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Journal Name

ARTICLE

Label-free detection of nicotinamide adenine dinucleotide based on ligation-triggered exonuclease III-assisted signal amplification

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

DOI: 10.1039/x0xx00000x

www.rsc.org/

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Effective detection of nicotinamide adenine dinucleotide (NAD^+) is crucial for better understanding its roles in biological process and further validating its function in clinical diagnosis. Herein, we developed a ligation-triggered exonuclease III (Exo III)-assisted signal amplification strategy for label-free and sensitive detection of NAD^+. We ingeniously designed an oligo1-oligo2-cDNA double-strand DNA (dsDNA) probe and a G-quadruplex (G4)-template dsDNA probe. In the presence of NAD^+, oligo1-oligo2-cDNA dsDNA probe containing a ligatable nick was ligated by E. coli DNA ligase and formed a complete trigger DNA (tDNA). Then, Exo III digested the ligated oligo1-oligo2-cDNA dsDNA probe and released the formed tDNA. Successively, tDNA hybridized with the G4-template dsDNA probe and initiated the Exo III-assisted cycling cleavage, ultimately releasing tDNA and G4 DNA. Finally, the released large amounts of G4 DNA interacted with N-methylmesoporphyrin IX (NMM) to form G4-NMM complex, generating a label-free and enhanced fluorescence signal. The proposed assay provided a detection limit as low as 3.0 pM and exhibited high selectivity against NAD+ analogues. Thus, the strategy provided a facile and convenient tool for sensitive quantification of NAD^+ in NAD^+ related biological process study and clinical diagnosis.

1. Introduction

Nicotinamide adenine dinucleotide (NAD^+), an important biological molecule, is found in most eukaryotic and prokaryotic organisms and plays significant roles in cell proliferation,[1] apoptosis,[2] transcriptional regulation,[3] and DNA repair.[4] It also serves as an essential cofactor for some enzymes such as NAD^+-dependent DNA ligases,[4] NAD^+-dependent oxidoreductases,[5] and sirtuins.[6] Moreover, recent studies have found that aberrant changes in NAD^+ level and NAD^+/NADH ratios are associated with diabetes, neurodegenerative diseases, and cancer.[7-9] Therefore, effective detection of NAD^+ is crucial for better understanding its roles in biological process and further validating its function in clinical diagnosis.

Currently, capillary electrophoresis,[10] nuclear magnetic resonance (NMR),[11] high-performance liquid chromatography (HPLC),[12] and electrospray ionization mass spectrometry (ESI-MS)[13] have been developed for NAD^+ detection. However, these methods are usually suffered from poor sensitivity or expensive equipment and complex operation process. Alternatively, enzyme-based cycling strategies exhibit high sensitivity, but they are unable to discriminate NAD^+ from its analogues, which limit their application in complex sample.[14, 15] To develop sensing system for NAD^+ with high sensitivity and selectivity, some fluorescent assays have been developed based on a strategy that ligation reaction catalyzed by E.coli DNA ligase is specific dependence on NAD^+. For example, Wang's group developed a molecular beacon (MB)-based biosensor for the detection of NAD^+.[16] Tan's group developed a ligation-triggered DNAzyme cascade amplification technique combined with MB for NAD^+ detection.[17] While high selectivity has been obtained, the above-mentioned assays depended on double labeled fluorescent probe, which not only face with high background caused by the incomplete quenching from quencher but also suffer from high cost and complicated synthesis process. Therefore, Yu's group developed a label-free fluorescence DNA probe based on ligation reaction with quadruplex formation.[18] Moreover, Yu and Jiang's group developed a label-free colorimetric assay for NAD^+ based on ligase-mediated inhibition of strand displacement amplification.[19] Although these methods have achieve certain advances toward NAD^+ assay, further improvement of the analytical performances, especially sensitivity, is still in demand.

Exonuclease-assisted signal amplification has been widely utilized for constructing high sensitivity biosensing system. Superior to other nuclease-assisted amplification strategies, especially endonuclease, exonuclease-assisted signal amplification is more universal because exonuclease does not need a specific recognition site. For example, exonuclease III (Exo III) is an exonuclease that specifically catalyzes the stepwise hydrolysis of mononucleotides from the blunt or recessed 3'-termini of duplex DNA. In addition to easy availability and excellent cyclic efficiency of

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Electronic Supplementary Information (ESI) available: [DNA sequences and supporting figures]. See DOI: 10.1039/x0xx00000x

Exo III, Exo III-assisted signal amplification strategy exhibits wide versatility and has been applied in the sensitive detection of various bimolecules including nucleic acids,[21] enzyme activity,[22] and metal ions.[23] However, there is no NAD^+ assay based on Exo III-assisted signal amplification strategy.

