Simultaneous Detection of Diverse Glycoligand-Receptor Recognitions Using a Single-Excitation, Dual-Emission Graphene Composite

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Ligand–protein interactions (LPIs) are fundamental biological processes that manipulate a variety of cellular events. While multiple LPIs can occur concurrently or concertedly at a cellular interface, techniques which are able to simultaneously probe these diverse interactions remain challenging. Here, to the best of our knowledge, the first fluorogenic composite material (FCM) is developed that can probe diverse LPIs at a single biomimetic interface. We determined that two glycoligands coupled with fluorescence dyes with different emission colors can coassemble to a graphene oxide platform, producing an integrated FCM with a quenched fluorescence. The fluorescence of each "glycodye" is uniquely elicited upon interaction with a pairing protein (lectin) that selectively recognizes the glycoligand. Importantly, the dual emission of the FCM can be produced, on a single excitation, while both proteins exist, providing a concise means for the simultaneous probing of diverse LPIs.

1. Introduction

Ligand–proteins interactions (LPIs) are fundamental biological processes governing a number of cellular events including cell–cell adhesion, cell development and motion, endocytosis, and cell death. A diverse range of signaling ligands coexist on the cellular membrane and multiple LPIs can occur concurrently to control cell fate.[1,2] However, the development of ingenious techniques that are able to probe multiple LPIs at a single biological interface has been challenging.

Indeed, multisignal detection of diverse biological recognitions is becoming a prevailing tactic in chemical biology over the past few years. The tactic is attractive because it provides an integrated platform for the more accurate investigation of complex biological processes. Fan and co-workers[3] constructed gold-nanoparticle (AuNP) based multicolor nanobeacons for analysis of three tumor-suppressor genes. Since, AuNP based multicolor sensors for simultaneous detection of multiple analytes have been developed.[4–6] Carbonaceous materials such as carbon nanotube and graphene oxide (GO), owing to their unique optical properties, have also been used as the substrate to construct fluorogenic composite materials (FCMs).[7–14] Multiplex sensors based on these materials for the concurrent analysis of multiple biologically important genes, enzymes, and pathogens have been reported.[14–26] Nevertheless, FCMs that can probe diverse LPIs occurring at a single biological interface have been elusive.

Glycoligand–protein interactions (glyco-LPIs) are pivotal biological events that may cause human diseases such as cancer, influenza, and inflammation.[27,28] Because of the rich structural diversity of glycoligands on the cell surface, it is believed that multiple glyco-LPIs can take place to promote a cellular event. Evidence also suggests the expression of multiple proteins with different glycoligand-binding specificities on a virus particle surface.[29–31] As a consequence, multiplex sensors that can probe diverse glyco-LPIs concurrently represent a promising tool for the precise delineation of glycobiology.

Here we develop a simple, integrated GO-based FCM for the simultaneous detection of two glycoligand-protein (lectin) recognitions. Notably, previous investigations as regards multitarget detection rely on fluorogenic systems that require multichannel excitation, which increases the complexity of detection. In the present study, we choose aminocoumarin (AC, λ_{ex} = 420 nm, $\lambda_{em(max)}$ = 500 nm) and dicyanomethylene (DCM, λ_{ex} = 460 nm, $\lambda_{em(max)}$ = 620 nm), whose emission bands are well-separated on a single excitation, to couple with two glycoligands (galactose-Gal and mannose-Man) (**Figure 1**). These glycodyes (glycosyl fluorescence dyes) can coassemble to a GO platform, producing an FCM with a quenched fluorescence probably due to the Förster resonance energy transfer (FRET).[7–14] We determine that the dual emission of the FCM can be produced while both Gal-selective and Man-selective lectins are added and only a single emission is generated while the corresponding lectin exists solely.

2. Results and Discussion

Synthesis of the glycodyes **DK1** (Gal-DCM), **DK2** (Man-AC), **DK3** (Man-DCM), and **DK4** (Gal-AC) was accomplished by a click reaction of alkynyl glycosides with azido dyes in good

