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# Novel Competitive Chemiluminescence DNA Assay Based on $Fe_3O_4$@$SiO_2$@Au-Functionalized Magnetic Nanoparticles for Sensitive Detection of p53 Tumor Suppressor Gene

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**Abstract** A simple, rapid response time and ultrahigh sensitive chemiluminescence (CL) DNA assay based on $Fe_3O_4$@$SiO_2$@Au-functionalized magnetic nanoparticles (Au-MNPs) was developed for detection of p53 tumor suppressor gene. In this study, 2′,6′-dimethylcarbonylphenyl-10-sulfopropyl acridinium-9-carboxylate 4′-NHS ester (NSP-DMAE-NHS), as a new kind of highly efficient luminescence reagent, was immobilized on the complementary sequence of the wild-type p53 (ssDNA) to improve the detection sensitivity. The optimal concentration of ssDNA-(NSP-DMAE-NHS) conjugates mixed with the wild-type p53 (wtp53) samples respectively. Then, the wtp53-Au-MNPs conjugates were added to continue the competitive reaction in the above solution. Subsequently, the Au-MNPs separated under magnetic field, measured by a homemade luminescent measurement system. Under optimal conditions, the method exhibited ultrasensitive sensitivity with a detection limit of 0.001 ng $mL^{-1}$ (0.16 pM), a wide range of liner response from 0.001 ng $mL^{-1}$~6.6 μg $mL^{-1}$. Therefore, the immunomagnetic nanocomposites-based detection strategy was rapid, low-cost, and highly sensitive that can be easily extended to the early diagnosis of cancer development and monitoring of patient therapy.

**Keywords** Chemiluminescence DNA assay · $Fe_3O_4$@$SiO_2$@Au magnetic nanoparticles (Au-MNPs) · Wild-type p53·NSP-DMAE-NHS

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## Introduction

The p53 tumor suppressor gene, well known as "the guardian of the genome," plays important roles in maintenance of genome integrity [1]. While, it is the most commonly mutated gene during cell stress (e.g., DNA damage induced by UV rays) in human tumors and has considerable impact on the "success" of the carcinogenic process (increasing the chances of a tumor cell surviving) [2, 3]. It is estimated that approximately 50% of all human cancers owing to DNA damage by dysfunction of genes involved in controlling the cell cycle, DNA repair, and apoptosis [4, 5]. Therefore, it is essential to understand the molecular basis of p53 inactivation in cancer; a sensitive and rapid detection of p53 gene is required for the early diagnosis and prompt operation.

Thus far, various analytical methods were employed for the determination of p53 gene including reverse transcription polymerase chain reaction (RT-PCR) [6, 7], the denaturing high-performance liquid chromatography (DHPLC) [8], the single-strand conformation polymorphism (SSCP) [9], the denaturing gradient gel electrophoresis (DGGE) [10], traditional nucleic acid probe [11], immunostaining [12], and immunohistochemistry [13, 14]. However, these traditional methods are time consuming for long incubation periods, less sensitive, and multiple steps. As an alternative, other techniques have been exhibited including matrix of polydimethylsiloxane (PDMS)-assisted bead [15], surface plasmon resonance (SPR) [16, 17], field-effect transistor biosensor [18, 19], and surface-enhanced Raman scattering [20]. Nevertheless, these methods typically require sample pre-treatment, skilled operators, and expensive instruments for high sensitivity. Two common methods, the electrochemical biosensor technology based on platforms [21, 22] and p53 antibody-based enzyme-linked immunosorbent assay (ELISA) [23–25], were employed later. The former is simple but still requires the sophisticated electrode materials, whereas the latter is sensitive but requires the use of p53 antibody and measures only the total p53.

