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Double stranded aptamer-anchored reduced graphene oxide as target-specific nano detector

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ABSTRACT

Here, we report a double-stranded, dual-anchored, fluorescent aptamer on reduced graphene oxide (rGO) for the sensitive, selective, and speedy detection of a target protein in biological samples. This nano detector is composed of a target protein-specific fluorescent aptamer with BHQ1 as one anchoring moiety that forms double-stranded sequences with a complementary oligonucleotide sequence with BHQ1 as the other anchoring moiety, anchored to rGO nanosheets. The double-stranded and dual-anchored aptamer on rGO nanosheets (DAGO) exhibited 7.3-fold higher fluorescence intensities compared to a single-stranded, single-anchored fluorescent aptamer on rGO. As a model target protein, interferon-γ was used. DAGO detected the target protein, with linearity over a five-orders-of-magnitude concentration range (0.1 ng/ml–10 μg/ml) in buffer and human serum. DAGO was highly specific for the target protein, exhibiting little changes in fluorescence intensity in response to the non-target proteins, interleukin-2 and tumor necrosis factor-α. Moreover, DAGO allowed rapid quantification of the target protein in human immunodeficiency virus-positive patient serum samples. DAGO-based detection was complete in less than 10 min. Our results indicate that the DAGO provides new opportunities for the rapid and specific detection of target proteins in biological samples and could be widely applied to quantitate various target proteins by replacing the aptamer sequences.

1. Introduction

Aptamers are short, single-stranded DNA (ssDNA) or RNA oligonucleotides that can bind a wide range of targets, including proteins [1], DNA [2,3] small molecules [4], and metal ions [5] with high specificity and affinity. There has been increased interest in the development of aptamer-based sensors because of the advantages they offer in terms of high thermal stability, ease of chemical modification, and capability of incorporating surface-binding or sensing moieties compared to antibodies [6,7]. In an attempt to develop such sensors, a number of detection strategies, including fluorescence, surface-enhanced Raman spectroscopy, microgravimetry, and electrochemistry have been explored. Among these approaches, detection by monitoring changes in fluorescence is an attractive option owing to features such as high sensitivity, reproducibility, and facile operation [8,9]. A conventional aptamer-based sensor detects fluorescence resonance energy transfer signals when a ligand-induced conformational change in a fluorophore- and quencher-labeled aptamer occurs. However, the sensitivity of such system is influenced considerably by the length of quencher-labeled DNA and temperature [10]. Thus, there remains a need for the development of new platforms that can selectively and sensitively detect target molecules.

Graphene, a two-dimensional nanosheet, has recently received considerable attention because of its remarkable electronic, mechanical, and thermal properties [11]. Reduced graphene oxide (rGO) can be chemically synthesized by placing graphene oxide (GO) in a solution of hydrazine [12]. Since rGO contains more crystalline graphene regions on the sheet than GO, aptamers are adsorbed more strongly onto a rGO surface through hydrophobic and π–π stacking interactions between the ring structures of nucleobases and carbon rings of rGO [13]. Moreover, rGO can act as a more effective distance-dependent fluorescence quencher than

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GO or graphene [14]. This combination of high fluorescence quenching efficiency and superb ssDNA adsorption ability makes rGO suitable for application to the detection machinery of aptamer-based nano detectors.

Cytokines are often secreted by immune cells in response to various pathogens [15]. Monitoring secreted cytokines can provide diagnostic information about various infectious diseases in patients. For example, in human immunodeficiency virus (HIV)-infected patients, T-helper and cytotoxic T-lymphocytes vigorously produce the cytokine protein such as interferon-γ (IFN-γ), resulting in low viremia and slow progression of the disease [16,17]. It is therefore important to detect cytokine levels for clinical diagnostic purposes. Conventional antibody-based immunoassays, such as enzyme-linked immunosorbent assays, are common methods for detecting and quantifying secreted cytokines. Although immunoassays are sensitive and specific for the target protein, they require multiple washing steps, several hours of reaction time, and the use of expensive reagents [18]. The development of a new assay system that saves time and reduces costs would thus be desirable.

