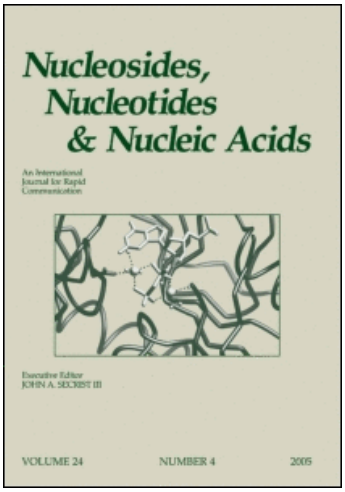

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OLIGONUCLEOTIDES AS CODING MOLECULES IN AN ANTI-COUNTERFEITING SYSTEM

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▫ *Due to the growing numbers of counterfeited products on the world market, there is a huge demand for new and forgery-proof marking systems. We developed a unique system using "molecular beacons" with well adapted thermodynamic parameters. This marking system consists of the three components: DNA tag (a label or directly printing), detection pen (contains the "molecular beacon" solution), and DNA-scanner (reads the specific signal triggered by the detection pen even at daylight). The vast coding capacity of DNA combined with the highly specific signal offers a degree of security that is unmatched by conventional identification technologies.*

INTRODUCTION

Counterfeited products cause huge financial damage to companies[1] and, what is even more serious, can create an enormous health risk for the consumer, especially if pharmaceutical products are affected. Therefore, the FDA recommended to apply new technologies as authentication features, which allow rapid authentication of products.[2] A possible marker for this purpose, which is virtually impossible to decode is DNA. Due to the enormous information density DNA as an anti-counterfeit marker is practically impossible to decode. The information is stored in the sequence of the DNA building blocks, namely the four bases adenine (A), cytosine (C), guanine (G), and thymine (T). Therefore, a DNA molecule built from 20 to 25 bases, which is called a oligonucleotide, can be arranged in more than one trillion different combinations.[3] This huge number of variations makes it impossible to find out a certain sequence by guessing or trial and error. Nevertheless, for the authentication, the DNA marker has to be detected and analyzed on the spot. Standard methods like PCR[4] are unsuitable for practical use, because they are too labor and time intensive. Therefore, new concepts had to be investigated. Today, several tools for homogeneous DNA monitoring are available. A smart test system can be achieved by using molecular beacons as detection

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molecules. Molecular beacons are hairpin-like structures with a linked fluorophore on one end and a quencher on the other end.[5] When a molecular beacon encounters its specific marker DNA molecule hybridization occurs between the two molecules and the fluorescence is restored, resulting in a detectable signal. Therefore the key-lock principle of molecular beacons could be used in an anti-counterfeiting system.

MATERIAL AND METHODS

Hybridization Kinetics

We used molecular beacons provided with 7, 8, or 10 bases in the stem (named B7, B8, B10 molecular beacon). The length of the target DNA was either 10 or 12 bases longer than the stem length (named +10, +12). The complementary sequence to the target DNA sequence was either located in the stem and reached in the loop or the fully loop was used as the target binding site (named loop). The target DNA was applied in a concentration of 4 pmol/mm^2 on cellulose dots and than dried. Afterwards, one microliter of the respective beacon solution (1 pmol/µl) was pipetted on the dot. The fluorescence signal was detected by excitation of the dot by a laser and monitoring the resulting fluorescence light with a filtered photodiode over time. In a second approach the specific quenching rate (QR) between the beacon and the target DNA was calculating using the following equation:

$$QR = \frac{Bo - Bg}{Bc - Bg} \tag{1}$$

where:

Bg (background): fluorescence signals of the wet cellulose dot
Bc (closed beacon): signal of beacon without target DNA
Bo (opened beacon): signal with beacon and applied target

Temperature Dependence

Target DNA was pipetted on dots as described above. The dots, the beacon solution, and the analytical device (laser, photodiode) were stored at the described temperature in a climatic exposure test cabinet. After half an hour the temperature equilibrium was reached and the beacon was applied on the dot. The fluorescence was measured 15 s later at the respective temperatures.

