ChemComm
Chemical Communications

Accepted Manuscript

This article can be cited before page numbers have been issued, to do this please use: X. Lu, L. Wang, Q. Chen, Y. Wang, Z. Cao, G. Zhou and L. Li, *Chem. Commun.*, 2020, DOI: 10.1039/D0CC01560A.

ROYAL SOCIETY OF CHEMISTRY

rsc.li/chemcomm

View Article Online
DOI: 10.1039/D0CC01560A

Authors

Xing Lu,‡[a] Liqian Wang,‡[b] Qingxue Chen,[a] Yulan, Wang,[a] Zhenxiong Cao,[a] Guobao Zhou*[a] and Lei Li*[a]

Affiliations

a College of Biological, Chemical Sciences and Engineering, Jiaxing University, Jiaxing 314001, China. Email: lei.li@mail.zjxu.edu.cn, gbzhou@mail.zjxu.edu.cn

b State Key Laboratory of Molecular Engineering of Polymers, Department of Macromolecular Science, Fudan University, Shanghai 200433, P. R. China.

‡ These authors contributed equally.

Acknowledgements

This work was supported by the National Natural Science Foundation of China (NSFC Grants 21677060), the Zhejiang Provincial Natural Science Foundation of China (Grants LQ19B050002) and the Program for Science and Technology of Jiaxing (Grants 2019AY11009). We also thank the Student Research Training program of Jiaxing University (Grants CD8517193176) for financial support.

View Article Online
DOI: 10.1039/D0CC01560A

COMMUNICATION

Biosensor based on multifunctional allostery for dynamic analysis of circulating tumor DNA

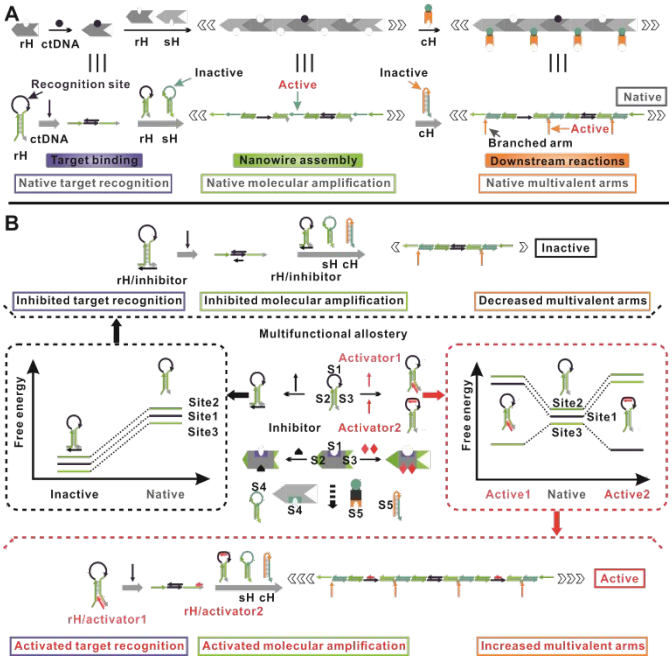
Received 00th January 20xx,
Accepted 00th January 20xx

DOI: 10.1039/x0xx00000x

Here, a new tool based on artificial multifunctional allostery (mFA) was explored to regulate the assembly of a DNA nanowire using circulating tumor DNA (ctDNA) as the initiator. Given its unique properties, the mFA-regulated versatile DNA nanowire was applied to engineer a single-step, amplified and dynamic biosensor for the quantitative analysis of ctDNA in serum samples with tunable sensitivity and selectivity.

