Self-Assembled Ti^{4+} @Biospore Microspheres for Sensitive DNA Analysis

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Supporting Information

ABSTRACT: Ti^{4+} can be chemically adsorbed and assembled on the surface of the modified spore to form highly monodispersed Ti^{4+} @spore microspheres. Moreover, we for the first time found that these biomicrospheres exhibit differential affinities toward ssDNA and dsDNA. As a principle-of-proof, we exploited the self-assembled Ti^{4+} @spore microspheres for a hybridization analysis. Interestingly, in the hybridization analysis, residual ssDNA probes are selectively adsorbed on Ti^{4+} @spore microspheres at pH 5.0 and then removed via centrifugation. By taking advantage of this property, the signal-to-noise ratio for DNA analysis was considerably increased by reducing the noise caused by the residual ssDNA probes. The proposed method features easy operation, high specificity, and sensitivity and thus exhibits potential for further applications on DNA biosensing.

KEYWORDS: spore, biomaterials, Ti^{4+} , adsorption, DNA analysis

1. INTRODUCTION

DNA biosensors are effective tools for disease diagnosis, drug screening, DNA damage detection, and forensic analysis^{1,2} because of their simplicity, rapidity, sensitivity, and specificity.^{3–6} By taking advantage of the hybridization reaction, molecular beacons (MBs) are often used for DNA analysis. However, the applications of MBs in common biosensing approaches are limited by their high cost and requirement for fluorophore–quencher pair optimization, sophisticated probe synthesis, and purification.^{7,8} To overcome these limitations, a range of nanomaterials were synthesized, including single-walled carbon nanotubes,^{8,9} graphene oxides,^{10–13} metal oxides,^{14–18} and transition-metal dichalcogenides,^{19–22} as a platform for DNA analysis. These nanomaterials can adsorb ssDNA via the van der Waals force, hydrophobic interactions, hydrogen bonding, and π – π stacking and possess an ultrahigh quenching efficiency for a wide spectrum of fluorescent dyes because of the prominent nanoscale-surface energy-transfer effect [e.g., Förster resonance energy transfer (FRET)]. Excessive fluorophore-labeled probes are adsorbed on the surface of the nanomaterials, and the fluorophores are quenched through FRET because of their close proximity to the nanomaterials. Adding these nanomaterials into the DNA hybridization system can effectively increase the signal-to-noise ratio (SNR). However, the preparation of these nanomaterials

is often very complicated and undergoes multiple exquisite steps. To the best of our knowledge, these methods when performed without signal amplification^{8–12,18–25} have detection limits ranging from nanomolar to picomolar levels because of their incomplete quenching of the nanomaterials. Moreover, the use of nanomaterials may lead to secondary pollution because they cannot be easily separated from the reaction system through traditional techniques, such as filtration and centrifugation.^{26–32} Therefore, eco-friendly and low-cost techniques with reduced noise and enhanced sensitivity were highly desired for the detection of nucleic acid with ultralow concentrations.

In this study, we demonstrated a simple approach for the preparation of new DNA-affinity microspheres and further developed a novel label-free fluorescent DNA biosensor with an enhanced SNR (Scheme 1). In the proposed strategy, we first prepared a class of bioderived spore-based highly monodispersed microspheres (SMMs) using *Bacillus* spores according to our previously reported method.^{33–35} After undergoing ultrasonication and trypsin-based protein removal, these SMMs can show some exposed natural functionalities, such as

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Scheme 1. Schematic of Ti^{4+} @Spore Synthesis (A) and Ti^{4+} @Spore-Based DNA Biosensor (B)

carboxylic, amino, and hydroxyl groups, which are potentially available to carry Ti^{4+} ions through self-assembly to form Ti^{4+} @spore microspheres. Second, after the DNA hybridization reaction, the unhybridized ssDNA probes with high affinity were adsorbed onto the surface of the metal ion-based affinity materials by using Ti^{4+} @spore microspheres as a model. The probes were then separated from a hybridization system through centrifugation. The dsDNA formed via ssDNA probes hybridized with complementary DNA targets could not be adsorbed on Ti^{4+} @spore microspheres because of weak affinity and retained in the reaction solution. As a result, the SNR considerably improved after the addition of the fluorescent dye SYBR Green I because of the minimal noise, leading to a high sensitivity for the detection of DNA targets. The proposed DNA biosensors demonstrated limits of detection as low as the femtomolar level and sequence specificity of single-mismatch discrimination. This study on Ti^{4+} @spore-based DNA biosensors provides new insights into the sensor design and in increasing the diagnostic capacity.

2. EXPERIMENTAL SECTION

2.1. Materials. *Bacillus amyloliquefaciens* was provided by the State Key Laboratory of Agricultural Microbiology, Huazhong Agricultural University (Wuhan, China). SYBR Green I and DNA oligonucleotides were purchased from Shanghai Sangon Biological Engineering Technology and Services Co., Ltd. (Shanghai, China). The DNA sequences are listed in the Supporting Information in Table S1. Trypsin and nucleobases were purchased from Sigma-Aldrich Shanghai Trading Co., Ltd. (Shanghai, China). Titanium sulfate and other reagents were from Sinopharm Chemical Reagent Co., Ltd. (Shanghai, China). All reagents were of analytical grade and used without further purification. Ultrapure H_2O (18.2 $\text{M}\Omega$ cm) was produced using a model Cascada IX laboratory ultrapure H_2O system (Pall Co., Ltd., Washington, NY, USA).

