Development of Two-Component Nanorod Complex for Dual-Fluorescence Imaging and siRNA Delivery

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Introduction

The field of nanomedicine holds great promise for the imaging and therapy of several diseases because of the unique physical and chemical properties of nanomaterials [32]. In recent decades, many nanomaterials have been developed and investigated extensively for applications in cancer treatment, including the use of viral vectors [30] and organic and inorganic materials [2, 13, 25]. Their high surface-area-to-volume ratios allow for the delivery of high concentrations of therapeutic agents, including anticancer agents [21, 23, 34, 35] (e.g., doxorubicin, cisplatin), antibodies [35] (e.g., trastuzumab), and genetic materials [12, 17] (e.g., small interfering RNA (siRNA)). However, many of these agents have severe side effects. Viral vectors have been shown to result in immune responses and mutagenesis [30]. Organic nanomaterials (e.g., liposomes) are often highly toxic to healthy cells [10]. In contrast, inorganic nanomaterials such as gold (Au), silicon oxide, iron oxide, and carbon nanotubes generally have beneficial properties suitable for cancer treatment, including multi-functionality, excellent biocompatibility, controllable release of therapeutic agents, and targeted cellular delivery; these features are not possible with viral- and lipid-based organic nanomaterials [2, 13, 31]. Moreover, inherent properties of inorganic nanoparticles, such as optical, magnetic, and other physical characteristics, show their great potential for use in the field of cancer diagnosis and therapeutics [6, 18, 22, 33].

Cancer theranostic agents require versatile functions, including active targeting, enabling medical imaging, and promoting cell death to treat the disease [3, 24]. Therapeutic nanomaterials have to be designed with at least two of these functions, active targeting and apoptogenic activity, which are challenging to implement in viral vectors or other anticancer agents. In general, active targeting minimizes adverse effects on healthy cells [3]. Unlike other agents, inorganic nanomaterials are relatively amenable to surface modification, encapsulation, and other methods that add functionality because these nanomaterials are simple to synthesize [14, 16, 29]. In addition, the physical properties of inorganic materials, such as the optical
properties of Au [14], the magnetic resonance of iron oxide [27], and the fluorescence of quantum dots [11], make implementing functionality feasible.

Even though inorganic nanomaterials have several advantages, producing multiple functions in nanomaterials is still quite difficult. Surface modification is a frequently employed technique because it is very efficient and simple to perform with various chemical linkers (e.g., EDC/NHS) [19] and biological moieties (e.g., avidin-biotin) [4]. However, it is challenging to functionalize nanomaterial surfaces with two or more features because these surfaces are typically uniform and compatible to only one type of inorganic component. This limitation is a critical barrier to synthesizing multipurpose nanomaterials. To solve this problem, Salem et al. [26] reported a potential gene delivery carrier using a two-component nanorod (consisting of Au and Ni), which was modified by green fluorescent protein (GFP) vectors and transferrin as a targeting moiety. However, this nanorod has never been implemented as a potential cancer therapeutic. In this study, we developed a suitable Au and Ni nanocomplex that produces at least two functions: active targeting and anticancer effects.

Among the various methods used for developing anticancer agents, siRNA has generated considerable interest as a component that can be designed into inorganic nanotherapeutic complexes [8]. The siRNA agent is a small 19–28-base pair, double-stranded RNA that is able to inhibit protein expression through selective suppression of mRNA by RNA interference (RNAi). Many studies have shown the therapeutic potential of siRNAs to treat several genetic diseases and in various cancer models. As a cancer treatment agent, siRNA has the ability to completely repair abnormal genes in cancer cells while having minimal toxicity compared with conventional chemotherapy agents [5]. However, siRNA alone is not effective because it lacks the ability to target and penetrate cancer cells. To address this issue, siRNA nanomaterials have been developed to be more selective. Vascular endothelial growth factor (VEGF) is critical for the survival of rapidly proliferating cancer cells and the retained growth of tumor tissue [20]. Therefore, targeting VEGF with siRNA (siVEGF) to inhibit angiogenesis has been suggested as a potential therapeutic strategy. Accordingly, we developed a two-component nanorod for effective anti-angiogenic gene therapy through the targeted delivery of siVEGF. In addition, we conjugated two kinds of fluorophores, one on each type of surface, for dual fluorescence imaging so that we could monitor the extent of the targeted delivery.

