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Detection of Anti-*Neospora* Antibodies in Bovine Serum by using Spiky Au-CdTe Nanocomplexes

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ABSTRACT: Detecting biomolecules via nanotechnology has become increasingly important in veterinary science. Neosporosis is an infectious disease that primarily affects cattle and it is caused by the intracellular parasite Neospora caninum. This microorganism now appears to a major cause of abortion in dairy cattle worldwide. We report herein on a rapid, sensitive, and inexpensive qualitative approach for detecting neosporosis based on photoluminescence (PL) enhancement between quantum dots (CdTe nanoparticles) and a unique form of spiky Au nanoparticles (SNP). We prepared anti-bovine IgG functionalized-SNPs, and a conjugated structure between quantum dots (QDs) and recombinant N. caninum protein that was expressed by silkworms. They bound easily when their common complementary target, anti-Neospora antibodies (ANABs) in bovine serum, was present. Binding was monitored by the PL enhancement of CdTe nanoparticles (NPs) in the PL spectrum that resulted from localized surface plasmons resonance (LSPR) of SNPs. The fluorescence intensities for samples from infected and healthy cattle were compared, for which significant differences in intensity were observed. The SNP-QDs sandwich nanocomplexes remained in solution and its optical properties allowed it to be easily quantified by using fluorescence spectra. More than 52% emission enhancement on the surface of the SNPs was attained compared with the CdTe NPs and the results were reproducible. Furthermore, the biosensor was suited for qualitatively analyzing ANABs in blood serum. The ease of operation of this system and its generality offer specific advantages over other immunoassay methods.

Keywords: neosporosis; quantum dots (QD); spiky Au nanoprobes; Au-QDs sandwich immunoassay; silkworm expression

Introduction

Neosporosis is an infectious disease that affects dogs, cattle, sheep, and under experimental conditions rodents, pigs, monkeys, and cats [1-5]. Neospora caninum is an obligate intracellular protozoan parasite and is a major cause of abortion in dairy cattle in many countries. Proteins that are displayed on the surface of this intracellular pathogen are believed to play critical roles in its infectivity. Several N. caninum parasite surface proteins have been reported. Surface-associated protein 1 (NcSAG1) and NcSAG1related sequence 2 (NcSRS2) are major surface proteins of N. caninum, and their antigenicity has also been documented [6,7]. The diagnosis of neosporosis by detecting anti-Neospora antibodies (ANABs) in bovine serum might be fast and practical [8,9]. For instance, an immunoperoxidase test that involves using specific antibodies can be employed to identify N. caninum in tissue sections or biopsy specimens. Alternatively, an indirect fluorescent antibody test and several ELISAs can be used to detect antibodies [10,11]. Even though a large number of bioassays are available nowadays, only one has been commercialized using an ELISA kit by Boehringer Ingelheim Svanova Inc. Therefore, it is still necessary to develop new detection methods that are rapid and highly selective to significantly improve the technology that is used for monitoring biological environments and in medical diagnostics [12].

Various bioreactor systems have been developed to mass-produce recombinant proteins that have biological sensor functions such as bio-probes that exhibit a high degree of specificity. Bacteria, especially *Escherichia coli*, are the most popular type of expression system, but are limited in terms of their poor capacity for modifying expression proteins and in many cases because the proteins that they express are in insoluble form [13]. Yeasts and insect cells are superior to bacteria since the proteins that they express can be glycosylated, which makes them more close to their native protein form. However, these protein production systems are costly. The silkworm *Bombyx mori* was first used by S. Maeda *et al.* [14] to produce human α -interferon and is now used as a bioreactor to produce many types of eukaryotic proteins that can be used for pharmaceutical purposes. In particular, an improved bacmid system, BmDH10Bac *CP⁻ Chi⁻*, can be used to express proteins in the milligram range in larvae [15].