2.2. Apparatus. Spores were ultrasonicated with a Sonifier Sonicator 450A (Branson Ultrasonics, Danbury, CT, USA). A Platform 50 constant-temperature (Shanghai Zhicheng Analytical Instrument Manufacturing Co., Ltd., Shanghai, China) instrument was used as a shaking incubator. The pH of all buffer solutions was measured by a PB-10 pH meter (Sartorius, Goettingen, Germany). Centrifugation was performed by a Sigma 1-14K refrigerated centrifuge (Sigma, Osterode am Harz, Germany). The Fourier transform infrared (FTIR) spectra in the range of 4500–250 cm^{-1} were recorded using an FTIR spectrometer (330 FTIR, Thermo Fisher Scientific, Waltham, MA, USA) in KBr-pressed disks. X-ray photoelectron spectroscopy (XPS) was performed on a VG MultiLab 2000 spectrometer (Thermo Fisher Scientific, Waltham, MA, USA) using monochromatic Al $K\alpha$ radiation as the X-ray source for excitation.

The DY CZ-24DN electrophoresis system was obtained from Liuyi Instrument Factory (Beijing, China). An Alphamager Mini gel imaging system was purchased from ProteinSimple (San Jose, CA, USA). A Fluoroskan Ascent FL Microplate reader (Thermo Fisher Scientific, Waltham, MA, USA) was used to measure fluorescence intensity. An RF-5301PC fluorescence spectrophotometer (Shimadzu, Kyoto, Japan) equipped with 1 cm quartz cells was used for the fluorescence spectrum.

2.3. Preparation of Ti^{4+} @Spore Microspheres. Spores were cultured and treated by our previously reported method.³⁴ Briefly, *B. amyloliquefaciens* (CCTCC AB 2013062) strains were grown on a lysogeny broth agar solid medium at 37.0 $^{\circ}\text{C}$ for 7 days to promote extensive sporulation and the autolysis of vegetative cells. Spores were then collected from plates and washed five times with deionized water by centrifugation (11 000 rpm) to remove vegetative cell debris. These spores were successively treated using sonicator, trypsin buffer (1.00% trypsin in phosphate-buffered saline, pH 7.6) on a shaking incubator (160 rpm, 37.0 $^{\circ}\text{C}$, overnight), and decoating buffer (0.10 mol L^{-1} NaCl, 0.10 mol L^{-1} NaOH, 1.00% sodium dodecyl sulfate, and 0.10 mol L^{-1} dithiothreitol) and were then inactivated using a high-pressure steam sterilizer (0.1 MPa, 121.0 $^{\circ}\text{C}$, 30.0 min). The number of the bioderived SMMs was determined by direct counting with a Burkert chamber under an optical microscope and adjusted to 2.00×10^9 particles mL^{-1} .

Ti^{4+} @spore microspheres were simply prepared by incubating SMMs with 100.00 mmol L^{-1} Ti^{4+} in sodium acetate buffer (25.00 mmol L^{-1} NaAc, 25.00 mmol L^{-1} HAc, and 250.00 mmol L^{-1} NaCl, pH 5.0) on a shaking incubator (100 rpm, 37.0 $^{\circ}\text{C}$, 40.0 min). These microspheres were then washed with sodium acetate buffer to remove the excessive Ti^{4+} ions and resuspended in sodium acetate buffer before further use.

2.4. Adsorption of the Phosphate Group on Ti^{4+} @Spore Microspheres. NaH_2PO_4 (1 mmol L^{-1}) was incubated with 2.00×10^9 particles mL^{-1} Ti^{4+} @spore microspheres in pH 5.0 sodium acetate buffer at room temperature for 120 min. After centrifugation at 11 000 rpm for 2 min, the supernatants were discarded, and the Ti^{4+} @spore microspheres were used for characterization (FTIR and XPS).

2.5. Adsorption of DNA on Ti^{4+} @Spore Microspheres. dsDNA and ssDNA samples were prepared by the hybridization of DNA probes and targets (Table S1) and the selection of DNA probes, respectively. dsDNA (1.0 $\mu\text{mol L}^{-1}$) and ssDNA (1.0 $\mu\text{mol L}^{-1}$) were incubated with Ti^{4+} @spore microspheres at the final concentration of 6.00×10^8 particles mL^{-1} in pH 5.0 sodium acetate buffer for 10 min at room temperature. Then, all solutions were centrifuged at 11 000 rpm, 4 $^{\circ}\text{C}$ for 2 min, and the supernatants were stored for gel electrophoresis. By contrast, microsphere-free DNA samples were also prepared. Each DNA sample (5 μL) was loaded into a 15% nondenaturing polyacrylamide gel electrophoresis (PAGE) gel, and electrophoresis analysis was performed in 1 $\times$ TBE buffer at room temperature, 100 V for 100 min. Then, gels were stained with SYBR Green I and photographed with an AlphaImager Mini gel imaging system.

