An amplified electrochemical strategy using DNA-QDs dendrimer superstructure for the detection of thymine DNA glycosylase activity

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\textbf{A B S T R A C T}

A triple-signal amplification strategy was proposed for highly sensitive and selective detection of thymine DNA glycosylase (TDG) by coupling a dendrimer-like DNA label with the electrochemical method and quantum dots (QDs) tagging. The DNA-QDs dendrimer-like superstructure was designed by DNA hybridization and covalent assembling. Benefiting from outstanding performance of the amplification strategy, this assay showed high sensitivity, extraordinary stability, and easy operation. The limit of detection could reach 0.00003 U μL\textsuperscript{-1} with a splendid specificity. The TDG content in different concentration of HeLa cell was also determined. This assay opens a new horizon for both qualitative and quantitative detection of TDG, holding great promise for potential application in cancer cell research and clinical diagnostics.

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1. Introduction

DNA glycosylases exist in almost all living organisms, from microbe to human (Dodson and Lloyd, 2002), which have two families of monofunctional and bifunctional glycosylases. Thymine DNA glycosylase (TDG) is a monofunctional enzyme, which plays a key role in the cellular defense against mutation (Solange et al., 2012). It excises thymine and uracil from G:T and G:U mismatched oligonucleotide substrates as well as 3’ N4-ethenocytosine from double-stranded DNA (Ulrike et al., 2003). Recent studies showed that it has multiple functions, such as link DNA repair, the control of epigenetic DNA modification, and the regulation of gene expression (Gallinari, Jiricny, 1996; Cortazar et al., 2007). Furthermore, it could also act as either a co-activator or co-repressor for a variety of genes. These findings highlighted the significance using TDG as a tool to promote the basic biomedical research. Thus, it is highly desirable to develop rapid, sensitive, and specific methods to detect the activity of TDG.

To date, only a few approaches were presented for the analysis of TDG activity including gel electrophoresis analysis using radioactive isotopes or fluorescent labels (Li et al., 2007b; Hashimoto et al., 2012), enzymatic coupled fluorescence assay (Fitzgerald and Drohat, 2008) and repair-mediated firefly luciferase expression analysis (Li et al., 2010a,2010b). However, these methods have the limitations of low sensitivity, poor accuracy, and sophisticated operations. In order to circumvent these problems, fluorescence assay was selected to evaluate the activity of TDG (Chen et al., 2013). Nevertheless, the expensive probe limited their application. Thus, it is potentially promising to explore convenient, sensitive and selective approaches to determine the activity of TDG.

Nowadays, electrochemically amplified methods have been proven to be powerful analytical techniques due to moderate cost, instrumental simplicity, and high sensitivity (Kim et al., 1998; Zhang et al., 2013). For acquisitions of high sensitivities and preferable specificity, various electrochemical strategies have been explored including the use of nanoparticles (Liu et al., 2013a,2013b; Wang et al., 2013b), catalyst enzymes (Wang et al., 2013a; Zhu et al., 2011), and conducting polymers (Rowe et al., 2011; Das and Yang, 2009). Among them, quantum dots (QDs) were well recognized as electroactive species for signal amplification due to their unique properties such as versatility in surface modification and high sensitivity. Thus, new schemes based on coupling QDs probe with additional amplification processes are highly desired.

Recently, a wide variety of well-defined DNA superstructures in one, two, and three dimensions have been used as efficient amplifying tags for the development of biosensors due to their unique properties, such as simplicity and predictability (Tang et al., 2012;
2. Experimental

2.1. Chemicals and materials

1-(3-(dimethylamino)-propyl)-3-ethylcarbodiimide hydrochloride (EDC), N-hydroxysulfosuccinimide (NHS), and tris(2-carboxyethyl) phosphine hydrochloride (TCEP) were purchased from Sigma-Aldrich. 3-Mercaptopropionic acid (99%), sodium borohydride (98%), and tris-(hydroxymethyl) aminomethane (Tris) were purchased from Nanjing Chemical Reagents Factory (Nanjing, China). Reagents for polyacrylamide gel electrophoresis and all purchased from Nanjing Chemical Reagents Factory (Nanjing, China). Reagents for polyacrylamide gel electrophoresis and all purchased from Nanjing Chemical Reagents Factory (Nanjing, China). Reagents for polyacrylamide gel electrophoresis and all purchased from Nanjing Chemical Reagents Factory (Nanjing, China). Reagents for polyacrylamide gel electrophoresis and all purchased from Nanjing Chemical Reagents Factory (Nanjing, China). Reagents for polyacrylamide gel electrophoresis and all purchased from Nanjing Chemical Reagents Factory (Nanjing, China).

