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Original Article

Cisplatin loaded albumin mesospheres for lung cancer treatment

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Abstract: The low solubility of cisplatin in aqueous solution limits the treatment effectiveness and the application of cisplatin in various kinds of drug-eluting devices. Although cisplatin has a high solubility in Dimethyl sulfoxide (DMSO), the toxicity of cisplatin can be greatly reduced while dissolved in DMSO. In this study, the solid powder of cisplatin-loaded albumin mesospheres (CDDP/DMSO-AMS), in a size range of 1 to 10 μm, were post-loaded with cisplatin and showed high cisplatin content (16% w/w) and effective cytotoxicity to lung cancer cells. Cisplatin were efficiently absorbed into the albumin mesospheres (AMS) in DMSO and, most importantly, the toxicity of cisplatin was remained at 100% after the loading process. This CDDP/DMSO-AMS was designed for the intratumoral injection through the bronchoscopic catheter or dry powder inhalation (DPI) due to its high stability in air or in solution. This CDDP/DMSO-AMS showed a fast cisplatin release within 24 hours. In the *in vitro* study, CDDP/DMSO-AMS showed high effectiveness on killing the lung cancer cells including the non-small cell lung cancer (NCL-H23 and A549), malignant mesothelioma (CRL-2081) and the mouse lung carcinoma (Lewis lung carcinoma) cell lines. The albumin based mesospheres provide an ideal loading matrix for cisplatin and other metal-based drugs due to the high swelling degree and fast uptake rate in the organic solvents with high polarity. In addition, to investigate the effects of polysaccharides, such as chitosan and chondroitin, on enhancing loading efficiency and lasting cytotoxicity of cisplatin, the polysaccharide-modified albumin mesospheres were synthesized and loaded with cisplatin in this study.

Keywords: Cis-diamminedichloroplatinum (II), DMSO, albumin mesospheres, non-small cell lung cancer (NSCLC), cytotoxicity, chitosan, chondroitin

Introduction

Cisplatin, cis-diamminedichloroplatinum (II), also known as CDDP, is the first metal coordination complex to have clinical anti-tumor activity. It is one of the most commonly used chemotherapeutic drugs nowadays in the treatment of a variety of cancer tumors including lung, testicular, ovarian, lymphoma and glioma [1, 2]. Clinically, cisplatin is generally used with other chemotherapeutic drugs as primary and secondary treatments for various cancers [3]. In a series of adjuvant trials in patients with resected non-small cell lung cancer (NSCLC), the combination of cisplatin with other agents, such as doxorubicin, etoposide and various kinds of vinca alkaloids including vinblastine, vincristin etc., had shown a significant improvement on 5-year survival rate of the NSCLC patients [4].

Cisplatin is transported across the plasma membrane through both passive diffusion and active transport [5]. Cisplatin is highly reactive in biological environment and binds to many biomolecules such as DNA, RNA, proteins and phospholipids [6-8]. The cause of the toxicity of cisplatin is difficult to determine since it binds to various kinds of biomolecules. However, the binding of cisplatin to DNA is thought as the most critical reaction leading to the toxicity for cancer tumors [9]. The Pt(II) of cisplatin remains coordinated to its chloride ligands while circulated in the blood with high chloride concentration. Upon entering into cells, the chloride ligands of cisplatin are replaced by water mole-

cules to form a positively charged species that can react with nucleophilic sites on DNA, RNA and proteins to form adducts [10]. To cause cellular apoptosis, cisplatin binds to the N7 atom of guanines in DNA to form intrastrand adducts, interstrand crosslinks or DNA-protein crosslinkes. It has been suggested that the major lesions produced by cisplatin are intrastrand adducts, and the cellular resistance to cisplatin may be associated with interstrand crosslinks [11].

Although cisplatin is one of the most commonly used chemotherapeutic drugs for cancer treatments, some tumors acquire resistance to cisplatin after the initial treatment and chronic cisplatin usage results in cisplatin accumulation in cancer cells and induces cisplatin resistance [12]. However, the higher dose of cisplatin is not clinically applicable to improve its efficiency due to the high toxicity of cisplatin. The usage of cisplatin combines with severe toxic side effects on cancer patients including neurosis, nephrosis, and hematosis, along with gastrointestinal and renal toxicity [13-17]. In addition, it has been shown that only 10% of the cisplatin introduced into blood stream enter into the cells, and 90% of them are bound to the plasma proteins outside the cells [18].

Platinol®, a FDA approved cisplatin for injection, is administered to patients as a 1 mg/ml concentration in aqueous solution containing 0.9% of sodium chloride and 1% of mannitol (Bristol-Myers Squibb, Princeton, NJ). It is injected intravenously to patients in the range of 20 to 150 mg/m^2 (concentration of cisplatin in body area) for daily dosage or one cycle, and which depends on the severity of patients. However, since the solubility of cisplatin is low in aqueous solution, the intravenous delivery through aqueous solution may result in cisplatin aggregation and cause the concentration uncontrollable in human body. Thus the antitumor effect of cisplatin might be significantly reduced while delivered through aqueous environment.

Cisplatin can be easily dissolved into DMSO to obtain an accurate solution concentration higher than 200 mg/ml. This has also been published as standard methodology by the National Cancer Institute for studying the biological effect of cisplatin [19]. While dissolved in DMSO, cisplatin binds rapidly to DMSO to form a cisplain-DMSO adduct and which has been reported to have reduced ability to bind to double-stranded DNA. The combination therapy of cisplatin and DMSO was proposed to enhance cytotoxicity and reduce systemic side effects in earlier studies [20, 21]; however, it has been recently reported that cisplatin in DMSO showed reduced toxicity to tumor cells [22-25].