Currently, chemiluminescence (CL) is one of the fast-growing analytical methods that have attracted the attention owing to enhancing emission intensity and improving selectivity for quantitative analysis [26]. Generally, the luminol or ABEI [*N*-(4-aminobutyl)-*N*-(ethylisoluminol)] as a luminescence reagent has been exploited to catalyze redox in the current CL reactions [26–29]. As an alternative, 2′,6′-dimethylcarbonylphenyl-10-sulfopropyl acridinium-9-carboxylate 4′-NHS ester (NSP-DMAE-NHS) demonstrates much more effective than the traditional chemiluminescence reagent such as luminol [30] and exhibits the high performance in the dilute alkaline hydrogen peroxide [31]. Meanwhile, the ability of coupling biomolecules by immobilization can improve the performance of biosensors and amplifying signal with functionalized magnetic nanoparticles (MNPs) as a carrier material [32]. MNPs have excellent abilities for biomolecule (proteins, DNA) immobilization due to their non-toxic nature, large surface-to-volume ratio, and biocompatibility. Moreover, they can also be easily separated from the sample matrix without damaging the biochemical activity of biomolecules.

To the best of our knowledge, 2′,6′-dimethylcarbonylphenyl-10-sulfopropyl acridinium-9-carboxylate 4′-NHS ester (NSP-DMAE-NHS) as an efficient luminescence reagent, this is the first report sensitive detection of the p53 gene based on the $Fe_3O_4$@$SiO_2$@Au magnetic nanoparticles. In this paper, a simple and ultrasensitive competitive chemiluminescence DNA assay based on Au-MNPs was developed for detection of the p53 gene. The wild-type p53 (wtp53) was immobilized on the surface of Au-MNPs through a series of chemical reaction. NSP-DMAE-NHS was immobilized on the complementary sequence of the wild-type p53 (ssDNA). Then, the optimal concentration of ssDNA-NSP-DMAE-NHS conjugates mixed

with the wild-type p53 (wtp53) samples respectively. Subsequently, the wtp53-Au-MNPs conjugates were added to continue the competitive reaction in the above solution. Finally, Au-MNPs were washed and separated under the magnetic field, and measured by a luminescent measurement system. The principle of chemiluminescence DNA assay detection based on Au-MNPs is schematically shown in Fig. 1.

## Experimental

### Materials and Reagents

The DNA oligonucleotides were obtained from Sangon Biotechology Inc. (China). Wild-type p53 (wtp53): 5′-$NH_2$-$(CH_2)_6$-GGCACAAACACGCACCTCAA-3′, mutant-type p53 (mtp53): 5′-GGCACAAACATGCACCTCAA-3′, complementary sequence of target ssDNA: 5′-$NH_2$-$(CH_2)_6$-TT GAG GTG CGT GTT TGT GCC-3′, three-base mismatched sequence of p53 DNA: 5′-GGCTCAAAGACGCACCACAA-3′. L-lysine and Sephadex G-25 were purchased from Seebio (Shanghai, China). Bovine serum albumin (BSA), 11-mercaptoundecanoic acid (MUA), 2-(*N*-morpholino)-ethanesulfonic acid (MES), and 3-aminopropyltriethoxysilane (APTES) were purchased from J&K Scientific Ltd. *N*-hydroxysuccinimide (NHS), 1-ethyl-3-(3-dimethylamino-propyl)-carbodiimidehydrochloride (EDC), and *N*,*N*-dimethylformamide (DMF) were purchased from Sigma Company (China). 2′,6′-Dimethylcarbonylphenyl-10-sulfopropyl acridinium-9-carboxylate 4′-NHS ester (NSP-DMAE-NHS) was purchased from MaterWin New Materials Co., Ltd. (Shanghai, China). $FeCl_3{\cdot}6H_2O$, tetraethoxysilane (TEOS), sodium acetate, sodium chloride, sodium phosphate monobasic ($NaH_2PO_4$), sodium phosphate dibasic ($Na_2HPO_4$), methanol, ethanol, ethylene glycol, HCl, NaOH, and aqueous ammonia (28 wt%) were of analytical grade and purchased from Tianjin Tianli Chemical Regents Ltd. All the chemicals were used without further treatment. Deionized water was used for all the experiments.

