Ultrasensitive Detection of Prostate-Specific Antigen and Thrombin Based on Gold-Upconversion Nanoparticle Assembled Pyramids

Tiantian Hao, Xiaoling Wu, Liguang Xu, Liqiang Liu, Wei Ma, Hua Kuang,* and Chuanlai Xu

Self-assembled nanostructures have been used for the detection of numerous cancer biomarkers. In this study, a gold-upconversion-nanoparticle (Au-UCNP) pyramid based on aptamers is fabricated to simultaneously detect thrombin and prostate-specific antigen (PSA) using surface-enhanced Raman scattering (SERS) and fluorescence, respectively. The higher the concentration of thrombin, the lower the intensity of SERS. PSA connected with the PSA aptamer leads to an increase in fluorescence intensity. The limit of detection of thrombin and PSA reaches $57 \times 10^{-18}$ and $0.032 \times 10^{-18}$ m, respectively. In addition, the pyramid also exhibits great target specificity. The results of human serum target detection demonstrate that the Au-UCNP pyramid is an excellent choice for the quantitative determination of cancer biomarkers, and is feasible for the early diagnosis of cancer.

1. Introduction

A cancer biomarker is a reflection of the existence of a tumor, and the presence and quantitative change in the biomarker may indicate the nature of the tumor. Thrombin, a serine protease,[1] is responsible for blood coagulation.[2] It is an essential biomarker in tumor diagnosis due to its concentration in human blood. Therefore, it is significant to achieve extremely sensitive detection of thrombin for the early diagnosis of cancer. Prostate-specific antigen (PSA) is an organ-specific biomarker thought to be associated with prostate cancer,[3] a common malignancy worldwide. Thus, the detection of this glycoprotein is of vital importance not only in the diagnosis, but also the prevention of prostate cancer progression.[4]

Many methods are used in the detection of these cancer biomarkers. Some traditional approaches, such as enzyme-linked immunosorbent assay[5] and radioimmunoassay, which are based on antibodies are expensive, difficult to operate and require more time and manpower, and the preparation and preservation of antibodies are difficult.[6] Other methods such as electrochemistry[7] and instrumental analysis also require considerable time and effort. In addition, these methods mostly recognize only one target, and some types of cancers are associated with more than one biomarker. For example, PSA is a biomarker of prostate cancer, but the level of thrombin is also an essential biomarker for prostate cancer.[8] Thus, a highly sensitive, convenient, controllable, and stable method for the simultaneous detection of two targets is required, which would save time and expense, and obtain accurate results in clinical diagnostics.[9] Nanoparticles (NPs) have more advantages than antibodies and many types of nanoparticle structures have been prepared and used as detection probes.[10] For example, optical analysis methods...
have been extensively used in the detection of targets.\cite{11}
Some types of nucleic acid and relevant nanostructures have been used in cancer detection and therapy, thus, the aptamer-based nanoparticles assembly is a perfect choice to meet the above requirements.\cite{12} Aptamers are single oligonucleotides, which are screened in vitro and possess high-affinity for the target.\cite{13} Their stability and storage properties are superior to those of antibodies, and different structures can be fabricated to recognize two or three tumor biomarkers, which facilitate the diagnosis and therapy of tumors which are difficult to identify.\cite{14}

Surface-enhanced Raman scattering (SERS), which is related to the coupling of polar oscillations in adjacent nanoparticles, provides a unique optical signal for the detection of cancer biomarkers due to their sensitivity to changes in structure.\cite{15} and is an essential tool for the structure characterization of assemblies.\cite{16} Upconversion luminescence arises from the sequential absorption of photons, whose emission
wavelength is shorter than the excitation wavelength.\cite{17} Fluorescence materials, especially lanthanide-doped-upconversion nanoparticles (UCNPs) and their assemblies, have attracted increasing attention in many fields due to their fluorescence stability and unique optical properties, and are capable of being excited by near infrared laser and causing less damage to organisms.\cite{18}

With these factors in mind, we designed a self-assembled Au-UCNP pyramid model based on different aptamers, which was capable of the simultaneous detection of two targets with Raman and fluorescence signals, respectively. If the targets were present in the solution, the specific recognition of aptamers caused a change in pyramid structure, and these changes were noted in SERS and fluorescence intensity. According to the variation in optical signals, the quantitative detection of targets was possible.