A fundamental limitation in the fabrication of artificial allosteric devices using DNA nanotechnology has been the restricted functional domain (always based on a single-function site) that produces the dynamic event.[1-8] Self-assembled multienzyme complexes comprising different protein subunits capable of diverse allosteric regulation can produce multiple biological functions.[9-12] As an example, the Slo1 α-subunit with multiple regulatory sites contains not only a Ca2+ binding site but also a voltage-dependent K+ channel. This permits BK channels to exert a variety of physiological roles including dynamic signal transmission and K+ channel switching regulated by cytosolic Ca^{2+} and Mg^{2+} concentrations.[10] Jänne and co-workers also found that a mutant inhibitor of EGFR can both inhibit cell proliferation and signalling in vivo and in vitro.[12] Imitation of these and other multiple regulatory mechanisms outside the cellular environment will allow the development of complex synthetic nanomachines with multiple functions that may eventually enable the embedded dynamic management of cells and organisms.

To construct synthetic nanodevices of higher complexity and to achieve the control of multiple functions using artificial allosteric nanomachines, it is worthwhile to add new allosteric regulation mechanisms to the current artificial allostery toolbox. To this end, we introduced a biomimetic multifunctional allostery design that allows versatile DNA nanowire assembly with a variety of effector binding sites and active sites for tuned target binding affinity, molecular assembly efficiency and downstream reaction kinetics. As shown in Scheme 1A, a recognition hairpin (rH) and a signal hairpin (sH), are kinetically protected by long stems until a specific ctDNA target strand, exon-5 (fragment of p53)[13, 14] binds with domain S1, breaking the rH stem and initiating their hybridization along domains S2 and S3. The opening of sH transforms domain S4 from an inactive looped strand to an active linear strand that further hybridizes with the capture hairpin (cH) through toehold displacement, whereby the domain S5 (branched arm) for multivalent capture is activated, to produce a multi-branched DNA nanowire. The binding of a cooperative activator or inhibitor to rH will induce a switch in its conformational equilibrium (Scheme 1B), which alters the affinities (or free energies) of multiple connected oligonucleotides at distant sites (direct control of site 1 for target recognition, site 2 and site 3 for molecular amplification), and the kinetics of downstream reactions (indirect activity control of site 4 in the signal hairpin and site 5 in the capture hairpin for multivalent capture).



Scheme 1 Schematic illustration of the principles of multifunctional allostery for dynamic DNA nanowire assembly.

The equilibrium concentrations, free energies and equilibrium probabilities of rH, rH with a two-fold excess of

Electronic Supplementary Information (ESI) available: See DOI: 10.1039/x0xx00000x

activator 1 (rH/activator) and rH with a 5-fold excess of inhibitor (rH/inhibitor) were estimated via NUPACK at 25°C (Figure S1). The 12-nt single-stranded DNA of activator 1 (Table S1), binds to one toehold of rH in such a way that it partially interrupts the double-stranded stem. Consequently, the equilibrium probability of the stem decreases, suggesting the opening of rH (Figure S1). However, the free energy of domain S3 is reduced due to the binding of activator 1. In order to positively regulate the activity of the DNA nanowire assembly, the 10-nt activator 2 was introduced to hybridize with a partial domain in S1 (rH/activator 2 cannot be estimated via NUPACK). The mechanical tension of the beacon increases the free energy of domains S2 and S3. The activator 2 should be added into the mFA system after ctDNA recognition due to the hybridization of activator 2 with domain S1 will decrease its free energy (the affinity of ctDNA binding is reduced). The inhibitor, a 14-nt single-stranded DNA that binds both toeholds simultaneously, hinders stem opening. The equilibrium concentrations of rH/activator and rH/inhibitor are enhanced with the increase of the concentrations of activator and inhibitor.