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DOI: 10.1002/adfm.201500448

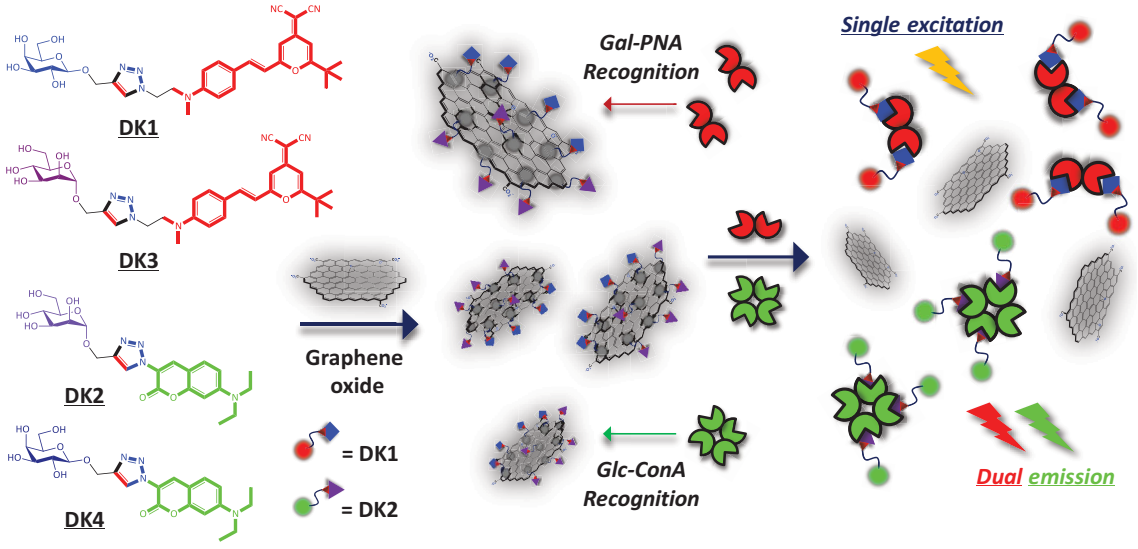

Figure 1. Structure of the glycodyes used for complexation with GO, and cartoon depicting the simultaneous detection of two proteins (lectins) using the single-excitation, dual-emission FCM. A galactosyl (**DK1** or **DK4**) and a mannosyl (**DK2** or **DK3**) dye with different emission wavelength are coassembled to a single GO platform, forming a composite with quenched fluorescence probably due to the FRET. Then, upon the simultaneous addition of the galactose-selective (PNA) and mannose-selective (ConA) lectins, both emissions can be recovered owing to the concurrently occurring sugar-lectin recognitions, with a single excitation.

yields (Scheme S1). With the compounds in hand, their composition with GO was carried out. From the emission spectra we observed that DCM-grafted (**DK1** and **DK3**) and AC-grafted (**DK2** and **DK4**) glycodyes showed strong and well-separated emission bands in a Tris–HCl buffer solution (pH 7.4) (**Figure 2**a). With increasing GO, the fluorescence of the glycodyes quenched gradually, and the quenching plateau of DCM and AC could be reached with 30 and 105 μg mL^{-1} GO, respectively (Figure 2a). The quenched fluorescence could probably be a result of FRET from the dyes to GO that has a broad adsorption band.[7–14] A series of techniques carried out confirmed the stacking of the compounds to the surface of the material (Figure S1, Supporting Information).

Then, we observed that the addition of the galactose-selective peanut agglutinin (PNA) and the mannose-selective Concanavalin A (ConA) to the **DK1**/**DK4** and **DK2**/**DK3** FCM solution caused concentration-dependent fluorescence recovery, respectively (Figure 2b). This could be ascribed to the binding of the selective protein receptor with the glycoligand, leading to dissociation of the FCM, and thus the enhanced fluorescence.[12] The limit of detection (LOD) of **DK1@GO**, **DK2@GO**, **DK3@GO**, and **DK4@GO** for the corresponding lectin was determined to be 92×10^{-9}, 27×10^{-9}, 76×10^{-9}, and 51×10^{-9} M, respectively (Figure S2, Supporting Information). A blueshift was observed upon glycodye–protein interactions (Figure 2b). This could probably be a result of the encapsulation of the hydrophobic dyes to a hydrophobic pocket of the protein, leading to blueshift-accompanied fluorescence enhancement because of the change of the microenvironment from hydrophilic to hydrophobic. This phenomenon has been similarly observed in a previous study.[32] A solvatochromic study corroborated this assumption that, in a nonpolar solvent (MeCN), the emission maxima of both dyes blueshifted with an enhanced fluorescence compared with that in Tris–HCl (Figure S3, Supporting Information). To test the selectivity of the FCMs, unselective lectins and proteins were added to the FCM solutions. To our delight, the FCMs showed insignificant signal variations with these proteins (Figure 2c), suggesting that the fluorogenic response was probably a reflection of selective LPIs.

