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# Aptamer-tagged green- and yellow-emitting fluorescent silver nanoclusters for specific tumor cell imaging

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ABSTRACT

Aptamer-functionalized fluorescent silver nanoclusters (Ag NCs) possessing specific recognition ability of aptamer and fluorescence emissions have been developed for applications in biorelated areas. However, only one color emission hampered their further use in multiplexed analysis. To address this issue, AS1411 aptamer-tagged Ag NCs with green color emissions and MUC 1 aptamer-tagged Ag NCs with yellow color emissions were synthesized by one-pot approach in this work. With the rational design of the DNA template and optimization of the synthetic buffer, as well as the $NaBH_4$-to-$Ag^+$ ratio, the growth time and purity of the obtained aptamer-functionalized Ag NCs were improved. Under the optimal conditions, the quantum yields of AS1411 aptamer-functionalized green Ag NCs and MUC 1 aptamer-functionalized yellow Ag NCs were 20.33% and 8.65% with 9,10-diphenyl anthracene and fluorescein as the reference standards, respectively. The green and yellow emission properties of Ag NCs were related to the surface oxidation states of the Ag NCs. Furthermore, the binding ability of AS1411 aptamer and MUC 1 aptamer to cancer cells enabled specific cellular imaging. Aptamer-functionalized Ag NCs with multicolor emissions could broaden the applications of Ag NCs in multiplexed analysis and multicolor imaging of tumor cells, especially with the selection of more targeted aptamers.



## 1. Introduction

Fluorescent metal nanoclusters (metal NCs), as a supplementary to quantum dots (QDs) and organic dyes, have attracted increasing attention from researchers due to their excellent properties, such as photostability, subnanometer size and biocompatibility. DNA oligonucleotide-stabilized fluorescent silver nanoclusters (Ag NCs) are a typical case. The high affinity of silver ions to cytosine bases on single-stranded DNA favors DNA oligonucleotides as an excellent template for the synthesis of fluorescent Ag NCs. $Ag^+$ binds to two different sites in DNA (N3 in pyrimidines and N7 in purines), whereas in RNA, $Ag^+$ binds to just N7, which favors the generation of Ag NCs after reduction of $NaBH_4$ [1]. Such easy preparation, good photostability, high luminescence quantum yield and excellent aqueous solubility of DNA-stabilized Ag NCs have made them promising materials for applications in chemical sensing [2,3], bioassays [4] and biological imaging [5–8]. In particular, the fluorescence features of DNA-stabilized Ag NCs were sensitive to the DNA sequence, base number, secondary structure, and DNA environment around them, which have been utilized for the analysis of DNA, RNA and other biomolecules [9–13]. Additionally, the tunable fluorescence emissions of DNA-Ag NCs could be controlled by adjusting the DNA template. In 2008, Dickson's group developed a DNA microarray for high-throughput selection of DNA oligonucleotides for Ag cluster encapsulation. The library was composed of various oligonucleotides containing combinations of cytosine, thymine, and adenine, and they were attached to the substrate by a T12 linker that did not contribute to the Ag NCs formation. After the reduction of $Ag^+$ by $NaBH_4$, the DNA microarray was excited with 543 nm light, and the special DNA sequences for the synthesis of Ag NCs with tunable fluorescence emissions throughout the visible and near-IR were obtained [14]. Such multicolor, DNA-templated Ag NCs enabled multi-analyte detection in a label-free manner. Zhang's group developed a sensitive and label-free/conjugation-free analysis system for multiplexed DNA detection based on two types of DNA-templated Ag NCs, with green emission (507 nm) and orange emission (597 nm). The DNA template for the stabilization of Ag NCs was blocked until the target DNA opened the molecular beacon structure. The total release of the DNA template could produce a fluorescence signal of Ag NCs, and the signal inten-

