Fluorescence-based immunosensor using three-dimensional CNT network structure for sensitive and reproducible detection of oral squamous cell carcinoma biomarker

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HIGHLIGHTS

- Fluorescence-based immunoassay using 3DN-CNTs for OSCC biomarker detection.
- Detection sensitivity enhances due to high surface area and structural properties of 3DN-CNTs.
- Reproducible detection of biomarker results from uniform surface modification.

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ABSTRACT

A hierarchical three-dimensional network of carbon nanotubes on Si pillar substrate (3DN-CNTs) was developed for the accurate detection of oral squamous cell carcinoma (OSCC) in clinical saliva samples. The 3DN-CNTs were uniformly coated with a layer of aluminum oxides to enhance structural stability during biomarker detection. Cytokeratin-19 antigen (Cyfra 21-1) was utilized as a model biomarker of OSCC for fluorescence-based immunosensor using 3DN-CNTs (3DN-CNTs sensor). The 3DN-CNTs sensor enhances the sensitivity of Cyfra 21-1 detection by increasing the density of immobilized antibody through high surface area of 3DN-CNTs and enhancing the accessibility of biomolecules through the ordered pathway of hierarchical structure. The reliable detection limit for sensing of Cyfra 21-1 was estimated as in the level of 0.5 ng/mL and the quantitative estimation of Cyfra 21-1 was analyzed by 4-parameter logistic (4-PL) model for curve-fitting analysis. Clinical applicability of 3DN-CNTs sensor was evaluated through correlation with the commercially available electrochemiluminescence (ECL) detection system in the hospital. The assay results of the two systems for clinical saliva samples showed a good agreement.
1. Introduction

Oral squamous cell carcinoma (OSCC) is the most common cancer of the oral cavity and the leading cause of death in the developing countries, and the mortality rate of OSCC patients has not significantly changed in the past 30 years [1–4]. The survival rate of the diseases is in the range of 80–90% at an early stage, whereas it is as low as only 15–50% at advanced stages [5]. Early detection of OSCC is a critical issue for the long-term survival of patients and successful treatment of cancer. Histopathological examination and biopsy have been used for a long time as a representative method of OSCC diagnosis. However, these processes are slow and time-consuming as well as critical diagnosis requires the professional interpretation by experienced pathologists if cellular or molecular changes were detected [4,6–8]. According to advances in molecular biology, clinical evaluation using cancer biomarker is considered to be useful for early diagnosis and prognostic monitoring with histopathological examination. Cancer biomarkers such as antigens, DNA, mRNA and enzymes are an important indicator for staging the pathological progression of the disease, and protein markers are most commonly used for cancer diagnosis [9,10]. The level of several biomarkers such as carcinoembryonic antigen, squamous cell carcinoma, immunosuppressive acidic protein and cytokeratin 19 fragment (Cyfra 21-1) in blood sample of OSCC patients led to sensitive and accurate diagnosis [11–13]. In recent decades, salivary analysis for OSCC diagnosis has become an alternative tool to the serum testing because saliva collection is simple, safe, painless, non-traumatic and can be taken repeatedly. Many research groups have been identified potential biomarkers in saliva of OSCC patients using the genomics or proteomics approach, and they have been applied for diagnosis and prognosis of OSCC [14–17].

Immunoassay-based methods have been widely used to determine the biomarkers in tumor tissues and body fluids such as blood, saliva and urine. Several types of immunoassay such as enzyme-linked immunosorbent assay (ELISA), fluorescence-based immunoassay, electrochemical sensor, chemiluminescence immunoassay, and multiplexed bead platforms have been explored for diagnostics of disease [18–22]. Although immunoassays can provide a simple, selective and cost-effective method for clinical diagnosis, they still have drawbacks such as long incubation time (hours-days), poor precision and limiting sensitivity which depends on the affinity of antibody-antigen. An analysis of the current state of the art reveals an unmet medical need in the management of oral carcinoma. Thus, novel strategies have been extensively worked out in the context of analytical platforms as well as diagnostic tools for rapid, sensitive and reproducible detection of biomarkers [23–26]. Nanomaterials, which have one spatial dimension less than 100 nm, have taken center stage as promising materials for catalyst, drug delivery, and sensing application in recent years due to their unique physical and chemical properties [27]. Recently, nanoscale systems such as nanostructured microfluidic array, three-dimensional carbon microarrays, zinc oxide nanowire arrays on hierarchical graphene, and silicon nanowires sensor arrays have been spotlighted as the noteworthy approaches for sensitive cancer diagnosis using high surface area, electrical and optical properties of nanomaterials [28–31]. Our group introduced a hierarchical three-dimensional network of carbon nanotubes on Si pillar substrates (3DN-CNTs) for filtration in microfluidic systems [32,33]. The 3DN-CNTs provide not only high surface area but also easy functionalization to immobilize antibodies on the modified surfaces of CNTs based nanostructures.

