Microfabrication of encoded microparticle array for multiplexed DNA hybridization detection†

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A strategy for the high-sensitivity, high-selectivity, and multiplexed detection of oligonucleotide hybridizations has been developed with an encoded Ni microparticle random array that was manufactured by a "top-down" approach using micromachining and microfabrication techniques.

Micrometer- and nanometer-dimensioned encoded particles or beads capable of biological molecule or cell attachment and identification are very valuable for miniaturizing and multiplexing array-based bioassays and screenings.[1,2] By using uniquely encoded particles tagged with specific recognition probes, a tiny amount of sample can be analyzed simultaneously for large numbers of targets. A variety of strategies to encode or label polymeric,[3–6] ceramic,[7] semiconductor,[8,9] and metallic[10,11] microparticles or beads have been previously reported. Current challenges to the encoding include development of strategies to increase the reliability of the coding elements, reduce nonspecific adsorption on the microparticle surface, and optimize the physical and chemical properties of the particles for better biomaterial immobilization and detection. Here we report a means of using microfabricated encoded metallic (Ni) particles for high-throughput sequence-specific oligonucleotide (DNA) hybridization assay.

In our procedure, we generated encoded Ni microparticles with nanometer precision using photolithographically created photoresist patterns as the template for microparticles that were replicated by electrodeposition technique. This resulted in well-defined rectangular particles carrying an engraved dot-and-space pattern code. A process diagram illustrating the fabrication of inexpensive encoded Ni microparticles can be found in the ESI.† The thickness (~2 μm) of the particles was determined by the current density and duration of electrodeposition, while the dimension (100 × 200 μm) of the particles and the coding patterns were controlled by a photomask. We used a pattern of 1–10 dots distributed along two rows on a part of particle surface as the coding element. A detailed description of the encoding scheme for the microparticles has been reported elsewhere.[12] Almost unlimited combinations of the total number and sequential order of the coding dots enable a massive number of unique codes to be created, and thus allowing a large number of particle types to be encoded.

A typical protocol proposed to perform multiplexed DNA hybridization assay is depicted in Fig. 1. The particles were first coated with a self-assembled monolayer (SAM) of 16-mercaptohexadecanoic acid by soaking each set of particles for 48 h in a 1 mM solution of the compound dissolved in ethanol. This surface passivation step is essential for improving the fluorescence detection sensitivity on the particle surface. Each set of particles were then treated individually with 50 μL of 10 μg mL^{-1} avidin in 10 mM phosphate-buffered saline (PBS), pH 7.4, for 3 h at ambient temperature with rotating agitation. The particles were subsequently treated with a 2% casein solution in PBS buffer containing 0.01% Tween 20 for 1 h to block the exposed particle surface. Biotinylated DNA probes were each attached to a given type of particle *via* biotin-avidin chemistry.

The synthetic oilgonucleotides related to genotypes of 5′-non-coding region genes of hepatitis C virus[13] were acquired from Fasmac (Kawaga, Japan) and had following sequences:

Probe A: biotin-5′CCAGGCATTGAGCGGGTTGAT. Target A: FITC-5′ ATCAACCCGCTCAATGCCTGG.

Probe B: biotin-5′GGCCGGGCATAGAGTGGGTTTAT. Target B: FITC-5′ATAAACCCACTCTATGCCCGGCC.

Probe C: biotin-5′GTCCAGGCATTGAGCGGGTTTAT. Target C: FITC-5′ ATAAACCCGCTCAATGCCTGGAG.

For attachment of the DNA probes onto the particles, each set containing 10–20 particles was added a specific biotinylated DNA probe (0.1 μM). After washing off the unbound probe, the particle sets were combined together. A 20 μL sample containing the fluorescein isothiocyanate (FITC)-labelled targets A, B, and C—diluted with 2% casein solution in 10 mM Tris buffer (pH 7.4), 1 M NaCl and 0.1 M EDTA—was added and incubated for 10 min. The particles were washed at 56 °C and then spread onto a ~5 × 5 mm area of a slide, the resulting pattern of fluorescence from the microscopic particle zones was imaged with a MZFLIII fluorescence microscope (Leica, Germany) equipped with a cooled charge-coupled device camera (AxioVision 3.0, Carl Zeiss Inc., Swiss). The signal amplification was achieved by acquiring the imaging with an integration time of 20 s.

