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PEGylated thermosensitive lipid-coated hollow gold nanoshells for effective combinational chemo-photothermal therapy of pancreatic cancer

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ABSTRACT

Pancreatic cancer has extremely poor prognosis with an 85% mortality rate that results from aggressive and asymptomatic growth, high metastatic potential, and rapid development of resistance to already ineffective chemotherapy. In this study, plasmonic hollow gold nanoshells (GNS) coated with PEGylated thermosensitive lipids were prepared as an efficient platform to ratiometrically co-deliver two drugs, bortezomib and gemcitabine (GNS-L/GB), for combinational chemotherapy and photothermal therapy of pancreatic cancer. Bortezomib was loaded within the lipid bilayers, while gemcitabine was loaded into the hydrophilic interior of the porous GNS via an ammonium sulfate-driven pH gradient method. Physicochemical characterizations and biological studies of GNS-L/GB were performed, with the latter using cytotoxicity assays, cellular uptake and apoptosis assays, live/dead assays, and western blot analysis of pancreatic cancer cell lines (MIA PaCa-2 and PANC-1). The nanoshells showed remotely controllable drug release when exposed to near-infrared laser for site-specific delivery. GNS-L/GB showed synergistic cytotoxicity and improved internalization by cancer cells. High-powered near-infrared continuous wave laser ($\lambda = 808$ nm) effectively killed cancer cells via the photothermal effect of GNS-L/GB, irrespective of cell type in a power density-, time-, and GNS dose-dependent manner. These results suggest that this method can provide a novel approach to achieve synergistic combinational chemotherapy and photothermal therapy, even with resistant pancreatic cancer.

1. Introduction

Pancreatic adenocarcinoma has a dismal prognosis, with a 5-year survival rate of less than 1% and a median survival of 4–6 months; it is responsible for 6% of all cancer-related deaths in developed countries [1,2]. There is no effective early detection or screening test for precancerous lesions. Pancreatic adenocarcinoma has highly aggressive growth, and the majority of patients are ineligible for surgical resection, because they present with early metastatic dissemination to distant organs due to asymptomatic initial progression [3]. Moreover, dense collagenous tumor stroma and high interstitial fluid pressure, which function as a barrier to drugs, as well as the emergence of chemo-resistant phenotypes following monotherapy, especially with the first-line drug, gemcitabine, has necessitated newer therapeutic approaches [4].

Gemcitabine is a deoxycytidine-analog antimetabolite that blocks DNA synthesis by inhibiting ribonucleotide reductase and by replacing cytidine during DNA replication and repair. It is pivotal to pancreatic cancer chemotherapy and is administered either alone or in combination with other agents. The effect of gemcitabine is impaired by extrinsic and/or intrinsic resistance [5]. Bortezomib, a dipeptide boronic acid derivative, has been shown to reverse resistance and sensitize pancreatic cancer cells to the gemcitabine therapy when administered in a schedule-dependent manner [3,6,7]. Bortezomib potently and reversibly inhibits the multicatalytic intracellular 26S proteasome (at the chymotrypsin-like enzyme site of the 19S particle) that is responsible for selective

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and non-lysosomal proteolysis of polyubiquitin-tagged proteins, resulting in the release of recyclable short peptides. This inhibition affects many key proteins of signaling cascades and cell cycle regulatory proteins, including those involved in cell proliferation, regulation of apoptosis, and gene transcription [6].

Gold nanostructures have attracted considerable attention as promising multifunctional platforms for cancer therapy (directly as plasmonic photothermal agents and indirectly as nanocarriers for drug and imaging molecules), diagnostics, imaging, and biosensors. They are capable of scattering and absorbing light in the visible and near-infrared (NIR) region because of collective resonant oscillations of conduction band electrons with respect to the metallic lattice when exposed to an electromagnetic field of resonant frequency, known as localized surface plasmon resonance (LSPR) [8]. Gold nanostructures can convert the incident photons having the same frequency as LSPR to heat that is sufficient to induce cell death. Gold nanostructures are photostable and possess a high photothermal energy conversion efficiency that is several fold higher than the traditionally used strong absorbing organic dyes, such as indocyanine green and rhodamine 6G. This makes gold nanostructures very useful in photothermal therapy (PTT) or photothermal ablation of cancer, which is highly selective and minimally invasive compared to radiotherapy and chemotherapy [9]. Among these nanostructures, hollow gold nanoshells (GNS) have added benefits, including ease of synthesis, use of non-toxic excipients, and modifiability to act as drug carriers; other gold-PTT agents require special surface modifications, including chemical conjugation of the drug via thiol ligands, mesoporous coatings for drug adsorption, and layer-by-layer assembly for loading water-soluble drugs [10–16]. Upon NIR irradiation, GNS can release drugs, thereby enabling precise spatial and temporal control of drug release at the targeted (irradiated) site. GNS also offer convenient LSPR tunability to the NIR region because chromophores in tissues are relatively transparent to NIR wavelengths (called the biological window, wavelength $\lambda > 700$ nm). This permits great tissue penetration and significantly less damage to irradiated, non-malignant surrounding tissues.

