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# Magnetically controlled immunosensor for highly sensitive detection of carcinoembryonic antigen based on an efficient "turn-on" cyanine fluorophore

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**ABSTRACT:** Early cancer diagnosis is critically important for early intervention and it can significantly enhance the treatment efficacy and the chance of cure. Herein, for the first time, the cyanine fluorophore namely, $SLSO_3$, was synthesized and found to possess a high binding affinity towards a typical biomarker, carcinoembryonic antigen (CEA) in which it served as an excellent turn-on labelling fluorophore for the detection of CEA. On this basis, the target CEA antigen is captured by a primary antibody conjugated on magnetic silica coated iron oxide nanoparticles and then followed by secondary antibody-loaded $SiO_2$ nanoparticles, which provide more binding sites for the labelling dyes resulting in 6-fold increase in fluorescence and thus heightened sensitivity. The immunosensor is capable of differentiating target analyte from other cancer-associated proteins and has been applied to quantify CEA in human serum sample and the result was consistent to that obtained from a commercial ELISA kit. This simple and direct detection assay has achieved a low limit of detection in the pg $mL^{-1}$ regime with small sample consumption (only 10 μL) and without any sample pre-treatment and purification.

***Keywords*:** Immunosensor; Turn-on fluorophore; Cancer biomarker; Carcinoembryonic antigen; Signal amplification

## 1. Introduction

In the past decade, detection of disease biomarkers has drawn remarkable attention for early disease diagnosis [1]. As biomarkers, including DNA, RNA, peptide, and metabolite, regulate the inter- and intracellular functions of the human body, alternation of expression profile of biomarkers often reflects the condition of the body. Profile of the biomarkers content can be generally used for screening and monitoring the progression of cancers [2-4]. Amongst all biomarkers, carcinoembryonic antigen (CEA) is the most commonly studied one which associates with several types of cancers, including colorectal [5,6], breast [7], lung [8] and liver [9] cancers. CEA is a glycoprotein present in early development of human embryo and fetal; CEA maintains in normal expression level throughout the entire life in healthy adults' tissue. During the malignant transformation, the level of CEA was found to be significantly overexpressed in colorectal and gastric carcinomas. CEA is highly responsive to cancer recurrence and its concentration in patient serum usually arises along with the progression of cancer [10,11]. Therefore, it has been considered as a promising biomarker for early cancer diagnosis and disease progression monitoring in recent decades.

Enzyme-linked immunosorbent assay (ELISA) is the most common method for antigen detection [12]. Benefiting from the bio-specific antibody-antigen interaction, ELISA facilitates a promising sensitive and specific detection for cancer-associated biomarkers. However, detecting trace amount of biomarkers in complex matrix, such as serum, ELISA often requires a relatively large amount of sample for multiple

pretreatment steps. It often hinders the throughput of the assay. A simple, direct, pretreatment-free yet sensitive and selective detection method is highly desired for early cancer diagnosis.

Nanomaterials have been emerged in the biosensing aspect for early disease detection and diagnosis. Taking advantages of the unique properties: inert, high surface area-to-volume ratio, high biocompatibility, surface plasmon resonance, superparamagnetic and so on, metallic nanoparticles (NPs), such as gold, silver, and iron oxide NPs, have been widely applied for biomarkers sensing, drug delivery, and bio-imaging [13-16]. Of all the metallic nanoparticles, iron oxide NPs are attractive for its unique magnetic properties that they can be simply magnetically isolated and so used to purify nucleic acids, proteins, metabolites, and even intact cells [17-20].

Herein, we have developed a magnetic immune-sensing platform for simple and direct detection of disease-related antigen (Ag). CEA was chosen to be the model biomarker as a demonstration. In the immunoassay, monodispersed magnetic silica coated iron oxide nanoparticles were firstly loaded with primary antibodies to probe the target antigens through the highly specific interactions. After that, an amplification probe, detection antibodies-conjugated silica NP, was introduced and was subsequently labelled by the tailor-made cyanine fluorophore, $SLSO_3$, which affords strong fluorescence upon binding resulting in high-sensitivity detection of the assay. The content of the target antigen CEA was quantified by measuring the resultant fluorescence signal.

