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Komikawa, T, Tanaka, M, Tamang, A et al. (3 more authors) (2020) Peptide-Functionalized Quantum Dots for Rapid Label-Free Sensing of 2,4,6-Trinitrotoluene. Bioconjugate Chemistry. ISSN 1043-1802

https://doi.org/10.1021/acs.bioconjchem.0c00117

Research Article

Peptide-Functionalized Quantum Dots for Rapid Label-Free Sensing of 2,4,6-Trinitrotoluene (TNT)

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ABSTRACT

Explosive compounds, such as 2,4,6-trinitrotoluene (TNT), pose a great concern in terms of both global public security and environmental protection. There are estimated to be hundreds of TNT contaminated sites all over the world, which will affect the health of humans, wildlife and the ecosystem. Clearly, the ability to detect TNT in soils, water supplies, and wastewater is important for environmental studies, but also important for security, such as in ports and boarders. However, conventional spectroscopic detection is not practical for on-site sensing because it requires sophisticated equipment and trained personnel. We report a rapid and simple chemical sensor for TNT by using TNT binding peptides which are conjugated to fluorescent CdTe/CdS quantum dots (QDs). QDs were synthesized in the aqueous-phase and peptide was attached directly to the surface of the QDs by using thiol groups. The fluorescent emission from the QDs was quenched in response to the addition of TNT. The response could even be observed by the naked-eye. The limit of detection from fluorescence spectroscopic measurement was estimated to be approximately 375 nM. In addition to the rapid response (within a few seconds), selective detection was demonstrated. We believe this label-free chemical sensor contributes to progress the on-site explosive sensing.

KEYWORDS

Peptide, Quantum dots, Explosive detection, Fluorescence chemical sensor

INTRODUCTION

Explosive detection has become important from the viewpoint of worldwide public security and food safety. 2,4,6-Trinitrotoluene (TNT) is one of the most commonly used nitroaromatic compounds for military purposes. It also causes serious contamination in ground soil and drinking water when leaked from landmines and weapons[1]. To date, various types of laboratory-based studies have been reported to achieve highly-sensitive and specific detection of TNT using conventional analytical methods (e.g. gas-chromatography mass-spectrometry (GC/MS), Fourier-transformed infrared spectroscopy and Raman spectroscopy)[2–6]. However, these techniques are difficult to apply to on-site sensing because they demand time-consuming and costly sample preparation. In addition, the subsequent processes of detection and analysis, require sophisticated equipment as well as trained personnel. For practical uses, especially at the places such as a minefield or security control in an airport, the sensing devices are required to be sensitive, specific, easy-to-use, inexpensive, and rapid. Several novel approaches including fluorescence chemical sensor, surface plasmon resonance (SPR), and electrochemical sensors may potentially be applicable to on-site explosive detection[7–10]. Among these, the fluorescence-based methods are attractive because only easy-handling and simple equipment is required (e.g. a light detector and a light source). The quenching of molecular probe-functionalized metal quantum dots (QDs) caused by energy transfer or electron transfer[11–13] and inner filter effect of carbon QDs or silicon nanoparticles were previously utilized for the explosive detection[14,15]. In addition, as other fluorescence materials, metal organic frameworks or nonporous polymers were also used[16,17]. However, from the viewpoint of the applicability for versatile types of target molecules and the

simplicity of the material preparation, QDs covalently functionalized with target recognition probe are promising materials for the detection.

For the selective entrapment and detection of small analyte, TNT, molecular recognition probes such as antibody-based recombinant proteins, peptides, or molecular imprinted polymers (MIP) were immobilized on the particle surface. In some cases, a pattern analysis technique is used to support the target determination among similar compounds[11–13,18,19]. Although antibody recombinant proteins, such as single-chain variable fragments, could perform specific binding properties to target molecules, they have some shortcomings related to the complicated production process requiring microorganisms or animal cells, and careful storage. Some researchers took advantage of MIP integrated with gold nanoparticles (AuNPs) for TNT detection; or the combination of MIP and DNA aptamers for enhancing both the sensitivity and selectivity[20,21]. In terms of direct binding, ease-of-synthesis, and stability, a chemically synthesized peptide is one of the promising probes for capturing a target molecule[22,23].

In previous studies, we produced an anti-TNT antibody[24] and screened TNT binding peptides from the complementarity-determining regions (CDRs) of the antibody[25]. CDRs are paratope sites in a variable region of antibody and three CDRs each exist in both light chain and heavy chain. Through the comparative TNT binding assay using these six CDRs, the heavy chain CDR3 (HCDR3) peptide was determined to be the strongest binder to TNT. The core 12-mer TNT binding peptide (TNT-BP, ARGYSSFIYWFF) was discovered by the optimization among a series of truncated sequences of HCDR3[25]. This TNT-BP was applied to SPR-based TNT detectors, and sensitive and selective detection of TNT was demonstrated[26,27].

