Using force spectroscopy analysis to improve the properties of the hairpin probe

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ABSTRACT

The sensitivity of hairpin-probe-based fluorescence resonance energy transfer (FRET) analysis was sequence-dependent in detecting single base mismatches with different positions and identities. In this paper, the relationship between the sequence-dependent effect and the discrimination sensitivity of a single base mismatch was systematically investigated by fluorescence analysis and force spectroscopy analysis. The same hairpin probe was used. The uneven fluorescence analysis sensitivity was obviously influenced by the guanine-cytosine (GC) contents as well as the location of the mismatched base. However, we found that force spectroscopy analysis distinguished itself, displaying a high and even sensitivity in detecting differently mismatched targets. This could therefore be an alternative and novel way to minimize the sequence-dependent effect of the hairpin probe. The advantage offered by force spectroscopy analysis could mainly be attributed to the percentage of rupture force reduction, which could be directly and dramatically influenced by the percentage of secondary structure disruption contributed by each mismatched base pair, regardless of its location and identity. This yes-or-no detection mechanism should both contribute to a comprehensive understanding of the sensitivity source of different mutation analyses and extend the application range of hairpin probes.

INTRODUCTION

Certain diseases, as well as disease resistance to curative medicines, can be attributed to the phenomenon of single base mutation. Therefore, the reliable detection of single base mutation is of great importance in the biological and medical sciences. Typically, sequencing is the preliminary technique for mutation detection (1,2). However, when the identity of the mutation-containing sequence is known, various oligonucleotide (ODN) probe techniques, based on hybridization, offer an attractive alternative (3,4). Among these alternatives, using a hairpin probe including a stem and a loop ODN portion was first employed by Tyagi et al. (5–7), who also successfully applied hairpin-probe-based fluorescence resonance energy transfer (FRET) to detect specific nucleic acids. Since then, the simple and novel architecture of hairpin probes has consistently received wide attention and prompted the widespread use of hairpin probes in both homogeneous and heterogeneous assays (8–11). Furthermore, continuing improvement of hairpin probe properties has focused on the development of strategies aimed at raising detection limits, selectivity and background discrimination (12–15). The high specificity, high signal-to-noise ratio and the capability of homogeneous detection all make possible a hairpin probe capable of revealing the presence of mismatched bases in target ODN using various detection techniques (16–18). Fluorescence is currently one of the most useful tools to accomplish this end. However, the application of fluorescence analysis in this field can be problematic. For instance, even if the length of the probe sequence and the identity of the stem base pairs have been constructed with great care, the performance of the same hairpin probe obviously still varies in its ability to distinguish a single base mismatch with a different position and identity in one target sequence. Consequently, this sequence-dependent effect becomes a limitation in mismatch analysis and probably restricts the application of hairpin probes for analyzing single nucleotide polymorphisms (19,20). It was found that the sequence-dependent effect could be minimized by a special sequence design, such as embedding the mismatched pairs in the same neighboring base pair (20). Notwithstanding this discovery, hairpin probe design is not as simple as attaching arbitrary arm sequences to a linear one (21), and

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constantly adapting the probe sequence to meet various single base mismatches increases design complexity. These flaws, therefore, call for other techniques liberated from the constraints imposed by the sequence-dependent effect.

Because it can detect the forces required to separate strands of DNA, atomic force microscopy (AFM) with ultra-sensitive force spectroscopy analysis has attracted enormous attention and has been applied to detect base mismatches (22–26). AFM can provide detailed information about base pair interaction and ODN conformational changes, and it is a potential way to achieve various mutation analyses on a base level scale. Alternatively, in our previous work, hairpin probes and force spectroscopy analysis have been successfully applied to detect a single base mismatch (18). However, up to now, it has been uncertain whether force spectroscopy analysis has the ability to diminish the sequence-dependent effect of hairpin probes.

Therefore, the purpose of this study is 2-fold: (i) to uncover the sensitivity source of different mutation analysis and (ii) to report a novel approach, which avoids the sequence-dependent effects of the hairpin probe. Fluorescence analysis and force spectroscopy analysis with the same hairpin probe were applied in order to ascertain the sensitivity of each in discriminating single base mismatches in various positions and of different identities. Interestingly, it was found that the sensitivity of force spectroscopy analysis is less dependent than fluorescence analysis upon the locations or identities of the mismatched base pairs, which proves that the ability of hairpin probes in discriminating single base mismatch could be improved by force spectroscopy analysis.