Over the past 2 decades, Au nanoparticles (NPs) have attracted increasing attention because of their unique nano-optical properties. These include surface plasmon bands (SPB), surface-enhanced Raman scattering (SERS), and Raleigh resonance scattering (RRS), and they are well documented. Efficient coating with biomolecules via various interactions enables Au NPs to act as signal transducers in biorecognition binding applications [16,17]. Moreover, in the proximity of a metallic surface the fluorescence emissions of molecules can be enhanced by localized surface plasmons resonance (LSPR) of metallic NPs. This is also the case for metallic nanostructures and NPs that are adjacent to quantum dots [18-21]. In noble metallic NPs, LSPR presents strong absorption and scattering cross-section and is sensitive to the local refractive index, size, shape, and chemical compositions of the nanostructures [22]. The presence of adjunct metallic nanoparticles not only enhances the fluorescence intensity but also stabilizes quantum dots against photo-bleaching, which further enhances their practical uses in bioimaging and biosensor applications [23]. For example, Ciftja *et al.* presented an updated review on the metal enhancement of fluorescence (MEF) of semiconductor, silicon and carbon quantum dots (QDs) that is achieved with LSPR [24]. Moreover, he also anticipated that the QDs/metal nanocomposites that can be dispersed in an aqueous solvent would be particularly suitable for the development of new bioanalytical methodologies where sensitivity and selectivity are the critical factor. Lin *et al.* studied that photoluminescence enhancement of CdS NPs via surface plasmon mode coupling, CdS-conjugated Ag nanoplates by forward-reverse cation exchange method for the application of cell imaging [25]. Oh *et al.* reported an inhibition assay method based on the modulation in fluorescence resonance energy transfer (FRET) efficiency between QDs and spherical Au NPs in the presence of the streptavidin-biotin that inhibit the interaction between QDs- and Au NP-conjugated biomolecules [26].

Recently, with the development of nanotechnology, researchers have been pushing the envelope to expand the applications of NPs with unique properties to construct novel receptor such as quantum dots, carbon nanotubes, nanowires, and magnetic NPs. However, the application of such receptor structure is often limited by their solubility and stability, requirements for expensive instruments, and most importantly, their uncontrollable responses to complex biological samples. Generally, practical sensors must work to minimize the complexity of the system. Therefore, it is a big challenge to detect proteins in real media by using newly developed detection platforms in clinical applications. Metal nanostructures, especially rough metallic nanostructures, exhibit remarkable optical properties due to the excitation of their surface plasmons by incident light, which significantly enhances the electromagnetic field at the nanoparticle surface [27]. Therefore, utilizing rough-surface metallic nanostructures that enhance quantum dot fluorescence may ultimately provide practical routes for enhancing light emission from a variety of materials systems and devices far beyond specific applications to bioimaging and biosensors.

In this study, we examined the properties of spiky Au-NPs (SNPs), which are threedimensionally branched NPs that have more than 20 epitaxially grown tips and exhibit high yields and narrow size distributions at room temperature. We demonstrated a proof of concept of using SNP-CdTe NPs conjugation as a novel fluorescence-sensor platform for detecting proteins. To the best of our knowledge, despite a number of bioanalytical applications of NPs [28], this work is unique in terms of optical and biological properties because it involves enhancing the fluorescence of QDs by LSPR of rough-surfaced metallic NPs. In addition, it uses recombinant proteins that are expressed by silkworms for immunoassay to develop an alert system for monitoring veterinary diseases. Antibody detection was accomplished as follows: anti-bovine IgG functionalized SNPs were bound to a complementary target (ANABs) in bovine serum. To achieve high sensitivity, recombinant *N. caninum* protein-conjugated quantum dots (rNcSAG1- or rNcSRS2-CdTe NPs) were employed to produce a fluorescence signal. Significant differences in intensity were observed between serum samples from infected and healthy cattle, which resulted from enhanced fluorescence of the Au-QD sandwich nanocomplex with its target proteins.

2. Experimental details

2.1 Spiky Au nanoparticle synthesis

All of the chemicals that we used were purchased from Sigma-Aldrich or Wako Pure Chemical Industries, Ltd. (Osaka, Japan) and used as received. Ultrapure Millipore water (>18.2 M Ω) was used as a solvent. All the glassware were cleaned with aqua regia (HCl/HNO₃ at a 3:1 ratio by volume) and rinsed with ethanol and ultrapure water (*Caution! Aqua regia is a very corrosive oxidizing agent that should be handled with great care*). The spiky Au NPs were synthesized by reducing HAuCl⁴ with 4-(2-hydroxyethyl)-1-piperazineethane-sulfonic acid (HEPES) and stabilized according to Wang's method [29] with slight modifications. An aqueous stock solution of 100 mM HEPES was prepared with ultrapure water and its pH was adjusted to 7.4 at 25°C by adding 1 M NaOH. In a typical experiment to synthesize SNPs, 1 mL of 100 mM HEPES (pH 7.4) was mixed with 9 mL of deionized water followed by the addition of 250 μ L of 20 mM HAuCl⁴. Without shaking, the color of the solution changed from light yellow to pink and finally to turbid blue at room temperature within 30 min.