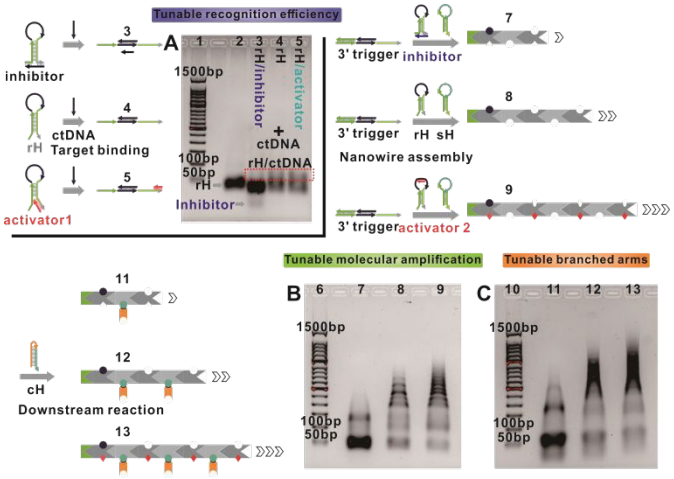


Fig. 1 Agarose gel electrophoresis of A) complexes between the receptors and the target. Lane 1, 50bp DNA ladder; Lane 2, 1 μM rH; Lane 3, 1 μM rH + 5 μM inhibitor + 0.2 μM Exon-5; Lane 4, 1 μM rH + 0.2 μM Exon-5; Lane 5, 1 μM rH + 2 μM activator + 0.2 μM Exon-5. B) Products of 3' directional DNA nanowire assembly. Lane 6, 50bp DNA ladder markers; Lane 7, 1 μM rH+5 μM inhibitor + 1 μM sH + 0.2 μM 3'trigger; Lane 8, 1 μM rH + 1 μM sH + 0.2 μM 3'trigger; Lane 9, 1 μM rH+2 μM activator + 1 μM sH + 0.2 μM 3'trigger. C) 3' directional multi-branched DNA nanowire assembly. Lane 10, 50bp DNA ladder markers; Lane 11, 1 μM rH+5 μM inhibitor + 1 μM sH + 1 μM cH + 0.2 μM 3'trigger; Lane 12, 1 μM rH + 1 μM sH + 1 μM cH + 0.2 μM 3'trigger; Lane 13, 1 μM rH+2 μM activator + 1 μM sH + 1 μM cH + 0.2 μM 3'trigger.

The regulation of ctDNA binding affinity was first demonstrated by agarose gel electrophoresis (AGE) as shown in Figure 1A. The product of the hybridization between the receptor (rH/inhibitor, rH or rH/activator) and ctDNA is related to the switching equilibrium constant of rH (allosteric regulation of domain S1). The average lengths of the resulting nanowires (Figure 1B) and multi-branched nanowires (Figure 1C) are related to the allosteric regulation of domains S2 and S3. This indicates that there is negative and positive control of the hybridization chain reaction (HCR) and downstream reaction kinetics. As expected, the hairpin monomers rH, sH and cH did not hybridize with each other in the absence of the target (Figures S2).

We then observed the kinetics of the mFA controlled versatile DNA nanowire assembly by monitoring the changes of fluorescence intensity due to the separation of the fluorophore FAM from the quencher dabcyl in F-sH-Q. The kinetic rate of fluorescence signal increase at the same ctDNA concentration varied significantly in response to changes of the initiator binding affinity and DNA nanowire assembly activity of the rH (Figure S3A-C). The AGE results verified the same tendency of the formation of bidirectional and multi-branched DNA nanowires. The products of DNA nanowires increased proportionally and became longer after the affinities of multiple hybridization sites were increased through mFA regulation (Figure S3D-F). We also wanted to determine whether the DNA nanowire assembly could be triggered along both the 3' and 5' directions. After using 5'termination (producing a 3'trigger) and 3' termination (producing a 5'trigger) to block one direction of the single strand initiator, we founded a similar rate constant for the HCR in the 5' and 3' direction of the F-sH-Q (Figures S4A and S4B). Furthermore, the loop-based displacement of the HCR in the 5' direction was much more recalcitrant than the toehold-based displacement in the 3' direction of HCR using F-sH'-Q (Figures S4D and S4E). The analysis by AGE confirmed the same tendency of the formation of HCR products in the 3' and 5' direction using F-sH-Q (Figure S4C) and F-sH'-Q (Figure S4F).

