentally cure the cancer at present.[1] As the pathological complexity of cancer, the desired therapeutic effects cannot be achieved effectively by treatment with only one type of drug.[2] The gene and drug codelivery system is much concerned and widely studied as a new strategy of antitumor treatment in the past decade.[3−5] The combinational use of gene and drug often produces better outcomes compared with single gene or drug therapy. Co-delivery of drug and gene in one system can reduce the number of injections, which can not only increase patient compliance but also improve their living quality.[2] Moreover, it achieves a synergistic or additive therapeutic effect at lower dose when both drug and gene are delivered into the same tumor cells or tissues, and the lower dose in therapy can suppress the severe adverse effect.[1,5,6]

The tumor suppressor gene p53 is widely used as a therapeutic gene, it can induce cell apoptosis or cellular senescence, and it plays a crucial role in inhibiting the cell proliferation.[7−10] The dysfunction of p53 gene happened in lots of human tumor cells, which led to decreased chemosensitivity and promoted resistance of tumor cells to chemotherapeutics.[11,12] Wild-type (wt) p53 maintains the stability of normal genom and induces apoptosis of DNA damaged cells. To cure cancer by gene therapy, reintroduction of wt p53 into tumor cells can realize the correction and replacement of the mutational p53 gene, which also can reduce drug resistance of tumor cells through inhibiting the P-glycoprotein (P-gp) expression encoded by MDR-1.[13−15] Moreover, it has been reported that the product of the tumor suppressor gene (p53 protein) can positively respond to some signals such as DNA damages caused by drugs, like doxorubicin (DOX).[16,17] The codelivery of wt p53 gene and DOX increases the therapeutic efficacy in cancer treatment.[1,18−20]

As is well-known, polyethylenimine (PEI) is widely used for gene delivery. PEI with molecular weight of 25 kDa (PEI25K) is

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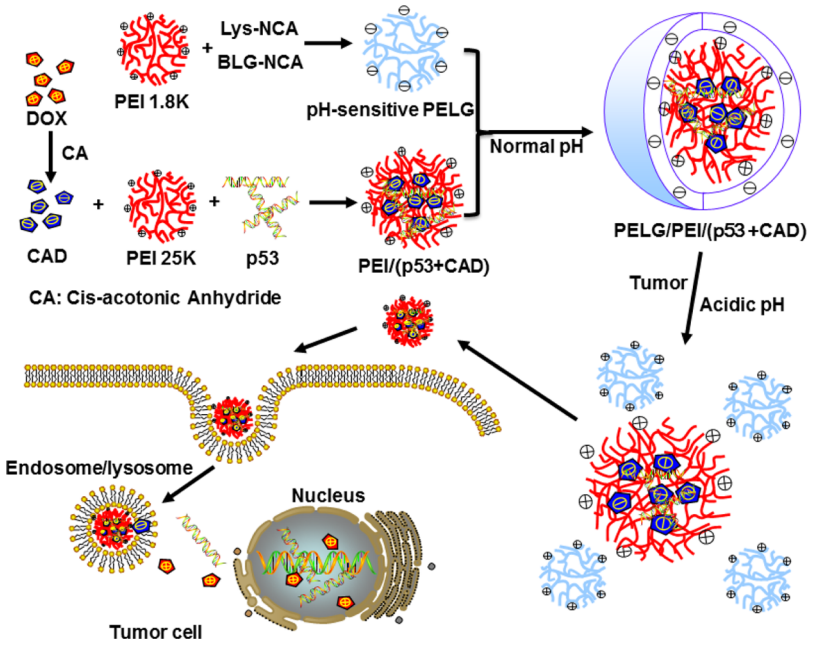

Figure 1. Design scheme of the codelivery system.

regarded as the "gold standard" for gene carriers due to its excellent transfection efficiency.[21−24] Positively charged PEI can be used for delivering negatively charged genes, proteins, and drugs, and the PEI/gene electrostatic complexes are widely used as a gene delivery system.[25−28] However, the positively charged PEI/gene complex may interact with negatively charged macromolecules or the cell membrane in vivo, which is unfavorable to arrive in the target tissues. So a negatively charged material is needed to shield the PEI/gene complex during transfer process in body fluid, it can shield the positive charge of PEI, decrease the toxicity, and prevent aggregation, and the material should possess the property that can be taken off from the PEI/gene complex when arriving in tumor area at lower pH.

The polyethylenimine-poly(L-lysine)-poly(L-glutamic acid) (PELG) is a copolymer that possesses pH-sensitive charge-reversal property, and it was designed as an intelligent shielding material that could reduce the positive charge of PEI because of its response to the different pH, and our previous studies showed that the intelligent shielding material PELG could make the nanocarrier systems more excellent and effective.[29,30] In this work, we further designed a PELG/PEI/(DNA+CAD) system to realize gene and drug codelivery (Figure 1). Negatively charged CAD and p53 gene were adsorbed by positively charged PEI to form the drug and gene coloaded complex, and the complex was further shielded by PELG; by doing this, a pH-sensitive charge-reversal codelivery system was obtained. This codelivery system could not only convert its surface charges in the acidic tumor area, but also intelligently release drugs depending on the different acidic pH. The system was investigated through a series of experiments. The properties such as pH-sensitive charge-conversion, DNA binding, drug release behavior, gene transfection, and codelivery of gene and drug into same cells were well-characterized. Furthermore, the p53 tumor suppressor gene was used as the therapeutic gene, and the cytotoxicity of the p53 and CAD codelivery system was investigated, and then the p53 gene expression and cell apoptosis were evaluated in detail. Two kinds of liver tumor models were established to explore the biodistribution and tumor accumulation of the codelivery system, which could offer a favorable evidence to verify the tumor accumulation ability of the codelivery system.

