論 文 の 内 容 の 要 旨

論文題目 Development of Targeted Unimer Polyion Complex-Assembled Gold Nanoparticles for Systemic Delivery of siRNA

（ユニマーポリイオンコンプレックス搭載金ナノ粒子の開発とがん標的siRNAデリバリーへの展開）

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Cancer is the rapid creation of abnormal cells in human body. Nowadays, it has become one of the leading causes of death all over the world, leading to 8.2 million deaths in 2012. As both cancer deaths and newly diagnosed cancer patients are increasing every year, it is urgent to find effective ways for cancer treatment. In recent decades, innovation in nanotechnology has made PEGylated therapeutic nanocarriers as one of the most promising candidates for cancer therapy. These nanomedicines with diameters between 10-100 nm are composed of anticancer drugs in the core and a biocompatible polyethylene glycol (PEG) shell, which can circulate long in bloodstream and accumulate in solid tumors via leaky vasculatures as well as impaired lymphatic drainage, namely enhanced permeability and retention (EPR) effect. Besides, recent studies have realized the importance of the size of nanomedicines for cancer therapy, demonstrating the deeply penetrating capability of sub-50 nm nanomedicines even in stroma-rich pancreatic tumor models. Till now, more than 10 nanocarrier-based anticancer drugs have been available on the market, and even more are under pre-clinical and clinical investigations. However, traditional anticancer nanomedicines still encounter the biological hurdles including systemic side effects due to the cytotoxicity of potent anticancer drugs and their non-specific accumulation in normal organs, and insufficient anticancer efficacy due to the lack of specific cancer targeting as well as cancer stem cell (CSC)-induced drug resistance and metastasis of cancers. Thus, innovation in nanomedicines to overcome these biological hurdles is highly desired.

As a milestone in biotechnology, RNA interference (RNAi) has emerged as a powerful strategy for treatment of various diseases including cancers. It is a sequence-specific mRNA silencing mechanism triggered by small interfering RNA (siRNA) in the cytoplasm. Through silencing specific genes associated with tumor development and progression, siRNA can be applied to cancer treatment. Compared with conventional anticancer drugs, siRNA is a highly safe therapeutic reagent for cancer with reduced side effects due to its low cytotoxicity and specificity for target mRNA. Besides, the huge variety of cancer-promoting proteins provide a wide range of targets for rational design of siRNA for cancer therapy, making it possible to overcome the multidrug resistance of cancers. However, the poor bioavailability of siRNA, due to the degradation by RNases and rapid renal clearance after systemic administration, has hampered its application in clinical use. Thus, siRNA delivery systems have been widely developed for RNAi-based cancer therapy, including liposomes, polymeric micelles and inorganic nanoparticles. An efficient siRNA

delivery system needs to protect siRNAs from clearance in bloodstream and selectively deliver them into the target cancer cells, which requires the siRNA nanocarrier to possess the abilities including 1) size control in sub-50 nm range, 2) long circulation in bloodstream, and 3) specific cancer-targeting. Herein, to satisfy these requirements for efficient systemic siRNA delivery for cancer treatment, we attempt a challenge to construct well-defined sub-50 nm-sized actively-targeted siRNA nanocarriers with long blood circulation for cervical cancer- and CSC-targeted tumor treatment.

