DNA hybridization on microparticles: determining capture-probe density and equilibrium dissociation constants

Priscilla Wilkins Stevens¹,²⁺, Michael R. Henry¹ and David M. Kelso¹

¹Department of Biomedical Engineering, Robert R. McCormick School of Engineering and Applied Science, Northwestern University, 2145 Sheridan Road, Evanston, IL 60208, USA and ²Center for Mechanistic Biology and Biotechnology, Argonne National Laboratory, 9700 South Cass Avenue, Argonne, IL 60439, USA

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ABSTRACT

Many DNA-probe assays utilize oligonucleotide-coated microparticles for capture of complementary nucleic acids from solution. During development of these assays, as well as in other particle-based nucleic acid applications, it is useful to know both the amount of duplex formation expected under various experimental conditions and the coating density of the capture oligonucleotide on the particle surface. We examined the simplest form of a DNA-probe microparticle assay: hybridization of a particle-bound capture oligonucleotide to its solution-phase complement. Fluorescein-labeled solution-phase oligonucleotide was hybridized to varying amounts of particles, and the amount of labeled oligonucleotide remaining in solution at equilibrium was measured. We present a simple two-state, all-or-none model for bimolecular hybridization of non-self-complementary sequences that can be used to calculate the equilibrium dissociation constant ($K_d$) from hybridization data. With experimental conditions where both the $K_d$ value and the concentration of capture probe in the reaction are small relative to the concentration of labeled complementary oligonucleotide in the reaction, density of the capture probe on the particle’s surface can also be determined. $K_d$ values for particle-based hybridization were different from those obtained from solution-phase thermodynamic parameters. At higher temperatures, hybridization on particles was more efficient than hybridization in solution.

INTRODUCTION

Recently oligonucleotides attached to solid supports such as microparticles and microchips have been utilized for a wide variety of applications including detection of genetic polymorphisms (1–3), disease screening and diagnosis (4–7), purification of biomolecules (8–11), monitoring gene expression (12,13), genome analysis (14–16), gene mapping (17) and nucleic acid sequencing (18,19). Although both microparticles and microchips make use of immobilized oligonucleotide sequences, modern DNA microchip technology generally employs a number of different oligonucleotide probes attached at discrete locations to a single support surface (20,21). Microparticles, on the other hand, are most often coated uniformly with a single oligonucleotide species (‘capture probe’) complementary to the nucleic acid of interest (‘target’) (22). Theoretical aspects of microparticle- and microchip-based hybridization of nucleic acids are just beginning to be elaborated (23–27).

Our research addresses two specific issues of DNA hybridization on microparticles: first, establishing a general method for quantitative comparison of microparticle-based duplex formation, and second, developing a reliable, straightforward method for determining the density of capture-probe oligonucleotide on the microparticle surface. The equilibrium dissociation constant, $K_d$, is an excellent parameter for quantitatively comparing hybridization on microparticles. Once this value has been determined at a given temperature for hybridization in a defined buffer system, the amount of duplex formed at equilibrium can be predicted for hybridization reactions containing varying amounts of particles or solution-phase oligonucleotide.

As an initial effort toward quantitative characterization of duplex formation in microparticle assays, we examined hybridization equilibria for a very basic experimental system: hybridization of particle-bound 22mer oligonucleotide capture probe to its fluorescein-labeled complement in solution. Applying a simple two-state, all-or-none model for bimolecular hybridization of non-self-complementary partners, we defined the fraction of labeled oligonucleotide remaining in solution at equilibrium, $f$, in terms of two unknowns: the equilibrium dissociation constant, $K_d$, and the surface density of capture probe on the particle surface. This simple mathematical model provides a reasonable first step for estimating equilibrium dissociation constants for microparticle-based duplex formation.

Due to their widespread use in screening assays and robotic applications, paramagnetic microparticles were chosen as the solid-phase material for our studies, even though we did not specifically utilize their magnetic-capture capability. For the solution-phase target, we selected a fluoresceinated oligonucleotide. Although radiolabeled target molecules have traditionally been

*To whom correspondence should be addressed at: Department of Biomedical Engineering, Robert R. McCormick School of Engineering and Applied Science, Northwestern University, 2145 Sheridan Road, Evanston, IL 60208, USA. Tel: +1 847 467 2560; Fax: +1 847 491 4928; Email: pwilkins@nwu.edu
used to monitor binding of target to the solid phase, we were unable to make satisfactorily accurate binding measurements with a $^{32}$P-labeled target oligonucleotide. Even when we used a vast excess of particles at room temperature to try to deplete all $^{32}$P-labeled target oligonucleotide from solution, a high proportion of the target (typically ~10–20% of the starting amount) remained unbindable, presumably due to autodigestion degradation. With a fluorescein-labeled target oligonucleotide, however, the unbindable fraction was negligible, typically <2%.

