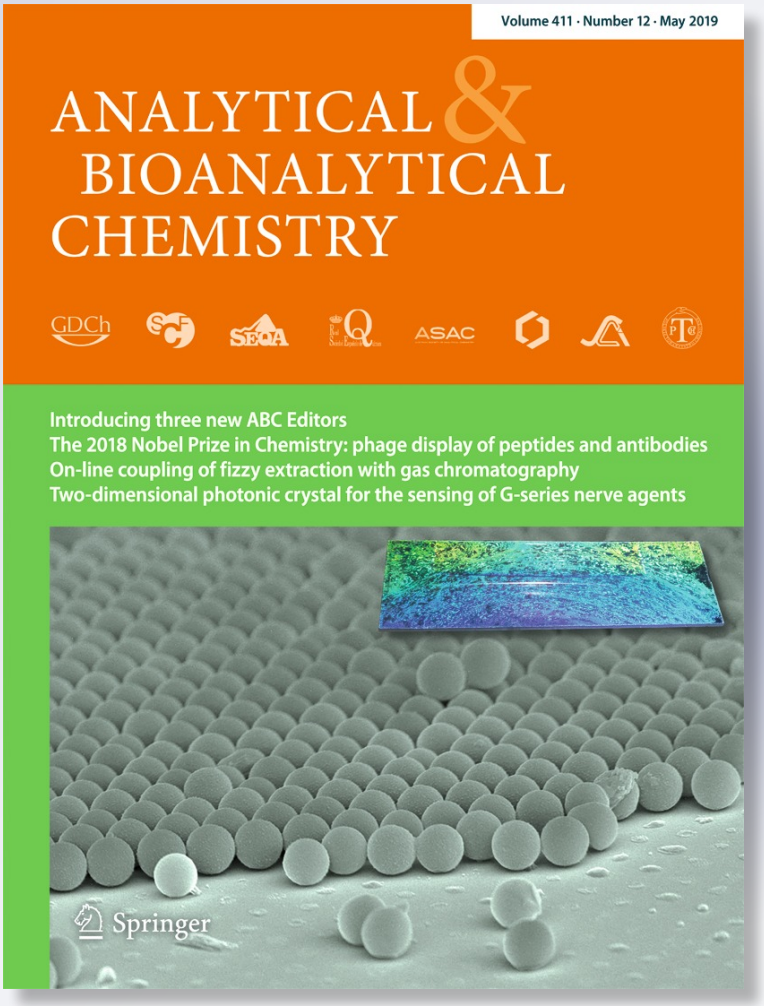
Detection of protease and engineered phage-infected bacteria using peptide-graphene oxide nanosensors

Juhong Chen & Sam R. Nugen

Analytical and Bioanalytical Chemistry

ISSN 1618-2642
Volume 411
Number 12

Anal Bioanal Chem (2019)
411:2487-2492
DOI 10.1007/s00216-019-01766-6



Springer

Analytical and Bioanalytical Chemistry (2019) 411:2487–2492
https://doi.org/10.1007/s00216-019-01766-6

COMMUNICATION

Detection of protease and engineered phage-infected bacteria using peptide-graphene oxide nanosensors

Juhong Chen[1,2] · Sam R. Nugen[1]

Received: 2 January 2019 / Revised: 1 March 2019 / Accepted: 7 March 2019 / Published online: 23 March 2019

Abstract

A peptide-graphene oxide nanosensor has been developed to detect tobacco etch virus (TEV) protease and bacteria infected with an engineered bacteriophage. In the detection strategy, a peptide (sequence: RKRFRENLYFQSCP) is tagged with fluorophores and graphene oxide (GO) is used to adsorb the peptides while quenching their fluorescence. In the presence of TEV protease, fluoropeptides are cleaved between glutamine (Q) and serine (S), resulting in the recovery of fluorescence signal. Based on the fluorescent intensity, the detection limit of TEV protease is 51 ng/μL. Additionally, we have utilized the sensing system to detect bacteria cells. Bacteriophages, which were engineered to carry TEV protease genes, were used to infect target bacteria (*Escherichia coli*) resulting in the translation and release of the protease. This allowed the estimation of bacteria at the concentration of 10^4 CFU/mL. This strategy has the potential to be developed as a multiplex detection platform of multiple bacterial species.