2.2 Synthesizing thiol-capped CdTe NPs

CdTe NPs were synthesized as described previously [30]. Briefly, 0.985 g (2.35 mmol) of Cd(ClO₄)₂ · 6H₂O was dissolved in 125 mL of water, and 5.7 mmol of thioglycolic acid (TGA) was added under stirring. The pH was adjusted to the appropriate value (11.4–11.6) by adding 1 M NaOH dropwise. The solution was placed in a three-necked flask and deaerated by bubbling in N₂ for 30 min. Under stirring, H₂Te gas that was generated by the reaction of 0.2 g (0.46 mmol) of Al₂Te₃ lumps with 15–20 mL of 0.5 M H₂SO₄ in the N₂ atmosphere was passed through the solution together with slowly flowing nitrogen for 20 min. The CdTe precursors that were formed were converted into CdTe nanocrystals by refluxing the reaction mixture at 100 °C under open air for 20 min with a condenser that was attached to the apparatus.

2.3 Preparing goat anti-bovine IgG conjugated SNP nanoprobes

The SNPs were first modified with 8-mercaptoocatanoic acid (MA) by adding 1.0 mL of MA to 9.0 mL of SNPs to reach a final concentration of 0.5 mM. A self-assembled monolayer (SAM) on the gold surface was formed after incubating the mixture overnight with gentle shaking at room temperature. Next, the mixture was centrifuged (4000 rpm for 15 min at 4°C). The supernatant was decanted and the pellet was rinsed with PBS (0.01 M at pH 7.4). This centrifuging/resuspending step was repeated more than 3 times to remove the unbound MA molecules. The final carboxyl-stabilized Au NPs were concentrated to 5.0 mL. Subsequently, 100 µL of 0.1 M N-hydroxysuccinimide (NHS) was immediately added after activating it with 400 µL of 0.1 M 1-ethyl-3-(3dimethylaminopropyl) carbodiimide (EDC) solution, and the mixture was incubated for 10 min. Afterwards, the goat anti-bovine IgG detection probe was added to the solution to reach a final concentration of 0.5 µM. The mixed solution was incubated for 8 h at room temperature with gentle shaking and the centrifuging/rinsing procedure was repeated three times to remove the unbound goat anti-bovine IgG detection probe. The final pellet was suspended in 5.0 mL of PBS buffer solution (0.01 M, pH 7.4) and stored at 4 °C for further use.

2.4 Expressing and purifying rNcSAG1 and rNcSAS2

The recombinant *N. caninum* proteins, rNcSAG1 and rNcSRS2 were expressed in silkworms by using a bacmid protein expression system and purified with an anti-FLAG affinity resin as described by Dong et al. [31] and Otsuki et al. [32]. As shown in Figure

S1 of supplementary information, the rNcSAG1 and rNcSRS2 genes were amplified by using the genomic DNA of *N. caninum* strain Nc-1 (ATCC no.50843) by the polymerase chain reaction (PCR) and cloned into a pENTR/D/TOPO plasmid (Invitrogen, USA) by employing the topoisomerase reaction. Using this plasmid, we inserted full-length rNcSAG1 or rNcSRS2 genes into pDEST8 by using Gateway technology (Invitrogen, USA) to construct plasmid pDEST-rNcSAG1 or pDEST-rNcSRS2, which were then used to transform *E. coli* BmDH10Bac CP^-Chi^-) [15]. *E. coli* colonies that contained recombinant *Bombyx mori* nucleopolyhedron virus bacmid (rBmNPV-rNcSAG1 or rBmNPV-rNcSAG1 or rBmNPV-rNcSAG