Herein, toward further development of simple and rapid detection system, fluorescence detection system using peptide-functionalized QDs was investigated. A new material (TNT-BP-C@QDs)

for TNT sensing comprising TNT-BP-C (TNT-BP supplemented cysteine at C-terminus) modified CdTe/CdS core/shell QDs was designed and was used as a TNT detector owing to its fluorescence quenching ability in the presence of TNT (Figure 1). We believe this technique encourages the development of rapid, simple, selective and low-cost on-site explosive detection in various fields.

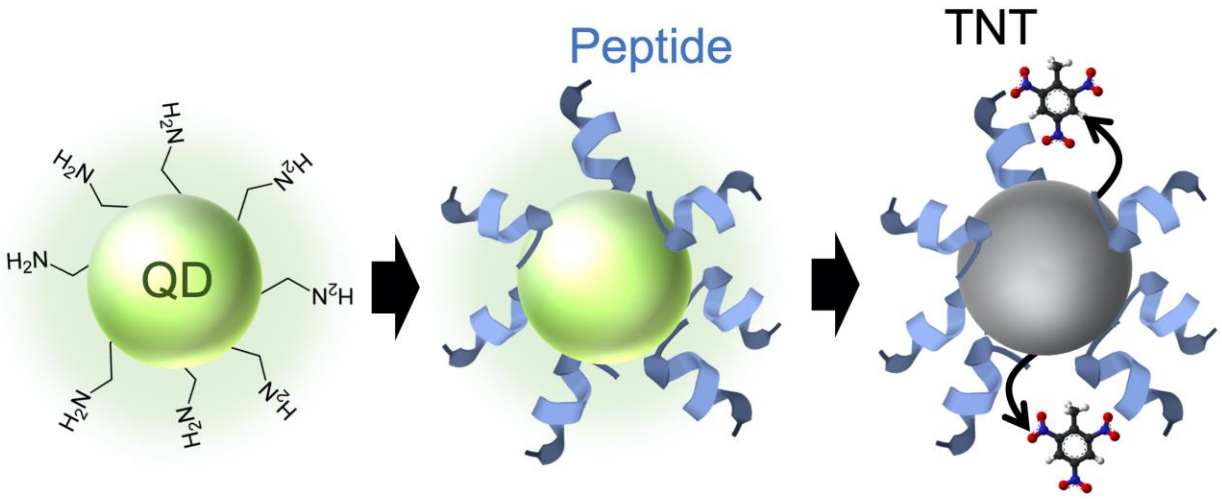

Figure 1 Schematic illustration of fluorescent quench detection by TNT with TNT-BP-C immobilized CdTe/CdS QDs.

RESULTS AND DISCUSSION

Preparation of CdTe/CdS QDs

CdTe/CdS QDs were prepared following aqueous phase method and the fluorescence emission and PLQY (photoluminescence quantum yield) were measured. A high-resolution transmission electron microscope (HR-TEM) image of the synthesized CdTe/CdS QDs is shown in Figure 2a. The histogram of the QD size distribution was obtained by analyzing 200 particles (Figure 2b). The mean and standard deviation of the QD diameters were determined by fitting a normal distribution, which resulted in 3.5±0.5 nm. The fluorescence emission spectra of core CdTe and CdTe/CdS QDs were measured by fluorescence spectroscopy (Figure 2c). Both of them emitted greenish-yellow fluorescence and the peak wavelength was red-shifted (from 546 nm to 551 nm) after shell coverage. The PLQYs of core and core/shell QDs were measured to be 8.5% and 11.3%, respectively. The PLQY of these samples are much lower than typically synthesized QDs for which thioglycolic acid is used as the stabilizing ligand. This suggests that the CdS shell growth using thiourea is not as effective at passivating surface trap states when cysteamine is used to coat the QDs.