Keywords TEV protease · Engineered phage · Bacteria detection · Graphene oxide · Bacteriophage · Biosensor

Introduction

Proteases, which is a group of hydrolytic enzymes, can break down specific amide bonds in peptides and play an extremely important role in multiple biological processes [1]. They also initiate and progress many important medical and disease conditions, including cancer, arthritis, and neurodegenerative diseases [2, 3]. Thus, there is an urgent need to develop a sensitive and rapid method to detect disease-related protease. Over the past few decades, several detection methods based on enzyme-linked immunosorbent assay (ELISA) and fluorescence resonance energy transfer (FRET) have been reported for protease detection. [4, 5]. In particular, FRET-based protease detection using hybrid advanced biological-nanomaterial sensors has gained increased attention due to a simple detection strategy and high sensitivity.

Graphene oxide (GO), synthesized by the oxidation of graphene, is a single-layer two-dimensional carbon nanosheet containing oxygenated groups in its molecular structure, such as hydroxyl and epoxide groups in the basal plane and carboxyl groups on its exposed edges [6, 7]. Due to these chemical groups, GO shows distinct advantages when used for biosensing applications, including large surface areas, good water solubility, and pragmatic surface modification [8–10]. Because of its strong capacity to adsorb biomolecules (e.g., nucleic acids, peptides, and proteins) and excellent fluorescence quenching efficiency, GO incorporated with fluorescence-labeled biomolecules has enabled the detection of DNA, metal ions, protease, and other analytes [11–16]. As a nanosensor, nucleic acid-GO hybrid nanoprobes have been well-studied. However, peptide-GO hybrid nanoprobes for protease detection are still in its early stages. Furthermore, the utilization of peptide-GO hybrid nanosensors for bacteria detection is yet unexplored.

Herein, we report the proof-of-principle for a novel protease and bacteria nanosensor based on the conjugation of GO and fluorescence-labeled peptides. To accomplish this, these fluoropeptides were adsorbed on the surface of GO, which can quench the fluorescence of fluoropeptides. In the presence of the appropriate protease (or released from engineered phage infection), the fluoropeptides were cleaved, resulting in the

✉ Sam R. Nugen
snugen@cornell.edu

[1] Department of Food Science, Cornell University, 241 Stocking Hall, Ithaca, NY 14853, USA

[2] Department of Mechanical Engineering, University of California Berkeley, 1113 Etcheverry Hall, Berkeley, CA 94720, USA

recovery of fluorescence signals. The intensities of fluorescence signals were directly related to protease concentration (or bacteria concentration). Bacteria were detected using bacteriophages (phages) which were engineered to carry a gene for the TEV protease. Phages are viruses which can specifically infect, replicate within, and then lyse host bacteria. Upon lysis, the TEV protease was released into the sample allowing peptide cleavage. To the best of our knowledge, it is the first study to detect bacteria concentration using peptide-GO nanoprobes combined with engineered phages.

Experimental

Materials and instrumentation

The engineered phage carrying tobacco etch virus (TEV) protease gene is described in a previous publication [17]. *Escherichia coli* (*E. coli*) BL21 was purchased from EMD Millipore (Billerica, MA). The peptides labeled with FITC (fluoropeptide: RKRFRENLYFQSCP-FITC) was synthesized by GeneScript (Piscataway, NJ). Sodium chloride, potassium chloride, sodium phosphate dibasic, potassium phosphate monobasic, tryptone, yeast extract, and agar were purchased from Fisher Scientific (Fair Lawn, NJ). All other chemicals were purchased from Acros Organics (Morris Plains, NJ). Aqueous solutions were prepared using Mill-Q water with 18.2 mΩ/cm at 25 °C from Thermo Fisher Scientific (Asheville, NC). All media, buffer, and plastic/glassware were autoclaved at 121 °C for 30 min. The graphene oxide was characterized using JEOL JEM-2000FX transmission electron microscopy (TEM, Hillsboro, OR) and a Veeco Dimension 3100 atomic force microscope (AFM, Signal Hill, CA). The fluorescence intensities were recorded using a UV-vis spectrophotometer from Synergy2 Biotek (Winooski, VT).