The silkworms expressed biologically active protein that was functionally similar to the native protein because they carried out post-translational modifications, including glycosylation. The recombinant bacmid rBmNPV-rNcSAG1 or rBmNPV-rNcSRS2 (10 µg each) was mixed with one-tenth volume of DMRIE-C (Invitrogen, USA) and incubated at room temperature for over 45 min. Each silkworm on the first day of the fifth instars larvae (Ehime Sansyu Co. Ltd., Ehime, Japan) was injected with 50 µL of the mixture and reared for 5–7 days, after which its hemolymph was collected. Recombinant protein rNcSAG1 or rNcSRS2 was purified from the silkworm larval hemolymph by using anti-FLAG M2 Affinity Gel (Sigma, USA), according to the instructions that were provided by the manufacturer. It was then conjugated with QDs and the product was used to detect anti-rNcSAG1 or anti–SRS2 antibodies in the sera of *N. caninum*-infected cattle.

2.5 Preparation of rNcSAG1- or rNcSRS2-captured CdTe nanoprobes

Pre-synthesized TGA-modified CdTe NPs were washed twice with ethanol. The CdTe NPs were centrifuged at 50,000 rpm for 1 h at 4 °C by using a microultracentrifuge (Himac CS120GXII, Hitachi, Japan) to remove the extra free TGA. Subsequently, 100 μ L of 0.1 M NHS was immediately added after it was activated with 400 μ L of 0.1 M EDC and then it was incubated for 10 min. It was then conjugated with rNcSAG1 or rNcSRS2 by using the same protocol for goat anti-bovine IgG modified SNP nanoprobes. The mixture was incubated for 8 h at room temperature with gentle shaking, washed twice (50,000 rpm for 1 h at 4 °C), and stored in a 1.0 mL PBS solution at 4 °C until further use.

2.6 Detecting antibodies in bovine serum by using SNP-CdTe nanocomposite

Figure S2 (as shown in supplementary information) illustrates the SNP-CdTe nanocomposite that was used to detect ANABs in bovine serum. In a typical experiment, 200 μ L of SNP nanoprobes that had been modified by goat anti-bovine IgG were added to three micro-centrifuge tubes and then 1 μ L of three different types of sample (control, negative, and positive) was added to these three micro-centrifuge tubes, respectively. The control sample was in PBS buffer, the negative sample was the serum of healthy cattle in which no anti-*Neospora* antibodies were present (denoted as negative serum), and the positive sample was the serum of *N. caninum*-infected cattle that contained the target

antibodies ANABs (denoted as positive serum). The exact concentration of the ANABs in the serum that we analyzed was not known since our sensor system was used to directly monitor the non-purified serum from the neosporosis-infected cattle. Finally, 20 µL of CdTe NPs that were modified by rNcSAG1 or rNcSRS2 were added to the above samples and the samples were divided into two groups (rNcSAG1 and rNcSRS2). Nanocomposite sensor experiments were conducted more than 20 times from two batches of sera for each group to collect reproducible and confidential data. The mixture was incubated for 1 h at room temperature to ensure all of the target segments were recognized. Then, an infinite® F500 microplate fluorescence reader (TECAN, Japan) was employed to measure the differences in fluorescence intensity of the QDs probes that were used to detect different serums in the presence of SNPs for the SNP-rNcSAG1-CdTe and SNP-rNcSRS2-CdTe systems. The absorbances of the SNPs were measured using UV-Vis spectroscopy (SCINCO, S310, Korea). The morphologies and sizes of the NPs were characterized using a high-resolution transmission electron microscope (HR-TEM, JEOL, JEM-3010, Japan). Their particle size distribution was monitored by Malvern zeta-sizer Nano S (Malvern Instruments Ltd., ZS Nano, UK) with a detection angle of 173°. All measurements in this study were taken at a temperature of 25 °C. The Nano S uses a 4 mW He-Ne laser operating at a wavelength of 633 nm. The intensity size distributions were obtained from analysis of the correction functions using the Multiple Narrow Modes algorithm in the instrument software. This algorithm is based upon a nonnegative least squares fit. These intensity particle size distributions were converted into number using Mie theory.