The two-component nanorod with Au and Ni blocks was fabricated by electrodeposition onto an aluminum oxide template. On the Au end, siVEGF and a red fluorophore (TAMRA) were conjugated to the nanorod surface. Luteinizing hormone releasing hormone (LHRH), used as a cancer-targeting moiety, and a green fluorophore (fluorescein isothiocyanate (FITC)) were conjugated to the Ni end. The advantage to this method of construction is that the conjugation steps employ facile techniques to produce multifunctional nanorods for cancer imaging and treatment.

Materials and Methods

Materials

The deposition solutions of Au, Ni, and Ag were purchased from Technic Inc. (Technic RTU Solution; TG-25 RTU, Silver 1025 and Nickel S, USA). Anodic alumina oxide (AAO) circles were purchased from Whatman Inc. (Anodisc circle, 13 mm, 0.02 μm; USA). The 3-(2-pyridyldithio)propionate (for use as a disulfide crosslinker) and all other chemicals, such as sodium hydroxide and phosphate-buffered saline (PBS, pH 7.4), were purchased from Sigma-Aldrich (St. Louis, MO, USA). Several kits for reverse-transcriptase polymerase chain reaction (RT-PCR) were purchased from Roche Inc. (Transcriptor First Strand cDNA synthesis and LC FS DNA Master PLUS SYBR Green kits, Switzerland). The heterobifunctional polyethylene glycol (PEG) linker (NH₂-PEG-SH) was purchased from Nanocs Inc. (Thiol PEG Amine, MW 3,400; USA). LHRH peptide for targeted delivery was synthesized from Peptron Inc. (Daejeon, Korea), and siVEGF was synthesized from Bioneer Inc. (Daejeon, Korea). The human VEGF enzyme-linked immunosorbent assay (ELISA) kit was purchased from Komabiotech (Seoul, Korea). Deionized (DI) water, obtained from a Millipore water system, was used throughout the experiments. All other chemicals were analytical-grade reagents.

Fabrication of Two-Component Au-Ni Nanorod

In order to fabricate the two-component nanorod, AAO was used as a template for the production of nanorods of uniform diameter size and desired lengths. Using the thermo-deposition method, Ag was deposited to a thickness of 200 nm onto one side of the AAO plate. In addition, pre-deposition was performed using an Ag solution to produce accurate sizes of nanorods through electro-deposition (~0.95 mV, 1 C). After several washing steps with DI distilled water, Au plating solution was added to the AAO plate and deposited through the application of a constant negative voltage (~0.95 mV, 0.15 C). Synthesis of the Ni component was also carried out using a similar method after the washing steps. The desired configuration and length of the two-component nanorod were controlled by the type of plating solution selected and the depositing coulomb value applied. To obtain template-free nanorods, the pre-deposited Ag and the AAO were dissolved using 50% nitric acid solution and 3 M sodium hydroxide, respectively.
Preparation of Dual-Functionalized Nanorods

Two-component Au-Ni nanorods were functionalized with a targeting ligand and siVEGF. First, the synthesized nanorod was washed in PBST (PBS, 0.1% Tween 20) three more times using centrifugation (centrifuge 5415R; Eppendorf, Germany). Au-Ni nanorods were incubated with LHRH-FITC-His (Pyr-HWSYkLRPK-FITC-(His)₉, 1 mg/ml) for 8 h at 4°C. LHRH peptides on the Ni side interact with the imidazole on histidine. LHRH plays a role in cancer cell targeting. After a binding reaction, the Au side of the nanorod was functionalized with thiolated siVEGF (SH-GAAGTTCATGGATGTCTAT-TAMRA) through thiol-Au-specific interactions after overnight incubation at 4°C.