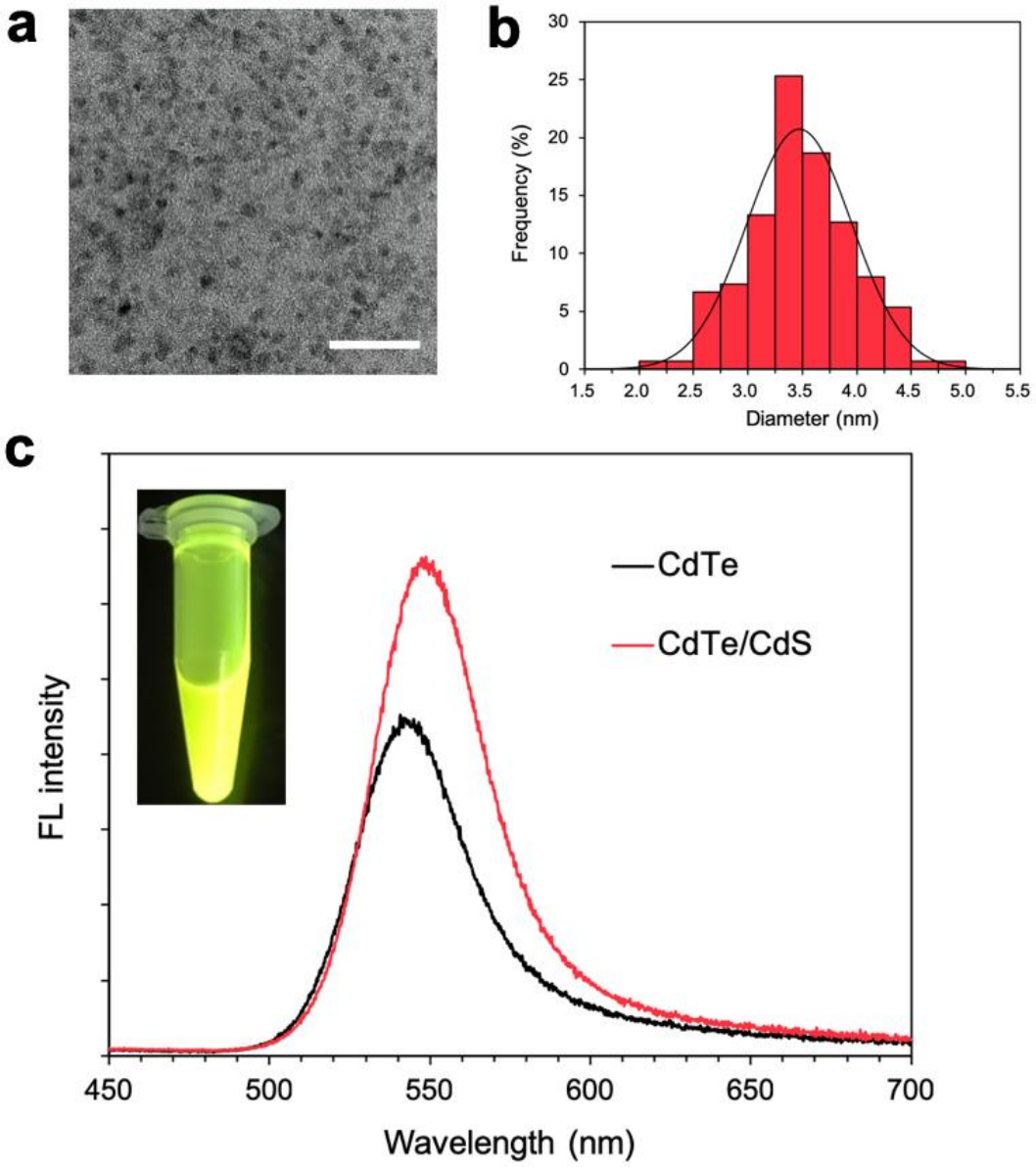

Figure 2 a Microscopic observation of synthesized CdTe/CdS QDs by HR-TEM (scale bar: 30 nm). **b** Size distribution of QDs obtained from TEM images. Mean particle diameter is calculated to be 3.5±0.5 nm. **c** Fluorescence spectra of QDs before and after CdS shell coverage. The inset shows the optical image of CdTe/CdS core/shell QDs under UV-lamp.

Fabrication and characterization of TNT-BP-C@QDs

Prior to the experimental evaluation of TNT binding, the QD functionalization condition was optimized by using different peptide concentration including, peptide:QD = 5:1, 10:1 and 20:1 for overnight. As the result, when the peptides were modified at the condition of 20:1, the aggregation of QDs with lower fluorescent intensity was observed. This is probably because the ligand exchange from cysteamine to cysteine tagged peptide (TNT-BP-C) caused side effect to the particle dispersibility. Based on the evaluation, the peptide modification was conducted at the condition of peptide:QD=10:1 in this study.

TNT-BP-C was synthesized following standard Fmoc-based protocol and immobilized on the surface of CdTe/CdS QDs via the thiol group. After overnight stirring of the mixture of TNT-BP-C and QDs, the peptide modification was confirmed by an increase in mean hydrodynamic diameter (Figure 3a) and a change in zeta potential that was shifted to a higher positive value (Figure 3b). Qualitatively, this can be understood because the peptide is positively charged (pI: 8.8). After peptide binding, the fluorescence emission peak was found to slightly blue-shifted (Figure S1). TNT-BP-C@QDs can be synthesized easily. In addition to the material synthesis process, this detection method is no-need of both expensive equipment and highly trained personnel, thus the sensing system is cost-effective compared to the conventional detection techniques (e.g. GC/MS or HPLC).

