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Different Strategies of Covalent Attachment of Oligonucleotide Probe onto Glass Beads and the Hybridization Properties

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Abstract The glass bead is a new biochip support material for immobilization biomolecules, due to its independence and convenient rearrangement. In order to optimize the immobilization efficiency of oligonucleotides onto glass beads and obtain the highest hybridization efficiency, three commonly used coupling strategies have been studied for covalently attaching oligonucleotides onto large glass beads. Glass beads with 250 μm diameter were amino-silaned with 2% 3-aminopropyltrimethoxysilane (APTMS) and then reacted separately with glutaraldehyde, succinic anhydride and 1,4-phenylene diisothiocyanate (PDITC) to derive CHO beads, COOH beads and isothiocyanate-modified beads (NCS-Beads) accordingly. Afterwards, amino-terminal oligonucleotides were covalently attached onto the surface of beads achieved by three strategies mentioned above. The immobilization efficiency were studied to compare the three strategies, which turned out 2.55×10^{13} probes/cm^2 for CHO-Beads, 3.21×10^{13} probes/cm^2 for COOH beads and 6.68×10^{13} probes/cm^2 for NCS beads. It meant that the immobilization efficiency based on NCS beads was most acceptable. And the method, developed by attaching amino-terminal oligonucleotides onto these cyanate active beads, could be regarded as an efficient one for immobilizing oligonucleotides onto a solid surface. Moreover, in this paper, the hybridization properties of NCS bead-based oligonucleotides have been studied by employing Cy5-tagged complementary oligonucleotides. It was found that the high probe density NCS beads led to low hybridization efficiency possibly due to the existence of steric crowding. In addition, the equilibrium binding constant K_A was determined by employing Langmuir isotherm model, which was 7.0×10^6 M^{-1} for NCS beads with the density of 6.7×10^{13} probes/cm^2. Furthermore, it only took 60 min to reach hybridization equilibrium. These large microspheres (>100 μm) can be employed in the mesofluidic systems for automated heterogeneous assays.

Keywords Covalent attachment · Immobilization · Oligonucleotides · Beads · Hybridization

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Introduction

DNA microarrays, especially oligonucleotide microarrays, have become an increasingly important biological tool in recent years. Because of their high throughput and efficiency, oligonucleotide microarrays have been utilized for drug development [1], DNA sequencing [2], clinical diagnostics [3], and environment detection [4]. However, the high cost for microarray fabrication [5] has limited this technology only to general laboratories and academies. Therefore, a new DNA-array platform with a reproducible, inexpensive, simply manipulating, high-throughput detection system [3] is required for genetic analyses in referred areas.

The bead array, which is based on beads as the major element and the support of biomolecules, has attracted attentions of biologists because of its flexible fabrication, low cost, and high efficiency. Compared with slide arrays based on glass slides, bead arrays have several advantages. First, beads, major element of the bead array, have a higher ratio of surface to volume than slides. Second, hybridization kinetics and thermodynamics are often improved, which in turn decreases analysis time. Third, the sub-array assemblage comprising beads with different kinds of probes is flexible, which overcomes the rechange limitation of the slide sub-array fabricated by spotting. These advantages have already been demonstrated in many applications, such as enzyme assay, immunoassays, and DNA detection.

Generally, attaching oligonucleotides to beads is a crucial step for the fabrication of bead arrays because it determines the hybridization efficiency and influences the signal strength in the following steps [6]. The basic forces between oligonucleotides and bead surface are generally electrostatic interaction and covalent interaction with chemical bonds [7, 8]. With the electrostatic interaction, oligonucleotides are susceptible to removal from the support under high salt or high temperature conditions. Thus, it results in low levels of attachment and high nonspecific binding to the support [9]. However, with the covalent interaction, oligonucleotides are immobilized onto the surface of the beads by forming specifically chemical bonds, which results in high levels of attachment and low nonspecific binding. Therefore, the latter interaction is much preferred in the bead array application. The strategies of covalent attachment of oligonucleotides onto bead surface include the following: (1) the carboxylated [9] or phosphorylated DNA [10] immobilized onto aminated supports; (2) amino-terminal oligonucleotides bound to carboxyl glass [11], or isothiocyanate-activated glass [12], or aldehyde-activated glass [13] or glass surfaces modified with epoxide [14]; (3) thiol-modified or disulfide-modified oligonucleotides coupled with aminosilane via a heterobifunctional crosslinker [15, 16] or on 3-mercaptopropylsilane [6, 17]. Here, we chose three most common strategies for immobilizing amino-terminal oligonucleotides onto chemically modified beads, to ultimately generate aldehyde-activated bead (CHO bead), carboxyl bead (COOH bead) and isothiocyanate-activated bead (NCS bead).

