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Electrochemical peptide sensor for diagnosing adenoma-carcinoma transition in colon cancer

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ABSTRACT

Colorectal cancer (CRC) is one of the leading causes of cancer-related deaths. Therefore, more sensitive and early diagnostic methods for CRC are urgently needed. In this study, an efficient electrochemical biosensor for early diagnosis of adenoma-to-carcinoma progression that employs a series of chemically modified affinity peptides was developed. A series of amino acid-substituted and cysteine-incorporated synthetic peptides with flexible linkers was chemically synthesized and immobilized to a gold sensor layer; performance of the sensor was monitored using cyclic voltammetry (CV) and electrochemical impedance spectroscopy (EIS). Potential affinity peptides (LRG1 BP1–BP4) specific for the LRG1 biomarker as a target protein were chosen according to a quantitative current decrease and dynamic impedance increase by CV and EIS, respectively. Using EIS, the K_d value of the LRG1 BP3 peptide was found to be 8.3 ± 2.7 nM. The applicability of the sensor to detect LRG1 proteins was confirmed in human plasma from colorectal adenomas and carcinomas (n = 20 in each group). The detection of LRG1 in accordance with the ΔR_{ct} value (electron-transfer resistance at the electrode surface) of the sensor layer incorporating LRG1 BP3 peptides showed a statistically significant difference ($p < 0.001$) between adenomas and carcinomas, indicating that the potential use of this biosensing platform for detecting the CRC biomarker, as well as for monitoring the colorectal adenoma-to-carcinoma transition in an electrochemically miniaturized biosensor (*e*-chem biosensor) in point-of-care testing, is possible.

1. Introduction

Colon cancer, a malignant cancer arising in the large intestine, is one of the most common cancers and is the third leading cause of death worldwide (Choi et al., 2013; Galamb et al., 2009; Sole et al., 2014; Zhang et al., 2007). Several clinical diagnostic methods are commonly used for the early detection of colorectal cancer (CRC), including sigmoidoscopy or virtual colonoscopy with biopsy confirmation of cancer tissue and blood (Choi et al., 2013; Galamb et al., 2009; Wu and Qu, 2015). Although miniaturized colonomicroscopy is the most powerful method for early diagnosis of CRC in hospitals, it requires multi-step processing with unfavorable conditions for the patients, requiring lengthy clinical processes. This technique also involves relatively high medical cost and invasive methods, and presents a small risk of perforation in the wall of the colon, though this statistical possibility is quite rare (Galamb et al., 2009; Kumar et al., 2011). Thus, there has been an increasing demand for the development of new diagnostic techniques that are capable of rapid and precise detection of cancer targets. Toward this goal, non-invasive and multi-functional diagnostic methods for the efficient prediction of early-stage CRC or pre-malignant lesions are being developed (Abdolahad et al., 2014; Albrethsen et al., 2005; Choi et al., 2013; Galamb et al., 2009; Kolitz-Domb et al., 2014; Schoen, 2002; Yi et al., 2012; Zhang et al., 2007). For example, a PCR-based assay (Clark-Langone et al., 2007; Galamb et al., 2009), a gene mutation assay (Yi et al., 2012), an antibody-based assay (Tao et al., 2012), and a biomarker-based method (Li et al., 2013) have been developed. However, these methods still possess some challenges for becoming ready-to-use in the early diagnosis of CRC. Recently, promising biomarkers from human plasma or blood suitable for diagnosing CRC have been proposed and well-characterized (Choi et al., 2013; Giusti et al., 2012; Ihi et al., 1997; Rangiah et al., 2009; Sole et al., 2014; Wu and Qu, 2015; Yi et al., 2012). Choi et al. (2013) reported that leucine-rich α-2-glycoprotein 1 (LRG1) was highly over-expressed up to a 2-fold increase in CRC patients. According to their

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studies, LRG1 may play an important role in apoptosis and cell survival. Additionally, the level of LRG1 in plasma from CRC patients was well-correlated with the progression of CRC from the adenoma to carcinoma stage. Furthering these findings, we have recently reported that unique and short linear affinity peptides specific for the target protein LRG1 were identified by an M13 phage display technique; their relative binding affinities were characterized by an enzyme-linked immunosorbent assay (ELISA) (Hwang et al., 2016). From these results, we observed that the phage-displayed peptides displayed sub-picomolar binding affinities for LRG1 proteins.

Affinity reagents that specifically bind to antigens are very important molecules in clinical and diagnostic applications. In particular, antibodies are the key reagents in many important fields, such as diagnosis of various diseases, therapeutics, and clinical or pharmaceutical analysis. Polyclonal antibodies are relatively cost-effective but possess heterogeneous binding properties, while monoclonal antibodies are expensive to produce from animal cells, but are homogeneous with high specificity (Hwang et al., 2015; Park et al., 2010, 2015; Wu et al., 2011). Antibody-like molecules such as single-chain fragment variables (scF_v) and fragment antigen binding (F_{ab}) are also promising alternatives monoclonal antibodies (Crivianu-Gaita and Thompson, 2016). Comparatively, aptamers have also been studied in biosensor development. Most aptamers are single-stranded nucleic acids that are identified using combinatorial techniques (e.g. SELEX) and selectively bind to various target molecules (Crivianu-Gaita and Thompson, 2016; Hu et al., 2014).