In view of these limitations including low efficacy and severe side effects, efforts have been taken to develop an ideal system for cisplatin delivery to continuously provide a prolonged high concentration of cisplatin locally on tumor sites. To achieve this goal, many delivery systems based on polymeric liposomes, micelles and microspheres had been designed for intravenous administration on the basis of the enhanced permeability and retention (EPR) effect [26-31]. It had been reported that the cisplatin entrapped or complexed in polymeric devices has improved efficacy and reduced systemic toxicity [32, 33].

Recently, some acrylic polymers containing a carboxyl group such as poly (acrylic acid) and poly (methyl methacrylate) have attracted attentions due to their high tolerance *in vivo* and most importantly, their high reactivity to interact with other therapeutic agents [34, 35]. The carboxyl groups on the polymer chains have a liable complexing ability towards cisplatin, therefore the polymeric matrix stabilizes cisplatin and protects it from binding to other biomolecules in a biological environment [32]. Due to the slow exchange rate of carboxyl groups with water molecules, the complexation provides prolonged release of cisplatin from the polymeric microspheres and further reduces systemic toxicity. However, the activity of cisplatin might be influenced by the complexation between cisplatin and the carboxylate polymers. It had been shown that the activity of cisplatin was slightly reduced after release from the cisplatin-loaded polymeric carriers [22, 32].

Among all the biocompatible and biodegradable polymeric microspheres, albumin microsphere has a high potential to be the ideal carrier for cisplatin delivery due to its abundant functional groups [36-38]. The amino acids in albumin provide a variety of reactive functional groups, including abundant carboxyl groups and other side groups on the amino acid residues, in the matrix and on the surface of micro-

spheres. The amphiphilic nature of protein molecules allows the albumin microsphere to have hydrophilic and hydrophobic domains in the matrix and enables the microsphere to load with both hydrophilic and hydrophobic drugs or biomolecules. Furthermore, the loading efficiency of metal ion containing drugs can be greatly improved by using albumin based microspheres as drug carriers due to the negatively charged nature of albumin proteins. The potential drug release behaviors of the cisplatin loaded albumin microspheres in a biological system are illustrated in **Figure 1B**.

The natural, biodegradable and highly biocompatible polysaccharides, chitosan and chondroitin have been proposed for use in various kinds of biomedical applications including drug delivery systems and tissue engineering [39-43]. Chitosan is a cationic biopolymer which is obtained by deacetylation of chitin. It has been reported that the cisplatin content increased with the increasing concentration of chitosan and chitin added into the drug carrying microspheres including chitosan-ablumin microspheres [44-47]. This may be explained by the intense ionic interaction between the negatively charged chitosan and chitin molecules and the cationic metal ions in cisplatin. Chondroitin sulphate is an anionic sulfated glycosaminoglycan which is obtained from mammalian connective tissue. Chondroitin microspheres have been made for various drug delivery purposes and controllable drug release had been achieved [43, 48].

Materials and methods

Synthesis of albumin mesospheres (AMS) and modified AMS

To prepare the modified albumin mesospheres, a solution of cellulose acetate butyrate (CAB) in 1,2-dichloroethane (DCE) was used as the continuous organic phase. Aqueous solution containing bovine serum albumin (BSA) was dispersed into CAB/DCE solution to form an emulsion solution. To prepare the chitosan and chondroitin modified albumin mesospheres (chitosan-MS and chondroitin-MS), aqueous solution containing bovine serum albumin and chondroitin sulfate (sodium salt from shark cartilage, Sigma, St Louis, MO) or chitosan glycol (MP Biomedicals, Solon, OH) was added into the continuous phase. The mixture solution in the tube was vortexed at 3000 rpm for 2 mins to create a stable emulsification. To solidify the mesospheres, 8% w/w EM grade glutaraldehyde to BSA was added through DCE into the emulsion solution. The tube was placed on rotator for 8 hours to complete crosslinking. Then the mesospheres were washed out by acetone and air-dried at room temperature. Detailed preparation and characterization of AMS were described in previous publication. The particle size of mesospheres used in this study has been characterized and determined previously [37]. The particles are majorly in the size range of 1 to 10 μm with a mean diameter about 5 μm. The appearance of mesospheres was investigated by using optical microscope in this paper.

Post-loading of cisplatin (CDDP)

As illustrated in **Figure 1A**, the post-loading of cisplatin into the mesospheres were performed in aqueous solution, PBS, and the polar aprotic organic solvent, dimethyl sulfoxide (DMSO). To prepare the mesospheres loaded with CDDP/PBS solution, a suspension solution containing about 10 mg cisplatin in 200 μg PBS was made as the loading solution. About 40 mg of mesospheres were dispersed in a minimal amount of acetone in the 1.5 ml micro-centrifuge tubes. In the mesospheres dispersion in the tubes, 200 μl of the loading solution containing cisplatin were added and well-dispersed by vigorously vortexing. The mesospheres dispersions were vortexed at room temperature for 72 hours and then centrifuged to remove the supernatant solution. The tubes containing residual mesospheres and precipitated cisplatin were filled with acetone, vortexed and centrifuged. The washes were repeated twice to remove the excessive cisplatin absorbed on the surface of mesospheres. After the last centrifugation, the layer of mesospheres loaded with cisplatin were separated from the layer of precipitated cisplatin and then collected. The collected cisplatin-loaded mesospheres were air-dried at room temperature and stored at 4°C for later analyses.

