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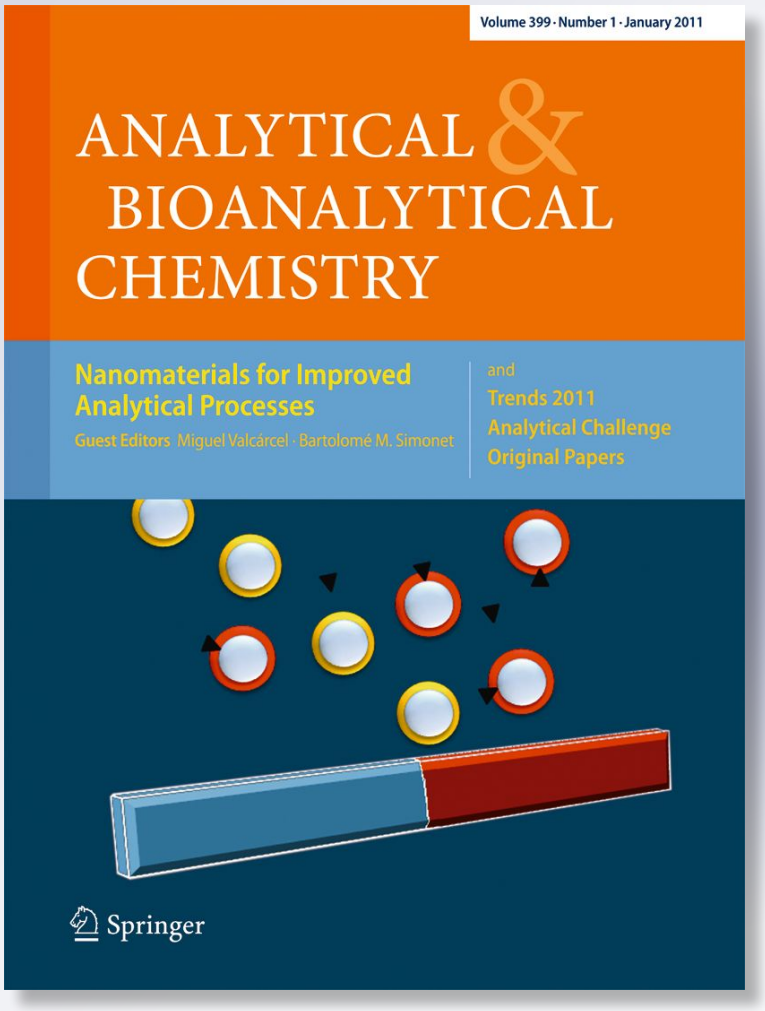

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ORIGINAL PAPER

Multianalyte immunoassay chip for detection of tumor markers by chemiluminescent and colorimetric methods

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Abstract Most cancers developed an elevation of the level of at least two markers associated with their incidence. Simultaneous detection of multi-tumor markers associated with a particular type of cancer plays an important role in cancer diagnostic. Here, a multianalyte immunoassay chip for simple and sensitive detection of tumor markers with chemiluminescent and colorimetric methods was proposed, in which carcinoembryonic antigen (CEA) and carbohydrate antigen (CA19-9) that associated with colorectal cancer were detected as model. The immunoassay chip was fabricated by co-immobilization of CEA/CA19-9 antibody on a glass slide with γ-glycidoxypropyltrimethoxysilane as linkage. Through sandwiched immunoreactions, CEA, CA19-9, and their corresponding enzyme tracers, alkaline phosphatase-labeled anti-CEA and horseradish peroxidase-labeled anti-CA19-9, were introduced on the chip. Then, they were sequentially detected by chemiluminescent method in the range of 0.5–80 μg/L and 0.5–80 kU/L with the detection limits of 0.41 μg/L and 0.36 kU/L at 3σ for CEA and CA19-9, respectively. They could also be detected by colorimetric method in the range of 1–200 μg/L and 5–200 kU/L with the detection limits of 0.25 μg/L and 1.25 kU/L at 3σ for CEA and CA19-9, respectively. All these results demonstrated that the present work provided a promising analytical method for tumor markers' analysis with the advantages of simple analytical procedure, small sample volume and lower cost, which made the proposed method potential for high-throughput detection.