With these data in hand, we further tested the cofunctionalization of both DCM- and AC-glycoligands to a single GO platform. We observed that the mixture of **DK2/1** and **DK4/3** (an optimized ratio of 1:2) showed two separate emission peaks on a single excitation of 430 nm (**Figure 3**a, emission maxima for AC and DCM at 500 and 620 nm, respectively). Following the addition of 50 μg mL^{-1} GO, both fluorescence emissions quenched adequately, which preliminarily suggests the coassembly of both glycodyes onto the GO.

We then employed several other techniques to characterize the material composition. In the UV spectra, the adsorption maxima (438 nm) of **DK2/1@GO** and **DK4/3@GO** (**DK** assembled on GO) redshifted comparing to those of the compounds alone (426 nm), which probably suggests π-stacking of the glycodyes to GO (Figure 3b). The I_D/I_G ratio of the composites increased (0.91 for both **DK2/1@GO** and **DK4/3@GO**) with respect to GO alone (0.86), suggesting that the complexation of the aromatic dyes to GO caused an increase of the carbon sp^2-hybridization of the FCM systems. Meanwhile, peaks characteristic of π-stacking were observed in the Fourier Transmission IR (FTIR) spectra of the composites (Figure S4, Supporting Information). Finally, atomic force microscope (AFM) showed that the composition of the glycodyes increased the height of GO from around 1.0 to 2.0 nm (Figure 3d and Figure S5, Supporting Information). These data together suggest the assembly of the glycodyes to the surface of GO.

Next, the ability of the FCMs to simultaneously probe PNA and ConA was tested. We first observed that addition of