Figure 1. Characterization of SMMs and Ti^{4+} @spore microspheres. (A) FTIR spectra of SMMs and Ti^{4+} @spore. (B) XPS of SMMs and Ti^{4+} @spore (B1), Ti 2p (B2), O 1s (B3), and N 1s (B4).

The role of phosphate backbones and nucleobases in the DNA adsorption process was investigated using displacement experiments. To test the interaction between phosphate backbones on DNA and microspheres, the displacement experiments of the phosphate group were performed as follows: $0.50 \mu\text{mol L}^{-1}$ dsDNA and ssDNA were incubated with 3.00×10^8 particles mL^{-1} Ti^{4+} @spore microspheres in pH 5.0 sodium acetate buffer for 10 min. Then, NaH_2PO_4 at the final concentration ranging from 0 to 500 mmol L^{-1} was added into the solutions and incubated for 10 min. After centrifugation at 11 000 rpm, 4°C for 2 min, the supernatants were incubated with SYBR Green I at the final concentration of 1.00×10^{-5} for 10 min, and their fluorescence intensities were recorded by using a microplate reader. The DNA adsorption rate was calculated by $(F_0 - F)/F_0$, where F_0 represents the fluorescence intensity of DNA samples without adsorption and F represents the fluorescence intensity of the supernatants containing the residual DNA after adsorption.

To test the interaction between nucleobases on DNA and microspheres, the displacement experiments of nucleobases were adopted. The displacement experiments of nucleobases (adenine, thymine, and cytosine) at the final concentration ranging from 0 to $50 \mu\text{mol L}^{-1}$ were implemented by the procedures similar to those of phosphate groups. Guanine was not used in nucleobase displacement experiments because it was needed to be dissolved in strong acid solutions, affecting DNA adsorption by Ti^{4+} @spore microspheres.

The affinities of Ti^{4+} @spore microspheres toward dsDNA and ssDNA were evaluated through isothermal adsorption experiments. The adsorption kinetics of DNA were performed as follows: 1 mL of 2.00×10^8 particles mL^{-1} Ti^{4+} @spore microspheres (0.11 mg mL^{-1}) was mixed with 1 mL $0.50 \mu\text{mol L}^{-1}$ dsDNA (molecular weight 12 234.1) and ssDNA (molecular weight 3157.1). The mixtures were incubated at room temperature for 0–120 min. After centrifugation at 11 000 rpm for 2 min, the supernatants were added with $1 \times$ SYBR Green I prior to the measurement of the fluorescent intensity by using a microplate reader.

The adsorption thermodynamics of DNA were performed as follows: 1 mL of 2.00×10^8 particles mL^{-1} Ti^{4+} @spore microspheres (0.11 mg mL^{-1}) was mixed with 1 mL dsDNA ($0.1\text{--}0.6 \mu\text{mol L}^{-1}$, molecular weight 12 234.1) and ssDNA ($0.25\text{--}2.5 \mu\text{mol L}^{-1}$, molecular weight 3157.1). The mixtures were incubated at room

temperature for 120 min. After centrifugation at 11 000 rpm for 2 min, the supernatants were added with $1 \times$ SYBR Green I prior to the measurement of the fluorescent intensity by using a microplate reader.

2.6. Discrimination of dsDNA and ssDNA by Ti^{4+} @Spore Microspheres. The ability of Ti^{4+} @spore microspheres to discriminate dsDNA and ssDNA was analyzed by gel electrophoresis. dsDNA and ssDNA samples were prepared by the hybridization of DNA probes and targets (Table S1) and the selection of DNA probes, respectively. DNA mixture samples [containing dsDNA ($0.5 \mu\text{mol L}^{-1}$) and ssDNA ($0.5\text{--}5.0 \mu\text{mol L}^{-1}$) in the concentration ratios of 1:1, 1:5, 1:8, and 1:10] were incubated with Ti^{4+} @spore microspheres at the final concentration of 6.00×10^8 particles mL^{-1} in pH 5.0 sodium acetate buffer for 10 min at room temperature. Then, all solutions were centrifuged at 11 000 rpm, 4°C for 2 min, and the supernatants were stored for gel electrophoresis. By contrast, microsphere-free DNA samples were also prepared. Each DNA sample ($5 \mu\text{L}$) was loaded into a 15% nondenaturing PAGE gel, and electrophoresis analysis was performed in $1 \times$ TBE buffer at room temperature, 100 V for 100 min. Then, gels were stained with SYBR Green I and photographed with an AlphaImager Mini gel imaging system.

2.7. DNA Analysis. The DNA probes ($10.00 \mu\text{L}$, $100.00 \text{ nmol L}^{-1}$) were hybridized with DNA targets ($10.00 \mu\text{L}$) at different concentrations for 30.0 min in $160.00 \mu\text{L}$ sodium acetate buffer. The products were incubated with Ti^{4+} @spore microspheres ($10.00 \mu\text{L}$, 2.00×10^9 particles mL^{-1}) and the fluorescent dye SYBR Green I ($10.00 \mu\text{L}$, 5.00×10^{-5}) for 10.0 min. The mixture was then centrifuged (11 000 rpm) to remove the excess ssDNA probes. The dsDNA remaining in the supernatant was measured using a Fluoroskan Ascent FL microplate reader (excitation at 485 nm, emission at 538 nm, and scaling factor 10/10).

