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Citation: CHEN, H. ... et al, 2016. Detection of matrilysin (MMP-7) activity using polypeptide functionalized reduced graphene oxide field-effect transistor sensor. Analytical Chemistry, 88 (6), pp. 2994-2998.

Additional Information:

- This article was published in Analytical Chemistry [© American Chemical Society] and the definitive version is available at: http://dx.doi.org/10.1021/acs.analchem.5b04663

Metadata Record: https://dspace.lboro.ac.uk/2134/20728

Version: Accepted for publication

Publisher: © American Chemical Society

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Letter

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Anal. Chem., Just Accepted Manuscript • DOI: 10.1021/acs.analchem.5b04663 • Publication Date (Web): 18 Feb 2016

Downloaded from http://pubs.acs.org on February 23, 2016

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Detection of Matrilysin (MMP-7) Activity Using Polypeptide Functionalized Reduced Graphene Oxide Field-Effect Transistor Sensor

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ABSTRACT: A novel approach for rapid and sensitive detection of matrilysin (MMP-7, a biomarker involved in the degradation of various macromolecules) based on polypeptide (JR2EC) functionalized reduced graphene oxide (rGO) field effect transistor (FET) is reported. MMP-7 specifically digests negatively charged JR2EC immobilized on rGO, thereby modulating the conductance of rGO-FET. The proposed assay enabled detection of MMP-7 at clinically relevant concentrations with a limit of detection (LOD) of 10 ng/mL (400 pM), attributed to the significant reduction of the net charge of JR2EC upon digestion by MMP-7. Quantitative detection of MMP-7 in human plasma was further demonstrated with a LOD of 40 ng/mL, illustrating the potential for the proposed methodology for tumor detection and carcinoma diagnostic (e.g. lung cancer and salivary gland cancer). Additionally, excellent specificity of the proposed assay was demonstrated using matrix metalloproteinase 1 (MMP-1), a protease of the same family. With appropriate selection and modification of polypeptides, the proposed assay could be extended for detections of other enzymes with polypeptide digestion capability.

Proteases are enzymes involved in the digestion of long protein chains into shorter fragments (a process known as proteolysis) by splitting the corresponding peptide bonds. Due to their significant roles in numerous physiological processes and involvement in several pathological conditions, proteases have attracted considerable attention recently. In particular, matrix metalloproteinases (MMPs) have been intensively studied over past decades as the dysregulation of MMP can be related to cancer, AIDS, inflammation, etc. Discovered in 1988, the matrix metalloproteinase matrilysin (MMP-7) was found to be involved in the degradation of various macromolecules. This molecule plays a key role in the regulation of lung defence and acts as an indicator of certain cancers (e.g. lung cancer and salivary gland cancer). Also, it is believed that MMP-7 contributes to invasive growth and metasis of tumors and carcinomas. Due to its various roles in physiological processes, MMP-7 has attracted intensive attention and various sensing approaches have been developed. Currently, sensing approaches for MMP-7 include western blot analysis, enzyme-linked immunosorbent assay (ELISA) and Förster resonance energy transfer (FRET) analysis. Nevertheless, these approaches have their respective limiting factors: ELISA suffers from limited throughput due to its resource intensive nature (sophisticated instrument and well-trained personnel required); western blot analysis is a non-quantitative method which provides limited information on the concentration of analyte targeted; FRET are limited by concerns such as photo-bleaching of probes and toxicity of quantum dots. Recently, Chen et al. described a colorimetric detection of MMP-7 based on polypeptide functionalized gold nanoparticles. In this assay, the gold nanoparticles were functionalized with synthetic polypeptides containing recognition/cleavage sites for MMP-7. Cleavage of the polypeptides induced nanoparticle aggregation and a concomitant color shift that enabled a direct and quantitative measurement of the concentration and activity of MMP-7. However, the limit of detection (LOD) achieved by this assay was 5 nM, approximately equivalent to the critical level of MMP-7 related to salivary gland cancer. Moreover, performance of this MMP-7 assay in clinical sample matrices was not demonstrated.

Graphene-based field-effect transistors (FETs) have been widely used as sensing platforms for ultra-sensitive and label free molecular detections. As a single layer of sp² hybridized carbon atoms, graphene has emerged to be an exciting class of nanomaterials for biosensing applications in virtue of its absolute 2D structure, as well as superior electrical and physical properties. FET is a transistor based on semiconducing material and the conductivity of the channel for charge carrier (either electron or hole) is controlled by an external electric field (gate voltage). FETs are ideal platforms for elec-
tronics as any changes in the physical parameters (such as charge density and electrical double layer composition/extension) of the graphene-based material induced by attachment of target molecules could have a significant effect on the source-drain current and generate detectable signals.\textsuperscript{26} To date, graphene based FET devices have been widely explored in electronic detections of DNA,\textsuperscript{27} enzymes,\textsuperscript{28} proteins \textsuperscript{29,30}and ions.\textsuperscript{31}

