Ultrasensitive label-free amplified colorimetric detection of p53 based on G-quadruplex MBzymes

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A novel label-free DNAzyme molecular beacon (MBzyme) strategy was for the first time developed for colorimetric amplification detection of target nucleic acids. The MBzyme, which is designed to contain peroxidase-mimicking DNAzyme that is locked by a common hairpin, was engineered to form a catalytically active MBzyme through hybridizing with the target p53 DNA. The MBzyme is a multi-functional label-free probe that can act as the target recognition element, catalytic DNAzyme and the primer of polymerization. The target p53 DNA hybridization can induce the isothermal circular strand-displacement polymerization even without any chemical modification and other DNA sequences. This unique amplifying strategy leads to the generation of multiple numbers of active MBzyme molecules even if one hybridization event occurs, achieving a dynamic range of seven orders of magnitude lower than those of related literature reports. These achievements might be helpful in the design of highly efficient enhancers for G-quadruplex-hemin DNAzymes to be applied on the fundamental research, biotechnology, and biomedical diagnosis.

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1. Introduction

Reliable, convenient, and sensitive analyses of nucleic acid sequences and other biomarkers assay have become important tools in drug screening, environmental and food safety monitoring, pathogen identification, forensic science, and especially in disease diagnosis (Sassolas et al., 2007; Sidransky, 1997; Levin et al., 1999). According to the report by World Health Organization in 2004, the malignant tumor (cancer) became the leading killer of human being. Up to now, the incidence and mortality of cancer is still considerably high in aggregate and even shows ascendant trend in the developing countries. In China, the number of people dying of lung cancer is estimated to be more than 100 million by 2025. Malignant tumor greatly affects our quality of health and lives. Nevertheless, if it is early detected, there is a great chance of cure. For example, when lung cancer is caught early, the 5-year survival rate is more than 90%. The poor prognosis is attributable to lack of efficient diagnostic methods for early stage screening, and most patients (> 75%) present with stage III or IV disease and are rarely curable with current therapies (Hirsch et al., 2001). Thus, early diagnosis and prompt operation are the keys of successful treatment for cancers. Because cancer markers, for example, mutant p53 gene, often exist in extremely small amounts, especially in the earliest stages, exploiting powerful technologies for the highly sensitive and reliable analysis of nucleic acids is always a central issue.

Among the techniques to amplify the signal induced by target recognition event, a newly emerging protocol, called isothermal circular strand-displacement polymerization (ICSDP), is attracting considerable attention in the nucleic acid-based sensing systems for the detection of nucleic acids (Capaldi et al., 2000; Qiu et al., 2011; Zhou et al., 2013) and nonnucleic acid targets (He et al., 2010; Ding et al., 2010; Zhu et al., 2009; Shlyahovsky et al., 2007a, b). For the common ICSDP reaction (Capaldi et al., 2000; Qiu et al., 2011), the target DNA hybridization can open the binding site for the primer. In the presence of dNTP/polymerase, the extension of bound primer can occur over the recognition probe as template, during which the hybridized target DNA is peeled off and induces the next hybridization/polymerization/displacement cycle. Although the ICSDP technique has been proved to execute signal amplification to a considerable extent and multiplexing properties for the development of sensitive biosensing systems, the detection sensitivity at the nanomolar or subnanomolar level seems to be the identical detection threshold for the detection of nucleic acid targets (Zhou et al., 2013) and

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nonnucleic acid targets (Qiu et al., 2011; He et al., 2010). However, even if the nicking enzyme is employed to cleave the double-stranded DNA to generate more products, the detection sensitivity is not obviously improved (Zhu et al., 2009; Shlyahovsky et al., 2007a, b). More efforts are still needed to meet the serious clinical challenge. Additionally, for the public and periodic disease screening, it is equally important to simplify the design of oligonucleotides and to employ cost-effective signal reporters because these improvements can make the sensing system readily available, affordable and amenable for various biomarker detection applications.

