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Sensitive multiplexed DNA detection using silica nanoparticles as the target capturing platform

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ABSTRACT

We present a simple and sensitive method for multiplexed DNA detection by simultaneously capturing two different DNA sequences with a same silica nanoparticle (NP) through a sandwich mode. This biobarcode assay method was demonstrated by using oligonucleotide sequences of 64 bases associated with human papillomavirus (HPV) 16 and 18 L1 genes as model systems. The nonfluorescent carboxyl-modified silica NPs were prepared using water-in-oil (W/O) microemulsion methods. Avidin was immobilized on the surface of the NPs by covalent binding to the carboxyl linkers. The binding capacity of the avidin-covered NPs for ligand biotin was quantified and the results show that about 8 avidin molecules are bound to one nanoparticle. The silica nano-platforms were prepared through the biotin–avidin interaction and the amounts of capture DNA strands for HPV-16 and HPV-18 (C-16 and C-18, respectively) conjugated to the surface of the same NPs were measured using fluorescent dye hoechst33258. The calculated result shows that the amounts of conjugated C-16 and C-18 on 1 mg of NPs (9.2 pmol) are about 13.5 pmol and 15.5 pmol, respectively. A one-step hybridization reaction was performed by mixing the silica nano-platforms, HPV-16 and HPV-18 target DNA (T-16 and T-18), fluorescein amidite (FAM) or 6-carboxyl-X-rhodamine (Rox) labeled HPV-16 and HPV-18 probes. The hybrid-conjugated NPs were separated by centrifugation, and T-16 and T-18 were detected by measuring fluorescence signals of FAM and Rox respectively. The results show linear dependence of the fluorescence intensity on target DNA concentration in the range from 0.5 to 9 nM, and the detection limit (3σ) of T-16 and T-18 is 0.17 nM and 0.78 nM, respectively.

1. Introduction

The development of a sensitive biosensor for multiplexed DNA detection is of great significance in life science research, which can exploit the most information from the smallest amount of sample volume at low cost [1–3]. Multiple tumor DNA markers (e.g., tumor-suppressor genes) have been proven valuable for the early-phase detection of cancers in asymptomatic individuals [4,5]. Although researchers take efforts to develop different DNA biosensors, some methods cannot be used for multiplexed DNA detection. Planar microarrays (protein and DNA microarrays, etc.) and suspension arrays are two multiplexed bioassay platforms. However, there are some limitations of planar arrays such as expensive, complex instrumentation, the necessity for target amplification, slow binding kinetics of target sequence to capture strand [5]. In comparison, suspension arrays, due to higher sample throughput and faster reaction kinetics are attracting increasing interest for detection and multiplex analysis of nucleic acid, proteins and other biomolecules [6–8].

Silica nanoparticles have many advantages such as straightforward synthesis, facile surface modification, easy separation, relatively low-cost, good biocompatibility and high hydrophilicity [9–13]. Immobilization of biomolecules (antibody, aptamer, etc.) on the surface of dye-doped silica NPs have been widely used for biosensing and bioanalysis, such as detections of human tumor marker protein and pathogens as well as recognition of tumor cells [14–18]. Our previous work has shown that carboxyl-modified silica nanoparticles can successfully be used for ATP detection by conjugating with amine-containing aptamer molecules [19], but the coupling between the amine-labeled aptamer and the carboxyl-modified silica is inefficient and the detection for target molecules is limited. In this work, in order to overcome this drawback, the NP capturing platforms were prepared through the biotin–avidin interaction due to the high affinity and specificity between avidin and biotin, and the actual number of the avidin molecules and capture DNA on each nano-platform is

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measured. Silica nanoparticles can protect DNA from nuclease cleavage owing to the steric hindrance effect, which is a promising method for DNA detection in cellular environments without any change in its property [9–11]. Thus we aim to develop a more sensitive approach for multiplexed DNA detection based on non-fluorescent silica nanoparticles as the platform.