Hybridization reactions were conducted with the same amount of fluorescein-labeled target oligonucleotide. The amount of fluorescein-labeled oligonucleotide remaining in solution at equilibrium was measured for each reaction and expressed as a fraction of the original concentration of labeled oligonucleotide. This approach avoided the potential difficulty of quenching of the fluorescence signal on the solid phase, which could confound measurements of particle-bound target. In addition, since the solution phase was used for measurements, this approach is generally applicable, not only for characterization of microparticle-based hybridization, but also for evaluation of hybridization on other types of solid-phase materials.

When measurements were made under appropriate experimental conditions, the maximum binding capacity of the particle for fluorescein-labeled oligonucleotide complementary to the capture probe could be calculated. This value for the surface density of capture probe was determined prior to estimation of $K_d$ and the value was then considered fixed in subsequent experiments to determine $K_d$ values, since the same lot of particles was used for all experiments.

**MATERIALS AND METHODS**

**Materials**

Capture-probe oligonucleotide was synthesized with a 5′ six-carbon long-chain amino linker (lca-CP) and purified by polyacrylamide gel electrophoresis at Chiron Corporation (Emeryville, CA). Seradyn paramagnetic latex particles, 0.75 μm in diameter, density 1.3 g/cm³, were coupled to a Jeffamine triethylen glycol diamine spacer (Huntsman Corporation, Houston, TX) with 1-ethyl-3-(3-dimethylaminopropyl)-carbodiimide hydrochloride (EDC; Pierce, Rockford, IL). Particles with gel-purified lca-CP attached to the Jeffamine spacer via a bis(sulfosuccinimidyl)suberate crosslinker (BS3; Pierce, Rockford, IL) were obtained from Chiron Corporation (lot 120C). The particle-bound capture probe oligonucleotide (p-CP) was separated from the particle surface by approximately 30 atoms in the long-chain linker, the BS3 crosslinking agent and the Jeffamine spacer.

A 5′-fluorescein-labeled oligonucleotide complementary to the capture probe ($fl$-CPc) was synthesized and purified by polyacrylamide gel electrophoresis at Promega Corporation (Madison, WI). Sequences of the oligonucleotides are 5′-lca-CAC TTC ACT TTC TTT CCA AGA G-3′ for lca-CP and 5′-fluorescein-CTC TTG GAA AGA AAG TGA AGT G-3′ for $fl$-CPc. Oligonucleotide concentrations were determined from extinction coefficients (260 nm) based on values in (28) as calculated by the program Extinct (kindly provided by Professor Robert Letsinger’s laboratory): 198.9 mM-cm for lca-CP and 226.5 mM-cm for both $CPc$ and $fl$-CPc.

Buffer A, 10 mM sodium phosphate, pH 7.0, 0.1 M sodium chloride, 0.1% lithium dodecyl sulfate was the particle hybridization buffer. The detergent lithium dodecyl sulfate was added to facilitate particle handling and to eliminate non-specific hybridization to the particles. Without detergent in the buffer, particles were not monodisperse, did not form a compact pellet during centrifugation, and yielded anomalous hybridization results. The hybridization buffer for thermal melting temperature studies was Buffer B: 10 mM sodium phosphate, pH 7.0, 0.1 M sodium chloride. To test fluorometer sensitivity, Buffer C, 50 mM carbonate buffer, pH 9.5, was used for fluorescein dilutions.

Chemicals were obtained from Sigma (St Louis, MO). Particle assays were conducted in 0.65 ml polyethylene Clickseal microfuge tubes. Fluorometric signals were measured in 6 × 50 mm Kimble disposable borosilicate glass culture tubes.

**Fluorometer**

In order to conserve reagents, each hybridization was conducted in a reaction volume of 220 μl. A fluorometer suitable for analysis of fluorescein in small sample volumes was therefore required for measuring fluorescence of experimental samples. While a number of commercial microplate-format fluorimeters or other small-volume fluorometers would be suitable, we constructed a fluorometer capable of analyzing samples with volumes of 150 μl. Ten mW, 488 nm light from an argon ion laser (Ion Laser Technology, model 5000) used as an excitation source was attenuated with a prism and steered with mirrors toward the sample tube. Fluorescence emission from the sample was collected with a lens, and the light was focused onto a photon-counting photomultiplier tube (Hamamatsu HC 135-01) interfaced to a Compaq Deskpro computer via the RS-232 serial port. Stray light was removed with a 535 ± 8 nm interference filter. A data acquisition program for the photomultiplier tube was written in Visual Basic.

Sensitivity, determined by the inverse slope of the best-fit line in the regression analysis, was 2.6 × 10⁻¹² and 2.0 × 10⁻¹² M/mv for fluorescein standards in Buffers A and C, respectively. For the fluorescein-labeled oligonucleotide in Buffer A, sensitivity was 8.4 × 10⁻¹³ M/mv. Detection limits (the concentration corresponding to the average signal of the buffer only plus three times the buffer-only standard deviation) were 2.4 × 10⁻¹² and 2.5 × 10⁻¹³ M for fluorescein in Buffers A and C, respectively, and 9.1 × 10⁻¹² M for $fl$-CPc in Buffer A.

