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Self-assembling Microfiber-like Biohydrogel for Ultrasensitive Whole-cell Electrochemical Biosensing in Micro-droplets

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ABSTRACT: A novel microfiber-like biohydrogel was fabricated by a facile approach relied on electroactive bacteria induced graphene oxide reduction and confined self-assembly in capillary tube. The microfiber-like biohydrogel (d=~1 mm) embedded high-density living cells and activated efficient electron exchange between cells and the conductive graphene network. Further, miniature whole-cell electrochemical biosensing system was developed and applied for fumarate detection under -0.6V (*vs.* SCE) applied potential. Taking advantages of its small size, high local cell density and excellent electron exchange, this microfiber-like biohydrogel based sensing system reached a linear calibration curve (R^2=0.999) ranging from 1 nM to 10 mM. The LOD obtained was 0.60 nM, which was over 1300 times lower than traditional biosensor for fumarate detection in 0.2 μL micro-droplet. This work opened up a new dimension for miniature whole-cell electrochemical sensing system design, which provided the possibility for bioelectrochemical detection in a small volume or three-dimensional local-detection at high spatial resolution.

Development of novel sensors in micro/nanoscale dimension is of great important for modern analysis. The miniaturized sensors provided the superior sensitivity and localized detection within small sample volume.[1] These unique properties were quite important for the scenario that with limited sample availability or high spatial resolution requirement.[2]

Therefore, various approaches to fabricate miniature biosensors had been developed in recent years. For example, sophisticated micro-fabricated devices (e.g., microelectrode, microchip, microfluidic, micro-droplet array) and highly sensitive detection instruments (e.g., surface plasma resonances, quartz crystal microbalance, atomic force microscope,) had been developed for construction of different miniature sensors.[2-6] Although these approaches achieved high sensitivity and small sample volume requirement, these traditional miniaturization approaches encountered the problems including complicated fabrication process, high operating cost, or expensive equipment requirement. Therefore, it is desirable to develop new miniature biosensor.

Whole-cell bioelectrochemical sensing systems, which integrated the advantages of electrochemical detection and the excellent sensing capability of the electroactive bacterial cell, provided unique tools for biosensing with electric output signalling.[7, 8] For the whole-cell bioelectrochemical sensing, the efficiency of electron exchange between cells and the electrode was quite essential to maximize the bio-signal transduction.[9] To miniaturize the whole-cell bioelectrochemical sensing system, traditional approaches usually encountered the problems of low local cell density, sluggish electron exchange, and/or poor conductivity. Thus, it is still quite challenge to fabricate miniature whole-cell bioelectrochemical sensing system.

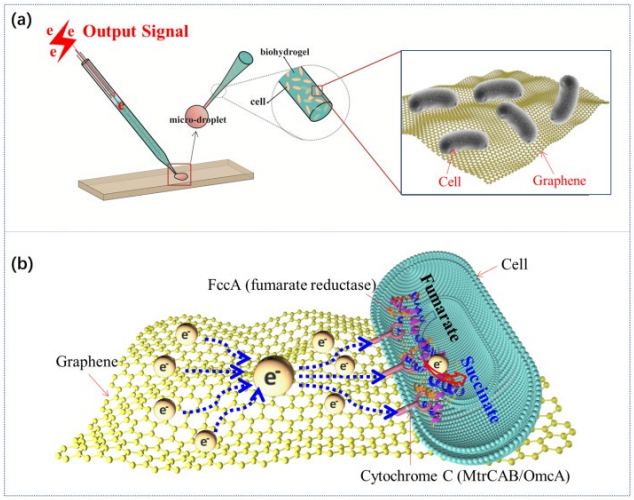

Figure 1. Schematic of the (a) biohydrogel based micro-droplet sensing and (b) the mechanism for fumarate detection with the cell-embedding biohydrogel.

