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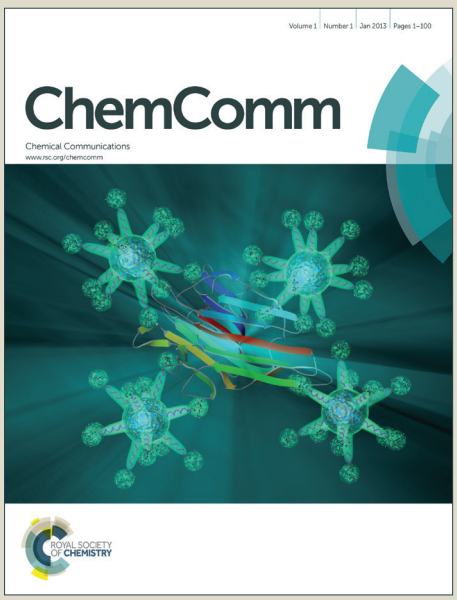

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Enzyme-driven *i*-motif DNA folding for logic operations and fluorescent biosensing

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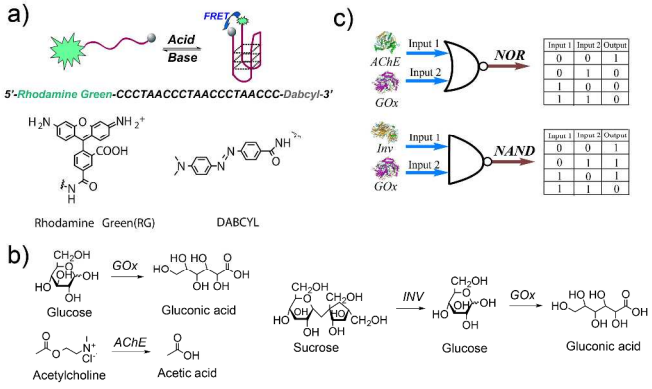
DNA nanodevices capable of "NOR" and "NAND" logic operations were developed using enzymatic reactions to generate acidic pH gradient and drive the conformation change of cytosine-rich DNA. Due to the high selectivity and sensitivity of the enzymatic reactions in driving DNA logic gates, novel fluorescent biosensors were further designed for enzyme activity assay and glucose sensing.

Molecular logic gates based on molecular recognition and reactions open a new avenue for information processing and communication in the future.[1-3] Meanwhile, molecular logic gates are also relevant to sensing. For instance, the normal fluorescent sensors correspond to either 1-input 'YES' logic gates or 1-input 'NOT' logic gates. [4] In principle, logic gates with more than one input can be employed to analyze more analytes simultaneously. [5] This may lead to intelligent diagnostics for medical purposes.

Biomolecular logic gates provide us novel modules to design molecular circuits and computers.[6] Since the introduction of DNA computer by Adleman two decades ago,[7] nucleic acids emerge prominently as versatile precursors to construct DNA logic gates and synthetic circuits with a myriad of biological or chemical inputs.[8-11] Further, the efficient computing and logic operations of biological inputs can enhance our ability to understand and control biological systems.[12] For example, the computing of proteins and enzymes within DNA logic devices provides a unique approach to mimic or probe the cell signaling inside cells,[6] where a large number of biological inputs are detected, processed, and integrated to make a decision for gene expression and/or enzyme regulation. Additionally, DNA logic devices hold great promise for biosensing and molecular diagnosis.[6,13-15] For instance, molecular logic gates on DNA origami nanostructure have been developed to detect microRNA for diagnosis diagnosis. [14]

In this communication, we report "NOR" and "NAND" logic operations by combining a conformational change of cytosine-rich DNA and enzymatic reactions, along with their applications for fluorescent biosensing. Cytosine-rich DNA is able to form C·CH+ base pair under a slight acidic condition, which stabilizes the folding of DNA into a quadruplex, also known as *i*-motif.[16] The unique and controllable folding of *i*-motif has been used to develop pH-driven DNA molecular motors. For example, by integrating photo-responsive spiropyran into cytosine-rich DNA and simultaneously control the physical property of DNA device using photo-irradiation and acid triggers, we previously reported a photo-pH dually modulated fluorescence molecular switch.[17] Herein, we design enzymatic reactions to generate pH gradient to drive *i*-motif folding for Boolean logical operations. Additionally, due to the high specificity of enzymes in catalyzing reactions to drive the DNA device, we further developed novel fluorescent biosensors for enzyme activity assay and glucose sensing with these logic gates.



