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A metabolic labeling way to in situ fabricate bacterial FRET Platform for innate immune defence molecule

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ABSTRACT

A simple and unique bacterial fluorescence resonance energy transfer (FRET) platform is developed through metabolic biosynthetic pathways for the sensitive and ratiometric detection of innate immune defence molecule. Using this bacterial FRET platform, immune molecule lysozyme could be sensitively monitored in buffer, in serum, and even in the secretion of infected immune cells. As an important defensive molecule of the innate immune system, lysozyme not only plays a significant role in mediating protection against microbial invasion, but also acts as a significant biomarker of leukemia, tuberculosis, meningitis and renal diseases. Considering the importance of perceiving lysozyme activity, this work sheds a light on the access of host's immune response of bacterial infection, as well as provides valuable guidance for the treatment of bacterial infection related diseases.

1. Introduction

Perceiving bacterial infection is critical for immune defence and proper antibiotics treatment [1,2]. Generally, host immune cells sense bacterial infection by identifying the microbial associated molecular patterns (MAMPs) through the surface germline encoded pattern recognition receptors (PRRs) [3,4]. The recognition of MAMPs will further activate host innate immune signaling and ultimately trigger the production of various defence molecules to mediate protection against bacterial invasion. Therefore, the level of defence molecules is commonly considered to be valuable information for understanding the status of bacterial infection [5,6]. Lysozyme (LYS) is one of such important defence molecules, which is also known as N-acetylmuramide glycan hydrolase that towards the cleavage of glycosidic bonds between N-acetylmuramic acid (NAM) and N-acetylglucosamine (NAG) of peptidoglycan to destruct bacterial cell wall [7]. Clinical studies indicated that the increase in the concentration of LYS in serum and urine is closely associated with acute bacterial infection, tuberculosis, meningitis, and even renal diseases and leukemia [8–10]. Therefore, accurate detection of LYS is not only able to assess the host's immune response of bacterial infection, but also capable to provides valuable guidance for the treatment of bacterial infection related diseases.

Hence, over the past few decades, enormous efforts have been devoted to develop various advanced techniques for LYS detection, including colorimetric, fluorescence, light scattering and electrochemical methods [11–18]. These efforts have greatly promoted our capacity to sensitively detect LYS in diverse physiological and pathophysiological conditions [19–23]. The recognition strategies of these LYS biosensors can be roughly divided into two modes, electrostatic interaction based non-specific recognition and biomolecules (e.g. antibody, aptamer, peptidoglycan) based specific recognition [24–29]. Although promising, both of these two modes possess evident drawbacks. For example, the non-specific recognition strategy is generally susceptible to other charged molecules, while the specific recognition strategy often requires high cost and cumbersome processes for the fabrication and detection [30–34]. Thus, the development of innovative method for inexpensive, sensitive and selective detection of LYS is still highly desired.

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Herein, we report a simple and unique bacterial metabolic labeling approach to fabricate fluorescence resonance energy transfer (FRET) based platform for the sensitive and ratiometric detection of LYS. As shown in Scheme 1, peptidoglycan, a natural target motif of LYS on bacteria cell wall, was acted as both the chromophores conjugation scaffold and LYS recognition site in our system. Through native metabolic biosynthetic pathways, a donor - acceptor fluorescence pair was in situ labeled on peptidoglycan motif to form the bacterial FRET platform, and the cleavage of peptidoglycan by LYS would widen the distance between the donor and the acceptor, resulting easily detectable FRET signal variation for effective bio-sensing.