2. EXPERIMENTAL SECTION

2.1. Materials. Doxorubicin hydrochloride (DOX·HCl) was purchased from Zhejiang Hisun Pharmaceutical Co. Ltd. *cis*-Aconitic anhydride (CA) was obtained from Alfa Aesar (Lancashire, UK). *cis*-Acontyl-doxorubicin (CAD) and polyethylenimine-poly(L-lysine)-poly(L-glutamic acid) (PELG) were synthesized according to our previous studies, and the chemical structures, preparation methods, and the detailed characterizations were shown in detail.[29,30] The tumor suppressor gene p53 (pCMV-p53, 6.3 kb) that encodes wild-type p53 tumor suppressor protein driven by CMV promoter was kindly provided by Jilin University (China). Branched PEI with molecular weight of 25 000 Da (PEI25K) was purchased from Aldrich. Calf thymus DNA was purchased from Sigma (St. Louis, MO, USA). Luciferase plasmid (pGL3-control), cell lysate and the luciferase reporter gene assay kit were purchased from Promega (Mannheim, Germany). Methyl thiazolyl tetrazolium (MTT) was purchased from Amresco (Solon, Ohio, USA). Dulbecco's modified Eagle's medium (DMEM) and fetal bovine serum (FBS) were purchased from Gibco (Grand Island, USA). FAM labeled RNA, Trizol and PCR Primer were purchased from Invitrogen (Carlsbad, CA, USA). AnnexinV-FITC/PI apoptosis detection kit was purchased from Nanjing KeyGEN Biotech Co. Ltd., China. Prime Script RT reagent kit with gDNA Eraser (Perfect Real Time) and SYBR Premix Ex *Taq*II (Tli RNaseH Plus) were purchased from TAKARA Biotechnology Co. Ltd. The other reagents were purchased from Sinopharm Chemical Reagent Co. Ltd., China.

2.2. Preparation of Complexes. The preparation of complexes was described in the following steps. First, the PEI/DNA, PEI/CAD and PEI/(DNA+CAD) were prepared by mixing DNA or (and) CAD aqueous solution with PEI aqueous solution in equal volume, and vortexed for 15 s. The solutions were then incubated at room temperature for 20 min. Afterward, a certain amount of PELG aqueous solution was added, and the final solution was adjusted to pH 7.4 to keep PELG in negative potential. The solution was vortexed for 15 s and incubated for 20 min. The gene-loaded PELG/PEI/DNA complexes, the drug-loaded PELG/PEI/CAD complexes, and the gene and drug coloaded PELG/PEI/(DNA+CAD) complexes were obtained separately.

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2.3. Zeta Potential and Particle Size. The zeta potentials and particle sizes of PELG/PEI/DNA, PELG/PEI/CAD and PELG/PEI/(DNA+CAD) complexes at different pH values (pH 7.4, 6.8, and 6.4) were measured at room temperature by using a zeta potential/BI-90Plus particle size analyzer (Brookhaven, USA). Data were shown as mean ± standard deviation (SD) based on triplicate independent experiments.

2.4. Gel Retardation Assay. The DNA binding ability of the system was tested by gel retardation assay. The agarose was added into TAE buffer (1/100, W/V), then heated and melted to obtain 1% agarose gel. The PELG/PEI/(DNA+CAD) complexes were prepared at different mass ratios, and mixed with loading buffer. Electrophoresis (DY-4C, Liuyi, Beijing, China) was carried out in TAE running buffer solution at 85−100 V for 1 h. The gel was carefully taken out and stained with ethidium bromide (EtBr). The gel was analyzed by UV gel imaging system (UVP EC3, UVP Inc., Upland, USA) to show the DNA position.

2.5. Drug Release Studies. In vitro drug release was studied in PBS at different pH values (pH 7.4, 6.8, 6.4 and 5.8). The PELG/PEI/(DNA +CAD) complexes were freshly prepared and 2 mL of the complex solution (containing 50 μg CAD) was migrated into a dialysis bag (MWCO 7000 Da), the dialysis bag was immersed into 58 mL PBS and shaken (70 rpm) at 37 °C. Then 2 mL of PBS was taken out from the release medium and 2 mL of fresh PBS was added at fixed time intervals. The released drug content was analyzed by UV spectrophotometry at 480 nm.

2.6. In Vitro DNA Transfection. The DNA transfection experiment of the system was carried out in a human hepatocarcinoma (HepG2) cell line. The luciferase plasmid DNA (pGL3-control) was used as the reporter gene. HepG2 cells were seeded in 96-well plates at a density of 8000 cells/well and then cultured at 37 °C in a 5% CO_2 atmosphere for 24 h. Before transfection, the PELG/PEI/pGL3 and PELG/PEI/(pGL3+CAD) complexes at various mass ratios were prepared. The plates were taken out and the culture medium (DMEM) was replaced with 180 μL/well fresh DMEM in different pH values (7.4 and 6.8). After the complexes were added to each well, the plates were returned to the incubator for 2 h. Then culture medium was replaced with 200 μL/well fresh DMEM, and the plates were returned to the incubator for another 46 h. After that, 50 μL of cell lysate was added to each well and the plates were frozen in −80 °C for 0.5−1 h. After thawing, the supernatant of the cell lysate (20 μL) was mixed with 100 μL of luciferase substrate. The relative light units (RLU) were measured using a luminometer (Turner Biosystems & Promega), and normalized to total protein content measured by BCA protein assay (Sigma). Luciferase activity was expressed as RLU/mg protein. One-way analysis of variance was performed to calculate the p-values.

