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A universal platform for amplified multiplexed DNA detection based on exonuclease III-coded magnetic microparticle probes†

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An amplified multiplexed DNA detection biosensor has been developed, which combines the unique cleavage function of exonuclease III (Exo III) with the separating ability of magnetic microparticles (MMPs). By using different fluorophores, the multiplexed detection of DNA is demonstrated.

The development of a sensitive and multiplexed DNA detection biosensor in parallel using a single sample is of great demand in clinical diagnostics, detection of genetic disorders, analysis of food and environmental pollutants, and homeland security.[1] Driven by the need to analyze an ever-increasing number of target sequences in a complex mixture, many efforts have been focused on the development of different DNA biosensors capable of measuring multiple targets in parallel.[2] Owing to its unique properties, fluorescence has shown great promise in achieving multiplexed DNA detection, which is difficult to achieve by conventional methods.[3–5]

Very recently, a number of approaches for DNA detection based on a signal amplification reaction were presented.[6] In the case of multiplexed DNA detection methods based on nuclease-induced signal amplification, Nam and coworkers recently developed a nicking endonuclease-assisted signal amplification system that can simultaneously detect three kinds of DNA sequences in one sample.[7] Despite high sensitivity and selectivity, this approach requires specific target sequence for nicking endonuclease recognition which cannot be fulfilled in all systems. Replacing the nicking endonuclease with a nuclease that does not require any specific recognition sequence may offer a universally adaptable system. In contrast, exonuclease III (Exo III) does not require a specific recognition site, so cleavage occurs irrespective of the sequence present at the blunt terminus.[8] With this property, Exo III could be used in a multiplexed DNA detection assay and solve the problems found in nicking endonucleases.[9] Interestingly, Willner and coworkers recently developed an Exo III-based signal amplification biosensor for simultaneously detecting two different DNA sequences in one sample.[9f] Unfortunately, however, in this biosensor, different-sized quantum dots required suited quenchers which limited the usage of this biosensor.

To solve the above problems, herein, a sensitive, multiplexed and universal DNA detection biosensor has been developed. We combine the unique cleavage function of Exo III with the separating ability of magnetic microparticles (MMPs) to develop a multicolor magnetic probe for simultaneous detection of two analytes: human immunodeficiency virus nucleotide sequence (T_{HIV}) and Ebola virus nucleotide sequence (T_{EV}).

As shown in Fig. 1, two different DNA strands (P_{HIV} and P_{EV}, probes for HIV and EV, respectively; sequences shown in ESI†) were mixed at equal molar ratio and co-assembled at the streptavidin-capped MMPs (1.02 μm). The surface coverage of P_{HIV} and P_{EV} on MMPs was determined by fluorescence spectroscopy (see ESI†). The choice of two dyes relies on the consideration of their spectral overlap, with P_{HIV} labeled with

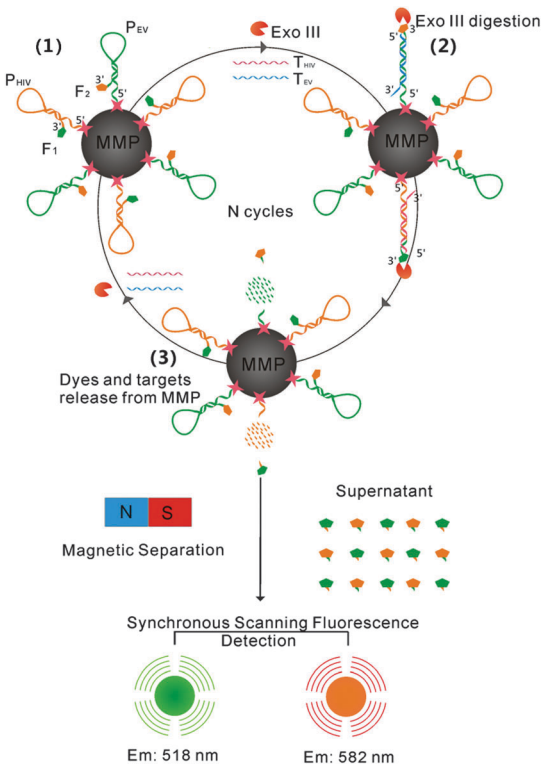

Fig. 1 Schematic representation of Exo III-coded magnetic microparticle-based multiplexed DNA detection.