The sensitivity of this particle-based DNA hybridization assay was assessed using a set of particles functionalized with probe A to hybridize the complementary target A at concentrations over the range from 0.01 nM to 10 μM. The fluorescence microscopy images of these particles are shown in Fig. 2a. The fluorescence signal was mainly observed at the edges of a particle, while the centre of a particle had very low intensity of fluorescence. This is due to the electronic quenching of fluorescence from those

† Electronic supplementary information (ESI) available: S1, process diagram for fabrication of encoded microparticles; S2, fluorescence signals on polymeric beads as a result of exposing to 0.1 μM target A. S3, enlarged views of the fluorescence and bright field images showing selective binding of a complementary DNA target (A) to the same encoded microparticles attached with the given DNA probe. See http://www.rsc.org/suppdata/cc/b5/b501146a/

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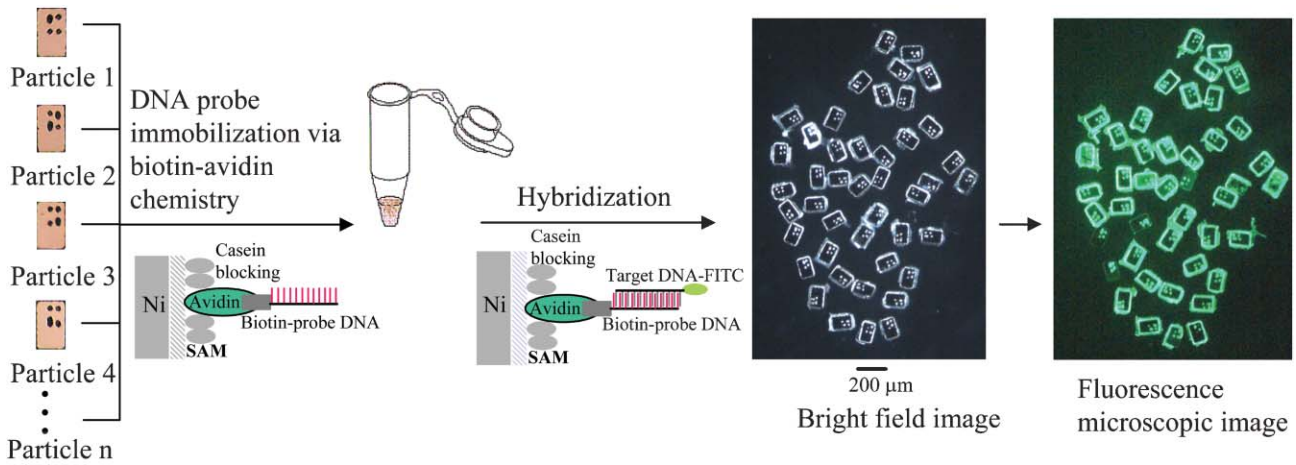

Fig. 1 Process diagram of the protocol proposed for multiplexed DNA hybridization assay using encoded microparticles. Selected encoding patterns are shown on the individual particles. Architectures of molecules built on the particle surface are also shown.

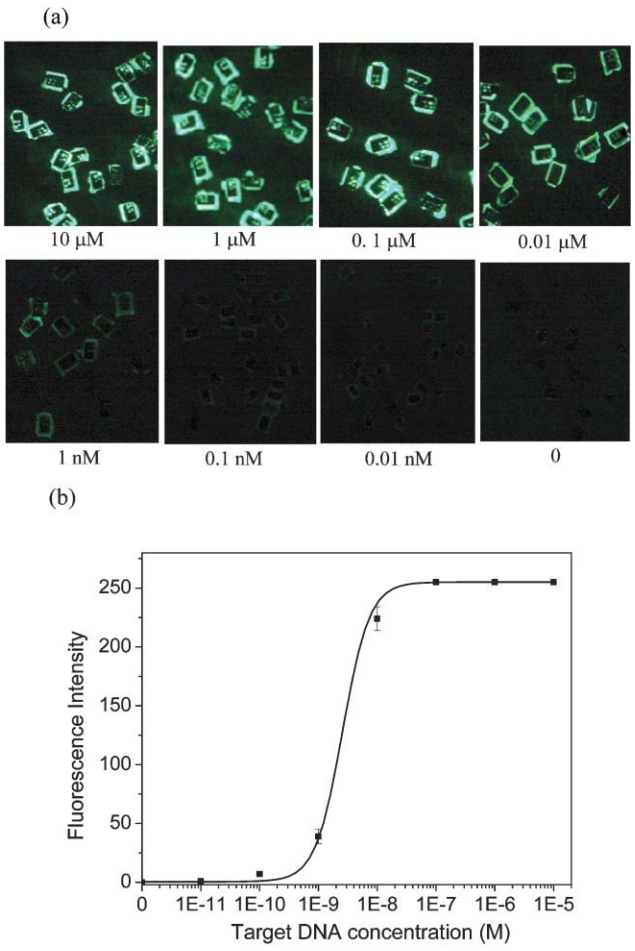

Fig. 2 (a) Fluorescence signals on particles (tagged with probe A) as a result of hybridization with different concentrations of FITC-labelled target A. (b) Overage value of fluorescence intensity measured at the edge zone of a particle as a function of target concentration.

FITC–DNA conjugates located at close proximity to the Ni surface, even through the surface was covered by a SAM. Nevertheless, the fluorescence signals near the edge zones could be easily detected at a concentration of ~1 nM, comparable to that reported elsewhere.[14,15] The detection limit was estimated at around 0.1 nM and for concentrations over 0.1 μM, the signals were virtually saturated (see Fig. 2b).