3. RESULTS AND DISCUSSION

3.1. Adsorption of Ti^{4+} on SMMs. First, we mixed inorganic Ti^{4+} with biospores under very mild conditions. The adsorption of Ti^{4+} on SMMs was investigated using FTIR, XPS, scanning electron microscopy (SEM), and energy-dispersive X-ray spectroscopy (EDX). FTIR spectra presented the surface

Figure 2. Adsorption of the phosphate group on $\text{Ti}^{4+}\text{@spore}$ microspheres. (A) XPS of $\text{Ti}^{4+}\text{@spore}$ microspheres and $\text{Ti}^{4+}\text{@spore}$ after the adsorption of the phosphate group ($\text{P@Ti}^{4+}\text{@spore}$). (A1) $\text{P@Ti}^{4+}\text{@spore}$; (A2) P 2p; (A3) C 1s; (A4) N 1s; (A5) O 1s; and (A6) Ti 2p. (B) FTIR spectra of $\text{Ti}^{4+}\text{@spore}$ and $\text{P@Ti}^{4+}\text{@spore}$ microspheres.

characteristics of SMMs and $\text{Ti}^{4+}\text{@spore}$ microspheres (Figure 1A), indicating the presence of carboxyl groups (3282.75 , 1651.02 , and 1319.27 cm^{-1}) and amino groups (1651.02 , 1541.08 , and 1203.87 cm^{-1}) on SMMs. An apparent wave-number shift of 3.86 cm^{-1} in 1651.02 cm^{-1} ($\text{C}=\text{O}$) and a weak shift of 0.71 cm^{-1} in 1203.87 cm^{-1} ($\text{C}-\text{N} + \text{N}-\text{H}$) were found after the SMMs were coated with Ti^{4+} ions.

Ti^{4+} ions adsorbed on SMMs were further investigated by XPS (Figure 1B1). The XPS of $\text{Ti}^{4+}\text{@spore}$ microspheres exhibited two special peaks with the binding energies of 458.65 and 464.50 eV , belonging to $\text{Ti } 2p_{3/2}$ and $\text{Ti } 2p_{1/2}$ electrons, respectively (Figure 1B2). Two clear peak changes occurred from SMMs to $\text{Ti}^{4+}\text{@spores}$, which were 1.00 eV at 531.25 eV (O element, Figure 1B3) and 1.02 eV at 399.50 eV (N element, Figure 1B4).

The SEM image (Figure S1A) indicated that $\text{Ti}^{4+}\text{@spore}$ microspheres possessed good homogeneity and dispersity. The EDX elemental mapping (Figure S1B) demonstrated that the elements C, N, O, and Ti were homogeneously distributed throughout the whole microspheres. Combining with those of

the FTIR, XPS, SEM, and EDX, these results revealed that Ti^{4+} ions were chemisorbed on the surface of SMMs through carboxyl and amino groups.

3.2. Adsorption of the Phosphate Group on $\text{Ti}^{4+}\text{@Spore}$ Microspheres. Although Ti^{4+} can be chemically adsorbed on the biospore, it is not known whether these Ti^{4+} ions on the surface of the self-assembled microspheres are available for interacting with phosphate-containing biomolecules. To this purpose, we characterized these microspheres using XPS and FTIR. Figure 2A1 presents the total XPS spectra of $\text{Ti}^{4+}\text{@spore}$ microspheres after the adsorption of the phosphate group ($\text{P@Ti}^{4+}\text{@spore}$). $\text{Ti } 2p_{1/2}$ and $\text{Ti } 2p_{3/2}$ in both $\text{Ti}^{4+}\text{@spore}$ and $\text{P@Ti}^{4+}\text{@spore}$ microspheres (Figure 2A2) were present at the binding energies of 464.18 and 458.51 eV , respectively, which were consistent with the results of previous reports.³⁶ P 2p binding energy (Figure 2A3) in the $\text{P@Ti}^{4+}\text{@spore}$ was observed at 133.42 eV corresponding to phosphorus in a pentavalent-oxidation state (P^{5+}), suggesting that no $\text{Ti}-\text{P}$ bonds occurred in which the characteristic binding energy was at 128.60 eV .³⁷ The O 1s (Figure 2A4) in