Nucleic acid sequences with the potential to form G-quadruplex structures are common in biologically important chromosomal features such as telomeres, gene promoters, and immunoglobulin switch region (Rachwal et al., 2007; Yang and Hurley, 2006; Seenisamy et al., 2004; Siddiqui-Jain et al., 2002; Palumbo et al., 2008), and thus they are often important research focuses (Neidle, 2009; Gatto et al., 2009; Kypr et al., 2009). The discovery that G-quadruplex–hemin complexes possess enzymatic catalysis similar to that of peroxidase is an important step toward the exploration of these structures (Chan and Khachigian, 2009; Rojas et al., 2007). G-quadruplex sequence is one of the fascinating DNAzymes. Compared to protein enzymes, DNAzyme possesses unique advantages such as high thermal stability, low cost, simple synthesis, easy modification, and sequence specific recognition characteristics in a very straightforward fashion (Chan and Khachigian, 2009; Rojas et al., 2007). To date, such peroxidase-like G-quadruplex–hemin DNAzyme has been utilized for the colorimetric, electrochemical, and/or chemiluminescence detection of a number of analytes, including telomerase activity (Pavlov et al., 2004; Xiao et al., 2004a, b), small molecules (Li et al., 2007; Elbaz et al., 2008; Kong et al., 2010), metal ions (Li et al., 2009a, b, c), DNA (Xiao et al., 2004a, b; Willner et al., 2008; Deng et al., 2008; Nakayama and Sintim, 2009), and proteins (Li et al., 2009a, b, c 2008; Shlyahovsky et al., 2007a, b; Li et al., 2009a, b, c; Tang et al., 2012; Huang et al., 2013; Zhou et al., 2012; Yuan et al., 2012). Moreover, the assay capability of G-quadruplex DNAzyme-based sensing systems can be improved by combining with protein enzyme-based amplification strategies, such as nicking enzyme-assisted DNA replication (Weizmann et al., 2006), rolling circle amplification (RCA; Tian et al., 2006; Cheglakov et al., 2007), and polymerase chain reaction (PCR; Cheglakov et al., 2006). The impressive research advances, as well as the extreme stability of G-quadruplex structure (Li and Mirkin, 2005) are the inspiration for the unique design of our convenient powerful promising colorimetric MBzyme probe for cancer gene diagnosis.

In the present work, a novel DNAzyme molecular beacon (MBzyme) was developed for the first time for colorimetric amplification detection of p53 DNA. The G-rich segment (anti-hemin aptamer) responsible for DNAzyme formation is introduced into the p53 gene recognition probe, not only acting as the signal reporter with the help of other reagents (e.g., hemin) but also triggering the strand-displacement amplification reaction without any additional oligonucleotides. Hence, the developed colorimetric sensing system involves only one oligonucleotide probe consisting of recognition region, primer and anti-hemin aptamer, and no any chemical modification is adopted. In the absence of target DNA, the designed probe can self-assemble into a large hairpin structure similar to molecular beacon (MB), while target DNA hybridization is able to force it to fold into the DNAzyme structure responsible for the subsequent strand-displacement amplification (SDA) and signaling reaction. Thus, this probe is called MBzyme. The usefulness of this technique was demonstrated by its application to the 25 fm level detection of target p53 DNA, implying a 3–7 orders of magnitude improvement in detection sensitivity compared to those previously reported, such as classical strand-displacement polymerization reaction (Qu et al., 2011), traditional molecular beacon (Martinez et al., 2009) and DNAzyme molecular beacon (Xiao et al., 2004a, b). The desirable detection capability and other features (e.g., only one oligonucleotide probe involved) indicate an exciting breakthrough in the development of signaling nucleic acid probe. MBzyme plausibly holds a significant potential in the reliable detection of trace target elements.

2. Experimental section

2.1. Materials and reagents

The oligonucleotides were purchased from Takara Biotechnol-ogy Co. Ltd. (Dalian, China) and their sequences are listed in Table S1. The secondary structures of all oligonucleotides, including the thermodynamic parameters, were calculated using bioinformatics software (http://mfold.rna.albany.edu/). DNA stock solution was prepared by dissolving oligonucleotides in 20 mM Tris–HCl buffer solution (pH = 7.4) containing 200 mM NaCl and 1 mM MgCl2 and was stored at 4 °C before use. SYBR Green I, H2O2, 2,2′-azino-bis(3-ethylbenzothiozoline)-6-sulfonic acid (ABTS), Tris(hydroxymethyl) methyl aminomethane, Triton X-100, hydrochloric acid, hemin, and the metal salts (NaCl and MgCl2) were obtained from Sigma. Klenow Fragment exonuclease (KF) was purchased from New England Biolabs (UK) Ltd. All chemical reagents were of analytical reagent grade and used without further purification. All solutions were prepared with ultrapure water, which was obtained through a Millipore Milli-Q water purification system (Billericia, MA) and had an electrical resistance of > 18.3 MΩ.

