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PEGylation of Carbonate Apatite Nanoparticles Prevents Opsonin Binding and Enhances Tumor Accumulation of Gemcitabine

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ABSTRACT

pH sensitives carbonate apatite (CA) has emerged as a targeted delivery vehicle for chemotherapeutics agent with tremendous potential to increase the effectivity of breast cancer treatment. The major challenge for intravenous delivery of drug-incorporated nanoparticles is their rapid opsonization, resulting in accumulation within the organs of reticuloendothelial system, such as liver and spleen. Therefore, surface modification by polyethylene glycol was implemented to improve the half-life of drug-particle complexes and enhance their uptake by target tissues. A simple, rapid, and sensitive triple quadrupole liquid chromatography–mass spectrometry method was developed and validated for quantification of gemcitabine in plasma, various organs and tumor tissues of mice with breast carcinoma, whereas sodium dodecyl sulfate-polyacrylamide gel electrophoresis, quadrupole-time of flight liquid chromatography–mass spectrometry and analysis by SwissProt.Mus_musculus database were performed for protein separation, identification, and homology search by comparing the *de novo* sequence tag. PEGylated CA exhibited almost 6-fold increase in gemcitabine accumulation in tumor with significant reduction in other organs within 1 h of intravenous administration, compared to free drug. In addition, plasma drug amount was found to be higher in PEGylated particles, implying their role in prolonging blood circulation time of particle-bound gemcitabine. Investigation of protein corona composition demonstrated notable reduction in opsonin interactions after PEGylation of CA particles. Overall, the results indicate that the composition and dynamics of protein corona subjected to alteration by PEGylation play crucial roles in affecting successful nanoparticle-based targeted delivery of a cytotoxic drug.

Introduction

Recent decades have seen the development of various organic and inorganic nanocarriers for drug delivery purposes. There are several key issues that emerge in relation to the features of nanoparticle-drug complexes, focusing on drug incorporation into carriers, stability of the complexes, their biocompability, biodistribution, targetability, and finally functionality.[1-3] Instability of nanocarriers could cause fast drug release before reaching the intended therapeutic sites, whereas too stable nanocarriers might delay drug release, resulting in extended exposure of the nanocarriers to the body with subsequent systemic toxicity. Hence, biodegradable nanoparticles with life span as long as it is required for therapeutic action, and high targeting specificity would be desirable.[2]

Polyethylene glycol (PEG) is widely used as a coating material especially for hydrophobic polymers and lipids to increase their solubility and prolong their blood circulation time.[4,5] The "stealth effect" of PEG could be explained by its highly hydrophilic backbone that creates steric repulsion, thus preventing binding of polymeric or liposomal particles with opsonins and their subsequent recognition by macrophages in the reticuloendothelial system (RES). The anti-opsonization effect largely depends on the length and density of PEG chains.[6]

Immediately after administration, nanoparticles encounter a dramatic challenge in biological environment (blood) which contains various biomolecules (proteins), creating "protein corona" on nanoparticle surface through hydrophobic and ionic interactions. Protein corona plays a key role in facilitating opsonin-mediated macrophage uptake and removal of the particles via RES, thus serving as a major barrier to targeted drug delivery.[7-10]

It should be noted that although overall protein adsorption is reduced, it could not be fully eliminated because interactions between plasma proteins and nanoparticles *in vivo* are dynamic in nature. Protein corona composition is influenced by a number of factors, such as the physical and chemical properties of nanoparticles including size, shape, and surface charges,[11,12] the characteristics of biological media including protein concentration,

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protein source (human, mouse, etc), and temperature,[13-16] and finally, the incubation time for interactions between nanoparticles and proteins.[17]

To date, protein corona investigation has largely been carried out on organic nanoparticles, such as polymers and liposomes, whereas inorganic nanoparticles remain far less explored. Carbonate apatite (CA) particles offer advantages in terms of simplicity of fabrication, biodegradability, and versatility compared to organic counterparts. pH-sensitive properties and naturally occurring constituents (calcium, phosphate and bicarbonate) of CA make it biodegradable and potentially safe to be used for human applications. In addition, the heterogenous charge distribution (i.e., existence of both anion- and cation-binding domains) enables it to be a versatile carrier of various chemotherapeutic drugs and genetic materials, with which it associates via electrostatic interactions.[1-3]

Surface modification of the CA particles by PEGylation and targeting ligand is necessary to enhance delivery efficiency of drugs into cancerous cells for *in vivo* applications. Our earlier *in vitro* study had proven that PEGylation help reduce size of drug-loaded CA particles, thus increasing cellular uptake of the drug molecules through endocytosis and cytotoxicity to cancer cells, in comparison to the free drug internalized by passive diffusion.[1,2] In this study, we have successfully developed PEGylated CA particles with nano-size diameters to effectively deliver gemcitabine, an anti-cancer drug to breast tumor through passive targeting in a syngeneic mouse model, and undertaken quantitative analysis of the *in vivo* distribution of drug-loaded, unmodified, and surface-modified CA nanoparticle in various mice organs, tumor, and plasma. Among all the bioanalytical techniques, triple quadrupole liquid chromatography coupled with mass spectrometry (QQQ LC/MS) was chosen for drug quantification in biological samples because of its high sensitivity, selectivity, and reproducibility.[18,19] Gemcitabine quantity obtained inside the tumor was found to be correlated to the effect on tumor regression observed in our previous *in vivo* study,[1] with PEGylation of CA nanoparticles drastically preventing opsonin binding to the nanoparticles and enhancing more retention in blood and higher accumulation in the tumor, compared to unmodified particles and free drugs. The study also provides insight in assessing the therapeutic efficacy and toxicity in normal organs/tissue, based on pharmacokinetic profiling of intravenously administered CA nanoparticles.