Figure 3. Adsorption of DNA on Ti⁴⁺@spore microspheres (A) 15% nondenaturing PAGE of dsDNA and ssDNA before and after DNA adsorption by Ti⁴⁺@spore microspheres. Lane M, DNA marker; lanes 1 and 3 were dsDNA and ssDNA, respectively, before adsorption by Ti⁴⁺@spore microspheres; lanes 2 and 4 were the supernatants of dsDNA and ssDNA, respectively, after adsorption by Ti⁴⁺@spore microspheres and centrifugation. Conditions: 1.00 μmol L⁻¹ dsDNA, 1.00 μmol L⁻¹ ssDNA, and 6.00 × 10⁸ particles mL⁻¹ Ti⁴⁺@spore microspheres were used. (B) Displacement experiment of the phosphate group. Conditions: NaH₂PO₄ ranging from 0 to 500 mmol L⁻¹, 0.50 μmol L⁻¹ dsDNA, 0.50 μmol L⁻¹ ssDNA probes, 3.00 × 10⁸ particles mL⁻¹ Ti⁴⁺@spore microspheres, 1.00× SYBR Green I, and 25.00 mmol L⁻¹ sodium acetate buffer, pH 5.0. (C) Displacement experiment of nucleobases. Conditions: Nucleobases ranging from 0 to 50 μmol L⁻¹, 0.50 μmol L⁻¹ dsDNA, 0.50 μmol L⁻¹ ssDNA probes, 3.00 × 10⁸ particles mL⁻¹ Ti⁴⁺@spore microspheres, 1.00× SYBR Green I, and 25.00 mmol L⁻¹ sodium acetate buffer, pH 5.0. The error bars represent standard errors (SE) of the means. The data represent mean ± SE (*n* = 3).

Ti⁴⁺@spore microspheres consisted of three peaks at 529.66, 531.05, and 532.24, which were Ti–O, Ti–O–H, and C–O/C=O, respectively.^{38,39} While the atom % of O 1s in the P@Ti⁴⁺@spore at 531.05 eV clearly increased from 28.35 to

37.60%, which might be related to O in Ti–O–P and P=O.³⁹ This finding could be explained by the occurrence of a ligand exchange with surface OH groups to form the Ti–O–P link, which means that the contribution of the Ti–O–H bond was replaced by the contribution of Ti–O–P and P=O bonds.³⁹ Therefore, the peak intensity was supposed to increase. C 1s in both Ti⁴⁺@spore and P@Ti⁴⁺@spore microspheres (Figure 2A5) was deconvoluted to three peaks at 284.58, 286.12, and 288.1 eV, which were C–C or C–H, C–O, and C=O, respectively.⁴⁰ N 1s (Figure 2A6) in Ti⁴⁺@spore microspheres consisted of two peaks at 399.78 and 401.77 eV, which were O–Ti–N and –NH₃⁺, respectively.^{41,42} The former was probably formed by the nucleophilic substitution reaction between the amino group and Ti⁴⁺,^{42,43} and the latter attributed to protonated amine groups on the surface of the spore.⁴⁴ Although the peak of N 1s in the P@Ti⁴⁺@spore at 401.77 disappeared, this peak is assigned to the electrostatic interaction between –NH₃⁺ and the phosphate group.^{45,46}

The FTIR spectra of Ti⁴⁺@spore microspheres and P@Ti⁴⁺@spores are shown in Figure 2B. The spectrum of Ti⁴⁺@spore microspheres indicated the presence of hydroxyl groups (3290.46, 1651.02, and 1419.57 cm⁻¹) and amino groups (1651.02, 1541.08, and 1207.40 cm⁻¹), which were consistent with the results (Ti–O–H and –NH₃⁺ on the surface of Ti⁴⁺@spore microspheres) of XPS. While the spectrum of P@Ti⁴⁺@spore indicated that after the adsorption of the phosphate group apparent wavenumber shifts of 3.87 cm⁻¹ at 1419.57 cm⁻¹ (–OH) and 27.01 cm⁻¹ at 1207.40 cm⁻¹ (–NH₃⁺) occurred, the variation of a broad peak at 900–1200 cm⁻¹ corresponding to the P–O bond was observed.^{39,47} Nevertheless, the variation of a broad peak at 1300–1400 cm⁻¹ corresponding to the P=O bond was not achieved,³⁷ indicating that the change of O 1s at 531.05 eV in XPS spectra was related to O in Ti–O–P but not P=O. These results, combined with those of XPS spectra, revealed that the phosphate group was adsorbed on the surface of Ti⁴⁺@spore microspheres through the formation of the Ti–O–P bond and the weak electronic interaction between –NH₃⁺ and the phosphate group.

3.3. Adsorption of DNA on Ti⁴⁺@Spore Microspheres.

We further examined the possibility of the Ti⁴⁺@spore microspheres binding to DNA. Both dsDNA and ssDNA before and after DNA adsorption by Ti⁴⁺@spore microspheres were investigated using PAGE. As shown in Figure 3A, bands belonging to dsDNA (lane 1) and ssDNA (lane 3, indicated by the red box) were observed. After treatment with Ti⁴⁺@spore microspheres, bands of both DNA (lanes 2 and 4) were weakened, especially the band of ssDNA which was nearly absent (indicated by the red box). These results indicated that Ti⁴⁺@spore microspheres worked well in the DNA adsorption.

The role of phosphate backbones and nucleobases in the DNA adsorption process was investigated using displacement experiments according to previous reports.^{8–25,48,49} As shown in Figure 3B,C, the DNA adsorption onto the surface of Ti⁴⁺@spore microspheres was significantly displaced by phosphate groups rather than free nucleobases. These results revealed that phosphate backbones played an important role in the interaction between DNA and microspheres.