## 2. Experimental section

### *2.1. Reagents*

N-(3-dimethylaminopropyl)-N'-ethylcarbodiimide (EDC), N-hydroxysuccinimide (NHS), tetraethylorthosilicate (TEOS), (3-aminopropyl)triethoxysilane (APTS), N,N-dimethylformamide (DMF), dimethyl sulphoxide (DMSO) and tetrahydrofuran (THF) were purchased from Sigma-Aldrich (St. Louis, USA). Carcinoembryonic antigen L2C010 (CEA), anti-human CEA multiclonal antibody L1C00205 (Ab1), anti-human CEA monoclonal antibody L1C00202 (Ab2), human alpha-fetoprotein (AFP), prostate-specific antigen (PSA), and Immunoglobulin G (IgG) were purchased from Shanghai Linc-bio Science Co. Ltd (China). IgG from rabbit serum was purchased from Sigma, USA. 50 mM phosphate buffer (PB) was prepared by mixing different ratios of 50 mM $NaH_2PO_4$ and $Na_2HPO_4$, yielding a buffer at pH 7.4. All other chemicals were of analytical grade and were dried by the standard methods wherever needed.

### *2.2. Apparatus*

$^{1}$HNMR spectra were recorded using a Bruker-400 NMR spectrometer and referenced to the residue DMSO-$d_6$ at 2.5 ppm. $^{13}$C NMR spectra were recorded using a Bruker-400 NMR spectrometer and referenced to the DMSO-$d_6$ (39.5 ppm). Mass spectroscopy (MS) measurements were carried out by using fast atom bombardment on the API ASTER Pulsar I Hybrid Mass Spectrometer or matrix-assisted laser desorption ionization-time-of-flight (MALDI-TOF) technique. The emission spectra were measured by PTI QM-4 Spectrofluorometer (Photon Technology International, Birmingham, NJ). The

size and morphology of the nanoparticles were monitored by transmission electron microscopy (TEM) (JEOL 2100, JEOL, Japan). UV-vis absorption spectra were measured by the Varian Cary 100-UV-Vis spectrophotometer (USA).

### *2.3. Binding assays*

20 μL of CEA solution (27.6 pM) was mixed with 20 μL of $SLSO_3$ with different final concentrations and incubated for 5 min at room temperature. The emission spectra of the mixture were recorded by the fluorescence spectrometer using excitation wavelength of 465 nm and scanning range of 550−800 nm. The dissociation constant ($K_d$) was calculated by GraphPad Prism 6.0 on the basis of the saturation experiments.

### *2.4. Preparation of the $Fe_3O_4$@$SiO_2$ nanospheres*

$Fe_3O_4$ nanoparticles were prepared according to our previously reported solvothermal method [21] with a reaction time of 2 h. Magnetic $Fe_3O_4$@$SiO_2$ ($mSiO_2$) NPs were obtained by coating $SiO_2$ layer on the surface of $Fe_3O_4$ NPs via a modified Stöber sol-gel method [22]. Briefly, an aqueous solution of HCl (0.1 M, 25 mL) was added to the collected $Fe_3O_4$ NPs and sonicated for 10 min. Then the activated $Fe_3O_4$ NPs was thoroughly washed with water and then redispersed in a mixture of ethanol (40 mL), water (10 mL) and ammonia (0.5 mL). Finally, TEOS (75 μL) was added dropwisely into the solution, which was stirred at room temperature for 6 h. After rinsing with ethanol and water, the isolated $mSiO_2$ NPs were dispersed in 4 mL of distilled water for later use.

### *2.5. Preparation of Ab1-coupled $mSiO_2$ and Ab2-coupled $SiO_2$*

To prepare Ab1-coupled $mSiO_2$ ($mSiO_2$-Ab1) as a probe, 2 mL of as-obtained $mSiO_2$ suspension was treated with 0.4 mL of APTES under stirring at 50 °C overnight. After centrifugation and rinsing with ethanol repeatedly for several times, the as-obtained amino-functionalized $mSiO_2$ nanospheres were dispersed in 4 mL of PB. Then 1 mL of Ab1 solution (0.2 mg $mL^{-1}$ in PB) was activated by 1 mL of EDC/NHS mixture (5 mM EDC, 10 mM NHS in PB) with gentle shaking for 40 min at room temperature. The $mSiO_2$-Ab1 bioconjugations were obtained by adding the solution of activated Ab1 into the amino-modified $mSiO_2$ suspension for the condensation reaction with gentle shaking at room temperature for 2 h. After removing the unbound Ab1 by a magnetic bar and rinsing with PB thrice, the resultant $mSiO_2$-Ab1 probes were obtained and dispersed in PB to a final volume of 2 mL and stored at 4 °C for later use. $SiO_2$ NPs of average diameter 60 nm (Fig. S1) were prepared with the previously reported method (Supporting Information) [23,24]. Ab2-coupled $SiO_2$ NPs (Ab2-$SiO_2$) probes were then prepared by using the same procedure except for the introduction of 2 mL of $SiO_2$ suspension (0.2 mg $mL^{-1}$), 1 mL of Ab2 (0.4 mg $mL^{-1}$) and 2 mL of EDC/NHS mixture. The resultant Ab2-$SiO_2$ probes were centrifuged and washed three times with PB, to remove the excess Ab2, and then re-dispersed in 4 mL of PB for further experiments.