2.2. Apparatus and characterization

UV–vis absorbance and fluorescence measurements were recorded on UV-3600 spectrophotometer and RF-5301PC (Shimadzu, Kyoto, Japan), respectively. Gel imaging was carried out with a Bio-Rad imaging system. Fourier-transform infrared (FTIR) spectroscopic measurements were performed on a Bruker-microspectrophotometer using KBr pressed disks. Electrochemical measurements were performed on a CHI 660B workstation (Shanghai Chenhua Apparatus Corporation, China) with a conventional three-electrode system composed of a platinum wire as the auxiliary, a saturated calomel electrode as the reference, and a gold (Au) electrode or a glassy carbon electrode (GCE) as the working electrode. Electrochemical impedance spectroscopy (EIS) was performed with an Autolab electrochemical analyzer (Eco Chemie, The Netherlands) in 10 mM K$_2$Fe(CN)$_6$/K$_4$Fe(CN)$_6$ (1.0 M KCl) as the supporting electrolyte at a bias potential of 0.18 V, within the frequency range of 0.01–100 kHz. Melting temperature (Tm) and the secondary structure of DNA were analyzed by OligoAnalyzer 3.1 (free online software from IDT).

2.3. Preparation of DNA1-QDs and DNA2-QDs

CdTe QDs were synthesized according to the literature (Qian et al., 2006; Zhang et al., 2006; Cui et al., 2009). The conjugation procedure for DNA1 and DNA2 to QDs was EDC/NHS method via the formation of amide between the carboxyl groups of QDs and the primary amine groups of DNA1 or DNA2. Briefly, QDs (100 μL), EDC (1 mg), and NHS (1 mg) were mixed in PBS (pH 7.4, 50 mM, 1 mL) and then incubated at room temperature for 30 min with continuous gentle mixing. Then, DNA1 or DNA2 solution (20 μL) was added and incubated for another 2 h under gentle stirring, then stored overnight at 4 °C. This allows the unreacted EDC to hydrolyze to lose its activity. After ultrafiltration with YM-30 ultrafilter, the DNA1-QDs and DNA2-QDs conjugates were redisolved in Tris buffer (pH 7.4, 50 mM).

2.4. Fabrication of the sensor

Before using, the Au electrode (3 mm in diameter) were cleaned with freshly made piranha solution (98% H$_2$SO$_4$:30% H$_2$O$_2$ = 7:3, v/v) for 10 min (CAUTION: piranha solution should be handled with great care) and then polished to a mirror using 1 and 0.05 μm alumina slurry followed by sonication in acetone, ethanol and water for 1 min each. After electrochemical cleaning in 0.5 M H$_2$SO$_4$, the electrode was allowed to dry at room temperature and a droplet of 10 μL MB (1 μM MB-DNA in 10 mM Tris–HCl (1 mM EDTA, 1 mM TCEP, 0.1 M NaCl, pH 7.4)) was dropped onto the electrode for 12 h at 37 °C in 100% humidity. Then the electrode was rinsed with deionized water and passivated with 10 μL MCH (1 mM MCH in 10 mM Tris–HCl, pH 7.4) for 2 h to remove nonspecific DNA adsorption. After rinsed with water, 10 μL TDG solution were dropped onto the above electrode for 2 h incubation at 65 °C to recognize G/T mismatch and excise the T base. Further rinsed with water, 10 μL TDG solution were dropped onto the above electrode for 2 h incubation at 65 °C to recognize G/T mismatch and excise the T base. Further rinsed with water, 10 μL TDG solution were dropped onto the above electrode for 2 h incubation at 65 °C to recognize G/T mismatch and excise the T base. Further rinsed with water, 10 μL TDG solution were dropped onto the above electrode for 2 h incubation at 65 °C to recognize G/T mismatch and excise the T base. Further rinsed with water, 10 μL TDG solution were dropped onto the above electrode for 2 h incubation at 65 °C to recognize G/T mismatch and excise the T base. Further rinsed with water, 10 μL TDG solution were dropped onto the above electrode for 2 h incubation at 65 °C to recognize G/T mismatch and excise the T base.