As shown in Figure 4A, the adsorption equilibrium of both dsDNA and ssDNA was achieved in about 40 min. The adsorption capacity (*Q*, mg g⁻¹) was calculated according to the following equation

Figure 4. Isothermal adsorption experiments of DNA. (A) Adsorption kinetics of dsDNA (A1) and ssDNA (A2) onto Ti⁴⁺@spore microspheres. Conditions: 2.00×10^8 particles mL⁻¹ Ti⁴⁺@spore microspheres, $0.50 \mu\text{mol L}^{-1}$ dsDNA, $0.50 \mu\text{mol L}^{-1}$ ssDNA, and $25.00 \text{ mmol L}^{-1}$ sodium acetate buffer, pH 5.0. (B) Adsorption thermodynamics of dsDNA (B1) and ssDNA (B2) onto Ti⁴⁺@spore microspheres. Conditions: 2.00×10^8 particles mL⁻¹ Ti⁴⁺@spore microspheres, 120 min adsorption time, $25.00 \text{ mmol L}^{-1}$ sodium acetate buffer, pH 5.0. The error bars represent SE of the means. The data represent means $\pm$ SE ($n = 3$).

$$Q = (C_0 - C_e)V/m$$

where C_0 (mg mL⁻¹) is the initial DNA concentration, C_e (mg mL⁻¹) is the final DNA concentration in the supernatant, V (mL) is the total volume of the mixture solution, and m (g) is the weight of Ti⁴⁺@spore microspheres. The adsorption kinetics of DNA were fitted by a pseudo-second-order kinetic model

$$Q_t = (K_2 Q_e^2 t) / (1 + K_2 Q_e t)$$

where K_2 (g mg⁻¹ min⁻¹) is the rate constant, Q_t (mg g⁻¹) is the adsorption capacity of DNA on the surface of the adsorbent at time t (min), and Q_e (mg g⁻¹) is the equilibrium adsorption capacity. The similar adsorption kinetic constant of dsDNA ($K_2 = 0.045 \text{ g mg}^{-1} \text{ min}^{-1}$) and ssDNA ($K_2 = 0.051 \text{ g mg}^{-1} \text{ min}^{-1}$) revealed that they had similar adsorption kinetics. The short adsorption equilibrium time and fast adsorption kinetics apparently benefited the rapid DNA analysis. The adsorption data of both dsDNA ($R^2 = 0.947$) and ssDNA ($R^2 = 0.965$) on Ti⁴⁺@spore microspheres were fitted well with the pseudo-second-order kinetic model.

The DNA adsorption of Ti⁴⁺@spore microspheres was fitted well by the Langmuir model and the Freundlich model, indicating that the adsorption process was monolayer chemisorption with more than one type of interaction between the DNA and the surface of Ti⁴⁺@spore microspheres (Figure 4B). The adsorption capacity (Q , mg g⁻¹) was calculated as above. The Langmuir model and the Freundlich model were listed as follows

$$Q = K_L Q_m C_e / (1 + K_L C_e)$$

where K_L is the Langmuir constant that directly relates to the adsorption affinity (L mg⁻¹), Q_m is the maximum adsorption capacity (mg g⁻¹), and C_e (mg mL⁻¹) is the final DNA concentration in the supernatant.

$$Q = K_f C_e^{1/n}$$

where K_f is the Freundlich constant, C_e (mg mL⁻¹) is the final DNA concentration in the supernatant, and $1/n$ is the heterogeneity factor. The Langmuir constants K_L of dsDNA and ssDNA were 0.983 and 2.502 L mg^{-1} , respectively. These results demonstrated that Ti⁴⁺@spore microspheres could be used for DNA adsorption, and interestingly, they had high affinity toward ssDNA and low affinity toward dsDNA.

3.4. Discrimination of dsDNA and ssDNA by Ti⁴⁺@Spore Microspheres. On the basis of the finding that Ti⁴⁺@spore microspheres had differential affinities toward dsDNA and ssDNA, we hypothesized that the Ti⁴⁺@spore microspheres have the discrimination ability for dsDNA and ssDNA. This discrimination ability for large amounts of ssDNA interference was then investigated using PAGE. The DNA mixtures were prepared in different concentration ratios, dsDNA/ssDNA from 1:1 to 1:10, to stimulate complex samples. The dsDNA concentration was kept at $0.50 \mu\text{mol L}^{-1}$, whereas the concentration of ssDNA varied in the range of 0.5 – $5.0 \mu\text{mol L}^{-1}$. As shown in Figure 5A, two bands belonging to dsDNA and ssDNA (indicated by the red box) were observed in the DNA mixtures (dsDNA/ssDNA: 1:1, lane 1; 1:5, lane 3; 1:8, lane 5; and 1:10, lane 7). Interestingly, after treatment with Ti⁴⁺@spore microspheres (dsDNA/ssDNA: 1:1, lane 2; 1:5, lane 4; 1:8, lane 6; and 1:10, lane 8), the bands corresponding to dsDNA were slightly weakened, whereas