Herein, we developed a ligation-triggered Exo III-assisted signal amplification strategy for label-free and sensitive detection of NAD^+. We designed an oligo1-oligo2-cDNA double-strand DNA (dsDNA) probe and a G-quadruplex (G4)-template dsDNA probe. Oligo1-oligo2-cDNA dsDNA probe, containing a ligatable nick, could be ligated by E. coli DNA ligase in the presence of NAD^+ and form a complete trigger DNA (tDNA). In addition, G4-template dsDNA probe, containing a G-quadruplex sequence (G4 DNA) and a template, could initiate the amplification process with the tDNA and eventually output signal. Here, we also took full use of Exo III. On one hand, Exo III could specifically hydrolyze ligated oligo1-oligo2-cDNA dsDNA probe and release the formed tDNA. On the other hand, Exo III could catalyze the Exo III-assisted signal amplification process, generating amplified fluorescent signal. Because Exo III has similar reaction conditions with NAD^+-dependence ligation reaction and excellent cyclic efficiency, the proposed NAD^+ assay not only could carried out in one-pot fashion under isothermal condition, but also exhibit high selectivity and sensitivity with a limit detection of 3.0 pM. Thus, the strategy provided a facile and convenient tool for sensitive quantification of NAD^+ in NAD^+ related biological process study and clinical diagnosis.

2. Experimental Section

2.1. Materials and Apparatus

Oligonucleotides were synthesized and purified by Invitrogen Biotechnology Co. Ltd. (Shanghai, China), and their sequences were listed in Table S1. Exo III and E. coli ligase were obtained from Takara Biotechnology Co., Ltd. (Dalian, China). Nicotinamide adenine dinucleotide (NAD^+), reduced form of nicotinamide adenine dinucleotide (NADH), reduced nicotinamideadenine dinucleotide phosphate (NADPH), adenosine triphosphate (ATP), and adenosine monophosphate (AMP) were obtained from Shanghai Sangon Biotechnology Co., Ltd. (Shanghai, China). All other chemicals used in this work were of analytical grade and obtained from standard reagent suppliers. All the solutions were prepared with ultrapure water (> 18.25 MΩ·cm).

2.2. Ligation reaction

The buffer containing 30 mM Tris-HCl (pH=8.0), 4.0 mM $MgCl_2$, 10 mM $(NH_4)_2SO_4$, and 1.2 mM EDTA was used for ligation reaction. To detect NAD^+, a 45 μL of mixture containing 0.3 μM of Oligo1, 0.3 μM of Oligo2, and 0.2 μM cDNA was denatured at 90 °C for 5 min and cooled down to 37 °C gradually. The mixture was incubated at 37 °C for 1.0 h. Then, 18 U of E. coli DNA ligase and varying concentrations of NAD^+ were introduced into the mixture to induce the ligation. The ligation reaction was performed at 37 °C for 1.0 h. Oligo1 DNA and Oligo2 DNA could be ligated to produce a complete trigger DNA.

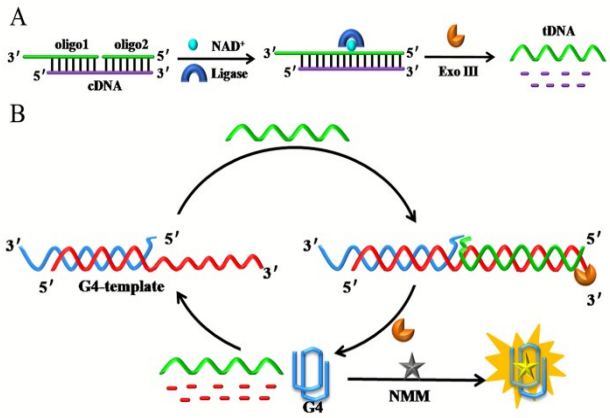
2.3. Exo III-assisted signal amplification process

Before the Exo III-assisted signal amplification process, 5 μL of 10 × Exo III buffer (500 mM of Tris-HCl, 50 mM of $MgCl_2$, 10 mM of DTT, pH = 8.0) and 50 U of Exo III were added to the as-prepared solution to digest the cDNA and release the tDNA. The digestion process was performed at 37 °C for 0.5 h. After that, the pre-prepared G4-template dsDNA probe at a final concentration of 1.0 μM was added to the digestion solution. The reaction mixture was incubated at 37 °C for 1.5 h to perform the signal amplification procedure.