In this article, a novel approach for rapid (lag time < 5 min) and sensitive electronic detection of MMP-7 using FETs based on polypeptide functionalized reduced graphene oxide (rGO), a p-type semiconductor,\textsuperscript{12,23} is described. In brief, rGO was deposited on Si/SiO\textsubscript{2} surface and then the electrodes were deposited using thermal evaporation through a mask. The polypeptide was attached to the rGO surface via a pyrene-maleimide linker. A small voltage bias (V\textsubscript{d}) of 10 mV was applied between the source electrode and the drain electrode, the current (I\textsubscript{d}) flowing through the transducing layer was recorded while sweeping the gate voltage (V\textsubscript{g}) from -600 mV to 0 mV. The rGO films obtained via GO reduction by hydrazine were deposited on Si/SiO\textsubscript{2} substrate, as reported previously.\textsuperscript{34} To achieve high sensitivity and specificity, the rGO was functionalized with a de novo designed synthetic polypeptide (JR2EC, sequence shown in Figure 1). Consisting of 42 amino acids, this polypeptide exhibits two recognition and digestion sites (Ala-Leu and Ala-Gln-Leu, as shown in Figure 1) for MMP-7.\textsuperscript{35} It has been shown that immobilized polypeptides could be hydrolyzed by MMP-7, resulting in decreased size, and more importantly, reduced net charge of the polypeptide from -5 to -1 upon hydrolysis, as schematically shown in Figure 1.\textsuperscript{19} As a result of the significantly reduced net charge carried by polypeptides, the source-drain current (I\textsubscript{d}) of the rGO-FET device decreased drastically and the change in I\textsubscript{d} could be readily detected in real time. It was observed that the change in I\textsubscript{d} is dependent on the concentration of MMP-7, thus enabling a quantitative monitoring of the concentration and activity of this protease. The principle of the proposed assay is illustrated in Figure 1. The results revealed that the detection using the proposed assay was rapid (lag time < 5 min) and highly sensitive. The LOD achieved for MMP-7 in buffer was 10 ng/mL (400 pM), which was one order of magnitude higher than the LOD achieved by the colorimetric approach reported.\textsuperscript{19} The high sensitivity could be attributed to the significantly reduced net charge (from 5 negative charges per peptide to 1 negative charge per polypeptide, at pH 7) carried by the immobilized polypeptides, as well as reduction of polypeptide size caused by MMP-7 digestion. The reduction of charges (Figure S3) leads to a reduction of electrostatic potential/gate voltage in the FET device. Besides kinetic measurements in buffer, detections of MMP-7 of different concentrations in human plasma were also demonstrated using the proposed assay. Note that before introduction of MMP-7, the sensing surface was thoroughly washed to remove free polypeptide in solution. This is critical, as free polypeptides in solution affects the sensitivity of the assay. The results indicated that I\textsubscript{d} decreased as a result of incubation with MMP-7 (Figure 3c) and the response increased exponentially with the concentration of MMP-7 (Figure 3d). The LOD of plasma test calculated using the 3σ/S approach was 40 ng/mL. Sensitivities of the proposed assay for detection of MMP-7 in both buffer and human plasma are higher than that of the colorimetric approach reported previously\textsuperscript{19} and comparable to ELISA and optical assays.\textsuperscript{36-38} Additionally, LODs achieved by this assay are within clinically relevant ranges (e.g. 0.1–5 µg/mL for salivary gland cancer\textsuperscript{39} and 1–10 ng/mL for bladder cancer patients\textsuperscript{40}). Therefore, the proposed assay shows a great potential for applications in tumor detection and carcinoma diagnostic.

RESULTS AND DISCUSSION

Figure S2 shows the FESEM image of the rGO flakes deposited on the Si/SiO\textsubscript{2} substrate. The bright regions are the substrate, while the dark regions are deposited rGO flakes. As-prepared rGO devices were incubated with pyrene maleimide for 30 min and rinsed with PBS, followed by PEG passivation and incubation with JR2EC for 30 min. Before kinetic measurements, the immobilization of JR2EC with pyrene maleimide as linker molecule was investigated with two control groups. In Group I (red), the rGO-FET device was passivated with PEG and incubated with JR2EC directly. In Group II (blue), the rGO-FET device was incubated with pyrene maleimide, passivated with PEG and then incubated with JR2EC. As shown in Figure 2(a), device in Group II exhibited a significant I\textsubscript{d} response as compared to that of device in Group I, demonstrating that the immobilization of JR2EC was achieved with pyrene maleimide as linker molecule. Figure 2(b) shows the transfer characteristics (I\textsubscript{d} vs. V\textsubscript{g}) of the rGO-FET devices before and after polypeptide functionalization. As observed, device before polypeptide functionalization exhibited a typical semiconductor behavior and polypeptide-functionalized device generated an I\textsubscript{d} vs. V\textsubscript{g} curve similar to that of non-functionalized device, demonstrating that both non-functionalized devices and polypeptide-functionalized devices showed typical FET responses.