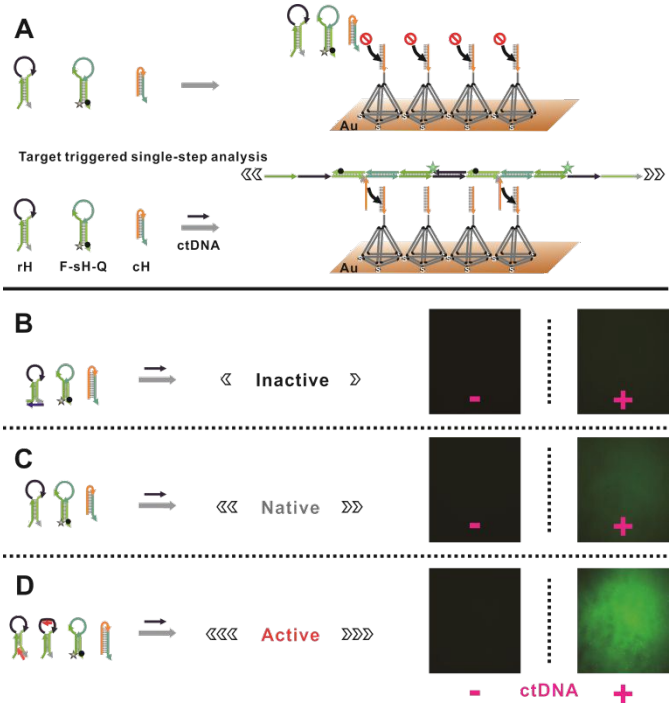


Fig. 2 A) Schematic illustration of the single-step fluorescence imaging. B-D) Imaging of single-step fluorescence triggered by ctDNA with mFA regulation.

A HCR is often employed in biosensors for signal amplification. However, current techniques, especially those

using electrochemistry, require extensive sample processing, which restrains their applications in on-site, real-time analytical devices.[15-18] The development of single-step electrocatalytic biosensors is challenging because of a high signal-to-noise ratio, problems with disrupted biomolecular recognition, as well as steric hindrance effects and low mass transfer rates that limit the surface capture efficiency of target-induced amplification conjugates.

The versatile DNA nanowire assembly with multiple functional domains designed here enables ctDNA analysis using single-step incubation via a cascade assembly of a signalling nanowire and multivalent capture, which are triggered only in the presence of the target molecule (Figure 2A). We constructed a gold chip modified with the framework nucleic acids probes (Figure S5 validates the formation of FANs) for the multivalent capture. In our previous work, we optimized the hybridization conditions and obtained a nearly saturated hybridization in 15 minutes, even with HCR products coupled to cancer cells.[15] Here, we incubated 100 nM Exon-5 to initiate DNA nanowire assembly and multivalent capture. We were able to obtain a positive signal on the 100 nM Exon-5 spots, while there was no signal at all on the spots with only probes (Figure 2C), indicating that the single-step ctDNA detection process based on the versatile DNA nanowire worked as anticipated. The bulk area fluorescent emission intensity of the spot could be regulated through mFA (Figure. 2B-D) and changed in line with expectations.

Next, we detected ctDNA using the single-step electrocatalytic assay with mFA regulation. We detected the catalytic current originated from the HRP enzyme that captured on the surface of electrodes. SA-HRP was pretreated with the versatile DNA nanowire assembly, and we found that 0.25 μg mL-1 SA-HRP was adequate to produce an optimal signal-to-noise ratio (Figure S6). We observed a significant increase in catalytic current from cyclic voltammetry and the i-t curve in the presence of 10 nM target, Exon-5 (Figure S7). We then tuned the dynamic range of the biosensor by regulating the target binding affinity and signaling nanowire assembly activity of rH. As expected, with the improvement of the affinities of multiple domains in the recognition hairpin, the detection limit was reduced from 0.5 nM (rH/inhibitor) to 10 fM (rH/activator) (Figures S8). With this rational mFA regulation, the useful detection range was increased to over 9 orders of magnitude.