2.7. Cytotoxicity Assay. The cytotoxicities of the materials and complexes were measured by MTT assay. HepG2 cells were seeded in 96-well plates at 8000 cells per well and cultured at 37 °C in a 5% CO_2 atmosphere for 24 h. Before the experiment, PELG, PELG/PEI, PELG/PEI/p53, PELG/PEI/CAD, and PELG/PEI/(p53+CAD) complexes at various mass ratios were prepared. The plates were taken out and 180 μL/well fresh DMEM in different pH values (7.4 and 6.8) was added. After the complexes were added to each well, the plates were returned to the incubator for 2 h. Then culture medium was replaced with 200 μL/well fresh DMEM, and the plates were returned to the incubator for another 46 h. MTT (20 μL, 5 mg/mL) was then added to each well. After 4 h of incubation, the medium was removed carefully and 200 μL of DMSO was added to each well for dissolving the formazan crystals of MTT. The samples were measured by using a Bio-Rad 680 microplate reader at 492 nm. The cell viability (%) was calculated as

$$\text{cell viability (\%)} = (A_{\text{sample}}/A_{\text{control}}) \times 100\% \quad (1)$$

where A_{sample} is the absorbency of the sample well and A_{control} is the absorbency of the control well. The data were shown as mean ± SD based on triplicate independent experiments.

2.8. Confocal Laser Scanning Microscopy. HepG2 cells were seeded on coverslips in 6-well plates at a density of 1.0×10^5 cells per well and grown for 24 h. Before the gene and drug coloaded complexes were added, the growth medium was replaced with fresh DMEM of pH 7.4 or 6.8, and then the complexes solution was added to each well. After 2 h of incubation, the cells were washed with PBS for three times and fixed with 3.7% paraformaldehyde for 15 min at room temperature. The cells were carefully washed three times with PBS after immobilization. The cell nuclei were stained by 4′-6-diamidino-2-phenylindole (DAPI, 1 mg/mL, 1 μL/well) for 15 min. The cells were then washed by PBS five times. At last, the coverslips were taken out from the wells and carefully placed on slides, enclosed with glycerol. The samples were observed by CLSM (ZEISS LSM780, Germany).

2.9. p53 Gene Expression. To measure the p53 gene expression level, quantitative real-time PCR (RT-PCR) technique was utilized to evaluate the relative mRNA quantity of p53 gene expression. HepG2 cells were seeded on 6-well plate at 2×10^5 cells per well. The cells were transfected with PELG/PEI/(p53+CAD) complexes in different pH (pH 7.4 and 6.8) for 2 h. Culture medium was then replaced with 2 mL/well fresh DMEM. After incubation for 24 or 48 h, total RNA was isolated from the cells. The isolated RNA was reverse transcribed to cDNA with Prime Script RT reagent kit with gDNA Eraser (Perfect Real Time) according to the instructions. Real-time PCR experiment was performed using SYBR Premix Ex *Taq*II (Tli RNaseH Plus) according to the instructions. The p53 primers used for the RT-PCR experiment were as follows: forward, 5′-CCGCAGTCAGATCCTAGCG-3′; reverse, 5′-AATCATCCATTGCTTGGGACG-3′.[1,31] The β-actin primers were as follows: forward, 5′-AATGTGGCCGAGGACTTT-GATTGC-3′; reverse, 5′-TTAGGATGGCAAGGGACTTCCTGT-3′. Amplification conditions are as follows: 40 cycles of denaturation at 95 °C for 30 s, annealing at 55 °C for 60 s, and extension at 72 °C for 60 s with Mx3005P instrument (Stratagene, USA).

2.10. Apoptosis Assay. The apoptosis assay of the system was detected by Annexin V-FITC/PI apoptosis detection kit. HepG2 cells were seeded in 6-well plates at 2×10^5 cells per well. Before the experiment, PELG/PEI/(p53+CAD) complexes were prepared. The plates were taken out and the culture medium (DMEM) was replaced with fresh DMEM in different pH values (7.4 and 6.8). After the complexes were added to each well, the plates were returned to the incubator for 2 h. Then culture medium was replaced with 2 mL/well fresh DMEM, and the plates were returned to the incubator for another 22 or 46 h. The cells were washed with 4 °C PBS and digested with EDTA-free trypsin. The cell suspension was collected and centrifuged twice (2000 rpm, 5 min). The supernatant was removed and the cells were dispersed in 0.5 mL of binding buffer, then 5 μL of annexin V-FITC and 5 μL of propidium Iodide (PI) were added into the cells in sequence and incubated for 5−15 min at room temperature in dark. The cells were tested with a FACS Calibur System from Becton-Dickinson (San Joes, CA) for apoptosis assay.

2.11. In Vivo Imaging. To evaluate the in vivo distribution of the codelivery system, two kinds of liver tumor models were used. In situ liver tumor model was established by orthotopic inoculation. HepG2 cells (2×10^6 cells/50 μL) were implanted into the left liver lobe of Balb/c nude mice (male, 4 week-old, about 20 g) to establish liver tumor. Subcutaneous xenograft tumor model was generated by injecting HepG2 cells (2×10^6 cells/50 μL) into the left flank of nude mice. After implantation, it needed about 3−4 weeks to develop into tumors that were about 0.5 cm in diameter. Then the mice were injected with 0.2 mL PELG/PEI/(p53+CAD) (1 mg/kg body weight on a DOX basis) via tail vein. At fixed time intervals, the animals were anaesthetized for in vivo imaging by a Maestro In Vivo Imaging System (Cambridge Research & Instrumentation, Inc., USA), DOX was excited by an excitation filter (445−490 nm), the fluorescence was detected through an emission filter (580 nm), and the exposure time was 2000 ms.[32−34] After 24 h, the mice were sacrificed and the major organs (heart, liver, spleen, lung and kidney) and tumors were excised and imaged in the same detection method.