First, well-defined 40 nm-sized unimer polyion complex (uPIC)-assembled gold nanoparticles (uPIC-AuNPs) were constructed from a two-step bottom-up strategy, including the formation of monodispersed uPIC and the conjugation of uPICs to a 20 nm AuNP template. The monodispersed uPICs were generated from single molecular pairs of siRNA bearing 40 negative charges and PEG-*block*-poly(L-lysine) with disulfide containing lipoic acid (LA) at the ω-end (PEG-PLL-LA) possessing ~40 positive charges through charge-matched polyion complex (PIC) formation. This PEG-PLL-LA containing ~40 units in PLL segment with narrow molecular weight (MW) distribution (M_w/M_n = 1.06) was synthesized using ring-opening polymerization of ε-trifluoroacetyl-L-lysine *N*-carboxy anhydride (NCA-Lys(TFA)) initiated by PEG-NH_2. The uPIC formation was evaluated by fluorescence correlation spectroscopy in terms of the change in diffusion coefficient of Alexa647-siRNA. The diffusion coefficient of Alexa647-siRNA progressively decreased with an increase in [polymer]/[siRNA] from 0 to 1 and leveled off at [polymer]/[siRNA] = 1, indicating that siRNA was bound to PEG-PLL-LA at [polymer]/[siRNA] < 1 and the binding was saturated at [polymer]/[siRNA] = 1. Thus, the MW of siRNA/PEG-PLL-LA complexes prepared at [polymer]/[siRNA] = 1 was further analyzed by analysis ultracentrifuge. The obtained MW (21.7 kDa) is apparently comparable to the sum of MWs of siRNA (13.3 kDa) and PEG-PLL-LA (7.6 kDa), demonstrating the selective formation of uPIC. Next, the uPICs were conjugated onto a 20 nm AuNP through Au-S bond. The capping amount of uPIC per AuNP was estimated by the fluorescence-based quantification using Alexa647-siRNA to be 90 at the feeding molar ratio of [uPIC]/[AuNP] = 500:1. To increase the surface PEG density on AuNP, short PEG-SHs with MW 800 were further grafted onto the uPIC-AuNP, resulting in a PEG loading number to be approximately 2300 per AuNP determined by Ellman's assay. The successful construction of uPIC-AuNP was verified by UV-Vis spectroscopy and dynamic light scattering (DLS). A red shift from 520 nm to 528 nm was observed in UV-Vis absorbance spectrum of AuNPs, indicating the change of surface plasmon resonance in AuNPs due to the surface modification of uPICs. The hydrodynamic diameter of uPIC-AuNP increased from 20 nm for bare AuNP to 40 nm with a narrow size distribution (polydispersity index (PDI) in DLS = 0.07). Also, the zeta-potential was significantly changed from –30.4 mV for bare AuNP to –11.3 mV for uPIC-AuNP. Due to the precise control in size and protection of PEG, this uPIC-AuNP exhibited prolonged blood circulation, as well as higher tumor accumulation in a subcutaneous luciferase-expressing cervical cancer (HeLa-luc) model compared with naked siRNA and uPIC, inducing significant luciferase gene silencing in subcutaneous HeLa-luc tumors through systemic administration. These results indicate the platform utility of uPIC-

AuNPs for further development of actively-targeted siRNA delivery for cancer treatment.

Absence of specific tumor targeting is a major obstacle for conventional nanomedicines to cancer treatment, leading to insufficient anticancer efficacy and side effects. To fully realize the advantages of uPIC-AuNP for efficient cancer treatment, an actively-targeted uPIC-AuNP was constructed by installing cyclic Arg-Gly-Asp (cRGD) peptide on the surface of nanocarrier. Accordingly, cRGD ligand, which is able to specifically recognize $\alpha_v\beta_3/\alpha_v\beta_5$ integrin on various cancer cell and tumoral endothelial cell surface, was conjugated in the *α*-terminus of PEG-PLL-LA (cRGD-PEG-PLL-LA) to form monodispersed cRGD-installed uPICs as building blocks. The obtained uPICs were further conjugated on a 20 nm AuNP followed by covering with short PEG, resulting in a uniform 42 nm-sized cRGD-installed uPIC-AuNPs (cRGD-uPIC-AuNPs) in a narrow size distribution (PDI in DLS = 0.10). The targetability of cRGD-uPIC-AuNP was evaluated in $\alpha_v\beta_5$ overexpressing HeLa-luc model by *in vitro* cellular uptake using flow cytometry and gene silencing assay using quantitative real-time PCR (qRT-PCR), as well as *in vivo* tumor accumulation by IVIS, inductively coupled plasma mass spectrometry (ICP-MS) and *in vivo* confocal laser scanning microscopy (IVCLSM). The significant enhancement of Alexa647-siRNA uptake for cRGD-uPIC-AuNP, as well as the reduce in enhancement once coincubation with an excess amount of free cRGD demonstrated more efficient cellular uptake of siRNA payloads delivered by the cRGD-uPIC-AuNP attributed to the specific recognition of cRGD ligand to $\alpha_v\beta_5$ integrin. Due to the facilitated cellular uptake, E6 siRNA (siE6)-loaded cRGD(+)-uPIC-AuNP achieved 49% downregulation of E6 oncogene in HPV-18 positive HeLa-luc cells from 28% downregulation for RGD(–)-uPIC-AuNP at siRNA concentration of 200 nM. Further, though intravenous injection, cRGD(+)-uPIC-AuNP exhibited significantly higher tumor accumulation of both Alexa647-siRNA and AuNP in subcutaneous HeLa-luc tumor-bearing mice compared with cRGD(–)-uPIC-AuNP. This was further supported by IVCLSM which showed that more Alexa488-siRNA-loaded cRGD(+)-uPIC-AuNPs were found in tumor tissues than Alexa647-siRNA-loaded cRGD(–)-uPIC-AuNPs at 4 h-post coinjection of these two nanoparticles. To evaluate the potential for cervical cancer treatment using siE6 to inhibit E6 oncoproteins for recovering the p53 tumor suppressor, therapeutic siE6-loaded cRGD-uPIC-AuNP was used for E6 oncogene silencing in subcutaneous HeLa-luc tumor model. It was found that cRGD-uPIC-AuNP exhibited significantly higher gene silencing ability than non-targeted uPIC-AuNP. Ultimately, antitumor efficacy of cRGD-uPIC-AuNP was evaluated by systemic delivery of siE6 to the subcutaneous HeLa-luc tumor, where each sample was injected 6 times into the tail vein of tumor-bearing mice (20 μg siRNA/injection). At day 12, the size of tumors treated with siE6-loaded cRGD(+)-uPIC-AuNP was significantly smaller than those treated with siE6-loaded cRGD(–)-uPIC-AuNP, as well as siCont-loaded cRGD(+)-uPIC-AuNP, indicating that cRGD ligands significantly enhanced the siE6-mediated antitumor activity of uPIC-AuNPs. In addition, the systemic administration of cRGD(+)/(–)-uPIC-AuNPs did not cause apparent body weight loss in the treated mice, suggesting the negligible systemic toxicity of cRGD(+)/(–)-uPIC-AuNPs. These results highlighted the potential utility of actively-targeted and size-regulated nanocarriers for cervical cancer treatment derived from the oncogene silencing.