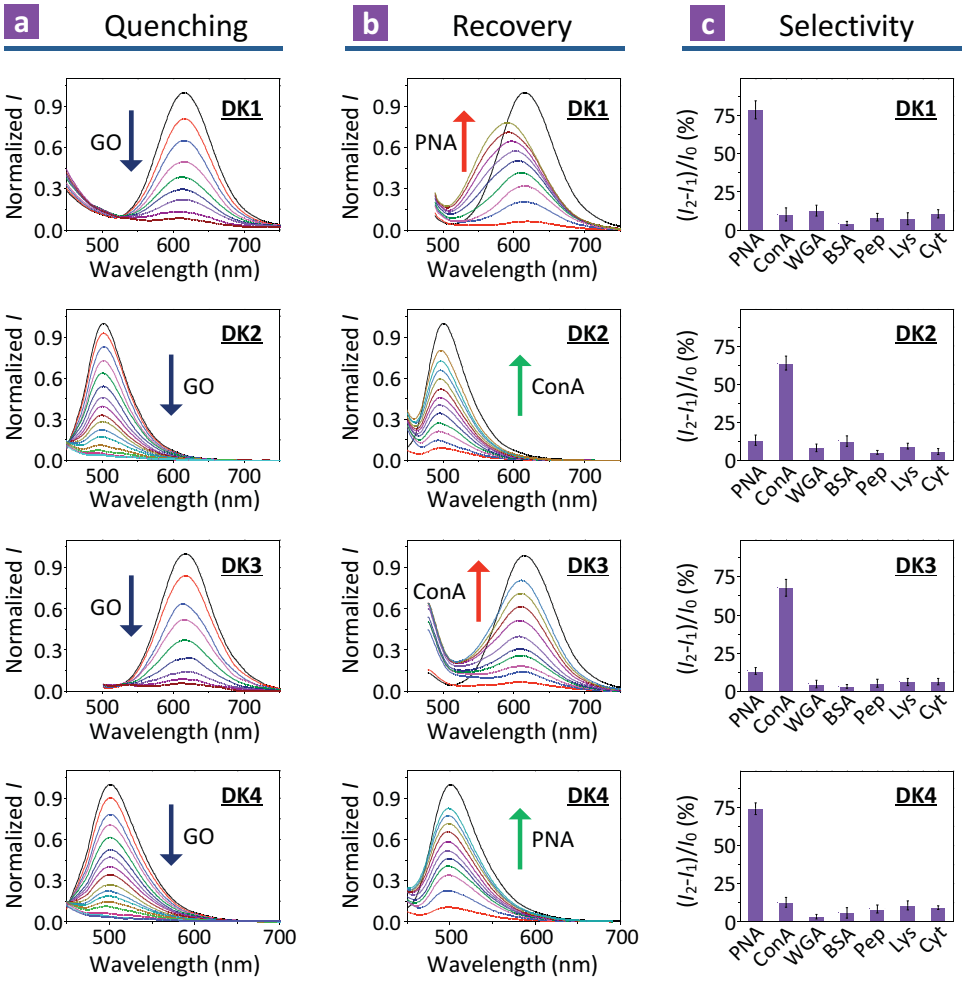

Figure 2. a) Fluorescence quenching of **DK** (1×10^{-6} M) in the presence of GO with a series of concentrations, 0–30 μg mL^{-1} for **DK1** and **DK3**, and 0–105 μg mL^{-1} for **DK2** and **DK4**, respectively. b) Fluorescence recovery of **DK@GO** in the presence of PNA (0–50×10^{-6} M) for **DK1**, Concanavalin A (ConA, 0–100×10^{-6} M) for **DK2**, ConA (0–90×10^{-6} M) for **DK3**, and PNA (0–87.5×10^{-6} M) for **DK4**. c) Selectivity (described as $(I_2 - I_1)/I_0$, where I_2 and I_1 are the fluorescence intensity of the FCM in the presence and absence of a lectin, respectively, and I_0 that of **DK** alone) of **DK@GO** in the presence of various proteins (WGA: wheat germ agglutinin, BSA: bovine serum albumin, Pep: pepsin, Lys: lysin, Cyt: cytochrome). All fluorescence spectra were recorded in 0.01 M Tris–HCl buffer (pH 7.4).

either lectin that recognizes the AC-glycoligand led to selective production of the green AC emission with no enhancement in the red emission band (**Figure 4**a). We also visualized the gradually intensified green fluorescence of **DK2/1@GO** with increasing ConA (that recognizes the Man moiety of **DK2**) (Figure 4d). Likewise, the red emission could be uniquely elicited upon the reverse addition of either lectin to the FCMs with no interference from the green emission band (Figure 4b) and a red-fluorescence intensification of **DK1** was visualized with increasing PNA for the **DK2/1@GO** system (Figure 4d). The LOD of **DK2/1@GO** for ConA and PNA was determined to be 91×10^{-9} and 57×10^{-9} M and that of **DK4/3@GO** for ConA and PNA 37×10^{-9} and 44×10^{-9} M, respectively. Eventually, we were delighted to observe that both emissions could be intensified upon the addition of both lectins, notably with a single excitation (Figure 4c, 430 nm). A mixed fluorescence color, similar to that of the **DK2/1** mixture, was visualized when ConA and PNA coexisted in the solution of **DK2/1@GO** (Figure 4d).

3. Conclusion

Since multiple LPIs can take place during a complex biological process, the ability to simultaneously probe diverse LPIs may improve our understanding towards biology. Whereas chemical tools for addressing this issue have been rare, we developed here, to the best of our knowledge, the first integrated FCM that showed a dual-fluorogenic response upon two simultaneous glycoligand-lectin recognitions. The dual FCMs were built through simple self-assembly of two separate glycodyes to a single GO platform. The FCMs showed a unique fluorogenic response to a selective protein receptor that recognizes either of the glycodyes with the other fluorogenic channel shut. Importantly, the dual emission of the FCMs could be elicited when the receptors of both glycoligands exist, with a single excitation. While there has been ever increasing interest in the development of graphene-based sensors,[33–37] this study provides a unique insight into the construction of GO composite materials