**Determination of time required for particle hybridizations to reach equilibrium**

To determine the time required for equilibration of the particle hybridization reactions, multiple reaction tubes were prepared with 200 μl of $fl$-CPc diluted to a single concentration (either 10⁻⁸ or 10⁻⁹ M) in Buffer A and incubated at the given temperature. To each tube was added 20 μl of 350 μg/ml particles (or 35 μg/ml particles for one 20°C study) or 20 μl Buffer A for no-particle controls. Tubes were quickly mixed and immediately returned to the given temperature. At 50°C, reaction mixtures were incubated in a hot water bath from 5 min to 6 h, with tubes manually inverted approximately every 30 min to prevent particle settling. At 40°C, tubes were incubated on a rotator in a hot-air incubator from 10 min to 24 h. The 20°C reactions were incubated on a bench-top rotator for times ranging from 7.5 min to 5 h or from 1 to 7 days.

At various time points, replicate tubes were removed and immediately centrifuged for 4 min at speed 4 in an Eppendorf 5415C microfuge. Centrifugation was used to separate particles.
from unhybridized fluoresceinated oligonucleotide remaining in solution because the separation was accomplished quickly, within the first few seconds. (Magnetic separation of particles from solution-phase labeled oligonucleotide is a slower process and is recommended only when magnetic separation can be performed at the same temperature as the hybridization reaction.) From each tube, 150 µl supernatant containing unhybridized \( \beta \)-CPc was transferred to a glass tube and read with fluorometer excitation and emission wavelengths of 488 and 535 nm, respectively. Standard curves were generated from known concentrations of \( \beta \)-CPc and buffer-only controls. The time point at which measurements were no longer statistically different from subsequent measurements was considered the time required for equilibration of those concentrations at the given temperature. Where the length of time between two time points was large, a time range is listed for the required equilibration time.

Hybridization reactions with varying particle concentrations

To determine capture probe surface density, we mixed 200 µl of \( 10^{-8} \) or \( 2 \times 10^{-9} \) M \( \beta \)-CPc in Buffer A with 20 µl particles at concentrations of 28, 14, 7.0 or 3.5 µg/ml for the higher level of target with additional concentrations of 1.8, 0.9 and 0.4 µg/ml for the lower target concentration. The no-particle control included 200 µl \( \beta \)-CPc and 20 µl Buffer A. Reaction tubes were incubated at 20°C for 2 h, 5 h or 2 days. Reaction mixtures incubated for 2 h were inverted every 30 min to prevent particle settling, while reactions incubated for 5 h or 2 days were continuously rotated. At the end of each incubation period, tubes were centrifuged and supernatant samples were transferred to glass tubes. After samples equilibrated to room temperature, fluorescence signals of the supernatants were measured as described above.

For \( Kd \) estimation, 200 µl \( \beta \)-CPc diluted to \( 10^{-8} \) or \( 2 \times 10^{-9} \) M in Buffer A were mixed with 20 µl particles in the same buffer at concentrations ranging from 2.5 mg/ml to 39 µg/ml (in 2-fold dilutions) or with 20 µl Buffer A for the no-particle control. The final concentration of \( \beta \)-CPc in each assay was \( 9.1 \times 10^{-9} \) or \( 1.8 \times 10^{-9} \) M, while final particle concentrations ranged from 50 to 0.78 µg/reaction mixture. Reaction tubes with the higher concentration of target were incubated for 2 days at 20°C, 5 h at 50°C, 7 h at 55°C and 2 h at both 60 and 70°C. Tubes with the lower concentration of \( \beta \)-CPc were incubated for 2 days at 20°C, 5 h at 60°C and 2.5 h at 70°C. For temperatures \( \geq 50°C \), tubes were incubated in a hot water bath and inverted approximately every 30 min to prevent particle settling. For room temperature (20°C) studies, reaction tubes were rotated continuously. At the end of each incubation period, tubes were centrifuged and fluorescence signals of supernatants measured, as described above.

Thermal denaturation analyses

Optical melting curves were measured for four concentrations of \( \text{lca-CP} \) and \( \text{CPc} \), ranging from \( 2.5 \times 10^{-6} \) to \( 3.2 \times 10^{-7} \) M total oligonucleotide/reaction. Equimolar amounts of the two oligonucleotides were mixed in Buffer B, denatured at 85°C for 15 min, and then reannealed during a slow reequilibration to room temperature. The hybrid was subjected to temperatures from 25 to 85°C in one-degree steps, allowing 2 min equilibration time per step. Measurements were made at 260 and 280 nm with a Perkin Elmer (Norwalk, CT) UV/VIS Lambda 2 spectrophotometer equipped with a PTP-1 Peltier temperature programmer and cooled with recirculating water from an ice-water bath. Identical melting curves were obtained for a sample analyzed with both 1 and 2 min equilibration times; we are therefore confident that the 2 min equilibration time was sufficient for attaining equilibrium at all temperatures tested (P.Wilkins Stevens, unpublished results).