2.5. Electrochemical detection of TDG

After bound with the reporter DNA-QDs, the electrode was rinsed with Tris–HCl buffer and immersed into HNO$_3$ (200 μL, 0.1 M) solution for 2 h to dissolve the residual CdTe QDs. Then, the concentration of TDG was determined by anodic stripping voltammetry (ASV) method. Briefly, the dissolved solution was added into a glass cell containing 4.8 mL acetate buffer (0.2 M, pH 5.2). The mercury film modified GCE was prepared by deposition at −1.0 V for 40 s and scanned from −0.9 V to −0.2 V in acetate solution.
buffer (0.2 M, pH 5.2) containing 40 μg mL⁻¹ Hg²⁺ under N₂ atmosphere. The anodic stripping voltammetric detection involved pretreatment at +0.6 V for 1 min, and electrodeposition at −1.1 V for 4 min, and stripping from −1.1 to −0.2 V under N₂ atmosphere using a square-wave voltammetry, with 4 mV potential steps, 25 Hz frequency and 25 mV amplitude.

2.6. Cell culture and sample preparation

HeLa cells were cultured in a flask with RPMI 1640 medium (Gibco, Grand Island, NV) supplemented with 10% fetal calf serum, penicillin (100 μg mL⁻¹), and streptomycin (100 μg mL⁻¹) in an incubator (5% CO₂, 37 °C). The density of cells was calculated using a Petroff–Hausser cell counter. The whole-cell extracts were prepared (Supporting Information).

3. Results and discussion

3.1. Principle of TDG detection based on DNA-QDs dendrimer superstructure

Scheme 1 illustrates the principle of the electrochemical biosensor for the detection of thymine DNA glycosylase activity using DNA-QDs dendrimer superstructure as probes. Since TDG is an enzyme in human which can selectively remove T from G/T mismatches through the DNA base excision repair pathway, a hairpin structure DNA (MB-DNA) with 5’ overhangs and one G/T mismatch in the stem part was designed as the DNA probe for TDG recognition. The MB-DNA probe was immobilized on the Au electrode surface through Au–S bond, followed by reacting with the TDG enzyme to recognize the G/T mismatch and excise the T base in the probe. EnIV was subsequently added onto the electrode surface to attack the abasic site in the stem part, producing a nick in the DNA probe as a result of the cleavage of the 5’ phosphodiester bond of the abasic site. Then, a double stranded DNA containing a free 5’ end in the short sequence was formed. In the assay, only these short sequences can supply as substrate for the DNA-QDs to form dendrimer superstructure. The reporter DNA1 functionalized by QDs could hybridize with the short sequence on the electrode surface, then the functionalized reporter DNA2 could further hybridize with the free reporter DNA1 to form nanoscale junctions. The DNA-QDs dendrimer superstructure could be formed after repetitive hybridizations of DNA1 and DNA2 to give an enhanced electrochemical signal. The amount of Cd²⁺ released by dissolving QDs was quantified by ASV method.

3.2. Characterization of the DNA1-QDs and DNA2-QDs

DNA1-QDs and DNA2-QDs probes played an important part in signal amplification by the formation of DNA-QDs dendrimer superstructure. Therefore, the fabrication of DNA-QDs is a critical step. The as-prepared CdTe QDs showed a broad absorption shoulder below 400 nm and a strong fluorescence emission at 548 nm (λex = 350 nm) as shown in Fig. 1A. According to the empirical equation (Yu et al., 2013):

\[
D = \frac{9.8127 \times 10^{-7}}{\lambda^3} + \frac{1.7147 \times 10^{-3}}{\lambda^2} + 1.0064 \lambda + 194.84,
\]

