Effective Chemotherapy of Lung Cancer Using Bovine Serum Albumin-Coated Hydroxyapatite Nanoparticles

Background: Successful chemotherapy of lung cancer relies largely on the use of a good drug delivery system (DDS). We successfully constructed a hybrid DDS comprised of hydroxyapatite (HAP) nanoparticles and bovine serum albumin (BSA).

Material/Methods: The HAP nanoparticles were selected as the core to encapsulate the anticancer drug doxorubicin (DOX), followed by surface modification of BSA as a stabilizer and shielding corona to finally prepare the hybrid DDS (BSA/HAP/DOX).

Results: The following characterizations revealed that BSA/HAP nanoparticles have high stability, high biocompatibility, and good DOX-loading capability to meet in vivo applications. Moreover, BSA/HAP/DOX can enhance the cellular uptake of drug in A549 cells (lung cancer cells). Most importantly, BSA/HAP had better in vivo tumor targetability than bare HAP nanoparticles, which resulted in stronger anticancer efficacy both in vitro and in vivo than free DOX or HAP/DOX, and greatly decreased the adverse effects of free DOX.

Conclusions: Our hybrid DDS shows potential to be applied in more advanced application of cancer therapy.

MeSH Keywords: Carcinoma, Non-Small-Cell Lung • Hydroxyapatites • Nanoparticles

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Background

Currently, although it has many inevitable flaws, chemotherapy is still the dominate approach for cancer therapy. As a result, the improvement of chemotherapy efficacy remains a great challenge for better anticancer performance [1]. Compared with free drugs, drug delivery systems (DDSs) composed of numerous nano-sized particles are believed to be preferable vehicles for drug delivery [2]. The nanoparticles can provide sufficient space and protection for the drug molecules. In addition, the nanoparticles with size under 200 nm can passively target tumor tissues through malformed tumor vessels, which can greatly reduce the notorious adverse effects of toxic chemical reagents [3,4]. Most importantly, recent advances in cancer therapy show that positive targeting of tumor tissues can also be achieved via the selected modification of targeting ligands, which can further reduce the bioavailability of drugs [5–7]. Among the various obstacles to designing cancer-related DDS, drug loading, drug targeting, and controlled release are the main obstacles [8,9].

To overcome these difficulties, many different kinds of DDSs have been introduced in previous research, among which diverse materials (ranging from organic to inorganic) were used [10–13]. Among these materials, hydroxyapatite (HAP) has unique merits such as high biocompatibility, good drug-loading capacity, and ease of preparation and modification, making it a promising candidate for use in cancer therapy [14,15].

Bovine serum albumin (BSA), as the major protein in bovine serum, has been widely used in biochemistry experiments [16]. Moreover, the preferable properties of BSA, such as being nontoxic, non-antigenic, and biodegradable, also enable it to be used as a biocompatible material for the construction of multiple nano- and microparticulate DDSs [17,18]. It was also suggested that due to the rapid division of cancer cells, they more easily take up the BSA than do normal cells, which provides a convenient approach for tumor-specific targeting.

In the present study, to combine the merits of both materials, HAP nanoparticles with appropriate size and surface charges (positive) were prepared and subjected to DOX loading (HAP/DOX). BSA was incubated with the HAP/DOX to produce a BSA corona on the surface of DOX/HAP to obtain BSA/HAP/DOX. We investigated the physicochemical properties of DOX/HAP, and then performed in vitro and in vivo experiments to compare the free drug vs. bare HAP/DOX. The BSA/HAP showed much higher stability and better tumor-targeting ability than HAP. Moreover, due to the high biocompatibility of BSA, the developed BSA/HAP exerted better biocompatibility than other cancer drug DDSs developed with HAP [19,20].

Material and Methods

Materials

Calcium chloride, sodium dihydrogen phosphate, (3-Aminopropyl) triethoxysilane (APTES), and chondroitin sulfate (20 kDa) were obtained from Sigma Chemicals (MA, USA). The other regents without further indications were all from the same company and were of analytical purity.

Cells and cell cultivation

Cells of the human lung carcinoma cell line A549 were cultured in DMEM with 10% (v/v) fetal bovine serum (FBS) and 100 U/mL streptomycin and penicillin. The medium was placed in a thermostat-controlled incubator (HeraCell 150i, Thermal-Fisher, USA) with constant temperature at 37°C and standard atmospheric condition [21].