MATERIALS AND METHODS

Sequences of probe and targets

A hairpin probe with the same sequence was used in all experiments. ODN 1 was the perfectly matched target. ODNs containing a single base mismatch, named as 2-1, 2-2, 2-3, 3-1 ... were designed according to the different mutation location and identity in ODN 1 and are shown in Table 1. Additionally, a drawing of the hairpin probe marked with a serial number of mismatched target ODNs, which reflects the distance from the mismatch position to the loop center, is given in the Supplementary Data (Scheme S-1).

Fluorescence analysis of probe–targets interaction

In this part, FAM and DABCYL, used to report the target DNA, were attached to the 5’ end and 3’ end of the hairpin probe, respectively (Takara Co., Dalian). The fluorescence intensity of a 100-μl solution containing 19.8 nM molecular beacon probe, 99 nM ODN target, 20 mM Tris-HCl and 4 mM MgCl2, pH 8, was monitored as a function of time, using a fluorescence spectrophotometer F2500 (Hitachi) to determine the initial velocity of the hybrids formed by the hairpin probe and its perfectly matched or mismatched target ODNs.

Force spectroscopy analysis of probe–targets interaction

The rupture forces between the biotinylated hairpin probe (Invitrogen Co., Shanghai) and various thiolated ODNs (Takara Co., Dalian) were measured with an SPA400 AFM (Seiko Instruments, Japan) in a liquid cell, filled with a hybridization buffer (20 mM Tris-HCl and 0.1 M MgCl2, pH 8). The spring constant of the commercial cantilevers is 0.02 N/m, which was not further calibrated. The same tip was employed for one set of experiments in the same solution condition, including perfectly matched and the differently mismatched ODNs. Moreover, three different new tips were individually modified to perform different sets of experiments. The loading rate of the force curve measurements, ranging from 50 to 250 nm/s, was preliminarily investigated, and 200 nm/s was finally chosen for all the experiments as the consistent loading rate, which is the premise of the comparative results.

A commercial silicon nitride cantilever (Seiko) with a pyramidal tip, which was functionalized with a biotinylated hairpin probe according to the procedure reported before (27), was used. After being immersed in acetone for 5 min and then irradiated with ultraviolet light for 30 min, the cantilever was immersed overnight at 37°C in 50 μl of 1 mg/ml biotinylated BSA solution (Sigma, St. Louis, MO), and then rinsed thoroughly with a phosphate buffer (PBS: 20 mM Na2HPO4, 150 mM NaCl, pH 7.2).

Streptavidin was coupled to the tip through incubation in 50 μl of 1 mg/ml streptavidin (Sigma, St. Louis, MO) and various thiolated ODNs, which is the premise of the comparative results.

For all the experiments as the consistent loading rate, ultra plane glass that had been precoated with chromium to improve adhesion of 99.99% purity gold (99.99%) onto the ultra plane glass that had been precoated with chromium to improve adhesion (200 nm of Au, 10 nm of Cr). Au substrates were cleaned in piranha solution (70% H2SO4; 30% H2O2) before exposure to the thiol-modified ODNs. (CAUTION: Piranha solution should be handled with extreme caution.) The surface was then modified with mixed self-assembled

Table 1. Probe and target ODN sequences

| Hairpin Probe | 3’GGATCG C A T T T C T C T C A C G C G CGATCC 5’ |
|---------------|-----------------------------------------------|
| ODN 1         | 5’ G T A A A G A G G G G T G C G C G C C 3’ |
| Mismatched ODNs’ number | 2 3 4 5 6 7 8 9 10 |
| Identity of mismatched base pair | 1 TT CT CC TT CT AG GA CT GA |
|               | 2 TG CA CA TG CA AC GT CA GT |
|               | 3 TC CC CT TC CC AA GG CC GG |
monolayers of HS-ODN and 6-Mercapo-1-hexanol (MCH, 97%, Aldrich), which were prepared by immersing the clean gold substrate in 250 nM HS-ODN solution for 3 h, then in 1.0 mM MCH solution for 1 h. After being rinsed thoroughly, the buffer used to prepare the HS-ODN solution was deoxidized with pure N2. MCH was used to minimize non-specific adsorption of thiol-modified ODNs and to control the molecular density of the self-assembled monolayers for free hybridization with the probe (28). Before force spectroscopy analysis commenced, the substrate was rinsed thoroughly with deionized water.