where \(D\) (nm) and \(l\) (nm) represent the diameter of QDs and the wavelength of the UV–vis absorption spectrum respectively, the average size of QDs is 2.7 nm. The DNA1 and DNA2 sequence were connected to the CdTe QDs by EDC/NHS reaction. In Fig. 1B, the fluorescence spectra of DNA1-QDs and DNA2-QDs are similar to that of pure QDs with Gaussian symmetry, suggesting the well stability of covalent conjugation between QDs and DNA. Slight red shifts of the maximum emission wavelength from 548 to 556 nm for DNA1-QDs, and from 548 to 554 nm for DNA2-QDs were observed after DNA functionalization. The red shifts can be ascribed to the QDs size increase after DNA functionalization (Li et al., 2010a, 2010b), while the different red shifts between two DNA-QDs structures were attributed to the different molecular weight of two DNA sequences. The obvious absorption peaks at 260 nm are characteristics of the DNA strands which come from the base of DNA, indicating the binding between DNA and QDs (Fig. 1C). Furthermore, FTIR was also used to verify the conjugation of DNA and QDs. In Fig. 1D, the peaks at 1640 and 3410 cm⁻¹ correspond to the stretching vibrations of C=O and –OH, respectively, which

![Scheme 1](image.png)

Scheme 1. The strategy for the electrochemical detection of thymine DNA glycosylase activity by DNA–QDs dendrimer superstructure.
demonstrates the carboxylic group capping on QDs. Compared with QDs, the characteristic absorption peaks of DNA2 at 1650, 1080, and 980 cm$^{-1}$ are preserved after the formation of DNA2-QDs conjugates, which further prove the successful connection. The FTIR spectra of DNA1 and DNA1-QDs are similar to the above mentioned results, showing the formation of DNA1-QDs.

3.3. Electrochemical characterization of the modified electrodes

EIS reflects the interface feature of the electrode surface, which can be used as an effective tool to study the stepwise sensor assembly (Li et al., 2009; Chen et al., 2008). The EIS spectrum includes a semicircle portion and a linear portion. The semicircle portion at higher frequencies corresponds to the electron-transfer-limited process, and the linear portion at lower frequencies represents the diffusion-limited process. The semicircle diameter corresponds to the electron transfer resistance ($R_{et}$), which controls the electron-transfer kinetics of the redox probe at the electrode interface. Fig. 2A illustrates the Nyquist plots for different modified electrodes in the presence of redox probe, Fe(CN)$_6^{3-}$/Fe(CN)$_6^{4-}$. Compared with bare Au electrode (curve a), the MB-DNA modified electrode shows a larger electron-transfer resistance (curve b), which is due to the electrostatic repulsion between negative charges of the DNA oligonucleotides backbone and the electroactive probe Fe(CN)$_6^{3-}$/Fe(CN)$_6^{4-}$. After immersing the modified electrode into the MCH solution (curve c), the resistance continually increased, which suggested that MCH was immobilized on the surface of electrode. Subsequently, after the modified electrode was treated by TDG (curve d) and EnIV (curve e), the $R_{et}$ decreased in turn, which was due to the excision of the T base in the probe by TDG and the formation of a nick in the probe by EnIV. Additionally, the access of the redox probe to the
electrode was further hindered after incubation with reporter DNA1-QDs probes because of the resistance of DNA (curve f). These results indicated the successful fabrication of the proposed biosensor.

Cyclic voltammogram (CV) of ferricyanide is an effective tool to monitor the barrier of the modified electrode and investigate the changes to the electrode behavior. Fig. 2B shows the CVs of Fe(CN)₆³⁻/Fe(CN)₆⁴⁻ on the modified electrode at different stages. As can be observed, stepwise modifications of the Au electrode were accompanied by changes in the amperometric response, as well as separation between the cathodic and anodic peak of the redox probe [Fe(CN)₆]³⁻/⁴⁻. On bare Au electrode, a pair of quasi-reversible redox peaks of [Fe(CN)₆]³⁻/⁴⁻ with a peak separation (ΔEp) of 71 mV were observed (curve a), showing good electron-transfer for Fe(CN)₆³⁻/⁴⁻. The amperometric response decreased and ΔEp was enlarged after the MB-DNA probes were immobilized on the Au electrode (curve b). The CV response slightly increased after MCH (curve c), TDG (curve d) and EnIV (curve e) treatment, successively. The CV response further decreased after the formation of the Watson–Crick helix between the MB-DNA and reporter DNA1-QDs probes (curve f). The results are in agreement with EIS results as confirmation of the modification for each step.

