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Redox- and pH-Responsive Nanoparticles Release Piperlongumine in a Stimuli-Sensitive Manner to Inhibit Pulmonary Metastasis of Colorectal Carcinoma Cells

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ABSTRACT

Redox-responsive nanoparticles having a diselenide linkage were synthesized to target pulmonary metastasis of cancer cells. Methoxy poly(ethylene glycol)-grafted chitosan (ChitoPEG) was crosslinked using selenocystine-acetyl histidine (Ac-histidine) conjugates (ChitoPEGse) for stimuli-responsive delivery of piperlongumine (PL). ChitoPEGse nanoparticles swelled in an acidic environment and became partially disintegrated in the presence of H_2O_2, resulting in an increase of particle size and in a size distribution having multimodal pattern. PL release increased under acidic conditions and in the presence of H_2O_2. Uptake of ChitoPEGse nanoparticles by CT26 cells significantly increased in acidic and redox state. PL-incorporated ChitoPEGse nanoparticles (PL NPs) showed similar anticancer activity *in vitro* against A549 and CT26 cells compared to PL itself. PL NP showed superior anticancer and antimetastatic activity in an *in vivo* CT26 cell pulmonary metastasis mouse model. Furthermore, an immunofluorescence imaging study demonstrated that PL NP conjugates were specifically delivered to the tumor mass in the lung. Conclusively, ChitoPEGse nanoparticles were able to be delivered to cancer cells with an acidic- or redox state-sensitive manner and then efficiently targeted pulmonary metastasis of cancer cells since ChitoPEGse nanoparticles have dual pH- and redox-responsiveness.

Introduction

Drug delivery systems based on stimuli-responsive behavior have been extensively investigated for several decades to concentrate cytotoxic agents into specific body sites to maximize drug efficacy at the target site and to minimize unwanted side effects.[1-5] Stimuli-responsiveness, such as temperature, pH, ion-sensitive, magnetic fields, ultrasound, and specific wavelengths of light have been applied to achieve these purposes.[1-7] Nanoparticle-based stimuli-responsive drug delivery systems have intrinsic superiority over conventional drug delivery.[8-11] For example, nanoparticles have large surface area for decoration with a targeting moiety and small diameter for parenteral administration and solubilization of lipophilic agents. Yoshida et al.[12] reviewed that pH- and ion-sensitive polymers for drug delivery can be used for site-specific delivery, that is, pH and ionic concentration gradients between various organs, blood stream, interstitial fluid, and intracellular components could exist, and these factors can be used to control behavior of polymeric drug delivery system. Sodium and potassium ion gradients between interstitial fluid and intracellular fluid are significantly changed, and then drug release from ion-exchange resins can be controlled by an equilibrium exchange reaction.[12] Reactive oxygen species (ROS)-sensitive drug delivery systems have been spotlighted for cancer targeting in recent decades because cancer cells in an advanced stage exhibit increased ROS

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production and have low pH values in the tumor tissue.[11-15] Especially, diselenide linkages are disintegrated by ROS; their intrinsic properties can then be applied to the intracellular targeting of cancer cells using polymer-diselenide conjugates.[15-17] Ma et al.[16] reported that diselenide bonds in the polymer backbone can be degraded by oxidants and reductants at very low concentration. They further reported that diselenide-linked block copolymer micelles have sensitivity against radiation, allowing for those micelles to be used for radiotherapy since ROS generation is substantial during radiation procedures.[17] Furthermore, increased ROS production of cancer cells induces an acidic environment in the tumor tissues; this property is also an opportunity for pH-sensitive drug delivery.[18] Zhang et al.[18] reported that the release rate of the anticancer drug increased under acidic conditions and in the presence of oxidants.

Piperlongumine (PL), a natural alkaloid extract from piper plants, has medicinal activity against some diseases, including tumors, malaria, gonorrhea, bronchitis, asthma, and cough.[19] Especially, PL is known to have selective anticancer activity in various cancer cells, attributable to induction of apoptosis by increasing ROS generation and DNA damage.[20-22] Furthermore, PL has antimetastatic activity against pancreatic cancer cells through suppression of STAT3, that is, PL-treated cells failed to metastasize in the lungs or liver.[23] Interestingly, PL has selective anticancer activity against various cancer cells, with minimal toxicity in normal cells, that is, PL showed significant antitumor activity in a tumor xenograft model with no apparent toxic effects in normal mice.[24] Despite this superiority, there are some drawbacks to the use of PL, such as poor aqueous solubility and instability under normal physiological conditions, which limit the clinical application of PL. Aodah et al.[25] reported that PL is labile in physiological solutions having a pH higher than 7.0 and shows marked photodegradation when exposed to an ultraviolet light source. Experimental value of n-octanol/water partition coefficient ($\log p$) value is known as 2.37 ± 0.12 at room temperature and is characterized as a weak base (p*K*a, 4.5).