In this study, we designed a double-stranded, dual-anchored, fluorescent (FAM-labeled) aptamer on rGO nanosheets for quantitation of target protein in biological samples. To prevent non-specific quenching of fluorescent aptamer upon interaction with rGO, we hybridized the fluorescent aptamer with a complementary sequence to form double strands. To fortify the anchoring on rGO, we labeled the double-stranded fluorescent aptamer with two anchoring moieties. As a model target protein specific for the aptamer, IFN-γ was used.

2. Materials and methods

2.1. Synthesis of rGO nanosheets

GO was prepared from graphite powder following a modified Hummer's method [12]. Briefly, graphite powder (0.5 g; Sigma–Aldrich, St. Louis, MO, USA) was added to cold H_2SO_4 (23 ml). While this mixture was gradually stirred on ice, $KMnO_4$ (3 g) and $NaNO_3$ (0.5 g) were added slowly. The resulting mixture was further stirred for 1 h at 35 °C. Subsequently, 46 ml of triple-distilled water (TDW) was added and the mixture was incubated at 90 °C for 1 h. The reaction was halted by adding 140 ml of TDW and 10 ml of 30% H_2O_2. The reaction product was washed and purified by repeated centrifugation, first with an aqueous 5% HCl solution and then with TDW (three times). Finally, the product was suspended in TDW and sonicated for 2 h to exfoliate the GO layers into GO nanosheets. Unexfoliated GO was removed by centrifugation at 1600 × *g* for 10 min. The supernatant containing GO nanosheets was collected and filtered through 0.2-μm polycarbonate membrane filters (Millipore Corp., Billerica, MA, USA) using an extruder (Northern Lipid, British Columbia, Canada).

GO nanosheets were subsequently reduced to generate rGO nanosheets according to the method of Li and colleagues [19], with slight modification. Briefly, 2.0 ml of homogeneously dispersed GO nanosheet solution was mixed with 8.0 ml of TDW, 0.5 ml of ammonia solution (28 wt% in water; Junsei Chemical Co., Tokyo, Japan), and 5.0 μl of hydrazine monohydrate (64% in water). The resultant mixture was stirred in a water bath (80 °C) for 10 min, and then removed from the water bath and allowed to cool to room temperature. Excess hydrazine and ammonia were removed by dialyzing the mixture (MWCO 100K; Spectrum Laboratories, Inc., Rancho Dominguez, CA, USA) with TDW for 2 d with four changes of TDW over a 12-h interval. The obtained rGO nanosheets dispersed in TDW were stored at 4 °C until use. The final concentration of the prepared rGO was 1 mg/ml.

2.2. Anchoring of target protein-specific fluorescent aptamers onto rGO nanosheets

Double-stranded aptamer structures dually anchored with Black Hole Quencher-1 (BHQ1) were designed for perpendicular anchoring of IFN-γ–specific fluorescent ssDNA aptamers (36 bp) to rGO nanosheets (Fig. 3A). Single-stranded IFN-γ–specific fluorescent DNA aptamers (ssAptamer, 25 μg/ml, Bioneer, Daejeon, South Korea) containing FAM dye at the 5′-end and BHQ1 at the 3′-end were hybridized in hybridization buffer (10 mM Tris–HCl, 1 mM EDTA, 100 mM NaCl, pH 8.0) with complementary ssDNA oligonucleotides singly labeled with BHQ1 (25 μg/ml). Double-stranded fluorescent aptamers (dsAptamer) were formed by heating the solution containing ssAptamers of various lengths and complementary oligomers to 95 °C for 5 min and slowly cooling to 30 °C to allow hybridization. Double-stranded, dual-anchored, fluorescent aptamers (dsDA-Aptamer; 50 μg/ml) were immobilized onto the rGO surfaces by adding 10 μg/ml of rGO to the resultant solution. In some experiments, ssAptamers were hybridized with complementary DNA sequences with or without BHQ1, to which 10 μg/ml of rGO was added. The mixture was vortexed vigorously and incubated at room temperature for 30 min to induce formation of dsDA-Aptamers on rGO nanosheets (DAGO). The solution was centrifuged at 6000 × *g* for 2 min to remove excess, unattached aptamers. The supernatant was discarded and the remaining pellet was suspended in hybridization buffer (200 μl).