Melting curve analysis

Both the hairpin probe and the target ODNs were used without further modification. They were synthesized by Shanghai Invitrogen Co. To investigate the thermal denaturizing profiles of the hybrids formed by the probe and its perfectly matched or mismatched ODN targets, a fluorescence intensity of 100 μl solution containing a 0.25 μM hairpin probe, a 0.25 μM ODN target, 1 × SYBR Green I, 20 mM Tris-HCl and 20 mM MgCl2, pH 8, was measured as a function of temperature. SYBR Green I could intercalate to the duplex of the ODN and induce fluorescent emission at 525 nm with excitation at 494 nm, which was measured in a spectrofluorometric thermal cycler (Applied Biosystems Prism 7000). The temperature was increased in gradations of 1 °C from 15 to 80 °C, and the melting profile was synchronously recorded. \( T_m \) is the highest point in the negative derivative of the thermal denaturizing profiles.

RESULTS

Mutation analysis with fluorescence analysis

The discrimination ability of the hairpin probe for the perfectly matched ODN, as well as ODNs containing a single base mismatch differing in identity and location, was analyzed by monitoring the fluorescence intensity as a function of time. The initial hybridization velocity was calculated on the basis of the increase of fluorescence intensity within the first 30 s. Figure 1 illustrates the sensitivity profile of this method in detecting various ODNs (from ODN 2 to ODN 10). As a consequence, the sensitivity difference resulted from sequence-dependent effect makes it difficult for fluorescence analysis to detect single base mismatch in some cases.

Mutation analysis with force spectroscopy analysis

A mutation analysis using AFM, in which the tip moved with approach-retract cycles in the Z-axis, was carried out in force spectroscopy analysis mode. AFM visual presentations of the Au film before and after modification are shown in Figure S2 in Supplementary Data. These images reveal the roughness of the surface at the nanometer level, making force measurement feasible.

As the hairpin probe modified tip approached the ODN-coated gold substrate individually (ODN 3, medium sensitivity; ODN 7, the highest sensitivity; and ODN 8, the lowest sensitivity), the probe and the target ODN were brought into close contact, and the hairpin probe hybridized with the ODNs to form the DNA duplex. When the tip was retracted, the duplex was separated. Force curves were obtained by plotting rupture force versus tip-sample distance during the approaching and receding process. The maximum force during the retraction course was related to the magnitude of the rupture force required to break the DNA duplex. It should be pointed out that all rupture forces detected in this experiment represented the total force of all contributing interactions, including specific and non-specific forces; however, base pair interaction dominates our results.
Typical force curves of perfectly matched ODN 1 and mismatched ODN 8 are shown in Figure 2. The rupture force between the hairpin probe and ODN 1 is much higher than that of the hairpin probe and ODN 8, which is in good agreement with the more stable DNA duplex formed by the hairpin probe and perfectly matched ODN (25, 30).

According to our previous work, those force curves originated from the specific hybridization between hairpin probe and ODNs 18. Here, force curves of BBSA-functionalized AFM tip on MCH and HS-ODN&MCH self-assembled monolayer are plotted in the Supplementary Data (Figure S-1). These data indicate that the non-specific forces are small and have only a minimal effect upon force measurement. The results further confirm that the interaction is specific.

We repeated hundreds of force measurements for each duplex hybridization. Mean rupture force was calculated, along with a 95% certainly confidence limit. The histogram of the four kinds of rupture forces with the same tip is displayed in Figure 3. Rupture forces show a wide distribution, which can be fitted with a Gaussian distribution curve. The wide distribution of rupture forces is due to the variation of the tip-surface contact area at different spots, the thermal fluctuations of the cantilever, and the uneven distribution of target ODNs on the substrate. The peak shifts to lower force in the case of mismatched ODNs. ODN 1 had the largest rupture force, measuring 1.63±0.06 nN. According to our previous work, a single duplex rupture force between hairpin probe and ODN 1 was in the same order of magnitude as those reported by other groups using the Poisson statistical method (18). The rupture forces of perfectly matched ODN and nine kinds of single base mismatched ones are given in the Supplementary Data (Table S-1). Unlike fluorescence analysis, the differences in rupture force among the nine kinds of single base mismatched ODNs were small, indicating only small sensitivity variations. For example, the sensitivity of ODN 7-2 was 1.5 times that of ODN 8-3.

**Mutation analysis with melting curve analysis**

There is a significant difference between fluorescence analysis and force spectroscopy analysis. Specifically, the hairpin probe in the former method experiences an open course due to hybridization, whereas, in the latter method, the hairpin probe—target DNA duplex denatures by force. To investigate whether the different sensitivity sources of these two methods derived from the different processes of hybridization and denaturing, a melting curve analysis, in which the hairpin probe—target DNA duplex denatures by elevated temperature, was carried out using the same probe-target pairs as those used for force spectroscopy analysis. Measurements to each duplex were repeated three times.