Synthesis of graphene oxide

Graphene oxide (GO) was prepared using a modified Hummers method [18, 19]. Briefly, graphite powder (3.0 g) was added into a solution consisting of concentrated H_2SO_4 (120 mL) and HNO_3 (60 mL) under gentle stirring at room temperature for 1 h. After chilling in an ice bath, $KMnO_4$ (15.0 g) was added slowly under gentle stirring to keep the temperature below 20 °C. The mixture was then stirred at room temperature for 2 h. After that, the mixture was kept in an ice bath and Mill-Q water (250 mL) was slowly added to keep the temperature below to 55 °C. Additionally, H_2O_2 (20 mL, 30%) was dropped into the mixture under gentle stirring, and the color of mixture solution changed into bright yellow with bubbling. After stirring for another 2 h, the solution was centrifuged and washed using 1:10 HCl solution. Finally, the solution was purified using MW 7000 dialysis

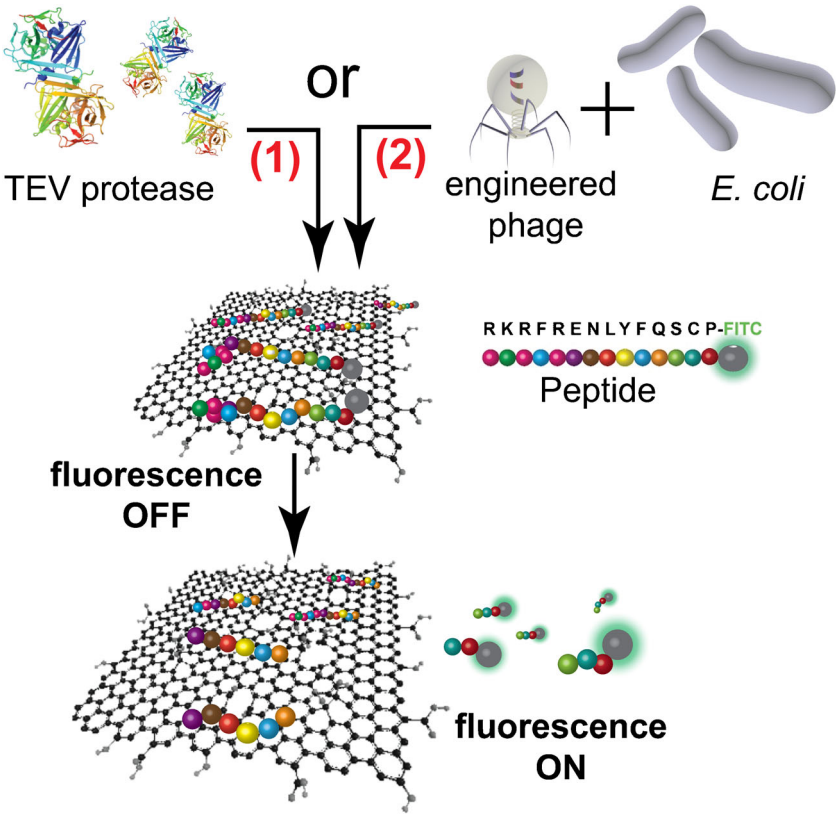


Fig. 1 Schematic illustration of the fluoropeptide-GO nanosensors to detect (1) TEV protease and (2) bacteria cells combining engineered bacteriophages. The peptide was labeled with FITC, which can be cleaved between glutamine (Q) and serine (S) amino acids by TEV protease