It is also very important to choose material used as the bead array support which must be satisfied with high chemical stability, high repeatability, and low nonspecific surface adsorption. Several prevalent materials nowadays used in bead array are gold particles, magnetic beads, glass beads, and polymer microspheres (polypropylene microspheres, polystyrene microspheres, poly vinyltoluene microspheres, etc.) [3, 18–23]. Here, glass beads were chosen as the support material because of their arbitrarily controllable and narrow size dispersion, mechanical strength, low cost [21], facile chemical modification, and resistance to high temperature. In addition, glass beads also provide a better signal-to-noise ratio because they show less background fluorescence and transparent. Some research

groups have applied glass beads to build analytical platform for the detection of oligonucleotides. Kohara et al. [5] placed oligonucleotide-conjugated glass beads in determined order into a capillary for hybridization detection. Seong et al. [20] packed streptavidin-coated microbeads into mircrochambers, which fabricated with photopolymerized hydrogels, to capture oligonucleotide targets. Monaghan et al. [23] detected Chlamydia trachomatis in a bead-based lab-on-a-chip format which showed several advantages over traditional laboratory methods, such as the rapid reaction of the array on-chip, the potential for sample clean-up, and the low volume of sample required.

In this paper, we compared three commonly used coupling strategies of amino-terminal oligonucleotide probe onto large glass beads to find out the most efficient one for both immobilization and hybridization. Glass beads with 250 μm diameter were chosen as solid support, which were silanized and chemically modified with glutaraldehyde, succinic anhydride, and 1,4-phenylene diisothiocyanate (PDITC), accordingly. Afterwards, amino-terminal oligonucleotides were immobilized onto these activated beads, and the immobilization densities were calculated to evaluate the covalent efficiency.

Materials and Methods

Chemical Reagents

Glass beads (average diameter 250 μm), 3-aminopropyltrimethoxysilane (APTMS; 97%), 2,4,6-trinitrobenzene sulfonic acid solution [TNBS; 5% (*w*/*v*) in H_2O], glutaraldehyde solution (25% in H_2O), succinic anhydride, and 1,4-phenylene diisothiocyanate (PDITC) were obtained from Sigma-Aldrich (St. Louis, MO, USA). *N*′-(dimethylaminopropyl)-*N*'-ehylcarbodiimide hydrochloride (EDC) was purchased from Pierce (Rockford, IL, USA). Morpholineethanesulfonic acid (MES) was purchased from Sinopharm Chemical Reagent (Shanghai, China). All other reagents required for use in the chemical modification, coupling, washing, or hybridization buffer were of analytical grade.

Oligonucleotides probes and targets were purchased from Shanghai Sangon Biological Engineering Technology & Services. The oligonucleotides were synthesized by standard phosphoramidite chemistry, and purified by reverse-phase HPLC. The sequences of oligonucleotides were listed in Table 1.

Glass Beads Cleaning and Silanization

Glass Beads Cleaning The glass beads (500 mg) were cleaned by rinsing in 1 ml 6 M HCl solution overnight with gently shaking at room temperature (RT). The beads were then extensively washed with distilled water until neutral. The beads were placed in a vacuum at 110°C for 2 h to dry.

Table 1 Sequence and nomenclature of oligonucleotides used in this work.

Name	Sequence
Probe A	5′ -NH_2 -TTT TTT TTT TTT TGG AGT AGA TTG GCC AAC CCT TTT
Target A	5′ - Cy5-AGG GTT GGC CAA TCT ACT CC
Target B	5′ - Cy5-GTC TCC ACA TGC CCA GTT TC
Cy5-tagged probe	5′ -Cy5-CCA CGT GGT ACC ACA GA-$(CH_2)_3$-NH_2

Target A complementary target of probe A, *Target B* non-complementary target of probe A

Amino-Silane Modification The pre-cleaned beads were immersed in a 2% solution of 3-aminopropyltrimethoxysilane (APTMS) in anhydrous toluene. Silanization was carried out with gently shaking at RT for 5 h. After five washes (3 min/wash) with anhydrous toluene, the beads were baked at 110°C in a vacuum for 3 h. Then, they were stored at RT.