To make the DMSO loading solution for mesospheres, 40 mg of cisplatin were completely dissolved into 160 μl of DMSO and loading solution was prepared freshly right before use. To load mesospheres with CDDP/DMSO, about

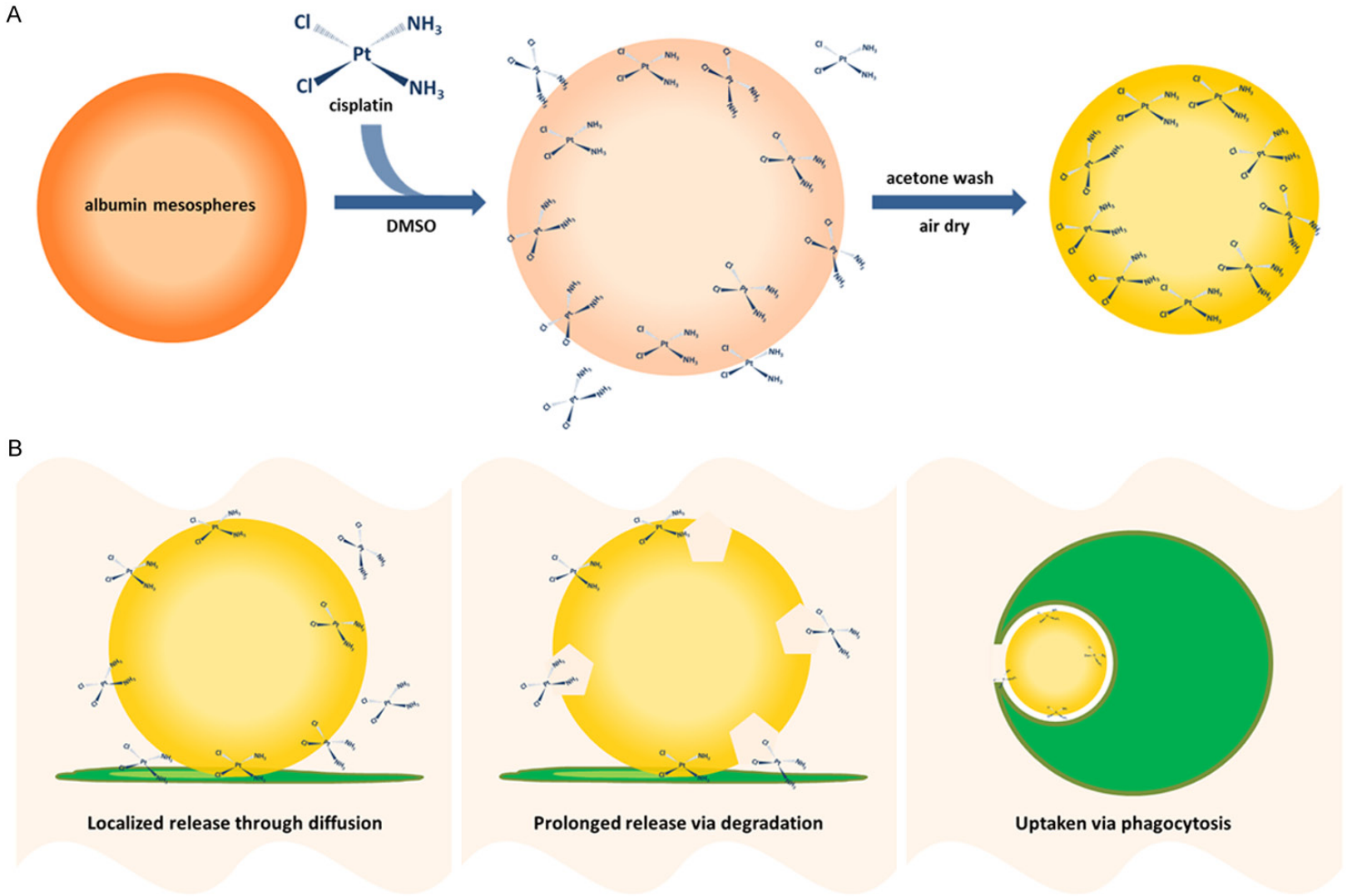

Figure 1. (A) Process of cisplatin loading in albumin mesospheres and (B) drug release behaviors of cisplatin loaded albumin mesospheres in biological systems.

40 mg of mesospheres were dispersed in acetone in the 1.5 ml centrifuge tubes and then the loading solution above was added. The mesospheres were well-dispersed in the loading solution and vortexed at room temperature for minutes to hours. Then the dispersions were centrifuged and the supernatant were removed. The tubes containing precipitated mesospheres were filled with acetone, vortexed and centrifuged. The acetone washes were repeated twice to remove the excessive cisplatin absorbing on the surface of mesospheres. After wash, the cisplatin-loaded mesospheres were air-dried at room temperature and stored at 4°C.

Measurement of loading efficiency and cisplatin release

To measure the loading efficiency of cisplatin in the mesospheres, about 1 to 2 mg of mesospheres were placed in a 15 ml centrifuge tube and then digested with at least 5 ml digest solution containing papain, protease K, ethylenediaminetetraacetic acid (EDTA) and cysteine. The suspension solutions containing mesospheres and enzymes were placed on a rotator in an incubator at 37°C for days. After digestion, the suspension solutions were centrifuged to precipitate the debris of MS and the supernatants were collected for analysis.