2. Experimental

2.1. Chemicals and materials

Triton X-100, n-hexanol, cyclohexane, tetraethylorthosilicate (TEOS), ammonium hydroxide (NH_4OH, 25–28 wt%), 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide hydrochloride (EDC), N-hydroxysulfosuccinimide sodium salt (sulfo-NHS), bisbenzimide(hoechst33258) were purchased from Sigma Chemical Co. (St. Louis. MO). Carboxyethlsilanetriol sodium salt (CEOS) was purchased from ABCR Chemical Co. (Germany). The DNA oligonucleotides were purchased from Shanghai Sangon Biological Engineering Technology & Services Co. (China), and have the following sequences:

Biotinylated capture DNA sequences of HPV-16 and HPV-18
C-16:5′-GTGTGGATAATAGAGAATGTATATCTATGG-$(CH_2)_6$-biotin-3′
C-18:5′-CTGAGGACGTTAGGGACAATGTGTCTGTAG-$(CH_2)_6$-biotin-3′
Probe DNA sequence:
P-16:5′-FAM-ACAGAAAATGCTAGTGCTTATGCAGCAAAT-3′
P-18:5′-Rox-ACTGAAAGTTCCCATGCCGCCACGTCTAAT-3′
Target sequences within the L1 gene of HPVS:
T-16:5′-CCATAGATATACATTCTCTATTATCCACACCTGCATTTGC-TGCATAAGCACTAGCATTTTCTGT-3′ (GenbankDQ469930)
T-18:5′-CTACAGACACATTGTCCCTAACGTCCTCAGAAACATTA-GACGTGGCGGCATGGGAACTTTCAGT-3′ (GenbankX05015)
Mismatched sequences:
MT1–16:5′-CCATAGATATACATACTCTATTATCCACACCTGCATTTG-CTGCATAAGCTCTAGCATTTTCTGT-3′
MT3–16:5′-CCATAGATAAACATTCTCTTTTATCCACACCTGCATTT-GCTGCATAAGGACTAGCATTTTCTGT-3′
MT1–18:5′-CTACAGACACATTGTCCCTAACGTCCTCAGAAACATTA-GACGTGGCGGCTTGGGAACTTTCAGT-3′
MT3–18:5′-CTACAGACAGATTGTCCCTTACGTCCTCAGAAACATTA-GACGTGGCGGCTTGGGAACTTTCAGT-3′
Random:5′-CGTTACATATACACTCTCTCTTATGCACGCGTGCATCT-GCTTCTTACGCTCTCGCATGTTCATG-3′

2.2. Instrumentation

The size and uniformity of carboxyl-modified silica NPs were measured with a transmission electron microscope (JEOL, JEM100CXII, Japan). The modified carboxyl on the surface of NPs was confirmed using the IR spectrum (FT-IR, Nicolet-5700, USA). Fluorescence spectra and fluorescence intensity were measured using a fluorescence spectrophotometer (Hitachi, F-4600, Japan) equipped with a 150 W xenon lamp.

2.3. Synthesis of carboxyl-modified silica nanoparticles

Nonfluorescent carboxyl-modified silica nanoparticles were synthesized using the water-in-oil (W/O) reverse microemulsion method [20,21]. The microemulsion consisted of a mixture of 1.77 mL of Triton X-100, 7.5 mL of cyclohexane, 1.6 ml of n-hexanol, 480 mL water and 100 mL of TEOS that was stirred for 30 min at room temperature, and then 60 mL of NH_4OH was added. The reaction was allowed to continue for 24 h at room temperature, then post-coating of carboxyl-modified silica was performed by adding 50 μL of TEOS and 50 μL of CEOS. This polymerization was allowed to proceed for 24 h. After the reaction completed, the silica nanoparticles were separated from the microemulsion by the addition of acetone, followed by centrifuging and washing with ethanol three times and deionized water one time. The NPs were finally resuspended in 4 mL deionized water and stored at 4 °C until use.

2.4. Immobilization of avidin onto silica NPs

1 mL of carboxyl-functionalized silica NPs was washed by centrifuging twice with 0.01 M PBS (pH 7.2). Then the NPs were resuspended in 1 mL of 0.01 M PBS (pH 7.2). 3 mg of EDC, 5 mg of NHS and 50 μL of 1 mg/mL avidin were added into the NPs solution, and the solution was then incubated for 4 h at room temperature with gentle shaking. Finally, the nanoparticles were washed three times with 0.01 M PBS (pH 7.2) and resuspended in 1 mL of 0.01 M PBS (pH 7.2).