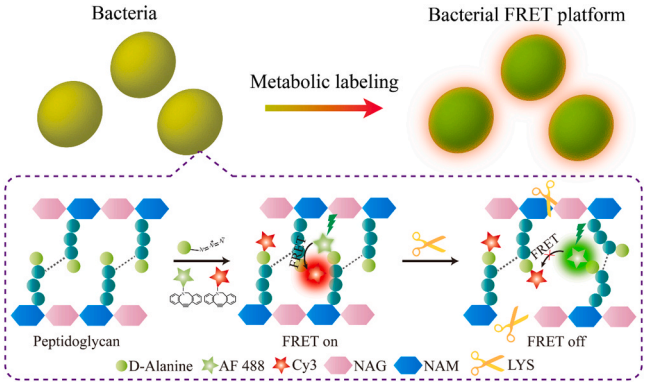
2. Experimental section

2.1. Metabolic labelling of Staphylococcus aureus

Staphylococcus aureus (*S. aureus*, ATCC 29213) were cultured in Luria-Bertani (LB) medium at 37 °C with shaking. *S. aureus* at the exponential growth phase were collected, ten times diluted, and co-cultured with 1 mM 3-Azido-D-alanine for another 2 h. Then, the azido modified *S. aureus* were harvested, washed with PBS buffer, and re-suspended in the buffer. After that, the azido modified *S. aureus* were labeled with fluorescence dye through a room temperature incubation. Typically, a certain volum of the stock solution of DBCO-AF488 (10 mM in DMSO) and DBCO-Cy3 (10 mM in DMSO) were added to the suspension of azido modified *S. aureus* with a final bacterial optical density of 0.5 at 600 nm. After incubation at room temperature for 2 h, the labled *S. aureus* were separated, washed, then dispersed in PBS buffer. We defined the AF488 and Cy3 labled *S. aureus* as SA-AF488/Cy3. Finally, SA-AF488/Cy3 were inactivated with 60% (v/v) ethanol for 1 h. After that, the inactivated SA-AF488/Cy3 were washed, resuspened in PBS and stored at 4 °C for further use.

2.2. Bacterial viability test

The bacterial viability of the ethanol inactivated *S. aureus* was measured by fluorescein diacetate (FDA) - propidium iodide (PI) double staining method. Typically, 5 μl of PI stock solution (100 μg mL^{-1} in deionized water) and 1 μl of the FDA stock solution (10 mg mL^{-1} in acetone) were added to 0.5 mL pH 7.2 PBS buffer containing bacteria with an optical density of 0.2 at 600 nm. After 30 min incubation at 37 °C, the stained bacteria were separated by centrifugation and washed three times with the PBS buffer. Then the bacteria were redispersed in PBS buffer and characterized by a confocal laser scanning microscope (ZEISS LSM 800).



Scheme 1. Schematic representation of the working principle of the bacterial FRET platform.

2.3. LYS activity assay

LYS activity were carried out by incubaton of 10 μl LYS containing soulution with 140 μl of the *S. aureus* - AF488/Cy3 (OD 0.5). After 5 h of incubation at 37 °C, the emission spectra under 450 nm excitation were collected. The same procedures were performed for the selectivity test.

In the inhibit experiments, the inbitor imidazole with the finnal concentration of 50 mM was incubated with LYS for 2 h before the lydolysis toward the bacterial probe.

2.4. Monitoring of LYS secreted from immune cells during bacteria infection

Murine macrophage cell line RAW264.7 was used as the model of immune cells. Gram positive bacteria *S. aureus* (ATCC 29213) and gram negative bacteria *Escherichia coli* DH5α (*E. coli*) strains were used as the bacteria models. RAW264.7 were cultured in Dul-becco's modified Eagle's (DMEM) (Gibco BRL) medium supplemented with 10% fetal bovine serum (FBS) in a 5% CO_2 humidified environment at 37 °C for an appropriate time. For the infection test, RAW264.7 cells were seeded at a density of 6×10^4 cells/well in 12-well assay plates and allowed to 24 h grow for attachment. Then 20 μl of *S. aureus* (1.0 OD600) or *E. coli* (1.0 OD600) were added respectively. After certain time incubation the culture medium was harvested and centrifuged to get the supernatant for LYS activity test. The test by our FRET platform was according to the method described above, and 10 μl of the supernatant was used for one time test. The tests by our FRET platform were performed according to the method described above, and 10 μl of the supernatant was used for one test. As comparison, LYS in the medium was also measured by a commercial LYS kit (LY0100, Sigma Aldrich), in which 100 μl of the cell medium supernatant was used for one test.