To determine the cisplatin release from the mesospheres, the mesospheres loaded with CDDP/DMSO and CDDP/PBS for a minimal loading time (< 5 mins) were used in the study. About 1 to 2 mg of cisplatin-loaded mesospheres were placed in a 10 ml centrifuge tube and dispersed with 50 μl acetone. The suspension solution was sonicated for 10 seconds to make the mesospheres well-dispersed in acetone. Then the tube was filled with PBS to 10 ml and placed on a rotator in an incubator at 37°C. After 24 hours of incubation, the suspension solution was centrifuged and 5 ml supernatants were collected and stored at 4°C for future analysis. The concentrations of cisplatin in aqueous solutions were determined by using ICP/AES, Perkin-Elmer Optima 3200 RL. The standard solutions were prepared by diluting the platinum standard for ICP (TraceCERT®, 1000 mg/L Pt in hydrochloric acid, Fluka).

Cytotoxicity of cisplatin released from cisplatin-loaded mesospheres

A549 cells (ATCC #CCL-185; human lung adenocarcinoma epithelial cell line), NCI-H23 (ATCC #CRL-5800; human lung adenocarcinoma epithelial cell line), CRL-2081 (malignant mesothelioma cell line), and Lewis lung carcinoma cells were obtained from American Type Cell Collection (Manassas, VA). The cells were cultured in RPMI-1640 medium (Sigma, St Louis, MO) containing 10% fetal bovine serum (FBS), penicillin (100 U/ml), streptomycin (100 μg/ml), fungizone (100 μg/ml), 0.25% D-glucose, 0.2% sodium bicarbonate and 1% sodium pyruvate as reported earlier [49, 50].

To determine the toxicity of cisplatin that was released out from the cisplatin-loaded mesospheres, the cisplatin released in PBS was collected first and reconstitute to desired concentration. About 10 mg of cisplatin-loaded mesospheres were dispersed in 2 ml PBS at room temperature to allow the release of cisplatin. After days of incubation, the dispersion solutions were centrifuged and the supernatant solution were collected and stored at room temperature. The concentration of the supernatant solution were determined by using ICP-AES and then diluted to 10 μg/ml using PBS. A 10 μg/ml cisplatin solution was also prepared by directly dissolving cisplatin into PBS.

About 6,000 H-23 (human lung adenocarcinoma epithelial cell line) cells were seeded into each well of the 96-well plate with 10% FBS culture media. After 24 hours of incubation at 37°C, PBS containing cisplatin was added into each well to acquire a final concentration of 10 μg/ml cisplatin in each well. After 48 hours of incubation, the proliferation of cells incubated with and without cisplatin was determined by using WST-1 reagents (Roche, Indianapolis, IN).

Cytotoxicity of cisplatin-loaded mesospheres

The cytotoxic effects of cisplatin-loaded mesospheres on lung cancer cells were determined by using the WST-1 reagent as reported earlier [49, 50]. About 1,500 to 3,000 cells were seeded in each well of the 96-well plate and incubated in media at 37°C. After 24 hours of incubation, PBS containing cisplatin-loaded mesospheres (minimal loading time) were added into

Table 1. Loading content and efficiency of cisplatin in AMS and modified AMS

Particles	Composition of Matrix	Loading solvent	Loading time (hrs)	CDDP Content (μg per mg of CDDP-MS[1])	Loading Efficiency (%)
AMS	100% BSA	DMSO	8	531	~100%
		PBS	72	72	14%
Chitosan-MS	80% BSA + 20% chitosan	DMSO	8	430	86%
		PBS	72	59	12%
Chondroitin MS	80% BSA + 20% chondroitin	DMSO	8	297	59%
		PBS	72	86	17%

[1]CDDP-MS: cisplatin-loaded mesospheres.

Table 2. Loading efficiency and cytotoxicity of CDDP-MS loaded through DMSO

Particles	Loading Time	CDDP Content (μg per mg of CDDP-MS)	Loading Efficiency (%)	Cytotoxicity of CDDP Loaded MS[1] (Cell Survival %)	Remaining Toxicity of Released CDDP[2] (Toxicity %)
CDDP/DMSO-AMS(0)	< 5 mins	163	33%	22%	100%
CDDP/DMSO-AMS(1)	1 hr	223	45%	46%	96%
CDDP/DMSO-AMS(4)	4 hrs	369	74%	69%	63%
CDDP/DMSO-AMS(8)	8 hrs	531	~100%	72%	46%

[1]To determine the cytotoxicity of CDDP loaded MS, H-23 cells were incubated with 100 μg/ml CDDP loaded MS for 48 hours. The cell proliferation were measured and compared to the untreated cells. [2]H-23 cells were incubated with10 μg/ml CDDP that were released from the CDDP loaded MS. Cell proliferation were determined after 48 hours of incubation and compared to CDDP which was dissolved in PBS.

each cell to make the concentration of cisplatin-loaded mesospheres in each well to be 100 and 200 μg/ml. The proliferation rates of the cells incubated with cisplatin-loaded MS were measured by using a microplate reader after 48 hours of incubation. To determine the cytotoxicity of cisplatin-loaded mesospheres after wash, the mesospheres were vigorously vortexed in daily changed fresh PBS for days to remove the cisplatin-loaded in matrix. Then the mesospheres were washed by acetone and dried at room temperature before use. Every experiment was done in triplicates.

Results

Loading content and efficiency of cisplatin in mesospheres

Table 1 shows the cisplatin content and loading efficiency of the AMS and modified mesospheres loaded with CDDP/DMSO and CDDP/PBS solutions. Among the mesospheres, AMS showed the highest loading efficiency of cisplatin when loaded with CDDP/DMSO, and chondroitin-MS showed the lowest efficiency about 60%. The loading efficiency of cisplatin in AMS and polysaccharide modified mesospheres loaded with CDDP/PBS was 10 to 20%.