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sity was closely related to the amount of target DNA, to provide a platform for multiplexed DNA analysis [15]. Willner's group proposed graphene oxide/nucleic-acid-stabilized Ag NCs as functional hybrid materials for optical aptamer sensing and multiplexed analysis of pathogenic DNAs. To realize the multiplexed analysis of pathogenic DNAs, red-emitting DNA-Ag NCs (616 nm) and near-infrared-emitting DNA-Ag NCs (775 nm) were employed as signal reporters. The DNA templates contained a sequence to complement with the target gene and a sequence to stabilize the Ag NCs. In the absence of a target gene, the adsorption of the complementary sequence in the DNA template on the surface of graphene oxide (GO) quenched the fluorescence emissions of DNA-Ag NCs. In the presence of the target gene, the hybridization between the target gene and the DNA template released Ag NCs from GO to provide fluorescence emissions. Based on this, multiplexed analysis of the hepatitis B virus gene (HBV) and the immunodeficiency virus gene (HIV) was demonstrated [16]. When the complementary part in the DNA template was substituted by the aptamer, other targets, such as ATP and cocaine, could be detected [17,18]. However, the lack of aptamer-functionalized multicolor Ag NCs hampered the multiplexed analysis of aptamer-related targets.

The integration of DNA aptamer and DNA template for Ag NCs synthesis enabled the preparation of aptamer-functionalized Ag NCs in a one-pot approach. The obtained Ag NCs produced fluorescence emission as signal reporters and had specific binding ability to the target. Aptamer-functionalized fluorescent Ag NCs have been widely used for the detection of small molecules [19], proteins [20,21], gene delivery [22] and tumor cell imaging [23] with rational design. In our previously study, we proposed a multifunctional probe comprising a cell-specific internalization aptamer, fluorescent Ag NCs, and therapeutic siRNA in one system for the specific delivery of siRNA into a target cell and for simultaneous and noninvasive imaging. This method demonstrated the promising potential of DNA templated Ag NCs as the fluorescence probe in cell imaging [22]. The successful application of aptamer-functionalized Ag NCs in cell imaging and gene delivery encouraged us to prepare aptamer-tagged Ag NCs with multicolor emissions for their potential use in multiplexed analysis and multicolor imaging of tumor cells. Thus, in this study, we design DNA templates for the synthesis of aptamer-functionalized, multicolor Ag NCs and investigate the possibility of their multicolor imaging of tumor cells *in vitro*.

## 2. Materials and methods

### 2.1. Materials and reagents

Silver nitrate (99+%), sodium borohydride, $NaBH_4$ (powder, 98%), disodium hydrogen phosphate, sodium dihydrogen phosphate, sodium chloride, sodium hydroxide, trisodium citrate, citric acid, acetate, sodium acetate, tris-(hydroxymethyl) aminomethane (Tris), 9,10-diphenyl anthracene, cyclohexane and fluorescein were purchased from Sinopharm Chemical Reagent Co., Ltd. (Shanghai, China). 4,6-Diamidino-2-phenylindole (DAPI) and cell cultures were purchased from Kangmei Biotechnology Co., Ltd. (Xuzhou, China). All DNA oligos were synthesized and purified by Shanghai Sangon Biotechnology Co., Ltd. (Shanghai, China), and the sequences are listed in Table 1. All chemicals involved in this work were analytical-grade, and all aqueous solutions were prepared with ultrapure water (≥18 MΩ, Milli-Q, Millipore).

### 2.2. Apparatus and characterization

Fluorescence measurements were conducted using a LS-45/55 Fluorescence/Phosphorescence Spectrometer (PerkinElmer, USA). UV–vis spectra were recorded on a Purkinje General Instrument T6 New Century spectrophotometer (Beijing Purkinje General Instrument, China). The size and morphology of Ag NCs were observed by high-resolution transmission electron microscopy (HRTEM) (JEOL JEM 200CX, Japan). MTT assays were performed with a microplate reader (Multiskon MK 3, USA), and fluorescence imaging was conducted with a confocal microscope (TCS SP5, Leica, Germany).