RESULTS AND DISCUSSION

Design of Molecular Beacon

One of the most important claims to an anti-counterfeiting identification system is the responding behaviour of the authenticity test. The test has to be fast and

reliable. This can be easily understood, if one imagines the rapidness and the complexity of modern distribution networks. The testing time is mainly determined through the hybridization kinetic. The kinetic is strongly influenced by the design of the molecular beacon. Basically, the beacon consists of two parts, namely the stem and the loop (see Figure 1). The stem is necessary for the formation of the hairpin loop, whereas the loop contains some or all bases that are complementary to the target DNA sequence. Therefore we analyzed the interactions between target DNA and Molecular Beacons, wherein the number of complementary bases and whose arrangement in the beacon sequence was different. The results are shown in Figure 2 and Table 1. A strong effect of the position of the complementary bases is obvious. If the bases are located in the loop only (T7 loop and T8 loop), almost no increase of fluorescence signal over the time and only small values for the quenching rate were detected. Only a little fraction of the molecular beacon and the target DNA formed a stable double strand. Another approach is to extend the binding region of the target DNA in the stem of the molecular beacon (stem targets), i.e., using longer target DNAs, that are for the most part complementary to the beacon sequence.[6] In this case, more bases of the molecular beacon take part in the hybridization event and the beacon opens easier, resulting in much faster hybridization kinetics and good quenching rates. The time constants of the kinetic plots together with the quenching rates (T7 + 12, T8 + 12 compared to T7 + 10, T8 + 10) are shown in Table 1. The hybridization kinetic between stem targets and

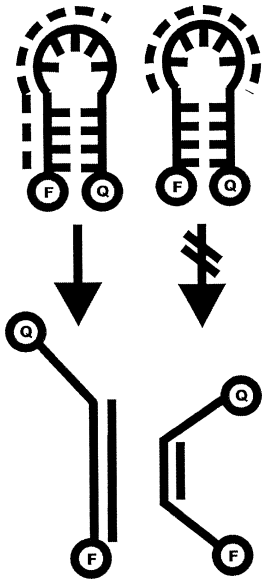

FIGURE 1 Schematic illustration of the hairpin structure of molecular beacons. The straight line shows the structure of the beacon with the stem forming bases (horizontal lines). The dotted lines show the different possibilities of location of the bases, which are complementary to the target DNA. Left side: bases are located in the stem and the loop. Right side: bases are located only in the loop.

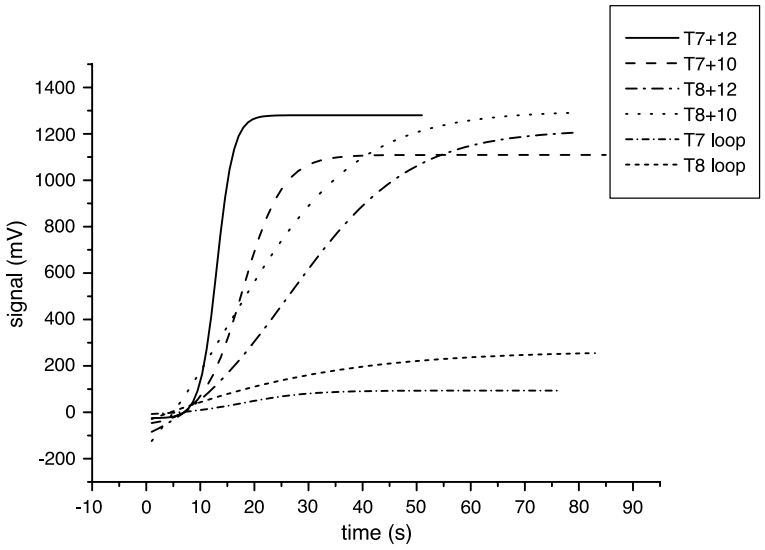

FIGURE 2 Kinetic of molecular beacons with different structures. B7: stem is built of 7 base pairs. B8: stem is built of 8 base pairs. +10: total number of complementary bases is 10 plus number in the stem. +12: total number of complementary bases is 12 plus number in the stem. Loop: all complementary bases are located in the loop. Experimental conditions: see Material and Methods.

molecular beacons (measured through the increase of the fluorescence signal over the time) is faster with increasing number of bases that are complementary to the molecular beacon sequence. Further, the velocity of the kinetic between target DNA and molecular beacon can be enhanced by using molecular beacons with short stem lengths.[7] Thus, for fast kinetic properties, molecular beacons should be designed with short stems and the number of complementary bases of the target DNA to the molecular beacon should be a as long as possible. The reduction of the stem length, however, is limited. If stems are too short, the hairpin structure is no longer stable, resulting in an undesirable high background signal and insufficient quenching rates of the molecular beacon. On the other side the size of the loop also limited. The stability of the hairpin structure is inversely proportional to the loop length.[8] This means that there is a much smaller chance for a 50-mer beacon to