3. Results and discussion

3.1. Construction of bacterial FRET platform

As a proof of concept, we used *S. aureus* as a bacterial model to construct the bacterial FRET platform. *S. aureus* is a gram-positive bacterium contains abundant peptidoglycan motif in the cell wall, which would facilitate the generation of strong FRET signal [35–39]. The fluorescence dye Alexa Fluor® 488 (AF488) and Cyanine-3 (Cy3) were chosen as the donor-acceptor pair owing to the broad overlap between their excitation and absorption spectra (Fig. S1, ESI†). The conjugation of the donor-acceptor pair to bacterial peptidoglycan motif was carried out through bio-orthogonal click chemistry coupled with a metabolic biosynthetic pathway (Fig. S2, ESI†) [40–43]. Briefly, we first incorporate a bioorthogonal azido tag functionalized D-alanine (3-Azido-D-alanine, D-Ala(N3)) into peptidoglycan of the bacterial cell wall through 2 h of feeding. Then, a copper-free click reaction was performed on the azido labeled *S. aureus* by employing the donor-acceptor pair (DBCO-AF488 and DBCO-Cy3) conjugated dibenzyl cyclooctyne (DBCO) as a bioorthogonal linkage. The covalent labeling of the peptidoglycan motifs on bacterial cell wall was monitored by confocal microscopy. As shown in Fig. 1, after the incubation of D-Ala(N3)-fed *S. aureus* with DBCO-AF488 (2 μM) and DBCO-Cy3 (10 μM) respectively, a bright green and yellow fluorescence rings appeared around the bacterial cell walls. Notably, no fluorescence signal was detected in *S. aureus* without the D-Ala(N3) feeding in control experiments (Fig. S3, ESI†), indicating the efficient bacterial peptidoglycan labeling through the native metabolic biosynthetic pathways. We next simultaneously labeled *S. aureus* with both the donor molecule (AF488) and the acceptor chromophore (Cy3). Encouragingly, a strong FRET-induced fluorescence signal was observed in the co-labeled *S. aureus* (Fig. 1), suggesting the high feasibility of fabricating bacterial FRET platform through the metabolic labeling way.

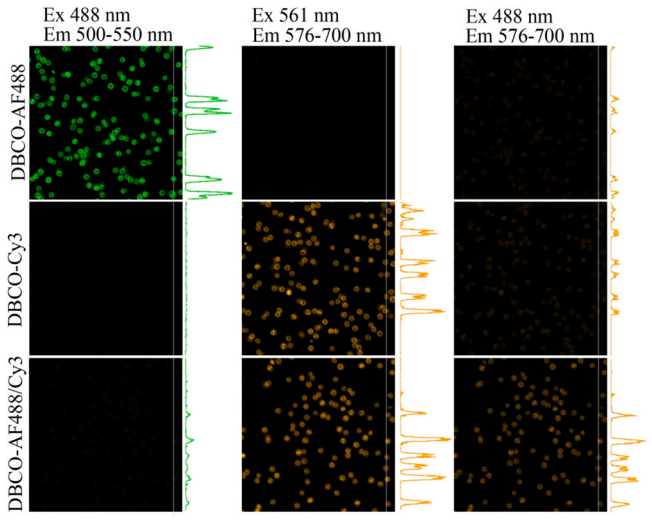


Fig. 1. Confocal images of *S. aureus* (D-Ala(N3) fed) incubated with DBCO dye under different conditions. The spectrum in the right indicates the fluorescence intensities of bacteria in the gray line.

3.2. Spectral Analysis of Metabolic labeled FRET platform

To gain better insight into the efficiency of the FRET platform, we further carried out spectral analysis of the metabolic labeled *S. aureus*. As shown in the fluorescence spectra (Fig. 2a), under 450 nm excitation, a strong emission peak at 520 nm was observed from the *S. aureus* that labeled with donor molecule AF488 (Olive line), while a weak peak at 568 nm was appeared from the bacteria labeled with acceptor chromophore Cy3 (Red line). However, when the bacteria simultaneously labeling with both AF488 and Cy3 (Orange line), the emission intensity at 520 nm reduced by 80%, while the emission intensity at 568 nm was enhanced over 200%, indicating the significant FRET effect between the donor and the acceptor. Notably, although the present metabolic labelling way cannot guarantee that each pair of donor and acceptor can be modified in the adjacent position, the peptidoglycan on the bacterial cell wall has high density, which could make a considerable number of donor and receptor pairs be modified close enough for FRET signal generation. The fluorescence signal and FRET efficiency can be further enhanced by adjusting the incubation ratio, and concentration of the donor - acceptor pair (Figs. S4–5, ESI†). We finally attained the optimum concentration ratio with 8 μM DBCO-AF488 and 20 μM DBCO-Cy3 as the bacterial feeding condition within 5 h of fabrication. Under this condition, the emission intensity ratio between 520 nm and 568 nm (I_{568}/I_{520}) of bacterial FRET platform is as high as 1.58 (Fig. 2b, black line). Considering that the live bacteria may divide during storage and detection, resulting in changes in the FRET signal, we correspondingly inactivate the donor - acceptor pair labeled bacteria with aqueous ethanol solution (Fig. S6, ESI†). As the ethanol treatment did not destroy the bacterial cell wall structure to cause significant changes in the FRET signal (Fig. 2b, red line), we used the inactivated *S. aureus* FRET platform for the followed biosensing studies.