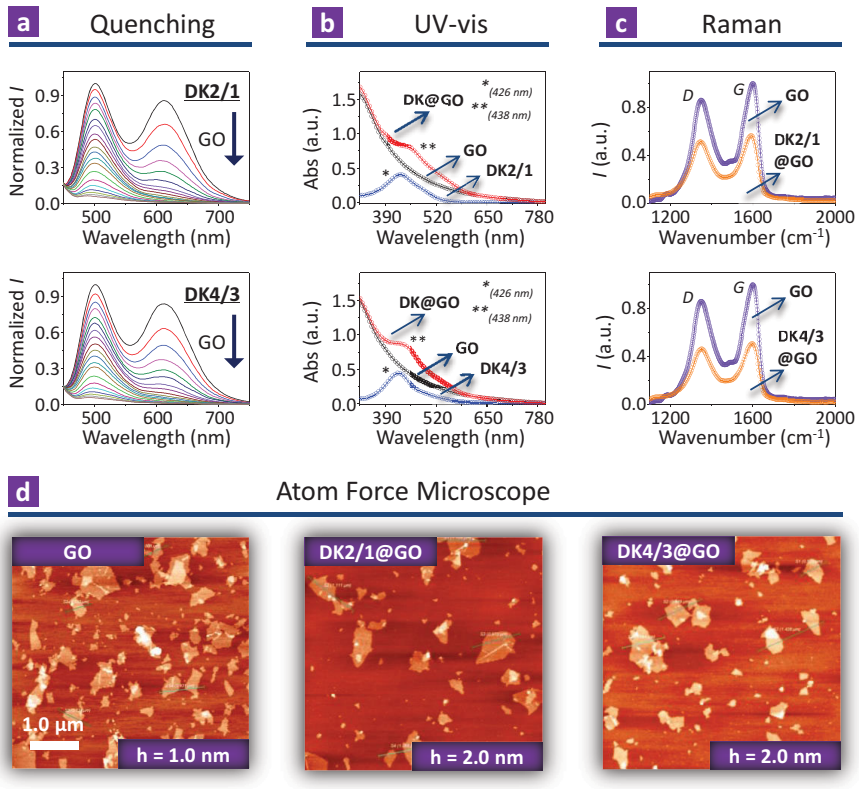

Figure 3. a) Fluorescence quenching of **DK2/1** ($1 \times 10^{-6}/2 \times 10^{-6}$ M) and **DK4/3** ($1 \times 10^{-6}/2 \times 10^{-6}$ M) in the presence of GO with a series of concentrations (0–130 μg mL^{-1}). b) UV spectra of GO and **DK2/1** or **DK4/3** in the absence and presence of GO (redshifts characteristic of π-stacking are observed for the material composites). c) Raman spectra of GO in the absence and presence of **DK2/1** or **DK4/3** (the increased I_D/I_G ratios of the material composite suggest the stacking of the compound to GO). d) Atomic force microscope images of GO and GO complexed with two glycodyes.

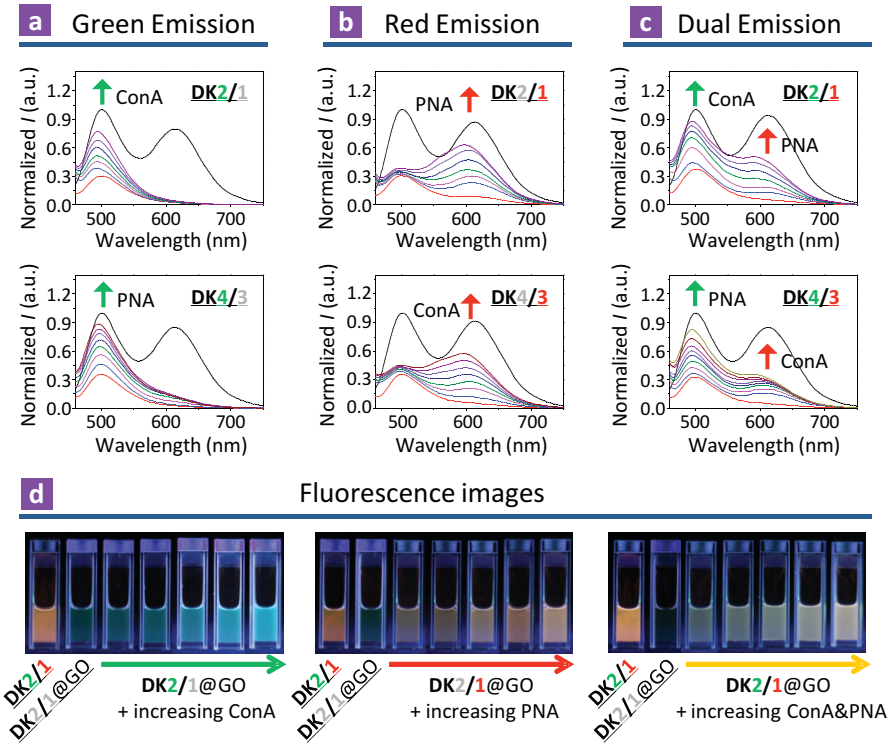

Figure 4. a,b) Fluorescence recovery of **DK2/1**@GO or **DK4/3**@GO in the presence of increasing ConA (0–25 × 10^{-6} M) or PNA (0–67.5 × 10^{-6} M) with a single emission. c) Fluorescence recovery of **DK2/1@GO** or **DK4/3@GO** in the presence of increasing PNA (0–67.5 × 10^{-6} M) and ConA (0–25 × 10^{-6} M) with dual emission. d) Fluorescence images of **DK2/1@GO** in the presence of a single lectin or both with increasing concentration.

for the simultaneous detection of diverse LPIs occurring at a single biological interface.

Supporting Information

Supporting Information is available from the Wiley Online Library or from the author.

Acknowledgements

The authors thank the 973 project (2013CB733700), the National Natural Science Foundation of China (21202045), the Key Project of Shanghai Science and Technology Commission (13NM1400900), and the Fundamental Research Funds for Central Universities (222201414010).

Received: February 3, 2015
Revised: April 3, 2015
Published online: May 4, 2015

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