In the present work, we offer a fluorescence-based immuno-sensor using 3DN-CNTs as a template (3DN-CNTs sensor) for the detection of Cyfra 21-1 which is one of representative OSCC biomarkers in saliva. The template was uniformly coated with Al2O3 to maintain the structural stability during solution drying process. The hydroxyl groups on an Al2O3-coated template was modified with an aminosilane reagent by self-assembled monolayer (SAM) formation for immobilization of biomolecules. The efficacy of 3DN-CNTs sensor was evaluated by the quantitative analysis of Cyfra 21-1 using a sandwich-type immunoassay method with a fluorescence-based corresponding antibody. In order to assess the feasibility of clinical diagnosis of OSCC, the Cyfra 21-1 concentration in clinical saliva samples measured by 3DN-CNTs sensor was compared with the results measured by electrochemiluminescence (ECL) assay.

2. Materials and methods

2.1. Materials

Fe(NO₃)₃·9H₂O and 3-(2-aminoethylamino)propyldimethoxymethylsilane (AEPDMS) were purchased from Junsei (Tokyo, Japan). Mo solution (JCP/DCP standard solution) was purchased from Aldrich Chemicals (Milwaukee, WI, USA). Phosphate buffer saline (PBS), bovine serum albumin (BSA), N-(3-dimethylaminopropyl)-N’-ethyloxycarbodimide hydrochloride (EDC), N-hydroxysuccinimide (NHS), 4-nitrobenzaldehyde and Tween 20 were purchased from Sigma Aldrich (St. Louis, MO, USA). Mouse monoclonal Cyfra 21-1 antibody (#10-2689, capture antibody; #10-2732, detection antibody; #61-1064, HRP-conjugated detection antibody) and partially purified Cyfra 21-1 protein (#30-AC69) were purchased from Fitzgerald Industries International (Action, MA, USA). Alexa Fluor 568 and tetramethylbenzidine (TMB) substrate solution were purchased from Thermo Scientific (Waltham, MA, USA). All other reagents were of analytical grade and used without further purification.

2.2. Fabrication of 3DN-CNTs

The fabrication of 3DN-CNTs was carried out as described in the previous reports [33,34]. The Si pillar-patterned substrate (diameter: 2 μm; height: 5 μm, and a gap of pillars: 1.5 μm) was used for fabricating 3DN-CNTs. 3DN-CNTs were synthesized by thermal chemical vapor deposition system using NH₃ gas for 10 min followed by C₆H₆ gas at 850 °C for 20 min. The deposition of Al2O3 onto 3DN-CNTs was conducted for 150 cycles by using atomic layer deposition (ALD, Cyclic 4000, Genitech). The morphology of 3DN-CNTs and Al2O3-coated 3DN-CNTs was examined by field-emission scanning electron microscope (FE-SEM, Hitachi S4800) operated at a beam energy of 15 kV. Raman spectra of 3DN-CNTs were detected by a Renishaw TM1000 Raman spectrometer (Renishaw, UK) using an Ar laser (laser excitation wavelength = 514.5 nm).
2.3. Amine functionalization of Al2O3-coated 3DN-CNTs