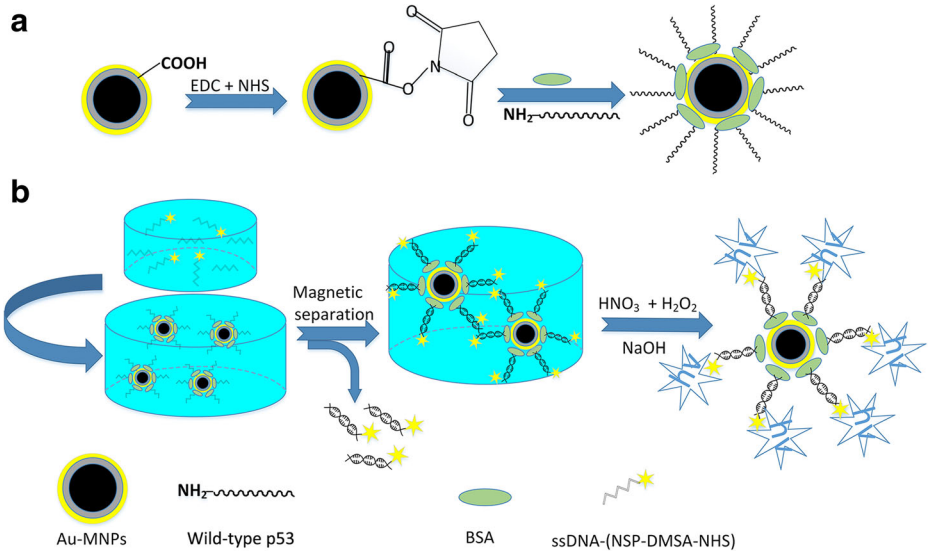


**Fig. 1** The principle of chemiluminescence DNA assay detection

## Synthesis of $Fe_3O_4$@$SiO_2$@ Au Magnetic Nanoparticles

Magnetic nanoparticles (MNPs) were prepared by solvothermal synthesis reaction [33] with some modifications. Briefly, $FeCl_3 \cdot 6H_2O$ (1.35 g) was dissolved in ethylene glycol (40 mL) to form a clear solution, followed by polyethylene glycol (1.0 g) and sodium acetate (3.6 g) as a stabilizing agent. The mixture was stirred vigorously for 30 min and then sealed in an autoclave (100-mL capacity). The autoclave was heated to 180 °C for 12 h and allowed to cool to room temperature (RT). The black products were washed three times with deionized water and three times with ethanol, and dried at 80 °C in vacuum for 2 h.

The above-prepared MNPs were stabilized by coating a layer of silica on the surface according to the Stober process [34]. Namely, dried MNPs (110 mg) were dispersed in 120 mL ethanol, followed by 30 mL deionized water, 2.4 mL ammonia, and 1 mL TEOS. The mixture solution was stirred at room temperature for 12 h. The silica-coated MNPs were washed and dried in the same way with the prepared MNPs.

Gold colloids with 20-nm size were prepared by reduction of $HAuCl_4$ with sodium citrate. Then, 30 mL of the abovementioned treated $Fe_3O_4$@$SiO_2$ nanoparticles was added dropwise to 100 mL of the citrate-stabilized gold colloids. The products were formed after 1 h, which were separated under the magnetic field and washed with deionized water (Fig. 2).

## Preparation of NSP-DMAE-NHS-Labeled Complementary Sequence of Target

The ssDNA-NSP-DMAE-NHS conjugates were prepared according to related reference with some modifications [35]. Purified complementary sequence of target (ssDNA) (100 μM dissolved in 200 μL of PBS, 0.1 M, pH 8.0) was labeled with NSP-DMAE-NHS (1 mg NSP-DMAE-NHS dissolved in 316 μL of dry DMF) (Fig. S1). The reaction mixture was shaken for 1 h at RT and quenched by 100 μL of L-lysine (10 mg $mL^{-1}$ of sterile distilled water) for 15 min. The labeled conjugates were separated from unbound NSP-DMAE-NHS by gel filtration on a Sephadex G-25 column (1 × 25 cm) equilibrated with PBS (0.1 M, pH 7.4). The peak nearby 8 mL demonstrated that the complementary sequence of target (ssDNA) has been labeled by NSP-DMAE-NHS. The conjugates have a strong absorption peak at 260 nm, and also a strong chemiluminescence peak. The results are shown in Fig. 3. It can be seen that the NSP-DMAE-NHS-labeled ssDNA eluted nearby volume 8 mL and collected in single-dose vials, enriched, and stored at 4 °C.

