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Drug-induced co-assembly of albumin/catalase as smart nano-theranostics for deep intra-tumoral penetration, hypoxia relieve, and synergistic combination therapy

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ABSTRACT

The abnormal tumor microenvironment (TME) featured with hypoxia, acidosis, dense extracellular matrix and increased tumor interstitial fluid pressure is closely related with the resistance of tumors to various therapies. Herein, a unique type of biocompatible nanoscale delivery system is fabricated by utilizing a chemotherapeutic drug, paclitaxel (PTX), to induce co-assembly of catalase and human serum albumin (HSA), the latter of which is pre-modified with chlorine e6 (Ce6), forming smart multifunctional HSA-Ce6-Cat–PTX nanoparticles via a rather simple one-step method. Upon intravenous injection, HSA-Ce6-Cat–PTX nanoparticles show high tumor accumulation and efficient intra-tumoral diffusion, likely owning to their changeable sizes that can maintain large initial sizes (~100 nm) during blood circulation and transform into small protein-drug complexes (<20 nm) within the tumor. Meanwhile, catalase within those nanoparticles could trigger decomposition of endogenic TME H_2O_2 to generate oxygen in-situ so as to relieve tumor hypoxia. This effect together with PTX-induced intra-tumoral perfusion enhancement is able to dramatically modulate TME to favor the anti-tumor effect in the combined photodynamic/chemotherapy with HSA-Ce6-Cat–PTX. Thus, our work presents a simple drug-induced self-assembly strategy to fabricate enzyme-loaded therapeutic albumin nanoparticles for synergistic cancer combination therapy.

1. Introduction

Solid tumors, which may be deemed as abnormal organs with heterogeneous structures and unique tumor microenvironment (TME), are composed by many different types of cells (e.g. tumor cells, fibroblasts, inflammatory cells, macrophages, and various types of immune cells) embed in the extracellular matrix and nourished by a vascular network with non-uniform structures [1–4]. The complex composition and structure of tumors contribute to compressed tumor blood vessels and increased tumor interstitial fluid pressure (IFP), which may severely hind the penetration of therapeutic agents, particularly nanoparticles with relatively large sizes, during cancer therapy [5–8]. Thus, for the next generation of nanomedicine, the development of size-changeable nanoparticles that could maintain relatively large sizes (50–100 nm) during the blood circulation for effective tumor accumulation via the enhanced permeability and retention (EPR) effect, and dissociate into small particles (below 20 nm) within the tumor for deeper penetration [9–13], would be of great interests. On the other hand, it has been proposed that modulation of TME such as reducing the neoplastic cell density to normalize the tumor blood vessels and lower the IFP in the TME, might be another alternative strategy to improve the penetration and distribution of therapeutic nanoparticles within solid tumors [14].

Another well-known feature of TME is insufficient oxygen supply, also known as hypoxia, within many types of solid tumors, particularly in the regions far from tumor blood vessels [15,16]. Hypoxia in the tumor is found to be related with the tumor angiogenesis and cancer metastasis, as well as limited therapeutic efficiency of several types of cancer therapies, especially for those with oxygen as a critical component during the cell killing process such as radiotherapy and photodynamic therapy (PDT) [17–22]. To date, various strategies including use of perfluorocarbon-based oxygen-carriers to transport oxygen into tumors [23,24] or in situ generation of oxygen from TME endogenous H_2O_2 (~50–100 μM), which is produced by the aberrant metabolism of cancer cells [11,25–29], have been explored to relieve the tumor hypoxia and improve therapeutic efficiency. Therefore, it would be of particular attractive to develop nano-theranostics, which on one hand are size-changeable for effective tumor accumulation and diffusion, on the other hand, can modulate tumor microenvironment (e.g. IFP, hypoxia) to achieve better therapeutic outcomes.

