Short communication

DNA diagnosis in a microseparator based on particle aggregation

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A B S T R A C T

A novel aggregation-based biosensing method to achieve detection of oligonucleotides in a pinched-flow fractionation (PFF) microseparator was developed. Employing functionalized polystyrene microspheres, this method is capable of the direct detection of the concentration of a specific DNA sequence. The label-free target DNA hybridizes with probe DNA of two kinds on the surface of the microspheres and causes the formation of an aggregate, thus increasing the average size of the aggregate particles. On introducing the sample into a PFF microseparator, the aggregate particles locate at a specific position depending on the size of the aggregate. Through a multi-outlet asymmetric PFF microseparator, the aggregate particles become separated according to outlets. Because the size of the aggregate particles is proportional to the concentration of the target DNA, a rapid quantitative analysis is achievable with an optical microscope. A biological dose–response curve with concentration in a dynamic range 0.33–10 nM has been achieved; the limit of detection is between 33 and 330 pM. The specificity of the method and the potential to detect single-nucleotide polymorphism (SNP) of known concentration were examined. The method features simple, direct and cheap detection, with a prospect of detecting other biochemical samples with distinct aggregation behavior, such as heavy-metal ions, bacteria and proteins.

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1. Introduction

Microfluidic systems have important applications in biomedical detection. Sensors on a miniaturized chip, taking advantage of its small size and large ratio of surface to volume, function satisfactorily with the merits of great sensitivity, so requiring only a small sample, and a brief interval for detection; they hence become promising tools for diagnostic detection (Yager et al., 2006; Mairhofer et al., 2009). Above all applications in biomedical usage, point-of-care (POC) diagnostics is a common objective because of prospective applications in developing areas and sites near patients. To attain this objective, a direct, simple, convenient and cheap method of detection is essential.

The analysis of nucleic acids (e.g., DNA sequencing, detection and genetic variation, etc.) is a critical issue in the field of diagnostic medicine; methods based on electrochemical (Wong and Gooding, 2003) and magnetic (Koets et al., 2009) properties, surface-plasmon resonance (SPR) (Tamada et al., 2007), fluorescence resonant-energy transfer (FRET) (Hsu et al., 2010) and so forth are extensively developed. Although these methods possess advantages of great sensitivity and efficiency (e.g., the detection limit is about 4 pM; the detection period is 3 min) (Koets et al., 2009), they require bulky or expensive apparatus (Wong and Gooding, 2003; Koets et al., 2009; Tamada et al., 2007). A complicated sample pretreatment (e.g., labeling of target DNA) might also be required (Hsu et al., 2010). DNA detection based on the aggregation phenomenon of beads on a micrometer or nanometer scale has been demonstrated (Elghanian et al., 1997; Li et al., 2007). Other than the detection of oligonucleotides, biosensors of other clinical analytes such as heavy-metal ions (Kim et al., 2001), E. coli (Hana et al., 2008), surfactant protein A (SP-A) (Heinrich and Griese 2010), and prostate-specific antigen (PSA) (Cai et al., 2011) have been developed. Various signal transductions include a shift of the absorption spectrum (Elghanian et al., 1997; Kim et al., 2001) a voltammetric signal difference (Li et al., 2007), or a an altered magnetic relaxation interval (Cai et al., 2011; Moser et al., 2009) after aggregation occurs. These methods might achieve a detection limit as small as, e.g., 0.1 pM DNA (Li et al., 2007), but they are inconvenient because of pretreatment of target analytes, a requirement of special instruments etc. Most such methods make use of gold or magnetic nanoparticles to generate an indirect signal change for target quantification; researchers have rarely measured the amount of target directly using the nature of an altered size of the particles after aggregation. Even if the size difference is observed directly under a microscope (Heinrich and Griese 2010), the detection...
performance is limited by sampling and measuring error; the efficiency also is limited by not being based on continuous detection on a chip.