Scheme 1 a) Modulating the FRET of dual-labeled cytosine-rich sequence DNA with acid and base treatment; b) enzymatic reactions to generate acidic gradient to induce DNA conformation change for logic operations; c) truth tables for the enzyme-driven "NOR" and "NAND" logic gates.

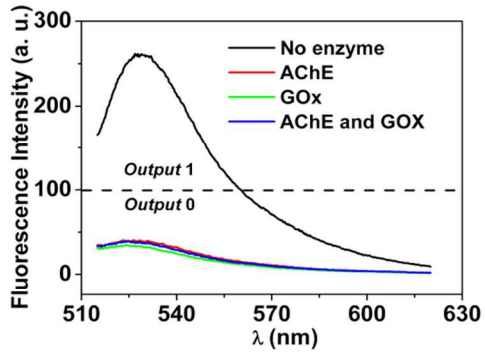
As shown in Scheme 1a, a 21-mer single-strand cytosine-rich DNA was first selected and dual-labeled with Förster resonance energy transfer (FRET) pair to facilitate the logic gates fabrication. The FRET between Rhodamine Green (RG) and Dabcyl (Scheme 1a) could be efficiently modulated by the DNA conformation

change. With a random-coiled DNA structure in a neutral solution, RG and Dabcyl are apart and thus the FRET is disrupted, the emission of RG can be detected in this case. However, with the folding of DNA and formation of *i*-motif under an acidic environment, the FRET pair is activated and the fluorescence of RG is quenched. Accordingly, the fluorescence output of the device is switched from "1" state in which the fluorescence of RG can be detected to "0" state in which the fluorescence of RG is quenched. We further designed the following enzymatic reactions to generate acidic gradient to drive the conformation change of DNA for logic operations: 1) acetylcholinesterase (AChE) catalyzed hydrolysis of acetylcholine into acetic acid, and glucose oxidase (GOx) catalyzed oxidation of glucose into gluconic acid (Scheme 1b). Therefore, to a DNA solution containing acetylcholine and glucose was added either AChE or GOx, or two enzymes at the same time, the resulted acidic gradient can drive *i*-motif formation and quench RG emission, which contributed to a "NOR" Boolean logic gate (Scheme 1c); 2) invertase (INV) catalyzed hydrolysis of sucrose into glucose, and subsequent oxidation of glucose into gluconic acid catalyzed by GOx. Accordingly, only with the presence of INV and GOx simultaneously gluconic acid could be generated in a DNA and sucrose mixture to drive *i*-motif folding, therefore, a "NAND" Boolean logic gate was developed with INV and GOx as two inputs (Scheme 1c).

We first studied the effect of solution acidity on the cytosine-rich DNA conformation and RG fluorescence by exposing the dual-labeled DNA to solutions with varied pH values. We hypothesized that an acidic solution can induce *i*-motif folding and quench RG emission. As shown in Figure S1, the fluorescence intensity of RG (20 nM DNA in 1.0 mM Tris buffer solution containing 0.1 M NaCl) decreased gradually when the solution acidity increased. With the solution pH decreased from 6.8 to 5.7, the RG emission intensity at 530 nm was quenched up to 87%, and no further emission quenching was observed when the solution pH was reduced below 5.5. This result is similar to our previous finding that dual-labeled cytosine-rich DNA can fold into *i*-motif under an acidic environment and quench the emission of labeled fluorescein.[17] Moreover, the RG emission intensity at 530 nm decreased linearly in the pH range of 6.8 to 5.7 (Figure S1b). Therefore, the solution pH-dependent *i*-motif folding and FRET efficiency allow us to design enzymatic reactions to modulate solution acidity and thus RG emission for logic operations and fluorescent biosensing.