Keywords Multianalyte immunoassay · Tumor markers · Chemiluminescence · Colorimetric method · Chip

Introduction

Sensitive detection of tumor markers is significant in early clinical diagnosis and evaluation of the recovery of patients [1–4]. However, most markers are not specific to a particular tumor (e.g., CEA is associated with colorectal cancer, gastric carcinoma, liver cancer, lung cancer, breast cancer, and pancreatic cancer) and sometimes they show elevated levels in patients without cancer. On the other hand, most cancers developed an elevation of the level of at least two markers in the patient's serum. Thus, the measurement of a single tumor marker has limited clinic diagnostic value due to its low specificity, while the simultaneous detection of multi-tumor markers associated with the same tumor can improve their diagnostics and has attracted considerable interest [5–7]. Compared with the traditional parallel single-analyte immunoassay, multianalyte immunoassay can provide higher sample throughput, shorter assay time, less sample consumption, lower cost of per assay, and more efficient diagnosis for the disease [8–10].

Efforts that have been conducted for multi-tumor markers' analysis focused mainly on multiple-label assays or channel distribution [11]. Matsumoto and co-workers reported a time-resolved fluoroimmunoassay for simultaneous detection of α-fetoprotein (AFP) and CEA in human sera. Eu-labeled anti-AFP antibody and biotinylated anti-CEA antibody that further complexed with Sm-labeled streptavidin were used for multi-analysis of clinically relevant AFP and CEA levels

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[12]. Terenghi and co-workers developed a method for the simultaneous determination of five tumor markers, which are as follows: AFP, CEA, CA19-9, ovarian tumor antigen (CA125/MUC16), and human chorionic gonadotropin [13]. The method was based on the incubation of a serum with five antibodies, each labeled with a different lanthanide (Pr^{3+}, Eu^{3+}, Gd^{3+}, Ho^{3+}, and Tb^{3+}, respectively) followed by the specific determination of the immunocomplex formed by size exclusion chromatography with inductively coupled plasma mass spectrometric detection. A series of channel-resolved approaches combined with semi-automated flow-through CL system were developed to achieve multianalyte immunoassay by Ju's group, in which the CL signals from different channels were sequentially collected with the aid of an optical shutter [6, 11, 14–17]. Based on a substrate zone-resolved technique, they developed a flow-through multi-analyte immunosensing system for sequential detection of CA 125 and CEA [11]. In their work, antibodies were immobilized on an UltraBind aldehyde-activated membrane. Paramagnetic microspheres [14] and polyethersulfone membrane [15] were [16] used. Recently, an automated CL dual-analyte immunoassay system was developed by immobilizing different capture antibodies on the inner walls of series-wound glass channels. AFP and CEA could be rapidly assayed in the ranges of 1.0–100 and 1.0–80 ng/ml with detection limits of 0.41 and 0.39 ng/ml, respectively [17]. A two-dimensional resolution system including channel- and substrate zone-resolved techniques was designed for sequential detection of CA 125, CA 199, CA 153, and CEA [6]. All of them were detected in the broad linear ranges with low detection limits.

In addition, the colorimetric method, in which the event is disclosed through a visual color change in the reaction medium and thus results can be seen with the human naked eye, has proven to be the most convenient [18, 19]. Thus, remarkable progress has been made on the design of colorimetric sensing systems for tumor marker detection over the years. Large quantities of colorimetric assays for detecting tumor markers are proposed based on enzyme-linked immunosorbent assay [20–23]. Liu et al. employed gold nanoparticles (AuNPs) as carriers of anti-CEA antibody. [20]. Ambrosi A et al. also used AuNPs as a multienzyme carrier for the detection of CA15-3 [21]. Wang et al. assemble of folate-polyoxometalate hybrid spheres for colorimetric immunoassay [22]. Chemiluminescent and colorimetric detection combined with enzyme-linked immunosorbent assays were used to detect ochratoxin A in food by Yu and co-workers [23].

In the present work, a multianalyte immunoassay chip for simple and sensitive detection of tumor markers with chemiluminescent and colorimetric method was prepared. According to Tomoyuki et al. [24] and Yakabe et al. [25], efficient evaluation of CEA and CA19-9, the characteristic tumor markers of colorectal cancer, is significant to early clinical diagnosis and evaluation of the recovery in postoperative follow-up. Thus, CEA and CA19-9 were chosen as the model to illustrate the chip design and the detection methods, mainly with CL and colorimetric detection. As compared with the previous methods, the adoption of an antibody chip as the immuno-reaction platform in the current method is novelty. On the other hand, the preparation of the immunoassay chip and their detection schemes were simple and inexpensive.