Data analysis

Microsoft Excel for Windows 97 (version SR-1) was used on a Gateway2000 computer for data analysis and spreadsheet calculations. The Solver tool in Microsoft Excel, which uses a generalized reduced gradient algorithm (29) to find parameter values that optimize an objective function, was employed to fit a two-state model of hybridization to experimental binding data. Optical melting curve data were analyzed with the Meltwin program (30), assuming a two-state, all-or-none transition of a bimolecular interaction of two non-self-complementary oligonucleotides. The Meltwin program calculated thermodynamic parameters \( \Delta H^\circ \) and \( \Delta S^\circ \) both from individual melting curves (31) and from a plot of the reciprocal of melting temperature \( (1/T_m) \) versus \( C_T/4 \) (32). The melting temperature, \( T_m \), is the temperature at which half the hybrids remain double stranded and half are dissociated; \( C_T \) is the total concentration of oligonucleotide strands in the reaction. Nearest neighbor parameters (33) were incorporated into a spreadsheet for calculating thermodynamic parameters from oligonucleotide sequences. Corrections for salt concentration were applied as in SantaLucia et al. (34,35).

RESULTS

Fluorescence signal as a function with two unknowns: particle capture-probe density and \( Kd \)

As a simple approximation of the hybridization reaction, we assumed that hybridization of the particle-bound capture probe (\( p-CP \); designated \( P \) in the equations) and the solution-phase fluorescent complement (\( \beta \)-CPc; designated \( F \) in the equations) could be represented as a two-state, all-or-none transition of a bimolecular interaction of two non-self-complementary oligonucleotides:

\[
[P] + [F] \longleftrightarrow [P:F]
\]

where \( P:F \) represents the particle-bound hybrid. At any given temperature, the equilibrium dissociation constant describing the hybridization of \( \beta \)-CPc to particle-bound \( CP \) is defined by:

\[
Kd = \frac{[F][P]}{[P:F]}
\]

Since \( \beta \)-CPc is free in solution while \( p-CP \) and hybrids are particle-bound, concentrations of \( \beta \)-CPc are expressed as moles per volume (mol/ml), while concentrations of \( p-CP \) and the hybrid are expressed as moles per surface area (mol/cm²). Mass-balance equations therefore take into account the assay volume, \( V \) (ml), and the total particle surface area in the assay, \( A \) (cm²):

\[
\]

\[
\]

Concentrations marked with a zero subscript represent the initial concentration of each oligonucleotide partner. Rearrangement of
these mass-balance equations, and substitution into equation 2 yields:

\[ K_d = \frac{[F]}{[F]_0} \left( \frac{[P]_0}{[F]_0} - \frac{1}{c} \right) \]

Equation 5 can be converted into a dimensionless form by dividing through by V/A and the initial concentration of fl-CPc to give:

\[ \frac{K_d}{[F]_0} = \frac{[F]}{[F]_0} \left( \frac{[P]_0}{[F]_0} - \frac{1}{c} \right) \]

Definition of dimensionless variables \( k, f \) and \( s \) that express the parameters as fractions of the original concentration of fluorescent oligonucleotide:

\[ k = \frac{K_d}{[F]_0} \]

\[ f = \frac{[F]}{[F]_0} \]

\[ s = \frac{A[P]_0}{V[F]_0} \]

leads to:

\[ k = \frac{f(s - (1 - f))}{1 - f} \]

This is a quadratic equation in \( f \):

\[ f^2 + (k + s - 1)f - k = 0 \]

whose physically meaningful solution is:

\[ f = \frac{-(k + s - 1) + \sqrt{(k + s - 1)^2 + 4k}}{2} \]

Assuming spherical particles, a particle’s area \( (a) \) and volume \( (v) \) can be calculated from its diameter \( (d) \):

\[ a = \pi d^2 \]

\[ v = \frac{4}{3} \pi d^3 \]

Particle density \( (\rho) \) is equivalent to particle weight \( (w) \) divided by particle volume \( (v) \):

\[ \rho = \frac{w}{v} = \frac{6w}{\pi d^3} \]

Since particle density and diameter are known, the relationship between particle area and particle weight is:

\[ \frac{a}{w} = \frac{6}{\rho d} \]

As long as particles are of uniform size, the same relationship holds for total surface area \( (A) \) and total particle weight \( (W) \) of a given number of particles. Substitution of this relationship into equation 11 gives:

\[ s = \frac{W}{V} \frac{6}{\rho d} \frac{[P]_0}{[F]_0} = c \cdot z \]

where \( z \) is the weight of particles per unit volume \( (W/V) \), which is known, and \( c \) is given by:

\[ c = \frac{6}{\rho d} \frac{[P]_0}{[F]_0} \]

The parameter \( c \) contains the known quantities \( \rho, d, [F]_0 \), as well as the surface concentration of the capture probe, \( [P]_0 \), which can be determined experimentally (see ‘Calculation of surface density of particle-bound capture probe’).

Rewritten in terms of these parameters, the experimentally measured fraction of labeled oligonucleotide remaining in solution at equilibrium, \( f \), is:

\[ f = \frac{-(k + c \cdot z - 1) + \sqrt{(k + c \cdot z - 1)^2 + 4k}}{2} \]

When the parameters \( c \) and \( k \) are estimated by fitting the above function to the observed data, as described below, capture-probe density can be calculated from \( c \) and \( K_d \) from \( k \). With the units identified above for each variable, the parameter \( k \) yields \( K_d \) with units of mol/ml, which can be easily converted to molarity, the standard unit for expression of \( K_d \) values.