Herein, we developed a facile approach to fabricate a cell-embedded microfiber-like biohydrogel (Fig. S1), and constructed a miniaturized capillary tube-based whole-cell bioelectrochemical sensing system for fumarate (a biomarker for kidney cancer and food spoilage) detection in 0.2 μL micro-droplet (Fig. 1a). Inspired by the finding that the electroactive bacterial cell had the ability to reduce graphene oxide and assemble to cell-embedding hydrogel,[10] a simple method for electroactive cell-induced graphene hybridized microfiber-like biohydrogel

fabrication was designed (Fig. 1a). The microfiber-like biohydrogel (~1 mm in diameter) that with the support of a microstainless steel wire (~100-150 μm in diameter) was formed under the confinement of a capillary tube (2 mm in diameter) (Fig. S1). The cells attached on the graphene surface, while the graphene nanosheets would serve as the electron collector to collect the electrons from the electrode and then serve as the electron distributor to distribute the electrons into the cells with the transmembrane electron transfer proteins (i.e., MtrCAB/OmcA) (Fig. 1). Next, the electrons would be passed to the periplasmic fumarate reductase (FccA) to power the fumarate reduction to succinate. Thus, a highway for electron flow from the electrode to the intracellular FccA would be constructed, while the electron flow could be considered as the indicator of fumarate for biosensing system design (Fig. 1b).

For confined assembly of the microfiber-like hydrogel in capillary tube, the cell suspension of *Shewanella oneidensis* MR-1 (OD600=8.0 in M9+LB medium) was mixed with the solution of graphene oxide (2 mg/L). Lactate (18 mM) was added into the mixture to serve as the electron donor for bacterial cell induced reduction of the graphene oxide.[10] After 24 hours incubation, the mixture turned from brown color to black and anchored onto the metal wires, which formed microfiber-like hydrogel (~1 mm in diameter) (Fig. S2). Strikingly, after taking the hydrogel out of the capillary tube, the residual medium turned to colorless, indicating nearly all the cells was embedded into the biohydrogel. Then, the total cell protein in the biohydrogel was quantified and the results indicated that nearly all the cells were included in the biohydrogel (Fig. S2), confirmed the excellent cell attraction ability of the biohydrogel. As the volume of the hydrogel was only half of volume of the cell suspension added, the biohydrogel would guarantee extremely high local cell density on the metal wire electrode. The confinement was also very crucial to make the hydrogel firmly anchored on the surface of the highly conductive metal wire, which served as the electron collector/distributor for biosensing. Without the capillary tube confinement, the biohydrogel never firmly anchored on the metal wire (Fig. S2c).

According to the SEM images, the bare metal wire showed smooth surface (Fig. S3), while the microfiber-like biohydrogel showed much rough surface (Fig. S4). After drying, the hydrogel layer only showed a thickness of about 10-15 μm that firmed anchored on the surface of the metal wire (Fig. 2a). It could be clearly observed that the cells aligned on the surface of the graphene nanosheets (Fig. 2b and Fig. S5). The cell viability was vital to the function of the biosensor. Thus, LIVE/DEAD staining was applied to evaluate the cell viability in the biohydrogel. The results showed that over 99% of the cell is alive (Fig. S6), guarantying the biosensing ability. The SEM observation and LIVE/DEAD staining clearly confirmed the presence of the cells on the graphene nanosheets. Further, XPS and Raman analyses were used to characterize the biohydrogel. By removing the surface attached cells with HCl and ethanol, the hydrogel was dried by lyophilisation and was subjected to analyses. The XPS spectrum of graphene oxide showed two clear peaks that could be assigned to C1s and O1s (Fig. S7a and Fig. 2c), where the atom ratio of C:O was 74:26. As expected, the oxygenated group of the biohydrogel decreased remarkably (the atom ratio of C:O was 83:7) (Fig. S7b and Fig. 2d), indicating the graphene oxide was reduced to reduced graphene oxide by the *S. oneidensis* MR-1 cells.[10] In addition, the Raman analysis showed that the I_D/I_G was increased from 0.95 (graphene oxide) to 1.12 (biohydrogel) (Fig. S8), confirming the graphene oxide was reduced. Therefore, all these results indicated that the living electroactive cells-embedded graphene-hybridized microfiber-like hydrogel was successfully fabricated.