3.4. Mechanism

In order to confirm the mechanism, we studied the gel electrophoresis results of the obtained products. As shown in Fig. 3A, two bands were obtained for the MB-DNA probe (lane 1), which corresponded to congeries of MB-DNA and itself. The formation of the congeries was attributed to the disulfide bond. After the MB-DNA probe reacted with TDG, the band appeared at a shorter distance which was due to the change in the DNA structure other than the base excise process (lane 2). Therefore, the double-stranded hybridization structure was formed because of the lyase activity from TDG. Subsequently, no further shift was observed after the addition of EnIV (lane 3).

3.5. Analytical performance of the electrochemical biosensor

Compared with other fluorescence or EIS protocols used in biosensors, the ASV technique has the advantage to increase the sensitivity and selectivity (Liao and Ho, 2009). After QDs which is exists in DNA-QDs dendrimer superstructure was dissolved with HNO₃, the dissolved cadmnic component was detected using ASV. Fig. 3B shows ASV curves of the modified electrodes. In the absence of TDG, only a low electrochemical response was observed (curve a) due to the nonspecific adsorption of QDs. After being treated with TDG and captured by DNA-QDs dendrimer superstructure probe, the QDs were bound to the electrode, and a distinct peak was observed (curve c). This phenomenon indicated that as-prepared biosensor had excellent electrochemical performance for TDG.

Furthermore, in order to study the enhanced sensitivity of the DNA-QDs dendrimer superstructure, comparative experiments involving DNA1-QDs or DNA-QDs dendrimer superstructure as signal probes were performed. Compared to DNA-QDs dendrimer superstructure probe, the current signal with DNA1-QDs showed a 3.1 times lower response (curve b). The results showed that the enhanced sensitivity came from the signal amplification of the DNA-QDs dendrimer superstructure probe.

3.6. Optimization of experimental conditions

To obtain the best analytical performance, some important parameters were optimized. Among them, the layer number of DNA-QDs is the most important factor for the improvement of electrochemical signal. Thus, the relationship between the electrochemical signals and the layers of DNA-QDs reporter were investigated. As shown in Fig. S1A, the peak current increased gradually while increasing the layers and started to level off after 4 layers. The DNA-QDs dendrimer probes will fall off due to the volume effect after adding more layers. Thus, the four layer modification of DNA-QDs dendrimer was chosen. For enzyme-mediated reactions, temperature is another important factor. Fig. S1B showed the relationship between the temperature for TDG reaction and peak current. The highest current was achieved at 65 °C, which was selected as the optimal temperature. The fixed quantity of MB-DNA on the Au electrode is also a key factor. Thus, the dependence of the MB-DNA concentration and fixed time on the ASV peak current was studied. In Fig. S1C, a significant increase of ASV peak current was observed between 50 and 500 nM, while no obvious difference was obtained for higher concentration. For convenience, 500 nM of MB-DNA was used for electrode modifications. Moreover, with the increase of fixed time for MB-DNA, the ASV peak current sharply increased and reached a steady value

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Fig. 3. (A) The gel electrophoresis results of the obtained products (lane M: DNA marker, lane 1: MB-DNA probe, lane 2: MB-DNA probe after treated with TDG, lane 3: MB-DNA probe after treated with TDG and EnIV, successively). (B) ASV measurements for the modified electrodes in the absence (a) and presence of TDG (b and c, which correspond to DNA1-QDs probe and DNA-QDs dendrimer superstructure probe, respectively).
Comparison of analytical performance for TDG detection.

<table>
<thead>
<tr>
<th>Method</th>
<th>Linear range (ng mL⁻¹)</th>
<th>Detection limit (ng mL⁻¹)</th>
<th>Specific features</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Fluorimetric</td>
<td>–</td>
<td>0.046–43.4</td>
<td>Convenient, high selectivity</td>
<td>Li et al. (2007a)</td>
</tr>
<tr>
<td>Colorimetric</td>
<td>0–1000</td>
<td>20</td>
<td>Intuitive</td>
<td>Chen et al. (2013)</td>
</tr>
<tr>
<td>Radioactive image</td>
<td>–</td>
<td>0.038–20</td>
<td>Intuitive</td>
<td>Method (2013a)</td>
</tr>
<tr>
<td>Electrochemical</td>
<td>0.023–23</td>
<td>0.0077</td>
<td>High sensitivity and selectivity</td>
<td>This work</td>
</tr>
</tbody>
</table>