2.2. Instrumentations

UV-vis absorption measurements were carried out at room temperature using a UV 2450 spectrophotometer (Shimadzu, Japan) with HYPER UV Version 1.50 software, and the absorbance spectra were collected from 400 to 500 nm. The absorbance intensity at 420 nm was used to evaluate the performances of the proposed assay strategy. Fluorescence spectra were determined using a Hitachi F-7000 fluorescence spectrometer (Hitachi Ltd., Japan) controlled by FL Solution software. Excitation and emission slits were all set for a 5.0 nm band-pass. The mixtures in square quartz cuvettes were excited at 494 nm, and the emission spectra were collected from 510 to 620 nm. A pH-3C digital pH meter (Shanghai Weije Instrument Plant, Shanghai, China) was used to measure the pH values of the solutions. Photos were taken using a Canon IXUS 245HS.

2.3. Trigger-displacement polymerization and p53 amplification detection

The target DNA determination could be briefly described as follows: at first, the MBzyme (2 μL, 10 μM) was added to the mixture of p53 sample (5 μL) at specific concentration, 8 μL of water and 10 × Klenow Fragment exonuclease (2 μL; KF buffer); after 2 h, 1 μL of 10 mM dNTPs and 1 μL of 5 U/μL Klenow Fragment exonuclease were successively injected into the resulting solution and incubated at 37 °C for 40 min. Subsequently, the polymerization reaction was stopped by keeping the resulting solution at 80 °C for 10 min. Then, 1 μL of 50 μM hemin was added and incubated at 37 °C for 1 h. To obtain the information on the amount of target DNA in the sample, 72.5 μL of 2 × HEPES (50 mM HEPES, 0.4 M NaCl, 20 mM KCl, 0.1% Triton X-100, 2% DMSO, pH = 5.2), 2.5 μL of 4 mM H2O2 and 5 μL of 0.04 M ABTS were added to the obtained solution and the absorbance spectrum was collected. The difference in the UV–vis peak between the target sample and blank was used to quantify target molecules in sample.
3. Results and discussion

3.1. MBzyme design and target-triggering signal amplification detection

Due to the fascinating advantages, for example, homogenous assay, isothermal amplification, design flexibility and convenient manipulation, ICSDP technique should play an important role in the sensitive detection of biomarkers and other trace species of interest. To improve the assay characteristics, some research efforts have been made to explore new signaling strategies that promote ICSDP development, for instance, the combination of nicking enzyme with three-way junction (Zhou et al., 2013) or conformational transition between hairpin structures (Shlyahovsky et al., 2007a, b). However, no substantial progress in the assay sensitivity is accomplished. Compared with a series of oligonucleotide probes based system, additional requirements need to be met for the single nucleic acid-based SDA (strand-displacement amplification) detection. For example, while the extension of primer can be self-blocked in the absence of target DNA, the SDA effect is capable of being induced by the target DNA hybridization. Naturally, to accomplish the double goal of substantially increasing assay sensitivity with significantly simplifying the probe design, the task remains very formidable. Very recently, as shown in Scheme S1, we tried to develop a bihairpin probe-based SDA system for DNA hybridization detection, but failed. No obvious difference in fluorescence intensity between target sample and blank was detected (sequences and data not shown). Presumably, in the presence of polymerase and dNTPs, unwanted polymerization could occur at the 3’ end even without target DNA. For the original single nucleic acid probe, there might be a conformational equilibrium between the hairpin 1-contained structure and hairpin 2-contained one. Although the hairpin 1 structure predominates, the hairpin 2-based polymerization could not be efficiently suppressed even in the absence of target DNA. This is reasonable taking into account the same properties of two hairpin structures. Seemingly, we cannot accomplish the development of a single nucleic acid-based SDA sensing strategy unless structural elements different from the hairpin structure of nucleic acids are introduced. Aided by the knowledge of G-quadruplex topology accumulated in our previous research works (Zhou et al., 2012; Wu et al., 2007; Zhang et al., 2010; Yim et al., 2010), we introduced the G-quadruplex DNAzyme into the hairpin probe for p53 gene, leading to an efficient MBzyme probe.