Methods and materials

Reagents

Bovine serum albumin (BSA) was provided by Sunshine Biotechnology Co. LTD. (Nanjing, China). γ-Glycidoxypropyltrimethoxysilane (GPMS) was obtained from Sigma-Aldrich Chemical Company (Shanghai, China). Mouse monoclonal anti-CEA (2.6 mg/L) and 500 μg/L of CEA antigen and alkaline phosphatase (ALP)-labeled mouse monoclonal anti-CEA were purchased from Xiamen Boson Biotechnology Co. Ltd. Mouse monoclonal anti-CA19-9 (2.45 mg/L), 1,200 kU/L of CA19-9 antigen, and 20 mg/L of horseradish peroxidase (HRP)-labeled mouse monoclonal anti-CA19-9 were provided by Autobio Diagnostics Co. Ltd. HRP substrate luminol–piodophenol–H_2O_2 for CL was supplied by Autobio Diagnostics Co. Ltd. ALP substrate disodium 3-(4-methoxyspiro-{1,2-dioxetane-3,2′-(5′-chloro) tricyclo[3.3.1.1^{3,7}]decan}-4-yl) phenyl phosphate (CSPD) for CL was provided by Xiamen Boson Biotechnology Co. Ltd. 3,3′,5,5′-Tetramethylbenzidine (TMB; ZhengZhou Biocell Biotechnology Co, Ltd, China) was used as a chromogenic substrate for HRP. Solution of 18.75 mg/ml nitro blue tetrazolium chloride and 9.4 mg/ml 5-bromo-4-chloro-3-indolyl-phosphate, toluidine salt in 67% (DMSO; *v*/*v*; NBT/BCIP stock solution; Sangon Biotechnology Co. Ltd, Shanghai, China) was used as a chromogenic substrate for ALP. Washing buffer (pH 7.4) was 0.05 M Tris buffered saline (TBS; Shanghai BioSun Sci & Tec Co. Ltd) spiked with 0.05% Tween-20, abbreviated TBST. Blocking buffer was 0.05 M TBS (pH 7.4) containing 1% BSA. All other chemicals were of analytical grade and used without further purification except toluene. Deionized water was used throughout the study.

Apparatus

The CL signal was detected with IFFM-E Luminescence Analyzer (Remax, China), and all manipulations were performed automatically by personal computer equipped with IFFM software package. The immunoassay chip was put just over the optical window and made a large surface

area exposed to the adjacent photomultiplier tube (PMT). The CL signal was detected without wavelength discrimination, and the negative high voltage (−800 V) was supplied to the PMT by a luminosity meter.

The morphology of the modified chip was confirmed by a scanning electron microscope (SEM, JEM-2100, JEOL, Japan) at an acceleration voltage of 30 kV. Fine F200EXR camera (Fujifilm, Japan) was used to record the color change of chromogenic reaction. The statistical distribution of the relative emission intensities, corresponding to color along the white lines, was extracted using a self-developed software. This system was able to count pels, referring to a certain color of a photo.

Preparation of immunoassay chip

The covalent attachment of organosilanes to glass surfaces is partially dependent on the number of hydroxyl groups exposed on the substrate. Thus, they were incubated in 1:1 MeOH/HCl for 30 min followed by an additional 30-min incubation in the high concentration of H_2SO_4 as that reported by Cras et al. [26, 27]. They pointed out that this cleaning method yielded the highest concentration of surface OH^- groups and provided the best surface for subsequent silanization. After washing thoroughly with deionized water and drying at 130 °C for 4 h, the activated glass slides reacted with 0.5% GPMS in dry toluene at room temperature under stirring overnight yielding the epoxy group covered surface. Then they were washed with the mixture of pure toluene and ethanol three times to remove the physical absorbed GPMS and dried under a nitrogen atmosphere for 1 h at 100 °C.