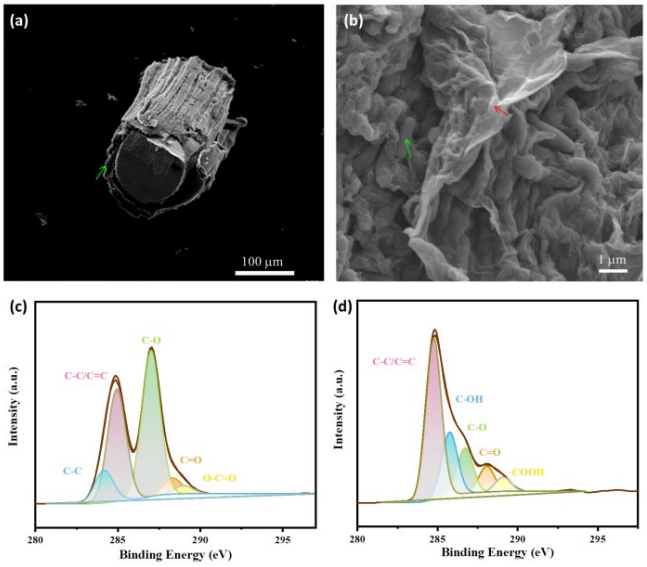

Figure 2. (a-b) The SEM images of the biohydrogel. The green arrow in (a) indicates the hydrogel layer, the green and red arrow in (b) indicate cell and graphene sheets, respectively. The XPS spectrum of graphene oxide (c) and biohydrogel (d).

With the aim to construct the electrochemical sensor with the biohydrogel, the bioelectrochemical activity of the microfiber-like biohydrogel was characterized. As shown in Fig. 3a, the biohydrogel showed two redox pairs with large capacitance, while the suspension cells did not show any obvious redox wave. Further, the DPV analysis was applied to further confirm the CV results. It was observed that the biohydrogel exhibited two dominant peaks that with the similar potential as the CV results (Fig. 3b). The two redox peaks could be assigned to the electron mediator of flavins and cytochrome C of *S. oneidensis* MR-1.[3,11,12] The peak at ~-0.1 to -0.2 V could be assigned to the cytochrome C based direct electron transfer (DET) pathway.[12] However, the DPV of the suspension cell showed a weak peak at ~ -0.3 to -0.4V due to higher resolution obtained by the DPV compared to the CV analysis. These results indicated that the suspension cells employed weak flavins mediated electron transfer pathway, while the biohydrogel activated cytochrome C based strong DET for electron exchange between cells and the electrode. These results implied that the close contact of the conductive graphene nanosheets networks with the *S. oneidensis* MR-1 cells activated the DET, which might greatly improve the electron exchange efficiency between cells and the electrode (stainless steel wires). In addition, the EIS results indicated that the biohydrogel had a much smaller charge transfer resistance than suspension cell (Fig. 3c and 3d), confirming the electron exchange efficiency between the electrode and the cells in biohydrogel was largely improved. Thus, the results proved that the biohydrogel exhibited excellent bioelectrochemical activity, suggesting it would be promising for biosensing application.

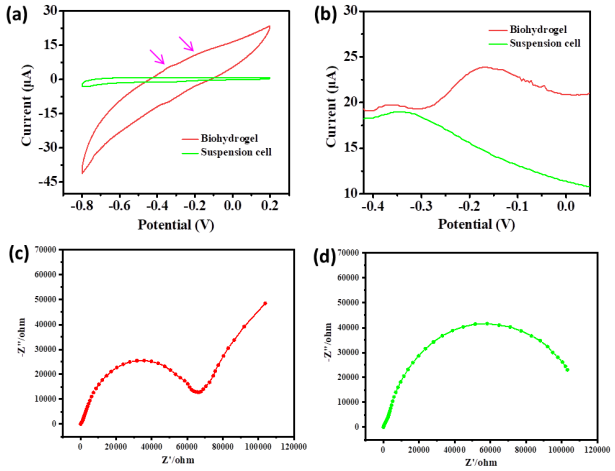

Figure 3. The CV (a) and DPV (b) curves of biohydrogel and suspension cell. The pink arrows indicate the redox waves. The EIS spectrum of biohydrogel (c) and suspension cell (d) in the capillary tube.