As a multifunctional oligonucleotide probe, the proposed colorimetric MBzyme integrates various abilities, including target DNA recognition, primer extension, SDA effect and signal generation, into one common oligonucleotide. Its working principle is shown in Scheme 1. Briefly, the oligonucleotide probe can self-assemble into a hairpin structure similar to the stem-loop configuration of molecular beacon, locking the active center of DNAzyme. Thus, in the absence of target p53 DNA, MBzyme is in the inactive state and cannot be employed as a catalytic unit to generate optical change. In contrast, hybridization of target p53 DNA to loop region opens the common hairpin structure and facilitates the formation of a new stem-loop configuration in the middle region of which is the G-quadruplex DNAzyme that can bury the hemin. By the way, after the stem of common hairpin is forced apart, the shorter stem formation at the 3’ end and G-quadruplex structure can promote each other. As a result, the extension from the 3’ end can take place in the presence of dNTPs and polymerase, and subsequent SDA effect is deemed to be accomplished. The resulting G-quadruplex structure in the SDA products can strongly bind hemin to form the aptamer–hemin DNAzyme complex with peroxidase-like activity, efficiently catalyzing the H$_2$O$_2$-mediated oxidation of ABTS. The enzymatic reaction product, ABTS$^*$, has a maximal absorbance of approximately 420 nm and endows the reaction mixture with a characteristic green color.

3.2. Feasibility of MBzyme-based SDA detection

Integration of target recognition, signal generation and amplification into an oligonucleotide probe without any chemical modification seems to be a great challenge for most researchers, and, to our knowledge, no such nucleic acid probe has been reported up to the present time. Thus, the feasibility of MBzyme probe should be firstly validated by comparative experiments. As shown in Fig. 1, when the system has no p53 target, the absorption peaks are very low in the absence (a) and presence (b) of dNTPs and KF polymerase, indicating that the enzymatic center of DNAzyme has been self-locked for the designed MBzyme probe. However, a slight increase in absorption peak is detected in line c, demonstrating that catalytic activity of DNAzyme can be activated by the target p53 gene. Nevertheless, low concentration (0.25 μM) of target DNA cannot lead to desirable optical signal in a one-to-one interaction between MBzyme probe and p53 target. In contrast, substantially amplified signal is observed in the presence of dNTPs and KF polymerase (line d), suggesting that target p53 hybridization not only activates the enzymatic activity but also triggers the SDA signal enhancement.
process. Meanwhile, colorimetric assay indicates that the information of target species in the sample can be obtained by naked eye. As shown in Fig. 1 inset, existence of p53 in sample can be easily detected based on the proposed sensing system without the need of any measuring instrument.

3.3. Exploration of MBzyme sequence

The MBzyme is designed to contain both target DNA recognition sequence with a common hairpin and DNAzyme sequence with G-quadruplex structure. In essence, their topologies are completely different from each other, and their thermodynamic parameters can be regulated via changing the base number and nature of the stem. Thus, the conformational transition of MBzyme from common hairpin to G-quadruplex is expected to be specifically induced by target DNA hybridization. To further confirm the function of regions introduced into MBzyme, different probes were designed and used to signal the target DNA in the sample. The results are shown in Fig. S1. Hybridization of target DNA to probes cannot induce the detectable change in absorption spectra if the anti-hemin aptamer sequence in MBzyme is changed as shown in group 2 of probes. Measured data from probes 6–11 indicate that the long stem of G-quadruplex-contained hairpin structure increases the absorption peak of blank, deteriorating the assay capability of sensing system. Even if the spacer length between G-quadruplex and stem is changed to facilitate the formational change, the assay characteristics cannot be still improved as illustrated in probes 6–9 of Fig. S1. Group 5 demonstrates that the assay ability of sensing system is obviously compromised even if a single-base mutation in anti-aptamer region occurs. In this case, although the blank absorption peak is sometime suppressed (e.g., probe 12), the difference (e.g., signal) between absorption spectrum corresponding to target DNA and that of blank is smaller than that of probe 1. All the results represented in this section illustrate that absorption spectrum originates directly from the H$_2$O$_2$–mediated oxidation of ABTS catalyzed by aptamer–hemin DNAzyme, and the design of stem and introduction of anti-aptamer region are essential elements for the development of MBzyme probe.