Figure 5. Discrimination of dsDNA and ssDNA by Ti^{4+} @spore microspheres. (A) 15% nondenaturing PAGE of DNA mixtures containing dsDNA and ssDNA at various concentration ratios before and after DNA adsorption by Ti^{4+} @spore microspheres. Lane M, DNA marker; lanes 1, 3, 5, and 7 were DNA mixtures (1:1, 1:5, 1:8, and 1:10) before adsorption by Ti^{4+} @spore microspheres; lanes 2, 4, 6, and 8 were the supernatants of DNA mixtures (1:1, 1:5, 1:8, and 1:10) after adsorption by Ti^{4+} @spore microspheres and centrifugation. Conditions: DNA mixture samples contained $0.50 \mu\text{mol L}^{-1}$ dsDNA and ssDNA of 0.50, 2.50, 4.00, and $5.00 \mu\text{mol L}^{-1}$; 6.00×10^8 particles mL^{-1} Ti^{4+} @spore microspheres were used. (B) Grayscale intensity of DNA bands in the image of PAGE. (C) Comparison of SNR before and after DNA adsorption by Ti^{4+} @spore microspheres. The error bars represent SE of the means. The data represent mean $\pm$ SE ($n = 3$).

those of ssDNA were nearly absent. The bands' digital information was acquired through grayscale intensity quantitatively calculated by software Quantity One (Figure 5B). We noticed that the grayscale intensities of dsDNA bands in lanes 1, 3, 5, and 7 were constant, whereas those of ssDNA were gradually increased because of the constant of dsDNA concentration and the increase of ssDNA concentration. After treating with Ti^{4+} @spore microspheres, the decreased range of the grayscale intensities of ssDNA bands was apparently larger than that of dsDNA in lanes 2, 4, 6, and 8, which were

Figure 6. Applications for DNA analysis. (A) Sensitivity and (B) specificity of a Ti^{4+} @spore-based DNA biosensor. Conditions: DNA target ranging from 0.50 to $2500.00 \text{ pmol L}^{-1}$ in the sensitivity test, and DNA target, single mismatch, double mismatch, and random sequences at 5.00 nmol L^{-1} concentration in the specificity test. Other conditions used in both tests include a 5.00 nmol L^{-1} ssDNA probe, 1.00×10^8 particles mL^{-1} Ti^{4+} @spore microspheres, $0.25 \times$ SYBR Green I, and $25.00 \text{ mmol L}^{-1}$ sodium acetate buffer, pH 5.0. The error bars represent SE of the means. The data represent mean $\pm$ SE ($n = 3$).

consistent with those shown in Figure 5A. We further analyzed the SNRs, which were defined as the ratios of the grayscale intensities of dsDNA to those of ssDNA. Results in Figure 5C indicated that SNRs were greatly increased from 5.36 (lane 1), 1.09 (lane 3), 0.77 (lane 5), and 0.67 (lane 7) to 244.56 (lane 2), 37.23 (lane 4), 6.64 (lane 6), and 4.87 (lane 8), respectively, by using Ti^{4+} @spore microspheres. These results further confirmed that our Ti^{4+} @spore microspheres significantly increased the sensitivity of dsDNA detection from large amounts of ssDNA interference through enhancing the SNRs.

Combining all the results above, we inferred that the adsorption of DNA by Ti^{4+} @spore microspheres was through phosphate backbones with two types of interaction (i.e., the formation of the Ti–O–P bond and the weak electronic interaction between $-\text{NH}_3^+$ and the phosphate group). As a result, both ssDNA and dsDNA had similar adsorption kinetics, which was not attributed to the differential adsorption. We believe that the discrimination ability of microspheres toward ssDNA and dsDNA was due to their different adsorption affinities because of the difference of DNA structures: ssDNA is a flexible linear structure, which is much easier to wrap around the Ti^{4+} @spore microspheres; dsDNA, however, is a rigid double helix structure, which makes it more difficult to effectively bend DNA.^{15,18} As a result, the rigid dsDNA binding

Table 1. Comparison of Different Fluorescent DNA Sensors without DNA Amplification^a

materials	limit of detection	linear range	refs	mechanism of discriminating dsDNA and ssDNA
MBs	25 nM	0–2 μ M	51	stem-loop structure and fluorophore–quencher pair
MBs	5 nM	50–1500 nM	52	
MBs	4.4 nM	10–200 nM	53	
MBs	3.5 nM	0–100 nM	54	
MBs	1.4 nM	5–160 nM	55	
SWNTs	4 nM	15–750 nM	8	π – π stacking between SWNTs and nucleobases and discrimination through different affinities
SWNTs	1 nM	0–20 nM	9	
GO	100 pM	0–20 nM	10	hydrophobic interactions, hydrogen bonding, π – π stacking, and discrimination through different affinities
GO	43 pM	0.05–50 nM	11	
GO	1 nM	5–75 nM	12	
GO	5 pM		23	
ITO	0.7 nM	0–10 nM	18	electrostatic interaction between phosphate and ITO and discrimination through different DNA structures
AuNPs	2 nM	0–20 nM	9	
PdNPs	3 pM	3–100 pM	24	coordination interaction between Pd and N on the nucleobase and discrimination through different affinities
Au-NRs	80 pM	0.17–11.67 nM	25	
MoS ₂	0.5 nM	0–15 nM	19	electrostatic interaction between phosphate and cationic-layer-coated Au-NRs and discrimination through different DNA structures
MoS ₂	0.315 nM	0.5–7.5 nM	20	
TaS ₂	50 pM	0–5.0 nM	21	
Ta ₂ NiS ₅	50 pM	0–5.0 nM	22	
Ti ⁴⁺ @spore	50 fM	0.5–2500 pM	this work	van der Waals force between nucleobases and TMDs and discrimination through different affinities
				coordination and electronic interaction of the Ti ⁴⁺ @spore with phosphate on DNA and discrimination through different DNA structures