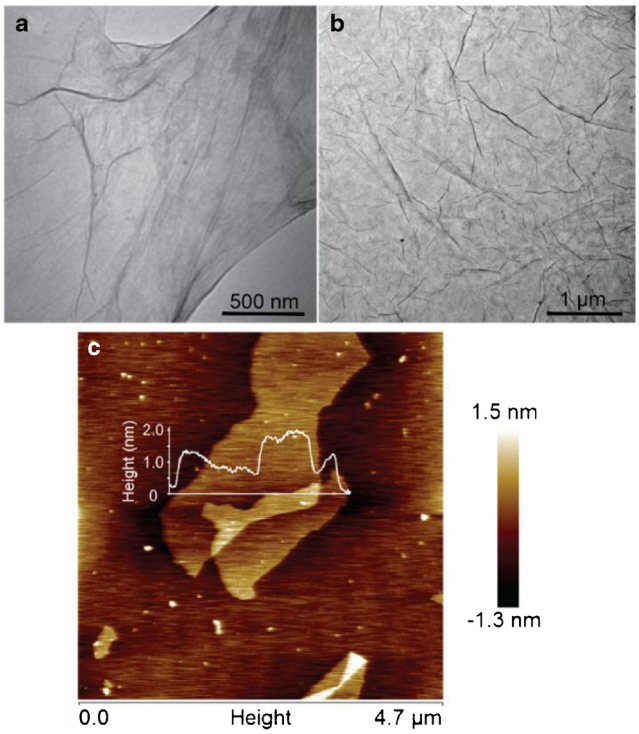


Fig. 2 The characterization of synthesized GO. **a**–**b** Transmission electron microscope (TEM) image and **c** atomic force microscope (AFM) image of the GO (insert: the thickness of GO)

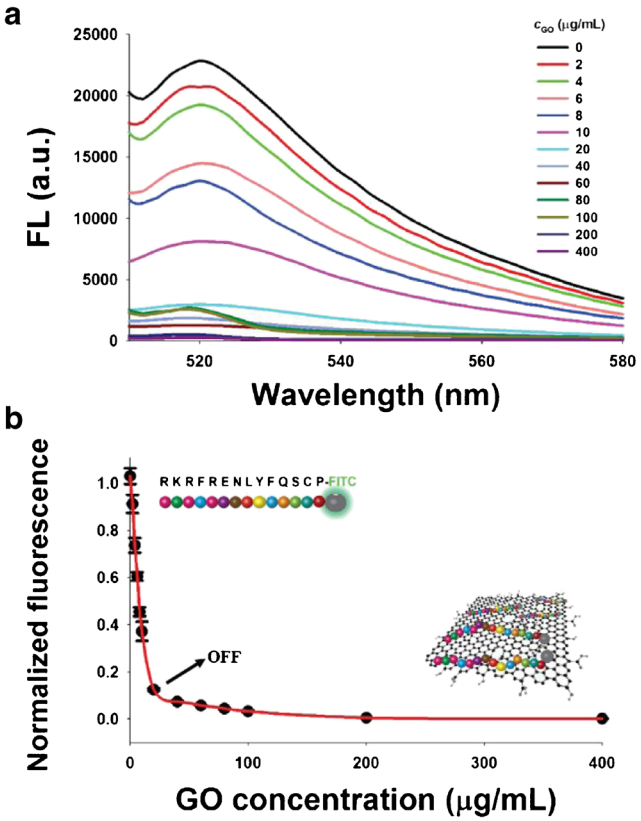


Fig. 3 **a** Fluorescence spectra of fluoropeptides in the presence of various concentrations of GO from 0 to 0.4 mg/mL. **b** Normalized fluorescence at the wavelength of 528 nm towards the GO concentration at the excitation wavelength of 485 nm

membranes for 2 days. The obtained solution was diluted to make a GO stock solution (2 mg/mL).

Fluorescence titration

Fluorescence titration was conducted using 96-well plates by titrating increasing of GO concentration against constant fluoropeptide concentration. The obtained lyophilized fluoropeptide (RKRFRENLYFQSCP-FITC) was dissolved and diluted using phosphate buffer (PB, 5 mM, pH 7.4) to a concentration of 10 μM. The fluorescence (FL) intensity of fluoropeptide (50 μL, 10 μM) at 200 μL fluid volume in the absence of GO quencher was determined at appropriately 25,000. With fluoropeptide concentration fixed, different GO concentrations (50 μL) were added and incubated for 10 min, while maintaining the fluid volume at 200 μL. The fluorescence intensities were then measured using Biotek at the excitation wavelength of 485 nm. All experiments were conducted in triplicates.

Monitoring of TEV protease activity

The TEV protease activity was monitored using fluoropeptide-GO hybrid nanoprobes. Fluoropeptide (50 μL, 10 μM), GO solution (50 μL, 20 μg/mL), and PB (80 μL) were incubated for 10 min. Different TEV protease concentrations (20 μL) were added into these mixture solutions in 96-well plates, respectively. The 96-well plate was read using Biotek following 30 min at an interval of 30 s at 30 °C at the excitation/emission of 485/528 nm. All experiments were repeated three times.