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fluorescein amidite (FAM) and P_{EV} with tetramethyl-6-carboxy-rhodamine (TAMRA). The functionalized MMPs were prepared using a modified protocol suggested by the manufacturer (see ESI†). Target DNA sequences hybridizes with fluorogenic probes to form double-stranded structure with a blunt 3′-terminus. It should be noted that the target hybridized only partially with probe, leaving a single-stranded overhang at its 3′-terminus that is resistant to the cleavage by Exo III because the resistance degree of Exo III on single-stranded DNA at 3′-protruding terminus are dependent on the length of the extension, with extension 4-nt or longer being essentially resistant to cleavage.[8b] Thus, in the presence of Exo III, only the 3′-terminus of the probe is subjected to digestion. Exo III catalyzes the stepwise removal of mononucleotides from this terminus, liberating the fluorophore before ultimately releasing the target. The released target then hybridizes with another probe, whence the cycle starts anew. Thus, a single copy of the target generates many fluorescent fragments. Released fluorescent fragments are recovered by applying a magnetic field to remove MMPs. The fluorescence signals of the supernatant are obtained with synchronous scanning fluorescence spectroscopy (SSFS). Owing to its particular property, Exo III provides a powerful tool for detecting a large number of different DNA strands in one solution.

We first explored the single-color mode. In the single-color mode, an oligonucleotide sequence associated with HIV was used as a model system. As shown in Fig. S1 (see ESI†), in the absence of T_{HIV}, P_{HIV} was in a stem-loop state with a single-stranded overhang at its 3′-terminus which prevented P_{HIV} from digesting by Exo III. The fluorescence signal of the supernatant was very weak (Fig S1,† curve a). Upon the addition of T_{HIV} which was hybridized with P_{HIV} to form a double-stranded structure with a blunt 3′-terminus, the probe-target DNA duplex was subsequently digested by the Exo III, releasing the fluorescent fragments and T_{HIV}. The released T_{HIV} then hybridized with another P_{HIV} to start a new cycle. Thus, a single copy of T_{HIV} generated many fluorescent fragments. Thus, the fluorescence signal of the supernatant was significantly enhanced (Fig S1,† curve b). As shown in Fig. 2, the fluorescence intensity increased when the concentration of T_{HIV} was raised from 0–8 nM, indicating that the release of fluorescent fragments was highly dependent on the concentration of the target DNA. This confirmed the working principle that the target DNA was hybridized with the stem-loop molecular beacon that triggered the Exo III digestion. Fig. 2 insert was shown the fluorescence signal changes of the supernatant as a function of the concentration of T_{HIV}. Under the optimal conditions, fluorescence restoration of the supernatant was linear over the range of 0.2–4.0 nM, with a detection limit of 70 pM. This DNA detection sensitivity is comparable to the existing signal amplification methods that utilized Exo III as a signal amplification nuclease.[9a,c,f] To evaluate the selectivity of single-color mode, we challenged the assay with single-base mismatched target ($MT1_{HIV}$), three-base mismatched target ($MT3_{HIV}$) and non-complementary target (NT). The results were shown in Fig. S2 (see ESI†). The results showed that this probe can effectively distinguish perfectly matched from mismatched DNA targets.

The performance of the multicolor mode was evaluated by fluorescence measurements as well. As shown in Fig. 3, the

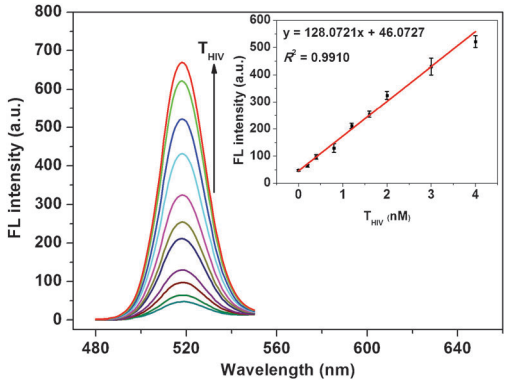

Fig. 2 Changes in the fluorescence spectra of the supernatant upon increasing the concentration of T_{HIV}: 0, 0.2, 0.4, 0.8, 1.2, 1.6, 2.0, 3.0, 4.0, 6.0 and 8 nM (from bottom to top). Insert: the linear curve of single determination for T_{HIV}. Experimental conditions: MMPs, 50 μg; Exo III, 75 U; at 37 °C for 90 min.

