Three-Enzyme Cascade Bioreactor for Rapid Digestion of Genomic DNA into Single Nucleosides

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ABSTRACT: Structure-based DNA modification analysis provides accurate and important information on genomic DNA changes from epigenetic modifications to various DNA lesions. However, genomic DNA strands are often required to be efficiently digested into single nucleosides. It is an arduous task because of the involvement of multiple enzymes with different catalytic activities. Here we constructed a three-enzyme cascade capillary monolithic bioreactor that consists of immobilized deoxyribonuclease I (DNase I), snake venom phosphodiesterase (SVP), and alkaline phosphatase (ALPase). By the use of this cascade capillary bioreactor, genomic DNA can be efficiently digested into single nucleosides with an increasing rate of ∼20 folds. The improvement is mainly attributed to dramatically increase enzymatic capacity and activity. With a designed macro-porous structure, genomic DNA of ∼5–30 Kb (∼1.6–10 million Daltons) can be directly passed through the bioreactor simply by hand pushing or a low-pressure microinjection pump. By coupling with liquid chromatography-tandem mass spectrometry (LC-MS/MS), we further developed a sensitive assay for detection of an oxidative stress biomarker 8-oxo-7,8-dihydro-2′-deoxyguanosine (8-oxodG) in DNA. The proposed three-enzyme cascade bioreactor is also potentially applicable for fast identification and quantitative detection of other lesions and modifications in genomic DNA.

Exposure to chemical carcinogens may result in DNA damages, a key step toward the onset of cancer.1–4 DNA damages can occur via structural modification of the nucleosides and phosphate moieties in DNA chains.5 It is difficult to directly search for these modifications in genomic DNA since DNA strands are long biopolymers consisting of a large number of normal nucleosides (deoxyadenosine, dA; deoxyguanosine, dG; deoxyctydine, dC; and thymidine monophosphates, dT) interspersing with a small quantity of modified nucleosides (e.g., 5-methylcytosine, 5mC) or trace modifications (e.g., 5-hydroxymethylcytosine, 5-formalcytosine, and 5-carboxylcytosine). To determine these DNA modifications, genomic DNA samples were often required to be efficiently digested into mononucleotides or single nucleosides.5–10 Liquid chromatography-mass spectrometry (LC-MS) can explore whether nucleotide is modified, and is sensitive enough to quantify these modifications on DNA chains.9,11–15 For example, during LC-MS/MS analysis of global DNA methylation and hydroxymethylation, genomic DNA was digested into the mixture of classical DNA nucleosides (dA, dT, dG, dC) and modified nucleosides.16–18 Recently, we discovered a new DNA modification in drosophila, N6-methyladenine.19 To accurately identify its structure and validate its identity, the Drosophila genomic DNA was also required to be digested into single nucleosides for high sensitivity analysis. Another example, identification and analysis of oxidative damaged DNA (e.g., 8-oxo-7,8-dihydro-2′-deoxyguanosine, 8-oxodG) completely digestion of genomic DNA to maximal release 8-oxodG was necessary for accurate 8-oxodG quantification.20–25 Analysis of nucleosides can provide increased sensitivity, accuracy and precision rather than detection of DNA polynucleotides or mononucleotides.

Of note, DNA digestion is very different from proteomic digestion, in which the proteins can be quickly digested into small peptides and amino acids by one enzyme (e.g., the potent enzyme trypsin), which can then be directly analyzed by LC-MS.26–32 Compared with the most commonly studied proteins, genomic DNA strands are much larger supramacromolecules, which possess molecular weight generally more than 10 million Daltons. To characterize chemical structural change of nucleotides in DNA or to quantify the stucturally changed nucleoties, long DNA chains are often required to be digested into single nucleosides facilitating followed MS analysis. Nevertheless, the digestion of genomic DNA into single nucleosides was required at least three enzymes, involving deoxyribonuclease (e.g., DNase P1 or DNase I), phosphodiesterase (e.g., snake venom phosphodiesterase, SVP), and alkaline phosphatase (ALPase).10,16–19,33–37 DNase I are applied to...
cleave DNA into small oligonucleotides and mononucleotides, phosphodiesterases attack the 3′- or 5′-terminal OH-groups releasing 5′- and 3′-mononucleotides respectively and ALPase are responsible for removing phosphate from the mononucleotides. However, this most commonly used approach has encountered some critical problems. Regarding the involvement of three enzymes in DNA digestion, the reaction buffer conditions, in particular pH, are hard to control to satisfy all three enzymes. This makes the DNA digestion a time-consuming and tedious process. For instance, incubation time of genomic DNA and three enzymes in solution is common 6–24 h. In terms of 8-oxodG analysis, digestion for long time is likely to induce excessive amount of artificial oxidation of DNA sample, for example, formation of 8-oxodG from dG. On the other hand, since the enzymes and the digested DNA products are in the same solution, the removal of enzymes in samples by denaturation and ultrafiltration was required prior to LC-MS analysis. The cleaved-nucleobases may change (oxidation and isomerization) during this period. To minimize artificial formation of 8-oxodG, several complicated manipulations involving oxygen removal, a lower workup temperature and the elimination of transition metals by nitroxides or deferoxamine mesylate (DFO) have been applied.21,22 The elimination of transition metals by nitroxides or deferoxamine mesylate (DFO) have been applied.21,22