Al2O3-coated 3DN-CNTs were functionalized with amine groups by self-assembly process of AEAPDMS for antibody immobilization. Briefly, the Al2O3-coated 3DN-CNTs were exposed to ultraviolet (UV)-activated oxygen in a UV-ozone chamber (Jeasung Engineering, UVC-30, Korea) for 30 min to remove organic contaminants and generate hydroxyl groups on the surface of Al2O3. Then they were dipped into 1% AEAPDMS solution (in non-hydrous toluene) for 3 h under nitrogen atmosphere followed by washing with non-hydrous toluene and absolute ethanol thoroughly. In order to confirm the formation of monolayer on the surface of templates after SAM modification, Al2O3-coated 2D Si substrates were also modified with the same procedures described above. The surface morphology of Al2O3-coated 2D Si substrates after SAM modification was identified by atomic force microscope (AFM, Park Systems, XE-100, KR) and the thickness of self-assembled aminosilane layer was measured by ellipsometer (Rudolph Research, Ellipsometer Auto EL-III, NJ, USA) with a wavelength of 632.8 nm. The amine group after SAM modification initiated by the imine formation reaction between 4-nitrobenzaldehyde and amine group was validated using a UV/Vis/NIR spectrometer (PerkinElmer, LAMBDA™ 1050, USA) as described in the previous report [33]. The number of amino groups was calculated using Beer-Lambert equation using individual absorbance of 4-nitrobenzaldehyde (at \( \lambda_{\text{max}} \) 267 nm) after hydrolysis of 4-nitrobenzaldehyde conjugated each 2D Si substrate and 3DN-CNTs.

2.4. Determination of pair antibodies against Cfya 21-1

The binding assay was performed on BLItz system (FortBio, Pall Life Sciences, NY, USA) in order to identify the binding selectivity of paired antibodies against Cfya 21-1. Prior to binding measurements, Anti-mouse IgG Fc Capture (AMC) biosensor (Fortebio, USA) was equilibrated for 10 min in PBS. After an initial baseline for 30 s, capture antibody was loaded onto the AMC-coated sensor for 120 s. The biosensor was washed for 30 s by flowing through PBS and Cfya 21-1 was then associated with for 120 s, followed by washing for another 30 s in PBS. The detection antibody was associated with for 120 s, followed by dissociation for 120 s in PBS. Pair antibodies were identified as suitable for assay development using a BLItz Pro software.

2.5. Quantification of Cfya 21-1 with 3DN-CNTs sensor

Capture antibody was immobilized onto the amine-functionalized Al2O3-coated 3DN-CNTs by EDC-NHS crosslinking reaction. Each 5 µL of 1 mM EDC and 4 mM NHS was added into 990 µL PBS (pH 7.4) containing 10 µg capture antibody for pre-reaction at room temperature for 15 min. The amine-functionalized Al2O3-coated 3DN-CNTs were placed in a 96 well plate and incubated in the 100 µL of antibody solution at 25 °C for 3 h. After the reaction, the 3DN-CNTs were washed with 0.1% Tween 20 in PBS (T-PBS) thoroughly to remove any excess reagents and non-binding capture antibodies. Capture antibody-immobilized 3DN-CNTs were incubated at 100 µL of eleven different concentration of Cfya 21-1 in the range from 0.1 to 10,000 ng/mL for 1 h, subsequently washed with T-PBS to remove unbound biomarkers. Next, each 3DN-CNTs was incubated in 100 µL of 10 µg/mL of Alexa 568-tagged antibody labeling kit, for 1 h and then non-binding antibodies were washed out. The fluorescence microscope images of 3DN-CNTs at different concentrations of Cfya 21-1 were obtained using a BX51WI upright fluorescence microscope (Olympus, Tokyo, Japan) with a 50 × objective lens at an exposure time of 500 ms. The fluorescence images were analyzed with ImageJ software (US National Institutes of Health). In order to compare the sensitivity of 3DN-CNTs, the sandwich ELISA was employed at the above-mentioned concentrations of Cfya 21-1 according to the general procedure suggested for sandwich ELISA (Abcam, Cambridge, MA, USA).

2.6. Assays of clinical saliva samples

The Cfya 21-1 in clinical saliva samples were analyzed to assess the diagnostic feasibility and capability of fluorescence-based 3DN-CNTs immunoassay method. The 11 saliva samples were collected from 4 healthy persons and 7 OSCC patients at the Department of Oral and Maxillofacial Surgery, Korea University Hospital. The clinical study was approved by the Institutional Review Board (IRB) at the hospital. Consent documents were also obtained from all patients included in this study.