3. Results and discussion

3.1 Characterizing protein-conjugated NPs

The quality of a self-assembled monolayer is crucial for nanoprobes, not only to maintain particle stability in highly strengthened ionic buffers but also to protect the surface from non-specific adsorption. MA was used as a linker between the bio-moiety and inorganic material to design a protein-sensor probe because of the excellent properties of the S-Au covalent bond. The MA-stabilized SNPs were conjugated with anti-bovine IgG by using one-pot EDC/NHS coupling. The UV-Vis spectra of unmodified and anti-bovine IgG modified SNPs are shown in Figure 1A. The unmodified SNPs in aqueous solution showed maximum absorption near 617 nm, which is a unique SNP plasmon band. Morphologically, SNPs are hybrids of spheres and branched particles [12,33,34]. According to the theoretical calculations of Hao et al. [35], Au tripods with a tip length of 27 nm exhibit plasmon resonance at \sim 700 nm. The hybridization of \sim 540 nm for spheres (~66 nm) and ~700 nm for branched particles should result in plasmonic absorption peaks between these limits—the experimental value of ~617 nm for the SNPs fell within this range. After modifying the surface, the surface plasmon band of antibovine IgG modified SNPs was red-shifted and was observed at 630 nm. Furthermore, we measured the size distribution curve of SNP and anti-bovine IgG modified SNP which were shown in Figure S5 (A and B) of supplementary information. The results indicated that the size of SNP increased after modification by anti-bovine IgG. Figure 1B shows a PL spectra for the unmodified CdTe NPs, rNcSAG1-, and rNcSRS2- modified CdTe NPs. We observed that the maximum absorption of the unmodified CdTe NPs in aqueous solution was 563 nm. The PL spectra of the rNcSAG1 and rNcSRS2 modified CdTe NPs

were slightly red shift, occurring at 568 and 566 nm, respectively. Their PL spectra and intensities had not broadened, nor were noticeably decreased. Concluding that the biological conjugation process on the CdTe NPs surface, had successfully occurred without any undue aggregation. Moreover, the size of modified CdTe NPs was also changed compare to the unmodified CdTe NPs (See in Figure S5 C, D and E).

On factor, detrimental for carrying out plasmon-induced PL enhancement, is the position of the plasmon and PL band [36]. In our nanocomposite design, >90% of the two bands had overlapped each other. SNP offer a great advantage, rather than using normal (small Au NPs), which generally exhibit a plasmon band near 520 nm. Figures 1C and 1D show TEM images of the SNPs and CdTe NPs after bioconjugation. The micrographs clearly show spiky Au NPs, about 50 nm in size, with approximately 20 branches per NP. The modified CdTe NPs were 5 nm in diameter on average. Therefore, results of UV-Vis and PL spectra and TEM images suggests that the SNPs and CdTe NPs were readily modified by anti-bovine IgG, rNcSAG1, and rNcSRS2.

In order to further confirm the function of anti-bovine IgG modified SNP and rNcSAG1- and rNcSRS2-captured CdTe Nanoprobes, we performed enzyme-linked immunosorbent assay (ELISA) before detecting ANABs. Experimental details and ELISA results are shown in the supplementary information. As shown in Figure S6A, higher signal was observed with anti-bovine IgG modified SNP than SNP, suggesting that anti-bovine IgG successfully modified SNP. The rNcSAG1- and rNcSRS2-captured CdTe gave higher signal than the CdTe which was used as a control. This suggests that the rNcSAG1 or rNcSRS2 was successfully conjugated with CdTe NPs (Figure S6B).

Each biologically conjugated NP was utilized to monitor the ANABs. Electron microscopic analysis was conducted to achieve a better insight on nanoscale clustering via an immunoassay of the nanomaterials. As can be seen in the high-resolution TEM images, the addition of rNcSAG1- and rNcSRS2-CdTe to the solution of anti-bovine IgG-SNP led to the formation of clusters in the positive serum (Fig. 2B and 2D). From these images, it is evident that the QDs and SNPs were close to each other due to the specific interaction between anti-bovine IgG, ANABs, and rNcSAG1 or rNcSRS2. This in turn, induced efficient PL enhancement of the QDs by the SNPs. In contrast, when the anti-bovine IgG-Au NPs were incubated with rNcSAG1- or rNcSRS2-CdTe in the negative serum, the formation of clusters between the QDs and Au NPs was negligible (Fig. 2A and 2C). This clearly supports the notion that externally added complementary target (ANABs) was conjugated with the QDs and SNPs and PL enhancement of the QDs owing to the LSPR of spiky Au NPs.