The results of cisplatin content and loading efficiency of the albumin mesospheres loaded with CDDP/DMSO solution in various loading time were shown in **Table 2**. By using DMSO as the loading solvent, the loading content of cisplatin in mesospheres increased with the incubation time in DMSO. As the loading time was minimized within seconds, the loading content of cisplatin in AMS was around 16%. After 8 hours of incubation in DMSO, the dissolved cisplatin in DMSO were completely absorbed into the mesospheres. The cisplatin content was improved to 500 μg in per milligram of cisplatin-loaded mesospheres and the loading efficiency was close to 100%.

In vitro release of cisplatin from cisplatin-loaded mesospheres

The *in vitro* release of cisplatin at 24 hours from the cisplatin-loaded mesospheres was determined in PBS. In **Figure 3**, the mesospheres loaded with CDDP/DMSO in a minimized loading time showed a range of cisplatin release from 80 to 130 μg per milligram of cisplatin loaded mesospheres. The chitosan-modified mesospheres loaded with CDDP/DMSO solution showed highest cisplatin release. The

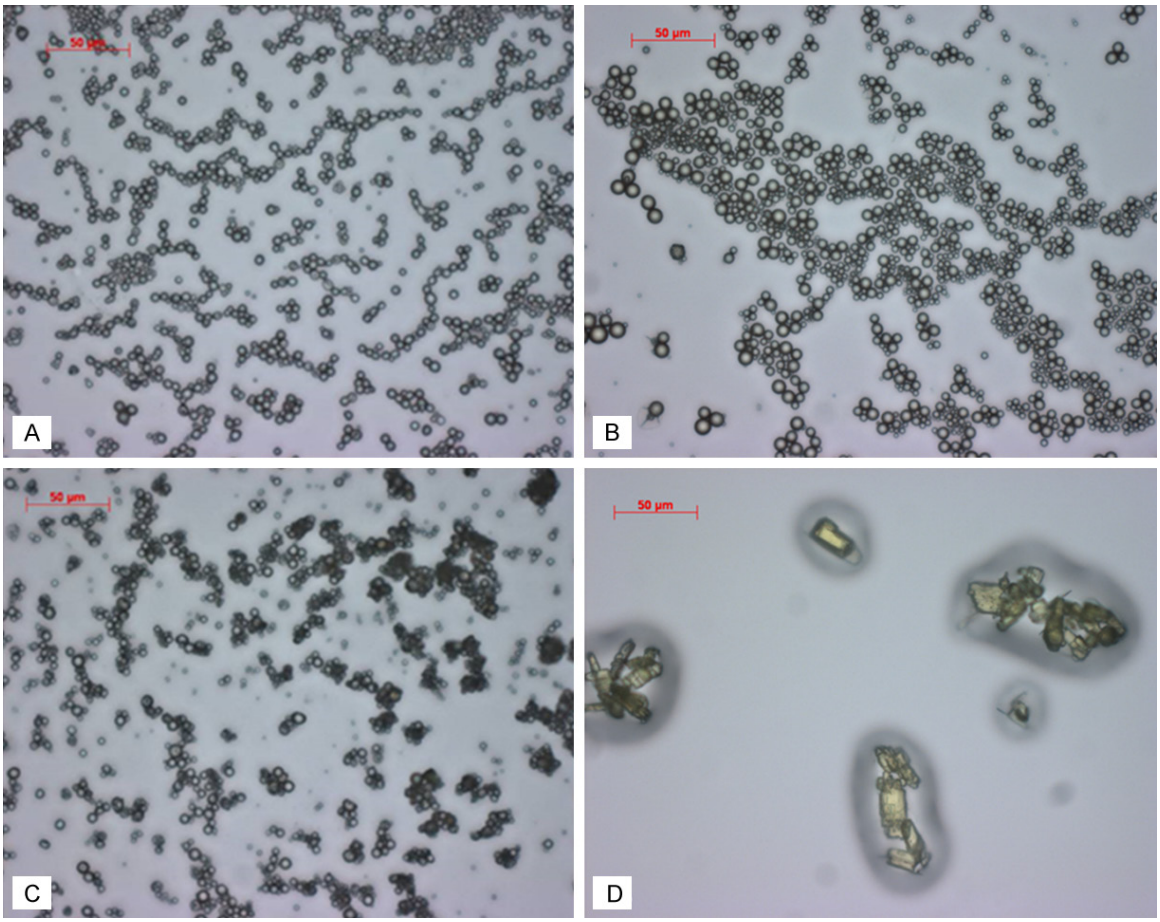

Figure 2. Optical images of stable cisplatin-loaded mesospheres. (A) CDDP/DMSO-AMS, (B) CDDP/DMSO-chitosan-MS and (C) CDDP/PBS-chondroitin-MS as well as (D) crystalline cisplatin. Clean solid particles without excessive cisplatin aggregation were observed under optical microscope in the mesospheres loaded in CDDP/DMSO.

mesospheres loaded with CDDP/PBS showed a release range of 2 to 20 μg per milligram of mesospheres. In the mesospheres loaded with CDDP/PBS, chondroitin-MS showed the highest cisplatin release which was up to 20 μg per milligram of mesospheres. However, the AMS and chitosan-MS showed a low cisplatin release under 10 μg per milligram.