Then, anti-CEA and anti-CA19-9 were immobilized on the glass slide by dropping a 10-μL mixture of anti-CEA and anti-CA19-9 (100 mg/L each in the sample) onto the prepared glass slide, followed incubation for 1 h at room temperature and aging in a refrigerator at 4 °C overnight. After thoroughly rinsed with TBST three times, the resultant chip was blocked with 0.05 M pH 7.4 TBS containing 1% BSA for 2 h to occupy the residual epoxy groups and non-specific binding sites. The modified chip was finally washed three times with TBST, dried in nitrogen, and stored at 4 °C for the next use. The whole process for immunoassay chip preparation was illustrated in Electronic Supplementary Material and Fig. S1(Electronic Supplementary Material).

Results and discussion

Sandwich immunoassay combined with CL for determination of CEA and CA 19-9

The determination of CEA and CA 19-9 was based on the sandwich immunoreactions. A 20-μL CEA and CA19-9 mixed solution and 5 μL of ALP-labeled mouse monoclonal anti-CEA and HRP-labeled mouse monoclonal CA19-9 were dropped on the chip and incubated at 37 °C for 30 min. Then, the chip was washed with TBST followed by dryness with nitrogen in order to remove the physically adsorbed enzyme tracer. The amount of immobilized ALP-labeled mouse monoclonal anti-CEA and HRP-labeled mouse monoclonal CA19-9 depended on the amount of CEA and CA 19-9. Thus, the assay CEA and CA 19-9 could be realized by detection of CL of ALP and HRP in the presence of their substrate.

The chip was delivered onto the photomultiplier and 15 μL of ALP substrate CSPD was dropped on the chip. After the stable baseline was recorded and followed by 5 min reaction, the next 10 s CL signals were recorded for CEA. Then, the chip was washed with TBST and deionized water to remove CSPD for the followed detection of CA19-9. Similar to the detection of CEA, 15 μL of HRP substrate luminol–piodophenol–H_2O_2 was delivered, after the baseline was recorded and followed by 60 s reaction, 10 s signals were recorded for determination of CA19-9.

In order to examine the optimal pre-incubation time, 25 μL of the mixture of 20 μg/L CEA, 20 kU/L CA19-9, and their enzyme tracers of ALP-labeled mouse monoclonal anti-CEA and HRP-labeled mouse monoclonal anti-CA19-9 was dropped onto the chip to react with the surface immobilized monoclonal antibody at 37 °C. The CL signals were determined at intervals of 10 min from incubation for 10 to 60 min, which shown in Electronic Supplementary Material Fig. S2. Both CL signals of the two tumor markers increased with the increasing pre-incubation time and tended to be the maximum value at incubation for 60 min, which indicated that the maximum of these sandwich complexes had been formed. Although the CL signals at 30 min were 76.5% and 77.9% of the maximum values for CEA and CA19-9, respectively, their sensitivity were enough for clinical diagnosis. Considering the speed of this detection method, 30 min of pre-incubation time was used in the next study.

The CL intensity for both CEA (Fig. 1 A) and CA19-9 (Fig. 1 B) increased with the increasing concentration of analytes. The dose–response curves showed linear ranges from 0.5 to 80 μg/L with a correlation coefficient of 0.9985 for CEA and from 0.5 to 80 kU/L with a correlation coefficient of 0.9981 for CA19-9. When the concentrations of analytes were over the linear ranges, an appropriate dilution of sample could extend the detectable concentration ranges.

The detection limit for CEA and CA19-9 was 0.41 μg/L and 0.36 kU/L, respectively, at a signal/noise ratio of 3. The cutoff value of the two tumor markers in clinical diagnostic is 3 μg/L and 37 kU/L, respectively [28]. Therefore, the described method was sensitive enough for practical application.