Cell Culture

MCF-7 cells that are specific to breast cancer and reliably overexpress LHRH receptor, and SK-OV-3 cells, which are ovarian carcinoma cells (a negative control cell line that expresses little to no LHRH receptor), were obtained from the Korean Cell Line Bank. The cells were cultivated in RPMI-1640 (Sigma-Aldrich, USA) supplemented with 10% fetal bovine serum (FBS; Biowest, USA), penicillin (100 units/ml), and streptomycin (100 µg/ml) at 37°C in a humidified 5% CO₂ atmosphere.

Targeted Delivery and Dual-Fluorescence Imaging for Cancer Cells

FITC-labeled LHRH and TAMRA-labeled siVEGF nanorods (~2 × 10⁸) were added to MCF-7 and SK-OV-3 cell cultures in 30 mm dishes for 3 h at 37°C in serum-free medium. Cells were washed three times with PBS (pH 7.4) and fixed with 4% paraformaldehyde. The fixed cells were imaged by confocal microscopy for dual-fluorescence imaging (488 nm and 557 nm excitation; EZ-C1; Nikon, Japan).

Flow Cytometry

MCF-7 and SK-OV-3 cells were seeded onto 6-well plates at a density of 2 × 10⁵ cells per well in 2 ml of RPMI-1640 medium (10% FBS, 1% penicillin and streptomycin) and cultured for 24 h. After the cells were treated with LHRH-siVEGF nanorods or unmodified nanorods for 3 h at 37°C in serum-free medium, they were carefully washed with PBS three times and then trypsinized and resuspended in PBS. The fluorescence intensity of the cells was measured by fluorescence-activated cell sorter (FACS) analysis (BD FACSCalibur). Untreated MCF-7 and SK-OV-3 cells were used as negative controls.

RT-PCR Analysis

The siVEGF-modified nanorods were applied at a 2 × 10⁶ cell density to 6-well plates for 3 h at 37°C in serum-free medium. MCF-7 cells were carefully washed with PBS three times, and then they were trypsinized and resuspended in PBS. To determine the amount of VEGF mRNA in MCF-7 cells, total RNA was isolated from the cells using Nucleospin RNA (Macherey-Nagel, USA) according to the manufacturer’s manual. RT-PCR was performed using the Transcriptor First Strand cDNA synthesis kit and LC FC DNA Master PLUS SYBR Green kit (Roche, UK) according to the manufacturer’s manual. The PCR primers to detect human VEGF were forward, 5'-AGGAGGGCAAGACATACACG-3'; and reverse, 5'-GATCCGATATCCTGATGTG-3'. The PCR primers to detect human beta-actin were as follows: forward, 5'-CGTCTTCCCCCTC CATCG-3'; and reverse, 5'-CTCGTAAATGTACCGCAC-3'; these were synthesized by Bioneer, Inc. (Daejeon, Korea).

ELISA

The amount of VEGF protein was measured by ELISA (Human VEGF ELISA kit; Komabio, Korea) following the manufacturer’s recommendations. In order to obtain VEGF protein in MCF-7 cells, which were treated with nanorod complexes, cell lysis buffer (Cell Signaling, USA) was spread on 2 × 10⁵ cells in 6-well plates. After incubation on ice for 5 min, the plates were scraped and cells were collected into 1.5 ml tubes and centrifuged to separate cell debris. The absorbance of final enzyme product was measured at 450 nm by spectrophotometry (EL 800; Bio-TEK, USA).

Cancer Cell Viability Test

A 3(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay was performed for two purposes. First, it was used to determine the cytotoxicity of the two-component nanorods; and second, it was used to measure the apoptogenic activity of the siVEGF-attached nanorods. The MTT assay was performed using MCF-7 cells in 96-well plates (5 × 10⁴ cells/well). Cells were incubated in fresh medium at 37°C and a 5% CO₂ atmosphere for 24 h. To determine the cytotoxicity of nanorods without siVEGF attached, LHRH-functionalized nanorods (~2 × 10⁸) were added to MCF-7 cells for 3 h in serum-free media in a cultured atmosphere. MCF-7 cells were washed with PBS, to which 100 µl of MTT solvent was immediately added for 4 h. To measure the apoptogenic activity of nanorod complexes, siVEGF-functionalized nanorods (~2 × 10⁸) were applied to MCF-7 cells over 3 h and incubated at 48 h under culture conditions after washing with PBS solution. After removing the MTT solvent, 200 µl of dimethyl sulfoxide was added to each well and the plate shaken for 20 min to dissolve the formazan. The absorbance of each well was measured at 530 nm with a microplate reader (EL800; BioTek, USA).