Preparation of the Monolith Column. DNase I was immobilized on monolith column using the glutaraldehyde as a cross-linking agent, as previously reported. A solution containing APPTMS, ethanol, and water (5:10:90, v/v) was injected into the monolith for 4 h and followed by deionized water rinsing for 1 h. Then, 20% glutaraldehyde solution in 25 mM phosphate buffer (pH 6.8) was filled into monolith column for 4 h at 4 °C and washed with phosphate buffer. Ultimately, 5 mg/mL DNase I in 25 mM phosphate buffer (pH 6.8) was filled into the monolith for 4 h. After immobilization of DNase I, the bioreactor was reduced with 2 mg/mL NaCNBH₃. DNase I bioreactor was stored in the 25 mM phosphate buffer containing 0.02% NaN₃ at 4 °C. The SVP and ALPase bioreactor was prepared by using the same method as DNase I bioreactor. Finally, DNase I (20 cm), SVP (15 cm) and ALPase (15 cm) bioreactors were cascaded in order with zero-volume steel unions (Valco, Switzerland).

Enzymatic Activity Assay. DNase I activity was determined by measuring the increase of absorbance response at 260 nm (A_{260}) using ct-DNA as the substrate. A solution of 200 ng/μL ct-DNA in phosphate buffer (pH 5.0, 2 mM MgCl₂ and 2 mM CaCl₂) was injected into the DNase I bioreactor for the substrate hydrolysis of 0.5, 1.0, 1.5, 2.0, 2.5 min. DNA samples were pushed out and the absorbance at 260 nm were measured using a NanoDrop 2000 type UV spectrophotometer. SVP activity was determined by measuring concentration of p-nitropheny1 obtained from the SVP catalyzed hydrolysis of bis(p-nitrophenyl) phosphate (NTP). A solution of 5 mM NTP in 100 mM Tris-HCl buffer (pH 9.8) was injected into the SVP bioreactor for substrate hydrolysis of 0.5, 1.0, 2.0, 3.0, 4.0 min. Then the solution was pushed out, and absorbance was measured at 400 nm using a Nanodrop 2000. ALPase activity was determined by measuring concentration of p-nitrophenyl phosphatase obtained from the ALPase catalyzed hydrolysis of p-nitrophenyl phosphate. The method was similar to SVP bioreactor.
Genomic DNA Enzymatic Hydrolysis. The DNA samples were dissolved in 20 mM Tris-HCl buffer (pH 7.6) containing 2 mM CaCl₂ and 2 mM MgCl₂, then injected into the bioreactor by a low-pressure microinjection pump, with injection volume of 12 μL. Then the reaction products were eluted from bioreactor by 20 mM Tris-HCl buffer (pH 7.6) at flow rate of 1.3 μL/min until 60 μL solutions were collected. Finally, 10 μL of the digested DNA samples were directly subjected to HPLC-MS/MS for analysis of 8-oxo-dG.

As a contrast, DNA samples were digested in solution at 37 °C for 12 h. 1 U DNase I, 1 U ALPase, 0.5 U SVP, and 1 mM DFO were added into 20 mM Tris-HCl buffer (pH 7.6) containing 2 mM CaCl₂ and 2 mM MgCl₂. The digestion solutions were then passed through an ultrafiltration tube (MW cut off 3 kDa, Millipore) by centrifugation at 12000 × g for 20 min.