TNBS Test The completeness of the Silanization reaction was checked by the methods described by Janolino and Swaisgood [24] with some modification. 15 μl 0.01 M PBS (PH 7.4) and 5 μl 5% aqueous 2,4,6-trinitrobenzene sulfonic acid solution (TNBS) were added to about 1 mg of aminopropyl glass beads at RT and reacted for 2 h. Then the beads were washed with distilled water and examined for color; whether the beads color becoming orange from transparent indicated successfulness or failure of the silanization.

Silanized Beads Coupling with Activated Groups

After derivation with APTMS to generate primary amino groups, the glass beads were then chemically modified with three different strategies (Fig. 1) before immobilization amino-terminal oligonucleotide probes. The glass beads were treated with glutaraldehyde to generate CHO beads, with succinic anhydride to generate COOH beads and with 1,4-phenylene diisothiocyanate (PDITC) to generate NCS beads.

Derivation of CHO Beads Aminopropyl beads (10 mg) were dispersed in 5% aqueous glutaraldehyde solution (500 μl). Then the beads were shaken for 60 min at RT and then washed five times with distilled water and dried in a vacuum at RT.

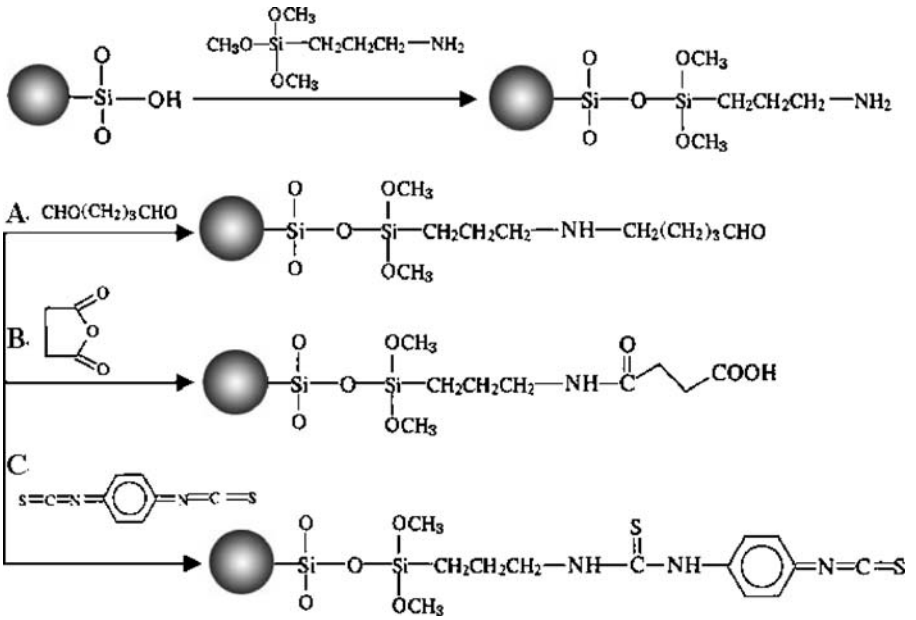

Fig. 1 Reaction scheme of different surface chemistries used to covalent attach oligonucleotide probe. After derivation with APTMS, glass beads were treated with (*A*) glutaraldehyde; (*B*) succinic anhydride; or (*C*) 1,4-phenylene diisothiocyanate (PDITC)

Derivation of COOH Beads Aminopropyl beads (10 mg) were immersed in a 10% succinic anhydride solution in 0.1 M sodium acetate, pH 4.5 (500 μl). Then the beads were shaken overnight at RT and then washed five times with 0.01M PBS (pH 7.4) and dried in a vacuum at RT.

Derivation of NCS Beads Aminopropyl beads (10 mg) were rinsed in a dimethylformamide (DMF) solution, including 10% pyridine and 0.2% PDITC (500 μl) and shaken for 2 h at RT. The beads were washed five times with DMF, three times with anhydrous ethanol, and 3 times with methylene chloride and then dried in a vacuum at RT.