TABLE 1 Quenching Rates and Kinetic Time Constants of Different Molecular Beacons and Target DNA Combinations

Name of beacon	Quenching rate	Time constant
T7 + 12	20.7	1.5
T7 + 10	18.2	3.7
T8 + 12	18.2	11.3
T8 + 10	15.7	11.9
T7 loop	2.8	6.1
T8 loop	3.8	20.9

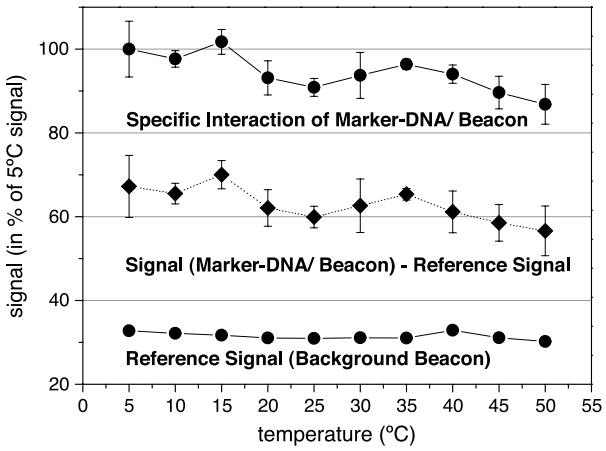

FIGURE 3 Kinetic of a T7 + 12 beacon at room temperature. Experimental conditions: see Materials and Methods.

form a hairpin loop than for a 30-mer. Considering these data we designed a B7 + 12 beacon and analyzed the kinetic of hybridization between the marker DNA and the molecular beacon. As obvious from Figure 3 about 90 percent of the total fluorescence signal is detected after less than 10 s. This exceedingly fast kinetic makes the molecular beacon system to a suitable instrument for an on-site anti-counterfeiting test system.

We also analyzed the temperature dependency of the system. As demonstrated in Figure 4, the system is quite independent from temperature influences in the temperature region between 5°C and 35°C. At temperatures over 35°C, the signal strength begins to decrease slightly with increasing temperature. This decrease is

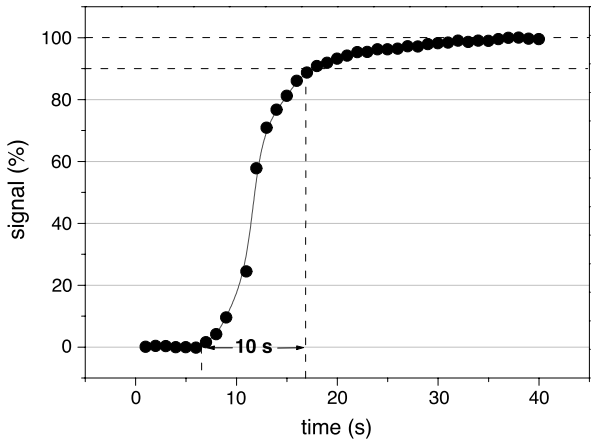

FIGURE 4 Temperature dependency of a T7 + 12 molecular beacon and target DNA interaction. Experimental conditions: see Materials and Methods.

not caused by a destabilization of the beacon structure (hairpin of the molecular beacon) or by destabilization of the molecular beacon/target-hybrid because the melting temperatures of both hybrids calculated much higher. As further experiments showed, the slight decrease of the fluorescence signal at temperatures higher than 35°C is caused by the temperature dependence of the laser module (data not shown). Considering this effect, the system works well at temperatures below 50°C. At higher temperatures, the beacon begins to open, which dramatically increases the background signal and decreases the quenching rate.

CONCLUSION

Well-designed molecular beacons as analytical instruments allow the specific detection of oligonucleotides at room temperature. For a fast and specific reaction, balanced lengths of the stem and loop regions of the molecular beacon are essential. Short stems led to fast kinetics but also to higher background. Longer loop regions also enhance the background. A good compromise to reduce the loop length is to use the stem also as the target binding region. Thus, the whole beacon structure contributes to the specific target binding. A beacon system that fulfils the requirements for an on-site test at room temperature consists of 7 stem bases and 12 loop bases. Such a system is already used for the detection of DNA marker on pharmaceutical products with very reliable results.

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