Cascade Bioreactor Coupled HPLC-MS/MS Analysis of 8-oxo-dG. HPLC-MS/MS analysis was performed on Agilent 1290 UHPLC system coupled with a G6410B triple quadrupole mass spectrometer (Agilent Technologies, Palo Alto, CA). A reversed-phase Zorbax Eclipse Plus C18 column (2.1 mm × 100 mm, 1.8 μm, Agilent) was used for isocratic elution at a flow rate of 0.3 mL/min. The mass spectrometer was operated in the positive ion mode. A multiple reaction monitoring (MRM) mode was adopted for selective detection of 8-oxo-dG: m/z 284.1 → 168.0 (collision energy, 5 eV) and [15N₅]-8-oxodG 289.1 → 173.0 (5 eV); dG: m/z 268.1 → 152.0 (5 eV) and [15N₅]-dG 273.1 → 157.0 (5 eV). Nitrogen was used for nebulization and desolvation gas. The desolvation gas was heated to 300 °C and delivered at a flow-rate of 9.0 L/min. The capillary voltage was set at 3500 V. Each sample was at least analyzed for three times with an injection volume of 10 μL. The oxidative damages frequency was calibrated internally by the stable isotopic standards.

Calibration Curves, Accuracy, and Precision. A calibration curve is built by fitting the peak area ratio of γ*8-oxo-dG/dG (γ = [15N₅]-dG/[15N₅]-8-oxo-dG) and concentration ratio of 8-oxo-dG/10⁶ dG. The ct-DNA containing high concentration level of 8-oxo-dG was prepared through Fenton reaction and then diluted by pristine ct-DNA to a series of concentrations of 8-oxo-dG (used as standard 8-oxo-dG solutions). The standard samples with different concentration of 8-oxo-dG (ranged from 32.9 to 1358.7 lesions per 10⁶ dG) were subjected to HPLC-MS/MS for analysis of 8-oxo-dG. The calibration curve was obtained by fitting the peak area ratio of γ*8-oxo-dG/dG (where γ = peak area of [15N₅]-dG/peak area of [15N₅]-8-oxo-dG) and the concentration of 8-oxo-dG/10⁶ dG. The levels of 8-oxo-dG in DNA samples were expressed as the frequency of 8-oxo-dG/10⁶ dG.

The accuracy represented by recovery of 8-oxo-dG was estimated by spiking 8-oxo-dG standard at three different concentration levels (5, 20, and 50 nM) into DNA samples before digestion. Recovery (%) = [(the measured - the background)/the added amount] × 100%. The intraday and interday precision was estimated by triplicate quantification of 8-oxo-dG and dG in DNA samples per day for three consecutive days.

Gel Electrophoresis. 0.8% Agarose gels were used to investigate the pattern and sizes of DNA fragments generated by enzyme digestion for DNase I-bioreactor, and 16% PAGE gels for the three-enzyme cascade bioreactor. The electrophoresis was conducted at 1 × TBE buffer containing Tris (10.8 g/L), boric acid (5.5 g/L), and EDTA (0.75 g/L) at 200 V for 1 h. Then the gels were stained with ethidium bromide solution for 10 min. The fluorescent bands induced by UV light, was recorded on Gel Documentation System.

MALDI-TOF Mass Spectrometry Analysis. MALDI-TOF MS analysis was performed on Bruker Autoflex III smart beam mass spectrometer (Bruker Daltonics, Bremen, Germany). At the mass range of m/z 100–10000, 90%–100% laser energy, 2 × 10⁻⁸ mbar vacuum, 200 Hz trigger frequency, linear positive-ion mode and 200 s extraction delay time was set to get best mass resolution and high sensitivity, and average of 0–200 laser shots was accumulated for each spectrum. Nicotinic acid (NA), Anthranilic acid (AA) and diammonium hydrogen citrate (DHC) were chosen as matrixes, and molar ratio was 2:1:0.006 (NA/AA/DHC). The DNA digestion solution was mixed with matrix solution (1:1, v/v) sufficiently and then deposited onto MALDI target plate for airing and crystallization.