In a microfluidic system, devices to differentiate and to sort particles according to size are common (Yamada et al., 2004; Takagi et al., 2005; Huang et al., 2004; Carlo et al., 2008). Several mechanisms have been developed, including pinched-flow fractionation (PFF) (Yamada et al., 2004; Takagi et al., 2005), deterministic lateral displacement (Huang et al., 2004), hydrodynamic filtration (Carlo et al., 2008), and inertial migration (Bhagat et al., 2009). Among these methods of separating particles, the approaches involving the PFF mechanism are utilized to control flexibly the results of particle separation by the modulation of the ratio of flow rates of inlets, or by means of the design of asymmetric outlets (Yamada et al., 2004; Takagi et al., 2005). PFF was applied to the detection of single nucleotide polymorphisms (SNP) (Larsen et al., 2008). In a PFF microseparator, a sample fluid with particles and a buffer without particles pass through a pinched segment in which the particles are pushed toward one side of the channel by a hydrodynamic force, then enter a broadened segment. The particles follow various streamlines in the lateral direction of the channel according to their size.

Here we report an on-chip aggregation-based microfluidic system for DNA detection utilizing a PFF microseparator. Hybridization between a label-free oligonucleotide target and a probe DNA modified on the surface of microspheres causes an aggregate to form, thus increasing the average size of the aggregate particles. The size of an aggregate particle is proportional to the target concentration, which facilitates a quantitative determination of the amount of analyte on examining, with a microscope, the lateral position of particles at the broadened segment of the channel. The results can be directly read in a device designed with multi-outlet channels. By detecting the DNA fragment of distinct sequences, such as a non-complementary sequence or a single base-pair-mismatch sequence, one can verify the specificity or the applicability of the method to detect mutations.

2. Experiments

The notion and detection in this work are illustrated schematically in Fig. 1. Streptavidin-coated polystyrene microspheres (PS beads) conjugate respectively with probe DNA of two kinds modified with biotin. On mixing the probe-labeled PS beads with a target DNA of which the two ends are respectively complementary to two DNA probes, hybridization occurs and causes the microspheres to aggregate. Asymmetric PFF microseparators of two types—two outlets and six outlets—are designed and applied to analyze the size of the aggregate particles and to determine the concentration of the target DNA (see Fig. S1 in Supporting information). For detailed information on the experiments, please refer to the supporting information.

3. Results and discussion

3.1. Relation between aggregate size and concentration of target DNA

The aggregation of particles formed from hybridization of target DNA and probe DNA is an essential factor of this aggregation-based detection. Through mixing and hybridization, the two ends of the target DNA bind with probes DNA-1 and DNA-2 respectively, thus causing the probe-labeled PS beads to aggregate and to form aggregate microparticles (see Fig. S2 in Supporting information). The results show that the size of aggregate particles increased in the range $C_{\text{target}}$ (the concentration of the target DNA) $= 0.33–10 \text{nM}$ (see Table S1 in Supporting information). The average size of the aggregate particle increased from 1.4 to 8.8 μm for $C_{\text{target}}$ from 0 to 10.0 nM: under these conditions the size of the aggregate microparticles indicated the target-DNA concentration. We also acquired images of particle aggregation with target DNA at varied concentrations with a field-emission scanning electron microscope (SEM, JEOL JSM-7600F). The results also indicate that the aggregate size increases with increasing concentration of target DNA (see Fig. S3 in Supporting information).

In general, for a fixed concentration of probe DNA, the binding number between the complementary base pairs was proportional to $C_{\text{target}}$, and attained a maximum value when $C_{\text{target}}$ became saturated, but the size of aggregate particles decreased to 7.9 μm when $C_{\text{target}}$ increased to 33 nM. In this case, the concentration of target DNA (33 nM) is 100,000 times that of the PS microspheres (0.33 pM); the number of target DNA is much larger than the label density of probe DNA (45,000 probe strands/microsphere). We suppose that, in this work, the saturation concentration of $C_{\text{target}}$ is about 15 nM (45,000 times that of the PS microspheres). When $C_{\text{target}}$ attains 33 nM, the superabundant target DNA might hybridize simultaneously with both probes DNA-1 and DNA-2 at the two ends, but it might hybridize with mostly either probe DNA-1 or probe DNA-2 at only one end, thereby blocking the two probes near each other and impeding the aggregation of particles. We assume that the favored concentration of detection of the target DNA, as a result of the size of aggregate particles, is in a range 0.33–10 nM.
3.2. DNA detection through the lateral position in the PFF microseparator