### 2.3. Cells and cell culture conditions

Human cervical carcinoma HeLa cells, NIH-3T3 mouse fibroblast cells and normal human umbilical vein endothelial cells (HLF-1) were purchased from the Cell Bank of the Chinese Academy of Sciences (Shanghai, China). HeLa cells and NIH-3T3 cells were cultured in HDMEM medium (Gibco, Grand Island, NY) supplemented with 10% fetal calf serum (Gibco Grand Island, NY), penicillin (100 μg/mL), and streptomycin (100 μg/mL) with incubation in a humidified incubator (Thermo, USA) at 37 °C under 5% $CO_2$ atmosphere. For HLF-1 cells, the cell culture medium was substituted with Ham's F12 nutrient medium (Gibco, Grand Island, NY) supplemented with 10% fetal calf serum (Gibco Grand Island, NY), penicillin (100 μg/mL), and streptomycin (100 μg/mL).

### 2.4. Preparation of fluorescence silver nanoclusters

DNA-stabilized Ag NCs were synthesized with $NaBH_4$ as the reducing agent. Briefly, 6 μL of 250 μM AS1411-NC Green was mixed with 20.8 μL of 20 mM PBS buffer (pH 7.4), and then 3.8 μL of 10 mM $AgNO_3$ solution was added. After cooling on the ice for 15 min, the mixture was reduced by the rapid addition of 9.4 μL of 4 mM $NaBH_4$, followed by vigorous shaking for 1 min. The reaction was kept at 4 °C for a suitable time to obtain the Ag NCs. Ag NCs stabilized with other DNA sequences, such as AS1411, NC Green, NC Green-AS1411, AS1411-Poly5A-NC Green, AS1411-Poly5T-NC Green, MUC 1, NC Yellow, MUC 1-NC Yellow, MUC 1-Poly5A-NC Yellow, and MUC 1-Poly5T-NC Yellow, were prepared with the same method, and the amounts of $AgNO_3$ and DNA were adjusted to obtain a base to $Ag^+$ ratio of 2:1. For the buffer optimization, 10 mM citrate buffer (pH 6.6), 20 mM citrate buffer (pH 6.6), 5 mM PBS (pH 7.4), 5 mM acetate/acetate acid (pH 5.5), 10 mM Tris-HCl (pH 7.4), 10 mM Tris-HAc (pH 8.0), 20 mM PBS (pH 7.4) and 20 mM PBS (pH 5.0) were introduced.

### 2.5. MTT assay

HeLa cells or HLF-1 cells ($1 \times 10^4$) were first seeded in each well of a 96-well plate for 24 h, respectively. After incubation with AS1411 aptamer-tagged green Ag NCs or MUC 1 aptamer-tagged yellow Ag NCs ranging from 5 nM to 1 μM for 24 h, the cells were washed twice with PBS buffer or Dhanks for HLF-1 cells before the addition of HDMEM or Ham's F12 nutrient medium containing MTT (5 mg/mL) and further incubation at 5% $CO_2$ and 37 °C for 4 h. Then, the medium containing MTT was replaced by 100 μL dimethyl sulfoxide (DMSO) to solubilize the formazan crystals. The absorbance for each well was measured by a microplate reader (Multiskon MK3, USA) at 490 nm.

### 2.6. Confocal laser microscopy assay

The specific optical images of the AS1411 aptamer-functionalized green Ag NCs and MUC 1 aptamer-functionalized yellow Ag NCs to HeLa cells with green and yellow color were examined by confocal laser microscopy. HeLa cells ($5 \times 10^4$) were seeded in special petri dishes. After 24 h, cells were incubated with AS1411 aptamer-functionalized green Ag NCs or MUC 1 aptamer-functionalized yellow Ag NCs at 4 °C for 30 min. The cells were then washed with PBS three times and placed above