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Self-fluorescent drug delivery vector based on genipin-crosslinked polyethylenimine conjugated globin nanoparticle

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ABSTRACT

A kind of self-fluorescent, biocompatible, and low-toxic Genipin crosslinked Globin-PEI nanoparticle (Gb-G-PEI NP) with high enzymolysis-stability and photo-stability was synthesized successfully. The properties of the Gb-G-PEI NP were characterized, including its particle size, surface zeta potential, morphology, paclitaxel (PTX) loading capacity and release. The Gb-G-PEI NPs as imaging probe were investigated by Confocal Laser Scanning Microscope (CLSM) *in vitro* and by fluorescence imaging system *in vivo*. Cell imaging results showed that the tumor cell line (HepG-2) had the faster cell uptake rate and metabolism rate than the normal cell line (L-O2), this difference showed its tumor selectivity. MTT assay revealed that the PTX-loaded Gb-G-PEI NPs showed almost the equal potence to tumor cell HepG-2 as the free PTX at the same PTX concentration, while a lower cytotoxicity to normal cell L-O2, suggesting its promising utilization as a drug delivery system. The imaging on mice demonstrated the possibility of the self-fluorescent Gb-G-PEI NPs as probe *in vivo*. So Gb-G-PEI NPs can be potentially utilized as both tracking marker and tumor cell selective drug delivery system in the biomaterial field.

1. Introduction

Protein-based nanoparticles with characteristics of good absorbability, non-toxicity, and non-antigenicity, have attracted special interest in biomaterial fields [1,2]. Proteins often used were gelatin, collagen, casein, albumin or whey protein, and these protein-based nanoparticles were usually studied for delivering drugs, nutrients, bioactive peptides and probiotic organisms, *etc.* [1–5] Globin (Gb), the protein component of Hemoglobin (Hb), was studied extensively as a kind of promising oxygen carriers [6,7], and the past chemical modification on globin included cross-linking of two lysine residues by glutaraldehyde [8] or bis(3,5-dibromosalicyl) fumarate [9],the PEGylation of the surface amino groups through urethane linkages [10], isopeptide linkage [11] or propyl maleimide linkage [12],*etc.* Besides its oxygen carrier ability, globin is also a bioactive "peptide pool" [13], its degradation would produce many active peptides, like hemorphin, ACE inhibitor, antibacterial peptides, so as a biomaterial it possesses more advantage compared with other proteins. However, little work on its potency as drug delivery system was studied.

For a delivery system, the surface properties, especially the charged groups, play an important role in their cellular internalization [14,15]. Positively charged particles generally had larger uptake amount than negatively charged or neutral ones [15]. Polyethylenimine (PEI), a cationic linear or branched polymer, with molecular weight ranging between 0.6 and 1000 kDa [16], could be modified on different kinds of proteins [1,3,17]to form a positive charged system, which enhances the material's cellular adhesiveness and uptake ability. Genipin, the aglycon of geniposide found in traditional Chinese medicine, is an effective naturally occurring crosslinking agent that can react with amino acids or proteins [18,19]. Numerous studies have been performed in genipin crosslinking amine-group-containing biomaterials, like chitosan, gelatin, silk protein, collagen, casein, *etc.* [18,20–23] Here we used genipin as crosslinker to conjugate PEI with globin.

Fluorescence imaging with advantages of simplicity, low-cost and noninvasive, could provide information of the bio-distribution, metabolic characteristics of the delivery systems by real-time tracking of these drug delivery vectors *in vitro* and *in vivo* [24]. The imaging fluorescent labels usually used are dyes [25] or quantum dots [26] which were conjugated to or encapsulated in the delivery system to produce optical imaging *in vitro* and *in vivo* [27,28]. However sometimes the spontaneous release of these often-toxic labels caused imaging ambiguity and gave the inaccurate information. In this paper, the external fluorescent label was omitted, instead the intrinsic pigment formed by genipin reaction with PEI and globin, was used as fluorescent label to provide accuracy information for the globin–PEI system *in vitro* and *in vivo*. Genipin is colorless but its reaction with amino acids or proteins could form blue fluorescent pigments, which was stable with regard to pH, temperature, and light conditions [29,30]. Up to now, most research about genipin was focused on its crosslink mechanism and efficiency, or the properties of the genipin-crosslinked product, like cytotoxicity, resistance to enzymatic degradation, mechanical properties, *etc.* The optical property of the genipin-crosslinked fluorescent product received less attention [31–33], some work used this pigment fluorescence to indicate the