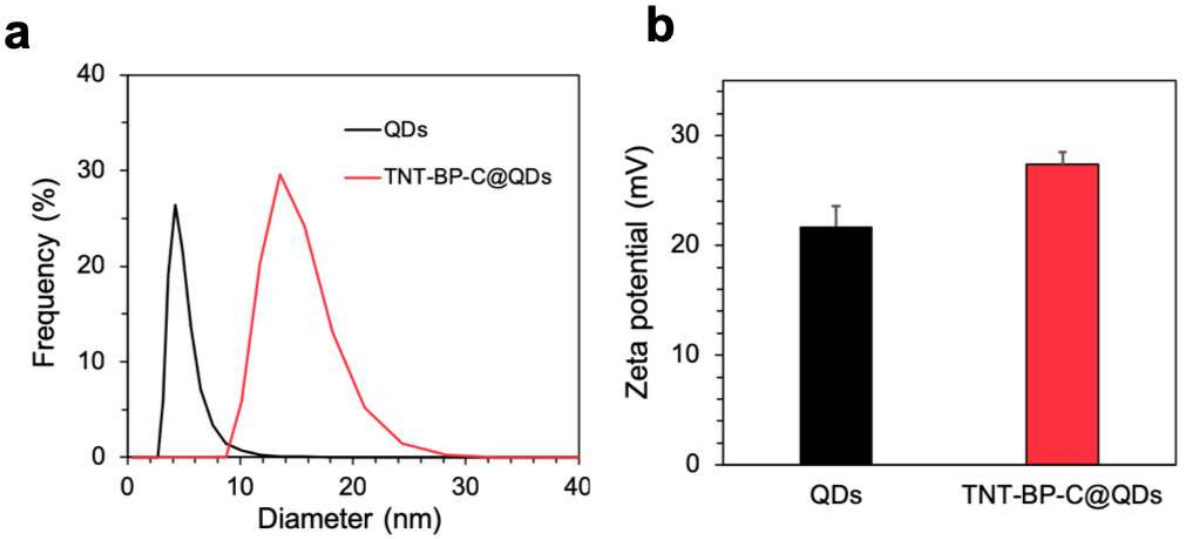

Figure 3 Optical diameter distribution change measured by dynamic light scattering (**a**) and zeta potential shift (**b**) before and after TNT-BP-C immobilization on CdTe/CdS QDs.

Fluorescence quench detection of TNT by TNT-BP-C@QDs

The fluorescent emission intensity of the TNT-BP-C@QDs was found to decrease in response to increasing TNT concentration (Figure 4a). The emission was almost 70% lower than initial intensity at the highest concentration of TNT added (90 μM). Then, the emission intensity, at the peak maxima of 560 nm, was measured against TNT concentration in Figure 4b. For making Stern Volmer plot, $(I_0/I)-1$, which indicates relative quenching of measured intensity (I) from the initial value (I_0), were plotted against TNT concentration, [TNT]. There is a linear relationship (R^2=0.97) in higher range (3-100 μM) of TNT concentration and the quenching coefficient, Ksv is estimated to 3.2×10^4 M^{-1}. This K_{SV} value is comparable with previously reported fluorescence-based explosive sensors (Table S1). Whereas, Stern-Volmer equation does not provide a good fit to the experimental data in lower concentration range. This is possibly because the limited access of the quencher caused the downwards trend of plot. In addition, the fluorescence lifetime measurement was conducted with different TNT concentration. As shown in Figure S2, significant change of fluorescence lifetime from 16.0 to 9.3 ns was observed before and after addition of TNT, which indicates that the TNT molecules chemically interacting with the peptide probe on the QDs surface and caused quench by energy transfer as a dominant quench mechanism. However, as the induction of QDs aggregation is also a possible theory and several mechanisms can be involved at the same time, further study is needed for clarifying the detailed quench mechanism.

To determine the limit of detection, the fluorescence intensity was plotted against TNT concentration (Figure S3). As this graph shows, the limit of detection (LOD) was 375 nM, which is 1.5 times higher than the concentration of donor QDs (250 nM). Although the limit of detection is required to be further improved, it is considered that the LOD depends on the QDs concentration

and there is a possibility to improve LOD by using another QDs with higher PLQY or more sensitive FL detector.

When it comes to the practical uses, the simplicity of detection can be as important as the detection sensitivity. Under the luminescence of UV lamp, the fluorescence quench in response to the amount of TNT was observed by naked-eye in relatively higher concentrations (>50 μM) (Figure 4c). This is promising for easy-to-use and on-site detection at the scene of requiring detection without any other equipment and preparation.