Catalase, an enzyme with specific catalytic behavior in decomposing H_2O_2 to produce O_2, has been explored in recent years to overcome the

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tumor hypoxia by in-situ producing oxygen inside the tumor, although its instability in the presence of protease during blood circulation after systemic administration may be a concern [25,30–34]. Thus, various strategies including encapsulation of this enzyme in inorganic nanoparticles or polymeric multilayer capsules have been developed to protect catalase from protease digestion to enable its tumor-targeted delivery [32,35]. However, the relative enzymatic activity of catalase loaded inside nanoparticles is often decreased after those complex synthetic processes [36–38]. In this work, inspirited by our previous systems in which hydrophobic molecules such as chemotherapeutics or imaging agents are able to induce self-assembly of human serum albumin (HSA) [39–47], a major component of serum proteins, to form nanoparticles for cancer theranostics, we design a simple and effective method utilizing hydrophobic drug to induce the co-assembly of albumin and catalase to fabricate multifunctional nanoparticles.

In our design, HSA pre-modified with chlorine e6 (Ce6), a clinically used photosensitizer, together with catalase, are mixed with an effective antitumor drug, paclitaxel (PTX), the latter of which would trigger co-assembly of proteins to form HSA-Ce6-Cat–PTX nanoparticles. Interestingly, the obtained HSA-Ce6-Cat–PTX nanoparticles show high EPR effect-driven tumor passive homing, together with efficient intratumoral permeability, likely owing to the unique concentration dependent dissociation behavior of those protein-drug nanoparticles. On the other hand, catalase encapsulated within those nanoparticles with largely retained enzyme activity and greatly enhanced stability against protease is able to trigger decomposition of endogenous H_2O_2 inside the tumor to produce O_2 and promote tumor oxygenation. Furthermore, PTX used in our system, not only is proven to be an effective chemotherapeutic drug, but also has the ability to decompress blood vessels and decrease intra-tumoral IFP, benefiting for intra-tumoral delivery of therapeutic agents and further relieve of tumor hypoxia. With those effects acting simultaneously and synergistically, impressive in vivo therapeutic effect is achieved using such HSA-Ce6-Cat–PTX nanoparticles for tumor treatment under a rather low single treatment dose. Therefore, this work presents a simple one-step approach to fabricate all-protein-based multifunctional nanoparticles, which not only show changeable sizes for enhanced intra-tumoral accumulation and diffusion, but also are able to modulate the TME such as hypoxia and IFP, so as to realize highly effective combination therapy of cancer.

2. Materials and method

2.1. Materials

Human serum albumin (HSA), dimethyl sulfoxide (DMSO), *N*-(3-Dimethylaminopropyl)-*N*-ethylcarbodiimide hydrochloride crystalline (EDC), *N*-Hydroxysuccinimide (NHS) and 3-(4.5-dimethylthiazol-2-yl)-2.5-diphenyltetrazolium bromide (MTT) was purchased from Sigma-Aldrich. Chlorine e6 (Ce6) was purchased from J&K Scientific Ltd. Catalase (CAT) solution (≥35,000 units/mg protein) was obtained from Aladdin. Paclitaxel (PTX) was purchased from Beijing ZhongShuo Pharmaceutical Technology Development Co., Ltd. All other chemicals were purchased from China National Pharmaceutical Group Corporation. RPMI-1640 medium was purchased from Thermo Fisher Scientific Inc.

2.2. Synthesis of HSA-Ce6

Ce6 used during this experiment was pre-dispersed in DMSO. To obtain HSA-Ce6, 0.01 ml Ce6 (20 mg/ml, 1 eq), 0.064 mg EDC (1 eq) and 0.042 mg NHS (1.1 eq) were mixed in dark for 0.5 h under room temperature. Then the activated Ce6-NHS was added into 2 mg (0.1 eq) HSA dissolved in phosphate buffered saline (PBS). The mixture was stirred overnight in the dark. HSA-Ce6 was obtained after centrifugation at 14,800 rpm for 5 min to remove possible aggregates and ultrafiltration by a centrifugal filter device (molecular weight cut-off MWCO = 10 kDa) for 3 times to remove free Ce6.