The saliva samples were centrifuged at 12,000 g for 5 min, and the supernatants were passed through a 0.45 µm pore-sized syringe filter (Millipore, Bedford, MA, USA). The clear supernatants were aliquoted and frozen for storage at –80 °C until thawed for analysis. All experiments were performed within 2 weeks after sampling. The samples were thawed in room temperature until completely thawed. Non-diluted clinical samples were assayed using a commercially available ECL assay system (ARCHITECT, Abbott Laboratories, USA). The clinical samples were diluted two fold with PBS buffer for the assay of the 3DN-CNTs sensor and the results were compared with those determined by the ECL assay method.

2.7. Statistical analysis

All data expressed as means ± SD are representative of at least two different experiments. To estimate the possible systematic bias and analytical agreement between the fluorescence-based 3DN-CNTs sensor and ECL assay, Passing–Bablok regression analyses were performed on clinical data using the MedCalc program (Ostend, Belgium).

3. Results and discussion

3.1. Structural properties and stabilization of 3DN-CNTs

The hierarchical CNT networks on Si substrates prepared as a sensor template to increase the sensitivity of biomarker measurement. As shown in Fig. 1a, CNTs were synthesized on Si pillar-patterned substrates and interconnected in a hierarchical shape between adjacent pillars. The interconnected CNTs between pillars were observed in various shapes such as straight, arch and double y-junction between pillars and the mean diameter of single CNTs bundles connected between pillars was about 10 – 15 nm (Fig. 51 c). The Raman spectra showed that the synthesized-CNTs were composed of single-walled carbon nanotubes (SWNTs) assembled in a bundled structure and the diameter of SWNTs was calculated to be about 0.9 nm as reported previously (Fig. 1c and d) [32,33].

The hierarchical morphology of CNTs between adjacent pillars was aggregated due to Van der Waals interaction and capillary force between adjacent CNTs during solution drying process [34]. Thin aluminum oxide layer was grown on the surface of 3DN-CNTs by atomic layer deposition (ALD) technique in order to achieve mechanical and structural stability of the hierarchical CNT networks. Fig. 1b showed the morphology of 3DN-CNTs after Al2O3 coating and the diameter of CNT bundle coated with Al2O3 was estimated to be 35 ± 5 nm (Fig. S1d). The hierarchical morphology of 3DN-CNTs was well maintained in aqueous solutions during the

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surface modification, immobilization processes or biomarker assay after Al2O3 coating (Fig. S2). These results indicated that Al2O3-coated 3DN-CNTs can be utilized as a template for biomolecule recognition under solution phases.

3.2. Surface functionalized template for antibody immobilization

Reliable biosensor platforms require the reproducibility and stability of antibodies, the uniform density of antibodies on the attached surface, as well as the uniform structure of sensor templates. Organosilane self-assembled monolayer (SAM) is commonly used for surface modification of Si substrates. In this study, one of dialkoxysilane monomers (AEAPDMS) was chosen to generate monolayer formation and amine functionalization on the surface of templates for uniform immobilization of antibody on the Al2O3-coated 3DN-CNTs [35,36]. In order to get a preliminary clue for expounding the formation of monolayer on the 3DN-CNTs, the surface properties of Al2O3-coated 2D Si substrate after SAM modification were characterized by using ellipsometer and AFM. The measured thickness of aminosilane layer on a Si substrate after 3 h of SAM modification was 11 ± 1 Å which was close to the theoretical thickness of AEAPDMS monolayer. Fig. 2a and b showed 1.0 μm × 1.0 μm AFM images of Al2O3-coated 2D Si substrate and AEAPDMS modified Al2O3-coated 2D Si substrate after SAM modification, respectively. Non-contact mode AFM images did not show any aggregated multilayer formation after SAM modification. The RMS value of Al2O3-coated 2D Si substrate was 0.186 nm and it was similar to RMS values after SAM modification. The amine group on the surface after SAM was characterized by imine formation reaction between the amine group and 4-nitrobenzaldehyde. The absorbance values of 4-nitrobenzaldehyde at aminosilanized 2D Si substrate and 3DN-CNTs were converted into 0.89 and 2.40 of the amine groups in 100 Å2, respectively. The absolute amine density of 3DN-CNTs after SAM modification was about 2.70 times higher than that of 2D Si substrate. On the basis of these results, the resultant layer on 3DN-CNTs after SAM modification was regarded as quasi-monolayer with successfully functionalized amine groups on the surface. The uniform modification of 3DN-CNTs can offer the reproducibility of antibody layer after immobilization of antibody.