## 2. Results and Discussion

Scheme 1 shows the principle of the assembly of the pyramid and quantitative detection of thrombin and PSA. First, Au nanoparticles and UCNPs were modified by the thrombin aptamer and PSA aptamer, respectively, then two DNA strands bare and two DNA strands functionalized with a Au nanoparticle and a UCNP, respectively, were hybridized together to form the frame of the pyramid. Finally, the nanoparticles functionalized with aptamers and the frame was combined, and the pyramid was successfully assembled. Part of the sequence of Apt 1 and Apt 2, not complemented with the frame and on both sides only several base sequences and the frame were complementary, therefore when the aptamer-modified nanoparticles encountered targets (thrombin or PSA), the target was recognized and the target bound to the aptamers competitively with the frame, resulting in a drop in the aptamer-modified nanoparticles from the pyramid. When thrombin bound to the aptamer, the Au nanoparticle modified with Apt 1 dropped from the pyramid, and SERS intensity decreased. When PSA was present in the solution, the UCNPs were released from the pyramid, and the quenched fluorescence recovered. The assembly and dissociation processes were characterized by transmission electron microscope (TEM) (Figure 1; Figure S1, Supporting Information). The Au-UCNP dimers are shown in Figure S2 (Supporting Information) on the frame of the pyramid. Figure 1 shows the successful assembly of the pyramid, the yield of the structure was up to 80%, and statistical analysis of the different products in the reactions involved in pyramid assembly can be seen in Figure S3 (Supporting Information). The hydrodynamic diameters of the assembly and building blocks (Figure S4, Supporting Information) also indicated successful assembly of the pyramid.

When the Au nanoparticles and UCNPs were assembled into the pyramid, the SERS signal increased, and the fluorescence of UCNPs was quenched. The Au nanoparticles possessed a strong surface-enhanced spectroscopic effect, thus, the Raman signal was enhanced due to this effect, and the fluorescence of UCNPs was quenched in the pyramid due to energy transfer. When PSA connected with the corresponding aptamer, the UCNPs modified by the PSA aptamer dropped from the pyramid, and the fluorescence recovered. When thrombin bound to the aptamer, this resulted in Au nanoparticle independence and the SERS signal decreased.

![Figure 2](image-url)

**Figure 2.** A) Raman spectra for different concentration of thrombin excited by 633 nm laser, and B) the calibration curve at 1074 cm\(^{-1}\) for thrombin detection. Raman reporter was 4-mercaptophenylacetic acid (4-ATP).
The use of SERS and luminescence signal had the advantages of gold nanoparticles and UCNPs, and realized the supersensitive detection of thrombin and PSA. The application of SERS and luminescence ensure more accurate detection and avoid interference signals.

The SERS spectra are shown in Figure 2. 4-aminothiophenol was chosen as the Raman reporter, which was modified in the pyramid following incubation for 4 h in the dark. The Raman intensity was highest at a Raman shift of 1074 cm$^{-1}$ when excited by a 633 nm laser, which was recorded as the signal peak. As seen in Figure 2, the solution without thrombin had the highest SERS intensity, and when the concentration of thrombin increased, more targets were linked to the aptamer, resulting in the disassembly of more Au nanoparticles from the pyramid leading to a decrease in Raman intensity. A standard solution of thrombin was injected into the pyramid solution, and the final concentration of the target was 0, 0.001, 0.005, 0.01, 0.05, and 0.1 $\times$ 10$^{-15}$ m. The above solution was incubated at 37 °C for 8 h. It can be seen from the standard curve that the logarithmic function of thrombin concentration and Raman intensity exhibited a good linear relationship from 0.001 $\times$ 10$^{-15}$ to 0.1 $\times$ 10$^{-15}$ m, with $R^2 = 0.998$, and the limit of detection was 57 $\times$ 10$^{-18}$ m which was more sensitive than most other methods.$^{[19]}$

The UCNPs showed three fluorescence peaks, and we chose 543 nm as the signal peak. For quantification of PSA, a standard solution was added to the Au-UCNP assembly solution with a final PSA concentration of 0, 0.04, 0.08, 0.1, 0.5, and 1 $\times$ 10$^{-18}$ m, respectively. The solution was then incubated at 37 °C for 8 h. The TEM image (Figure S1, Supporting Information) shows that the pyramid was dissociated step by step. The fluorescence spectra were obtained following excitation with a 980 nm laser (Figure 3), which showed that fluorescence of UCNPs was lowest without added PSA in the solution, and the higher the concentration of the target, the more nanoparticles dissociated from the pyramid. A standard curve was plotted according to the logarithmic function of PSA concentration and the corresponding intensity of luminescence of UCNPs, which exhibited an excellent linear relationship in the range of 0.04 $\times$ 10$^{-18}$ to 1 $\times$ 10$^{-18}$ m with $R^2 = 0.996$. The limit of detection of PSA was 0.032 $\times$ 10$^{-18}$ m. The sensitivity of our method was superior to that of other methods.$^{[20]}$