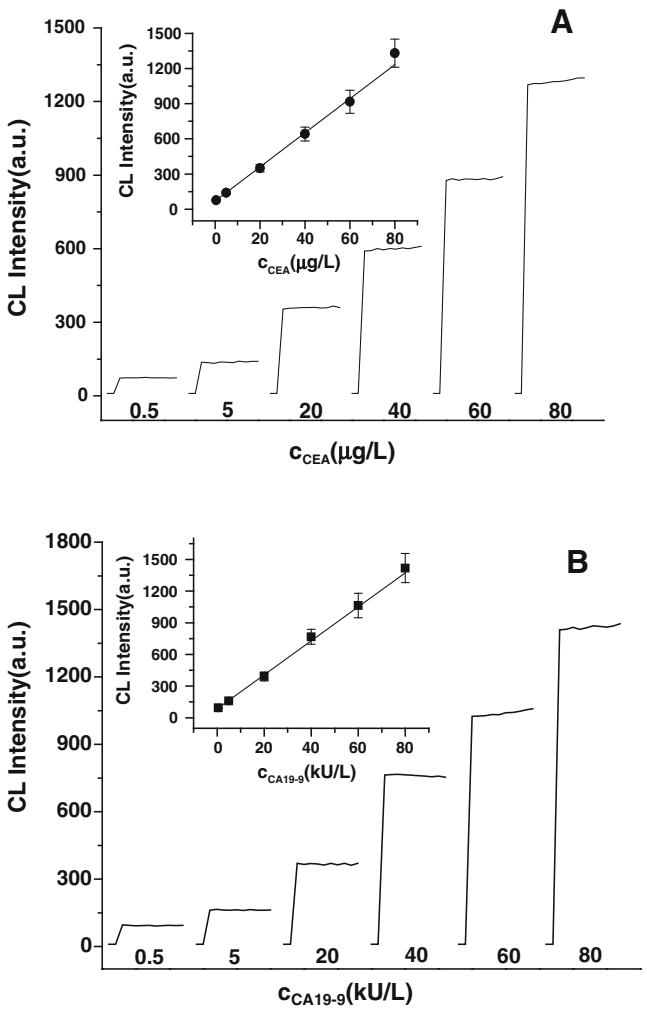

Fig. 1 Dose–response and calibration curve for the CL immunoassay of (A) CEA and (B) CA 19-9

Evaluation of cross-reactivity

It is essential to evaluate the cross-reactivity for specificity and reliability of the proposed method. The cross-reactivity between CEA and CA19-9 was examined by detecting the change of CL signal at a definite concentration of analyte with the increasing concentrations of coexistent another analyte. Variable amounts of CA19-9 were spiked in the mixture of 20 μg/L CEA and ALP-labeled anti-CEA, or CEA was spiked in the mixture of 20 kU/L CA19-9 and HRP-labeled anti-CA19-9, respectively. The spiked samples were dropped on the chip to incubate for 30 min at 37 °C. The CL intensity obtained for a constant CEA that varied the CA19-9 concentration, or a constant CA19-9 that varied the CEA concentration, which shown in Electronic Supplementary Material Fig. S3. In the presence of 100 kU/L of CA19-9, the CL signal of CEA decreased only 4.0%. This suggested that the presence of CA19-9 did not change the CL signal of CEA and cross-reaction existed between CA19-9 and anti-CEA could be negligible. On the other hand, the presence of CEA up to100 μg/L did not change the CL signal of CA19-9. Thus, CEA and CA19-9 could be assayed sequentially without noticeable interference to each other.

Colorimetry for determination of CEA and CA19-9

In addition to CL immunoassay, both of the model tumor markers of CEA and CA19-9 were also detected with color determination. Procedures for determination of CEA and CA19-9 with colorimetric method were similar to that with CL method. However, the CL substrates of CSPD and luminol–piodophenol–H_2O_2 for ALP and HRP were substituted by their chromogenic substrate of NBT/BCIP and TMB. The color information was recorded by a digital camera.

First, 20 μL of NBT/BCIP was dropped on immunoassay chip to react with ALP-labeled anti-CEA for 10 min and their color was recorded by digital camera. Photos of chip in the presence of 1, 10, 20, 50, 100, and 200 μg/L of CEA were shown in Fig. 2A (bottom). The color became more and more deep with the increasing concentration of CEA because more CEA captured more ALP-labeled anti-CEA. The statistical distribution of the relative intensities corresponding to the different colors shown in the blue lines was extracted using our self-developed software. The results were plotted in the top of Fig. 2A. The average intensity is illustrated by red lines. This result revealed the relationship between the intensity and CEA concentration, as shown in Fig. 2B. The inset in Fig. 2B showed that the intensity decreased linearly with the logarithm of CEA concentration in the range of 1–200 μg/L, which was suitable to detect CEA. The linear equation could be represented by I=182.92–33.35 log(c/μg/L) with the correlation coefficient of R=0.9911, where I was the intensity and c was the CEA concentration. The detection limit of 0.25 μg/L was obtained at a signal to noise ratio of 3.