3.3. The quantitative analysis of Cyfra 21-1 using 3DN-CNTs sensor

Scheme 1 illustrated the fluorescence-based immunoassay using 3DN-CNTs for the quantitative analysis of Cyfra 21-1. Paired antibodies against Cyfra 21-1 were selected by the binding assay using BLItz system which measures the shift in the interference pattern of the reflected light between the biomolecule-binding layer and an internal reference layer on a disposable fiber optic-based surface (Fig. 3). Various concentrations of Cyfra 21-1 in PBS solution were captured on the surface of 3DN-CNTs through
antibody-antigen interaction, and Alexa-fluor 568-tagged antibodies were formed sandwich immunocomplexes. Fig. 4a showed that the fluorescence intensity was significantly enhanced with increasing concentrations of Cyfra 21-1 in the range from 1 to 1000 ng/mL. The fluorescence intensity values on each fluorescence image were converted into fluorescence enhancement ratio (F/F0) and then plotted on a calibration curve with log scale of concentration (x-axis). As shown in Fig. 4b, the calibration curve is the sigmoidal in shape ranged from 0.1 to 10,000 ng/mL and the calibration curve was used for quantitative estimation of Cyfra 21-1 by 4-parameter logistic (4-PL) model for curve-fitting analysis [37]. The adjusted R-squared of the experimental data according to 4-PL model is 0.993. The limit of detection (LOD) of Cyfra 21-1 measured by 3DN-CNTs sensor was found to be 0.5 ng/mL and it was...
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4. Conclusions

In this study, sensitive and reproducible 3DN-CNTs sensor was developed for the detection of Cyfra 21-1 as OSCC biomarker. The surface of A12O3-coated 3DN-CNTs was uniformly modified with amine groups for facilitating antibody immobilization. Potential application 3DN-CNTs as fluorescence-based immunosensor was investigated through quantitative analysis of Cyfra 21-1. The LOD of the 3DN-CNTs sensor was estimated to be 0.5 ng/mL and the calibration curve of Cyfra 21-1 was sigmoidal in shape ranged from 0.1 to 10,000 ng/mL with r square = 0.993. The sensitivity of 3DN-CNTs sensor was approximately 20 times higher than that of the conventional sandwich ELISA system. The 3DN-CNTs sensor improved the detection sensitivity of Cyfra 21-1 because of unique structural properties such as increase of total binding sites, hierarchical

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<th>Cyfra 21-1 (ng/mL)</th>
<th>Fluorescence enhancement ratio (F/F₀)</th>
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<tr>
<td>Mean value</td>
<td>Standard deviation</td>
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<tr>
<td>0.1</td>
<td>1.54</td>
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<td>0.5</td>
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<td>1</td>
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<td>5000</td>
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※ CVs: coefficient of variations.

Table 1

Fig. 4. Representative fluorescence images of (a) 3DN-CNTs sensor at different concentrations of Cyfra 21-1. (b) The calibration curve for Cyfra 21-1 on 3DN-CNTs sensor. (c) The standard calibration curve from a conventional sandwich ELISA.
structures for the easy accessibility of biomolecules. The clinical feasibility of 3DN-CNTs sensors was assessed by comparing with commercial available ECL assay system in the hospital. The results of Cyfra 21-1 in saliva samples measured by 3DN-CNTs sensor showed a linear relationship and good conformity with those measured by ECL assay, and the mean CVs values of 3DN-CNTs sensors were less than 15% (CV cut-off value). The 3DN-CNTs sensor could be expected as a feasible biosensor for diagnosis of OSCC in clinical saliva samples. However, since the number of clinical samples analyzed by 3DN-CNTs biosensors is small and the commercially available ECL assay for OSCC diagnosis is not reliable enough to completely complement, further experiments will increase the reliability by using more patient samples in future study.

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Appendix A. Supplementary data

Supplementary data related to this article can be found at https://doi.org/10.1016/j.jaca.2018.04.025.

References


Fig. 5. Passing and Bablok regression analysis (a) between 3DN-CNTs sensor and ECL assay.