Detection of *E. coli* combined with engineered phage

An overnight culture of *E. coli* cells was washed three times and finally suspended in phosphate-buffered saline (PBS, 10 mM). The washed *E. coli* cells were diluted into different concentrations using lysogeny broth (LB). Engineered phages carrying TEV gene (100 μL, 10^3 PFU/mL) were incubated with different *E. coli* concentrations (900 μL) at 37 °C with 200 rpm shaking for 3 h. LB without *E. coli* cells was used as negative control. Each lysis solution (20 μL) was added into fluoropeptide-GO hybrid solution containing fluoropeptide (50 μL, 10 μM), GO solution (50 μL, 20 μg/mL), and PB (80 μL). After incubation at 30 °C for 30 min. The fluorescence intensities were then measured using a Biotek Fluoresence plate reader at the excitation wavelength of 485 nm. All experiments were conducted in triplicates.

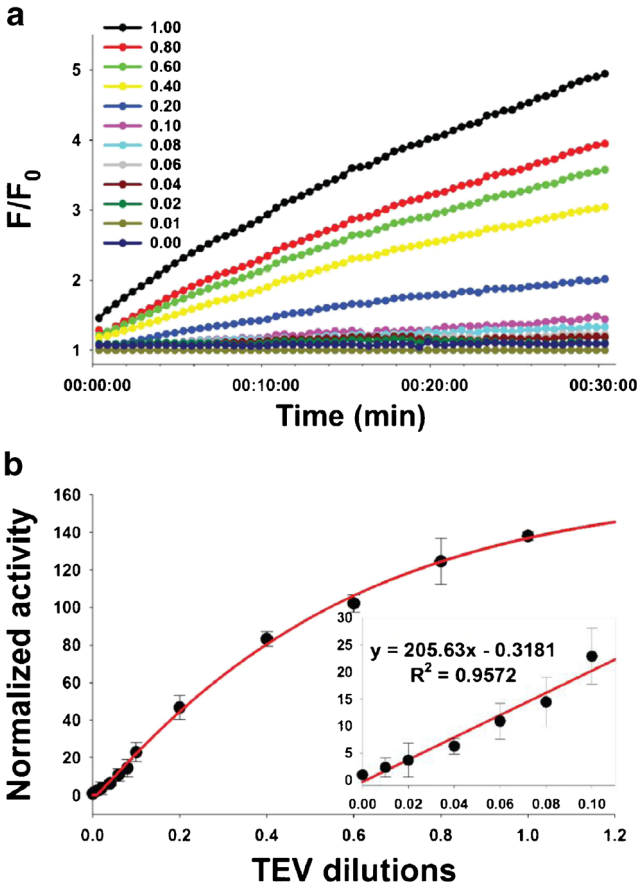


Fig. 4 **a** Kinetic fluorescence intensity response of the fluoropeptide-GO nanosensors in the presence of different TEV protease concentrations at the excitation/emission wavelength of 485/528 nm. **b** Plot of normalized TEV protease activity towards TEV protease concentration

Statistical analysis

Statistical significance was determined using Student's t test. All data were presented as mean ± standard deviation (SD) from a minimum of three replicates, and the error bars in all figures represent one SD of at least three independent experiments. The test set of data was compared with the control set, with one asterisk (*, $0.01 < P$ value < 0.05) indicating difference and two asterisks (**, P value < 0.01) indicating significant difference.

Results and discussion

Principle of protease/bacteria detection using peptide-GO nanosensors

The scheme for the detection of protease/bacteria is shown in Fig. 1. A synthetic TEV-substrate fluoropeptide (sequence: RKRFRENLYFQSCP) was labeled with a fluorescein isothiocyanate (FITC). The net charge of the peptide at pH 7 is + 2.9, allowing a strong attractive interaction with the strongly negatively charged GO [20, 21]. After the fluoropeptides were adsorbed on the surface of GO sheets, the fluorescence was quenched via the efficient electron transfer. Upon adding TEV protease solution, the TEV protease can recognize and cleave the bond between glutamine (Q) and serine (S), resulting in the recovery of the fluorescence signal [17, 22]. The strategy was used to measure the concentration of TEV protease. In