Fig. 4. (A) ASV of the biosensor incubated with different concentrations of TDG; (B) the corresponding linear calibration plots. The TDG concentrations from a to f are 0, 0.0001, 0.0002, 0.001, 0.01, and 0.1 μL⁻¹. The illustrated error bars represent the standard deviation of three independent measurements.

after 16 h (Fig. S1D), indicating the attachment of MB-DNA on the electrode. Extending time would not enhance the response, where 16 h was chosen for the incubation of MB-DNA.

3.7. Detection of TDG activity

As demonstrated above, after the modified electrode was treated by TDG, the ASV peak current dramatically increased. The signal was directly related to the activity of TDG. The higher the TDG concentration was, the stronger the ASV signals got. Fig. 4 illustrates the ASV responses of different TDG concentration and the corresponding standard calibration curve for TDG detection. The ASV signal was proportional to the logarithmic value of the TDG concentration ranging from 0.0001 to 0.1 μL⁻¹ with a correlation coefficient R of 0.9962 (n=5). The detection limit was 0.00003 μL⁻¹ (S/N=3). In comparison with other existing approaches (as listed in Table 1), this proposed system exhibited the lowest detection limit and the widest range, which could attribute to the signal amplification of dendrimer superstructure probe with more loading of QDs on the electrode.

3.8. Specificity, reproducibility and stability of the biosensor

Specificity is an important criterion in analyzing biological samples (Wang et al., 2004). Some other base-specific glycosylase hOGG1 and UDG as well as several proteins such as IgG, CEA, BSA were used as the possible interferences to evaluate the specificity of the proposed biosensor. The concentration of TDG, UDG, hOGG1 and IgG, CEA, BSA used in this experiment was 0.1 μL⁻¹ and 20.0 ng μL⁻¹, respectively. No remarkable signals were observed in comparison with TDG, indicating no response to the DNA probe. Furthermore, the perfectly matched G/C pair DNA sequence was used as a control to disclose the specificity of the proposed biosensor, and no remarkable signal was observed, either. These results indicated that the proposed biosensor has good selectivity for TDG, and further detection of TDG in complicated samples is possible.

The reproducibility was evaluated by the relative standard derivations (RSD) of intra- and inter-assays, which was done by measuring same TDG sample with three duplicate measurements made on same or different electrode. The RSDs of intra- and inter-assay for 0.017 μL⁻¹ TDG were 5.9% and 7.2%, respectively, indicating that the precision and the reproducibility of the biosensor were acceptable. Moreover, the stability was assessed. After the biosensor was stored at 4 °C for 20 days, no significant change was observed for the same TDG concentration, indicating that the biosensor has good stability.

3.9. Measurement of TDG in Cancer cells

TDG from HeLa cells extract was determined using the proposed biosensor. As shown in Fig. S2, a dramatic current increase was obtained with cells extract while a low background was observed with lysis buffer. It means that the TDG was present in HeLa cell, which is consistent with the previously reported results (Chen et al., 2013). The successful responses for HeLa cell extracts illustrated a good prospect of the current method for the detection of TDG in clinical applications and extensions.

4. Conclusion

In conclusion, a novel DNA-QDs dendrimer-based triple-signal amplification strategy was developed to design electrochemical TDG biosensor. The high sensitivity of anodic stripping voltammetric method combined with the remarkably amplified effect of the DNA-QDs dendrimer superstructure enables ultra-high sensitivity of this biosensor. Moreover, this method exhibited a broad dynamic range, excellent specificity, and low matrix effect. The assay can be used for the determination of TDG in HeLa cell as well. Consequently, this assay has the potential to provide a sensitive, selective, convenient, and cost-effective approach for the accurate detection of TDG and relative research.

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### Appendix A. Supplementary Information

Supplementary data associated with this article can be found in the online version at [http://dx.doi.org/10.1016/j.bios.2015.04.048](http://dx.doi.org/10.1016/j.bios.2015.04.048).

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