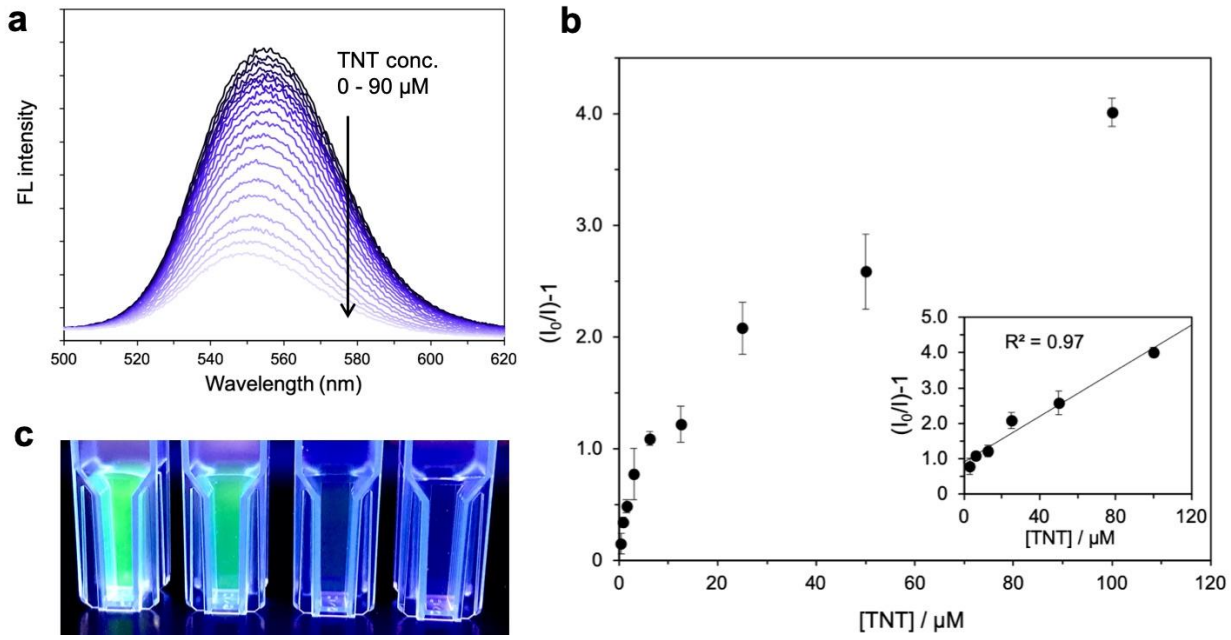

Figure 4 a Representative fluorescence spectra of 100 nM TNT-BP-C@QDs solution in response to increasing concentration of TNT. **b** (I_0/I)-1 is plotted as a function of TNT concentration for confirming the Stern-Volmer equation. The linear dynamic range of Stern-Volmer plot is estimated to 3-100 μM (inlet). **c** The optical image of 250 nM TNT-BP-C@QDs under UV-lamp with 0, 50, 100, 200 μM of TNT, respectively, from left to right.

Rapid and selective detection of TNT

The reaction time is also an important parameter for the chemical sensing, and both shorter response time and longer duration are preferable. The result in Figure 5 shows the quench rate, which stands for 1-(I/I_0), at each time point before and after the injection of TNT to TNT-BP-C@QDs solution. From this result, fluorescence quenching occurred within 1 min and was maintained for at least 30 min after starting reaction. This quick reaction phenomenon is further demonstrated in Supplementary Movie 1, and it is observed that the fluorescence emission was rapidly quenched by addition of TNT sample within a few seconds. Thus, this quench-based TNT detection has a great advantage in rapidity and simplicity for the on-site practical uses.

Furthermore, to evaluate the selectivity of TNT detection by TNT-BP-C@QDs, 2,4-dinitrotoluene (DNT), 2,6-DNT, 2-nitrotoluene (NT), toluene and amyl nitrate were used as TNT analogues. 2,4-DNT, 2,6-DNT and 2-NT were selected as representatives with aromatic ring and different number of nitro groups. Toluene was chosen as a representative of non-nitro aromatic compounds and amyl nitrate was selected as a non-aromatic nitro compound. To evaluate the selectivity property of the developed material, the quench rate for each analyte at the same concentration (100 μM) is shown in Figure 6. The responses to toluene and amyl nitrate were more than 3-fold lower than that to TNT. Whereas, 2,4-DNT, 2,6-DNT and 2-NT showed relatively higher response than toluene. The number and position of nitro groups were suggested to have important role for the binding between TNT-BP-C@QDs and analytes. TNT-BP was considered to have specificity for TNT attributed to both its aromatic and basic amino acid residues, promoting π-electron interaction and electrostatic interaction, respectively. Also this specific binding property was consistent with previous studies using an array-based and SPR-based TNT binding assay [25,26]. Thus, these results affirm that the molecular recognition property of peptide probe has

been maintained on QDs. Although, other military explosives such as picric acid or 1,3,5-Trinitroperhydro-1,3,5-triazine (RDX) should be tested in the future, this material could contribute to selectivity of fluorescence quench-based TNT detection. In addition, through further optimization of TNT detection condition (e.g. QD concentration), the selectivity and sensitivity will be improved in the future.