Figure 4 shows the duplex melting curves of the perfectly matched ODN and mismatched ODN with the hairpin probe, respectively. The temperature at which fluorescence intensity rapidly falls is the denaturing (or ‘melting’) temperature ($T_m$) of the probe-target duplex and is visualized as a peak in the plot of the negative derivative of fluorescence versus temperature. The $T_m$ listed in inset shows that the single base mismatched ODN was lower than that of the perfectly matched ODN. The intercalator gradually departs from the duplex as it becomes a single strand. Where there is a mismatch, the melting point is lower, and thus the intercalation departs more rapidly (31). The normalized $T_m$ of ODN 3, ODN 7 and ODN 8 with the probe, which is given in Figure S-3 and is compared with the error bar from the calculation of
error propagation, indicates that those mismatched bases could only be discerned with difficulty by using this approach. However, even ODN 7, the easiest one to detect in fluorescence analysis and force spectroscopy analysis, showed a very small $T_m$ difference from the perfectly matched one.

**DISCUSSION**

The sequence-dependent effect limits the application of the hairpin probe in discriminating a single base mismatch (20, 21). A hairpin probe designed to diminish this effect appears to be quite difficult to manufacture, and there has been no report of a system that could avoid the effect without a change in probe sequence being required. Force spectroscopy analysis was highly significant and very useful in nucleic acid interactions (25), and it is now reported to be a novel approach in avoiding the sequence-dependent effect through its high and even sensitivity.

To make the sensitivity difference between fluorescence analysis and force spectroscopy analysis clear, the results are plotted in Figure 5. The black bar indicates the
normalized rupture force, and the gray bar denotes the initial velocity of the normalized fluorescence. It is clear that the sensitivity of force spectroscopy analysis of every kind of ODN is high and almost at the same level, while the sensitivity of fluorescence analysis fluctuates. This result indicates that force spectroscopy analysis does always work once mismatched ODN occurs.

The relatively high and even sensitivity of force spectroscopy analysis might be explained in two ways. First, DNA duplex interaction is monitored at a molecular level using force spectroscopy analysis, which is different from homogenous fluorescence analysis in nature. The collective signal of all molecules is recorded, and a small change in the signal can easily be submerged. The sequence-dependent effect of the hairpin probe has an obvious influence. However, the force spectroscopy analysis signal results from the interaction of a few molecules immobilized on the both tip and substrate (22). The percentage of rupture force reduction, therefore, could be directly and dramatically influenced by the percentage of secondary structure disruption to which each mismatched base pair contributes, in spite of their mismatched location and identity. This is the key reason for the even sensitivity in the force spectroscopy analysis. Additionally, Strunz et al. and Stattin et al. (24, 25) both reported an approximate 10–20% reduction induced by a single base mismatch in line DNA probe. The discrepancy between their results and ours—which yielded an almost 50% reduction—could be explained, in part, by our previous work, which indicated that the specific discrimination capability of the hairpin probe is higher than that of the linear DNA probe. Certainly, a different base sequence design and secondary structure could contribute to this difference.

The second aspect is the increased probability of molecular collision in heterogeneous hybridization using AFM by controlling the two molecular-modified-surfaces. This high probability of molecular collision is a key issue for single base mismatch detection based on DNA hybridization in both homogeneous fluorescence analysis and heterogeneous force spectroscopy analysis. Hence, force spectroscopy analysis is anchored to the both probe and target, which gives our binding reaction higher efficiency.

Compared with the other two methods, the sensitivity of a melting curve analysis is the lowest. This could be attributed to a lack of specificity for any particular duplex (31). The stem-loop structure of the hairpin probe also did not show any advantages in this case. Both melting curve analysis and force spectroscopy analysis are based on the denatured course of the DNA duplex, and the much lower sensitivity of our melting curve analysis indicates that there is no obvious relationship between the analysis sensitivity and the course of denaturing or hybridization. The sensitivity of the force spectroscopy analysis was derived from the nature of the method.

In terms of its sensitivity level when detecting a single base mismatch with the same hairpin probe, we found that force spectroscopy analysis resulted in the identification of all kinds of mismatched target ODNs. These encouraging findings both shed new light on the nature of different mutation analysis methods and provide evidence that the approach would be very helpful to the extension of the applied range of the hairpin probe through diminishing the sequence-dependent effect.

SUPPLEMENTARY DATA
Supplementary Data are available at NAR Online.

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Conflict of interest statement. None declared.

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