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biodegradation or morphological structure of the materials. No work used this pigment as fluorescent probe to track nanocarrier *in vitro* or *in vivo*. This paper would explore the safety of genipin function as natural crosslinker and its product as fluorescent marker *in vitro* and *in vivo*.

In this research paper, a kind of self-fluorescent, biocompatible, and low-toxic Genipin crosslinked Globin-PEI nanoparticle with high enzymolysis stability and photo-stability was synthesized successfully (Scheme 1). The self-fluorescent was used as a bio-imaging probe to track the cellular uptake and metabolism of Gb-G-PEI nanoparticle by Confocal Laser Scanning Microscopy (CLSM). Also, the self-fluorescent nanoparticle was monitored by *in vivo* imaging system with mice as models. The cytotoxicity of the developed paclitaxel-loaded Gb-G-PEI nanoparticle was assessed in comparison to free PTX at varying drug concentrations. Results were promising and suggest its application in fluorescent imaging and drug delivery.

2. Experimental section

2.1. Materials

The branched Polyethylenimine (PEI, Mw = 1.8 or 10 kDa) was purchased from Aladdin Industrial Corporation (Shanghai, China). Hemoglobin from swine (Hb) was obtained from Hewons Biochem Technologies (Tianjin, China). Genipin (98%) was purchased from Xi'an Plant Bio-engineering Corporation (Xi'an, China). Paclitaxel (PTX) was obtained from Huafeng United Technology Company (Beijing, China). All other chemical reagents were purchased from Tianjin Jiangtian Chemical Technology Corporation (Tianjin, China). Acetonitrile (CH_3CN) and trifluoroacetic acid (TFA) (HPLC grade) were purchased from Concord Technology Corporation (Tianjin, China). All reagents were used as received without further purification.

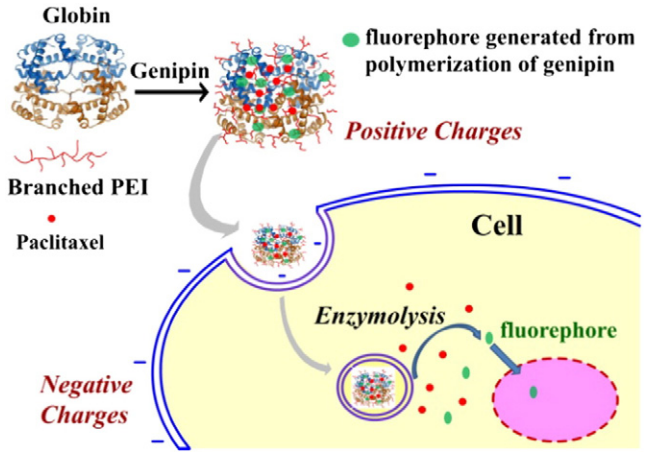
Hepatoma carcinoma cell line (HepG-2)and hepatic cell line (L-O2) were purchased from American type culture collection (Maryland, USA) and cultured in DMEM medium enriched with 10% fetal bovine serum (FBS) and 100 U/mL penicillin-streptomycin. Cells were incubated at 37 °C with 5% CO_2.

ICR mice (4-weeks, male, 20–25 g) were used to evaluate the *in vivo* imaging. All animal procedures are approved and controlled by the local ethics committee and carried out in accordance with the guidelines Principles for the Care and Use of Laboratory Animals, Peking Union Medical College, People's Republic of China.