For a more detailed dynamic analysis of the mFA-regulated biosensor, we constructed a set of three receptors using the traditional molecular beacon assay (monofunctional allostery) with affinities spanning 3 orders of magnitude (Figures 3A).[6] The dynamic range of the mFA-regulated molecular beacon assay (multifunctional allostery) was increased considerably by introducing dynamic target recognition, dynamic signal amplification and dynamic capture of HCR products. However, it should be noted that it is challenging to stoichiometrically quantify the contribution from allosteric regulation, amplification and multivalent capture effects. The dynamic range of detection increased 10000-fold after integrating a molecular beacon with dual-function allostery, which was 20-fold greater than the traditional molecular beacon assay (Figure 3B). The dynamic detection range further improved 5-fold by using a molecular beacon with multifunctional allostery, mainly due to the varied multivalent capture efficiency caused by the different lengths of signalling DNA nanowires (Figure 3C).

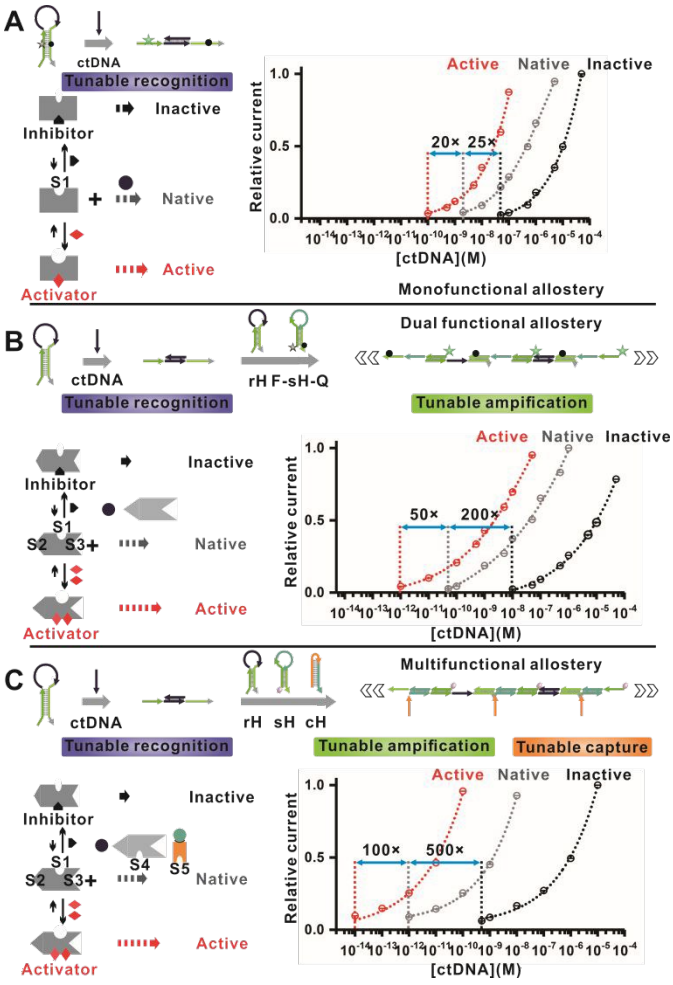


Fig. 3 Rational design of allosterically inhibited and activated biosensors. A) Dynamic detection range tuned though monofunctional allostery regulation. B) Dynamic detection range tuned though dual functional allostery regulation. C) Dynamic detection range tuned though multifunctional allostery regulation.

Interestingly, not only the dynamic detection range, but also the selectivity of the developed biosensor could also be tuned through mFA regulation. As shown in Figures 4C-E, the disparity of signal generation between a perfectly matched ctDNA and ctDNAs with single mismatches in five locations varied significantly when using rH/inhibitor, rH or rH/activator as receptor. Furthermore, both AGE analysis and electrochemical measurements agree that the HCR initiation based on the conformational switch provides higher single-mismatch selectivity than traditional HCR initiation based on toehold displacement (using 5' termination as trigger). This is caused by the higher energy required to open the stem by ctDNA in strand displacement within the loop region (Figure 4).