Oligonucleotides Probes Immobilization

Oligo Immobilization on CHO Beads CHO beads (2 mg) were placed in a 5-μM Probe A or Cy5-tagged probe (listed in Table 1) aqueous solution in 3×SSC (20 μl) with shaking overnight at RT and then washed one time with 3×SSC, two times with 0.2%SDS, two times with distilled water and then dried in a vacuum at RT. Before hybridization, the beads were reduced with sodium borohydride solution for 10 min, and then washed two times with 0.2% SDS, two times with distilled water, and then dried in a vacuum at RT.

Oligo Immobilization on COOH Beads COOH beads (2 mg) were immersed in a 5-μM Probe A or Cy5-tagged probe aqueous solution in 0.1 M MES buffer, pH 6.5 (18 μl). An 18-mg/ml EDC solution (prepared in 0.1 M, pH 6.5 MES buffer in 15 min before the reaction) was added during immobilization: 1 μl at 0 min, 0.5 μl 60 min and 0.5 μl 90 min, to a final concentration of 1.8 mg/ml EDC. The beads were shaken for 105 min at RT, then washed five times with 0.01 M PBS (pH 7.4), and then dried in a vacuum at RT.

Oligo Immobilization on NCS Beads Probe A or Cy5-tagged probe solution containing 5 μM oligonucleotides (for generate low probe density NCS beads) or 10 μM oligonucleotides (for general high probe density NCS beads) in 20 μl 0.05 M, pH 8.5 sodium borate buffer, was added to 2 mg activated beads. The beads were shaken overnight at RT in the dark, then washed five times with distilled water and then dried in a vacuum.

DNA Probe Density on Glass Beads

To estimate the DNA probe density on glass-bead surfaces, 5′-amino/3′-Cy5 probes were immobilized on beads by the same method used in 5′-amino oligo probe A immobilization. For control experiment, without adding activated beads to the immobilization solution containing Cy5-tagged Probe, was for the measurement of the initial concentration of Cy5-tagged Probe. The average probe density on different activated glass beads was calculated over three repeated bead experiment under the same conditions. The total number of DNA probes immobilized on beads was estimated by observing the fluorescence intensity change in the DNA probe solution through the immobilization process. The difference was due to the immobilization of the probes on the bead surfaces. A ND-1000 Spectrophotometer (NanoDrop Technologies, Wilmington) with an excitation wavelength of 635 nm and an emission wavelength of 650 nm was used. Because of only 1–2 μl sample volume was needed for the spectrophotometer, the supernatant of Cy5-tagged Probe solution was collected and dried in a vacuum at RT and then diluted with 2.5 μl distilled water. The probe density on the bead surfaces was estimated from the decreased amount of DNA probes in the solution and the total surface area of beads.

The average diameter of a bead was 250 μm, which is the average surface area of a glass bead (S_1) was 1.96×10^{-3} cm^2. The number of beads in the array (N) could be counted through the photograph taken by the scanner (Gene Pix 4000B, Axon). The total surface area of glass beads (S_2) was supposed to be $N\times S_1$. Finally, the DNA probe density on glass-bead surfaces was evaluated by dividing the number of the Cy5-tagged probes to the bead surface by the estimated surface area of beads.

$$\text{Probe density}\left(\text{probe}/\text{cm}^2\right)=\frac{(C_1-C_0)\times V\times N_A}{M\times S_2} \quad (1)$$

in which, C_1 = final concentration (ng/μl), C_0 = initial concentration (ng/μl), V = volume of sample, 2.5 μl, M = molecular weight of Cy5-tagged probe, N_A = the Avogadro constant, 6.02×10^{23} mol^{-1}, S_2 = total surface area of glass beads.

DNA Hybridization

Hybridization was performed as follows: 0.5 mg of beads were immersed in a 4 × SSC–0.1% SDS buffer (4 μl) containing 1.25 μM target A or target B at 42°C for 1 h. After hybridization, the beads were washed sequentially one time with 1 × SSC–0.03% SDS, 0.2 × SSC, and 0.05 × SSC. They were then dried in a vacuum before fluorescence intensity measurement.

