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

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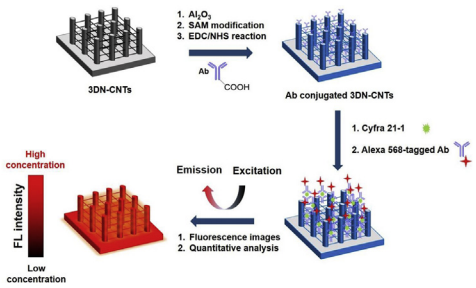
HIGHLIGHTS

- 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.

GRAPHICAL ABSTRACT



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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

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linear correlation. The 3DN-CNTs sensor offers great potential for accurate diagnosis of OSCC using Cyfra 21-1 biomarker in clinical fluids.

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 carcinoembyonic 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 substrate**s** (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 immunosensor 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 Al_2O_3 to maintain the structural stability during solution drying process. The hydroxyl groups on an Al_2O_3-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

$Fe(NO_3)_3 \cdot 9H_2O$ and 3-(2-aminoethylamino)propyldimethoxymethylsilane (AEAPDMS) were purchased from Junsei (Tokyo, Japan). Mo solution (ICP/DCP standard solution) was purchased from Aldrich Chemicals (Milwaukee, WI, USA). Phosphate buffer saline (PBS), bovine serum albumin (BSA), *N*-(3-dimethylaminopropyl)-*N*′-ethylcarbodiimide 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 NH_3 gas for 10 min followed by C_2H_2 gas at 850 °C for 20 min. The deposition of Al_2O_3 onto 3DN-CNTs was conducted for 150 cycles by using atomic layer deposition (ALD, Cyclic 4000, Genitech). The morphology of 3DN-CNTs and Al_2O_3-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).

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