View Article Online
DOI: 10.1039/D0CC01560A

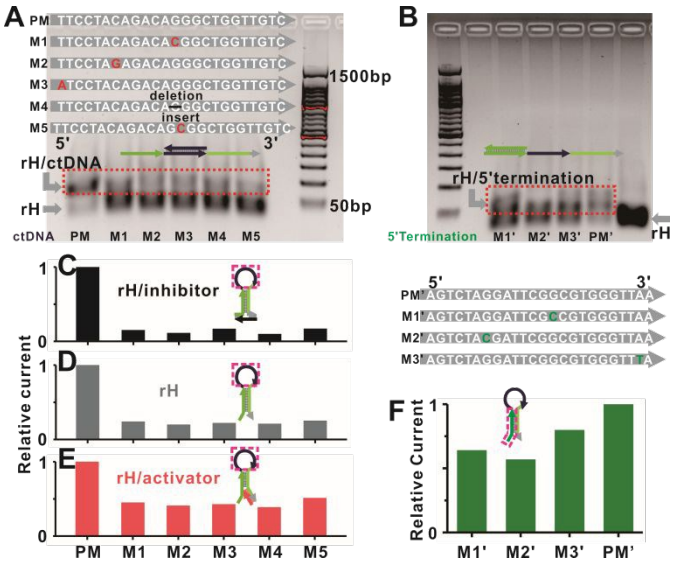


Fig. 4 Schematic of traditional molecule beacons assay for ctDNA dynamic analysis. Analysis by AGE of A) selective recognition of receptors with perfectly matched ctDNA and five ctDNAs with single mismatches in different locations. B) Selective recognition of receptors with perfectly matched 5' termination and that with three single mismatches. C-E) The differentiation between perfectly matched and mismatched ctDNAs can be tuned by multifunctional allostery regulation. The selectivity varied significantly when using (C) rH/inhibitor, (D) rH and (E) rH/activator as receptors. F) The differentiation between perfectly matched and mismatched 5' termination using rH as receptor. The electrochemical data were collected with 10 nM of Exon-5 or 5' termination.

We also tested the single-step electrocatalytic biosensor with direct ctDNA detection in serum samples and optimized the detection conditions (Figure S9). Figure S10 shows the excellent detection performance for the four different ctDNA targets in serum with 25% of 2× SPSC buffer. Moreover, to generalize our single-step electrocatalytic biosensor application, we changed the recognition sequence to an aptamer responsive to ATP (Figure S11). We observed a clear signal response and good detection performance in buffer, with a tunable dynamic range. With the decrease of recognition hairpin stability, the detection limit was reduced from 500 nM (rH/inhibitor) to 1 nM (rH/activator) (Figures S11 A-C).

Conclusions

By using a mFA-regulated versatile DNA nanowire, we have developed a single-step, amplified and dynamic biosensor that enables the robust detection of ctDNA with tunable sensitivity and selectivity. The mFA strategy updates the toolbox for artificial allosteric regulation based on nucleic acid nanotechnology. It has the potential to enrich the design of DNA complexes to achieve dynamic control of DNA nanostructures with multifunctional events. It also offers inspiration to other allosteric regulation mechanisms, and can therefore be applied in combination with other functional DNA domains to build complex DNA machines or devices. For example, the mFA biosensor component can be adapted for use in precision switches or genetic regulators by employing other functional nucleic acid structures such as aptamers, DNAzymes and ribozymes. The mFA platform offers design principles for the development of a new generation of artificial nanomachines that may have the potential to embed dynamic regulation within cells and organisms.

Conflicts of interest

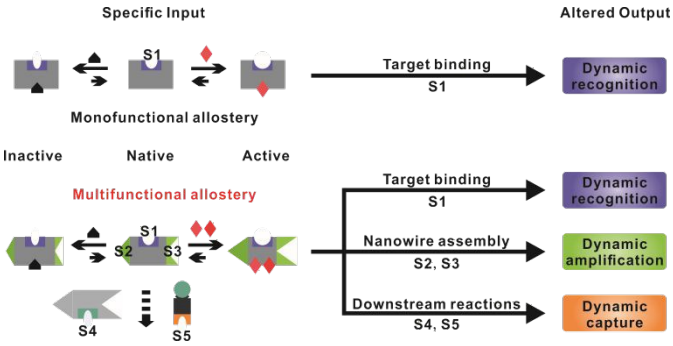
There are no conflicts to declare.