3. RESULTS AND DISCUSSION

3.1. Zeta Potential and Particle Size. The zeta potentials and particle sizes of the complexes are shown in Figure 2. The PELG/PEI/CAD, PELG/PEI/DNA, and PELG/PEI/(DNA +CAD) complexes were negatively charged in pH 7.4, when the pH decreased to 6.8, the zeta potentials of the complexes

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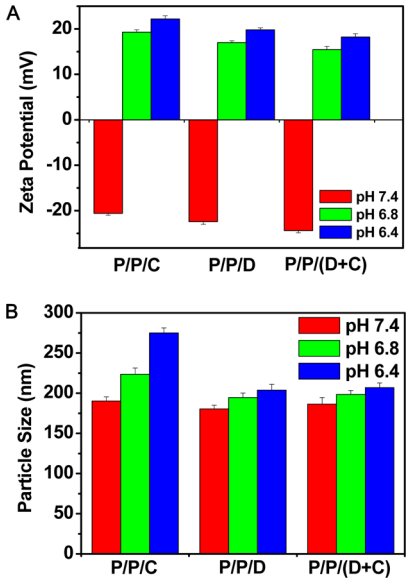

Figure 2. (A) Zeta potential and (B) particle size of the complexes: P/P/C, PELG/PEI/CAD (wt/wt/wt = 5:2.5:1); P/P/D, PELG/PEI/DNA (wt/wt/wt = 5:2.5:1); P/P/(D+C), PELG/PEI/(DNA+CAD) (wt/wt/wt/wt = 5:2.5:1:1). Data are shown as mean ± SD (n = 3).

were reversed to positively charged, and with the pH further decreased to 6.4, the potentials were relatively increased (Figure 2A). These results were in good agreement with our original intention, and the PELG shielding system showed excellent pH-sensitive charge-reversal property. When transported in body fluid (pH 7.4), the complexes were negatively charged and barely interacted with the negatively charged proteins and cell membranes. When the complexes reached acidic tumor area (pH 6.8, 6.4), their surface was positively charged which was conducive to interact with cell membranes for higher uptake efficiency. The PELG/PEI/CAD complexes were about 190 nm in pH 7.4, and the size of the complex increased with the pH decreasing as shown in Figure 2B. The particle sizes of PELG/PEI/DNA and PELG/PEI/(DNA+CAD) were smaller than PELG/PEI/CAD because of the DNA condensing effect of the system, producing a compacting complex structure.

3.2. Gel Retardation Assay. Gel retardation assay was used to test the DNA binding ability of the codelivery system, and the results were showed in Figure 3. Lane 1 was naked DNA; Lane 2 was PEI/DNA, wt:w t= 2.5:1; Lane 3 was PEI/(DNA+CAD), wt:wt:wt = 2.5:1:1; Lanes 4−8 were PELG/PEI/(DNA+CAD), wt:wt:wt:wt=2.5:2.5:1:1, 5:2.5:1:1, 7.5:2.5:1:1, 10:2.5:1:1, and 20:2.5:1:1, respectively. Considering the negatively charged PELG and DNA were both interacted with the positively charged PEI by electrostatic binding, the DNA might be likely to compete with PELG. So it was important to elucidate whether the DNA binding ability decreased with the PELG amount increased. The mass ratio of PELG/PEI was changed from 2.5:2.5 to 20:2.5 for the verification. The results showed that complete retardation of DNA mobility was achieved (Lanes 2−8) in the range of tested mass ratios, indicating that the complexes had efficient DNA binding capability, and the PELG did not damage the stability of the PEI/(DNA+CAD) complexes.

3.3. Drug Release Studies. The drug cumulative release of the codelivery system is shown in Figure 4. The cumulative drug release of the PELG/PEI/(DNA+CAD) complexes increased with the decreasing pH, and the cumulative release reached to 80% in pH 5.8. This kind of acid-responsive drug release was due

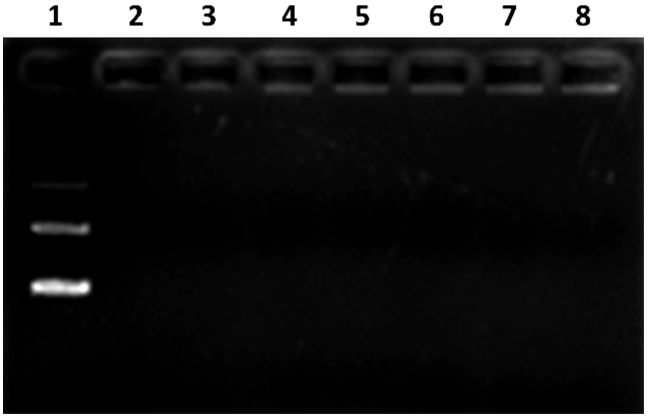

Figure 3. DNA gel retardation assay. Lane 1, naked DNA; Lane 2, PEI/DNA, wt:wt = 2.5:1; Lane 3, PEI/(DNA+CAD), wt:wt:wt = 2.5:1:1; Lanes 4−8, PELG/PEI/(DNA+CAD), wt:wt:wt:wt = 2.5:2.5:1:1, 5:2.5:1:1, 7.5:2.5:1:1, 10:2.5:1:1, and 20:2.5:1:1.

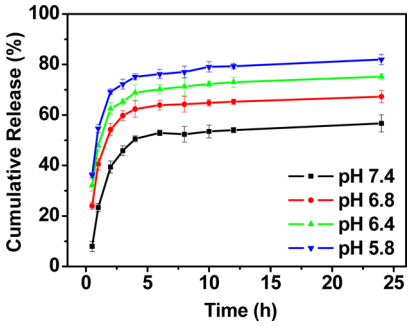

Figure 4. Cumulative release of drug from PELG/PEI/(DNA+CAD) complexes at different pH values. Data are shown as mean ± SD (n = 3).

to the acid-sensitive CAD, the cis-aconityl linkage of CAD could be cleaved in acidic environment.[29,35,36] The CAD was cleaved to positively charged DOX, which could not interact with PEI, so that DOX easily released from the complexes. This result demonstrated that the codelivery system possessed the acid-sensitive drug release property, which caused more drug release in acidic tumor area, and the system was suitable for cancer therapy.