2.5. Quantitative detection of avidin on the silica NPs surface

A method for avidin quantitative detection based on the theory that avidin quenches the fluorescence of biotin(5-fluorescein) conjugate was introduced [22]. 50 μL of 1.0 μg/mL biotin(5-fluorescein) conjugate in 0.01 M PBS (pH 7.2) was added into the centrifuge tube. Various volumes of 1 mg/mL avidin in 0.01 M PBS (ranging from 0 to 1.2 μg) and 0.01 M PBS were then added to keep the final volume at 2 mL in each solution. After incubating for 30 min in the dark, the fluorescence of the solution was measured at $\lambda_{ex}/\lambda_{em}$=495 nm/520 nm with a spectrofluorometer, which the excitation and emission slits were set at 10 nm and 5 nm, respectively. The fluorescence intensity (ΔF) was plotted as a function of the concentrations of avidin. The number of immobilized avidin on the NPs can be quantitatively calculated from the regression equation of free avidin molecules.

2.6. Immobilization of capture DNA onto silica NPs (CNPs)

C-16 and C-18 were added to the solution with a final concentration ratio of biotin–DNA:avidin–NPs=4:1. The solution was incubated for 2 h, then washed and centrifuged to remove the free biotin–DNA. Finally the resultant silica NPs were resuspended in Tris buffer solution (200 mM NaCl, 20 mM Tris, pH 8.2) and stored at 4 °C until use.

2.7. Quantitative detection of captured DNA on the surface of silica NPs (CNPs)

25 μL of 0.1 μM T-16 and different concentrations of standard C-16(ranging from 0 to 5 nM) were added to the hybridization solution (200 mM NaCl, 20 mM Tris, pH 8.2) to keep the final volume at 500 μL in each solution. After incubating for 60 min at 60 °C, 50 μL of aqueous solutions of 2 μM hoechst33258 were added into the solution. The fluorescence was measured at $\lambda_{ex}/\lambda_{em}$=360 nm/450 nm with the excitation and emission slits set at 10 nm and 5 nm, respectively. The fluorescence intensity was plotted as a function of the concentrations of C-16. The number of immobilized C-16 DNA on the silica NPs can be quantitatively calculated from the regression equation of standard C-16.

The detection of C-18 on the silica NPs followed the same procedure for C-16 except that the concentration of T-18 was changed to 1 μM and the amounts of standard C-18 were ranging from 0 to 40 nM.

2.8. Hybridization procedure and detection of target DNA

50 μL of 5 pmol probe DNA (P-16 and P-18) and various amounts of target DNA (T-16 and T-18) were added into 50 μL of CNPs and incubated for 90 min at 60 °C. Then the hybrid-conjugated CNPs were washed three times with buffer solution (10 mM Tris, pH 8.2, 200 mM NaCl and 0.1% Tween 20). Finally, the hybrid-conjugated CNPs were resuspended in 100 μL Tris buffer solution (200 mM NaCl, 20 mM Tris, pH 8.2). Detections of T-16 and T-18 were done by measuring the fluorescence signals of FAM at $\lambda_{ex}/\lambda_{em}$=495/520 nm and Rox at $\lambda_{ex}/\lambda_{em}$=570/608 nm, respectively.

2.9. Specificity of the design

1.5 pmol target DNA (T-16 or T-18) and the same amount of other mismatched strands were added into the same hybridization system. Then, the fluorescence intensities were measured.

3. Results and discussions

3.1. Working principle

The detection principle for multiplexed DNA with silica NPs is depicted in Scheme 1. Firstly, the carboxyl-modified silica NPs (60 ± 4 nm) were prepared by the reverse microemulsion method; then avidin was immobilized on the surface of the NPs by covalent binding to the carboxyl linkers, and then biotinylated capture DNA (C-16 and C-18) that are complementary to a nucleotide region within the targets (T-16 and T-18) were immobilized on the surface of silica NPs. Afterwards, a one-step hybridization reaction was performed by mixing the silica nano-platforms, HPV-16 and HPV-18 target DNA (T-16 and T-18), FAM or Rox labeled HPV-16 and HPV-18 probes, and the hybrid-conjugated nanoparticles were separated by centrifugation. Finally T-16 and T-18 were detected by measuring fluorescence signals of FAM and Rox, respectively.