Cytotoxicity of released cisplatin

Figure 4 shows the remaining cytotoxicity of the cisplatin released out from the CDDP/DMSO-AMS. The CDDP/PBS solution showed high toxicity and reduced the cell proliferation to 6%. The cisplatin released from the CDDP/DMSO-AMS(0) showed the same toxicity to the cisplatin dissolved in PBS. After one hour of incubation in CDDP/DMSO, the cisplatin released form CDDP/DMSO-AMS (1) showed slightly reduced toxicity to about 95% (proliferation to 10%) as shown in **Table 1**. The cytotoxicity of cisplatin-loaded in the mesospheres decreased to about 50% (proliferation to 64%) after 8 hours of incubation in DMSO.

In vitro cytotoxicity of cisplatin-loaded mesospheres

Figure 5 shows the cytotoxicity of cisplatin-loaded AMS before and after PBS wash. The CDDP/DMSO-AMS(0) showed the highest toxicity and reduced the cell proliferation of the lung cancer cells to about 20%. However, the toxicity of cisplatin-loaded AMS decreased with increasing incubation time in DMSO during loading process. After 4 hours of incubation in DMSO, cisplatin-loaded AMS showed great reduction in toxicity and the cell proliferation was increased to 70%. For the cisplatin-loaded AMS washed by PBS, the mesospheres without further release of cisplatin still showed 20 to

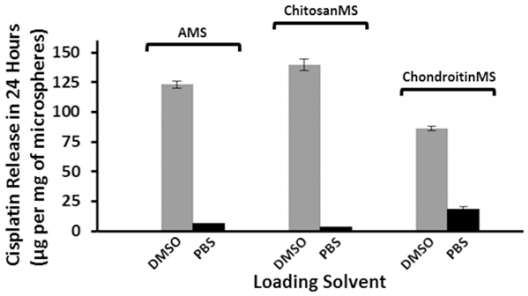

Figure 3. Cisplatin release from mesospheres within 24 hours. The release of cisplatin from the mesospheres loaded with cisplatin via DMSO (grey column) and PBS (black column) were measured.

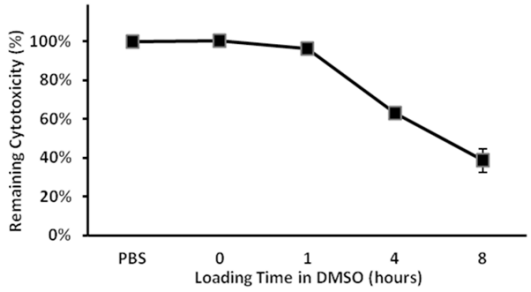

Figure 4. The cytotoxicity of cisplatin released from the cisplatin-loaded albumin mesospheres (CDDP/DMSO-AMS). The cisplatin, which were released out from the CDDP/DMSO-AMS prepared with different loading time in DMSO, were used in this test. The figure showed the percentage of remaining toxicity of cisplatin with various loading time. Cisplatin with loading time in 1 hour showed similar cytotoxicity (above 90%) to cisplatin in PBS. The cytotoxicity of cisplatin was determined by the cell death caused by cisplatin incubation. The cytotoxicity of cisplatin in PBS was 100%.

35% of cell death percentage. The unloaded AMS showed no cytotoxicity to lung cancer cells at a low concentration such as 100 μg/ml.

The cytotoxicity of cisplatin-loaded AMS and modified mesospheres were tested by using A549, CRL-2081 and LLC cells. In **Figure 6**, the CDDP/DMSO-AMS showed most efficient inhibiting effect on the proliferation of lung cancer cells. The CDDP/DMSO-chitosan-MS showed similar toxicity to CDDP/DMSO-AMS and CDDP/DMSO-chondroitin-MS showed reduced toxicity. However, for the mesospheres loaded with CDDP/PBS, CDDP/PBS-chondroitin-MS showed the most effective inhibition on the growth of lung cancer cells. The unloaded mesospheres including AMS and the modified mesospheres

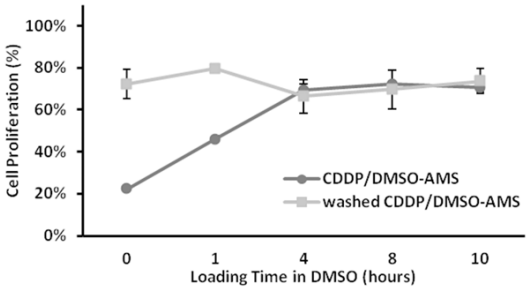

Figure 5. Cytotoxicity of cisplatin-loaded albumin mesospheres before and after PBS wash. Reduction in toxicity of cisplatin was found with increasing loading time in DMSO. The PBS-washed CCDP/DMSO-AMS had almost no further cisplatin release but showed apparent toxicity compared to unloaded AMS. The proliferation of H-23 cells incubated with unloaded albumin mesospheres was around 110%.

showed negligible toxicity toward the lung cancer cells.

Discussion

A new formulation of cisplatin, lipoplatin, has been recently successfully administered in clinical trials including mainly NSCLC [51]. Lipoplatin is the liposomal encapsulation of cisplatin into nanoparticles and it has been reported to show high efficacy and lower side effects of cisplatin in NSCLC. However, the cisplatin-loaded albumin microspheres introduced here allow a different approach for lung cancer treatment by different treatment strategies such as bronchoscopic catheter injection and dry powder inhalation [52, 53]. The dry powder of cisplatin-loaded mesospheres can be applied on the tip of bronchoscopic catheter and directly placed onto tumor sites to provide a localized release of cisplatin. In addition, due to the high stability and well-dispersion of AMS in aqueous solution, these cisplatin-loaded mesospheres can also be applied in solution by being dispersed with saturated cisplatin solution to provide a prolonged release.