The above ssDNA-NSP-DMAE-NHS conjugates solution was serially diluted from 1:10 to $1:10^{-5}$ in the purification buffer to determine the sensitivity. Then, the dilute ssDNA-NSP-DMAE-NHS conjugates were measured with a homemade luminescent measurement system. The trigger solution was injected into each well tube through a peristaltic pump and well at 2 s.

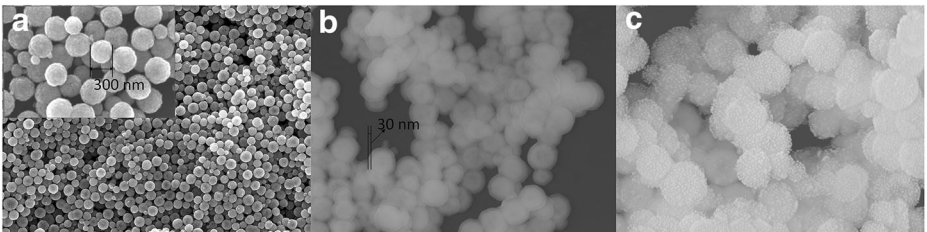


**Fig. 2** SEM images of $Fe_3O_4$ (**a**), $Fe_3O_4$@$SiO_2$ (**b**), and $Fe_3O_4$@$SiO_2$@Au (**c**)

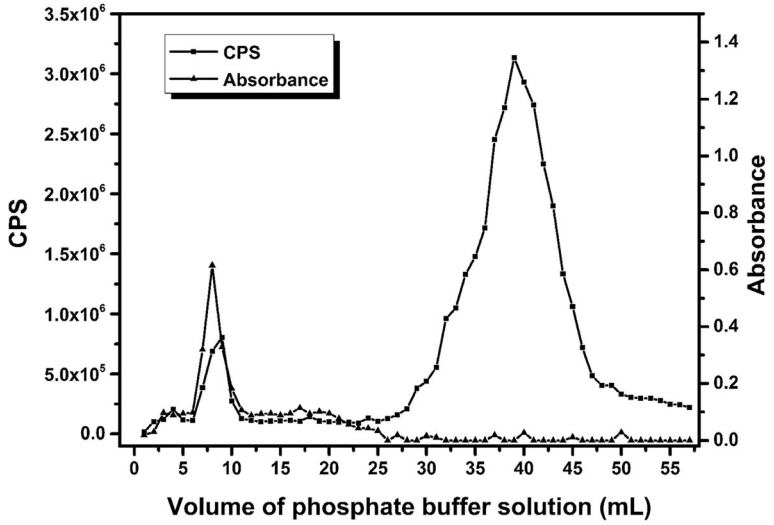


**Fig. 3** Results of complementary sequence of ssDNA

The number of photons detected in unit time by the detector and its intensity were counted. The signals were monitored and handled in real time by a computer. Figure 4a showed the moles of complementary sequence of target (ssDNA) were plotted against the emitted relative light unit (RLU). Figure 4b showed the change of chemiluminescent intensity over time. The sensitivity was determined to be at least $5 \times 10^{-17}$ mol.

## Immobilization of Wild-Type p53 via BSA on Au-MNPs

$Fe_3O_4$@$SiO_2$@Au magnetite nanoparticles were dispersed in 5 mL MUA (5 mM) ethanol solution for 16 h at room temperature. The carboxyl-Au-MNPs were formed and washed twice with 2-(*N*-morpholino)-ethanesulfonic acid (MES) buffer solution. The carboxyl-Au-MNPs were activated by incubation with 100 μL of a 1:1 ratio mixture of EDC (200 mM) and NHS (50 mM) in MES buffer at 37 °C for 1 h. Then, the activated carboxyl-Au-MNPs were washed three times with PBS (0.01 M, pH 7.4) in a magnetic field and mixed with 100 μL BSA (0.1 mg $mL^{-1}$ in deionized water) with gentle stirring for 2 h. The obtained Au-MNPs-BSA conjugates were separated under the magnetic field [36, 37].