**Calculation of surface density of particle-bound capture probe**

Examination of equation 19, which defines \( f \) in terms of the parameters \( k, c \), demonstrates that for \( k \) values very much less than 1, the value of \( k \) does not have much effect on the value of \( f \). The statement \( k \ll 1 \) is equivalent to \( K_d \ll [F]_0 \), i.e., the equilibrium dissociation constant is much less than the concentration of fluorescein-labeled target oligonucleotide (see equation 7). In that case, when the concentration of particles is high, i.e., \( c \cdot z > 1 \), essentially all of the added fl-CPc hybridizes to particle-bound capture oligonucleotide. When the ratio of particle-bound capture probe to solution-phase complement is less than one, however, i.e., \( c \cdot z \ll 1 \), there is not enough capture probe to combine with all the complement. The response is a straight line, which is insensitive to values of \( k \). Formally these relationships are stated: When \( k \ll 1 \),

\[ f = 0 \quad \text{for} \quad c \cdot z > 1 \]

\[ f = 1 - c \cdot z \quad \text{for} \quad c \cdot z \leq 1 \]

For small \( k \) values, then, at low concentrations of particles, the amount of fl-CPc depleted from the supernatant is directly proportional to the concentration of particles in the reaction. Extrapolation of this straight-line relationship to the horizontal axis (i.e., the value of \( z \) where \( f = 0 \)) indicates the concentration of particles required for complete depletion of labeled oligonucleotide from the supernatant. At this concentration of particles \( (c f = 0) \), the amount of p-CP in the reaction would be equivalent to the total amount of fl-CPc in the reaction. Combining equation 21 with equation 18 above, the total concentration of particle-bound capture probe can be calculated in terms of moles of oligonucleotide per surface area of particles:

\[ [P]_0 = [F]_0 \frac{\rho d}{6c [f = 0]} \]
Calculation of \( K_d \) values

For each temperature investigated, \( K_d \) values were calculated based on experiments in which different concentrations of particles were each hybridized to a single concentration of fluorescein-labeled complementary oligonucleotide. The fluorescein-labeled oligonucleotide remaining in solution at equilibrium was measured and expressed as \( f \), the fraction of the initial concentration of labeled oligonucleotide. For each data set, we estimated the parameter \( k \) (defined in equation 7) by fitting the quadratic function for \( f \) (equation 19) to the observed data using the Solver tool of Microsoft Excel for curve fitting. The objective function minimized was the sum of squares of the differences between observed and calculated \( f \) values.

Predicted hybridization curves for various experimental conditions

Figure 1 illustrates hybridization curves predicted by equation 19 for equilibrium \( K_d \) values from \( 10^{-7} \) to \( 10^{-12} \) M. The calculations assume a reaction volume of 0.22 ml with an initial concentration of fluorescein-labeled target of \( 10^{-8} \) M (Fig. 1A and C) or \( 10^{-9} \) M (Fig. 1B and D). Particles are assumed to have diameter 0.75 \( \mu \)m and density 1.3 g/cm\(^3\), with the surface concentration of capture probe being either 3 pmol/cm\(^2\) (Fig. 1A and B) or 0.3 pmol/cm\(^2\) (Fig. 1C and D).

For any given \( K_d \) value, the initial slope of \( f \) as particle concentration increases is much steeper when \([\beta-CPc]\) is decreased 10-fold from \( 10^{-8} \) to \( 10^{-9} \) M (compare Fig. 1A and B, and C and D). When the particle coating is dense (\([p-CP]\) = 3 pmol/cm\(^2\)), \( K_d \) values spanning six orders of magnitude, from \( 10^{-6} \) to \( 10^{-11} \) M, can be distinguished with both initial concentrations of \( \beta-CPc \) (Fig. 1A and B). With the sparse particle coating, however, even the highest concentration of particles is insufficient to deplete \( >50\% \) of the \( \beta-CPc \) when the initial concentration is \( 10^{-8} \) M. In that case, only four orders of magnitude of \( K_d \) values can be distinguished, from \( 10^{-7} \) to \( 10^{-10} \) M (Fig. 1C). With the sparse particle coating and a 10-fold decrease in \([\beta-CPc]\), however, six orders of magnitude of \( K_d \) values are again discernible, in this case from \( 10^{-7} \) to \( 10^{-12} \) M (Fig. 1D).

Practical considerations for planning experiments

Practical aspects of experimental design limit both the range of concentrations that can be tested and the range of \( K_d \) values that can be measured. As illustrated in Figure 1, when particle capture-probe density is sparse and the concentration of fluorescein-labeled target oligonucleotide high, the large quantity of particles required for differentiation of \( K_d \) values is impractical (Fig. 1C). Lowering the concentration of target, however, allows discrimination of a larger range of \( K_d \) values within a reasonable range of particle concentrations (Fig. 1D). It is useful to prepare graphs of \( f \) (the fraction of labeled oligonucleotide remaining in solution at equilibrium) such as those in Figure 1 when evaluating what concentrations are reasonable to use or what \( K_d \) values are reliably measured.