In this study, core cross-linked ChitoPEG copolymers were synthesized using selenocystine (ChitoPEGse) as a ROS-sensitive moiety. PL was then incorporated into the pH/ROS-sensitive core of the nanoparticles. Especially, cancer cells are known to produce more ROS during the advanced stages than during the initial stages of cancer progression, which specifically and frequently induces metastasis of cancer cells.[11,26] This distinctive behavior of cancer cells also can be applicable in the targeted drug delivery against metastatic cancer cells, that is, ROS-sensitive nanoparticles such as PL-incorporated ChitoPEGse nanoparticles (PL NPs) would be effectively target metastatic cancer cells. The anticancer activity of PL NPs was also studied in an *in vitro* cell culture model using A549 lung carcinoma cells and CT26 colorectal carcinoma cells, and *in vivo* using a pulmonary metastasis CT26 cell model.

Materials and Methods

Materials

Water-soluble chitosan (deacetylation degree $\geq$ 97%, molecular weight (M.W.) = 7000 g/mol) was purchased from Kittolife Co. (Pyeongtaek, Gyeonggi-do, Korea). Methoxy poly(ethylene glycol) (MePEG)-succinimidyl glutarate (M.W. = 5000 g/mol) was purchased from Sunbio Co. (Seoul, Korea). N-acetyl-L-histidine (Ac HIS), N-hydroxysuccinimide (NHS), N-(3-dimethylaminopropyl)-N′-ethylcarbodiimide hydrochloride (EDAC), chloroform, dimethyl sulfoxide (DMSO), 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), and selenocystine were purchased from Sigma-Aldrich Chemical Co. PL was purchased from LKT Labs. Co. (St. Paul, MN). The dialysis membrane or dialysis device (M.W. cutoff [MWCO] size: 500, 8000, and 12,000 g/mol) was purchased from Spectrum/Por Lab., Inc. (Rancho Dominguez, CA). All organic solvents and other chemicals were used as extra-pure grade.

Synthesis of ChitoPEGse Nanocomposite

Selenocystine-Ac HIS Conjugates

Ac HIS (78.8 mg) was mixed with 1.0 equivalent of NHS and EDAC in 10-mL DMSO following magnetic stirring for 6 h (NHS-activated Ac HIS). A 0.5 equivalent of selenocystine (66.8 mg) was separately dissolved in HCl solution (1 N, 15 mL) and mixed with 25-mL DMSO. This solution was mixed with the NHS-activated Ac HIS solution. The mixed solution was further stirred for 2 days and dialyzed against an aqueous solution using dialysis equipment (500 g/mol) and against HCl solution (0.01 N) for 6 h with exchange of water at 2 h intervals followed by dialysis against deionized water for 24 h. The resulting solution was then lyophilized for 3 days and precipitated into an excess amount of a chloroform/methanol solution (volume ratio: 7/3) to remove unreacted Ac HIS. Precipitates were then dried *in vacuo* for 24 h. Solids were stored at 4°C until used for the next synthesis step. The final yield was approximately 94.6% as a solid (yield [w/w, %] = [weight of final products/{weight of Ac-histidine + weight of selenocystine}] × 100).

ChitoPEG Copolymer

The ChitoPEG copolymer was synthesized as previously described.[27] Briefly, 360 mg water-soluble chitosan was dissolved in 5 mL deionized water, and 1 g MePEG-succinimidyl glutarate in 10-mL DMSO were then added (10/1, glucosamine/MePEG). This solution was stirred for 2 days and then dialyzed against a plentitude of water using a dialysis membrane (MWCO: 8,000 g/mol) for 2 days to remove unreacted byproducts. The solution was then lyophilized for more than 3 days, and the solid powder was precipitated into chloroform to remove unreacted MePEG. Precipitates were dried under vacuum for 2 days and then stored at 4°C until use or analysis. At ^{1}H nuclear magnetic resonance (NMR) spectra measurements, substitution degree of PEG was calculated as 9.6 (substitution degree of MePEG = ([proton integration ratio of methyl group of MePEG/3]/[proton integration ratio of H1 of chitosan + {proton integration of acetyl group of chitosan/3}]) × 100).

ChitoPEGse Nanocomposites

Ac HIS-selenocystine conjugates (70 mg) were dissolved in 10 mL DMSO/water (9/1, v/v) and then mixed with 2 equivalents EDAC and NHS following magnetic stirring for 6 h. ChitoPEG copolymer (400 mg) dissolved in 15 mL of DMSO/water (4/1, v/v) was added to this solution and then magnetically stirred for 2 days. The solution was then dialyzed using dialysis membrane (MWCO: 8000 g/mol) against water to remove unreacted chemicals for 2 days following lyophilization for 3 days. Lyophilized solids were precipitated in methanol and then filtered with filter paper (Whatman No. 6). This procedure repeated 3 times, and then, the samples dried *in vacuo* for 3 days. The yield of final product was greater than 97% (w/w). (yield (w/w, %) = [weight of final products/(weight of Ac-histidine-selenocystine conjugates + weight of ChitoPEG copolymer)] × 100). The conjugation yield of selenocystine-Ac HIS conjugates was approximately 79.9% (w/w). (conjugation yield of selenocystine-Ac HIS conjugates = [(weight of final product-feeding weight of ChitoPEG copolymer)/feeding weight of selenocystine-Ac HIS conjugates] × 100). The content of selenocystine-Ac HIS conjugates in the final product (ChitoPEGse copolymer) was calculated via mass measurements with the following equation: content of selenocystine-Ac HIS conjugates =