Fluorescence Intensity Detection

The fluorescence intensity of each bead is measured with Gene Pix 4000B (Axon). The beads were placed on a non-fluorescence adhesive tape attached on a glass slide. The slide was scanned with the scanner.

The hybridization fluorescence intensity or the Cy5-tagged probe fluorescence intensity on a bead was represented by the average signal from the bead surface subtracted by the signal from the bead before the hybridization experiment or before the probe immobilization experiment. The average and standard deviation were calculated over the fluorescence intensity of 15 beads under the same experiment conditions.

Results and Discussions

Optimization of Glass Beads Silanization

Silanization was a crucial step for chemical modification of glass beads. In order to achieve a better efficiency of glass beads silanization, various solvents for the silanization reaction were tested, such as water, methanol, anhydrous ethanol, 95% ethanol/water, acetone, acetate, and anhydrous toluene; TNBS test were employed to determine the quantity of amino group on the glass beads, which were induced by silanization reaction. Usually, the deeper orange color indicated more amino groups on the surface of glass beads. Here, it had been found that anhydrous toluene appeared the best stability (data were not shown) among all of those solvents. Therefore, five different concentrations of APTMS in anhydrous toluene were further tested. As shown in Fig. 2, the color gets darker when the concentration of APTMS increased from 1% to 2%, while the color turned into lighter orange when the concentration intermittently increased to 3%, 5%, and 10%. Thus, among

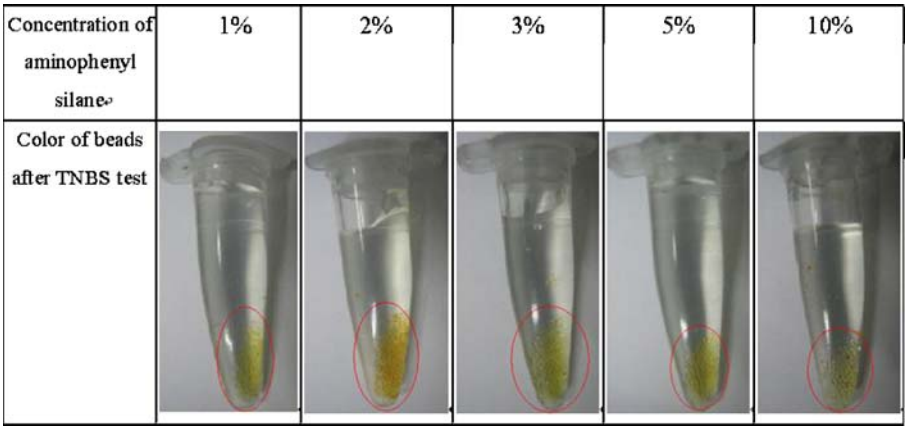

Fig. 2 Pre-cleaned beads were immersed in a 1%, 2%, 3%, 5%, 10% concentration of APTMS in anhydrous toluene for 5 h and then tested with TNBS solution in 200 μl tubes for 2 h. They were washed five times with distilled water

all these samples examined, 2% APTMS in anhydrous toluene was the best choice for the silanization solution.

Oligonucleotides Probe Density on Different Chemical Modification Beads

There were lots of oligonucleotide immobilization strategies. Here, three different chemically modified beads, aldehyde-activated bead (CHO bead), carboxyl bead (COOH bead) and isothiocyanate-activated bead (NCS bead) were fabricated because they were used most frequently in amino-terminal oligonucleotide immobilization. To choose the best strategy for amino-terminal oligonucleotides covalent attachment onto glass beads, oligonucleotide densities on these three kinds of activated beads were determined by the method mentioned in "Materials and Methods." The higher the densities, the more efficient the immobilization strategy was.

According to the Eq. 1, oligonucleotide densities on these three kinds of activated beads were calculated, which were 2.6×10^{13} probes/cm^2 for CHO beads, 3.2×10^{13} probes/cm^2 for COOH beads, and 6.7×10^{13} probes/cm^2 for NCS beads. In other words, the order of beads for efficient oligonucleotide immobilization was NCS beads > COOH beads > CHO beads. The fluorescence intensity of these different beads, which immobilized with Cy5-tagged oligonucleotide, further confirmed this result (as indicated in Fig. 3).