3.2. Synthesis of carboxyl-modified silica nanoparticles

The reverse microemulsion method that yields monodisperse particles in the nanoscale is a robust and efficient method for the preparation of NPs. The NPs were uniform in shape and size with average diameter 60 ± 4 nm as characterized by TEM (Fig. 1). The IR spectrum of carboxyl-functionalized silica NPs, compared with the pure silica, shows a new peak at $\sim$1712.5 cm^{-1} (data not shown) [19]. Due to the electrostatic force between the NPs, the functionalized NPs (isoelectric point near pH 4.7) are dispersed very well in aqueous solution and no aggregation is observed [23]. Before we know the molar number of NPs contained by a certain mass of NPs ($\sim$1 mg), the size and the density of one NP should be measured first. The mass of surface-modified silica NPs was weighed after freezing and drying in a high vacuum. Based on its size (60 ± 4 nm) and the density (1.6 g cm^{-3}), we can calculate the mass of an NP and estimate that 1.0 mg of the synthesized NPs contains $\sim 5.54 \times 10^{12}$ NPs (9.2 pmol) using the following equation:

$$\frac{4}{3}\pi r^3 \rho n N_A = m$$

$$n(mol) = \frac{3m}{4\pi r^3 \rho N_A}$$

(m: a certain mass of NPs; r: the radius of NPs; N_A: Avogadro's constant; ρ: the density of NPs; n: the number of moles).

3.3. Quantitative detection of avidin on the silica NPs surface

In order to quantify the amounts of avidin immobilized on the NPs, a fluorescently labeled avidin ligand, biotin(5-fluorescein) conjugate was used. When biotin(5-fluorescein) conjugate binds to avidin due to the affinity binding by molecular recognition of its

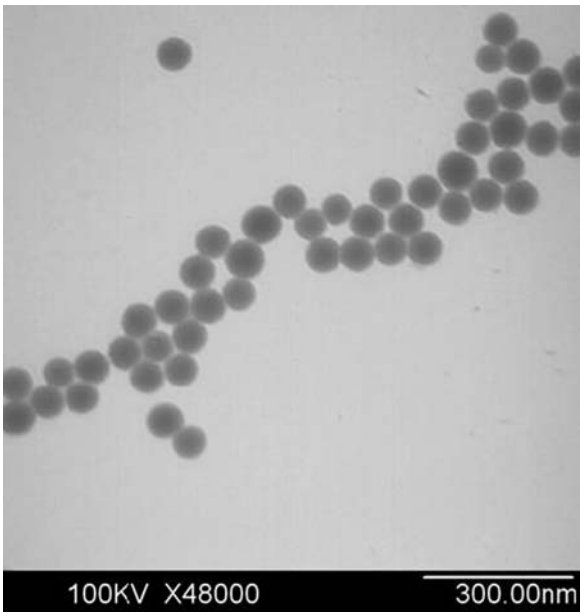
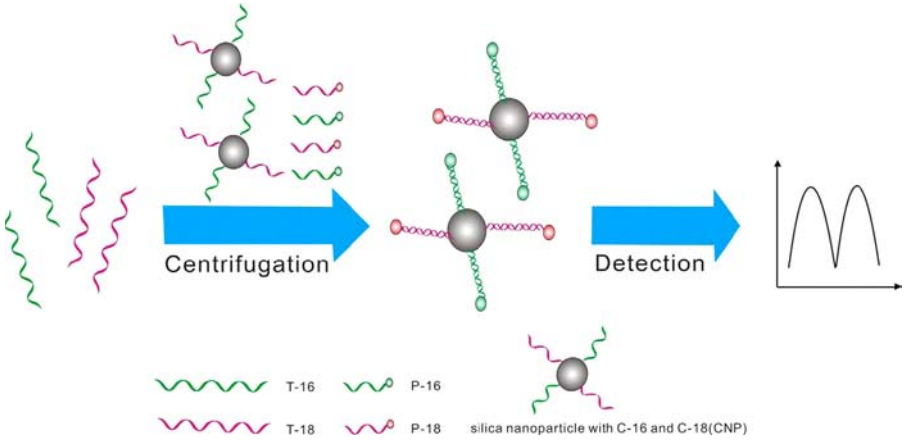


Fig. 1. TEM image of the carboxyl-modified silica nanoparticles (60 ± 4 nm).