Results and Discussion

Characterization of Two-Component Nanorod Complexes

In order to produce two-component nanorods, the AAO plate was used as a nanorod template as shown in Fig. 1. This AAO template-based fabrication method has several advantages compared with the seed-mediated method, including the capability of bulk synthesis (yielding 10⁷–10¹⁰ per batch), ease of producing the desired lengths and diameters, and a relatively short synthesis time [7, 28]. In addition, the size and composition can be fine-tuned to
tailor the electrical, optical, and magnetic properties. The size of nanomaterials, such as nanorods, can be tailored to improve the pharmacokinetic and pharmacodynamic parameters and their active intracellular delivery. AAO template-based synthesis is based on the electro-deposition mechanism, using a three-electrode configuration composed of working, counter, and reference electrodes. Before depositing metal ions onto the AAO frame, it is necessary to block one side of the AAO with conducting materials that have many nano-sized holes. Hence, Ag, which can later be dissolved by nitric acid, was deposited on one side of the AAO plate through thermo-deposition. Then, Ag deposition was performed again by electro-deposition before the deposition of Au. This sequence protects the rod components from being dissolved by nitric acid and obtains uniform rod distribution.

The basic principle of metal deposition on an AAO plate is as follows: when applying a negative charge on the working electrode, positive metal ions in several plating solutions are deposited to the bottom of the AAO plate and elongated through the AAO frame. To protect the Ni from the dissolving step, Au is the first metal to be deposited. After the deposition of Ni, the AAO plate was treated with nitric acid and sodium hydroxide to remove residual Ag. The metal-deposited AAO can then be used as a nanorod template. The two-component nanorod was purified through several washing steps using magnetic separation to retain Ni’s magnetic property. Magnetic separation using a washing step is superior to the conventional method of centrifugation, because centrifugation results in lower yields, deformation, and/or aggregation of the nanorods. As shown in Fig. 2, the two kinds of gold-nickel (Au-Ni) nanorods exhibited a length of 600 nm and 10 µm, respectively, with a consistent diameter of 300 nm, respectively. In scanning electron microscopy imaging, the two components of the nanorods were confirmed: (i) the dark side of the rod comprised Ni, and (ii) the opposite side was discerned from the optical properties of Au. As shown in Fig. 2A, approximately 600-nm-sized nanorods were used for delivering siRNA in cancer cells. The longer nanorods shown in Fig. 2B were used in fluorescence imaging to separately confirm the functionalization of both targeting ligands and siRNAs.

Selective Functionalization of Two-Component Nanorods

As previously mentioned, a 10-µm-long two-component nanorod was synthesized to investigate selective functionalization by a fluorophore-labeled targeting ligand and siRNA. As shown in Fig. 3, several steps were needed to functionalize the Au side with siRNA and the Ni side with a peptide sequence, the targeting moiety. The selected therapeutic agent was TAMRA-labeled siVEGF, which inhibited angiogenesis through the knock-down of overexpressed VEGF protein. For conjugation of siVEGF with the nanorod, heterobifunctional PEG (SH-PEG-NH₂) was used as a linker through an Au-thiol interaction. The tailed amine groups of PEG on the surface of the nanorods were converted into pyridyldisulfide groups by the reaction of N-succinimidyl-3-(2-pyridyldithio) propionate. Then, the TAMRA-labeled siVEGF was attached to the Au surface through disulfide bonds that are later released by the reducing enzyme in the cytoplasm of the cancer cell [15].