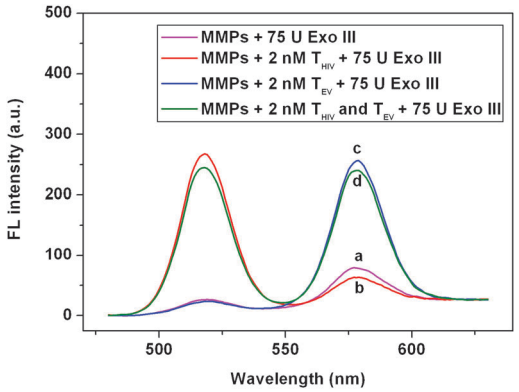

Fig. 3 Fluorescence spectra of the supernatant in the absence (a) and presence of 2 nM T_{HIV} (b), 2 nM T_{EV} (c), 2 nM T_{HIV} and 2 nM T_{EV} (d), respectively. Experimental conditions: MMPs, 50 μg; Exo III, 75 U; at 37 °C for 90 min.

dual-colored fluorophores emitted weak fluorescence in the absence of target DNAs (curve a). When T_{HIV} alone existed in the sample solution, increasing fluorescence emission of FAM was observed (curve b). In contrast, when T_{EV} alone existed in the analysis sample, only TAMRA fluorescence emission intensity increased (curve c). When both T_{HIV} and T_{EV} existed in the sample solution, the two distinguishing colored fluorophores fluorescence emission increased at the same time (curve d). With the increased concentrations of T_{HIV} and T_{EV}, the fluorescence intensities were increased at the same time, respectively (Fig. 4). The DNA concentration was quantified by monitoring the change in the fluorescence intensity. The fluorescence signals of FAM and TAMRA can be simultaneously obtained by SSFS. This method can avoid the interference of Raleigh light scattering signal and improve the detection sensitivity.[2g] T_{HIV} can be determined at 60 pM, whereas T_{EV} can be quantified at 81 pM, respectively.

We further evaluated the specificity of the multicolor mode. The assay was challenged with single-base mismatched target and non-complementary target. As shown in Fig. 5, the fluorescence intensity for $MT1_{HIV}$ was about 50% of that for perfect target T_{HIV} at the same concentration on the addition of DNA.

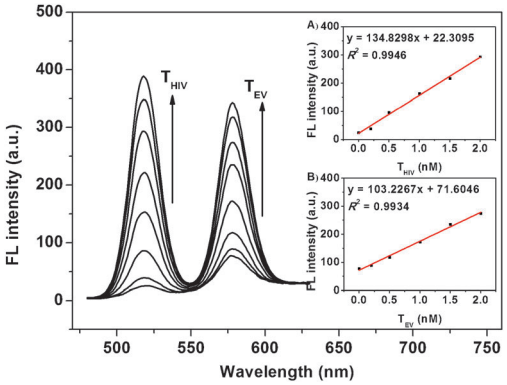

Fig. 4 Changes in the fluorescence spectra of the supernatant upon simultaneously increasing the concentration of the target DNAs (T_{HIV} and T_{EV}): 0, 0.2, 0.5, 1.0, 1.5, 2.0, 3.0 and 4.0, respectively (from bottom to top). Insert: the linear curve of determination for T_{HIV} (A) and T_{EV} (B). Experimental conditions: MMPs, 50 μg; Exo III, 75 U; at 37 °C for 90 min.

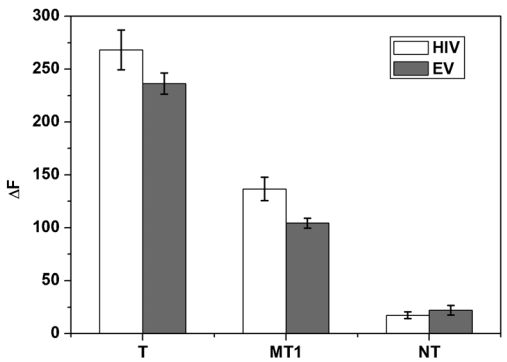

Fig. 5 Fluorescence intensities *vs.* different DNA sequences. Target DNA and other mismatched strands were all 2 nM. Experimental conditions: MMPs, 50 μg; Exo III, 75 U; at 37 °C for 90 min.

Of note, the fluorescence of NT reached to the background. The similar results were obtained from detecting T_{EV}. These results indicated that this multicolor probe can provide an excellent capability in differentiating between perfectly matched and mismatched DNA targets, demonstrating the good selectivity of this platform. This high specificity was owing to the weak hybridization of mismatched DNA with the stem-loop structure of the probe. This simultaneous detection method not only can realize DNA differentiation but also DNA determination at the same time with good sensitivity and selectivity.

In conclusion, we have developed a highly sensitive, multiplexed and universal DNA detection assay using Exo III signal amplification reaction, functionalized MMPs and SSFS. As a proof of concept, we demonstrate that the proposed assay is effective for detecting HIV and EV nucleotide sequences simultaneously with high sensitivity and specificity without cross reaction. This biosensor can be achieved with SSFS, which makes the assay much easier to achieve multiplex detection. Because Exo III does not require specific recognition site, this assay offers great versatility and could detect simultaneously many more types of targets in parallel with SSFS. Therefore, we expect this platform will become a practical and powerful tool for molecular diagnostics.

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