3.3. LYS responsive activity

Having the established bacterial FRET platform, we then studied its LYS responsive activity (Fig. 2b, blue line). As expected, after incubation with LYS, an obvious fluorescence change appeared in the bacterial FRET platform, in which the emission intensity at 520 nm increased by 65%, while the intensity at 568 nm decreased accordingly. Consequently, the significant decline of the I_{568}/I_{520} value from 1.58 to 0.84 was observed. Under the same condition, LYS did not cause any detectable alternation in the spectrum of the bacteria labeled only with either AF488 or Cy3 (Fig. S7, ESI†). Moreover, the response of the FRET platform to LYS was substantially inhibited in the presence of a standard LYS inhibitor (e.g., Imidazole) (Fig. S8, ESI†) [44]. These results suggested that the cleavage of the peptidoglycan by LYS is responsible for the change of FRET signal, which is coherent with the rationale in our design.

3.4. FRET signal variation study under different incubation conditions

We further examined the FRET signal variation under different concentrations of LYS. As shown in Fig. 3a, the I_{568}/I_{520} value of the probe decreased gradually with the increasing of LYS concentration in the range of 5–500 nM. A good linear relationship between the ratiometric intensity of I_{568}/I_{520} and the logarithmic concentration of LYS was identified (Fig. 3b). The detection limit (triple signal-to-noise ratio) was calculated to be as low as 0.06 nM, which is sensitive than most presently reported optical method (Table S1, ESI†) and sufficient for sensitive analysis of LYS in most physiological and pathophysiological conditions [45,46]. The responses of the bacterial FRET platform to other different proteins (e.g., trypsin, myoglobin, BSA, HSA) were subsequently investigated. Notably, these interferents could not contribute to significant changes in the FRET signal (Fig. S9, ESI†), clearly indicating the high stability and selectivity of our biosensing platform.

3.5. LYS analysis feasibility in serum sample

Consequently, in order to verify the lysozyme analysis feasibility of the bacterial FRET platform in complex biological media, we investigated the detection accuracy in serum sample through a conventional

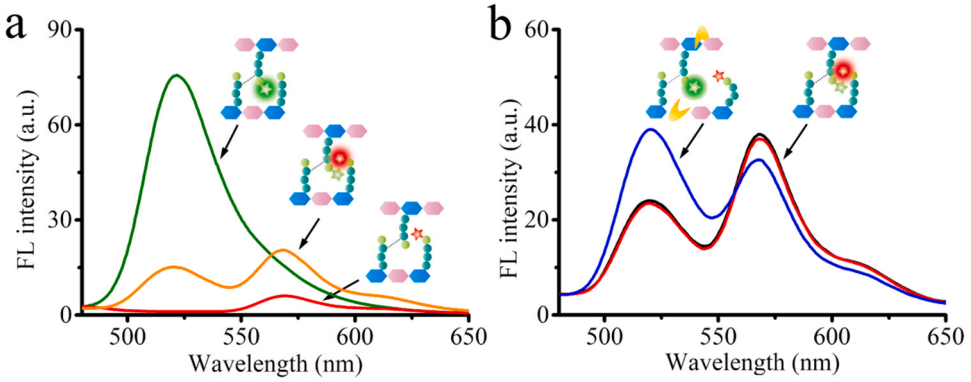


Fig. 2. a) Fluorescence spectra of *S. aureus* labeled with AF488 (Olive line), Cy3 (Red line), AF488 and Cy3 (Orange line); b) Fluorescence spectra of *S. aureus* labeled with AF488 and Cy3 in an optimized condition (Black line), the labeled bacteria inactivated with ethanol aqueous solution (Red line) and then incubated with 500 nM LYS for 2 h (Blue line). (Ex = 450 nm).