2.3. Synthesis of different protein nanoparticles

HSA-Ce6-CAT-PTX nanoparticles were prepared by PTX-induced self-assembly of proteins. 10 mg HSA-Ce6 and 0.1 ml catalase (10 mg/ml measured by BCA) were mixed in 1 ml PBS, into which 65 μl PTX pre-dissolved in methanol (20 mg/ml) was added. The obtained HSA-Ce6-CAT-PTX nanoparticles were purified by centrifugation at 14,800 rpm for 5 min to remove insoluble PTX and then by ultrafiltration using a centrifugal filter device (MWCO = 100 kDa) for 3 times to remove methanol.

As the control, HSA-Ce6-PTX without catalase were prepared by the same procedure except that 1 mg plain HSA was used to replace catalase. Furthermore, HSA-Ce6-CAT nanoparticles for control experiments were obtained by cross-linking HSA-Ce6 and CAT (w/w = 10:1) with glutaraldehyde [48]. 10 mg HSA-Ce6 and 1 mg catalase were first dispersed in 1 ml water, and then added with 1 ml ethanol at the rate of 1 ml/min. Afterwards, 10 μl glutaraldehyde (2.5%) was added to the above-mentioned mixture, which was then stirred at room temperature overnight. HSA-Ce6-CAT nanoparticles were obtained after dialyzing in a dialysis bag (MWCO 3500 Da) to remove ethanol and glutaraldehyde.

2.4. Characterization

Transmission electron microscopy (TEM, FEI Tecnai F20, acceleration voltage = 200 kV) was used to characterize the morphology of HSA-Ce6-CAT-PTX nanoparticles. UV–vis-NIR absorbance spectra were recorded by PerkiinElmer Lambda 750 UV–vis-NIR spectrophotometer. Fluorescence spectra of different samples were measured with a FluoroMax 4 luminescence spectrometer (HORIBA JobinYvon). The hydrodynamic diameters of different samples were determined by a Zetasizer Nano-ZS (Malvern Instruments, UK).

2.5. In vitro release of PTX in Hank's solution

The release of PTX from HSA-Ce6-CAT-PTX dialyzed against Hank's solution (pH 7.4) was carefully studied. 1 ml of HSA-Ce6-CAT-PTX (10 mg/ml HSA, 0.65 mg/ml PTX) was packaged in a dialysis bag (MWCO = 14 kDa) and then immersed in 40 ml Hank's solution. At different time points (2 h, 6 h, 12 h, 24 h, 48 h), 2 ml of the Hank's solution was collected and the same volume of fresh Hank's solution was added. Then the collected solution was extracted with 1 ml of dichloromethane, evaporated and then followed by adding 1 ml of the mixture of acetonitrile and water (50:50, v/v). The PTX concentration was measured by high performance liquid chromatography (HPLC).

2.6. Catalase activity assay

Catalase activity was determined by the Góth method [49]. Briefly, 1 ml of hydrogen peroxide (50 mM) was added with 0.2 ml of free catalase or HSA-Ce6-CAT-PTX (0.5 μM) for 1 min at 37 °C, and then terminated by adding 1 ml of ammonium molybdate (32.4 mM) and cooling down to 25 °C. The catalase activity can be determined by the absorbance of the above complex at 400 nm. To measure the stability of catalase, both free catalase and HSA-Ce6-CAT-PTX were incubated with protease K at 37 °C with the final concentration of protease K at 0.4 mg/ml. At predetermined time points, aliquots of sample were removed for immediate catalase activity assay.

2.7. Detection of singlet oxygen

The method for the singlet oxygen detection was based on the protocol reported previously [50]. In brief, singlet oxygen sensor green reagent (SOSG, molecular probes, USA) dissolved in methanol was