Current immunoassays rely mainly on antibodies as affinity reagents, are relatively expensive, require multiple sample preparations (Hwang et al., 2015; Park et al., 2010, 2015), and sometimes lead to false-positive results. Compared to the use of antibodies, linear and unique short peptides have attracted attention as effective affinity reagents in the development of new biosensors (Wu et al., 2011). One of the most exciting properties of the peptides is their small size and cost efficiency in the creation of a new biosensing platform (Hwang et al., 2016; Park et al., 2015). Generally, peptides are either linear or in a cyclic form, indicating that peptides are more amenable than antibodies to engineering at the molecular level. In addition, targeting peptides can be attached easily to the surfaces of materials such as nanoparticles or liposomes for bio-imaging and new bioassay systems (Wu et al., 2010, 2011).

The evolutionary M13 phage display technique is widely utilized for identifying unique affinity peptides that selectively bind to nearly limitless targets (Kehoe and Kay, 2005). This procedure includes the following steps: i) the phage displaying peptide interacts with the chosen targets, and unbound phages are washed away; ii) bound phages are eluted at low pH (pH 2.2), neutralized, and then amplified in *E. coli*; iii) the individual phage clones are sequenced, and a phage ELISA is performed. There have been some reports in which the entire phage clones, or the free peptides identified from these selections, were used as recognition elements on various types of biosensors (Hwang et al., 2017, 2015; Sidhu, 2001). Using this strategy, our group has recently reported a new biosensor for detecting troponin I (a cardiac biomarker) (Park et al., 2010), norovirus (foodborne pathogen) (Hwang et al., 2017), procalcitonin (a kidney biomarker) (Park et al., 2015), alanine aminotransferase (a liver function biomarker) (Wu et al., 2011), and a CRC biomarker (Hwang et al., 2016).

Meanwhile, a number of remarkable electrochemical detection platforms have been developed for clinical and bioanalytical applications (Abdolahad et al., 2014; Hwang et al., 2017; Wu et al., 2010). Among electrochemical sensing methods, cyclic voltammetry (CV) and electrochemical impedance spectroscopy (EIS)-based detections have been widely used because they have the potential for rapid response, label-free detection, and robust reliability (Hwang et al., 2017; Wu et al., 2011). Both methods can measure either the change of current or the impedance by applying voltage. When applied on an electrode, the method measures binding of molecules on the electrode layer by mass adsorption which obstruct the access of an electroactive redox probe to the electrode, therefore decreasing the redox current in CV or increase impedance in EIS. Additionally, one of the exciting benefits of these approaches is that they do not require labeling for detection. However, there are some considerations for aspects further refinements (Daniels and Pourmand, 2007). For example, the size of the electrode can affect the actual impedance and can be chosen with respect to instrument's frequency range. Increasing frequency will be less affected by drift and noise in the measurement. In addition, reduction of the electrode area or thickness may also affect impedance change upon target binding. In this study, we demonstrate the first use of synthetic affinity peptides identified through phage display for the development of an electrochemical biosensor toward the early diagnosis of CRC from plasma in patients with both adenoma and carcinoma. The basic principle of our study is as follows; once the most promising affinity peptide for target was selected, a cysteine-incorporated peptide was chemically synthesized and transferred to the gold electrode. Thereafter, the peptide-modified gold electrode was used to detect LRG1 and evaluated the performance of the sensor using CV and EIS.

2. Materials and methods

2.1. Chemicals

Bovine serum albumin (BSA) was purchased from Sigma-Aldrich (St. Louis, MO, USA). Human recombinant LRG1 proteins were purchased from Randox Life Science (Crumlin, UK). A series of synthetic peptides modified with a C-terminal cysteine and a flexible linker (-GGGGS-) were chemically synthesized (> 95% purity) by Peptron (Daejeon, Korea), as shown in Table S1. The quartz crystals deposited with gold were obtained from Biolin Scientific (Stockholm, Sweden). Phosphate-buffered saline (PBS, pH 7.4) and Tris-HCl buffer solutions were used to make all proteins, human plasma samples, and synthetic peptide solutions for CV and EIS measurements. Unless otherwise stated, all reagents were analytical grade.

2.2. Preparation of human patient plasma samples

A total of 40 patients with adenomas (n = 20) and carcinomas (n = 20) were used (Table S2). The study protocol was reviewed and approved by the College of Medicine, Keimyung University. In detail, blood samples from the two groups were collected, and then the crude blood plasma was separated by centrifugation (3,000×g, 10 min), as demonstrated previously (Choi et al., 2013). Finally, the fractionated plasma was directly used for electrochemical analysis without any purification.

2.3. Circular dichroism (CD) spectroscopy

Circular dichroism (CD) spectra of all synthetic peptides at a concentration of 50 μM were recorded on a CD spectrometer (J-715, JASCO, Tokyo, Japan) using a UV cell with 0.1 cm optical path length at 25 °C. For the CD analysis, PBS solution (pH 7.4) was used, and CD spectra were scanned 4 times for every sample, as previously reported (Hwang et al., 2017).

2.4. Preparation of the affinity peptide-modified electrode

The peptide-functionalized gold electrode (14 × 20 mm in size) was prepared by the following steps. First, the gold electrode was immersed into piranha solution (H_2SO_4:H_2O_2 = 4:1, v/v) for 10 min to remove residual dust and impurities on the surface layer of the gold electrode. The electrode was then rinsed with deionized (DI) water several times. This pre-functionalized electrode was dried by blowing with N_2 gas for several seconds. After these polishing steps, the electrode and voltammetric cell were assembled, and 100 μL of thiol-modified synthetic