3.4. In Vitro Transfection. Before the DNA transfection of the codelivery system was discussed, PELG/PEI/DNA should be studied for DNA transfection on HepG2 cells to optimize the mass ratio of PELG/PEI/DNA in transfection. The pGL3 was used as reporter gene. The mass ratios of PELG/PEI/DNA were chosen as 0:2.5:1, 2.5:2.5:1, 5:2.5:1, 7.5:2.5:1, and 10:2.5:1, and the transfection experiment was carried out in different pH (pH 7.4 and 6.8). As was seen in Figure 5, the transfection efficiency increased when PEI/DNA was shielded with PELG at 2.5:2.5:1 both in pH 7.4 and 6.8, and a significant transfection efficiency difference can be seen between pH 7.4 and pH 6.8 (much higher in pH 6.8). And when the mass ratio increased to 5:2.5:1, a more significant difference happened. This was caused by the PELG shielding. When PEI/DNA was shielded by PELG, the positive charge of PEI/DNA was covered and the PELG/PEI/DNA complex was negatively charged in pH 7.4, which may have lower cytotoxicity than PEI/DNA. Although the shielded complexes were not easy to interact with the negatively charged cell membranes as PEI/DNA did, finally the decreased cytotoxicity might be beneficial for PELG/PEI/DNA having relatively higher transfection efficiency than PEI/DNA in pH 7.4. The trans-

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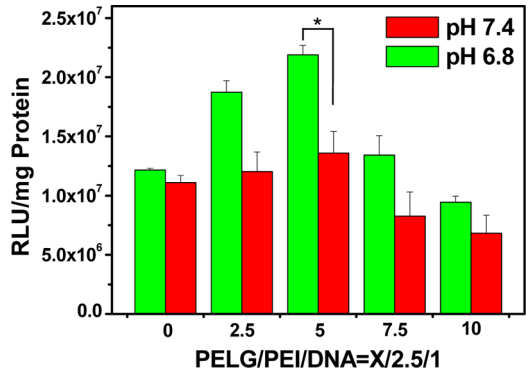

Figure 5. Transfection efficiency of PELG/PEI/DNA at different pH values (7.4 and 6.8) in HepG2 cells. Data are shown as mean ± SD (n = 3). p^* < 0.01.

fection efficiency of the shielded complexes in pH 6.8 increased a lot, which was caused by the pH-sensitive charge-conversion of PELG. The PELG made the complexes be positively charged in pH 6.8 and might result in higher cell uptake and transfection efficiency. However, when the mass ratio further increased to 7.5:2.5:1 and 10:2.5:1, the transfection efficiency decreased. This result might be caused by two reasons. First, when the mass ratio increased to 7.5:2.5:1 or 10:2.5:1, more PELG would make the complexes become more negatively charged in pH 7.4, which might barely interact with the negatively charged cell membranes, leading to low cell uptake and transfection efficiency. Second, when the complexes were under pH 6.8, PELG changed to positively charged, which would make the complexes become more positive, the positive charge might increase the cytotoxicity to the cells, resulting in a decreased transfection. By this study, we can know that the optimal PELG/PEI/DNA mass ratio for transfection efficiency was 5:2.5:1, the following studies were carried out according to this mass ratio.

After confirming the optimal mass ratio of PELG/PEI/DNA in transfection, we further investigated the transfection of the codelivery system, the mass ratios of PELG/PEI/DNA/CAD were 5:2.5:1:0, 5:2.5:1:0.1, 5:2.5:1:0.2, 5:2.5:1:0.5, 5:2.5:1:1, 5:2.5:1:2, 5:2.5:1:5, and 5:2.5:1:10, the final concentrations of CAD were respectively 0, 0.1, 0.2, 0.5, 1, 2, 5, and 10 μg/mL, the experiment was carried out in different pH (7.4 and 6.8). As shown in Figure 6, the transfection efficiency was appeared to be reduced in the presence of CAD. This phenomenon was

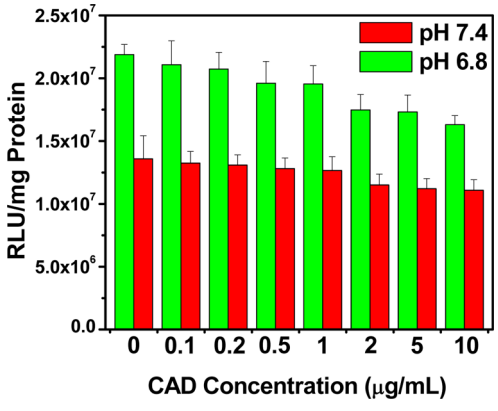

Figure 6. Transfection efficiency of PELG/PEI/(DNA+CAD) at different pH values (7.4 and 6.8) in HepG2 cells. Data are shown as mean ± SD (n = 3).

consistent with the reported results,[6,37] the reduced luciferase expression might be influenced by the cytotoxicity of DOX. DOX can inhibit biomacromolecule synthesis by intercalating DNA molecules,[30,38,39] the cytotoxicity of DOX might impede the gene expression of the transfected cells.[6] The luciferase expression level decreased a little in the CAD concentrations of 0.1−1 μg/mL, and a further decline was observed in the CAD concentrations of 2−10 μg/mL. In the range of tested CAD concentrations, the transfection in pH 6.8 was higher than that of in pH 7.4, this was attributed to the pH-sensitive charge-conversion of PELG, the complexes were positively charged in pH 6.8, leading to a higher cell uptake and transfection efficiency. Considering this result and previous studies, the mass ratio of PELG/PEI/DNA/CAD was chosen as 5:2.5:1:1 for further studies.

3.5. Cytotoxicity Assay. The cytotoxicity of the materials against HepG2 cells was studied and showed in Figure 7. It showed that the cytotoxicity of PELG (Figure 7A) was slightly increased with the concentration getting higher, and the cell viability was above 80% even at the maximum tested concentration (100 μg/mL). That was to say the cytotoxicity of PELG was really low. As we know, low toxicity was very important for the carriers, it guaranteed the safety of the carriers while applied in vivo. As the low cytotoxicity and good biocompatibility of PELG, it can be safely used in vivo. In Figure 7B, it was found that both PEI and the carrier PELG/PEI showed decreased cell viability with the PEI concentration increasing, and the PEI was really toxic to the cells. However, the PELG/PEI carrier showed much lower cytotoxicity than PEI at the same PEI concentration. This might be because when PEI was shielded with PELG, the PELG could shield the positive charges of PEI, which would be conducive to reduce cytotoxicity. So the PELG is an effective shielding material, and the PELG/PEI is safe to use as a nanocarrier.