The DNA logic gates were constructed by adding enzymes to DNA solutions supplemented with enzyme substrates, followed by RG emission monitoring and intensity gating to define logic operations according to the truth table of Boolean logic gates. To develop a "NOR" logic gate, to a solution containing DNA (20 nM), glucose (25 mM), and acetylcholine (1.0 mM) was added GOx (40 U) or AChE (50 mU), or both GOx and AChE simultaneously, the solution fluorescence was measured after incubation for 15 min. at 37 ^{o}C. As shown in Figure 1a, with the addition of either AChE or GOx or both of them, the RG emission was quenched down to 15% of that in the absence of enzymes, suggesting that GOx or AChE, or AChE and GOx could drive *i*-motif formation to activate FRET. By defining GOx as input 1 and AChE as input 2, the absence and presence of enzymes were considered to be input "0" and input "1", respectively. With regard to the output, the following definition was made: the output was regarded as "0" when the fluorescence intensity of RG at 530 nm was lower than 100 (see Fig. 1), whereas it was '1" when the fluorescence intensity was higher than 100. As depicted in Fig. 1b, the variation of RG fluorescence intensity at 530 nm in the absence and presence of enzymes can be represented by a "NOR" Boolean logic gate.

a)



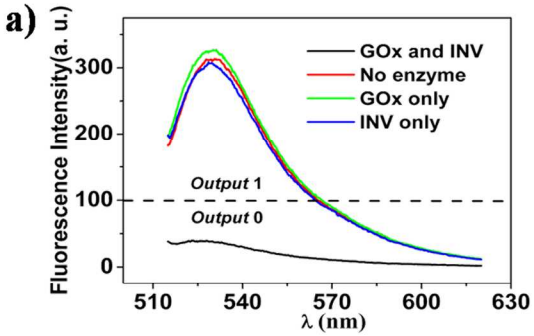
b)

Input		Output
AChE	**GOx**	**I_{RG} (530 nm)**
0 *(No AChE)*	**0** *(No GO_x)*	**1** *(I_{RG} > 100)*
1 *(AChE)*	**0** *(No GO_x)*	**0** *(I_{RG} < 100)*
0 *(No AChE)*	**1** *(GO_x)*	**0** *(I_{RG} < 100)*
1 *(AChE)*	**1** *(GO_x)*	**0** *(I_{RG} < 100)*

Fig. 1 Fluorescent spectra of DNA (20 nM in 1.0 mM Tris buffer, pH = 6.8), acetylcholine (1.0 mM), and glucose (25mM) mixed solutions in the presence and absence of AChE (50 mU) and GOx (40 U). The RG fluorescence was measured with an excitation at 503 nm.

A "NAND" logic gate requires the two inputs of the device to be true simultaneously. To design a DNA device with "NAND" logic operation, INV and GOx were added to a solution composed of DNA (20 nM) and sucrose (25 mM) to produce solution pH change and induce *i*-motif folding. Sucrose can be converted to gluconic acid via a two-step reaction (Scheme 1b): INV catalyzed the transformation of sucrose into glucose, and GOx catalyzed the oxidization of glucose into gluconic acid. The emission of DNA and sucrose mixture was monitored in the presence of INV (100 U) or GOx (40 U), or two enzymes simultaneously. As shown in Figure 2a, the addition of only INV or GOX had minor effect on quenching RG emission, due to neither enzyme can directly convert sucrose to gluconic acid. However, with the addition of INV and GOx simultaneously, RG fluorescence intensity was deceased down to 15% of that without enzyme addition or only with one enzyme addition. Therefore, by defining the absence of INV or GOx to be input "0" and the presence of INV or GOx to be input "1", a Boolean logic gate with "NAND" operation was achieved in which the output (the fluorescence intensity at 530 nm) was defined as "0" when the fluorescence intensity was lower than 100, and the output was "1" when the fluorescence intensity was higher than 100 (see Fig. 2b).[18]