LHRH, which binds to the LHRH receptor and is
Overexpressed in breast, ovarian, and prostate cancer cells, was selected as the targeting ligand [9]. Peptide fragments have favorable properties for functionalizing nanomaterials, including good stability and ease of adding functional groups compared with antibodies. In order to attach LHRH peptide on the Ni side of the two-component nanorods, a poly-histidine (His$_6$) tag was added to the C-terminus of the peptide [1] and FITC was labeled to confirm ligand functionalization on the Ni side and to image the targeted cancer cell. As shown in Fig. 4, we verified selective conjugation of siVEGF and LHRH peptide on the two-component nanorods. In Fig. 4A, an optical image of the nanorods is shown, where the lower-left side in blue is the Ni component and upper-right side in orange is the Au component. FITC-labeled LHRH peptide was successfully immobilized on the Ni side, as seen through the green fluorescence (Fig. 4B), but not on the Au side. In contrast, siVEGF was attached on the Au side separately, which is represented by red in Fig. 4C. Functionalization must occur separately through different conjugation methods to immobilize two kinds of functional groups without producing steric hindrance. These separate conjugation methods on nanorods are sufficient to immobilize the targeting ligand and therapeutic agents so that each function can efficiently operate without problems.

**Selective Targeting of Two-Component Nanorod Complexes for Cancer Cells**

Efficient and active targeting delivery is particularly important to prevent adverse effects on healthy cells during cancer treatment. LHRH receptors are overexpressed in several cancer cell lines, but are not detected in healthy human cells and some cancer cells. In this study, MCF-7, a breast cancer cell line, was targeted for delivery of the nanorod complexes through the interaction with siVEGF. In contrast, SK-OV-3, an ovarian cancer cell line that does not express LHRH receptors, was selected as a negative control. This allowed us to select the LHRH peptide and LHRH receptor as a targeting moiety to specifically deliver siRNA to a cell through receptor-mediated endocytosis. The green fluorescence showed that the FITC-labeled LHRH peptide was successfully conjugated to the two-component nanorod, which penetrated the MCF-7 cells. The confocal microscopy observation shown in Fig. 5 demonstrated that the presence of LHRH on nanorods considerably increased the cellular uptake by MCF-7 cells (Figs. 5D–5F) compared...
with the SK-OV-3 cell line (Figs. 5A–5C). Furthermore, red fluorescence in Fig. 5E indicates that siVEGF, the therapeutic agent, was also delivered into MCF-7 cells. Hence, dual-fluorescence imaging was possible for use in receptor-positive cancer cells using the nanorod complexes. To gain additional evidence in supporting that targeted delivery occurs in this system, FACS analysis was conducted to determine the exact extent of cellular uptake of nanorod complexes in cancer cells, as shown in Fig. 5G. Experimental conditions were selected for two variables: the presence of LHRH peptide on the Ni surface, and the degree of the LHRH receptor expressed on the cell surface, in which MCF-7 versus SK-OV-3 were compared. As shown in Fig. 5G, LHRH-conjugated nanorod complexes with receptor-positive cells exhibited obvious green fluorescence compared with the other cases. These results demonstrate that LHRH peptide used as a targeting ligand can enable the selective delivery of anticancer agents through receptor-mediated endocytosis to target tumor cells and prevent damage to normal tissues.

In order to determine the toxicity of the nanorods, cell viability tests of the cargo material is required. In particular, multimodal theranostic nanocomplexes composed of inorganic materials might have some toxic effects on normal cells because their components do not contain natural biomaterials. For this reason, the MTT assay, a colorimetric assay for measuring the viability of cells through the enzymatic reaction of tetrazolium dye, was performed with a concentration range up to $\sim 8 \times 10^8$ nanorod complexes without siVEGF. In Fig. 6, MCF-7 cells with $\sim 8 \times 10^8$ nanorod complexes had a >80% survival rate. This result provides critical evidence for the almost complete lack of toxicity of nanorod complexes, and injection of $2 \times 10^8$ nanorod complexes showed no toxicity, demonstrating the good potential of these complexes for targeting and therapeutic experiments. Therefore, the multifunctional Au-Ni nanorod is suitable for targeted imaging and delivering genetic material and anticancer agents.