Animals and animal experiment

Male BALB/c nude mice from Nanjing University were raised in an SPF II lab according to previously reported protocols. The A549 tumor xenograft mice model was established on the basis of a previous report with some changes [22]. All experimental procedures complied with our institutional guidelines and were approved by the Ethics Committee of GuiHang Guiyang Hospital.

DDS preparation

The preparation of DOX-encapsulated HAP was based on previously reported methods [23]. Briefly, DOX (1 mM) and CaCl$_2$ (100 mM) were added to 3 mL of water-in-oil microemulsion. After being agitated for 30 min to achieve a transparent solution, 300 μL of NaH$_2$PO$_4$ (100 mM) and ammonium hydroxide (20 μL) were added to initiate the reaction. At 30 min after the reaction, 300 μL of APTES (2 mg/mL) was added, followed by another 12 h of reaction time. The product was collected by centrifugation (Thermo A68, Thermo-Fisher, USA) to get obtain functionalized HAP/DOX.

To achieve BSA modification on the surface of functionalized HAP/DOX, the aqueous BSA was dropped slowly into the solution of amine-functionalized HAP/DOX under agitation. After adding BSA, the mixture was left standing at room temperature for 1 h to allow the construction of BSA/HAP/DOX. The product was collected by centrifugation and stored at 4°C.

Drug loading

The encapsulated DOX within BSA/HAP/DOX was extracted using specific medium (ethanol: 0.6 M HCl, 1:1, v/v) as reported
previously [24]. The DOX content was determined by HPLC using a protocol reported previously [25].

Characterizations

The size distributions and zeta potential of nanoparticles were assessed using a Zeta/Size analyzer (Malvern Instruments Corp., UK). The morphology was imaged using a transmission electron microscope (TEM, LVEM25, Quantum Design, China).

Stability/hemolysis assay

The nanoparticle stability was evaluated by measuring size changes in 20% FBS during storage (48 h). The hemolysis of nanoparticles was determined using 2% red blood cells (RBCs) of mice. Briefly, the RCB was incubated with various concentrations of nanoparticles at 37°C for 1 h with saline/distilled water as negative/positive controls. Afterwards, all the samples were centrifuged to obtain supernatant, and the UV absorption (OD545) was determined using a UV spectrophotometer (Orion AquaMate, Thermo-Fisher, USA) [26]. The drug release of BSA/HAP/DOX under various pH conditions was studied using a previously reported method [27].

Results

Characterizations

According to DLS measurement, the as-prepared amine-functionalized HAP/DOX was homogeneous particles that were narrowly distributed at 50 nm. The size of amine-functionalized HAP/DOX was 55.6 ± 2.3 nm, with a small poly-dispersion index (PDI) of 0.127 (Figure 1A).

In vitro uptake of nanoparticles

The quantitative intracellular transportation of BSA/HAP/DOX was investigated in A549 using flow cytometry (FCM, Invitrogen iSort, Thermo Scientific, USA). In detail, A549 cells were incubated with different formulations (with or without BSA pretreatment) for different time intervals. Afterwards, cells were detached and subjected to FCM analysis of intracellular DOX content [28].

In vitro anticancer assay

The in vitro anticancer effect of different formulations was investigated. In brief, A549 cells were incubated with different formulations at various DOX concentrations for another 48 h. Finally, cell viability was assessed using MTT assay, as mentioned previously [29].

In vitro tumor targeting

The in vivo targeting of different nanoparticles to the tumor tissue was revealed by monitoring the location of the ICG probe that encapsulated the nanoparticles. In brief, HAP/ICG or BSA/HAP/ICG were intravenously administered to A549 xenograft nude mice at the ICG dosage of 5 mg/kg. After 12 h, mice were sacrificed to obtain major organs and tumors, and the distribution of nanoparticles was assessed using a real-time imaging instrument (IVIS Lumina LT, PerkinElmer, USA) as reported before [30].

In vitro antitumor efficacy

In vivo antitumor efficacy of different formulations was assessed using the A549 tumor xenograft model. In detail, 24 mice were equally divided into 4 groups and subjected to different treatments: 1) saline; 2) free DOX; 3) HAP/DOX, and 4) BSA/HAP/DOX nanoparticles. The administration was repeated every 2 days (DOX dosage of 5 mg/kg) and the variations in tumor volume and body weight were recorded and plotted against time [31]. Finally, mice were sacrificed and the harvested tumor tissues were subjected to HE staining.