RESULTS AND DISCUSSION

Fabrication and Characterization of DNase I, SVP, and ALPase Bioreactors. The DNase I, SVP, and ALPase-immobilized bioreactors were fabricated in capillary silica monolith according to our previous work. In brief, three major steps were included (Scheme 1). First, the silica monolith was in situ synthesized in a fused-silica capillary (i.d. 75 μm) using the sol–gel polymerization of TMOS. Then the silylation reagent (3-aminopropyltriethoxysilane) was used to introduce amino groups to the surface of the capillary monolith by APTMS silylation, and (iii) immobilization of enzymes (DNase I, SVP, ALPase) via Schiff-Base reaction using glutaraldehyde to link the amino groups between the modified monolith and enzymes, finally reduction by sodium cyanoborohydride.

Scheme 1. Synthesis of Silica Monoliths and Immobilization of Enzymes in Capillary Monoliths

- (i) Preparation of silica monolith in capillary by sol–gel polymerization of TMOS; (ii) introduction of amino groups to the surface of the capillary monolith by APTMS silylation, and (iii) immobilization of enzymes (DNase I, SVP, ALPase) via Schiff-Base reaction using glutaraldehyde to link the amino groups between the modified monolith and enzymes, finally reduction by sodium cyanoborohydride.
Tris-HCl bu SYPRO orange dye (1:5000) for 15 min and then rinsed with 20 mM ALPase-immobilized bioreactors. The capillaries were stained with the DNase I-immobilized, (d) the SVP-immobilized, and (e) the ALPase-immobilized bioreactors. The capillaries were stained with SYPRO orange dye (1:5000) for 15 min and then rinsed with 20 mM Tris-HCl buffer for 10 min to remove the unbound dyes prior to imaging.

**Figure 1.** Characterization of DNase I, SVP, and ALPase-immobilized bioreactors. (a) Scanning electron microscopic image of the porous silica monolith within the capillary bioreactors. Bright field (the upper rows) and fluorescence images of (b) the capillary silica monolith, (c) the DNase I-immobilized, (d) the SVP-immobilized, and (e) the ALPase-immobilized bioreactors. The capillaries were stained with SYPRO orange dye (1:5000) for 15 min and then rinsed with 20 mM Tris-HCl buffer for 10 min to remove the unbound dyes prior to imaging.

**Figure 2.** Digestive performance of DNase I-immobilized bioreactor. (a) MALDI-TOF MS analysis of the pristine ct-DNA, (b) MALDI-TOF MS analysis of the products ct-DNA digested by DNase I bioreactor (15 min), and (c) the agarose gel electrophoresis (AGE) image of ct-DNA (lane 1) and its digested products adopted at 15 (lane 2), 30 (lane 3), and 45 min (lane 4).

To verify whether enzymes had immobilized on the monolith, SYPRO orange dye was used to stain the enzymes and detected by fluorescence inverted microscope with blue light (450–490 nm). As can be seen in Figure 1a, DNase I, SVP, and ALPase immobilized monoliths show obvious fluorescence compared with monolith without enzyme immobilization, and enzymes were evenly distributed in the monoliths.

**Construction of the Cascade Capillary Bioreactor.** The cascade bioreactors were assembled by tandem connecting the enzyme-immobilized capillary microreactors in an order of DNase I–SVP–ALPase with zero-volume steel unions. Being different from solution digestion that three hydrolytic enzymes were mixed together, the connection sequence of DNase I, SVP, and ALPase bioreactors has influences on the digestion performance. Removal of SVP bioreactor or put it in front of DNase I bioreactor will deteriorate the digestion efficiency to 59.8–83.7%. For instance, ranging the bioreactors in an order of SVP–DNase I–ALPase led to a decreased enzymatic efficiency of 83.7 ± 1.0%. This is probably because that both of DNase I and SVP are capable of generating mononucleotides and oligonucleotides, but DNase I tends to digest double-strands DNA to generate small DNA fragments (2–10 nucleotides) whereas SVP bioreactor seems more efficient on enzymatic hydrolysis of oligonucleotides to release mononucleotides. Thus, the connection sequence of DNase I–SVP–ALPase ensures high efficiency for genomic DNA digestion.

**Fast Digestion of Genomic DNA into Single Nucleosides.** To verify the cleavage of ct-DNA by DNase I bioreactor, 500 ng/μL of ct-DNA was pumped into this bioreactor and incubated for 15, 30, and 45 min, then the digestion products were pumped out and resolved by 0.8% agarose gel. A diffused band with the size of 5–30 Kb appears in the gel, which refers to the pristine ct-DNA was used as the control. No distinct bands can be seen after the digestion process for 15, 30, and 45 min (Figure 2c). We speculated that ct-DNA has been digested to small DNA fragments (less than 0.1 Kb) due to the highly enzymatic performance of DNase I bioreactor.