The capability of AChE in catalyzing acetylcholine hydrolysis to drive a "NOR" logic gate provides us a unique strategy to design a novel AChE activity assay method. AChE that catalyse the hydrolysis of acetylcholine is related to Alzheimer's disease (AD),



b)

Input		Output
GOx	INV	I_{RG} (530 nm)
0 (No GOx)	0 (No INV)	1 (I_{RG}> 100)
1 (GOx)	0 (No INV)	1 (I_{RG} > 100)
0 (No GOx)	1 (INV)	1 (I_{RG} > 100)
1 (GOx)	1 (INV)	0 (I_{RG} < 100)

Fig. 2 Fluorescent spectra of DNA (20 nM in 1.0 mM Tris buffer, pH = 6.8) and sucrose (25 mM) mixed solutions in the presence and absence of INV (100 U) and GOx (40 U). The RG fluorescence was measured with an excitation at 503 nm.

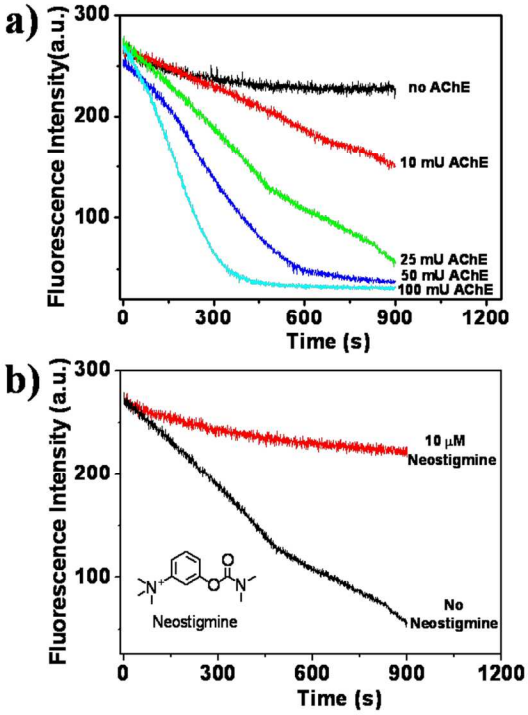


Fig. 3 a) AChE activity assay using AChE-driven "OR" logic gate. RG emission intensity (at 530 nm) was monitored by incubating DNA (20 nM in 1.0 mM Tris buffer, pH = 6.8) and acetylcholine (1.0 mM) in the presence of varied amounts of AChE; b) AChE inhibitor screening using DNA logic gate. A mixed solution of DNA (20 nM), acetylcholine (1.0 mM), and AChE (50 mU) was incubated for 15 min. in the absence and presence of 10 μM neostigmine.

and a large number of AChE inhibitors have been designed and screened as drug candidates for the clinical treatment of AD.[19] Therefore, a facile and convenient method for AChE activity assay is desired for the high throughput screening of AChE inhibitors for AD treatment.[20-22]To validate the use of DNA logic gate we developed above for AChE activity assay, a mixed solution of DNA and acetylcholine was co-incubated with varied concentration of AChE, followed by monitoring the RG emission to correlate the fluorescence intensity with the concentration of AChE. As shown in Figure 3a, the RG emission of mixed solution was barely changed without AChE after incubation for 15 min.. However, upon the addition of AChE, the fluorescence intensity of the solution decreased rapidly; and in the presence of 100 mU of AChE, the solution emission was quenched up to 85% in 5.0 min. compared to that without AChE. Moreover, the RG fluorescence intensity was dependent on the concentration of AChE present in the reaction mixture. With the amount of AChE decreased from 100 to 10 mU, less acetylcholine was hydrolyzed and a slower RG emission quenching was observed. The efficient correlation of RG emission intensity to AChE activity suggesting that the DNA logic gate can be used as a convenient yet accurate AChE assay sensor.