To obtain a high loading efficiency of cisplatin in the albumin mesospheres (AMS), the high solubility of cisplatin and high swelling degree of the AMS in the loading solvent are necessary. Solid cisplatin powder can be completely dissolved by DMSO. Compared to the other organic solvent, the swelling degree of AMS in DMSO is relatively high due to the high solubility of albumin in DMSO. However, the reduction in

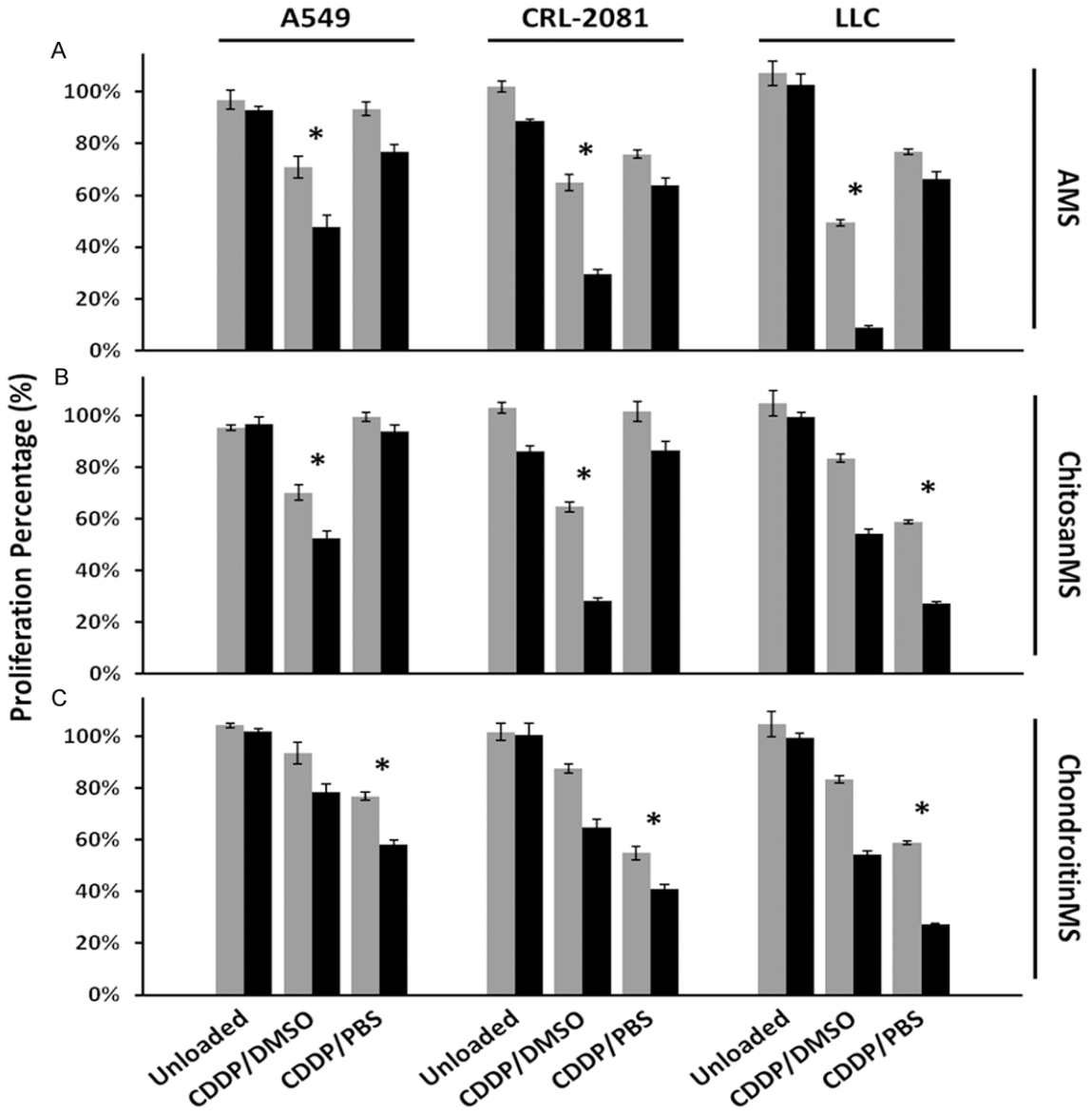

Figure 6. Cell proliferation of A549, CRL-2081 and LLC cells after 48 hours of incubation with 100 (grey column) and 200 (black column) μg/ml of cisplatin-loaded (A) AMS, (B) Chitosan-MS and (C) chondroitin-MS. The CDDP/DMSO-AMS and CDDP/DMSO-chitosan-MS showed high efficiency on killing lung cancer cells. Among the mesospheres loaded through PBS, the chondroitin modified mesospheres (CDDP/PBD-chondroitin-MS) showed the highest cytotoxicity. All the unloaded mesospheres showed low cytotoxicity. (Proliferation percentage = cell proliferation of mesospheres-treated cells /proliferation of untreated cells %; $*P < 0.05$ is considered significant when compared with cells without treatment).

toxicity of cisplatin caused by the adduction with DMSO has been reported. In the previous studies, cisplatin were loaded into microspheres composed of albumin, chitosan or PLGA [45-47, 54-56]. These cisplatin-loaded microspheres were mostly prepared in situ in the organic solvent in which cisplatin has low solubility, such as acetic acid, methylene chloride and dimethylformamide. Cisplatin were usually sonicated and mixed with matrix molecules to form a dispersion solution in the discontinuous phase in emulsion systems. As a result, even high contents of cisplatin in microspheres up to 20% has been reported, the aggregation of cisplatin trapped in the synthesized microspheres was unavoidable. The uneven loading of cisplatin within the microspheres may cause unexpected cisplatin

release in the body of cancer patients. However, the post loading method by using DMSO in this report provides a molecularly even loading of cisplatin within albumin microspheres.