Figure 1. Particle size distribution (A) of HAP/DOX and BSA/HAP/DOX nanoparticles. Mean particle size, zeta potential, and poly-dispersion index (PDI) measurements (B) of HAP/DOX and BSA/HAP/DOX nanoparticles. The inserted image is the TEM picture of BSA/HAP/DOX nanoparticles. Data are shown as mean ± S.D. (n=3).
with further decreased PDI (0.095). The zeta potential results in Figure 1B also revealed that amine-functionalized HAP/DOX was positively charged nanoparticles (27.2 ± 3.3 mV). However, the BSA modification reversed this potential to a negative one of −25.1 ± 2.8 mV. The resulting encapsulation efficiency and loading efficiency of BSA/HAP/DOX were 90.31% ± 4.35% and 15.26% ± 1.87%, respectively.

**Colloidal stability, hemolysis, and drug release**

As displayed in Figure 2A, the size of BSA/HAP/DOX remained almost unchanged during the whole time interval, which suggested that BSA/HAP/DOX maintains stable in the circulation system [32]. As shown in Figure 2B, the hemolysis ratio of BSA/HAP/DOX at the highest concentration of 1 mg/mL was only 1.12%, which was lower than the generally recognized threshold of 5%. Finally, the drug release profile of BSA/HAP/DOX under different pH conditions was explored and results are summarized in Figure 2C. It was concluded that BSA/HAP/DOX showed a pH-responsive release of DOX, which is beneficial in DDS for cancer therapy.

**Intracellular uptake of BSA/HAP/DOX**

As demonstrated in Figure 3, the transportation of both HAP/DOX and BSA/HAP/DOX was a time-dependent process. In detail, the cellular accumulation of DOX was positively
related to the incubation time. Moreover, higher intracellular DOX accumulation was observed in all time intervals in the BSA/HAP/DOX group, which was 1.78 and 1.26-fold of that in HAP/DOX and free DOX groups (at 2 h post incubation), suggesting that BSA/HAP/DOX is preferably taken up by A549 cells.

To further verify this, the competitive assay was conducted by pretreating A549 cells with excess BSA before the uptake experiment. Interestingly, after BSA pretreatment, DOX accumulation in the BSA/HAP/DOX group had a significant drop, while no obvious difference was observed in the HAP/DOX group.

Anticancer efficacy in vivo

As shown in Figure 4A, A549 cells treated with BSA/HAP showed more than 95% viability at the highest nanoparticle concentration (150 µg/mL). As shown in Figure 4B, free DOX and BSA/HAP/DOX showed comparable anticancer efficacy, which was much stronger than that of HAP/DOX.

In vivo distribution

As shown in Figure 5A, the fluorescence signal in tumor tissue of the BSA/HAP/ICG group was 5.96-fold higher than in the HAP/ICG group. In addition, it was noted that the accumulation of BSA/HAP/ICG in the liver was only 0.66-fold that in the HAP/ICG group, suggesting that the BSA modification could also assist the escape of liver capture to increase the drug bioavailability. The blood-persistence properties of the BSA/HAP/DOX compared to free DOX were also investigated. As shown in Figure 5B, the half-life of free DOX was approximately 15 min, while the half-life of BSA/HAP/DOX was almost 6 h.

Anticancer efficacy in vivo

As displayed in Figure 6A, unlike its strong anticancer efficacy in MTT assay, the anticancer outcome of free DOX was far inferior to that of BSA/HAP/DOX under the same condition. In detail, BSA/HAP/DOX exerted the most powerful anticancer outcome among all formulations, with final tumor dimension at...
323 ± 52 mm³. This conclusion was also verified by the results of HE staining in Figure 6C. In addition, time-dependent variations of body weight of all formulations are summarized in Figure 6B. In line with a previous report [33], free DOX exerted strong toxicity on the subjects, as supported by the steady weight loss after day 4, which was correlated to the severe off-target distribution of DOX.

**Discussion**

Microemulsion is a widely recognized method for the preparation of size-controlled nanoparticles. It was reported that microemulsion accommodates many nano-sized water pools for reaction and the size of these pools can be easily regulated by changing the microemulsion formulation [5,32]. As a result, the microemulsion used in our study was not only able to produce nano-sized HAP, but was also capable of separating the neighboring nanoparticles from aggregation. According to a previous report, the negatively charged nanoparticles can avoid absorption of other nanoparticles, especially negatively charged proteins, which offers the potential for long circulation and high biocompatibility [34]. The changes in size and PDI suggest the modification of BSA can increase the stability of HAP/DOX and further retard the neighboring nanoparticles from assembling. The reverse in zeta potential provides decisive evidence of the successful modification of BSA on the surface of HAP.