Next, the whole-cell electrochemical sensing system was established in the capillary tube with the biohydrogel. A three-electrode system was constructed in the capillary tube by using the microfiber-like biohydrogel as the working electrode, Ag/AgCl wire as the reference electrode, and the Pt wire as the counter electrode (Fig. 1a). A 100 μL M9+LB medium was injected into the capillary tube and served as the electrolyte. Due to the capillary effect, the micro-droplet sample on the glass sheet could be spontaneously and quickly up-taken into the tube and subjected for detection (Fig. 1a). It was reported that *S. oneidensis* MR-1 could be used for fumarate sensing (a biomarker for disease and food contamination),[7] thus fumarate was selected as the model analyte here. It was observed that, with the capillary tube system, 0.2 μL fumarate micro-droplet sample (500 μM) could induce significant signal output (Fig. 4a). In comparison, suspension cells were also injected into the capillary tube with the bare stainless steel wire as the working electrode for fumarate detection. This suspension cell system could also respond to this micro-droplet sample. However, the output signal of the suspension system was only 0.11±0.03 μA, which was 16 times lower than that from the biohydrogel (1.87 ±0.09 μA). Biofilm was also considered as another promising sensing element for sensitive and small sample detection.[13] However, it was quite difficult to form thick biofilm on the metal wire (after 24 h incubation, the cell protein on the wire was only ~20 μg, Fig. S2d). As a result, the signal output for biofilm based sensing system was only 0.03±0.01 μA, which is 61 times lower than that from the biohydrogel (Fig. 4a). These results substantiated that this microfiber-like biohydrogel-based whole-cell bioelectrochemical sensing system was successfully constructed.

Then, the analytical performance of this microfiber-like biohydrogel-based electrochemical sensing system was evaluated. Strikingly, a peak like signal output was obtained when the sample concentrations in 1 nM, 500 nM and 100 μM (Fig. 4b). It was in good agreement with previous report that the output signal resulted from the fumarate reduction to succinate would decrease to the baseline once the fumarate was fully reduced.[7] Impressively, the signal output in response to fumarate addition exhibited obvious concentration dependent manner. In addition, as the fumarate added could be quickly reduced to succinate, it was expected that the sensing system could be used for successive detection. Thus, successive detection with the single biohydrogel system was tested and a uniform peak array was obtained (Fig. 4c). The result implied this system could be reliable for on-line/continuous monitoring. Theoretically, the charge injected into the biohydrogel was in proportional to the amount of fumarate reduced (Fig. S9). Thus, it was likely to quantify fumarate with this biosensing system by using the peak area as the output signaling.

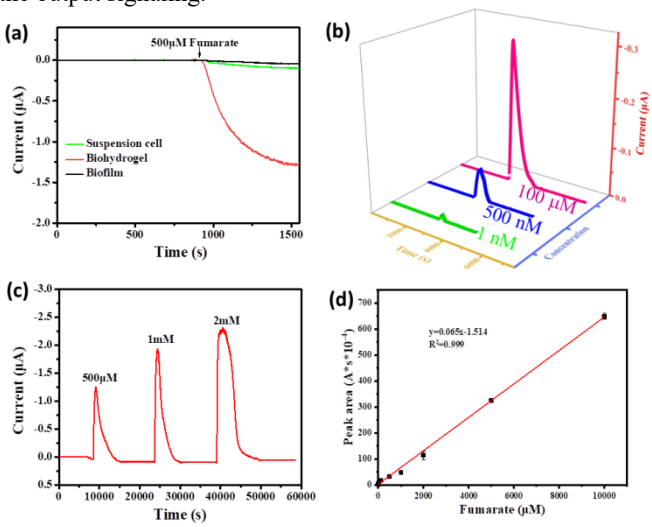

Figure 4. (a-c) Amperometric response of the biosensing system to fumarate. (d) Calibration curve for fumarate quantification with the biohydrogel.

Therefore, the calibration curve was determined to evaluate the possibility for fumarate quantification in micro-droplets. As expected, the peak area of the signal output showed good linear relationship (R^2=0.999) with the fumarate concentrations ranging from 1 nM to 10 mM (Fig. 4d). The limit of detection (LOD, s/n=3) determined was 0.60 nM, while the limit of quantification determined (LOQ, s/n=10) was 25.6 nM. Fumarate was a key intermediate of tricarboxylic acid cycle and was considered as the biomarker for fumarate hydratase-associated cancer and food spoilage.[7] Thus, various quantification methods have been developed (Table S1, Fig. 5).[7,13-17] Compared with the physicchemical methods, the current biohydrogel based system showed ~5000 times and ~50000 times lower LOD than that of HPLC and chemometric method,[14,15] respectively. Compared with the most sensitive method reported previously (whole-cell bioelectrochemical sensing system),[7] the LOD for fumarate detection was decreased ~1380 times by the current biohydrogel based electrochemical sensing system (Fig. 5). Although the sensing mechanism for current biohydrogel based system was similar to previous whole-cell bioelectrochemical sensing systems,[7, 13] the embedding of the cells into the graphene-hybridized biohydrogel largely increased the local cell density and dramatically improved the electron exchange efficiency by activating the DET (Figs. 2 and 3). The enhanced local cell density and reinforced electron exchange might be the explanation for this high sensitivity. Moreover, the sample volume required for the current method was the lowest among all the reported methods (Fig. 5). To the best of our knowledge, this is the only