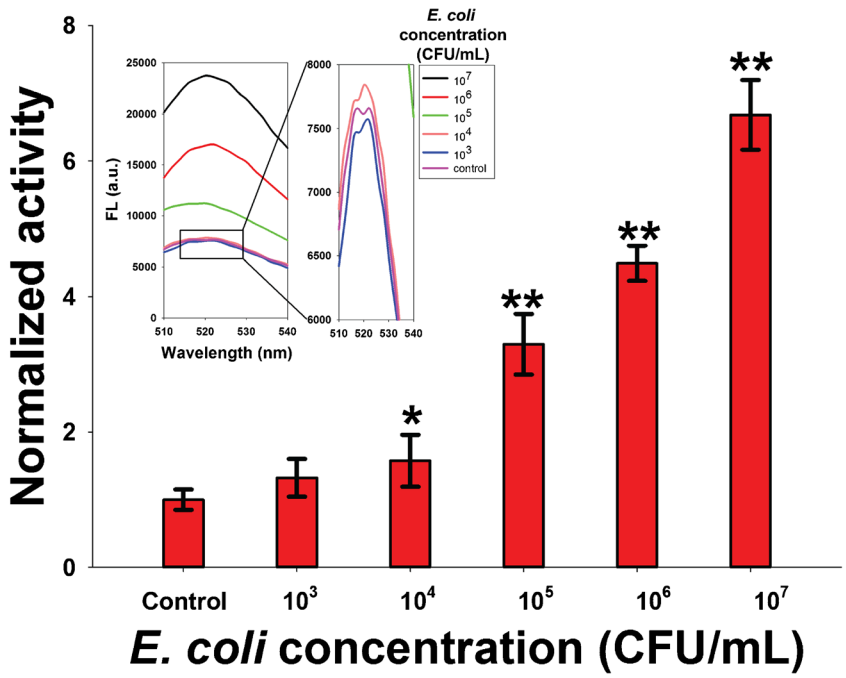


Fig. 5 Plot of normalized activity of TEV protease towards various *E. coli* concentrations (insert: fluorescence spectra of fluoropeptide-GO nanosensors in the presence of various *E. coli* concentrations at the excitation wavelength of 485 nm)

order to apply this technology to detect bacterial cell concentration, genetically engineered bacteriophages (phages) carrying the genes for the TEV protease were used to infect bacterial cells, resulting in the expression and release of the TEV protease. The detection steps can be divided into two steps: (1) engineered phages infect the bacteria cell to express TEV protease, which are then released into the sample media following cell lysis, (2) the released TEV protease recognizes and cleaves the fluoropeptides quenched by GO, resulting in the recovery of fluorescence signal. Based on the obtained fluorescence readout, bacteria concentration can be estimated.

Characterization of synthesized GO

To assemble the peptide-GO hybrid nanosensors, GO was first synthesized using a modified Hummers method [18, 19]. The as-synthesized GO was purified using membrane dialysis and dispersed in Mill-Q water to a concentration of 2 mg/mL. GO was then characterized using a transmission electron microscope (TEM) and an atomic force microscope (AFM). The TEM image provided more detailed morphological information of GO sheet (Fig. 2a–b). The thickness of GO was characterized using the AFM. As shown in Fig. 2c, the thickness of the GO sheets was appropriately 1.0 nm, suggesting the formation of a single-atom thickness layered GO nanosheet.

Fluorescence titration to determine the ratio of fluoropeptide and GO

Prior to detecting TEV protease, fluorescent titration was conducted to determine the fluorescence turn-off ratio between fluoropeptides and GO nanosheets. Fluoropeptides at the concentration of 2.5 μM in 200 μL fluid volume were measured in the absence of GO nanosheets (FL intensity: approximately 25,000). With fluoropeptide concentration fixed, varying concentrations of GO were added and incubated for 10 min. The fluorescence intensity at the excitation wavelength of 485 nm was measured. As shown in Fig. 3a, the fluorescence peak at 528 nm decreased with the increasing of GO concentrations. The normalized fluorescence was then plotted against the GO concentration (Fig. 3b). Upon the addition of GO at 20 μg/mL, the fluorescence quenched gradually up to 90% and then plateaued, suggesting that the fluoropeptides adsorbed strongly to the GO, and the fluorescence was efficiently quenched. Based on the quenching efficiency, a GO concentration of 20 μg/mL was selected for further experiments.