Several other chemically covalent strategies and corresponding immobilized probe densities were also studied by other groups. For example, Kohara et al. [5] immobilized 5′ thiol-modified 18-mer oligonucleotide probe onto maleimide-modified glass bead surface and observed a density of 1.8×10^{13} probes/cm^2. Rogers et al. [17] attached 5′ disulfide-modified 26-mer oligonucleotides to the surface of mercaptosilane-modified glass slide and observed the density of 3.0×10^{13} probes/cm^2. Fixe et al. [25] immobilized 5′ NH2 or SH groups modified 18-mer oligonucleotides on chemically aminated PMMA substrates and observed an immobilized density of 6.0×10^{12} probes/cm^2. It meant that the immobilization efficiency based on NCS beads was acceptable, and the method, developed by attaching amino-terminal oligonucleotides onto these cyanate active beads, could be regarded as an efficient one for immobilizing oligonucleotides onto a solid surface.

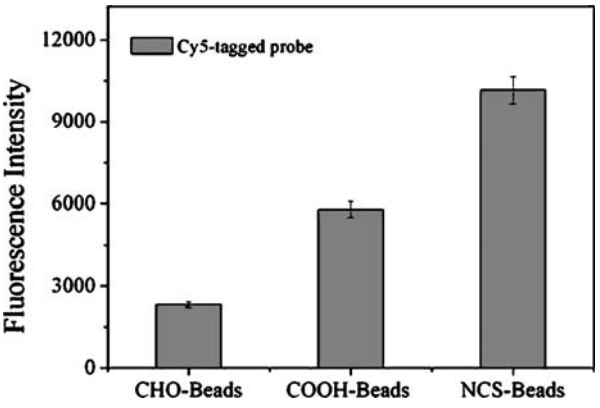

Fig. 3 Mean fluorescence intensity of different activated beads attached with Cy5-tagged probe. The order of beads for efficient oligonucleotide immobilization was NCS beads > COOH beads > CHO beads. 5 μM Cy5-tagged probe in immobilization solutions. The fluorescent intensity of beads was measured with Gene Pix 4000B and was represented by subtracting the average signal before immobilization

The Effect of Different Oligonucleotide Densities on NCS-bead Base Hybridization

Surface hybridization depends strongly on probe density. The hybridization efficiency was studied between two different oligonucleotide densities of NCS beads. Briefly, NCS beads were immersed in 5 and 10 μM probe A solutions, and shaken overnight. Afterwards, oligonucleotide densities of NCS beads were determined by the method mentioned above. It was found that the density was 6.7×10^{13} probes/cm^2 for NCS beads immersed in 5 μM probe A solution, and the density was 4.6×10^{14} probes/cm^2 when beads were kept in 10 μM probe A solution.

To our surprise, the high oligonucleotide density on the surface of NCS beads did not lead to high hybridization efficiency. In Fig. 4, only about 50% fluorescence intensity was observed for beads with the density of 4.6×10^{14} probes/cm^2 when compared with 6.7×10^{13} probes/cm^2 of beads. It seemed that only about 50% oligonucleotide on the surface was available for high-density oligonucleotide beads. We believed that this was possibly due to steric crowding. On the other hand, non-complementary oligonucleotide targets were tested, and very low hybridization signals were observed. It meant that hybridization based on oligonucleotide beads was very specific.

Equilibrium Binding Isotherms Studied for Low Probe Density NCS Bead

Since 6.7×10^{13} probes/cm^2 of oligonucleotide density beads showed better hybridization efficiency, they were chosen for equilibrium binding isotherms studies. We hypothesized that all probe sites were energetically equivalent, independent, and available for binding; the measured saturation level for target binding was equal to the coverage of immobilized probe molecules. The data obtained from Fig. 5 were fit to Eq. 2, which was the well-known Langmuir model.

$$\Gamma_{\text{target}}=\Gamma_{\max}\frac{K_{\text{A}}\bullet C}{1+K_{\text{A}}\bullet C} \tag{2}$$

where Γ was fractional coverage of target binding sites on the probe-attached NCS beads, namely hybridization efficiency, $\Gamma_{\max}$, fully coverage of target binding sites at saturation, K_{A} was the equilibrium binding constant, C was the concentration of target in solution. In this work, the equilibrium binding constant, K_{A} was determined from isotherms to be 7.0×10^6 M^{-1}.