Although DOX is widely used as a versatile anticancer drug, its application was strictly hindered by its hydrophilic nature. As a result, in order to be encapsulated in currently applicable DDSs, DOX is transferred to its hydrophobic form, with the sacrifice of efficacy. Therefore, a DDS capable of effectively delivering the hydrophilic DOX is urgently needed [35]. HAP is the phosphate of calcium, which can encapsulate DOX via the complexation between calcium and DOX [36]. By this effect, the DOX was easily loaded into HAP, with good loading efficacy.

The ideal DDS for effective drug delivery is expected to withstand hostile extracellular and intracellular environments. In general, the colloidal stability and hemolysis are 2 basic requirements to be satisfied, since the in vivo fate of DDS is greatly affected by them [37]. The preferable colloidal stability of nanoparticles requires the DDS to maintain its morphology under physiological environments without significant changes in size. As a result, 20% FBS was selected, and the size changes of BSA/HAP/DOX in the medium were recorded.

In addition, hemolysis as another important parameter used to evaluate performance, since DDS with high hemolysis might trigger undesired adverse effects [38]. Therefore, hemolysis of...
BSA/HAP/DOX at different nanoparticle concentrations was investigated. Moreover, it is well known that when applied in vivo, due to the dilution of blood, the concentration of nanoparticles would be lower than the tested concentrations. Our results suggest that the as-prepared BSA/HAP/DOX is a safe DDS that does not induce significant hemolysis.

DOX, a commonly used fluorescent molecule, was used in our study to indicate the cellular uptake of BSA/HAP/DOX. According to a previous study, it was expected that the BSA on the surface of BSA/HAP/DOX can be recognized and transported more rapidly due to the mass nutritional requirements of cancer cells [39]. As a result, the A549 cells were used to verify our hypothesis. As a result, it was concluded that the BSA on the surface of BSA/HAP/DOX was responsible for enhanced uptake of DOX into A549 cells, which was beneficial in cancer treatments [40].

To evaluate the anticancer assay of various formulations, the cytotoxicity of blank nanoparticles was determined to eliminate potential interference. We found that BSA/HAP is highly biocompatible and exerted no effects on the viability of A549 cells in the anticancer assays. Subsequently, A549 cells were treated with free DOX and DOX-loaded formulations at various DOX concentrations. It was suggested that the fast diffusion of DNA molecules into the cell nucleus is responsible for the stronger anticancer effect of free DOX [24], and the comparable effect of BSA/HAP/DOX suggested that the encapsulated DOX could also be readily released to exert cytotoxicity effects. By contrast, HAP/DOX exerted inferior cytotoxicity on cells, which might be due to its impaired cellular uptake profile.

The surface modification of BSA in BSA/HAP/DOX was confirmed to increase its cellular uptake into A549 cells. However, whether BSA modification could facilitate the in vivo tumor targeting of nanoparticles remains unexplored. Therefore, ICG was loaded into BSA/HAP and HAP to indicate the in vivo accumulation of nanoparticles. The ICG-loaded nanoparticles were administered to A549 xenograft mice. After 12 h of distribution, the mice were sacrificed to study the accumulation of the various formulations. The in vivo results strongly suggested that BSA/HAP/DOX greatly extends the blood persistence of the loaded drug, which is beneficial for cancer therapy.

The in vivo anticancer efficacy of both nanoparticles was investigated in A549 xenografted model mice using HAP-DOX nanoparticles and free DOX as controls. The results in Figure 6A agree with previous reports that that introduction of DDS can enhance the anticancer outcome compared to free DOX. Our results suggest that BSA/HAP/DOX nanoparticles are a highly safe DDS, without producing significant weight loss in the same condition. This might be related to the high tumor-targetability of BSA/HAP/DOX nanoparticles, which not only enhance the anticancer effect of the encapsulated drug, but also increase the safety of the DDS by reducing off-target distributions.

**Conclusions**

A highly biocompatible DDS composed of BSA and HAP was successfully developed and investigated as a DDS for DOX delivery. The BSA/HAP/DOX has ready uptake by the rapidly developing cancer cells to achieve enhanced in vitro uptake in A549 cells and in vivo tumor targeting in the A549 xenograft model for improved chemotherapy of lung cancer as compared with unmodified HAP/DOX and free DOX, and it appears to be a promising platform for more advanced application in cancer therapy.

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**Conflict of Interest**

None.

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