CSCs are defined as a kind of cells that contain self-renewal and multilineage differentiation abilities. They are demonstrated to play important role in drug resistance and metastasis of cancers, where the latter causes more than 90% of cancer-related deaths. Given that the presence of CSCs is a primary obstacle to treat sold tumors, targeting CSCs is a promising strategy to improve the efficacy of cancer treatment. CSCs in solid tumor are believed to residue in hypoxia CSC niches, where the CSCs are reported to have a high glucose (Gluc) metabolism with enhanced Gluc transporter 1 (GLUT1) expression, suggesting GLUT1 is a potential target for CSCs treatment using Gluc as a targeting ligand. In this regard, Gluc-installed uPIC-AuNP (Gluc-uPIC-AuNP) was prepared from Gluc-conjugated PEG-PLL-LA (Gluc-PEG-PLL-LA) for targeting CSCs. Artificially modified human oral squamous carcinoma (HSC-2) cells expressing CD44 variant isoforms (HSC-2/CD44v) culture in hypoxia (2.5% O_2) was chosen as a CSC candidate, in which CD44v is reported to maintain the properties of CSCs and serve as a marker for identifying CSCs. HSC-2 cells cultured in normoxia, originally expressing CD44v, were used as a control cell. The targetability of this well-defined Gluc-uPIC-AuNP (45 nm in size, PDI = 0.12) against HSC-2/CD44v cells under hypoxia was confirmed by *in vitro* cellular uptake using flow cytometer, showing that Gluc(+)-uPIC-AuNP induced significantly enhanced cellular uptake compared with Gluc(–)-uPIC-AuNP whereas the enhancement was much slighter in cultured HSC-2 cells under normoxia. This may be due to the overexpression of GLUT1 on the surface of HSC-2/CD44v cells under hypoxia. The specific recognition between Gluc and GLUT1 was further validated by the inhibition assay, where the coincubation with an excess amount of GLUT1 inhibitor significantly reduced the cellular uptake of Gluc(+)-uPIC-AuNP to the similar level of Gluc(–)-uPIC-AuNP, indicating that the enhanced cellular uptake of Gluc(+)-uPIC-AuNP was derived from the specific binding between Gluc and GLUT1. To demonstrate the potential of therapeutic effect for CSCs treatment, the delivery efficacy of Gluc-uPIC-AuNP to downregulate polo-like kinase 1 was evaluated by qRT-PCR. Compared with Gluc(–)-uPIC-AuNP, the Gluc(+)-uPIC-AuNP exhibited significantly enhanced gene silencing effect to HSC-2/CD44v cells under hypoxia while this enhancement was not observed in HSC-2 cells under normoxia, suggesting promising potential of Gluc-uPIC-AuNP for targeted CSCs treatment to solve the problems of the drug resistance and metastases of the cancers.

In conclusion, we developed a novel size-controlled 40 nm uPIC-AuNP for systemic delivery of siRNA to solid tumor from a two-step bottom-up approach. This nanocarrier with narrow size distribution displayed long blood circulation and enhanced tumor accumulation. To fully take the advantages of uPIC-AuNP for targeted cancer treatment, cRGD and Gluc were separately installed on the surface of uPIC-AuNP for tumor- and CSC-targeting. Benefited from the targetability of ligands, cRGD-uPIC-AuNP achieved more efficient antitumor efficacy for cervical cancer treatment by inhibiting the expression of E6 oncoproteins, meanwhile the Gluc-uPIC-AuNP displayed enhanced gene silencing ability in CSCs. These results highlighted the strategy of well-defined nanoconstruction using uPIC and AuNP as building blocks, as well as the versatility of ligand-installed uPIC-AuNPs for actively-targeted siRNA delivery, demonstrating the promising applications of actively-targeted uPIC-AuNPs for efficient cancer treatment.