3.4. Characterization of colorimetric MBzyme biosensor using fluorescence spectroscopy

For the present MBzyme probe, target DNA-induced SDA reaction could lead to the additional ds DNA segments, which might be reflected by the fluorescence intensity change of fluorescent intercalators. Thus, the operation of proposed sensing system was investigated by fluorescence spectroscopy using SYBR Green I as a reporter (He et al., 2010), and the fluorescence spectra were collected at room temperature in pH 7.4 Hepes buffer. Fig. 2 validates the assay capability of MBzyme probe to detect target p53 DNA where SDA technique does enhance the fluorescence signal. SYBR Green I alone has almost no fluorescence emission (line a). An obvious fluorescence peak appears in the mixture of SYBR Green I and MBzyme (line b), indicating the existence of ds DNA segment. Polymerase without target DNA induces the fluorescence increase (line c) to some extent, which is distinct from the change in UV–vis absorption peak shown in Fig. 1. This should be attributed to the different supporting buffer and fluorescence intercalator whose signaling mechanism is different from that of colorimetric assay. Target p53 DNA hybridization in the absence of polymerase and dNTPs causes only a slight fluorescence change (line d), while introduction of SDA reaction process is able to result in a substantial increment in fluorescence peak intensity (line e), demonstrating more ds DNA products into which SYBR Green I intercalates and emits fluorescence. More precisely, the SDA amplification makes the fluorescence intensity to increase from 468 to 1456 au, suggesting a substantial enhancement in the fluorescence response to target DNA. Those measured data indicate the success in converting target DNA/MBzyme hybridization into the SDA-based enhanced signal.

3.5. Performance of the colorimetric biosensor for target p53 DNA

The detection sensitivity of conventional MB is often limited to the nanomolar level. Although improved MBs, for example, pyrene excimer signaling MB (Conlon et al., 2008) and locked nucleic acid based MB (Martinez et al., 2009), have been described, only target nucleic acids at the concentration more than subnanomolar level can be reliably detected. Additionally, to prepare those new MBs, specifically designed synthetic methods are needed. For the present MBzyme probe, MB and DNAzyme were for the first time elegantly integrated into one oligonucleotide to develop a powerful SDA-based sensing system, indicating seemingly the simplest probe for the label-free ultrasensitive detection of DNA hybridization. To validate its assay capability, p53 gene samples at the concentration of 0–2.0 × 10$^{-6}$ M were detected using the identical procedure. As shown in Fig. 3A, monotonic increment of absorption spectrum is observed with increasing target p53 concentration in both low and high concentration ranges. The change of the absorption spectrum induced by target DNA at the concentration of 25 fM is easily detected compared with the blank (shown in Fig. 3 inset). Thus, this concentration value is defined as the detection limit, implying an improvement in assay sensitivity over classical strand-displacement polymerization reaction (Jiu et al., 2011), traditional MB (Martinez et al., 2009), adapted MB (Conlon et al., 2008) and catalytic beacon (Xiao et al., 2004a, b) by more than 1000-fold, nearly 6, 5 and 7 orders of magnitude, respectively. If the experimental conditions, for instance, the volume ratio of the solutions involved, are properly optimized, the assay sensitivity is expected to be possibly further improved. Fig. 3B–D demonstrates the favorable dynamic response in a relatively wide quantification range and good measurement reproducibility. It is noteworthy that two different dynamic response ranges are involved. In the target concentration range of 2.5 × 10$^{-11}$–2.5 × 10$^{-7}$ M, the regression equation is expressed as $Y = 0.1688 \log X + 3.086$ with a correlation coefficient of 0.9592 (shown in Fig. 3C), while, in the lower concentration range (from 2.5 × 10$^{-14}$ to 2.5 × 10$^{-9}$ M), the absorption peak value versus the logarithm of target concentration is fitted to the regression equation $Y = 0.1688 \log X + 3.086$ with a correlation coefficient of 0.9806 (described in Fig. 3D). In the two
equations, Y indicates the absorption peak and X represents the p53 gene concentration. The former relationship between optical signal and target concentration, as well as the assay sensitivity, is similar to literature results of classic MB-based assay (Martinez et al., 2009) without the signal amplification process. Apparently, the capability of proposed sensing system to detect the lower concentration of target DNA should be attributed to the introduction of G-quadruplex DNAzyme. Namely, not only can the integration of DNAzyme and MB be successfully designed, but also the detection limit can be easily improved by 5 orders of magnitude, indicating an ultrasensitive detection of DNA hybridization based on the simplest multifunctional oligonucleotide probe.