### *2.6. Detection of CEA with the developed immunosensor*

20 μL of $mSiO_2$-Ab1 suspension was incubated in 10 μL of CEA solution at 37 °C for 40 min to capture CEA with the first immunoreaction ($mSiO_2$-Ab1/Ag). After

magnetic separation and washing thoroughly with PB, the resultant $mSiO_2$-Ab1/Ag were exposed to 20 μL of Ab2-$SiO_2$ suspension at 37 °C for 40 min, to capture Ab2-$SiO_2$ with the secondary immunoreaction. After removing excessive Ab2-$SiO_2$ by magnetic separation and washing thoroughly with PB, $mSiO_2$-Ab1/Ag/Ab2-$SiO_2$ immunocomplex was obtained and re-dispersed in 20 μL of PB, followed by the introduction of 20 μL of 10 μM $SLSO_3$ and incubated at room temperature for 5 min. Then the mixture was transferred to a mini-cuvette and subsequently the fluorescence spectra were recorded. The serum sample of donors (GeneMay, USA) stored at −78 $^oC$ was brought to room temperature before use. After slightly shaking for 5 min, the human serum sample was diluted by 10 and 2 times for the measurements using the developed immunosensor and ELISA kit (Sigma-Aldrich, USA), respectively. The quantification of CEA in serum sample by ELISA kit was conducted in accordance to the manufacture's instruction. The absorbance at 450 nm of the resultant was recorded by Benchmark Plus Microplate Reader.

## 3. Results and Discussion

### *3.1. Design and photophysical properties of the new fluorophore, $SLSO_3$*

The cyanine fluorophore, $SLSO_3$ was synthesized by Knoevenagel reaction (Scheme S1). The molecular structure of $SLSO_3$ was characterized by $^1H$ NMR (Fig. S2), $^{13}C$ NMR (Fig. S3) and high-resolution mass spectrometry. The newly synthesized $SLSO_3$ was highly water soluble and exhibited a very strong charge-transfer absorption band with the absorption peak at 487 and 465 nm in DMSO and PB, respectively (Fig. S4). Upon excitation at its absorption maximum,

$SLSO_3$ showed strong fluorescence emission with a large Stokes shift of ~164 nm and the emission maximum in the range of 625–675 nm. The fluorescence quantum yield of $SLSO_3$ in PB is much lower than that obtained in organic solvent, DMSO, due to the fast non-radiative decay resulting from the strong and dynamic adhesive interactions with water molecules [25]. The optical properties of $SLSO_3$ were measured in DMSO and PB and the results are summarized in Table S1. Despite a low fluorescence quantum yield (<1%) in PB, a dramatic fluorescence increase was observed upon mixing of $SLSO_3$ with a host molecule, for instance, a typical cancer biomarker CEA, accompanied by a slight blue shift of the emission peak of 2–7 nm (Fig. 1A). This zwitterionic fluorophore, $SLSO_3$ is very lipophilic in nature with estimated lipophilicity (Log P value) of 2.86. The enhanced fluorescence could be ascribed to the restricted rotation of the bound $SLSO_3$ on protein via the hydrophobic and π–π stacking interactions and consequently leading to a large reduction in the non-radiative decay [25,26]. These results suggested that $SLSO_3$ would be an excellent "turn-on" labelling fluorophore for the protein-based detection. The binding affinity of $SLSO_3$ towards a typical cancer biomarker, CEA, was evaluated by fluorescence saturation assays (Fig. 1B) which gave the dissociation constant ($K_d$) of 10.19 μM, suggesting high binding affinity between $SLSO_3$ and CEA. In addition, $SLSO_3$ showed a stronger fluorescence enhancement upon binding to the secondary antibodies (detection antibodies Ab2) of CEA as compared to that of our previously reported fluorophore, SLAce (Fig. S4) [25]. This would be greatly beneficial to remarkably enhancing the detection sensitivity of the assay as silica particles loaded

183 with secondary antibodies (Ab2-$SiO_2$) with $SLSO_3$ labelled can serve as the amplified
184 signal reporter.