On use of the two-outlet pinched-flow fractionation (PFF) microseparator (Fig. S1a, Supporting information), the size of the tested sample is differentiated according to the lateral position of the streamline at the broadened segment (see Fig. S4 in Supporting information). According to the mechanism of separation in the PFF microseparator (Yamada et al., 2004), the sample solution in the pinched segment was squeezed to a width 0.77 μm when the ratio of flow rates of sample and buffer was 25:1. Particles of radius greater than 0.77 μm were arranged in varied lateral locations according to their size; the lateral location (y) of the particles at the broadened segment is calculated with (Yamada et al., 2004)

\[ y = \frac{Dw_b}{\sum w_p} \quad (if \quad D \geq 1.54 \mu m) \]  

where D is the diameter of the particle. The streamline pertaining to larger particles is nearer to the center of the channel.

On respective introduction of the buffer and sample solutions into the two-outlet PFF microseparator, the experimental results of the aggregate particles with varied target DNA concentration are shown in Fig. 2a and b. When \( \text{C}_{\text{target}} = 0 \), the fluorescent signals (indicating the streamlines of the particles) are focused in a position in a range 0–75 μm, near the side wall. According to Eq. (1), the calculated mean and maximum lateral locations of \( \text{C}_{\text{target}} = 0 \) (mean aggregate size 1.4 μm; maximum aggregate size 3.2 μm) are about 35 and 80 μm, respectively. The result of the lateral position in the PFF microseparator is similar to the value calculated from the aggregate size, but the lateral position \( \text{C}_{\text{target}} = 33 \text{ pM (0–90 μm)} \) shows no clear difference from the situation for \( \text{C}_{\text{target}} = 0 \). The reason might be that the sizes of aggregates at these two concentrations are too near to each other; the results are thus difficult to distinguish under our experimental conditions. When the concentration of target DNA increased to 0.33 nM, the distribution of the fluorescent signal also enlarged: the non-uniform size of aggregates caused a discontinuous distribution of the particle streamline. In general, the fluorescent signal was farther from the side wall with increasing \( \text{C}_{\text{target}} (\text{C}_{\text{target}} = 10 \text{ nM}) \). The concentrations agree with the supposition in the preceding section; the concentration for detection of the target DNA is optimal in a range 0.33–10 nM. The size of the aggregate particles must be distinguishable and proportional to \( \text{C}_{\text{target}} \).

On analysis of the average lateral position of the farthest streamline of several images, we obtained a dose–response curve (Fig. 2c) for aggregation-based DNA detection in the PFF chip. The value at the right side of the figure signifies the calculated size of aggregates according to the lateral location (at the figure left). The dose–response curve is applicable directly to evaluate \( \text{C}_{\text{target}} \), which is important in applications. On the basis of that curve (Fig. 2c), the behavior of the detection system becomes analyzed. For our aggregation-based system, the limit of detection (LOD) is between 33 and 330 pM; the linear dynamic range is 0.33–10.0 nM. The sensitivity of detection was calculated from the slope of the curve. The detection system becomes analyzed. For our aggregation-based system, the concentration of target DNA was readily and directly examined with use of the two-outlet and six-outlet asymmetric PFF microseparators. In the two-outlet PFF microseparator, one outlet collects particles of size larger than a particular value; the existence of a target DNA in the tested sample can hence be rapidly screened. The six-outlet PFF microseparator was used to determine rapidly the concentration of the target DNA (\( \text{C}_{\text{target}} \)). Because of the asymmetric design of the outlets, most buffer flow that squeezed the sample flow was collected into the drain outlet, thus having the ability to enhance the resolution of particle separation (Takagi et al., 2005).

The experimental results of varied concentration of target DNA in the asymmetric PFF microseparators are shown in Fig. S5 (see Supporting information). For the two-outlet PFF chip (Fig. S5a), all the samples without target DNA (\( \text{C}_{\text{target}} = 0 \)) would flow into the narrow outlet channel. Once the sample contained target DNA, the fluorescent signal appeared in the wide outlet channel. The amount of flow of fluorescent particles into the wide channel increased with increasing \( \text{C}_{\text{target}} \). The results show that the two-outlet PFF chip readily distinguishes whether a sample includes a target of concentration greater than a standard value; this property is useful for rapid screening in a clinical application.