^aAbbreviations: SWNTs, single-walled carbon nanotubes; GO, graphene oxide; ITO, indium tin oxide; AuNPs, gold nanoparticles; PdNPs, palladium nanoparticles; Au-NRs, gold nanorods; TMDs, transition-metal dichalcogenides.

to Ti⁴⁺@spore microspheres was less favorable as compared to the flexible ssDNA.

3.5. Applications for Sensitive DNA Analysis. The results shown in Figure 5 also revealed that the Ti⁴⁺@spore microspheres had the ability to adsorb excessive ssDNA probes from the hybridization system. Therefore, the use of Ti⁴⁺@spore microspheres for DNA analysis could significantly reduce the noise caused by the excessive ssDNA probes. As a result, the detection sensitivity of DNA target was greatly improved through enhancing the SNR. The effects of various conditions, including pH, concentrations of SYBR Green I and Ti⁴⁺@spore microspheres, and adsorption times, on the detection of DNA target were investigated (Figure S2). Under the optimal conditions (pH 5.0, 0.25 $\times$ SYBR Green I, 1.00×10^8 particles mL^{−1} Ti⁴⁺@spore microspheres, and 10.0 min adsorption time, see section S2 in the Supporting Information), the linear response to the concentration of DNA targets ranged from 0.50 to 2500 pmol L^{−1}, and the linear regression equation was $Y = 0.018X + 2.840$ ($R^2 = 0.998$), where Y is the fluorescence intensity and X is the DNA target concentration (Figure 6A). Furthermore, the limit of detection for DNA assay was 50.0 fmol L^{−1} (SNR = 3), which is remarkably improved as compared to different DNA fluorescent sensors without DNA amplification (Table 1). A series of repeatability measurements of 500 pmol L^{−1} gave reproducible results with a relative standard deviation of 4.15% ($n = 11$). The developed DNA biosensor was used for distinguishing single mismatch, making the detection of single nucleotide polymorphism (SNP) possible. As shown in Figure 6B, the fluorescence intensity of the single- and double-mismatched duplex DNA and random sequence was obviously decreased when compared to that of the perfectly matched duplex DNA (DNA targets/probes). The

high-sequence specificity of distinguishing mismatches was quite inspiring, demonstrating that this developed method possessed great promise for further application in a drug-resistant and mutation-related genetic disease study.^{3,50}

4. CONCLUSION

In summary, this work revealed that novel functional monodispersed microspheres composed of spores and Ti⁴⁺ exhibited differential affinities toward dsDNA and ssDNA. Inspired by this finding, we constructed a label-free fluorescent DNA biosensor for sequence-specific DNA detection. This Ti⁴⁺@spore-based DNA biosensor possessed several advantages. First, spores could be easily prepared on a large scale at low cost and steadily stored for a long time, making high-throughput detection possible and showing a potential commercial perspective. Second, the rapid adsorption properties of Ti⁴⁺@spore microspheres toward DNA provides opportunities to develop label-free biosensors and complete the DNA assay (including the preparation of Ti⁴⁺@spore microspheres and DNA hybridization and measurement) in less than 1 h. Third, the developed sensor showed not only high sensitivity down to 50 fmol L^{−1} without any DNA amplification due to the minimal background fluorescence but also high specificity toward the sequence-specific DNA, making SNP detection possible. Moreover, through doping magnetic nanoparticles into the Ti⁴⁺@spore microspheres in the future, the separation of ssDNA from the DNA hybridization reaction system would become simpler and more convenient using magnetic separation instead of centrifugation. The study represents our first attempt to apply Ti⁴⁺@spore microspheres in a DNA biosensor design, and we hope that these green

materials will have widespread applications in biology and other fields.

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acsami.7b10478.

Optimization of experimental conditions and DNA sequences (PDF)

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Notes

The authors declare no competing financial interest.

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■ ABBREVIATIONS

MBs	molecular beacons
FRET	Förster resonance energy transfer
SNR	signal-to-noise ratio
single nucleotide polymorphism	
SMMs	spore-based highly monodispersed microspheres
FTIR	Fourier transform infrared
XPS	X-ray photoelectron spectroscopy
PAGE	polyacrylamide gel electrophoresis
SNR	signal-to-noise ratio
single nucleotide polymorphism	

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