185 **Here Fig. 1**

186 ***3.2. Detection principle and characterization of the sandwich immunocomplex***

187 The developed detection assay of CEA is mainly based on the specific
188 immuno-reaction. Briefly, capture antibody was firstly immobilized on the surface of
189 magnetic $mSiO_2$ acting as a capture probe. These $mSiO_2$ NPs have a homogenized
190 size with an average diameter of 125 ± 10 nm and a silica shell of thickness ~8 nm
191 (Fig. S6). These $mSiO_2$ NPs not only act as a pre-concentration platform for accurate,
192 simple, and direct detection, but also provide an on-line purification to magnetically
193 separate the target analyte from the bulk solution (Fig. S7). In addition, bare $mSiO_2$
194 does not influence the emission wavelength and fluorescence intensity of $SLSO_3$ (Fig.
195 S8A).

196 To amplify the detection signal, the detection antibody conjugated on $SiO_2$ NPs
197 and labelled with the newly developed turn-on fluorophores was adapted. Through the
198 specific immuno-interaction, the target CEA was firstly captured by the $mSiO_2$-Ab1
199 probe followed by forming a sandwich immunocomplex with Ab2-$SiO_2$ (Fig. S8B).
200 Afterwards, $SLSO_3$ was applied and the fluorescent signal of the immunocomplex
201 which is highly correlated to the target antigen content was recorded by a
202 spectrofluorometer. Upon the formation of the $mSiO_2$-Ab1 (Fig. 2A), FL intensity of
203 $SLSO_3$ increased from 7900 to 12300 a.u. upon the binding to the capture antibodies
204 as compared to that of bare $mSiO_2$ and $SLSO_3$ mixture (curve b, Fig. S8A). By

capturing the target CEA, on the surface of the magnetic probe, a further fluorescence enhancement of $SLSO_3$ (Fig. 2B) was observed indicating the successfully coupling of CEA on $mSiO_2$-Ab1 interface through the first immuno-reaction. A further increase in fluorescence intensity (Fig. 2C) confirmed the formation of $mSiO_2$-Ab1/CEA/Ab2-$SiO_2$ immunocomplex (Fig. 2D). The 6.2-fold enhancement supported the significance of the signal amplification strategy contributed by $SLSO_3$ labelled Ab2-$SiO_2$.

**Here Fig. 2**

### *3.3. Optimization of the assay parameters*

To achieve ultimate performance of the immunosensor, we systematically investigated several parameters that govern its sensitivity. The optimized applied concentration of Ab1 conjugated onto $mSiO_2$ NPs was firstly studied (Fig. 3A). The fluorescence intensity increased as the concentration of Ab1 increased and ceased to increase beyond 0.2 mg $mL^{-1}$. Therefore, 0.2 mg $mL^{-1}$ was anticipated to be the saturation concentration of Ab1 to prepare $mSiO_2$-Ab1. The effect of the $mSiO_2$-Ab1 probe concentration used for the immunosensor was also investigated as shown in Fig. 3B. It is noted that the probes diluted by 10-fold and 2-fold were obviously inadequate to capture 10 ng $mL^{-1}$ CEA. While 1×, 3× and 5× probes concentration gave similar fluorescence signal, while the background signal of the 1× probe was the lowest but yielded the largest fluorescence response.

As the incubation time also plays an important role on the assay performance. As shown in Fig. 3C, the fluorescence signal increased along with the incubation time

(from 10 to 50 min) but no further increase after 50 min, therefore 50 min was set as the optimal immune-reaction time. Besides, the influence of reaction temperature on the detection performance was evaluated as depicted in Fig. 3D. It suggested that 37 °C is the optimal temperature for the immune-reaction between antibody and target antigen. Furthermore, the fluorophore $SLSO_3$ is the key player for the fluorescence response, the optimal concentration of $SLSO_3$ not only allows sufficient fluorophores to bind with the target protein and detection antibodies but also gives minimal background signal due to the unbound excessive probe. As shown in Fig. 3E and Fig. 3F, dye concentration of 10 μM was determined to be optimal as it yielded the highest fluorescence response and thus was applied in the following detection.