For the six-outlet PFF chip (Fig. S5b), about 80% of the fluid flowed into the drain outlet because of an asymmetric channel design; the results thus show that the particles of varied size were eventually collected into separate outlets. All the samples without target DNA (\( \text{C}_{\text{target}} = 0 \)) would outflow into outlet 1; a sample with target DNA would flow into outlets 2, 3, 4 and 5 for \( \text{C}_{\text{target}} = 0.33, 1.0, 3.3 \) and 10.0 nM, respectively. Within the linear dynamic range (0.33–10.0 nM), the aggregate particles were collected from the next outlet when the concentration of the target DNA increased 3.3 times in each case. The multi-outlet design enables a rapid diagnosis and quantitative determination of the concentration of the target DNA from a solution collected at an outlet, which requires no analysis of the lateral position in the PFF chip; it combines advantages of convenience and accuracy.

3.3. DNA detection in the asymmetric PFF microseparator

In addition to precise detection of the aggregate size of a particle from the lateral position in the PFF microseparator, the concentration for detection of the target DNA is optimal in a range 0–75 μm, indicating the streamlines of the particles) are focused in a

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3.4. Test of specificity of aggregation-based detection

To verify that the detection system works well in a realistic environment in which biomolecules other than the target coexist in the sample, we tested the specificity on mixing both a perfectly complementary oligonucleotide target (target DNA) and non-complementary DNA strands (test DNA) with the probe-labeled PS beads. After hybridization overnight in an Eppendorf tube, the sample fluid was introduced into the PFF microseparator, and the results were analyzed.

Fig. 3(a) illustrates the result of this test of specificity of the hybridization-induced aggregation. The lateral positions of the largest aggregate particles were measured ten times (\( N = 10 \)) independently on analysis of the fluorescent streamline farthest from the side wall of the broadened segment from images recorded with a monochromatic CCD camera (exposure duration 10 s). The result shows that the largest aggregate formed in either a sample with only target DNA or a sample with target DNA and test DNA have nearly the same size; a non-complementary oligonucleotide sequence would hence not interfere with the formation of aggregate particles induced on hybridization between a target DNA and complementary probe DNA sequences at its two ends. As a result, the hybridization-induced aggregation phenomenon is proved specific.

3.5. Detection of single base-pair mismatched DNA

In the field of gene analysis, research has focused on gene variants in disease. Among nucleotide variations of all kinds,
single-nucleotide polymorphism (SNP) attracts the greatest attention as it is the most common form of genetic variation. For this reason we assessed also the applicability of this aggregation-based detection method for the analysis of SNP. To test the capability of SNP detection in our system, we replaced a perfectly complementary target with oligonucleotide targets with one mismatch (1-mismatch).

![Image of aggregate particles with varying concentrations](image)

**Fig. 2.** (a) Optical microscopic image of aggregate particles with \( C_{\text{target}} = 0, 0.033, 0.33, 1.0, 3.3, 10.0 \) and 33.0 nM. The images were captured at a location near the sidewall (dashed line in white) of the broadened segment of the PFF microseparator with a monochromatic CCD camera (exposure duration 10 s). (b) Normalized distribution of fluorescent intensity of varied \( C_{\text{target}} \) along the lateral direction of the broadened segment. (c) Dose–response curve for aggregation-based DNA detection on a PFF chip. The lateral position of particles farthest from the wall of the broadened segment is measured on image analysis. Error bars correspond to one standard deviation of five nominally identical measurements. Inset: for small concentrations (< 1.0 nM), a quasi-linear response (adjusted R-square \( \sim 0.9956 \)) is observed.
DNA) in a mixture of probe-labeled PS beads to assess the extent of the size of aggregation in the presence of imperfect hybridization.

Fig. 3(b) demonstrates the result of SNP detection in the aggregation-based detection system. The mean value and the error bars (one standard deviation) are derived from ten independent measurements (N=10) of the lateral position of aggregates. Analysis of the images of samples of both kinds exposed for a long period shows that the size of aggregate particles in the presence of 1-mismatch DNA is smaller than the aggregation caused by target DNA (both the concentrations of the 1-mismatch DNA and of the target DNA are 2.5 nM). The laterally shifted distance decreased by approximately 21%, which is a difference evidently reliable to measure mutations.