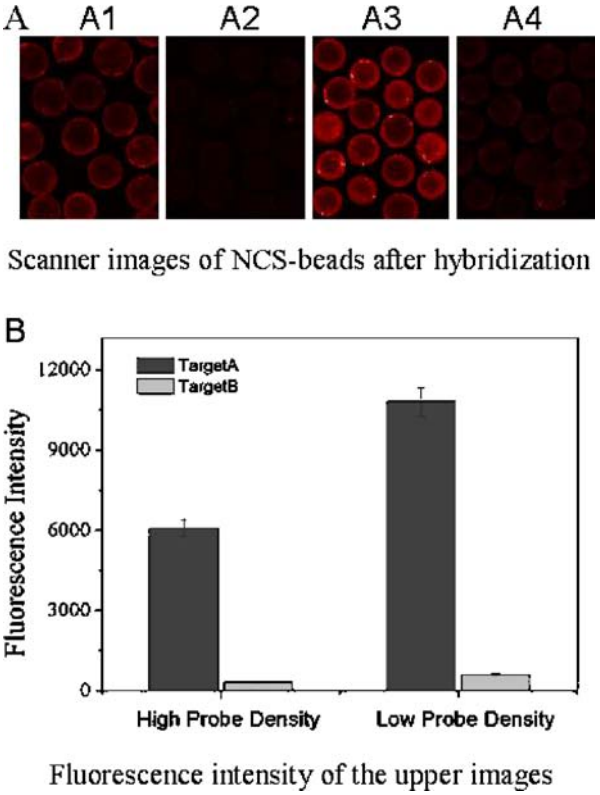

Fig. 4 Comparison of hybridization fluorescence intensity for complementary targets (target A) and non-complementary targets (target B) tagged with Cy5 onto probe A-attached NCS beads of two different surface densities. **a** Image *A1*, high probe density NCS-beads hybridized with target A; image *A2*, high probe density NCS-beads hybridized with target B; image *A3*, low probe density NCS beads hybridized with target A; image *A4*, low probe density NCS beads hybridized with target B. They were measured with Gene Pix 4000B (Axon). **b** Fluorescence intensity of corresponding images. Several NCS beads immersed in 5 μM Probe A solution shaken overnight derived low probe density NCS beads (6.7×10^{13} probes/cm^2) and in 10 μM Probe A solution derived high probe density NCS beads (4.6×10^{14} probes/cm^2). Hybridization was performed at 42°C for 60 min and the concentration of targets tagged with Cy5 was 1.25 μM

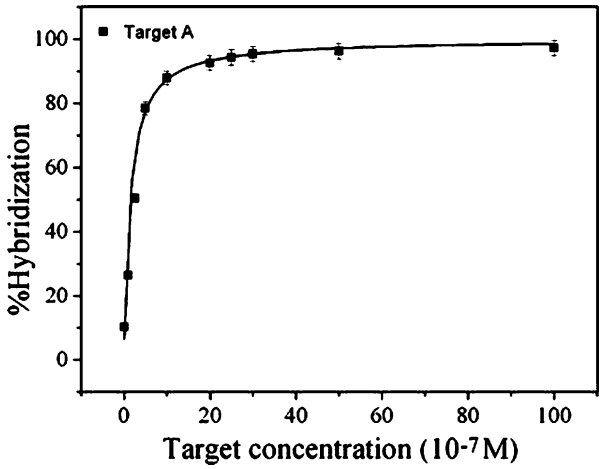

Fig. 5 Equilibrium binding isotherm for hybridization of complementary target A. Low probe density (6.7×10^{13} probes/cm^2) of probe A-attached NCS beads was used. Hybridization efficiency was determined on the basis of fluorescence intensity as compared to intensity at saturation. Langmuir isotherm (*solid line*) fits to the equilibrium binding isotherm with $\Gamma_{max}=100\%$, yield binding constants, K_A, equaled to 7.0×10^6 M^{-1}. Hybridization was performed at 42°C for 60 min. The concentration of target A tagged with Cy5 was varied from 0.1×10^{-7} to 100×10^{-7} M. The sample volume was 4 μl. The error bars are the 98% confidence interval

Because of differences in immobilization strategies, the limited availability of probe density data and the different measurement of hybridization signals, it is difficult to make directly comparisons between the literatures. However, our equilibrium binding constant is in general agreement with the results of other research groups. Nelson et al. [26] reported $K_A=1.8\times10^7$ M^{-1} for a perfectly matched 18-mer DNA oligonucleotide duplex on a gold substrate using SPT measurement. Okahata et al. [27] reported $K_A=1.2\times10^6$ M^{-1} for a perfectly complementary 10-mer oligonucleotides immobilized on a 27-MHz quartz crystal microbalance (QCM).