**Here Fig. 3**

### *3.4. Analytical performance of the immunosensor*

To determine the detection efficiency of the immunoassay, a calibration curve with the fluorescence intensity as a function of target CEA concentration was constructed. As depicted in Fig. 4A, the fluorescence intensity at ~650 nm increased successively with concentration of CEA. By analysing the fluorescence intensities with response to the concentrations of CEA (Fig. 4B), a standard calibration curve for CEA detection was obtained (inset in Fig. 4B). It is noted that the linear range was in the concentration range of 0.05–100 ng $mL^{-1}$ with a correlation coefficient ($R^2$) of 0.9956. And the limit of detection (LOD) was estimated to be 3.7 pg $mL^{-1}$ which gives a signal-to-noise ratio of 3.

**Here Fig. 4**

The analytical performance (i.e. linear range and LOD) of the developed assays shows great improvement when compared with those reported immunosensors based on utilization of different signal reporters and methods (Table 1). Besides a broader linear range, its LOD has at least 5 orders of magnitude improvement compared to most of the reported works and is better than or comparable to most of those fabricated by using the expensive enzymes labels [27,28]. Coupling with $SiO_2$-assisted signal amplification labelled with the turn-on fluorophore $SLSO_3$, its sensitivity of CEA detection was enhanced by approximately 30-fold as compared to the previously reported immunosensor (110 pg·mL$^{-1}$) [25].

**Here Table 1**

### *3.5. Selectivity, reproducibility, and stability of the immunosensor*

To assess the selectivity of the immunosensor, three cancer-associated antigens (AFP, PSA and IgG) were introduced at a 10-fold higher concentration as CEA. As shown in Fig. 4C, the detection assay successfully differentiated CEA from AFP, PSA and IgG. This was attributed to the fact that the m$SiO_2$-Ab1 and Ab2-$SiO_2$ have a high specificity towards the target CEA and can selectively discriminate the target antigen from the other potential proteins interference from the sample matrix.

The reproducibility of the immunosensor was also evaluated by assaying one CEA level at 10 ng mL$^{-1}$. The relative standard deviation (RSD) for six replicate measurements was calculated to be 5.9%, suggesting that the developed method exhibited an acceptable reproducibility. Fig. 4D displayed the fluorescence responses recorded from the free $SLSO_3$ and $SLSO_3$ labelled immunosensor under consecutive

scanning in an hour (with 5 min interval). Stable fluorescence signals were observed, implying both the free dye and the dye labelled immunosensor possessed good photostability. Storage stability of the $mSiO_2$-Ab1 and Ab2-$SiO_2$ bioconjugations were also investigated by detection of fluorescence response after a sandwich immunoreaction at 10 ng $mL^{-1}$ of CEA and followed by dye labelling. There was a 7.3% decrease in intensity observed after 4 weeks of storage in PB at 4 °C, indicating that the designed immunosensor possessed good long-term storage stability. Practically, to determine the CEA content, one would simply mix and incubate the as-prepared $mSiO_2$-Ab1 and Ab2-$SiO_2$ bioconjugations with the test serum samples, followed by $SLSO_3$ labelling and fluorescence measurement.

### *3.6. Real sample analysis*

Matrix effect is a crucial aspect in clinical assay. The feasibility of the developed immunosensor in clinical applications was evaluated by using a concurrent human sample of donors and the results were compared with those obtained by a commercial ELISA kit. The results by these two methods agreed very well and were summarized in Table 2. As can be seen, the developed immunosensor showed good recovery results and accuracy, with RSD values 5.3–6.1% as compared with 4.7–5.6% for ELISA results. Besides, this immunosensor exhibited a deviation of −3.1–0.8% from those determined by ELISA kit. These results suggested that the developed immunosensor possessed high practicability in clinics.

**Here Table 2**

## 4. Conclusions

We reported here a simple, pre-treatment-free, sensitive and highly specific assay for detection of serum CEA using antibodies conjugated magnetic nanoparticle platform in which an efficient turn-on labelling fluorophore, $SLSO_3$ and detection antibody-conjugated silica nanoparticles serving as an amplification reporter for target antigen were developed to afford high detection sensitivity of the assay. The newly developed assay achieved a sensitive detection of CEA with a LOD of 3.7 $pg \cdot mL^{-1}$ and was capable of differentiating the target antigen, CEA, from other proteins in serum. The assay has also demonstrated its capability of quantifying CEA in serum samples where the result agreed well with those obtained from ELISA. The assays could be readily modified to detect other proteins of interests by using the corresponding pair and antibodies. It is of a high potential to practically serve as an analytical tool for early disease diagnosis and monitoring of the disease treatment progress.

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**Appendix A. Supplementary material**

Supplementary data associated with this article can be found in the online version at

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