To demonstrate the selectivity of the assay, different interfering chemicals (α-fetoprotein, mucin 1, human serum albumin, immunoglobulin G, and their mixture) were tested under the same conditions.$^{[2,21]}$ The concentration of PSA was 1 $\times$ 10$^{-18}$ m, thrombin was 10 $\times$ 10$^{-15}$ m, and other interfering chemicals were 1 $\times$ 10$^{-6}$ m. As shown in Figure 4, both the spectra pattern and histogram showed significant differences between the target and other proteins, and the SERS and fluorescence signals showed no obvious changes after the addition of interfering chemicals, although the concentrations of these chemicals were much higher than those of the targets. In order to further confirm the specificity of the method, all the interfering chemicals were mixed together as the substrate for PSA and thrombin detection, and the results indicated that they had no influence on detection of the targets. The above results indicated that the pyramid modified with

![Figure 3. A) The upconversion fluorescence spectra from 500 to 700 nm of different concentration of PSA, excited by 980 nm laser and B) the calibration curve at 543 nm for thrombin detection.](image)

### 3. Conclusion

In conclusion, Au-UCNP pyramid nanostructures were fabricated for the simultaneous detection of PSA and thrombin with satisfactory results. The SERS intensity was enhanced and UCNPs fluorescence was quenched following successful assembly of the Au-UCNP pyramid, while SERS intensity decreased accompanied by fluorescence recovery when the aptamers met the targets. The application of this structure in the detection of targets in human serum indicated that the
ultrasensitive quantitative detection method is useful for the early diagnosis of cancer. We believe that the assembly based on aptamers is promising for accurate and distinct identification of disease biomarkers in clinical practice.

4. Experimental Section

Synthesis of Gold Nanoparticles: Gold (Au) nanoparticles were synthesized according to a routine method: 1.25 mL HAuCl$_4$ solution ($10^{-3}$ M) was added to 48.75 mL water and heated to boiling with vigorous stirring. Then 0.9 mL sodium citrate (1% w/w) was quickly injected into the mixture. The mixture was boiled until the resulting solution turned wine red. The solution was then cooled to room temperature with continuous stirring.

Synthesis of DNA-Functionalized Gold Nanoparticles: The sequences of all DNAs are shown in Table S1 (Supporting Information). Au nanoparticles were modified with Aptamer 1 using a previously reported method. Briefly, 1 mL of the as-prepared Au nanoparticles were concentrated by centrifugation (7500 g for 15 min), the supernatant was then removed and the precipitate redispersed in 0.1 mL 1× TBE buffer. The above solution was mixed with ssDNA at a ratio of 1:4.1 µL and NaCl solution (5 M) was added. The resulting mixture was incubated at 37 °C for 8 h. The mixture was then centrifuged (7500 g for 15 min) to remove the uncoupled ssDNA, and the pellet was redispersed to 0.1 mL 1× TBE buffer.

Synthesis of DNA-Functionalized UCNPs: The UCNPs were purchased from Beijing Oneder-Hightech Co., Ltd., with maleimide groups on the surface which can bind with thiol. The UCNPs were diluted with 1× TBE buffer to a final concentration of $10^{-9}$ M, and then ssDNA was added at a ratio of 1:4. 1 µL NaNO$_3$ solution (5 M) was added to the resulting solution and the mixture was incubated at 37 °C for 8 h. The sample was centrifuged at 13 000 g for 30 min to remove the uncoupled ssDNA, and the pellet was redispersed in 1× TBE buffer.

The Assembly of Au-UCNP Pyramids: First, Au-UCNP dimers were assembled. The as-prepared functionalized Au nanoparticles and UCNPs with complementary ssDNA (DNA 1 and DNA 2) of the same volume were mixed together, and DNA 3 and DNA 4 were injected into the mixture (the ratio of DNA 1, DNA 2, DNA 3, and DNA 4 was 1:1:1:1). Then NaNO$_3$ and Mg(NO$_3$)$_2$ solution were added at final concentrations of $50 \times 10^{-3}$ and $10 \times 10^{-3}$ M, respectively. The mixture was heated in a 90 °C water bath for 5 min and cooled to room temperature slowly with the steam from the water bath, and the frame of the pyramid was then formed. The Au nanoparticles and UCNPs functionalized with Apt 1 (thrombin aptamer) and Apt 2 (PSA aptamer) at the same concentration of nanoparticles were hybridized with the frame for 8 h to achieve assembly of the pyramid.

Analysis of PSA and Thrombin in Human Serum: In order to examine the reliability of the sensor, human serum, a complex biological matrix, was analyzed, which was obtained from the Second Hospital in Wuxi, China. As shown in Table S2 (Supporting Information), the results indicated that use of the sensor was feasible for the detection of cancer biomarkers and for clinical applications.

Supporting Information

Supporting Information is available from the Wiley Online Library or from the author.
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