Graphs of the function \( f \) do not tell the whole story, however. One might assume from Figure 1, for example, that experiments with low concentrations of labeled target oligonucleotide are in general the most practical. However, the use of low concentrations of target may be difficult due to decreased resolution or imprecision of measurement. In addition, the time required for microparticle-based nucleic acid hybridization reactions to come to equilibrium can be quite long, especially with low concentrations of oligonucleotides at low temperatures (where \( K_d \) is low). In all cases, preliminary experiments should be conducted to determine the equilibration time required for the particular experimental conditions (temperature and concentrations).

Fluorescence signals measured from experimental samples are converted to \( f \) using the signal generated from buffer-only samples as 0 and the signal from the starting concentration of fluorescein-labeled target oligonucleotide as 1. Accurate determinations of \([p-CP]\) and \( K_d \) therefore require that each experiment include sufficient replicates of both of these no-particle control samples so that signals corresponding to \( f = 0 \) and \( f = 1 \) can be known with precision.

Time required for microparticle hybridizations to reach equilibrium

DNA hybridization on microparticles is temperature-dependent, with reactions coming to equilibrium faster at high temperatures than at low temperatures. We determined the time required for
equilibration of hybridization reactions on our particles at three different temperatures: 40 and 50°C, temperatures which represent lower bounds for the temperature range at which microparticle DNA-probe assays are likely to be run, and 20°C, the temperature at which experiments for calculating \([p-CP]_0\) were conducted.

Times required for equilibration were determined from hybridization reactions containing 200 \(\mu\)l \(\beta\)-CPc diluted to either 10\(^{-8}\) or 10\(^{-9}\) M and 20 \(\mu\)l of 0.20 or 0.05 \(\mu\)g/ml. Thus each reaction contained either 2 or 0.2 pmol \(\beta\)-CPc and either 7 or 0.7 \(\mu\)g particles. With the higher concentration of both \(\beta\)-CPc and particles, a 1 h incubation time was sufficient at 50°C, and 4 h sufficed for hybridization at 40°C, but an overnight incubation was required for equilibration of the hybridization reaction at 20°C. When the lower concentrations were used for both \(\beta\)-CPc and particles, the time required for equilibration at 20°C lengthened to >2 days. When the higher concentration of particles was used in the reaction but the lower concentration of \(\beta\)-CPc, the time required for equilibration of the particle-based hybridizations shortened considerably, to 90 min for hybridizations carried out at 40°C and to 2 h for the 20°C reaction (Table 1).

<table>
<thead>
<tr>
<th>Temperature (°C)</th>
<th>[particles] ((\mu)g/reaction)</th>
<th>([\beta\text{-CPc}]_0) (pmol/reaction)</th>
<th>Time required for reaction to reach equilibrium</th>
</tr>
</thead>
<tbody>
<tr>
<td>50</td>
<td>7</td>
<td>2.0</td>
<td>1 h</td>
</tr>
<tr>
<td>40</td>
<td>7</td>
<td>0.20</td>
<td>1.5 h</td>
</tr>
<tr>
<td>40</td>
<td>7</td>
<td>2.0</td>
<td>4 h</td>
</tr>
<tr>
<td>20</td>
<td>7</td>
<td>0.20</td>
<td>2 h</td>
</tr>
<tr>
<td>20</td>
<td>7</td>
<td>2.0</td>
<td>5–24 h</td>
</tr>
<tr>
<td>20</td>
<td>0.7</td>
<td>0.20</td>
<td>2–3 days</td>
</tr>
</tbody>
</table>

Table 1. Equilibration times for hybridization reactions at different temperatures with various concentrations of particles and \(\beta\)-CPc.

Determination of density of capture probe on microparticle surface

Since \(K_d\) values are temperature-dependent, and oligonucleotide duplexes are less likely to dissociate at lower temperatures, \([p\text{-CP}]_0\) was determined from data obtained at lower temperatures where the condition \(k << 1\) holds (see ‘Calculation of surface density of particle-bound capture probe’). In our experimental system, \([p\text{-CP}]_0\) was measured at room temperature (20°C).

Two-fold dilutions of particles were prepared from 312 to 39 \(\mu\)g/ml, and 20 \(\mu\)l of each particle dilution was incubated with 200 \(\mu\)l of 10\(^{-8}\) M \(\beta\)-CPc at room temperature for 2 h. At the end of the incubation period, fluorescein-labeled oligonucleotide remaining in solution was measured, experimental measurements were converted to \(f\), and the values plotted against the concentration of particles in the reaction. At these low particle concentrations in which the labeled target was in excess over capture-probe oligonucleotide, there was a straight-line relationship between the concentration of particles in the reaction and the fraction of labeled oligonucleotide remaining in solution. For each set of data, this straight-line relationship was extrapolated to zero labeled target remaining in solution, indicating the concentration of particles required to completely deplete the fluorescent oligonucleotide (Fig. 2). From equation 22, \([p\text{-CP}]_0\) was calculated to be 3.0 pmol/cm\(^2\).