Monitoring of TEV protease activity

Next, we examined the fluorescence response of TEV protease using the turn-off fluoropeptide-GO hybrid nanosensors. Varying concentrations of TEV protease were mixed with the turn-off nanosensors, and the fluorescence intensities were monitored over 30 min at 30-s intervals. As shown in Fig. 4a, the fluorescence intensities increased with the increasing of reaction time. Higher TEV protease concentrations resulted in a more rapid fluorescence response. Furthermore, the TEV protease activities were normalized towards TEV protease concentrations (Fig. 4b). The calibration plots displayed a good linear relationship between normalized activity and TEV protease concentration in the range from 0 to 0.40 μg/μL. The detection limit of 51 ng/μL was calculated using the mean of the control replicates plus three times the standard deviation.

Analytical performance for *E. coli* detection using engineered phages

In addition, the investigation of the fluoropeptide-GO hybrid nanosensors to detect bacteria cells was performed. *Escherichia coli* (*E. coli*) BL21 was selected as a model analyte, and T7 phage, which was genetically engineered to carry TEV protease gene, was reported to infect *E. coli* cells [17, 23–25]. In this proof-of-concept assay, the turn-off fluoropeptide-GO nanosensors was combined with the engineered $T7_{TEV}$ phage, to detect *E. coli* BL21. As shown in Fig. 5, the normalized activity of TEV protease was plotted against *E. coli* concentration from 10^3 to 10^7 CFU/mL. Lysogeny broth (LB) without *E. coli* cells was used as negative control. The normalized activity increased with the increasing of *E. coli* concentration. Additionally, the fluorescence peak at 528 nm for the varying *E. coli* concentrations is shown in Fig. 5 insert. These results indicated that *E. coli* cells at the concentration of 10^4 CFU/mL (P value < 0.05) can be detected using our proposed fluoropeptide-GO nanosensors combined with an engineered phage infection.

Conclusions

In summary, we successfully employed a novel switchable fluoropeptide-GO hybrid nanosensor to detect TEV protease and then used to detect bacteria cells through the addition of an engineered phage. With this system, TEV protease concentrations as low as 51 ng/μL can be detected within 30 min. Combined with engineered phages carrying the TEV protease gene, our proposed strategy provided a potential tool to detect bacteria cells with detection limit of 10^4 CFU/mL. Future efforts will be made to translate this proof-of-principle to other phage-protease-peptide combinations, enabling the multiplex detection of multiple bacterial species.

Acknowledgements The authors would like to acknowledge the National Science Foundation (1705797). Additionally, this material is based upon work that is supported by the National Institute of Food and Agriculture, U.S. Department of Agriculture, Hatch under 1013507.

Compliance with ethical standards

Conflict of interest The authors declare that there are no conflicts of interest.