Scheme 1. The detection principle for multiplexed DNA using silica nanoparticles as the platform.

binding site, its fluorescent activity is effectively quenched. The calibration curve (Fig. S1, inset) shows a good linear relationship between fluorescence intensity ΔF ($\Delta F = F_0 - F$) of biotin(5-fluorescein) conjugate and concentration of avidin (C) in the range of 0–0.6 μg/mL. The fitted regression equation was $\Delta F = 13624.3C + 445.0$ with a correlation coefficient of 0.9971. The number of immobilized avidin molecules on NPs could be quantitatively calculated from the regression equation of standard avidin. So, the calculation shows that about 0.074 nmol of avidin was immobilized on 1.0 mg of NPs (1.0 mg of NPs equals about 9.2 pmol; the molecular weight of avidin is 66 kD). Therefore, averagely 8 avidin molecules are conjugated to one silica NP.

3.4. Quantitative detection of capture DNA on the surface of silica NPs (CNPs)

A novel indirect fluorescent method based on hoechst33258 for ssDNA detection was developed to quantitatively calculate the number of immobilized DNA molecules on the NPs [24,25]. Some methods such as zeta potential and agarose gel electrophoresis are usually used to confirm the conjugation between NPs and DNA [26,27]. However, they can only be used for qualitative not for quantitative detection, and the procedure is time-consuming. Although absorbance measurement (A260) is usually used to calculate immobilized DNA molecules on the NPs [28], it is not sensitive for the determination of low concentration of DNA. Hoechst33258 can selectively intercalate dsDNA and show highly sensitive fluorescent enhancement. Therefore, quantitative detection of dsDNA can be realized on the basis of fluorescence intensity enhancement by adding various amounts of capture DNA to the solution containing a fixed amount of complementary target DNA. The calibration curves (Fig. S2 and S3, insets) show good linear relationship between fluorescence intensity ΔF ($\Delta F = F - F_0$) of hoechst33258 and concentration of capture DNA (C). For C-16, the fitted regression equation is $\Delta F_1 = 297.27C_1 + 5.92$ with a correlation coefficient of 0.9899, Similar results were found in the detection of C-18, and the fitted regression equation of C-18 is $\Delta F_2 = 51.9C_2 - 0.52$ with a correlation coefficient of 0.9734, which are shown in the supporting information (Fig. S2 and Fig. S3). The number of immobilized DNA molecules on the silica NPs could be quantitatively calculated from the regression equation. The calculation shows that the conjugated C-16 on 1 mg of NPs (9.2 pmol) is about 13.5 pmol and the conjugated C-18 on 1 mg of NPs (9.2 pmol) is about 15.5 pmol and the efficiency of DNA immobilized on the NPs (0.074 nmol of avidin molecules was immobilized on 1 mg of NPs) is about 9.8%.