\(K_d\) values at different temperatures

Table 2 reports microparticle hybridization \(K_d\) values for six different temperatures. Figure 3 reports experimental and fitted data for five of these experiments. \(K_d\) values ranged from 10\(^{-9}\) M at 50°C to 10\(^{-7}\) M at 70°C.

<table>
<thead>
<tr>
<th>Temperature (°C)</th>
<th>([\beta\text{-CPc}]_0) (M)</th>
<th>(K_d) (M)</th>
</tr>
</thead>
<tbody>
<tr>
<td>70</td>
<td>1 × 10(^{-8})</td>
<td>1.4 × 10(^{-7})</td>
</tr>
<tr>
<td>70</td>
<td>2 × 10(^{-9})</td>
<td>1.3 × 10(^{-7})</td>
</tr>
<tr>
<td>65</td>
<td>1 × 10(^{-8})</td>
<td>8.8 × 10(^{-8})</td>
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<tr>
<td>60</td>
<td>1 × 10(^{-8})</td>
<td>6.6 × 10(^{-8})</td>
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<td>60</td>
<td>2 × 10(^{-9})</td>
<td>4.4 × 10(^{-8})</td>
</tr>
<tr>
<td>55</td>
<td>1 × 10(^{-8})</td>
<td>1.3 × 10(^{-8})</td>
</tr>
<tr>
<td>50</td>
<td>1 × 10(^{-8})</td>
<td>1.2 × 10(^{-9})</td>
</tr>
<tr>
<td>20(^a)</td>
<td>2 × 10(^{-9})</td>
<td>&lt;1.0 × 10(^{-11})</td>
</tr>
<tr>
<td>20(^a)</td>
<td>1 × 10(^{-8})</td>
<td>&lt;1.0 × 10(^{-11})</td>
</tr>
</tbody>
</table>

\(^aK_d\) value for this temperature is below the resolution of the method for this experimental set-up.
Table 3. Thermodynamic parameters for duplex formation of the lca-CP and CPc oligonucleotides in solution in Buffer B calculated from thermal denaturation data with the Meltwin program (30) or calculated by nearest neighbor analysis with the unified parameters in 1 M NaCl (33) and corrected for salt concentration (34,35)

<table>
<thead>
<tr>
<th>Method of calculating parameters</th>
<th>ΔH° (kcal/mol)</th>
<th>ΔS° [cal/(K·mol)]</th>
<th>Tm° (°C)</th>
<th>AG° 37 (kcal/mol)</th>
<th>AG° 60b (kcal/mol)</th>
<th>Kd60c (M)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Melting curve fits</td>
<td>–150.2 ± 1.4</td>
<td>–419.4 ± 4.2</td>
<td>67.9</td>
<td>–20.12 ± 0.13</td>
<td>–10.48 ± 0.10</td>
<td>1.17 × 10⁻⁷</td>
</tr>
<tr>
<td>Tm° versus ln(Cp)</td>
<td>–179.9 ± 5.6</td>
<td>–508.5 ± 16.8</td>
<td>66.7</td>
<td>–22.24 ± 0.40</td>
<td>–10.54 ± 0.13</td>
<td>1.06 × 10⁻⁷</td>
</tr>
<tr>
<td>Nearest neighbor</td>
<td>–169.94</td>
<td>–481.3e</td>
<td>67.0f</td>
<td>–19.46f</td>
<td>–9.57</td>
<td>5.27 × 10⁻⁷</td>
</tr>
</tbody>
</table>

a Calculated from $T_m = \Delta H^0 / [\Delta S^0 + R \ln (C_p1/4)]$ at $C_T = 10^{-4}$ M.
b Calculated from $AG^{0}_{60} = \Delta H^0 - T \Delta S^0$ at $T = 60$°C.
c Calculated from $Kd_{60} = \exp (AG^{0}/RT)$ at $T = 60$°C.
d Considered independent of salt concentration, so calculated for 1 M NaCl (34).
e Corrected to 0.1 M NaCl with equation 6 of (34).
f Corrected to 0.11 M NaCl with equation 7 of (35).
g Corrected to 0.11 M NaCl with equation 7 of (34).

Figure 3. Experimental and fitted particle hybridization data for various temperatures. 200 µl 10⁻⁸ M fl-CPc and 20 µl particles were mixed and incubated at a constant temperature: 20°C (—), 50°C (—), 55°C (—), 60°C (—), or 70°C (—). Reactions were incubated for 2 days at 20°C, 5 h at 50°C, 7 h at 55°C and 2 h at both 60 and 70°C. Symbols represent $f$ values corresponding to individual experimental measurements; lines indicate $f$ values calculated from optimal fits of equation 19 to the experimental data.

At 20°C, but not at other temperatures investigated in this experiment, the higher particle concentrations tested were capable of depleting virtually all fl-CPc from the supernatant. A Kd of 3 × 10⁻¹⁴ M was calculated for the 20°C data sets, but this value was not considered accurate. Based on fluorometer resolution, as well as concentrations of fluoresceinated oligonucleotide and capture probe tested, the lowest Kd value for which the method provides accurate resolution is ~10⁻¹¹ M; (compare with Fig. 1A). In Table 2, therefore, exact Kd values for 20°C data sets are not provided but instead are listed as <1 × 10⁻¹¹ M.