2.4. Fluorescence assay and Gel Electrophoresis.

The signal amplification product was mixed with 5 μL of KCl (100 mM) and 2 μL of NMM (75 μM). After incubated at 37 °C for 20 min, the mixture was added into a 100 μL quartz cuvette. The fluorescence assay was carried out by F-7000 spectrometer (Hitachi, Japan). The spectrometer parameters were set as follows: λex = 399 nm, λem = 610 nm, voltage of PMT detector = 700 V. A 15% polyacrylamide gels analysis was carried out in 1×TBE buffer (89 mM Tris, 89 mM boric acid, 2.0 mM EDTA, pH 8.3). The gels were stained with ethidium bromide, and then photographed under UV imaging system (Bio-RAD Laboratories Inc. USA).

3. Results and Discussion



Scheme 1 Schematic illustration of the Exo III-assisted amplification strategy for the NAD^+ detection.

3.1. Principle of NAD^+ detection based on ligation-triggered Exo III-assisted signal amplification

The design of the sensing system for NAD^+ detection was illustrated in Scheme 1.The oligo1-oligo2-cDNA dsDNA probe with a ligatable nick was obtained by hybridizing oligo1 and oligo2 with cDNA. Here oligo1 contains a phosphorylated 5′ end for ligation reaction and a short single strand overhang at 3′ to inhibit digestion of Exo III. The G4-template dsDNA probe was prepared with a G-quadruplex sequence and a template to aid the amplified sensing system. In the presence of target NAD^+, oligo1 was ligated to oligo2 by E. coli DNA ligase, forming a complete tDNA. Then, the ligated oligo1-oligo2-cDNA dsDNA probe was selectively hydrolyzed by Exo III, releasing formed tDNA. Since the released tDNA was complementary with the region of single strand of the G4-template dsDNA probe, it could hybridize with G4-template dsDNA probe,

generating a new hybrid with a stable blunt 3'-terminus. Next, the new hybrid was again digested from 3' to 5' by Exo III, ultimately releasing more tDNA and G4 DNA. The released tDNA would hybridize with another G4-template dsDNA probe to form another hybrid with 3'-termini, thus triggering the Exo III assisted cycling cleavage process. Due to the cycling cleavage, large amounts of G4 DNA were released and then folded into G-quadruplex structure in the presence of K^+. Finally, NMM, which have a pronounced structural selectivity for G4 structures but not for dsDNA or ssDNA forms,[24] interacted with the G-quadruplex to form G4-NMM complex generating a label-free and enhanced fluorescence signal. However, in the absence of target molecule NAD^+, the DNA ligation could not proceed. Thus, tDNA could not be produced to initiate the Exo III-assisted signal amplification, resulting in weak fluorescence background signal. Because the NAD^+ is the indispensable cofactor for ligation reaction, our method could detect NAD^+ with high selectivity. Furthermore, it is noteworthy that we have taken full use of Exo III, including the cleavage of oligo1-oligo2-cDNA dsDNA probe to release tDNA and the subsequent Exo III-assisted signal amplification, which could make the method convenient and sensitive.

3.2. Feasibility study of the sensing system for NAD^+

To confirm the feasibility of this signal amplification strategy, fluorescent spectra in different conditions were investigated. As shown in Fig. 1A, the system only containing G4-template dsDNA probe showed very weak fluorescence intensity (curve green), suggesting that G4 sequence was well hidden by template sequence, and thus ensured a low background. When oligo1-oligo2-cDNA dsDNA probe was added into the solution containing E. coli DNA ligase, G4-template and Exo III, no appreciate increase of the fluorescence intensity was observed (curve glue), because in the absence of the target NAD^+, the ligation of oligo1-oligo2-cDNA dsDNA probe did not occur, resulting in no free G4 DNA and thus no amplified fluorescence signal. However, with the further addition of target NAD^+, significant fluorescence enhancement was observed (curve red), indicating the occurrence of ligation reaction, as well as the Exo III-assisted signal amplification. Moreover, control experiment which contained all the reagents but Exo III was also conducted. As shown in Fig. 1 (curve pink), a low fluorescence signal was detected, which indicated the release of tDNA and the subsequent signal amplification step could not be conducted without Exo III. Furthermore, gel electrophoresis experiment was also carried out to confirm the feasibility. Fig. 1B showed the electrophoresis analysis results. One obvious band was observed in the middle of lane 1, suggesting that ligation reaction was happened in the presence of NAD^+ and formed a stable duplex structure. Compared to lane 1, the band of ligation product was missing and a new band with faster migration rate appeared. These indicated that ligation product was selectively hydrolyzed by Exo III and tDNA was released. Compared lane 4 to lane 3, a new band representing G4 was observed in lane 4, indicating the occurrence of the Exo III assisted cycling cleavage process to release G4 DNA. The gel results were consistent with the fluorescence experiments and commonly demonstrated the feasibility of the proposed fluorescence sensing system for NAD^+ detection.