3.2 Detecting ANABs in bovine sera

The feasibility of using SNP-CdTe NPs nanocomposite as a fluorescence sensor platform for detecting ANABs was shown by using anti-bovine IgG, rNcSAG1, or rNcSRS2. The detection of ANABs could be translated by using the PL signal of the CdTe nanoprobes. When cattle are infected by *N. caninum*, they produce anti-*N. caninum* antibodies. NcSAG1 and NcSRS2 are located on the surface of the *N. caninum* parasite and are highly antigenic [37-39]. Therefore, anti-NcSAG1 and anti-rNcSRS2 antibodies are produced by the adaptive immune system of cattle during the early stage of infection.

By detecting the anti-NcSAG1 or anti-NcSRS2 antibodies through two nanocomposite sensor systems, we could recognize whether or not cattle are infected with *N. caninum*.

Figure 3 shows the differences in the fluorescence intensity of the CdTe QDs probes for detecting different sera in the presence of SNPs by an infinite F500 microplate fluorescence reader at SNP-rNcSAG1-CdTe and SNP-rNcSRS2-CdTe nanoprobes under excitation/emission wavelength of 485/535 nm. The [(F/F₀)-1] values showed that the PL difference that was generated by the sensor platform was directly related to the differentiation between the complementary and mismatched antibodies, where F₀ and F were the fluorescence intensities in the absence and presence of the target, respectively. Figure 3A shows the results for the SNP-rNcSAG1-CdTe system. The PL intensity with PBS was detected to be 20374±1287 A.U., while with negative and positive serum sample, 23019±1172 A.U. and 26769±1327 A.U. were obtained. Compared with the control sample in PBS, the PL intensity increased for the negative and positive serum samples. The fluorescence of CdTe was enhanced by about 31% (p=0.0255) by the addition of the positive serum while 13% (p=0.0724) enhancement was seen for the negative serum. Meanwhile, Figure 3B shows the experimental results for the SNPrNcSRS2-CdTe system. The PL intensity of CdTe in which PBS, negative and positive serum samples were added were 19316±749 A.U., 19806±470 A.U. and 29450±749 A.U., respectively. PL intensity with the positive serum sample was enhanced by 52% (p=0.0036) compared to the negative serum. However, the intensity of the negative serum was similar to that of the control sample to which PBS was added. This is because in the control and negative serum both the SNP and CdTe biosensor probes did not bind without anti-NcSRS2 antibodies, which resulted in the distance between the SNP and CdTe was

not changed. However, in the presence of the positive serum, the anti-NcSRS2 antibodies bound to both the anti-bovine IgG modified SNP and rNcSRS2-CdTe, thereby permitting the appropriate distance to develop between the SNP and QDs. This in turn resulted in enhanced fluorescence emission. The distance between two NPs was considered to be approximately 20–30 nm since two whole IgG type antibodies were used in the immunoassay and the hydrodynamic diameter of IgG is 10–13 nm [40]. Over this range, PL enhancement or quenching can occur effectively between semiconductor and metallic NPs via dipole-dipole induced interactions [41]. Furthermore, in geometric terms, many tips on the spiky NPs possessed hot spots of plasmonic energy that enhanced the effects of adjacent semiconductor NPs [42]. Our results also indicated that anti-bovine IgG modified SNPs can strongly absorb complementary target protein (ANABs) and conjugate with rNcSRS2 modified CdTe NPs to enhance the fluorescence of CdTe NPs very effectively. The binding of rNcSAG1 and rNcSRS2 to the ANABs were also reported by Otsuki *et. al.* with a traditional ELISA, and the results were very similar [32].