Having demonstrated an efficient AChE assay using the DNA logic gate, we further extended this novel assay to screen AChE inhibitor for potential AD drug discovery. The addition of AChE inhibitor to DNA, acetylcholine and AChE mixture can inhibit enzyme activity and thus retard the variation of the fluorescence intensity of RG. In this study, neostigmine, a well-known AChE inhibitor was selected as a model drug. The fluorescence intensity at 530 nm of RG for the ensemble solution of DNA (20 nM), acetylcholine (1.0 mM), and AChE (50 mU/mL) was monitored in the absence and presence of 10 μM neostigmine. As shown in Figure 3b, the addition of neostigmine to the reaction mixture significantly retarded RG emission quenching. This is due to the capability of AChE to catalyze acetylcholine hydrolysis was inhibited, and as a result the *i*-motif folding was prohibited. Moreover, the retarded RG emission quenching is dependent on the concentration of neostigmine present in the mixture. The higher concentration of neostigmine, the slower RG emission quenching was observed (Figure S2, ESI). By incubating the reaction mixture with varied concentration of neostigmine, followed by correlating RG emission to neostigmine concentration, the IC_{50} (half maximal inhibitory concentration) of neostigmine against AChE was determined to be 3.1 μM (Figure S2, ESI).

GOx can catalyze glucose oxidation and induce *i*-motif folding to modulate RG emission, therefore, the DNA logic gate could be similarly used for fluorescent glucose assay. To an assay mixture containing DNA (20 nM) and GOx (40 U) was added glucose, the RG emission intensity decreased significantly (Figure 4). When the concentration of glucose added to the mixture reached 525 μM, the RG emission was reduced down to 15 % of that without addition of glucose. Meanwhile, the RG emission intensity is linear to glucose concentration in the range of 0 to 450 μM. Moreover, due the high specificity of GOx in oxidizing glucose, the DNA logic gate based biosensor had a very high selectivity toward glucose assay. In the presence of other saccharides (the concentration of each saccharide was 750 μM), including glucose, fructose, mannose, maltose and

galactose, only the addition of glucose resulted in a significant RG emission quenching (Figure S3, ESI).

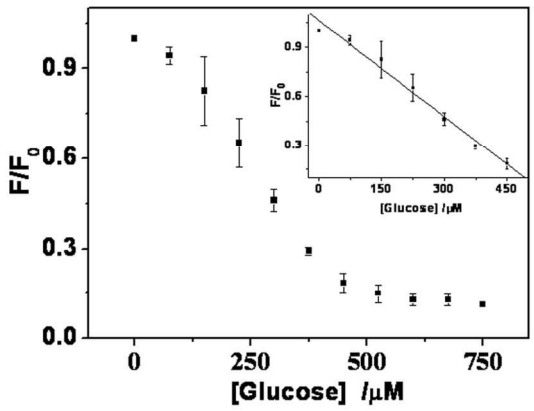


Fig. 4 The RG emission intensity variation of DNA (20 nM) and GOx (40 U) mixed solution in 1.0 mM Tris buffer (pH = 6.8) in the presence of increased concentration of glucose. RG emission intensity was monitored at 530 nm, F_0 and F represented the emission intensity of RG in the absence and presence of glucose, respectively.

In summary, we report the design of enzymatic reactions to produce acidic gradient to drive cytosine-rich DNA conformation change for logic operations. By coupling dual-labeled DNA with enzymatic reactions, the fluorescence emission of DNA could be efficiently modulated by GOx and AChE, or a combination of INV and GOx, which allowed for "NOR" and "NAND" logic operations. Further, we developed fluorescent biosensors for AChE activity assay and drug screening, as well as a novel glucose assay method, by making use of the enzyme-driven logic gates. We believe that the use of enzymatic reactions to drive nucleic acid nanodevices could mimic the function of cellular machine, and fabrication of these logic gates could further advance the design of biomolecular device to compute biological inputs inside cells for disease diagnosis.

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Notes and references

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