To verify the above speculation, we then analyzed the digestion products at 15 min by MALDI-TOF mass spectrometry to identify DNA fragments. As shown in Figure 2a, there are no signals of small DNA fragments of ct-DNA were found from m/z = 900 to 4000. On the contrary, by application of DNase I bioreactor, mass ladder was generated from m/z = 600 to 4000 with the digestion sample at adopted 15 min (Figure 2b), which suggested that ct-DNA can be digest to mononucleotides (≏90%) and small oligonucleotides (≏10%).

Similarly, the enzymatic performance of SVP bioreactor was tested by using a 20-mer oligodeoxynucleotides probe (5′-CCCATTCAGCCGCTAAT-3′). An aliquot of 500 ng/μL 20-mer oligonucleotides was pumped into the SVP bioreactor and incubated for 5 min. The enzymatic digestion products of 20-mer oligodeoxynucleotides were then examined by MALDI-TOF mass spectrometry (Figure 3). A series of desired mass ladders detected by MALDI-TOF mass spectrometry ensures that the SVP bioreactor can be applied to release mononucleotides from 3′-hydrox-terminated oligonucleotides. This result is well in accordance with obviations of our previous study.

Then the enzymatic efficiency of three-enzyme cascade bioreactor was evaluated. To this purpose, 12 μL of 200 ng/μL ct-DNA was pumped through the cascade bioreactor and digested for 5, 10, and 15 min, respectively, until 60 μL of products were collected. The digestion products were analyzed by 16% PAGE gel for evaluation of enzymatic performance of the cascade bioreactor (Figure 4a). By application of 3-enzyme
Table S1. The immobilized capacity and enzyme activity of DNase I, SVP, and ALPase were investigated and results are listed in Table S1. The immobilized capacity of enzyme was determined based on Bradford assay. The application of three-enzyme cascade bioreactor in genomic DNA digestion provides a higher digestion rate than another 2−6 h digestion reaction. This result indicates that the cascade bioreactor is more efficient than traditional solution digestion processes in respect of DNA digestion.

HPLC-MS/MS analysis provided more sensitive and accurate results. To investigate the digestion efficiency of the cascade bioreactor, the collected digestive products of ct-DNA at incubation time of 15, 30, and 45 min were injected into HPLC-MS/MS for quantitative analysis of nucleosides. The enzymatic digestion efficiency (%) was calculated according to the measured nucleosides being divided by theoretical calculated nucleosides. As can be seen from Figure 3c, the enzymatic digestion efficiency at 15 min ranges from 73.4 to 76.8% for dC, dG, and dT, while it can reach 97.1 ± 2.4% for dC, 99.3 ± 1.6% for dG, and 103.7 ± 0.8% for dT with digestion of 45 min (Figure 4c). dA was not considered in our calculation because it has been converted to dI (>80%) by adenosine deaminase which was an impurity contaminant in ALPase. This finding is in accordance with what has been reported. The above results demonstrate that the cascade bioreactor can be used to digest genomic DNA and release nucleosides effectively at short time less than 1 h. In this experiment, the nucleoside dG was selected as the marker to evaluate the digestion efficiency of enzymes under different digestion conditions. A representative chromatogram of dG by HPLC-MS/MS analysis is shown in Figure 4b.

The digestion reaction also conducted in solution, the routinely used condition in our laboratory. Enzymes including 1 μL of DNase I, 1 μL of SVP, and 0.5 μL of ALPase were added to 50 μL of 200 ng/μL ct-DNA solution at 37 °C. The digestion efficiency reached 93.0 ± 1.6% until 6 h in solution enzymatic system (Figure S1), whereas a higher enzymatic efficiency (99.3 ± 1.6%) could be obtained within 45 min by using the three-enzyme cascade bioreactor. To achieve digestion efficiency near 100%, the solution system needs another 2−6 h digestive reaction. This result indicates that the three-enzyme cascade bioreactor is more efficient than traditional solution digestion process in respect of DNA digestion.

The enzymatic digestion efficiency and digestive rate are probably attributed to the high local concentration of enzymes in the cascade bioreactor and enhanced stability.