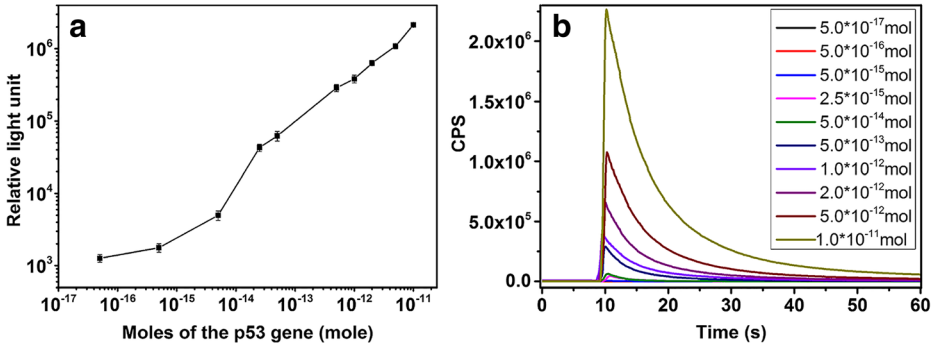


**Fig. 4** Moles of the complementary sequence of ssDNA and the change of chemiluminescent intensity

The Au-MNPs-BSA-wtp53 conjugates were prepared as follows: the above obtained Au-MNPs-BSA were dissolved in 2 mL PBS (0.01 M, pH 7.4) with adding 4.6 mg of *N*-hydroxysuccinimide (NHS) and 7.7 mg of 1-(3-dimethylamino-propyl)-3-ethylcarbodiimidehydrochloride (EDC). The solution was stirred for 30 min at 37 °C. Then, wild-type p53 (wtp53) were added and stirred for another 2 h. Subsequently, the remaining carboxyl group was blocked with ethanolamine (1 M, pH 8.5) and stirred for 30 min. Finally, the Au-MNPs-BSA-wtp53 conjugates were separated with magnet.

### Establishment of CLIA Method Based on Au-MNPs for Wild-Type p53 Detection

The process of reaction is shown in Fig. 1b. Wild-type p53 was dissolved in buffer solution to prepare the standard samples in which the concentrations of the wild-type p53 were 0.001, 0.0066, 0.066, 0.66, 6.6, 66, 660, and 6600 ng $mL^{-1}$ respectively. The above solutions were mixed with the optimized amount of ssDNA-NSP-DMAE-NHS conjugates, which incubated at 37 °C for 1 h. Then, the mixture reacted with 100 μL of wild-type p53 immobilized Au-MNPs-BSA at 37 °C for 1 h with gentle shaking. Finally, the particles were washed by PBS in a magnetic field. Here, $HNO_3 + H_2O_2$ and NaOH acted as the pre-trigger and trigger respectively. A volume of 100 μL of the pre-trigger was previously added to the tube containing Au-MNPs and 100 μL of trigger was injected into the tube by a peristaltic pump. At the same time, the chemiluminescence emission intensity was measured versus time by the luminescent measurement system (Fig. S2).

## Results and Discussion

### Determination of the Optimized Amount of NSP-DMAE-NHS-Labeled ssDNA

Before construction of standard curves for different concentrations of wild-type p53 (wtp53), the amount of NSP-DMAE-NHS-labeled ssDNA should be optimized. Different amounts (1, 5, 10, 20, 35, 40, 50, 60 μL) of ssDNA-NSP-DMAE-NHS conjugates were bound on Au-MNPs-BSA-wtp53 conjugates. Figure 5 showed the chemiluminescence intensity corresponding to different amounts of ssDNA-NSP-DMAE-NHS conjugates. The concentration of ssDNA-NSP-DMAE-NHS was 0.01 mg $mL^{-1}$. It is clear that the chemiluminescence intensity