References

1. López-Otín C, Bond JS. Proteases: multifunctional enzymes in life and disease. J Biol Chem. 2008;283(45):30433–7.
2. Concha N, Abdel-Meguid S. Controlling apoptosis by inhibition of caspases. Curr Med Chem. 2002;9(6):713–26.
3. Puente XS, Sánchez LM, Overall CM, López-Otín C. Human and mouse proteases: a comparative genomic approach. Nat Rev Genet. 2003;4(7):544–58.
4. Boeneman K, Mei BC, Dennis AM, Bao G, Deschamps JR, Mattoussi H, et al. Sensing caspase 3 activity with quantum dot–fluorescent protein assemblies. J Am Chem Soc. 2009;131(11):3828–9.
5. Morales-Narvaez E, Merkoci A. Graphene oxide as an optical biosensing platform. Adv Mater. 2012;24(25):3298–308. https://doi.org/10.1002/adma.201200373.
6. Kim J, Cote LJ, Huang J. Two dimensional soft material: new faces of graphene oxide. Acc Chem Res. 2012;45(8):1356–64.
7. Du D, Wang L, Shao Y, Wang J, Engelhard MH, Lin Y. Functionalized graphene oxide as a nanocarrier in a multienzyme labeling amplification strategy for ultrasensitive electrochemical immunoassay of phosphorylated p53 (S392). Anal Chem. 2011;83(3):746–52. https://doi.org/10.1021/ac101715s.
8. Huang X, Qi X, Boey F, Zhang H. Graphene-based composites. Chem Soc Rev. 2012;41(2):666–86.
9. He Q, Wu S, Yin Z, Zhang H. Graphene-based electronic sensors. Chem Sci. 2012;3(6):1764–72.
10. Jang H, Lee J, Min D-H. Graphene oxide for fluorescence-mediated enzymatic activity assays. J Mater Chem B. 2014;2(17):2452–60. https://doi.org/10.1039/c4tb00199k.
11. Li F, Chao J, Li Z, Xing S, Su S, Li X, et al. Graphene oxide-assisted nucleic acids assays using conjugated polyelectrolytes-based fluorescent signal transduction. Anal Chem. 2015;87(7):3877–83.
12. Gao L, Lian C, Zhou Y, Yan L, Li Q, Zhang C, et al. Graphene oxide–DNA based sensors. Biosens Bioelectron. 2014;60:22–9.
13. Huang J, Gao X, Jia J, Kim J-K, Li Z. Graphene oxide-based amplified fluorescent biosensor for Hg2+ detection through hybridization chain reactions. Anal Chem. 2014;86(6):3209–15.
14. Kwak SY, Yang JK, Jeon SJ, Kim HI, Yim J, Kang H, et al. Luminescent graphene oxide with a peptide-quencher complex for optical detection of cell-secreted proteases by a turn-on response. Adv Funct Mater. 2014;24(32):5119–28.
15. Wang H, Zhang Q, Chu X, Chen T, Ge J, Yu R. Graphene oxide–peptide conjugate as an intracellular protease sensor for caspase-3 activation imaging in live cells. Angew Chem Int Ed. 2011;50(31):7065–9.
16. Chou SS, De M, Luo JY, Rotello VM, Huang JX, Dravid VP. Nanoscale graphene oxide (nGO) as artificial receptors: implications for biomolecular interactions and sensing. J Am Chem Soc. 2012;134(40):16725–33. https://doi.org/10.1021/ja306767y.
17. Alcaine SD, Tilton L, Serrano MAC, Wang M, Vachet RW, Nugen SR. Phage-protease-peptide: a novel trifecta enabling multiplex detection of viable bacterial pathogens. Appl Microbiol Biotechnol. 2015;99(19):8177–85. https://doi.org/10.1007/s00253-015-6867-8.
18. Hummers WS Jr, Offeman RE. Preparation of graphitic oxide. J Am Chem Soc. 1958;80(6):1339–9.
19. Kjeldsen L, Bjerrum OW, Hovgaard D, Johnsen AH, Sehested M, Borregaard N. Human neutrophil gelatinase: a marker for circulating blood neutrophils. Purification and quantitation by enzyme linked immunosorbent assay. Eur J Haematol. 1992;49(4):180–91.
20. Zhang M, Ye BC. A reversible fluorescent DNA logic gate based on graphene oxide and its application for iodide sensing. Chem Commun. 2012;48(30):3647–9. https://doi.org/10.1039/c2cc17906g.
21. Zhang M, Yin BC, Wang XF, Ye BC. Interaction of peptides with graphene oxide and its application for real-time monitoring of protease activity. Chem Commun. 2011;47(8):2399–401. https://doi.org/10.1039/c0cc04887a.
22. Gray DC, Mahrus S, Wells JA. Activation of specific apoptotic caspases with an engineered small-molecule-activated protease. Cell. 2010;142(4):637–46.
23. Chen J, Jackson AA, Rotello VM, Nugen SR. Colorimetric detection of Escherichia coli based on the enzyme-induced metallization of gold nanorods. Small. 2016;12(18):2469–75.
24. Chen J, Alcaine SD, Jackson AA, Rotello VM, Nugen SR. Development of engineered bacteriophages for Escherichia coli detection and high-throughput antibiotic resistance determination. ACS Sensors. 2017;2(4):484–9.
25. Chen J, Picard RA, Wang D, Nugen SR. Lyophilized engineered phages for detection in food matrices. ACS Sensors. 2017;2(11):1573–1577.