In this study, spiky Au NPs caused high plasmon-induced PL enhancement because of many plasmonic hot spots and a high coupling ratio (>90%) between the plasmonic band of SNP and the excitonic band of CdTe NPs. Coupling between excitons and plasmons is another crucial factor for obtaining efficient sensor devices because the coupling effect becomes stronger when the exciton energy is located closer to the plasmon peak. On the other hand, proteins play very important roles in detecting biomoieties. Silkworm-expressed proteins are biologically active and most similar functionally to their native forms because of post-translational modifications, including glycosylation. Through these two specific optical and biological advantages, we constructed two systems for quickly detecting neosporosis in cattle by employing antibovine IgG modified spiky Au NPs and silkworm-recombinant *N. caninum* protein (rNcSAG1 or rNcSRS2). In contrast, in traditional assays such as ELISA, many steps such as blocking to decrease non-specific signals, at least three washing steps after blocking, adding a primary antibody, and then sometimes a secondary antibody are necessary to detect anti-*N. caninum* antibodies. These types of assays are cumbersome and take more than 3 h. However, with our novel assay system, we do not need to perform washing steps, and our assay can be carried out quickly.

The results that were generated by our Au-rNcSAG1-CdTe system were similar to those of the Au-rNcSRS2-CdTe system. This suggests that in both systems, rNcSAG1 and rNcSRS2 on CdTe bound to their complementary antibody (anti-NcSAG1 antibody or anti-NcSRS2 antibody) steadfastly during consecutive self-assembly and bioconjugation. Compared with the rNcSAG1 system, the rNcSRS2 system gave more ideal results i.e., higher enhancement and obvious optical discrimination with respect to the positive and negative samples. This is because rNcSRS2 may have resulted in a better response in the bovine immune system. Even though the concentration and volume of rNcSRS2-modified CdTe NPs was the same in the Au-rNcSRS2-CdTe system, the fluorescence of the free CdTe NPs was scarcely influenced by adding the negative serum in the absence of a target protein. It is likely that the Au-rNcSRS2-CdTe system may have exhibited more specificity than the Au-rNcSAG1-CdTe system. From these proofof-concept results, the system that uses rNcSRS2 will be further developed as a biosensor system to minimize bio-moiety related variants.

All of the above observations suggest that both systems exhibited good reproducibility in distinguishing between complementary and mismatched target proteins. Even though we successfully identified neosporosis-positive sera by using these two systems, the increase in the PL intensity was still low compared with other advantageous conditions such as a high band overlapping ratio, geometrical uniqueness of the plasmonic NPs, and the high specificity of proteins that were produced by silkworms. For example, only 55% fluorescence enhancement was observed for the rNcSRS2 system. More factors should be examined to further enhance the fluorescence. The first factor that should be considered should be the distance between two NPs. In this study, the SNP and CdTe particles were linked by two antibodies (anti-bovine antibody and bovine anti-Neoposra antibody) and they may have been separated by tens of nanometers. Even this distance enhanced the fluorescence signal enough to be able to detect the anti-Neospora antibodies in the sera. Thus, increased enhancement may be obtained by changing the distance between the particles. By employing biotechnological techniques, we also can make small antibody units such as fragments for antigen binding (Fab) that exhibit the same binding affinity as that of an antibody but are only half the molecular weight, or single chain variable regions (scFvs) that are only one-sixth the size of an antibody. In addition, the distance could be lengthened by adding a linker.

4. Conclusions

CdTe QD-SNP nanocomplexes were used successfully as a novel fluorescencesensor platform for detecting designated proteins by immunoassay. SNPs are promising NPs in terms of being an effective universal sensor platform for fluorescence-enhanced detection because of their unique optical and geometrical properties. Both proteins, rNcSAG1 and rNcSRS2 that were expressed from silkworm were linked on CdTe QDs. Then, they were bound to their complementary antibody (anti-NcSAG1 antibody or anti-NcSRS2 antibody) steadfastly during consecutive self-assembly and bio-conjugation, resulting in higher enhancement and obvious optical discrimination with respect to the positive and negative samples. Therefore, since this sensing system is easy to operate, inexpensive, and versatile, with further study on quantitative analysis, it may be incorporated into next generation protein detection tools that will be used to detect the early stages of the disease.

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Figure Captions

Fig. 1 (A) Comparison of UV-Vis spectra for SNPs in aqueous solution and anti-bovine IgG modified SNPs in PBS solution. (B) Comparison of PL spectra for CdTe NPs in aqueous solution, and rNcSAG1 or rNcSRS2 modified CdTe nanoprobes in PBS solution. (C) TEM images of anti-bovine IgG modified SNPs and (D) TEM images of rNcSRS2 modified CdTe NPs.