Notes and references

1. J. L. Vinkenborg, N. Karnowski and M. Famulok, *Nat. Chem. Biol.*, 2011, **7**, 519-527.
2. M. Rossetti, S. Ranallo, A. Idili, G. Palleschi, A. Porchetta and F. Ricci, *Chem. Sci.*, 2017, **8**, 914-920.
3. B. Lin, Y. An, L. Meng, H. Zhang, J. Song, Z. Zhu, W. Liu, Y. Song and C. Yang, *Chem. Commun.*, 2019, **55**, 12223-12226.
4. Y. Yao, S. Li, J. Cao, W. Liu, K. Fan, W. Xiang, K. Yang, D. Kong and W. Wang, *Chem. Commun.*, 2018, **54**, 4774-4777.
5. X. Zheng, J. Yang, C. Zhou, C. Zhang, Q. Zhang and X. Wei, *Nucleic. Acids. Res.*, 2018, gky1245-gky1245.
6. F. Ricci, A. Vallee-Belisle, A. Porchetta and K. W. Plaxco, *J. Am. Chem. Soc.*, 2012, **134**, 15177-15180.
7. A. J. Simon, A. Vallee-Belisle, F. Ricci and K. W. Plaxco, *P. Natl. Acad. Sci. USA.*, 2014, **111**, 15048-15053.
8. X. H. Mao, A. J. Simon, H. Pei, J. Y. Shi, J. Li, Q. Huang, K. W. Plaxco and C. H. Fan, *Chem. Sci.*, 2016, **7**, 1200-1204.
9. J. D. Rabinowitz, J. J. Hsiao, K. R. Gryncel, E. R. Kantrowitz, X.-J. Feng, G. Li and H. Rabitz, *Biochemistry-Us.*, 2008, **47**, 5881-5888.
10. X. M. Xia, X. Zeng and C. J. Lingle, *Nature.*, 2002, **418**, 880-884.
11. S. J. Edelstein, O. Schaad, E. Henry, D. Bertrand and J.-P. Changeux, *Biol. Cybern.*, 1996, **75**, 361-379.
12. C. To, J. Jang, T. Chen, E. Park, M. Mushajiang, D. J. H. De Clercq, M. Xu, S. Wang, M. D. Cameron, D. E. Heppner, B. H. Shin, T. W. Gero, A. Yang, S. E. Dahlberg, K. K. Wong, M. J. Eck, N. S. Gray and P. A. Janne, *Cancer Discovery.*, 2019, CD-18-0903.
13. L. Gorgannezhad, M. Umer, M. N. Islam, N.-T. Nguyen and M. J. A. Shiddiky, *Lab. on. a. Chip.*, 2018, **18**, 1174-1196.
14. J. Wang, K. M. Koo, E. J. H. Wee, Y. Wang and M. Trau, *Nanoscale*, 2017, **9**, 3496-3503.
15. G. B. Zhou, M. H. Lin, P. Song, X. Q. Chen, J. Chao, L. H. Wang, Q. Huang, W. Huang, C. H. Fan and X. L. Zuo, *Anal. Chem.*, 2014, **86**, 7843-7848.
16. Z. L. Ge, M. H. Lin, P. Wang, H. Pei, J. Yan, J. Y. Sho, Q. Huang, D. N. He, C. H. Fan and X. L. Zuo, *Anal. Chem.*, 2014, **86**, 2124-2130.
17. W. J. Wang, J. J. Li, K. Rui, P. P. Gai, J. R. Zhang and J. 4J. Zhu, *Anal. Chem.*, 2015, 87, 3019-3026.
18. J. Zhao, C. Chen, L. Zhang, J. Jiang and R. Yu, *Biosens. Bioelectron.*, 2012, **36**, 129-134.

View Article Online
DOI: 10.1039/D0CC01560A

Table of contents entry



Multifunctional allostery regulated DNA molecule beacon assay was applied to engineer a single-step, amplified and dynamic biosensor for controllable analyses of ctDNA.