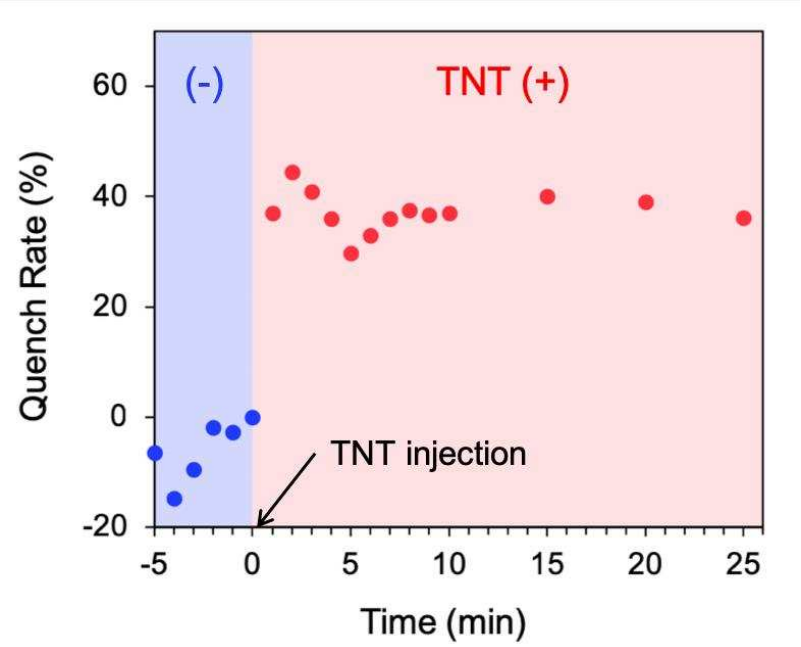

Figure 5 The quench rate, 1-(I/I_0), of 100 nM TNT-BP-C@QDs is plotted as a function of time before and after the injection of 50 μM of TNT.

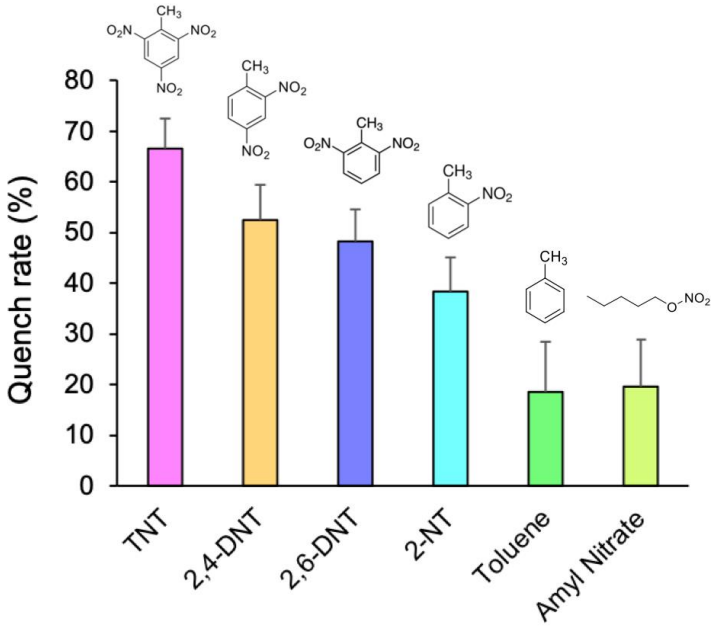

Figure 6 Selectivity assay with 2,4-DNT 2,6-DNT, 2-NT, toluene and amyl nitrate. 100 μM of analyte was added to 250 nM TNT-BP-C@QDs.

The developed technique of a rapid and simple chemical sensor for TNT could be applied to on site explosive detection. Especially, as the TNT samples utilized in this study contains acetonitrile derived from purchased TNT reagent, the wipe test of contaminated surface seems to suite in practical use. For further challenging samples (e.g. soil, air, and water-based environments), various sample dependent optimization of the detection parameters with sample preparation should be addressed. It should be also noted that the development of one-pot QD functionalization process would be able to improve the sensor stability, although the prepared functionalized QD is also stable for more than a week.

Herein, as the peptide function expression on nanoparticle surface was confirmed, the peptide probe would also function on other nanoparticles utilized for other applications. The functionalized magnetic nanoparticle may be useful for the magnetic recovery of TNT from contaminated samples. Furthermore, using plasmonic nanoparticles such as gold and silver nanoparticles, there

is a potential to develop the colorimetric sensor, which can determine by naked eye without any light irradiation. In that case, the fluorescent quenching mechanism by the binding with TNT and peptide-QD should be elucidated.

CONCLUSION

In this research, we demonstrated a rapid, selective and easy-to-use chemosensor material, TNT-BP-C@QDs for TNT. TNT-BP selected from antibody paratope region was chemically synthesized and conjugated CdTe/CdS QDs via thiol ligand exchange reaction without complicated procedure or other materials. The fluorescence emission of this material proportionally decreased depending on the concertation of TNT. The linear dynamic range and the limit of detection were estimated to 3-100 μM and 375 nM, respectively, and simple detection by naked-eye was also attained under UV-lamp irradiation. In addition, the response time was confirmed to be less than 1 min and the specific response to TNT was affirmed. The rapid and selective response property of this material was an attractive candidate for novel chemosensor in various fields including on-site explosive detection.