The cytotoxicity of PELG/PEI/p53 gene delivery system, PELG/PEI/CAD drug delivery system and PELG/PEI/(p53+CAD) gene and drug codelivery system against HepG2 cells were investigated and showed in Figure 8. The mass ratio of PELG/PEI/p53 and PELG/PEI/CAD was 5:2.5:1, PELG/PEI/p53/CAD was 5:2.5:1:1, Figure 8A was in pH 7.4 and Figure 8B was in pH 6.8. The cell viability of all groups decreased gradually with the PELG concentration increase. The cell viability of PELG/PEI/p53 was higher than that of PELG/PEI/CAD, and the lowest one was PELG/PEI/(p53+CAD) which owned highest killing effect on tumor cells. In pH 7.4, the half inhibitory concentration (IC_{50}) of PELG/PEI/p53 gene delivery system, PELG/PEI/CAD drug delivery system and PELG/PEI/(p53+CAD) gene and drug codelivery system was 40.6, 25.1, and 18.3 μg/mL, respectively. However, the IC_{50} of the three systems was 25.6, 17.0, and 11.6 μg/mL in pH 6.8 which significantly reduced comparing with pH 7.4. The pH-sensitive charge-reversal PELG made the complexes positively charged in pH 6.8, leading to a higher cell uptake and cytotoxicity. This result indicated clearly that the cytotoxicity of the systems increased in acidic environment (pH 6.8), and the codelivery system had high killing effect on tumor cells.

3.6. Confocal Laser Scanning Microscopy. To verify the intracellular colocation of gene and drug, we exploited the CLSM to observe the cells treated with gene and drug codelivery system. As shown in Figure 9, the cell nucleus was stained blue with DAPI, the green fluorescence was from FAM (6-carboxyfluorescein) labeled RNA (replace DNA), the red fluorescence was from DOX. The gene and drug related fluorescent signals were

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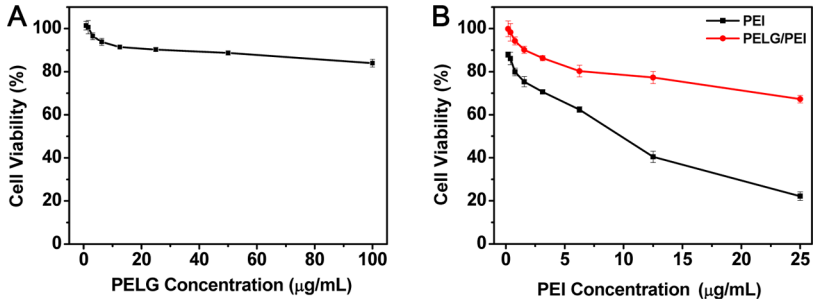

Figure 7. Cytotoxicity of the materials at various concentrations, (A) PELG, (B) PEI and PELG/PEI (wt/wt = 5/2.5).

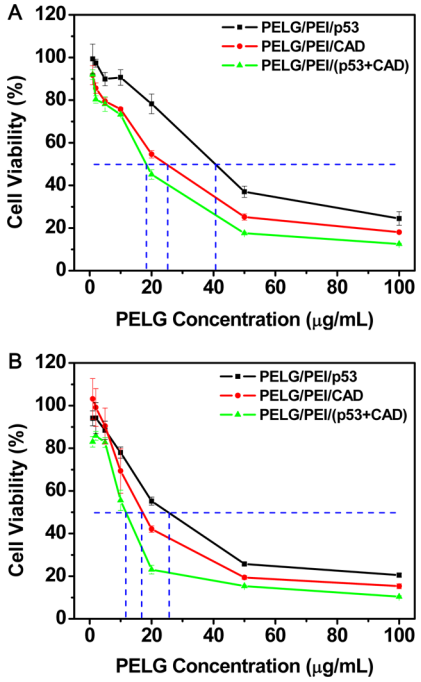

Figure 8. Cytotoxicity of PELG/PEI/p53, PELG/PEI/CAD, and PELG/PEI/(p53+CAD). (A) pH 7.4, (B) pH 6.8.

observed in HepG2 cells in pH 7.4 and pH 6.8, which indicated that the gene and drug could be delivered into the same cells. In pH 6.8, the gene and drug related fluorescent signals were much stronger than pH 7.4, a relative high amount of gene and drug was observed locating in the same cells. The result showed that the codelivery system could simultaneously transport gene and drug into one tumor cell, and better cell uptake efficiency could be achieved in acidic tumor area.

3.7. p53 Gene Expression. The RT-PCR was carried out to evaluate the p53 gene expression of the HepG2 cells treated by PELG/PEI/(p53+CAD) codelivery system. As shown in Figure 10, the normal HepG2 cells were used as control, and the relative

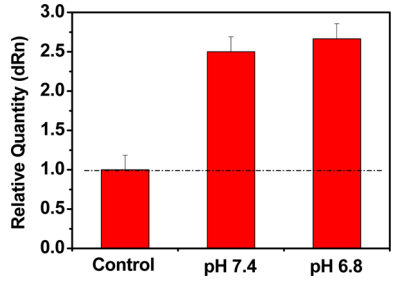

Figure 10. Relative mRNA quantity of p53 gene expression of the HepG2 cells treated by PELG/PEI/(p53+CAD) codelivery system.