2.2. Genipin crosslinked Globin-PEI Nanoparticles (Gb-G-PEI NP)

Globin was prepared from hemoglobin by acid-acetone extraction method [34] and characterized by UV–Vis spectra and far-UV CD spectra. The Gb-G-PEI NP was prepared by genipin cross-linked reaction between globin and PEI. The experiment was as follow: 20 mg globin and 120 mg PEI (Mw = 1800 Da) was dissolved in 7 mL 0.1 mol/L HCl solution, the pH value was adjusted to 5.0 with 0.65 mL 1 mol/L NaOH solution. Then 1.91 mL genipin solution (50 mmol/L) was added, and the final genipin concentration in the system was 10 mmol/L. The system was thoroughly stirred and at time point of 5, 10, 24, 36, 48 or 72 h, 0.5 mL solution was taken out to monitor the reaction progress by both the UV–Vis spectrum and Fluorescent spectrum respectively. The color of the system changed from slight yellow to dark blue, and the solution was extensively dialyzed (MWCO 12,000–14,000 Da) against de-ionized water for 24 h, the water was totally changed 5 times. Dialyzed product was lyophilized to give the Gb-G-PEI_{1800} NP. To investigate the rate of the crosslink reaction dependence on the concentration of genipin, the following experiments was conducted: 0.32 mL genipin solution was added to Gb and PEI_{1800} system, the procedure was remained the same as above.

Gb-G-$PEI_{10,000}$ NP (PEI Mw = 10,000 Da) was prepared in the same method as Gb-G-PEI_{1800} nanoparticles except that the final genipin concentration in the reaction system was 10 mmol/L and reaction time was 48 h at 37 °C.



Scheme 1. Scheme for the synthesis of Gb-G-PEI nanoparticles and its cellular uptake process.

2.3. The stability of Gb-G-PEI NPs' self-fluorescence

Both the photo-stability and enzyme-stability were evaluated for nanoparticle's self-fluorescence. The procedure was as following: To monitor the photo stability, Gb-G-PEI NPs sample (1 mg/mL in water) was irradiated with a 630 nm semiconductor laser (7404, Intense, USA) *via* an optic fiber (5-cm diameter, 250 mW/cm^2), its absorbance and fluorescence were recorded at different time points. To measure the enzyme-stability, Gb-G-PEI NPs sample (1 mg/mL in water) was treated with different concentration of trypsin solution, 1000 μg/mL or 40 μg/mL respectively. UV–Vis and fluorescence spectra were recorded at different times to evaluate the stability of self-fluorescence.

2.4. The paclitaxel loaded Gb-G-PEI_{1800} NPs

100 mg Gb-G-PEI_{1800} NPs was suspended in 10 mL H_2O–DMSO (1:1 v/v) and kept at 37 °C for 1 h under continuous stirring to swell completely, then 1 mL of the PTX solution (10 mg/mL in DMSO) was added and stirred for 4 h for encapsulation. The resulting assembly suspension was dialyzed (MWCO 3500 Da) against distilled water for 36 h, and the water was totally changed 6 times. The obtained solution in dialysis tube was filtered through 0.45 μm filter and lyophilized to give PTX-loaded nanoparticles. To obtain the PTX-loaded nanoparticles with different drug loading capacity, 3 or 5 mL of the PTX solution (10 mg/mL in DMSO) was added instead.

The drug-loaded capacity (LC) and encapsulation efficiency (EE) of PTX was analyzed by HPLC (Waters e2695 Separations Module liquid chromatography, column: VP-ODS C18, 5 μm, 4.6 × 150 mm; acetonitrile/water 70/30 (v/v), 1.0 mL/min, UV detector at 227 nm) and calculated as follow:

$$Loading\ \ capacity(LC) = \frac{mass\ \ of\ \ loaded\ \ drug\ in\ carrier}{mass\ \ of\ \ carriers} \times 100\%$$

$$Encapsulation\ \ efficiency(EE) = \frac{mass\ \ of\ \ loaded\ \ drug\ \ in\ \ carriers}{mass\ \ of\ \ initially\ \ added\ \ drug} \times 100\%$$

2.5. In vitro drug release

A solution of PTX-loaded Gb-G-PEI_{1800} NPs (1 mL, 5 mg/mL, LC was 8.55 ± 0.18%) was dialyzed (MWCO 3500 Da) against 12 mL of phosphate buffer (10 mmol/L phosphate, 150 mmol/L NaCl, 0.5%