EXPERIMENTAL PROCEDURES

Reagents

All chemicals studied were analytical grade. TentaGel Resin (capacity: 0.25 mmol g^{-1}) was purchased from Intavis AG (Cologne, Germany). N-Fluorenyl-9-methoxycarbonyl (Fmoc) protected L-amino acids, *O*-benzotriazole-*N,N,N',N'*-tetra-methyl-uronium-hexafluoro-phosphate (HBTU), diisopropylethylamine (DIEA), trifluoroacetic acid (TFA), triisopropylsilane (TIPS), *N,N*-dimethylformamide (DMF) and 20% piperidine solution in DMF were obtained from Watanabe Chemical Ind., Ltd. (Hiroshima, Japan). Dichloromethane (DCM), ethanol, diethyl ether, acetonitrile, 1-methyl-2-pyrrolidone (NMP), toluene and acetonitrile were purchased from FUJIFILM Wako Pure Chemical Corp. (Osaka, Japan). TNT solution solution (1mg mL^{-1} in acetonitrile), 2-propanol, sodium hydrate, cadmium perchlorate hydrate, cysteamine and thiourea were purchased from Sigma-Aldrich (St. Louis, USA). Aluminum telluride were provided from ABSCO Limited (Haverhill, UK) and stored under N_2 atmosphere. Sulfuric acid was purchased from Fisher Scientific (Loughborough, UK). 1,2-Ethanedithiol (EDT), thioanisole, 2,4-Dinitrotoluene (2,4-DNT), 2,6-dinitrotoluene (2,6-DNT), 2-nitrotoluene (2-NT) and amyl nitrate were purchased from Tokyo Chemical Industry Co., Ltd. (Tokyo, Japan). All aqueous solutions were prepared with MilliQ grade deionized water.

Preparation of TNT-BP-C

The sequence of 12-mer TNT binding peptide (TNT-BP, ARGYSSFIYWFF) was previously screened and optimized from the complementarity determining regions of anti-TNT antibody [25]. Cysteine residues were added to the C-terminus of the peptide sequence (TNT-BP-C, ARGYSSFIYWFFC) to enable the immobilization of the peptide to the QD surface via thiol

coupling. The peptide was synthesized following the standard Fmoc-based solid-phase protocol with Respep SL automatic peptide synthesizer (Intavis AG, Germany). Briefly, Fmoc protected amino acid residues were applied to the TentaGel Resin by stepwise for elongation of peptide chain. The synthesized peptide was deprotected by 20% piperidine in DMF and cleaved from the scaffold resin by the cleavage cocktail containing TFA, water, thioanisole, phenol, EDT and TIPS (82.5:5:5:2.5:1). The peptides were precipitated in cold diethyl ether and dissolved in 30% acetonitrile for the storage in a form of freeze-dried powder. Purification was performed by ODS-80TS column (Tosoh Corp., Tokyo, Japan) and the high-performance liquid chromatography (HPLC) system (LC-20AR, CBM-20A, SIL-20AC, CTO-20AC, SPD-20AV, Shimadzu Corp., Kyoto, Japan) before measuring the molecular weight by matrix assisted laser desorption / ionization mass spectrometry (AXIMA-CFRPlus, Shimadzu Corp.) (Figure S1a). The final purity of the peptide was confirmed to be >85% by ODS-100Z column (Tosoh Corp.) and the HPLC system (Shimadzu Corp.) (Figure S1b).

Synthesis and characterization of CdTe/CdS QDs

Core CdTe QDs were prepared following a previously reported protocol [28]. Briefly, 0.985 g of Cd(ClO4)2·6H2O and 0.488 g of cysteamine were dissolved in 100 mL of MilliQ water, and the pH was adjusted to 5.6 by dropwise addition of 1 M sodium hydroxide. The solution was placed into a three-neck flask with N2 bubbling through it to remove oxygen. The core precursor synthesis was initiated by injection of H2Te gas provided by adding 20 mL of 1 M sulfuric acid to 200 mg aluminum telluride powder in separate three-neck-flask which is connected by tubing. CdTe nanocrystal growth was achieved by refluxing at 100 ℃ for 30 minutes. 200 mg of thiourea was dissolved in 1.5 mL MilliQ and added to the QDs solution in three times (0.5 mL each) with an

interval of 1 min under refluxing for CdS shell growth. The QDs were cleaned by dialysis with Mini dialysis kit 8 kDa (GE Healthcare, Chicago, USA) for 2 h just before use.

UV-vis. absorption spectra and photoluminescence emission spectra were recorded by Cary 5000 UV/Vis/NIR (Agilent, Cheadle, UK) and FLS1000 Photoluminescence Spectrometer (Edinburgh Instruments, Edinburgh, UK), respectively. The photoluminescence quantum yield (PLQY) and fluorescence lifetime were also measured by the FLS1000 Photoluminescence Spectrometer with the integrating sphere accessory. To obtain the size distribution, CdTe/CdS QDs were observed by high resolution transmission electron microscope (HR-TEM) FEI Tecnai T20 (FEI company, Oregon, USA). Dynamic Light Scattering (DLS) analysis and zeta potential measurements were performed using Malvin Zetasizer Nano (Malvin, UK).

Preparation of TNT-BP-C@QDs by immobilization of peptide on QDs surface

The concentration of the CdTe/CdS QDs were determined using the previously reported extinction coefficient-based method [29]. The QDs solution was diluted to 1 μM with deionized water and then mixed with 10 μM TNT-BP-C peptide solution before being incubated overnight in the dark at room temperature. The TNT-BP-C peptide, having a thiol group on the side chain of its C-terminus residue, can be immobilized on the CdS shell by thiol ligand exchange reaction. After peptide conjugation, excess peptide molecules were removed with Mini dialysis kit 8 kDa (GE Healthcare, Chicago, USA).