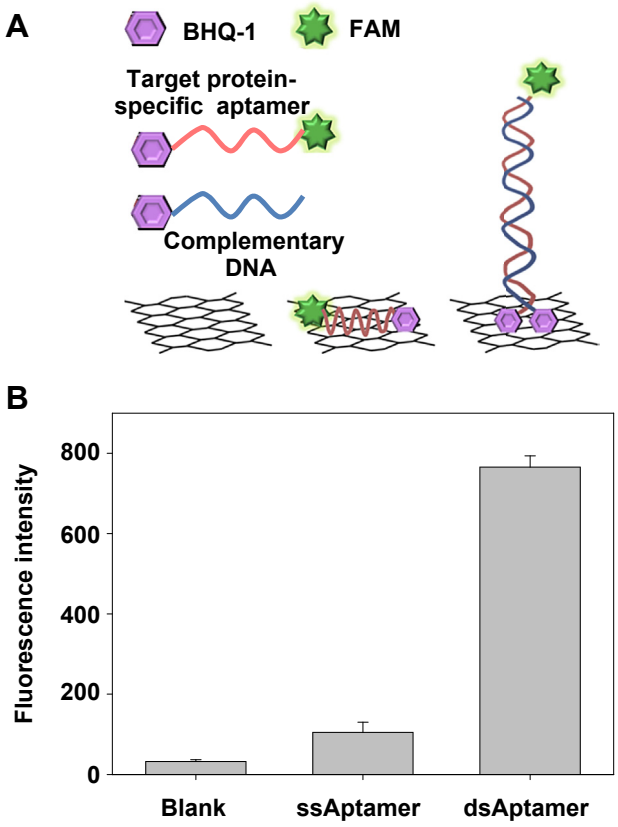

Fig. 1. Fluorescence intensity of ssAptamers and dsAptamers on rGO. (A) Schematic representation of ssAptamers and dsAptamers anchored on a rGO nanosheet. (B) Fluorescence intensities of rGO blank, ssAptamers on rGO, and dsAptamers on rGO were determined at an excitation wavelength of 485 nm and an emission wavelength of 520 nm ($n = 4$).

2.3. Target protein sensitivity and specificity test

The sensitivity of DAGO for its target protein was evaluated by incubating 10 μg/ml of DAGO with different concentrations of human IFN-γ (R&D Systems, Inc., Minneapolis, MN, USA) ranging from 100 pg/ml to 10 μg/ml. Specificity was demonstrated by challenging DAGO with 10 μg/ml of human tumor necrosis factor-α (TNF- α; R&D Systems), interleukin-2 (IL-2; R&D Systems), or the analyte of interest (1 μg/ml of IFN-γ). After incubating for 3 min at room temperature, the solution was centrifuged at 6000 × *g* for 2 min to remove any excess, unreacted materials and the remaining pellet was resuspended in hybridization buffer (200 μl). The binding of IFN-γ to DAGO was determined by exciting at a wavelength of 485 nm and measuring fluorescence intensity at an emission wavelength of 520 nm using a spectrophotometer (Molecular Devices, Sunnyvale, CA, USA).

2.4. Assay of target protein in human serum

To detect IFN-γ in human serum using DAGO, we spiked serum samples from healthy individuals (Scipak Ltd, Kent, UK) with different concentrations of IFN-γ (100 pg/ml to 10 μg/ml) and then added DAGO (10 μg/ml) to each spiked serum sample. After incubating for 3 min at room temperature, the solution was centrifuged at 6000 × *g* for 2 min to remove any excess, unreacted materials and the remaining pellet was resuspended in hybridization buffer (200 μl). The concentrations of IFN-γ in human serum were determined by exciting at a wavelength of 485 nm and measuring fluorescence intensity at an emission wavelength of 520 nm using a spectrophotometer (Molecular Devices, Sunnyvale, CA, USA). In some experiments, Quantitkine enzyme-linked immunosorbent assay (ELISA) human IFN-γ (R&D Systems, Inc., Minneapolis, MN, USA) was used to detect IFN-γ in normal serum samples according to the manufacturer's protocol.