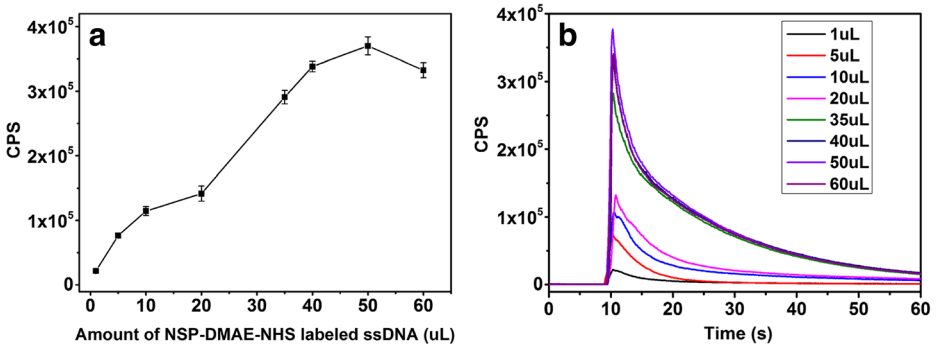


**Fig. 5** Chemiluminescence intensity corresponding to different amounts of ssDNA-NSP-DMAE-NHS conjugates

was proportional to the amount of ssDNA-NSP-DMAE-NHS in the range of 1–50 μL and reached saturation value at 50 μL. However, it is interesting to note that signals showed a drop when the ssDNA-NSP-DMAE-NHS conjugates were beyond 50 μL. This extraordinary result may be explained by steric effect. Simply, the steric effect could influence the reaction between the ssDNA-NSP-DMAE-NHS and Au-MNPs-BSA-wtp53 conjugates. Once the amount of ssDNA-NSP-DMAE-NHS conjugates was beyond 50 μL, the mass of them affected each other to generate conformational changes, which was unfavorable to make the antibodies recognize antigens. To ensure higher sensitivity, 25-μL ssDNA-NSP-DMAE-NHS conjugates were chosen as the optimal volume for the competitive reaction, which is the 50% inhibition concentration ($IC_{50}$).

## Calibration Curve for Wild-Type p53 Analytes

Different concentrations of wild-type p53 (0.001, 0.0066, 0.066, 0.66, 6.6, 66, 660, 6600 ng $mL^{-1}$) were mixed with 25-μL ssDNA-NSP-DMAE-NHS solution and the mixture were incubated at 37 °C for 1 h. Then, 100 μL of the Au-MNPs-BSA-wtp53 conjugates were added and stirred for another 30 min at 37 °C. After the reaction was complete, the particles were washed three times with PBS buffer (containing 0.1% Tween 20). In Fig. 6a, it can be seen that chemiluminescence intensities were linearly decreased according to the increases of wild-type p53 concentrations. That is due to the competitive immune binding of the analyte and tracer to the immobilized antibodies. Figure 6b showed the calibration curve of wild-type p53 detection. The peak of photon count was found to be linear with the logarithm of wtp53 concentration in the range from 0.001 to 100 ng $mL^{-1}$. The equation for the resulting calibration plot was $y = 85{,}445.6–21{,}831.5x$ (x was the concentration of wtp53, y was the photon count); correlation coefficient $r^2$ was 0.990. The limit of detection (LOD) was 0.001 ng $mL^{-1}$ (0.16 pM), which was estimated using $3\sigma$ (where $\sigma$ is the relative standard deviation of a blank solution, $n = 6$) [38]. The $IC_{50}$ values (the competitor concentration that causes 50% growth inhibition) were about 0.68 ng $mL^{-1}$. Table 1 shows the comparison between this method and other typical detection methods. The result is comparable or better than that of previously reported p53 assays [39, 40, 43] and is more sensitive and advantageous in wider linear range than the former [41, 44]. That might be greatly contributed by the increased binding ratio of the ssDNA-NSP-DMAE-NHS conjugates coupling Au-MNPs-BSA-wtp53 conjugates and the high emitting efficiency of NSP-DMAE-NHS.