Then, the chip was thoroughly washed with distilled water to remove the chromomeric reagent NBT/BCIP. TMB (20 μL) were added to the immunosensors to react with HRP labeled on CA19-9 for 10 min. Figure 3A (bottom) showed photos of different immunosensors containing 5, 10, 20, 50, 100, and 200 kU/L of CA19-9. The color became more and more deep with the increasing concentration of CA19-9 due to the similar reason for CEA. Relative intensities of them were also extracted by our self-developed software and their relation to the different concentration of CA19-9 was shown in Fig. 3B. The inset displays a calibration curve suitable for CA19-9. The linear range was from 5 to 200 kU/L with a detection limit of 1.25 kU/L at a signal to noise ratio of 3. The linear equation was I=301.78–80.43 log(c/kU/L) with the correlation coefficient of R=0.9965, where I was the intensity and c was the concentration of CA19-9.

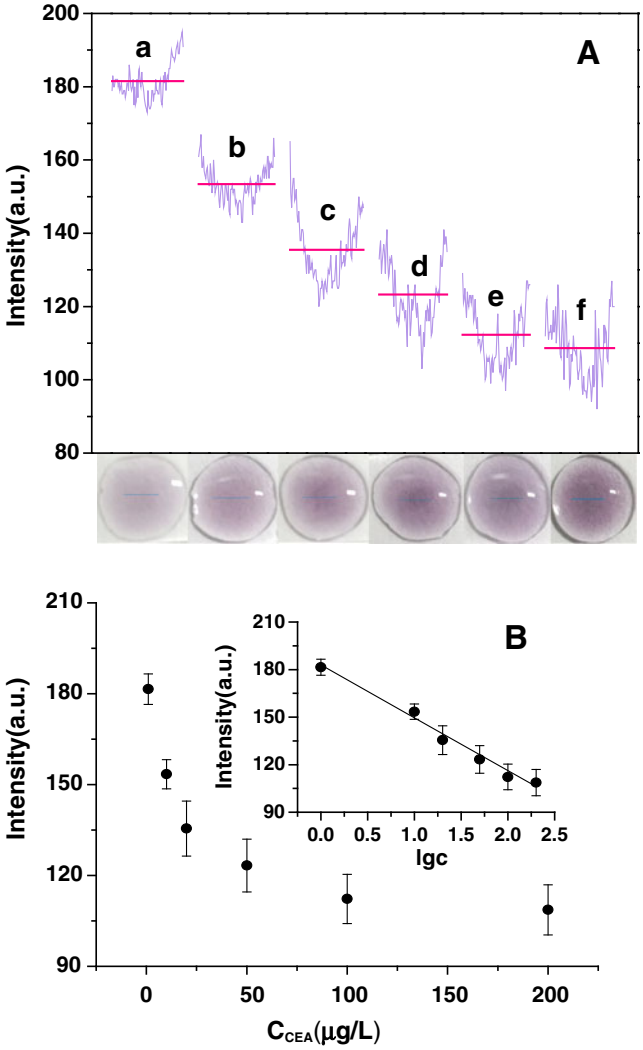

Fig. 2 (A) Photo of colormetric result for CEA (*bottom*) and the intensity statistics along the *blue lines* in the photos (*top*) of samples. The *red lines* are the average values of intensity. The concentrations of CEA are (a) 1, (b) 10, (c) 20, (d) 50, (e) 100, and (f) 200 μg/L, respectively. (B) Plot of intensity vs CEA concentration in the incubation solution. *Inset* in panel B: linear calibration plot

Reproducibility and accuracy of the immunoassay chip

The reproducibility of the multianalyte immunoassay system was assessed by intra- and inter-assay coefficient of variation (CV). The intra-assay CV was the variation between eight determinations of one sample on the same chip with repeat procedure between each measurement. The inter-assay CV was the variation between the measurements of the same sample on eight immunoreactors prepared in batch. The 20 μg/L CEA and 20 kU/L CA19-9 were used in the reproducibility evaluation. The intra- and inter- assay CVs obtained were 3.4% and 5.9% for CEA and 4.6% and 8.3% for CA19-9, respectively.