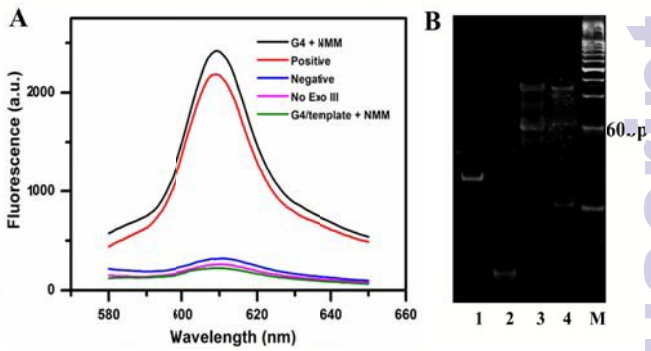


Fig. 1 (A) Fluorescence emission spectra of the system under different conditions. The concentration of NAD^+ is 100 nM. (B) Nondenaturing polyacrylamide gel (15%) electrophoresis analysis with different reactions. Lane 1: oligo1 + oligo2 + cDNA + E. coli DNA ligase + NAD^+; Lane 2: oligo1 + oligo2 + cDNA + E. coli DNA ligase + NAD^+ + Exo III; Lane 3: tDNA + G4/template probe; Lane 4: oligo1 + oligo2 + cDNA + E. coli DNA ligase + NAD^+ + Exo III + G4/template probe.

3.3. Optimization of the reaction conditions

Experimental conditions will have great effects on sensing performance, so the value of F/F_0 was used to optimal the reaction conditions, among which the F and F_0 were the fluorescence intensity of the solution with and without target NAD^+, respectively.

In our sensing system, the length of template DNA sequence played an important role. On one hand, template DNA was hybridized with G4 DNA to hide the G4 sequence and lower the background signal. On the other hand, the template was hybridized with the released tDNA to initiate Exo III-catalyzed recycling process in which the template was digested by Exo III and released G4 DNA to generate fluorescent signal. Therefore, a short length template DNA would lead to more guanine bases exposed, which could partly interact with NMM and increased the background signal. But a long length of template DNA might influence the digestion efficiency of Exo III. As shown in Fig. 2, three template DNAs with different lengths were designed and optimized. With the decreasing of template DNA length, fluorescence response of F and F_0 increased ultimately, and the template (40 nt) gave the best F/F_0 response, so template (40 nt) was chosen for the following experiments.

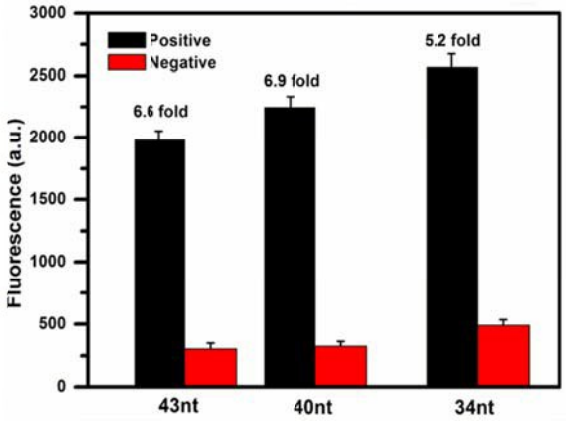


Fig. 2 The influences of three different template sequences on th

intensity of fluorescence signal. Error bars are the standard deviation of three measurements. The concentration of NAD^+ is 10 nM.