mRNA quantity of p53 gene expression of control group was set as 1, the relative mRNA quantity of the HepG2 cells treated with PELG/PEI/(p53+CAD) was 2.50 in pH 7.4 and the mRNA quantity further increased to 2.66 in pH 6.8. The result showed that the p53 gene expression of the HepG2 cells was significantly improved after the tumor cells were treated by PELG/PEI/(p53+CAD) codelivery system, and the p53 gene expression was higher in acidic environment (tumor area) than neutral environment. This experimental result verified that the codelivery system could significantly increase the expression of

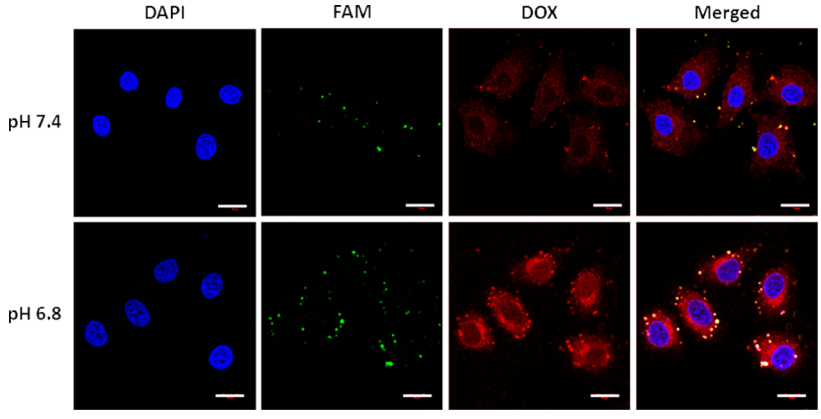

Figure 9. CLSM images of HepG2 cells incubated with gene and drug codelivery system for 2 h in pH 7.4 and 6.8. DAPI: cell nucleus (blue); FAM: RNA (green); DOX (red). Scale bars = 20 μm.

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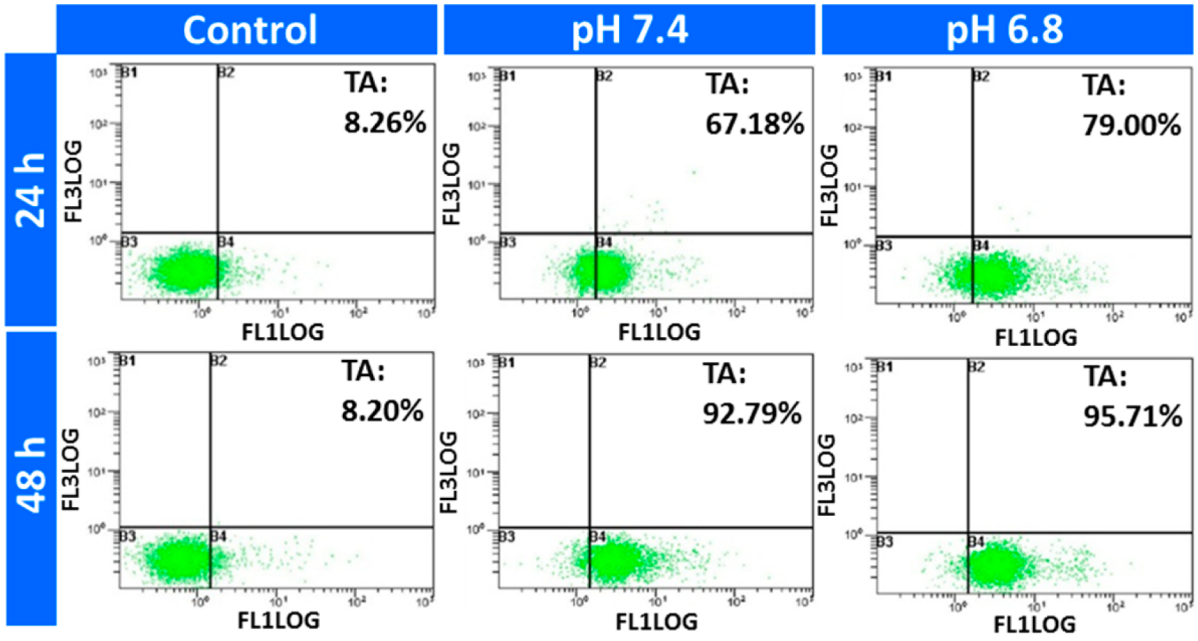

Figure 11. Apoptosis of HepG2 cells treated with PELG/PEI/(p53+CAD) codelivery system. The upper row: apoptosis in 24 h, the lower row: apoptosis in 48 h. TA: total apoptosis (the sum of early- and late-phase apoptosis shown in lower-right and upper-right quadrants, respectively).