Materials and Methods

Reagents

Dulbecco's Modified Eagle Medium (DMEM) powder and ethylenediamine tetraacetate (EDTA) were obtained from Gibco BRL (Carlsbad, CA). Calcium chloride dihydrate ($CaCl_2$-$2H_2O$), sodium bicarbonate, ammonium bicarbonate, formic acid, acetonitrile, dithiothreitol, and iodoacetamide were from Sigma-Aldrich (St. Louis, MO). Streptavidin from *Streptomyces avidinii* and poly(-ethylene glycol) 2-aminoethyl ether biotin were obtained from Sigma-Aldrich. The chemotherapy drug, gemcitabine was purchased from Sigma-Aldrich. Pierce Trypsin Protease, MS Grade was obtained from Thermofisher Scientific (Waltham, MA), and Blue-BANDit protein stain was purchased from AMRESCO.

CA Nanoparticles Preparation

One hundred milliliters of DMEM was prepared using 1.35 g of DMEM powder and 0.37 g of sodium bicarbonate with the pH subsequently adjusted to 7.5 using 0.1 M hydrochloric acid. The prepared DMEM solution was filtered using 0.2 μm syringe filter in laminar flow hood, followed by transferring 200 μL of the filtered medium into 1.5-mL microcentrifuge tubes. Gemcitabine was then added to the microcentrifuge tubes, followed by the addition of 4 mM of $CaCl_2$ and incubation at 37°C for 30 min. For PEGylation of particles, 5 μM of streptavidin was added prior to incubation for additional 10 min, followed by addition of 5-μM biotinylated PEG (with the resultant ratio of streptavidin to biotin−PEG being 1:1) and incubation for 10 min.

Field Emission Scanning Electron Microscope Imaging of Modified and Unmodified CA Nanoparticles

Modified and unmodified gemcitabine-loaded CA nanoparticles were prepared with 1 mL of DMEM. Drug was then added at 1 μM into the microcentrifuge tubes (1 μM concentration), followed by addition of 5 mM of $CaCl_2$ and incubation at 37°C for 30 min. Afterward, surface modification of drug-loaded CA nanoparticles using streptavidin and biotin-PEG (both at 2 μM concentrations) was done with 15 min incubation at first with streptavidin, and with additional 15 min incubation with biotin-PEG. Samples were kept on ice before microscopic observation. One microliter of sample was placed onto mica substrate tape of sample holder and dried at room temperature for 15 min, followed by platinum sputtering of each samples for 60 s. Sputtered samples were observed at approximately 10-15 kV using field emission scanning electron microscope (Hitachi SU8010).

In Vivo *Distribution Study of Gemcitabine-Loaded CA Nanoparticles*

Female Balb/c mice (6- to 8-week old) of 15-20 g of body weights (obtained from School of Medicine and Health Science animal facility, Monash University) were maintained in 12:12 light:dark condition and provided with ab libitum and water. All the experiments conducted were carried out in accordance with the protocol approved by MONASH Animal Ethics Committee (MARP/2016/126). Approximately, 1×10^5 4T1 cells (in 100 μL phosphate buffered saline) were injected subcutaneously on the mammary pad. For biodistribution study, mice were administered with 30 mg/kg dosage of gemcitabine either in free or nanoparticle-bound form through tail vein injection using 30 G needle when the tumor volume reached 13.20 ± 2.51 mm^3. For synthesis of CA particles, exogenous calcium salt was added at 4 mM to 100 μL of DMEM before incubation at 37°C for 30 min. For surface modification of CA particles, generated particles were subjected to further incubation sequentially in the presence of 5 μM streptavidin and biotin PEG. One hour after intravenous injection through tail vein, mouse was anasthesized prior to blood collection by cardiac puncture, using a 1 mL syringe and a 25G needle. A secondary method of euthanasia was performed after blood collection to ensure that the animal is deceased. Blood samples were collected in EDTA-coated blood tubes. The tubes were placed on ice and immediately transported to the laboratory, where they were centrifuged at 4000 rpm for 15 min at 4°C. Afterward, the plasma was stored at −80° until it was assayed. Organs (liver, kidney, spleen, lung, brain, and tumor) were collected immediately and washed in chilled 1× phosphate buffered saline for 2 times and kept in −150°C freezer until further analysis. In addition, untreated mice were also be sacrificed when the tumor volume reached 13.20 ± 2.51 mm^3 for controls.

Preparation of Organ and Plasma Samples for QQQ LC/MS

A highly sensitive and rapid LC tandem mass spectrometry (LC-MS/MS) method was developed and validated for gemcitabine from mice plasma and organs using protein precipitation technique. Organs were homogenized using homogenizer in ice cold 50%