It was widely accepted that the concentration of the probe had an important effect on the sensing process. In our study, oligo1, oligo2 and cDNA were used to construct oligo1-oligo2-cDNA dsDNA probe, which had a ligatable nick for ligation reaction. As shown in Fig. S1 and S2, the value of F/F_0 increased to a maximum and then gradually decreased with the increased oligo DNA and cDNA concentration. Here oligo DNA contained oligo1 and oligo2, and the ratio of oligo1 to oligo2 was 1:1. The optimized concentration of oligo1 and oligo2 DNA were 300 nM, and cDNA was 100 nM. Furthermore, G4-template dsDNA probe, which was used in the process of signal amplification and signal output, was optimized and the concentration of 1.0 μM was selected (Fig. S3).

E. coli DNA ligase and Exo III played key roles in the ligation process and signal amplification process. 18 U of E. coli DNA ligase and 50 U of Exo III were chosen for the subsequent experiments based on the optimization experiment results (Fig. S4, Fig. S5). Moreover, the influence of different pH on the sensing system was also investigated, and pH value of 8.0 was selected (Fig. S6). Finally, the concentration of NMM was also investigated. As shown in Fig. S7, the fluorescence response reached its maximum with 2.0 μM and then decreased at higher concentration. Thus, 2.0 μM was chosen as the optimal concentration of NMM.

3.4. Analytical performance of the sensing system for NAD^+

Under the optimal conditions, the sensitivity and dynamic range of the proposed strategy were performed with various NAD^+ concentrations. As shown in Fig. 3A, the fluorescence intensity increased with the NAD^+ concentration increasing from 0 to 100 nM, which demonstrated that the release of G4 DNA sequence from G4-template dsDNA probe assisted by Exo III was highly dependent on the concentration of NAD^+. A good linearity was obtained in 3 orders of magnitude from 5.0 pM to 1.0 nM (Fig. 3B) with a detection of 3.0 pM (3σ/slope), which was lower than that of most reported assay and was also comparable to the target-triggered ligation-RCA.[25] The high sensitivity was attributed to the following reasons. Firstly, the signal amplification effect of the proposed Exo III-assisted recycling strategy was indeed realized. Moreover, the G4-template dsDNA probe was well designed to hide the G4 sequence, which ensured our strategy a low background signal.

A great challenge for the NAD^+ assay was to distinguish it from its analogs, since analogs such as NADH had a very similar structure with NAD^+ and might disturb the NAD^+ assay. In order to evaluate the specificity of this proposed strategy, the fluorescence response of several potential interferes including NADH, NADPH, ATP and AMP was investigated, respectively. As shown in Fig. 4, the NAD^+ analogs exhibited almost the same signal intensity with the negative experiment, while NAD^+ increased the fluorescence signal significantly. Thus, the strategy we proposed here exhibited good performance for discriminating NAD^+ against its analogs, and held great potential in clinical applications. This high selectivity was because of the intrinsically extreme fidelity of E. *coli* DNA ligase toward NAD^+.

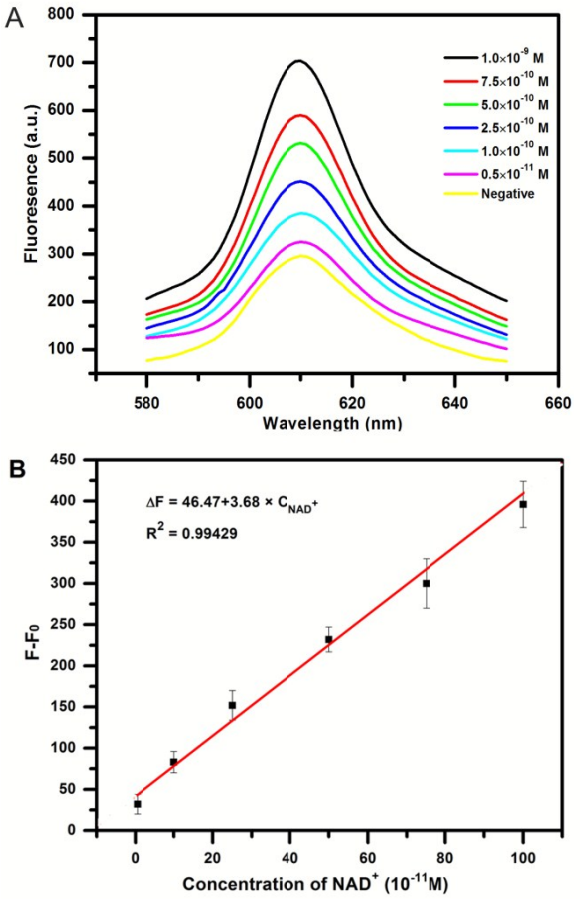


Fig. 3 (A) Fluorescence emission spectra of amplification strategy in various concentration of NAD^+; (B) linear responses between fluorescence intensity and NAD^+ concentration in the range from 5.0 pM to 1.0 nM. Error bars are the standard deviation of three measurements.