The specificity and multi-analyte capability of this particle-based multiplexed assay were tested with 3 model targets. In the assay, three sets of DNA probe (A, B, C)-functionalized particles were combined and then subjected to a treatment with 0.1 μM target A, 0.1 μM target B, and a mixture of 0.1 μM each of targets A, B, and C, respectively. As shown in Fig. 3, particles treated with a sample containing target A yielded fluorescence signals from only those particles tagged with the corresponding probe (Fig. 3a). In contrast, particles with immobilized probe B and C showed no signal, reflecting substantially low non-specific adsorption for non-target DNA on the particle surface. This result is in direct contrast to a polymeric bead-based DNA hybridization assay where a strong non-specific adsorption was evidenced (see ESI†). Elucidation of the target identities that bound on a randomly located particle was done by reading the signature of the particle in a bright field image (Fig. 3a′). For enlarged clear images, see ESI.† A similar degree of discrimination was observed in an experiment in which the particles were exposed to a sample containing target B at a concentration of 0.1 μM; as was evidenced by a signal detected for only those particles with immobilized probe B (Fig. 3b). On the other hand, in the combined particles exposed to a cocktail of sample containing 0.1 μM of all three targets, a fluorescence signal was detectable for all those particles with immobilized individual probes (Fig. 3c). These results indicate that no false positive or false negative signals were encountered in this multiplexed assay.

In conclusion, we have demonstrated a proof-of-concept of an encoded microparticle-based multiplexed assay for DNA hybridizations. The assay was highly sensitive, easy-to-use, kinetically fast, and showed effective discrimination against different kinds of targets in a sample. The assay scheme is highly flexible and can be scaled: by incorporating additional particles functionalized with other DNA probes, more hybridization/binding events could be tracked and analysed in an assay. However, a single-base mismatch discrimination has not been achieved with the procedure reported here. We envision that the ability of identification of single base mismatch for this encoded particle-based assay can be improved by implementing more sequence-specific hybridization

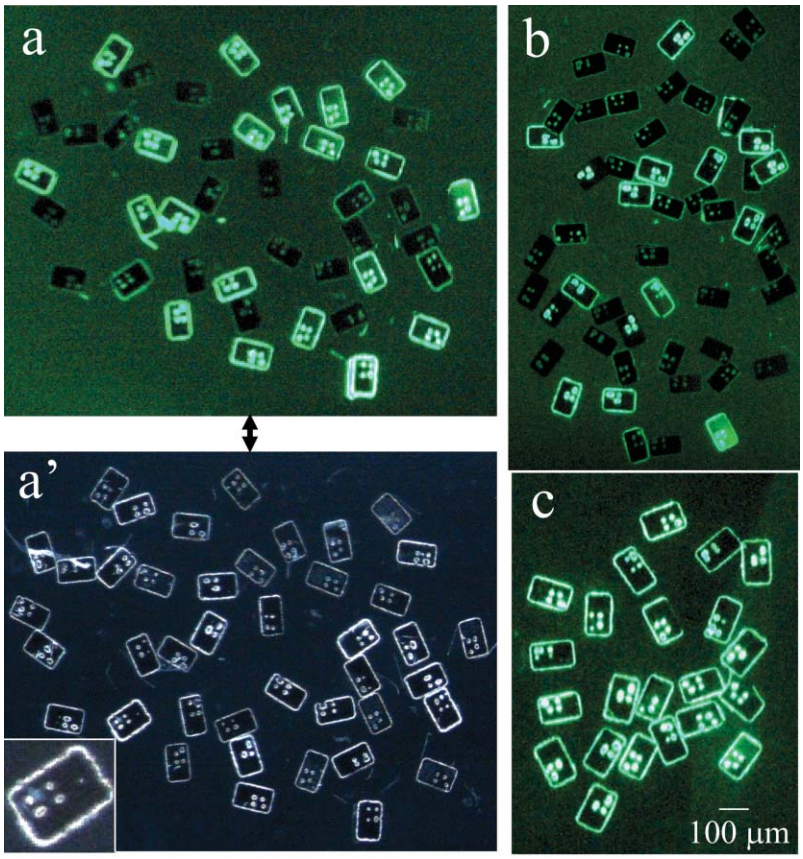

Fig. 3 Multiplexed DNA hybridization assay with encoded particles using a fluorescence-labelling detection scheme. Case (a) shows the fluorescence microscopy image for detection of 0.1 μM target A; fluorescence signals indicate the on-particle hybridization events. The panel below (a′) shows a bright field microscopic image, revealing the location and identity of particles on the substrate; inset: enlarged view of a particle, the coding pattern is clearly observed. Case (b) shows the image for detection of 0.1 μM target B. Case (c) shows the image for simultaneous detection of 0.1 μM targets A, B, and C. Note only fluorescence images are given for cases (b) and (c).

formats such as those reported by Kerman *et al.*[16] and Yershov *et al.*[17] Finally, the fact that inexpensive Ni particles are magnetic greatly increases the methods available for particle handing and recovery.

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