In this study, plasmonic GNS coated with PEGylated thermosensitive lipids were prepared for co-delivery of two drugs. The PEGylated thermosensitive lipid was composed of dipalmitoyl-sn-glycero-3-phosphocholine (DPPC) and 1,2-distearoyl-sn-glycero-3-phosphoethanolamine-*N*-[methoxy(polyethylene-glycol)-2000] (DSPE-PEG2000). DPPC, with a gel-to-fluid phase transition temperature (Tc) of 41 °C, imparted thermosensitivity to the GNS for localized, laser-triggered drug release. The hydrophilic PEG component of DSPE-PEG2000 ensured long blood circulation of the GNS by preventing opsonization by the reticuloendothelial system. Hydrophobic bortezomib was loaded within the lipid bilayers, while water-soluble gemcitabine was loaded into the hydrophilic interior of GNS via an ammonium sulfate-driven pH gradient method. Various physicochemical characterizations and biological studies were performed to investigate the synergistic chemotherapy and photothermal effects.

2. Materials and methods

2.1. Materials

Bortezomib free base was purchased from LC Laboratories (Woburn, MA, USA). DPPC was obtained from NOF America Corporation (White Plains, NY, USA). DSPE-PEG2000 was purchased from Avanti Polar Lipids (Alabaster, AL, USA). Tetrachloroauric acid ($HAuCl_4$), silver nitrate ($AgNO_3$), polyvinylpyrrolidone 55000 (PVP 55000), and ethylene glycol (EG) were purchased from Sigma-Aldrich (St. Louis, MO, USA). The pancreatic cancer cells, MIA PaCa-2 and PANC-1, were obtained from the Korean Cell Line Bank (Seoul, South Korea). All other chemicals were reagent grade and were used without further purification.

2.2. Synthesis of silver nanoparticles (AgNPs)

PVP-stabilized AgNP was synthesized by the polyol method [17]. Briefly, 500 mg of PVP in 15 mL EG was heated to 180 °C at the rate of 1 °C/min under vigorous stirring until the color turned golden yellow. The temperature was maintained for 30 min, and the reaction mixture was left to cool to 140 °C. $AgNO_3$ (100 mg) dissolved in 5 mL EG was added dropwise to the above solution over 5 min, and the reaction was continued for another 10 min. The viscous brown dispersion was cooled to 25 °C, 50 mL of acetone was added, and the mixture was left in the dark for 5 h. The AgNP precipitate was separated and washed by repeated centrifugation/resuspension in water. The final volume was brought to 50 mL with deionized water.

2.3. Synthesis of GNS

GNS were prepared by the galvanic replacement method. Briefly, $HAuCl_4$ was reduced onto AgNP templates [18]. AgNP solution (10 mL) was mixed with 90 mL water and heated to 60 °C followed by dropwise addition of 500–900 μL of 24 mM $HAuCl_4$. The reaction was continuously monitored using UV–vis spectroscopy until the Ag peak at ~400 nm disappeared or until the required LSPR was attained. Stoichiometrically, the Ag peak should vanish when the molar ratio of silver to gold is approximately 1:0.37. The reaction was continued for an additional 15 min. Next, the sample was cooled and the precipitated byproduct, silver chloride (AgCl), was removed. The formed GNS was washed with saturated sodium chloride and deionized water several times and stored at 4 °C. As $AuCl_4^-$ signals (~285 nm) disappeared in the spectra, it was assumed that all gold salt was reduced. The amount of GNS was measured in gold equivalents (Au_{equiv}), which is the weight of elemental gold present in the GNS.

2.4. Lipid coating and drug loading

One milligram of Au_{equiv} GNS was centrifuged (14,000 rpm, 20 min) and the pellet was resuspended in 2 mL 250 mM ammonium sulfate ($(NH_4)_2SO_4$) for 4 h, followed by intermittent (20/20 s on/off cycles) probe sonication for 5 min at 80% amplitude (Sonics vibracell, USA). Separately, 1 mg of lipids (DPPC:DSPE-PEG2000 in the molar ratio 9.5:0.5) and 0.2 mg of bortezomib were dissolved in chloroform:methanol (9:1 vol ratio). The solution was dried in a round-bottom flask using a rotary evaporator (Eyela SB-1200, China) overnight to remove the solvent and form thin lipid films. The thin films were rehydrated for 6 h with $(NH_4)_2SO_4$-loaded GNS solution prewarmed to 45 °C. Next, the solution was sonicated at 50 °C for 10 min. Subsequently, 10 μL of 0.8 mM 1-octadecanethiol (ODT) was added and the mixture was stirred for 10 min. Then, the mixture was passed through a 100-nm polycarbonate filter, followed by an 80 nm filter for at least 10 times, using a mini-extruder set maintained at 60 °C on a hotplate. The unentrapped $(NH_4)_2SO_4$ and untethered ODT was removed by centrifugation at 13,200 rpm and 4 °C for 30 min.

The lipid-coated GNS (GNS-L) pellet was resuspended in 500 μL of 2–20 mg/mL aqueous gemcitabine solution overnight for pH gradient-driven loading. During this incubation, the neutral free base, gemcitabine, diffused to the interior of the GNS-L where it was protonated due to the low pH and formed gel-like precipitates with $(NH_4)_2SO_4$. This will prevent transmembrane escape of gemcitabine and facilitate better gemcitabine uptake during cell treatment. The mixture was subjected to 3 cycles of alternate warming (at 60 °C in thermostat-controlled water bath for 30 min)