3.6. Specificity study

In order to evaluate the selectivity of the sensing system, we compared the optical response obtained from absorbance peak induced by DNA strands containing the noncomplementary-based fragment with that of target p53 DNA. All results are shown in Fig. 4. The completely matched DNA target triggered remarkably larger absorbance enhancement than those of target DNA2 and target DNA3, indicating that no interference was caused by the two partly complementary target DNA. This result might be attributed to the high hybridization specificity of hairpin structure and the high fidelity of polymerase.

Fig. 3. The capability of MBzyme probe to detect target p53 gene. A) UV–vis absorption spectra of MBzyme-based sensing system in the presence of target p53 gene at various concentrations. Inset: the absorbance spectra from 0 to 2.5 x 10^{-14} M; B) the dynamic relationship between UV–vis absorption peak at 420 nm and target DNA concentration from 0 to 5 x 10^{-7} M, which is a complex of dynamic response curves including dynamic range1 (2.5 x 10^{-14} to 2.5 x 10^{-7} M) and dynamic range2 (from 2.5 x 10^{-14} to 2.5 x 10^{-9} M). The relative standard deviations for all datum points are between 0.9% and 6.8%. To exhibit the signal change at the concentration beyond the dynamic range, two dose-response points at higher concentration (1.0 and 2.0 μM) are included. The dynamic range1 and dynamic range2 are detailed in Fig. 3C and D, respectively. Experimental conditions: [MBzyme] = 1 μM, [hemin] = 2.5 μM, [H2O2] = 0.1 mM, [ABTS] = 2 mM, [KF] = 0.25 U/μl, [dNTPs] = 0.5 mM, Time1 = 40 min, and Time2 = 8 min.

Fig. 4. Optical response recorded from UV–vis absorption spectra of sensing system toward p53 gene and other DNA strands with about 50% of bases similar to p53 target. Their concentration is 0.25 μM. The experimental conditions are identical to those in Fig. 3. A0: absorption peak in the absence of target DNA and A: absorption peak in the presence of target DNA.
4. Conclusions

In this study, we have developed a new simple, label-free and ultrasensitive MBzyme probe for the visual detection of target p53 DNA. The MBzyme is for the first time designed not only to execute the isothermal circular strand-displacement polymerization (ICSDP), but also to combine the allosteric molecular beacon for target DNA recognition and G-quadruplex-hemin DNAzyme for signal amplification. For the MBzyme probe alone, the catalytic activity of peroxidase-mimicking DNAzyme is locked by a stable hairpin structure. However, the hybridization between target DNA and recognition fragment of MBzyme is capable of promoting the formation of G-quadruplex enzyme and inducing the ICSDP amplification reaction, providing a positive readout signal and achieving the desirable analytical characteristics. The developed colorimetric assay possesses the detection limit of 25 fM and dynamic response range of 7 orders of magnitude without any additional nucleic acids and chemical modification. Besides, the proposed probe exhibits an acceptable detection selectivity, and incompletely matched target DNAs can be readily detected. Regarding the powerful signal amplification of colorimetric system and the delicate concept in designing an efficient nucleic acid probe, this ultrasensitive, selective, simple, rapid and visual detection method based on DNAzyme molecular beacons would be seemingly known for its wide perspective for fundamental research, biotechnology, and biomedical diagnosis.

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Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at http://dx.doi.org/10.1016/j.bios.2013.06.041.

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