Enzymatic Activities of the Cascade Bioreactors. The immobilized capacity and enzyme activity of DNase I, SVP, and ALPase were investigated and results are listed in Table S1. The capacity of enzyme was determined based on Bradford assay. DNase I, SVP, and ALPase bioreactor were incubated with 0.1 M of NaOH for 2 h to release enzyme from the bioreactors. Then, the enzyme solution was pumped out of monolith and mixed with 500 μL of Bradford reagent. After incubation at room temperature for 5 min, the absorbance of 595 nm was
measured by microplate reader. The amount of immobilized enzyme was calculated by standard curve. Result exhibits that the immobilized enzyme is about 1.15 mg/mL for DNase I, 1.27 mg/mL for SVP, and 1.48 mg/mL for ALPase respectively, showing high immobilization capacity (Table 1). Correspondingly, the bound amount of each enzyme was 0.91 µg for DNase I (20 cm), 0.75 µg for SVP (15 cm), and 0.87 µg for ALPase (15 cm) bioreactors.

Table 1. Immobilization Capacity and Enzyme Activity of the Immobilized Enzymes in the Cascade Bioreactor and Free Enzymes in Solution System

| enzyme | immobilization capacity (mg/mL) | enzymatic activity (U/mL) |
|--------|--------------------------------|--------------------------|
| DNase I | 1.15                          | 1117                     |
| PDE    | 1.27                          | 0.022                    |
| ALPase | 1.48                          | 4.210                    |

The activity of immobilized and free enzymes was measured with UV–vis spectrophotometry. More details are described in the Supporting Information.

DNase I activity was determined by monitoring the increasing absorbance at 260 nm (A260) and SVP; ALPase activity were estimated by measuring the absorbance at 400 nm (Supporting Information). The enzymatic activity in the cascade bioreactor is 1117 U/mL for DNase I, 0.023 U/mL for SVP, and 4.20 U/mL for ALPase, whereas it is 130, 0.007, and 0.06 U/mL for DNase I, SVP, and ALPase in solution condition, respectively. In a word, compared to the enzymes in free solution condition, immobilized enzymes in bioreactor enhanced the enzymatic activity by 9-fold for DNase I, 3-fold for SVP, and 70-fold for ALPase, respectively. The high activity together with the high capacity of the immobilized enzymes enables the fast and complete digestion of ct-DNA into nucleosides within 45 min.

Reproducibility and Reusability of the Cascade Bioreactors. The reproducibility of bioreactor was studied by using ct-DNA as the substrate, and dG was detected to characterize the DNA digestion efficiency. Result shows that RSD for enzyme activity by batch-to-batch analysis was 3.9% (n = 3) and run-to-run analysis was 6.6% (n = 3), which proved that the reproducibility of the silica monolith based bioreactor is acceptable for the routine analysis.

The longevity of the bioreactor was investigated by storing it in 10 mM Tris-HCl buffer (pH 7.4, 0.05% BSA, 0.02% NaN₃) at 4 °C. The monolith was not destroyed during the repeated measurement. In addition, there was less than 20% variation of enzyme activity after bioreactor was used for 20 times or stored at 4 °C for 30 days (Figure 5). However, a mixture solution of DNase I, SVP, and ALPase, which were stored at same condition, almost completely lost its activity within 1 day. The result indicated that stability of enzymes was greatly enhanced after immobilized on the silica matrix because the matrix could prevent autolysis of the enzyme effectively. That means the cascade bioreactor possesses a unique superiority in stability and reutilization.

Development of the Cascade Bioreactor Off-line Coupled HPLC-MS/MS Method for 8-oxodG Detection. By advantage of the cascade bioreactor, we further developed an immobilized enzymes-based DNA digestion coupled UHPLC-MS method for fast screening of the oxidative stress marker 8-oxodG in genomic DNA. Oxidative DNA damage has been supposed to be closely associated with the occurrence of cancer and degenerative diseases.51–54 8-Oxo-7,8-dihydro-2′-deoxyguanosine (8-oxodG) as a stable biomarker of oxidative DNA damage products has been extensively detected in human tissues, blood and urine.21–23