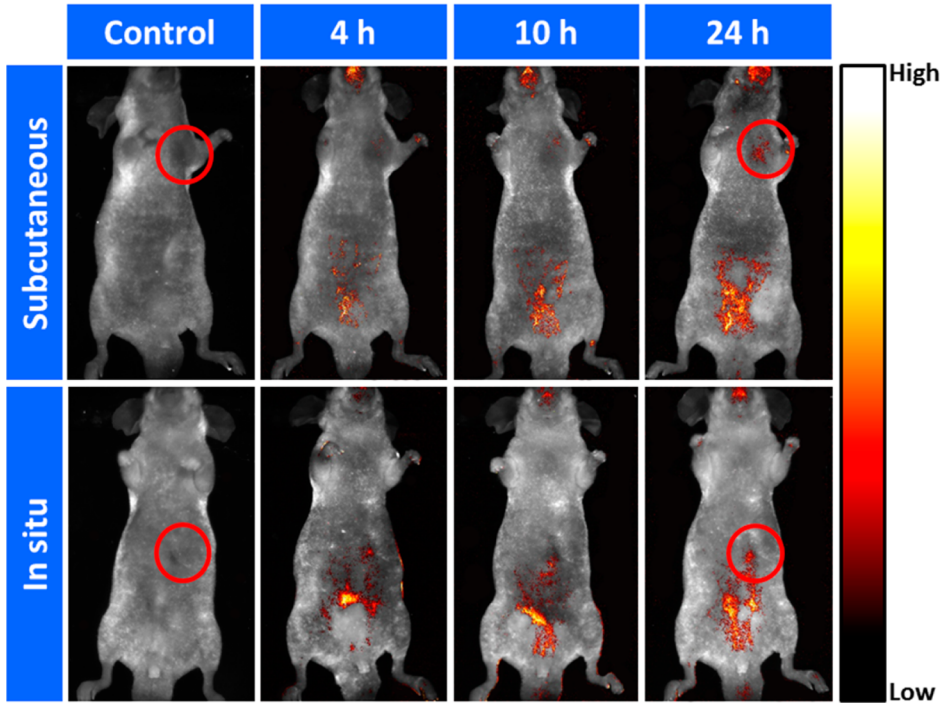

Figure 12. In vivo images showed the distribution of PELG/PEI/(DNA+CAD) after the codelivery system was injected into the mice via tail vein. Circles indicate the sites of the tumors.

the tumor suppressor gene p53, which was beneficial for the treatment of cancer.

3.8. Apoptosis Assay. The apoptosis of the HepG2 cells treated with codelivery system was detected by flow cytometry (FCM). In Figure 11, the upper row was cell apoptosis in 24 h and the lower row was apoptosis in 48 h, the total apoptosis (TA) was calculated and marked. There were 67.18% HepG2 cells apoptosis in 24 h after treated with codelivery system in pH 7.4, the apoptosis increased to 79.00% in pH 6.8; higher apoptosis (92.79% in pH 7.4 and 95.71% in pH 6.8) of HepG2 cells occurred when the time extended to 48 h, which showed that almost all HepG2 cells underwent apoptosis in pH 6.8 within 48 h. This result indicated clearly that the gene and drug codelivery system could lead to an effective apoptosis on tumor cells.

3.9. In Vivo Imaging. In vivo imaging was used to evaluate the distribution of the codelivery system and the results are shown in Figure 12. For subcutaneous xenograft tumor model, the DOX fluorescence was very weak at 4 h postinjection, but the fluorescence signal became stronger with the time prolonged, and the strongest fluorescence signal can be observed in the

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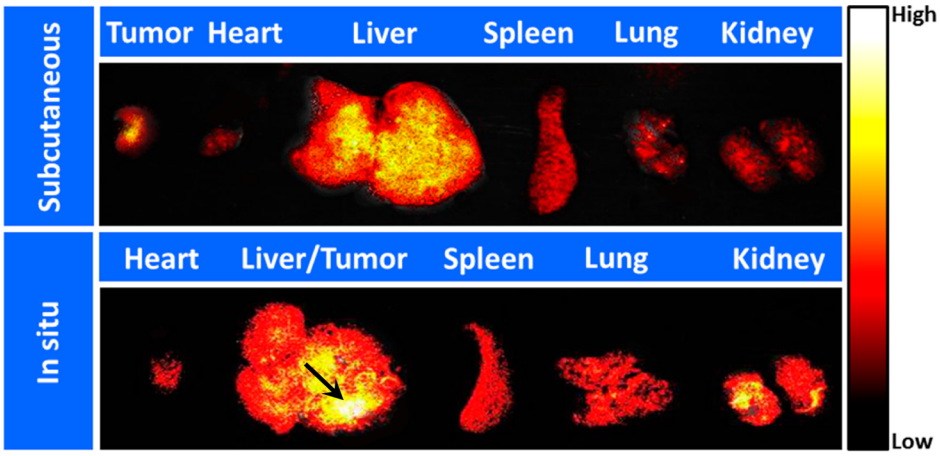

Figure 13. Distribution of PELG/PEI/(DNA+CAD) codelivery system in the main organs; the mice were sacrificed 24 h postinjection. Arrow indicate the site of tumor.

subcutaneous tumor at 24 h. In situ liver tumor model was established on the left liver lobe of nude mice. An obvious fluorescence signal was seen in the liver tumor at 4 h postinjection, and the signal maintained strong during the test time (24 h). From the above results we can conclude that the PELG/PEI/(DNA+CAD) codelivery system can accumulate in tumors. For cancer treatment, whether drugs can accumulate in the tumor site at effective concentration is a critical premise for antitumor therapy. The major organs and tumors were also excised and imaged (Figure 13). The strong DOX fluorescence signal can be detected in both subcutaneous xenograft and in situ tumor, the result was consist with the in vivo image, which all indicated that the codelivery system can efficiently accumulate in tumors.

4. CONCLUSIONS

In this study, a gene and drug codelivery system PELG/PEI/(DNA+CAD) was successfully designed and studied. The system had pH-sensitive charge-reversal and acid-responsive drug release properties, could simultaneously deliver drug and gene into the same cells. The codelivery of CAD and tumor suppressor gene p53 could achieve an excellent killing effect on HepG2 cells when compared to individual treatments of CAD delivery system and p53 delivery system. The p53 gene expression increased significantly after HepG2 cells treated by PELG/PEI/(p53+CAD) codelivery system. The apoptosis assay showed that almost all HepG2 cells underwent apoptosis in pH 6.8 within 48 h after being treated with the codelivery system, which indicated clearly that the codelivery system could lead to an effective apoptosis on tumor cells, which was beneficial for the treatment of cancer. Two kinds of liver tumor models were used to explore the biodistribution and tumor accumulation of the codelivery system via in vivo imaging, and the results showed that the codelivery system can accumulate efficiently in tumor for both subcutaneous xenograft and in situ tumor. Because of these advantages mentioned above, the codelivery system was believed to offer new opportunities and hold great potential for cancer therapy.

AUTHOR INFORMATION

Corresponding Authors

*E-mail: liyanhui@ciac.ac.cn. Tel: +86-431-85583105.
*E-mail: thy@ciac.ac.cn. Tel: +86-431-85262539.

Notes

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