To confirm the peptide modification, the zeta potential and the hydrodynamic diameters were analyzed before, and after, conjugating peptide ligands. For the zeta-potential measurement, TNT-BP-C@QDs were diluted to 100 nM with 1 mM sodium chloride solution. The mean of three experiments is reported.

TNT detection assay by fluorescence quenching using TNT-BP-C@QDs

The fluorescence spectroscopic measurements for obtaining QDs spectra were performed with the sample in a disposable cuvette. TNT-BP-C@QDs were diluted to 100 nM with deionized water and TNT solution was added and mixed by pipetting. Fluorescence emission spectra with 380 nm excitation were acquired by the FLS1000 Photoluminescence Spectrometer. For estimating the limit of detection and selectivity for TNT, POWERSCAN 4 micro plate reader (DS Pharma Biomedical, Osaka, Japan) was also used. The TNT-BP-C@QDs were adjusted to a concentration of 250 nM and added to a 96 well-plate. Fluorescence emission intensity at 560 nm were measured at the excitation wavelength of 380 nm. The optical images of the quenching using concentrations of 0, 50, 100, 200 μM of TNT were taken with 250 nM of TNT-BP-C@QDs under the luminescence of UV-lamp. The optical movie of 1 μM TNT-BP-C@QDs mixed with 200 μM TNT was filmed under UV-lamp by XT-20 camera (Fujifilm, Tokyo, Japan). The response time measurement was also performed in a cuvette and each intensity was measured at each time point for 30 min after the addition of TNT by FLS1000 spectrometer. For the TNT selectivity assay, 100 μM of 2,4-DNT, 2,6-DNT, 2-NT, toluene and amyl nitrate were used as control analytes and performed by POWERSCAN 4 micro plate reader.

ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: XXX.

Fluorescence spectra of synthesized QDs before and after peptide conjugation, Calibration curve for TNT detection, Characterization of TNT-BP-C, Movie of rapid fluorescence quench of TNT-BP-C@QDs

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Notes

The authors declare no competing financial interest.

ACKNOWLEDGMENT

This work was supported by Cross-ministerial Strategic Innovation Promotion (SIP) Program and Impulsing Paradigm Change through Disruptive Technologies (ImPACT) Program (Cabinet Office, Government of Japan) and the international collaboration research projects (the JSPS and the Royal Society) (IEC/R3/170038). This work was also partially sponsored by Grant-in-Aid for Scientific Research (Ministry of Education, Culture, Sports, Science and Technology, Japan) (Grant Numbers: 18H01795, 18K18970 and 18K04848). The authors thank Suzukakedai Materials Analysis Division, Technical Department, Tokyo Institute of Technology, for mass spectrometry analysis. In addition, T. K. thanks the Keidanren for the financial supports under the Keidanren Global Fellowship's scheme.

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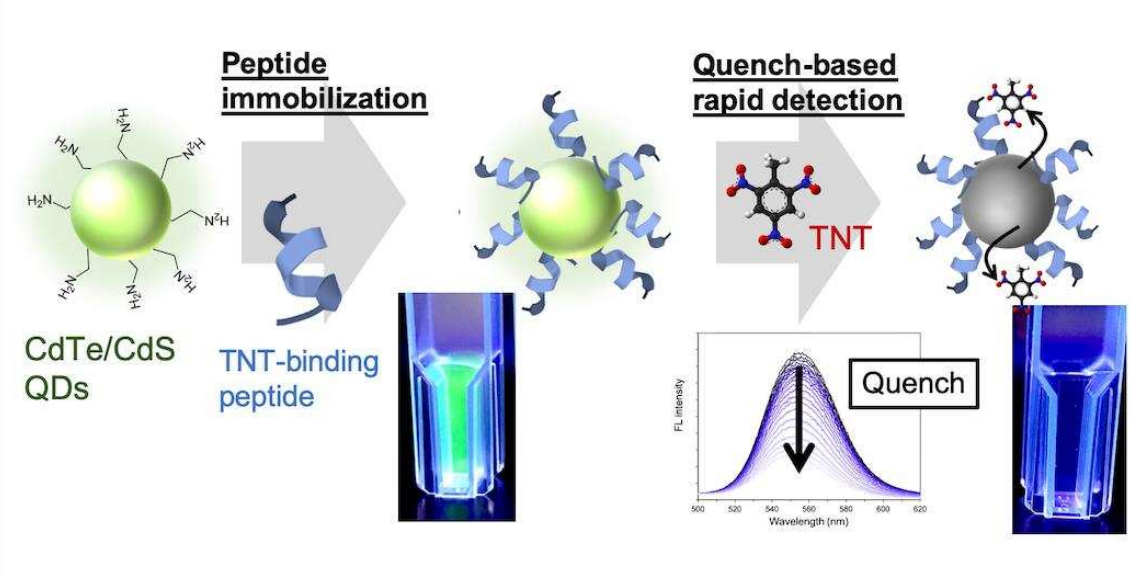

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