**Anticancer Effect of RNAi-Based Nanorod Complexes**

The main purpose of two-component nanorod complexes is to use siRNA as the anticancer agent, because it affects proteins involved in cancer proliferation, apoptosis, or...
angiogenesis. In this study, VEGF, which is a critical regulator of angiogenesis, was targeted in cancer by RNAi to inhibit angiogenesis and suppress the growth and metastasis of malignant cancers [20]. Of all the numerous anticancer agents, siRNA requires relatively fast delivery and reaction times with specific genes in targeted cells. The agent is unstable and takes a relatively longer time to initiate genetic degeneration and induce cellular death as compared with chemotherapeutic agents. Our siRNA nanorod complexes were advantageous to treat cancer, specifically for intratumor injection, when compared with the encapsulation of nanocomplexes. Here, encapsulated nanomaterials requires relatively more time for disassembly of outer materials and release of siRNA into the cytoplasm. In this study, thiolated siVEGF was functionalized on the Au surface of the two-component nanorod by disulfide linkage, which is cleavable after endocytic uptake and can then induce gene silencing with siVEGF. Notably, the determination of siRNA effects was confirmed at the mRNA level. The normalized VEGF mRNA level was compared through real-time PCR analysis (Fig. 7A). Scrambled siRNA with Lipofectamine and LHRH-functionalized nanorods showed similar mRNA levels, and produced an almost 100% mRNA ratio, indicating their suitability as a negative control. In contrast, the nanorod complexes developed in this study suppressed VEGF mRNA levels to less than 5% of normal amounts, and the data suggest that there was a huge difference in the transfection rate of siVEGF, which contained the same sequence attached on the nanorod. This provides evidence that siVEGF delivery with two-component nanorods is superior to Lipofectamine-based siVEGF delivery. The results can be inferred from the relatively higher concentration of siVEGF near the nanorod complexes than observed for the transfected siVEGF by Lipofectamine. Additional data for the effect of siVEGF-conjugated nanocomplexes came from the determination of VEGF protein levels by ELISA in the cytoplasm. As shown in Fig. 7B, siVEGF-conjugated two-component nanorod complexes significantly suppressed VEGF protein levels compared with untreated MCF-7 cells. Moreover, siVEGF with Lipofectamine exhibited less of a knock-down effect than the two-component nanorods, as expected. Finally, the influence of siVEGF treatment on cell viability was determined by the MTT assay (Fig. 7C). Similar to the previous results shown in Figs. 7A–7B), using nanorod complexes with siVEGF showed the lowest cell viability at >20%, which markedly differed with the other treatments, especially for siVEGF with Lipofectamine. The LHRH-functionalized nanorods without siVEGF did not decrease cell viability substantially, which confirms the results of the toxicity test (Fig. 6). In Lipofectamine-treated cells with
siVEGF, cell viability was approximately 70% (Fig. 7C). This may be due to the synergistic effect of siVEGF with the toxicity of Lipofectamine. However, the Lipofectamine-treated cells with siVEGF exhibited a much weaker effect on cancer treatment as compared with two-component nanorod complexes. Therefore, siVEGF-functionalized two-component nanorod complexes resulted in efficient cancer treatment through the degradation of mRNA, the knockdown of protein levels, and the induction of death in cancer cells.

In this study, we demonstrated the generation of excellent two-component nanorod complexes for dual fluorescence imaging and cancer treatment for siRNA-based gene therapy. The Au-Ni bimetallic nanorod provided active targeting to cancer cells using LHRH peptide ligands that promoted their selective uptake on LHRH receptor-overexpressed cancer cells through receptor-mediated endocytosis. This approach also resulted in efficient delivery and induction of cell death based on RNAi through the activation of siVEGF. In particular, the unique advantage of this two-component nanorod is represented by the selective functionalization on each metallic surface of the nanorod using separate interactions, including Ni-His interactions and Au-thiol conjunction, without any steric hindrance. We minimized side effects originating from poor targeting and maximized the silencing effect of antiangiogenic siVEGF, leading to successful suppression of cell viability in breast cancer cells. Notably, this surface-positioned structure of siRNA can be expected to have synergistic effects with other therapeutic agents, such as chemotherapeutic agents, owing to its relatively rapid reaction before other therapeutic agents work. The two-component and multifunctional nanorod complexes developed in this study are novel and differ from other previously reported polymer and lipid-based nanocomplexes. The approach holds great potential for clinical applications of siRNAs and their use in cancer gene therapy.

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