Clinical serum samples from colorectal cancer were detected using the CL method. When the levels of tumor markers were over the calibration ranges, serum samples were appropriately diluted with 0.01 M pH 7.4 PBS beforehand. CEA and CA 19-9 were detected to be 5.4 μg/L and 41.2 kU/L for the early stage cancer patient, which was in agreement with 4.8 μg/L and 45.6 kU/L that from the reference ECLIA method. The relative error was +12.5% and −9.6%. Another sample was from late stage cancer patient, which was detected to be 22.8 μg/L CEA and 164.9 kU/L CA 19-9 by reference ECLIA method. CEA (21.3 μg/L) and 155.8 kU/L of CA 19-9 were detected by this proposed method. The relative error was −6.6% and −5.5%, showing an acceptable agreement between the two methods.

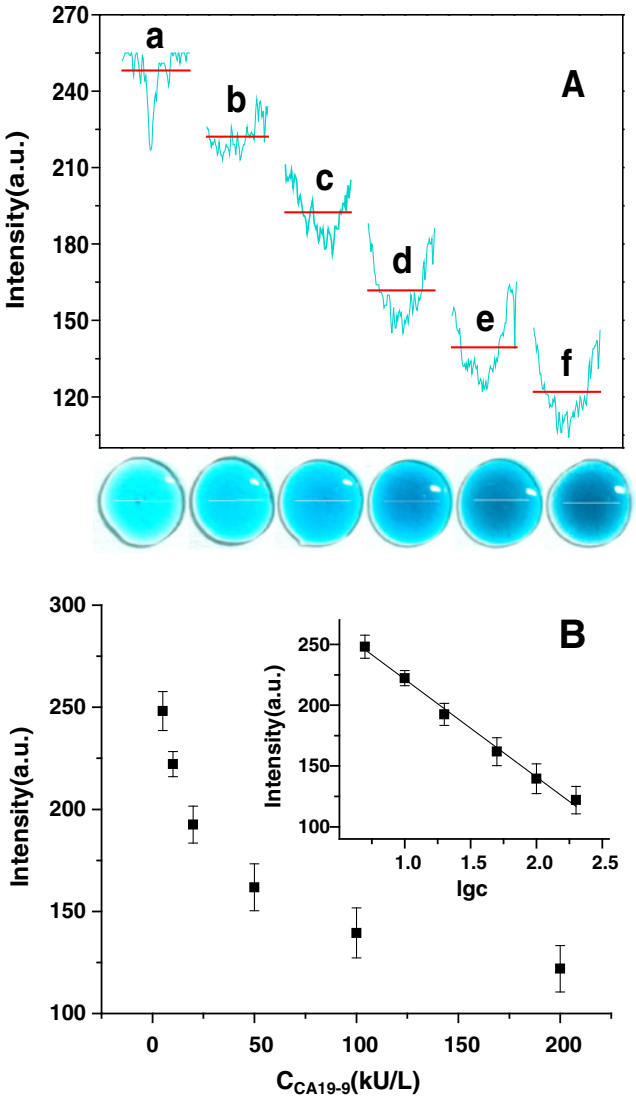

Fig. 3 (A) Photo of colormetric results for CA19-9 (*bottom*) and the intensity statistics along the *white lines* in the photos (*top*) of samples. The *red lines* are the average values of intensity. The concentrations of CA19-9 are (a) 5, (b) 10, (c) 20, (d) 50, (e) 100, and (f) 200 kU/L, respectively. (B) Plot of intensity vs CA19-9 concentration in the incubation solution. *Inset* in panel B: linear calibration plot

When the chips were not in use, they were dipped in TBS (0.05 M, pH 7.4) with 0.05% BSA and stored at 4 °C. No obvious change was observed after storing for at least 7 days.

Conclusions

A novel strategy for simultaneous CL and colorimetric detection of CEA and CA19-9 that associated with colorectal cancer was demonstrated. SEM images confirmed the successfully immobilization of monoclonal CEA and CA19-9 antibodies on the glass slide though GPMS linkage, which could be worked as immunoassay chip for simultaneous detection of CEA and CA19-9. Both CL and colorimetric signals showed a high sensitivity with the proposed methods. The linear ranges for CEA and CA19-9 were 0.5–80 μg/L and 0.5–80 kU/L with CL method, and 1–200 μg/L and 5–200 kU/L with colorimetric method. The present work provided a promising analytical method for tumor marker analysis with the advantages of simple analytical procedure, small sample volume and lower cost, which made the proposed method to be potential used for high-throughput detection.

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