Fig. 2 TEM image of bioconjugation of CdTe NPs and SNPs by adding negative (A and C) and positive serum (B and D) to the Au-rNcSAG1-CdTe system (A and B) and Au-rNcSRS2-CdTe system (C and D). The CdTe NPs are outlined with guidelines (white circle).

Fig. 3 Fluorescence intensities of CdTe NPs for detecting different sera (PBS, positive, and negative-serum) in the presence of SNPs under excitation/emission wavelengths of 485/535 nm that were produced by using an infinite F500 microplate fluorescence reader. (A) SNP-rNcSAG1-CdTe system, (B) SNP-rNcSRS2-CdTe system, and (*) p < 0.05, (**) p < 0.01.



Zhou et al., Figure 1



Zhou et al., Figure 2



Zhou et al., Figure 3

Supplementary Information

Detection of Anti-*Neospora* Antibodies in Bovine Serum by Spiky Au-CdTe Nanocomplex

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Figure S1. Schematic presentation of the bovine immune system reacting against the *N. caninum* parasite and recombinant parasite protein expression in silkworms.



Figure S2 .Schematic representation of the synthesis of antibody-functionalized SNP and CdTe nanocomposite, and the detection mechanism that the biosensor immunoassay employed to capture targets in control, negative serum, and positive serum samples.



Figure S3. The TEM image of rNcSAG1-modified CdTe NP



Figure **S4**. The TEM image of the mixture of SNP and CdTe before conjugation



Figure S5. the size distribution curves of SNP (A); anti-bovine IgG modified SNP (B); CdTe NPs (C); rNcSAG1 modified CdTe NPs (D); rNcSRS2 modified CdTe NPs (E).

Confirmation of functional conjugation using ELISA

In order to confirm the function of anti-bovine IgG modified SNP and rNcSAG1- and rNcSRS2-captured CdTe Nanoprobes, we performed enzyme-linked immunosorbent assay (ELISA) as follow:

Experimental detail

1. Confirmation of anti-bovine IgG modified SNP

Fifty microliter of SNP or anti-bovine IgG modified SNP were prepared in a 1.5 ml tube and goat bovine IgG-HRP (horseradish peroxidase) (Santa Cruz Biotechnology, CA) was then added with a final concentration of 1 μ g/ml. After incubation at room temperature for 1 h, samples were washed three times by centrifuge at 10,000 rpm for 10 min. HRP was developed with 50 μ L TMBZ substrate solution (100 μ g ml-1 3,3',5.5'tetramethylbenzidine (Sigma) and 0.04% H₂O₂ in 100 mM NaOAc, pH 6.0) for 5-30 min at 25°C. The reaction was stopped by adding 25 μ L 10% H₂SO₄ and the absorbance was read at 450 nm with a reference at 655 nm using a microplate reader Model 680 (Bio-Rad, Tokyo, Japan).

2. Confirmation of rNcSAG1- and rNcSRS2-captured CdTe nanoprobes

Fifty microliter of CdTe, rNcSAG1-CdTe, or rNcSRS2-CdTe were prepared in a 1.5 ml tube and Monoclonal ANTI-FLAG M2-HRP (Sigma) was then added with a final concentration of 1 μ g/ml to probe the FLAG tag fused in protein rNcSAG1 or rNcSRS2. After incubation at room temperature for 1 h, samples were washed three times with a 100K Nanosep Centrfugal Device (PALL, Tokyo, Japan). The samples were recollected with 50 μ L TMBZ substrate solution and developed as described above.

<mark>Results</mark>

As shown in Figure S6A, higher signal was observed with anti-bovine IgG modified SNP than SNP, suggesting that it was successfully conjugated. rNcSAG1-CdTe and rNcSRS2-CdTe gave higher signal than CdTe which was used as a control, suggesting the rNcSAG1 or rNcSRS2 was successfully conjugated with CdTe NPs (Figure S6B).



Figure <mark>S6</mark>. ELISA results for: (A) anti-bovine IgG modified SNP; (B) rNcSAG1- and rNcSRS2-CdTe nanoprobes