The site of the mismatched base pair locates at one end of the target strand, decreasing the probability of hybridization between the target and one oligonucleotide probe. Calculation of the Gibbs energy of formation of the DNA duplex according to the nearest-neighbour method (Santa Lucia, 1998) shows that this energy altered from ΔG_{27} = -118.8 kJ mol^{-1} to ΔG_{27} = -60.8 kJ mol^{-1} when the target sequence was replaced with one mismatched sequence; this decrease of the binding probability causing the decrease of hybridization-induced aggregation of microspheres is large. The obvious size difference verifies the capability of detection of SNP in our system.

Our proposed microfluidic system might have difficulty with an unknown sample if the target DNA has a lower concentration or the SNP has a higher concentration, but the concentration of DNA sample is readily controllable with the existing DNA pretreatment. For the clinical DNA diagnosis, the DNA was extracted with a commercial kit (Aljanabi and Martinez, 1997; Chien et al., 2010). The extracted DNA was quantified on testing the UV absorbance at 260 and 280 nm with a spectrophotometer, followed by amplifying the DNA extracted with the primers specific for the genetic mutated region utilizing a PCR process. The concentration of the DNA (target DNA) was adjusted to the required range (e.g. 0.33–10.0 nM in our aggregation-based detection system).

Through injecting the DNA sample of known concentration into our proposed microfluidic system, we are able to determine whether the DNA is normal-type (complementary target DNA) or SNP-type (1-mismatch DNA) by reading the lateral position.

Previous work has shown that the hybridization-driven aggregation depends on temperature (Leslie et al., 2012) and demonstrated that the aggregation method has the ability to differentiate between the complementary DNA and SNP by heating experiments (Elghanian et al., 1997). In general, the melting temperature of the target DNA (T_{m,t}) is higher than the dissociation temperature (T_{m,s}) of the SNP DNA, such that the SNP DNA is unable to conjugate with probe DNA on the particles. For some temperature T between T_{m,t} and T_{m,s}, the particle aggregate involving the SNP DNA would thus become disintegrated, whereas that involving the target DNA would remain stable. We tried also to find a critical temperature at which the target aggregate is stable whereas the SNP aggregate disintegrates. We analyzed and compared the melting curve of target-DNA aggregated particles and SNP-DNA aggregated particles (Fig. S6). The melting temperatures are 74 °C for the target and 73 °C for the SNP aggregate. A possible temperature to differentiate aggregated particles with these two DNA types might hence locate between 73 and 74 °C. To detect SNP of an unknown concentration, we consider that heating (with temperature T set between T_{m,t} and T_{m,s}) is a better way to control the DNA binding and to distinguish the type of DNA.

4. Conclusion

We propose a method of direct detection of oligonucleotides based on aggregation using a PFF microseparator. Beside an advantage that the target DNA is free of a label, the aggregation-based method features simplicity and immediacy. In clinical SNP diagnosis, the extracted DNA can be readily adjusted to an equivalent concentration during the pretreatment. With the same sample concentration, the normal-type DNA (target DNA) and the SNP-type DNA can be recognized and quantified using the proposed chip as their aggregation sizes differ. We thereby differentiate the target DNA and SNP-type DNA of similar concentration based on a calibrated lateral location of the aggregate particles. According to the specificity and the SNP tests, the method shows the prospect for practical applications. When probe DNA-labeled fluorescent microspheres of two kinds mix with a solution of a nucleic-acid target, hybridization of two complementary DNA strands occurs, and large aggregate particles form. Depending on their size, the microparticles separate in a PFF microseparator, enabling subsequent analysis of the concentration of target DNA.

With a fluorescence microscope, we obtained significant results in the proposed device. If the device is modified and equipped with particular optical components (e.g., a few LED, photodiodes and filters), it becomes potentially a portable point-of-care (POC) diagnostic tool. Obtaining the results with a naked eye can be achieved also by means of the use of color microspheres instead of fluorescent particles. This method can serve for the detection of other biochemical samples with distinct aggregation behavior. The present method has the ability to be applicable in the fields of disease diagnosis, home care, environmental monitoring and otherwise.

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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.2013.06.017.

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