reported approach for micro-droplets detection of fumarate, which would be promising for in-situ detection with high spatial resolution or disease diagnosis with very limited sample.

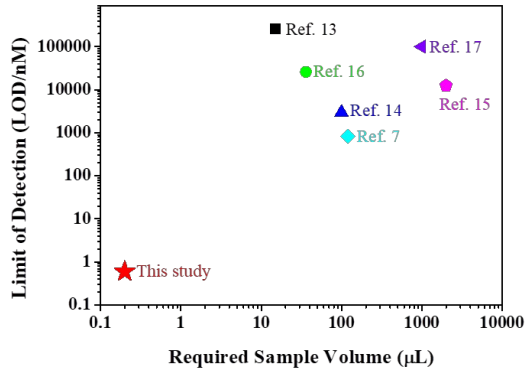

Figure 5. Performance comparison between various methods.

To establish the credibility of this newly developed micro-droplet sensing system, the stability and interferences resistance were tested. As the *S. oneidensis* MR-1 cell had excellent low temperature resistance, it was found that the sensing system could be stored at 4 ºC for 8 days, while the signal output was still maintained over 85% (Fig. S10). Further, this sensing system also showed good reusability. After 30 times reusing, the signal output of the sensing system was also retained over 85% (Fig. S11). The sensing response to other chemicals possible co-existed in the samples such as glucose, citrate, succinate, or maleate was tested respectively. Due to the highly selective interaction between fumarate reductase with the fumarate, other chemicals only resulted in marginal or baseline signal output (Fig. S12). Further, the interference from bacterial contamination was a serious problem for biosensing. Thus, the samples contaminated by *Escherichia coli* or *Pseudomonas aeruginosa* were tested. Impressively, both of the contamination resulted in negligible effect on the biosensing output (Fig. S13). These results indicated the developed sensing system was in good stability and interference resistance, which would be promising for practical application.

Finally, the sensing system was applied to detect the fumarate in different samples. Synthetic water samples with different amount of authentic fumarate were tested. The fumarate concentrations quantified by this developed system were in good agreement with the real concentration spiked (Table S2). The recovery of all samples was between 95% and 108% with the coefficient of variation lower than 8% (n=3). Moreover, synthetic food spoilage samples and mouse kidney samples were prepared, tested with the sensing system and compared with the HPLC analysis. It was found that the sensing results were in good agreement with the HPLC results, which also showed high recovery (92%-105%) and low coefficient of variation (<8.5%, n=3) (Table S3). These low coefficients of variation obtained with biohydrogels fabricated under different batches and/or different samples substantiated good reproducibility of the developed sensing system (Table S1 and Table S2), suggesting this developed system was reliable for real application.

In summary, a microfiber-like graphene-hybridized cell-embedding biohydrogel was fabricated by using bacteria-induced self-assembly under the confinement with a capillary tube. The obtained biohydrogel showed small size, high local cell density and activated DET. The activation of DET by the graphene hydrogel enabled excellent electron exchange efficiency between cells and the electrode. As a results, the biohydrogel exhibited ultra-high sensitivity for fumarate detection with an LOD of 0.60 nM. In addition, needle-like sensing apparatus enabled direct detection with small sample volume of 0.2 μL. In comparison with other miniature biosensors, the simple fabrication process made it promising for application, which would enable sensitive bioelectrochemical detection in micro-droplet or in-situ detection with high spatial resolution.

ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website.
Additional experimental details; Supplementary data (Fig. S1-S13), including biohydrogel characterization and sensing system validation; Analytical performance (Table S1-S3) (PDF).

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Notes

The authors declare no competing financial interest.