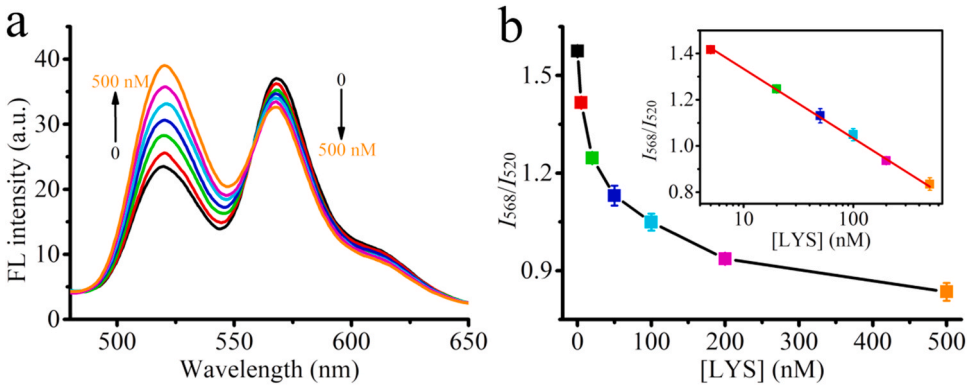


Fig. 3. a) The spectra of the bacterial FRET platform response to different concentrations of LYS (0, 5, 20, 50,100, 200, and 500 nM). d) The plot of I568/I520 as a function of LYS concentration.

spike recovery test. As expected, good recoveries of the spiked lysozyme in serum ranging from 96% to 105% with the relative standard deviation (RSD) values less than 5% were observed (Table S2, ESI†), indicating a high accuracy of the FRET system for biosensing in complex biological conditions.

3.6. LYS analysis feasibility in bacteria-infected cell secretion sample

Finally, the secretion of LYS from bacterial infected immune cell was investigated by using this bacterial FRET platform (Fig. 4a). In the in vitro bacterial infection experiments, a commonly used macrophage cell line Raw 246.7 was chosen as a model immune cell, meanwhile, a typical Gram-negative strain *E. coli DH5α* and Gram-positive strain *S. aureus* (ATCC 29213) were used as model bacteria strains. After incubating the bacteria with Raw 246.7 for a certain period of time, the culture medium of the infection system was harvested and centrifuged to obtain a supernatant for LYS analysis. As shown in Fig. 4b, after 36 h of cultivation without bacterial infection, no LYS was detected in the culture medium of immune cells. However, after cultivation of the immune cell that infected with either *E. coli* or *S. aureus* for 4 h, obvious LYS was detected in culture medium at the concentrations around 6 nM. Upon elongation of the incubation time to 12 h, the LYS concentrations in both of the infected medium reached over 20 nM. These results demonstrate that both *E. coli* and *S. aureus* can induce significant immune response of Raw 246.7 to up-regulate the expression and secretion of the immune defence molecule LYS (Fig. 4a).

Considering defence of Raw 246.7 to bacterial infection is a synergistic process which usually expresses other immune molecules (e.g., TNF-α, IL-6) to cooperate with LYS, we further investigated immune molecule TNF-α generation during bacterial infection to confirm the immune response phenomenon. As shown in the immune fluorescence images (Fig. 5), a significant increase of TNF-α expression was observed upon bacterial infection, which is consistent with the stimulated expression of LYS, thus strongly confirming the occurrence of immune response of Raw 246.7. Furthermore, to demonstrate the reliability of our bacterial FRET platform, the detection of LYS in the infected cell culture medium was performed with a standard commercial LYS assay kit. Remarkably, the similar detection results were obtained while the