Compared to PBS, DMSO as the loading solvent showed an extremely high efficiency loading cisplatin into the AMS. Within seconds of loading time, above 30% of loading efficiency and 160 μg of cisplatin content in per milligram of mesospheres can be achieved. In a prolonged loading time up to 8 hours, eventually the CDDP/DMSO solution was completely absorbed into the AMS. It reveals that the AMS can be an ideal carrier for those metal based drugs which can be soluble in DMSO.

In order to clearly investigate the interaction between the polysaccharides and cisplatin, the loading time was prolonged to 8 and 72 hours for DMSO and PBS. In the modified mesospheres, chitosan-MS showed a similar loading efficiency to AMS in DMSO and the chondroitin-MS showed a significantly lower loading efficiency compared to AMS. This difference in loading efficiency in DMSO may be caused by the lower swelling degree of the chondroitin domain in the mesospheres. However, the chondroitin-MS showed a higher loading efficiency in PBS than AMS, whereas the chitosan-MS showed a lower one. This implies that the negatively charged chondroitin sulfate may be more effective on entrapping the positively charged cisplatin into the mesospheres in aqueous environment.

The release of cisplatin from the albumin based mesospheres can be influenced by the matrix materials, crosslink density and the release environment. In addition, the release profile of cisplatin from the surface area of the mesospheres can be totally different from the release prolife from bulk. Generally, cisplatin are mainly loaded in the surface area of AMS while using post-loading method with short loading time. In the modified mesospheres, CDDP/DMSO-chitosan-MS showed the highest cisplatin release; even it has lower cisplatin content. The release percentage of cisplatin from chitosan-MS was around 35%, and that from AMS was 23%. Thus, it reveals that chitosan can be added into AMS for controlling the release rate of cisplatin. In the modified mesospheres loaded with cisplatin through PBS, CDDP/PBS-chondroitin-MS showed the highest cisplatin content and highest release rate; however in the optical image in **Figure 2C**, the chondroitin-MS showed slightly aggregation of the mesospheres after acetone wash, and which may hinder the release of cisplatin from mesospheres.

Cytotoxicity is the most important characteristic to determine the efficacy and effectiveness of the drug loaded mesospheres. In our study, cisplatin showed decreasing toxicity when incubated with DMSO over time. However, the remaining toxicity of the released cisplatin was maintained above 90% within 1 hour of loading time. In **Figure 5**, it shows that the cytotoxicity of CDDP/DMSO-AMS decreased with loading time in DMSO. However, it was also noticeable that the CDDP/DMSO-AMS showed toxicity even no further cisplatin release from the mesospheres after washed by PBS. This implies that the cisplatin conjugated on the surface of AMS may also have some degree of ability to kill the cancer cells via chemical or physical interactions.

In the modified mesospheres, the chitosan and chondroitin did not show significant influence on cytotoxicity of mesospheres. The unloaded AMS, chitosan-MS and chondroitin-MS showed low cytotoxicity and did not cause visible cell death in the *in vitro* studies. In **Figure 6**, it shows that the CDDP/DMSO-chitosan-MS and CDDP/DMSO-AMS both showed high effectiveness on killing the lung cancer cells. From the similar toxicity of CDDP/DMSO-chitosan-MS and -AMS, it can be assumed that the polysaccharides in the mesospheres showed no significant effect on stabilizing the cisplatin in DMSO during the post-loading process. For the mesospheres loaded in PBS, the CDDP/PBS-chondroitin-MS killed CRL-2081 and LLC cells effectively, whereas the other two mesospheres showed low toxicity to all these lung cancer cell lines.

The CDDP/DMSO-AMS were designed to be applied intratumorally through the bronchoscopic needle catheter or for dry powder inhalation. The highly stable mesospheres can provide a controllable release of cisplatin with the absence of particle aggregation. While applied in solution, high concentration of CDDP/DMSO-AMS is convenient to be injected into the tumor sites due to its well-dispersion in aqueous solution. The CDDP/DMSO-AMS can be dispersed

in saline solution saturated with cisplatin to prevent the early release of cisplatin before injection. In addition, injecting CDDP/DMSO-AMS with the saturated cisplatin solution may lower cisplatin release rate and prolong the continuous release of cisplatin. Besides, not only cisplatin, the AMS can also be an ideal loading matrix for the other metal-based chemotherapeutic drugs, such as zinc protoporphyrin (ZnPP) and cobalt protoporphyrin (CoPP), which have a high solubility in DMSO. The loading efficiency of metal-based drugs in AMS can usually reach almost 100% and high drug content can be obtained.

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Disclosure of conflict of interest

The authors declare that they have no competing interests.

Abbreviations

CDDP, Cis-diamminedichloroplatinum (II); DMSO, Dimethyl sulfoxide; AMS, Albumin mesospheres; DPI, Dry powder inhalation; NSCLC, non-small cell lung cancer; EPR, Enhanced permeability and retention; CAB, Cellulose acetate butyrate; DCE, 1,2-dichloroethane; BSA, Bovine serum albumin; EDTA, Ethylenediaminetetraacetic acid; ZnPP, zinc protoporphyrin; CoPP, cobalt protoporphyrin.

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