For kinetic measurements in buffer, MMP-7 at different concentrations was measured using the method described previously. The leakage current of the FET device is typically very small (1-10 µA). In addition, the effect of varying leakage current was accounted for by normalizing the current (normalized current = (drain current-leakage current)/leakage current × 100%), Figure 3. As shown in Figure 3a, I\textsubscript{d} decreased as a result of incubation with MMP-7 and the magnitude increased exponentially with the concentration of MMP-7 (Figure 3b). The results indicated that MMP-7 could be quantitatively measured by the proposed methodology. The limit-of-detection (LOD) calculated using the 3σ/S approach (see supporting information) was 10 ng/mL (400 pM), which is one order of magnitude higher than the LOD achieved by the colorimetric approach reported.\textsuperscript{19}
ure 4. In the first experiment (control group A), no polypeptides were immobilized on rGO prior to device incubation with MMP-7 in order to investigate the non-specific binding of MMP-7 on rGO films. No significant L responses were triggered by MMP-7 in the absence of polypeptides, demonstrating that non-specific binding is minimized by PEG surface passivation. In the second experiment (control group B), the polypeptide-functionalized rGO devices were incubated with deactivated (realized by heating at 100 °C for 10 min) MMP-7. In virtue of the robustness of the synthetic polypeptides used and possible miniaturized structure (a portable electronic circuitry) of the FET platforms, the proposed assay could be employed for point-of-care diagnostic applications. It is also envisioned that the proposed approach could be extended for detections of other polypeptide-digesting enzymes upon appropriate selection or modification of polypeptides.

CONCLUSIONS

A novel approach for rapid (lag time < 5 min) and sensitive (LOD in buffer = 10 ng/mL, equivalently 400 pM) detection of MMP-7 based on polypeptide functionalized rGO-FET is reported. The superior sensitivity could be attributed to the significant reduction of net charge of the polypeptides induced by MMP-7 digestion. More importantly, detection of MMP-7 in human plasma has been demonstrated by the proposed assay with LOD = 40 ng/mL. Additionally, this assay enables detection of both activity and concentration of MMP-7 and excellent specificity of the proposed assay was demonstrated using MMP-1, a protease of the same family. Hence, the proposed assay shows a great potential for applications in tumor detection and carcinoma diagnostic.

ASSOCIATED CONTENT

Supporting Information

The FESEM and AFM images are shown. 3σ/S approach for LOD calculation is described. This material is available free of charge via the Internet at http://pubs.acs.org.

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The manuscript was written through contributions of all authors. All authors have given approval to the final version of the manuscript. ‡These authors contributed equally.

ACKNOWLEDGMENT

This research was funded by the Institute for Sports Research, Nanyang Technological University.

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The device showed negligible responses to deactivated MMP-7, indicating that the proposed assay is capable of providing essential information on the activity of MMP-7. In the third experiment (control group C), the polypeptide-functionalized rGO devices were incubated with MMP-1tio investigate the specificity of the polypeptide proposed. Negligible L responses as compared to the responses in kinetic measurements were observed, demonstrating that the polypeptide is specifically digested by MMP-7.


Figure 1. The working principle of the proposed assay. The presence of target molecules (MMP-7) leads to cleavage of polypeptides immobilized on the rGO surface, resulting in reduced size and net charge of the immobilized polypeptides. Therefore, decreases in $I_d$ could be detected. The cleavage sites for MMP-7 are the 11th and the 26th amino acids.

Figure 2. (a) $I_d$ responses of the proposed rGO-FET in two control groups. In Group I (red), the PEG-passivated rGO-FET device was incubated with JR2EC; in Group II (blue), the rGO-FET device was incubated with pyrene maleimide, PEG and JR2EC consecutively (Error bars refer to the standard deviations of data collected on n devices, n > 3). (b) Transfer characteristics ($I_d$ vs. $V_g$) of the rGO-FET devices before and after polypeptide functionalization.
Figure 3. (a) Kinetic measurements of MMP-7 at different concentrations (0.01 µg/mL, 0.05 µg/mL, 0.1 µg/mL, 0.5 µg/mL and 1µg/mL) in buffer; (b) $I_d$ response of rGO device vs. MMP-7 concentration in buffer tests using devices from different batches (n>3); (c) Kinetic measurements of MMP-7 at different concentrations (0.01 µg/mL, 0.05 µg/mL, 0.1 µg/mL, 0.5 µg/mL and 1µg/mL) in human plasma; (d) $I_d$ response of rGO device vs. MMP-7 concentration in plasma tests using devices from different batches (n>3). Error bars are the standard deviation of at least three repetitive experiments.

Figure 4. $I_d$ responses to three control groups as compared to that of kinetic measurements: Control group B = non-functionalized rGO device incubated with active MMP-7; Control group B = JR2EC functionalized rGO device incubated with deactivated MMP-
Control group C = JR2EC functionalized rGO device incubated with MMP-1. (Error bars are the standard deviation of at least three repetitive experiments)