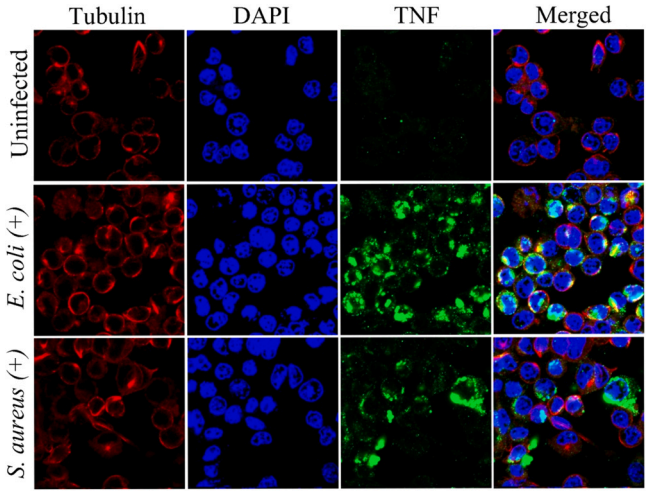


Fig. 5. Immune fluorescence images display the bacterial infection induced TNF-α expression in immune cell RAW 246.7.

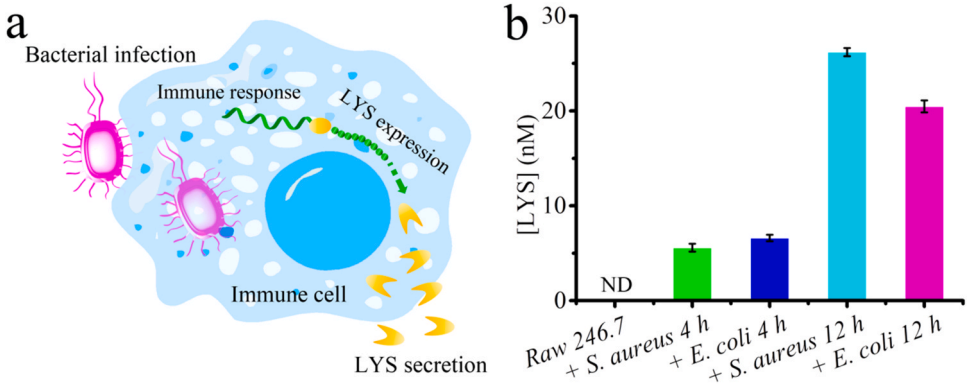


Fig. 4. a) Schematic illustration of bacterial infection induced LYS expression and secretion by immune cell RAW 246.7. b) LYS in the medium of bacteria-infected Raw 264.7 measured by the bacterial FRET platform.

required medium volume for detection by the bacterial FRET approach is ten times smaller than that by the commercial kit (Fig. S10, ESI†), strongly indicating the capability of our fabricated bacterial FRET platform as a robust biosensing tool for LYS.

4. Conclusion

In summary, we presented a facile and unique bacterial FRET platform for the precise and sensitive monitoring of innate immune defence molecule LYS in diverse physiological and pathological conditions. The FRET platform was fabricated through metabolic labeling of bacterial peptidoglycan, in which peptidoglycan was functioned as both the donor-acceptor chromophores conjugation scaffold and LYS recognition site. Such distinctive FRET platform fabrication method is simple, low cost, and robust. Moreover, the FRET probe could provide sensitive response to LYS to give a remarkable ratiometric detection signal with the detection limit as low as 0.06 nM. Using this platform, the LYS in serum could be precisely quantified. Furthermore, this FRET platform could indeed be used for monitoring LYS secretion from immune cells during bacterial infection. This work not only provides a simple and reliable approach for the sensitive and selective analysis of LYS in diverse samples but also affords a new idea to fabricate bacteria based biosensing platforms.

CRediT authorship contribution statement

Zhijun Zhang: Conceptualization, Methodology, Investigation, Data curation, Writing – review & editing. **Qinyu Han:** Investigation, Data curation, Writing – review & editing. **Jun Wei Lau:** Investigation, Data curation. **Zhimin Wang:** Investigation, Data curation. **Ming Hu:** Formal analysis. **Hao Qiu:** Formal analysis. **Thang Cong Do:** Formal analysis. **Bengang Xing:** Conceptualization, Methodology.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Acknowledgements

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Conflict of Interests

There are no conflicts to declare.

Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.snb.2021.130913.

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