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REFERENCES

(1) Huang, Y. X.; Chen, P. Nanoelectronic Biosensing of Dynamic Cellular Activities Based on Nanostructured Materials. Adv. Mater. 2010, 22, 2818-2823.
(2) Wang, X. W.; Dong, X. C.; Wen, Y. Q.; Li, C. M.; Xiong, Q. H.; Chen, P. A graphene-cobalt oxide based needle electrode for non-enzymatic glucose detection in micro-droplets. Chem. Commun. 2012, 48, 6490-6492.
(3) Ammam, M. Electrochemical and electrophoretic deposition of enzymes: Principles, differences and application in miniaturized biosensor and biofuel cell electrodes. Biosens. Bioelectron. 2014, 58, 121-131.
(4) Dahlin, A. B. Size Matters: Problems and Advantages Associated with Highly Miniaturized Sensors. Sensors 2012, 12, 3018-3036.
(5) Song, Y.; Xu, T.; Xiu, J.; Zhang, X. Mini-pillar microarray for individually electrochemical sensing in microdroplets. Biosens. Bioelectron. 2020, 149.
(6) Xu, T.; Xu, L.-P.; Zhang, X.; Wang, S. Bioinspired superwettable micropatterns for biosensing. Chem. Soc. Rev. 2019, 48, 3153-3165.
(7) Si, R. W.; Zhai, D. D.; Liao, Z. H.; Gao, L.; Yong, Y. C. A whole-cell electrochemical biosensing system based on bacterial inward electron flow for fumarate quantification. Biosens. Bioelectron. 2015, 68, 34-40.
(8) Yang, Y.; Yu, Y.-Y.; Shi, Y.-T.; Moradian, J. M.; Yong, Y.-C. In Vivo Two-Way Redox Cycling System for Independent Duplexed Electrochemical Signal Amplification. Anal. Chem. 2019, 91, 4939-4942.
(9) Wang, J.-X.; Yang, X.-J.; Wang, Y.-Z.; Yang, K.; Chen, H.; Yong, Y.-C. Bio-Nanohybrid Cell Based Signal Amplification System for Electrochemical Sensing. Anal. Chem. 2022, 94, 7738-7742.

(10) Yong, Y.-C.; Yu, Y.-Y.; Zhang, X.; Song, H. Highly Active Bidirectional Electron Transfer by a Self-Assembled Electroactive Reduced- Graphene-Oxide-Hybridized Biofilm. Ang. Chem. Int. Ed. 2014, 53, 4480-4483.
(11) Yong, Y.-C.; Cai, Z.; Yu, Y.-Y.; Chen, P.; Jiang, R.; Cao, B.; Sun, J.-Z.; Wang, J.-Y.; Song, H. Increase of riboflavin biosynthesis underlies enhancement of extracellular electron transfer of *Shewanella* in alkaline microbial fuel cells. Bioresource Technol. 2013, 130, 763-768.
(12) Baron, D.; LaBelle, E.; Coursolle, D.; Gralnick, J. A.; Bond, D. R. Electrochemical Measurement of Electron Trans-fer Kinetics by *Shewanella oneidensis* MR-1. Journal of Bio-logical Chemistry 2009, 284, 28865-28873.
(13) Atci, E.; Babauta, J. T.; Ha, P. T.; Beyenal, H. A Fumarate Microbiosensor for Use in Biofilms. J. Biol. Chem. 2017, 164, 3058-3064.
(14) Baati, T.; Horcajada, P.; Gref, R.; Couvreur, P.; Serre, C. Quantification of fumaric acid in liver, spleen and urine by high-performance liquid chromatography coupled to photodiode-array detection. J. Pharm. Biomed. Anal. 2011, 56, 758-762.
(15) Sokullu, E.; Palabiyik, I. M.; Onur, F.; Boyaci, I. H. Chemometric methods for simultaneous quantification of lactic, malic and fumaric acids. Eng. Life Sci. 2010, 10, 297-303.
(16) Roehlen, D. L.; Pilas, J.; Schoening, M. J.; Selmer, T. Development of an Amperometric Biosensor Platform for the Combined Determination of l-Malic, Fumaric, and l-Aspartic Acid. Appl. Biochem. Biotechn. 2017, 183, 566-581.
(17) Ganesh, I.; Ravikumar, S.; Lee, S. H.; Park, S. J.; Hong, S. H. Engineered fumarate sensing *Escherichia coli* based on novel chimeric two-component system. J. Biotechn. 2013, 168, 560-566.

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