Hybridization Time

Figure 6 showed the time required for NCS bead-based hybridization. It was found that the fluorescence intensity reached a plateau within 60 min for a complementary target (target A); whereas the fluorescence intensity for a non-complementary target (target B) was very low, even 240 min given to reaction.

It seemed that beads appeared to have higher rapid hybridization rate than slide-based microarray. Usually, traditional slide arrays needed several hours for hybridization. Therefore, the method developed in this paper can be applied to some fast detection systems.

Conclusion

Glass beads with average diameter 250 μm have been chosen as support materials. The glass beads were amino-silane modified with APTMS first. In this step, we optimized the silanization conditions and found that 2% APTMS in anhydrous toluene was the best choice for APTMS silanization of glass beads. For immobilizing 25-mer amino-terminated oligonucleotides, the glass surface was required further chemical modification. And three different reagents, glutaraldehyde, succinic anhydride, and 1,4-phenylene diisothiocyanate, were used to derive CHO beads, COOH beads and NCS beads, accordingly. These three covalent attachment strategies of amino-terminated oligonucleotides were the most commonly used in bead array fabrication. We found out that the NCS beads provide the

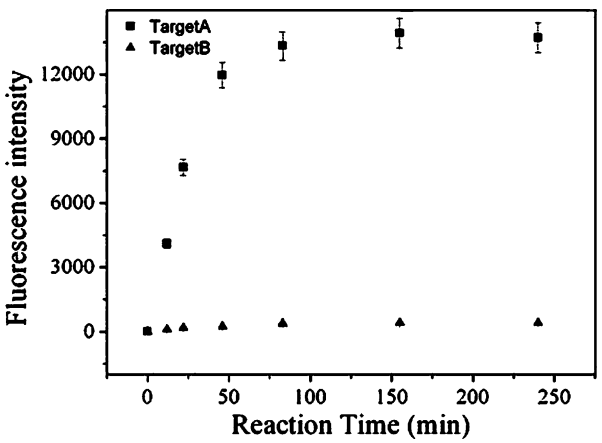

Fig. 6 Time course of NCS bead-based hybridization. Low probe density (6.7×10^{13} probes/cm^2) of probe A-attached NCS beads was used. Hybridization was performed at 42°C and the concentration of targets tagged with Cy5 was 1.25 μM. The sample volume was 4 μl. The reaction time was varied from 0 to 240 min

best chemical support surface for attaching oligonucleotides when comparing immobilized oligonucleotide probe binding densities. Thus, the strategy of immobilization amino-terminated oligonucleotide probe onto NCS beads was the best method to generate highest attaching efficiency. Subsequently, we studied the hybridization properties of this best attachment strategy on NCS beads. We found that the probe density on glass beads surface affected the hybridization, and high probe density NCS beads (4.6×10^{14} probes/cm^2) appeared to have low hybridization efficiency, maybe relating the existence of steric crowding. Therefore, low oligonucleotide density NCS beads were used in equilibrium binding isotherms studies, and the equilibrium binding constant, K_A, was determined to be 7.0×10^6 M^{-1} by employing the Langmuir isotherm model, which was in general agreement with the results of other research groups. It was also found that the hybridization showed rapid rate and was finished within 60 min.

As it was well known, small microspheres (low-μm to sub-μm diameters, <10 μm) have been used as solid supports in bead array assays based on flow cytometry or microfluidic system known as suspension array technology (SAT) [28]. However, MEMS technology and flow cytometry equipment are expensive and inconvenient on a routine basis. The mesofluidic systems for automated heterogeneous assays using large microspheres (>100 μm) but not employing flow cytometric detection would provide an economical and more convenient alternative for high throughput bioassay development. We expect the three covalent attachment strategies and methods of NCS bead-based immobilization and hybridization can accelerate development and industrialization of bead-based mesofluidic systems.

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