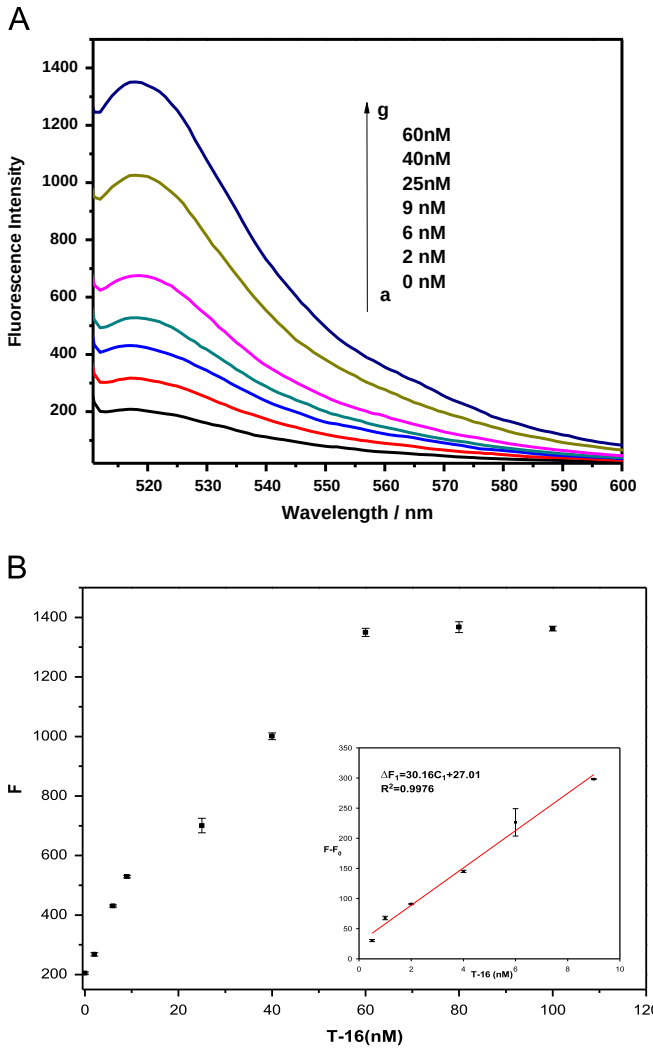


Fig. 2. (A) Fluorescence spectra of FAM on P-16 upon the addition of the different concentrations of T-16 ranging from 0 to 60 nM. (B) Relationship between fluorescence intensity of FAM and concentrations of T-16. Inset: The calibration curve for T-16 detection. Concentration of T-16: 0.5, 1.0, 2.0, 4.0, 6.0 and 9.0 nM.

3.5. Detection of target DNA

Under the optimum conditions (Figs. S4 and S5), the relationship between the fluorescence intensity ΔF ($\Delta F = F - F_0$) and the concentration of target DNA (C) was investigated (Figs. 2 and S6). The calibration curves (Fig. 2B, insets and Fig. S6B, insets) show good linear relationship between ΔF and C in the range of 0.5–9.0 nM for both T-16 and T-18. For T-16, the fitted regression equation is $\Delta F_1 = 30.16C_1 + 27.01$ with a correlation coefficient of 0.9976, and that of T-18 is $\Delta F_2 = 26.91C_2 + 3.8$ with a correlation coefficient of 0.9935. The detection limit ($3\sigma/S$, in which σ is the standard deviation for blank solution, S is the slope of the calibration curve) of T-16 is estimated to be 0.17 nM and that of T-18 is 0.78 nM, respectively.

3.6. Specificity of the design

To evaluate the specificity of this design in the detection of target DNAs, the fluorescence intensities of the different DNA

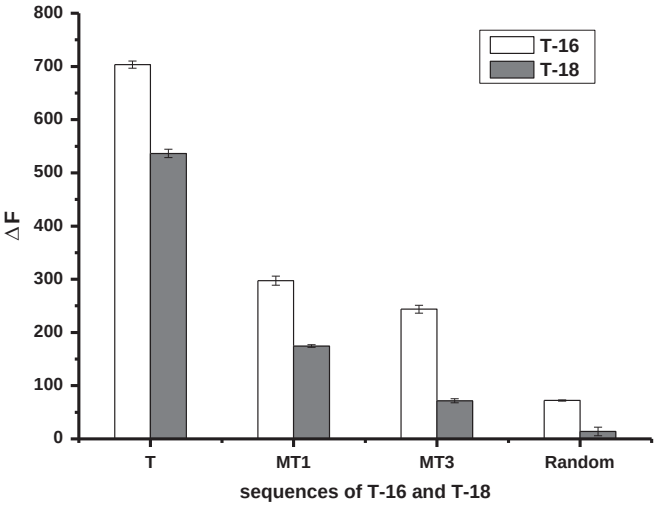


Fig. 3. Fluorescence intensity vs. different DNA sequences. 1.5 pmol target DNA and other mismatched strands or random strands are used for comparison.

sequences with the same amount (1.5 pmol) were investigated. As shown in Fig. 3, the ratio of the fluorescence intensities of T-16 and mismatched sequences (T-16:MT1–16:MT3–16:Random) was 100: 42.3:34.7:10.3, and that of T-18 and mismatched sequences (T-18: MT1–18:MT3–18:Random) was 100: 32.5:13.3:2.6. The results indicate that the proposed method is quite sensitive and can be used markedly to distinguish one-base mismatched sequences from the perfectly complementary sequences.

4. Conclusions

In conclusion, we developed a simple and sensitive method for multiplexed DNA detection using silica NPs as the platform. In order to quantitatively detect the procedure, the binding capacity of the avidin covered NPs for ligand biotin was determined using the quenching of the fluorescence upon binding due to molecular recognition. Moreover, the number of the capture DNA on silica nano-platforms was detected using fluorescent dye hoechst33258. Besides, this detection method can serve as a universal platform by simply changing the DNA sequences for other nucleic acid-based assays.

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Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at http://dx.doi.org/10.1016/j.talanta.2014.05.011.

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