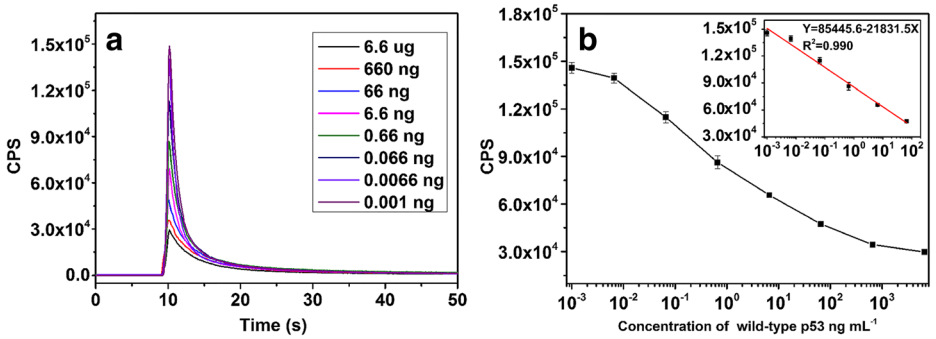


**Fig. 6** Chemiluminescence intensities and calibration curves of wild-type p53

**Table 1** Comparison of this method and other typical methods

| DNA assay method | Detection limit | Measurement range | References |
|---|---|---|---|
| Surface Raman scattering | 500 fM | – | [20] |
| Non-competitive electrochemical nanofiber biosensor | 50 fM | – | [38] |
| Non-competitive electrochemical peptide nucleic acid (PNA) probe biosensor | 0.682 nM | – | [39] |
| Competitive luminol DNA assay | 3.8 pM | 0.01–0.5 nM | [40] |
| Non-competitive electrochemical DNA sensor | 0.8 nM | 1.0–1000 nM | [41] |
| Non-competitive electrochemiluminescence | 22.8 fM | 0.2–200 pM | [42] |
| Competitive improved CL DNA assay | 0.16 pM | 0.001–6600 ng $mL^{-1}$ | This work |

### Selectivity of CLIA Detection Wild-Type p53

The selectivity was investigated by hybridize with ssDNA-NSP-DMAE-NHS sequences related to p53 tumor suppressor gene using Au-MNPs as a carrier. Three the same concentrations (0.66 ng $mL^{-1}$) of wild-type p53 were prepared. Then, one-base mismatched wtp53 (6.6 ng $mL^{-1}$) and three-base mismatched wtp53 (6.6 ng $mL^{-1}$) were introduced into the 1 and 2 detection system, respectively, while the three-detection system was replaced by a blank buffer solution [45]. Further, 25-μL ssDNA-NSP-DMAE-NHS solution was mixtured with the above-incubated solution for 1 h, and then repeated the previous steps. The experiment results showed that the peaks of photon count did not decrease with the addition of mutated gene interference respectively (Fig. S3). Those signals in the presence of wtp53 were basically consistent. The results indicated that ssDNA-NSP-DMAE-NHS conjugates could clearly distinguish wtp53 from one-base mismatched wtp53 and three-base mismatched wtp53. That confirmed the high selectivity of this assay, importantly providing a novel sensing platform for the early detection diagnosis of cancer through mutated genes [42, 46].

## Conclusions

In this study, a simple and ultrahigh sensitive chemiluminescence (CL) DNA assay based on $Fe_3O_4$@$SiO_2$@Au-functionalized magnetic nanoparticles (Au-MNPs) has been established for the detection of p53 tumor suppressor gene. In this proposed strategy, application of integration of the advantages of NSP-DMAE-NHS and Au-MNPs in immunoassay was first adopted in p53 tumor suppressor gene. The calibration curve detection has been obtained for wild-type p53 with a wider linear measurement range through competition experiments. Furthermore, this proposed method exhibited high sensitivity for detection of wild-type p53 with a detection limit of 0.001 ng $mL^{-1}$ (0.16 pM), and the results demonstrated that the chemiluminescence (CL) DNA assay has ultrahigh sensitivity and good selectivity. We also anticipate that the novel biosensor platform could be expanded for the cancer diagnosis and early detection of cancer and to make this commercialized for detection of other biomarkers in the future.

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**Compliance with Ethical Standards**

**Conflict of Interest** The authors declare that they have no competing interests.

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