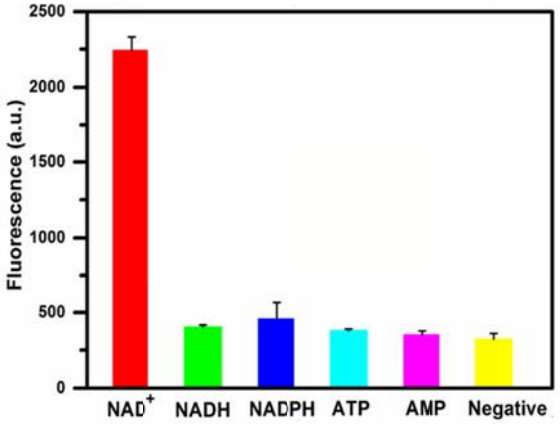


Fig. 4 Selectivity of the Exo III-assisted amplification strategy for NAD^+. Error bars are the standard deviation of three measurements. The concentration of NAD^+ and its analogues are 100 nM.

3.5. Precision and reproducibility

The precision and reproducibility were investigated to ensure the genuineness and reliability of the results in the sensing system. As the experiment results suggest, the relative standard deviation (RSD) obtained from the same batch were 3.9 %, 3.2 % and 3.4 % (

the target concentration of 1.0 nM, 500 pM and 10 pM. Three batches were obtained through measuring the same samples in 3 days at the same experimental conditions, and the RSD were 5.2 %, 4.8 %, and 3.5 %. The results demonstrated that the developed sensing system has satisfactory precision and reproducibility.

3.6. Feasibility in complex biological samples

To verify the applicability in real sample of the proposed sensor, spike and recovery experiments were performed in cell culture fluid. The cell culture fluid was first diluted in 1:5 ratio with reaction buffer and then spiked with target NAD^+ at three different concentrations (1.0 nM, 500 pM and 10 pM). As shown in Fig. 5, the fluorescence intensity obtained from the two different solutions showed little difference, demonstrating that the interference effect of complex matrix was negligible. The concentrations of target in cell culture fluid were determined by using calibration method and the obtained recoveries of three samples were all about 95 %. The analytical results indicated that our method was reliable and had the potential for real sample application.

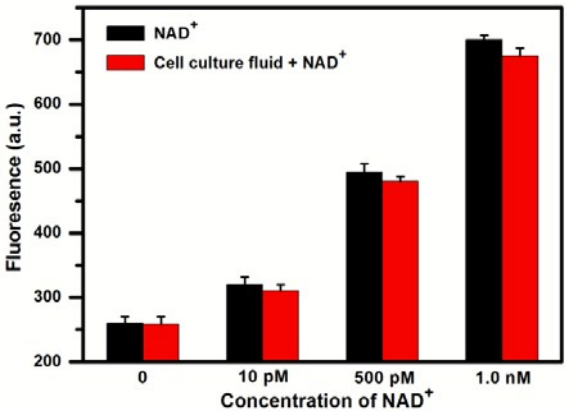


Fig. 5 Feasibility of the approach in complex biological matrices. The cell culture fluid was diluted with buffer in 1:5 ratios. Error bars are the standard deviation of three measurements.

4. Conclusions

In conclusion, we successfully developed a ligation-triggered isothermal Exo III-assisted signal amplification strategy for label-free and sensitive detection of NAD^+. Firstly, because the DNA ligation reaction is specific dependent on the indispensable cofactor NAD^+, the proposed strategy significantly showed high selectivity toward target NAD^+ and its analogues. Furthermore, by taking full use of Exo III that has similar reaction conditions with NAD^+-dependence DNA ligation reaction, the proposed NAD^+ assay not only exhibit high sensitivity with a limit detection of 3.0 pM, but also possess additional advantages of simplicity and convenience. The high selectivity and sensitivity makes it possible to dilute complex real samples, which will have reduced interference of the matrix. Thus, the proposed strategy would be more applicable for complex real samples and further provide a facile and convenient tool for better understanding the roles of NAD^+ in biological process and further validating its function in clinical diagnosis.

Acknowledgements

This project was supported by the National Natural Science Foundation of China (Grant Nos. 21175081, 21175082, 21375078, and 21475077).

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