In this experiment, multiple reaction monitoring (MRM) mode was adopted for selective detection of 8-oxodG (m/z 284.1 → 168.0) and [15N₁]₃-8-oxodG (289.1 → 173.0). The untreated dG was chosen as internal standard in order to calibrate the deviations of sample preparation and monitored by transition ion of m/z 268.1 → 152.0, and [15N₁]₃-dG 273.1 → 157.0. The linear equation for 8-oxodG was obtained as y = 0.0019x + 0.0848 (R² = 0.998), presenting a good linear correlation of signal response of y=8-oxodG/dG with the concentration 8-oxodG/10⁶ dG. This result revealed that 8-oxodG in genomic DNA could be released effectively by using cascade bioreactor. The linear equation can be used to calculated concentration of 8-oxodG in DNA samples digested in bioreactor.

The limit of detection (LOD, S/N ≥ 3) was 0.3 nM and limit of quantification (LOQ) was 1.0 nM for 8-oxodG. The recovery was measured by spiking DNA samples with three concentration levels (5, 20, and 50 nM) of 8-oxodG. The estimated recovery is about 109.4 ± 4.9% for 5 nM 8-oxodG, 95.4 ± 7.4% for 20 nM 8-oxodG, and 84.5 ± 1.0% for 50 nM 8-oxodG, respectively. The precision of this method was evaluated by performing replicate determination of 8-oxodG in DNA sample treated by Fenton reaction. The intraday precision for 8-oxodG was 2.1% (n = 5) and interday precision was 7.2% (n = 3), respectively.

Screening of 8-oxodG in ct-DNA Induced by Fenton Reaction. We further demonstrated the applicability of the cascade bioreactor to determine DNA damages in genomic DNA using 8-oxodG as an example. Transition metals Fe³⁺ and
Cu’ can react with \( \text{H}_2\text{O}_2 \) via Fenton reaction to generate ROS, which is associated with 8-oxodG formation in DNA.\(^{55,56}\) In this experiment, 200 ng/\( \mu \)L of ct-DNA (120 \( \mu \)L) were reacted with 10 \( \mu \)M Fe\(^{2+}\) and 50 \( \mu \)M \( \text{H}_2\text{O}_2 \) at 37 °C for 2 h. Aliquot of 12 \( \mu \)L of the resultant products (2.4 \( \mu \)g ct-DNA were subjected to the cascade bioreactor for enzymatic digestion and thereafter HPLC-MS detection. As a contrast, these samples were also digested in free solution conditions. Figure 6a shows MRM chromatogram of 8-oxodG in ct-DNA induced by \( \text{H}_2\text{O}_2 \) and Fe(II)/\( \text{H}_2\text{O}_2 \) treatment using HPLC-MS/MS. The analysis of μ\( \text{H}_2\text{O}_2 \) treated ct-DNA, and 230.1 lesions/10\(^6\) dG in Fe(II)/\( \text{H}_2\text{O}_2 \) which is associated with 8-oxodG formation in DNA.\(^{55,56}\) In DNA (20 accurate quantitation than 99.3%) and releasing of 8-oxodG, which is important for bioreactor works well on complete digestion of DNA (no less heat denatures of enzymes, ultra-short digestion time, anoxic condition in bioreactor and free of artificial addition of 8-oxodG. By taking advantages of its good permeability and mechanical strength, DNA samples can be injected into bioreactor easily by a low-pressure microinjection pump without destroying the structure of the cascade bioreactor. The collected product could be directly injected into HPLC-MS/MS without tedious ultrafiltration process. In addition, the remarkable stability of the immobilized enzymes guarantees the reusability and longtime preservation of the cascade bioreactor. By the advantages of the cascade bioreactor, 8-oxodG induced by Fe(II)/\( \text{H}_2\text{O}_2 \) was accurately detected in ct-DNA. Our work demonstrated that the cascade monolithic bioreactor was powerful for HPLC-MS/MS analysis of 8-oxodG and are promising in rapid identification and highly sensitive detection of adducts in genomic DNA samples.

**ASSOCIATED CONTENT**

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acsnano.6b001682.

DNase I activity assay, SVP activity assay, ALPase activity assay, HPLC-MS/MS analysis of the cleavage products, and digestion efficiency of ct-DNA (PDF)

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**Notes**

The authors declare no competing financial interest.

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**REFERENCES**

(1) Farmer, P. B.; Singh, R. Mutat. Res., Rev. Mutat. Res. 2008, 659, 68−76.