Hybridization of capture probe and its complement in solution

To obtain solution-phase dissociation constants to compare with Kd values obtained for particle-based hybridization, we analyzed the hybridization of lca-CP to CPc in thermal denaturation experiments. At the oligonucleotide concentrations used in these solution-phase studies ($C_T$ from 2.5 × 10⁻⁶ to 3.2 × 10⁻⁷ M), Tm values for the experimental melting curves ranged from 59.5 to 62.0°C. With the Meltwin program (30), thermodynamic parameters were calculated from individual melting curves (31) as well as from the plot of Tm⁻¹ versus ln(Cp) (32). Parameters obtained by both methods of calculation are listed in Table 3.

According to the criteria of Allawi and SantaLucia, Jr (33), the oligonucleotides used in these studies are classified as ‘molecules with marginally non-two-state transitions’, since values of ΔH° calculated by the two methods differ by 20%, as do the two values calculated for ΔS°. That this oligonucleotide pair does not strictly adhere to a simple two-state transition is not unexpected, since the assumption of a two-state transition is rarely adequate for oligonucleotide hybrids longer than 20 bp (35). We feel that marginal compliance of this pair of 22mers with the two-state transition justifies use of this simple model for these preliminary analyses of particle-based hybridization. In addition, since errors in ΔH° and ΔS° compensate, at temperatures close to the experimental Tm values, ΔG° values calculated from optical melting curves tend to be reliable even for oligonucleotide pairs that do not exhibit two-state transitions (36,37). Therefore ΔG° 60 and Kd60 were determined (at 60°C) from thermodynamic parameters calculated by both methods. These values are listed in Table 3 and demonstrate excellent agreement for both methods of calculation. For reference, parameters derived from nearest neighbor analyses (33) and corrected for salt concentration (34,35) are also included in Table 3.

Comparison of Kd values for solution-phase and particle-based hybridization

For hybridization of the capture-probe oligonucleotide with its complement at temperatures from 50 to 70°C, Kd values calculated from solution-phase parameters ranged over about seven orders of magnitude. For the same temperature range, however, Kd values measured in particle hybridization experiments extended over only about three orders of magnitude (Fig. 4). This muting of Kd values across the temperature range means that at lower temperatures, hybridization on particles was less efficient than solution-phase hybridization, while at higher temperatures, particle hybridization was more efficient than solution-phase hybridization.

DISCUSSION

By applying a simple bimolecular, all-or-none model for hybridization, we calculated both the density of capture-probe
oligonucleotide on the particle surface and the equilibrium dissociation constant for hybridization of particle-associated capture-probe oligonucleotide to its labeled solution-phase complement.

We compared $K_d$ values measured from particle hybridizations with those predicted from solution-phase thermodynamic parameters, but we were careful to limit our comparisons to a temperature range close to the $T_m$ values of the experimental curves from which the solution-phase parameters were derived. In this way we hoped to avoid large errors introduced by extrapolating data from a marginally two-state system across a wide temperature range. Within this limited range (50–70°C), there was only a narrow range of temperatures for which $K_d$ values of both particle-based and solution-phase hybridizations were similar (Fig. 4). At higher temperatures, $K_d$ values for particle hybridization were lower than comparable solution-phase values, while at lower temperatures, $K_d$ values for particle hybridization were somewhat higher than analogous solution-phase values.

One explanation for the divergence of these values may be differences between the distribution of capture probe in the two systems. For solution-phase hybridization, capture-probe oligonucleotide is uniformly distributed throughout the entire reaction volume. For particle-based hybridization, capture probe is uniformly distributed on each particle, and both particles and fluoresceinated complement are evenly distributed throughout the reaction mixture. However, local concentrations of capture probe vary.

At 70°C in low salt (0.11 M), a particle would be only sparsely populated with fluoresceinated complement. A labeled oligonucleotide that dissociates from the particle at this temperature will thus encounter a very high local concentration of available capture-probe oligonucleotides, thereby increasing the probability of that time of available capture-probe oligonucleotides is lower and thus is not such a driving force for reassociation as at 70°C. At these lower temperatures where particles are more highly populated with duplexes, steric or orientational requirements for particle hybridization may be more stringent than for solution-phase hybridization. Thus a lower proportion of fluoresceinated oligonucleotide would be in duplexes, resulting in a higher $K_d$ value than calculated from solution-phase parameters.

It is reasonable to assume, therefore, that at lower temperatures, particles with less dense coating of capture-probe oligonucleotide would increase the equilibrium level of hybridization (resulting in a lower $K_d$ value), while at higher temperatures, increased density of capture probe may improve the level of hybridization at equilibrium. Further studies will be required to test these predictions and to explore other possibilities.

Our model for hybridization on particles assumes that capture probe is uniformly distributed and positioned away from the particle surface via a linker. Furthermore, the model assumes that neither oligonucleotide is self-complementary and that bridging of single target molecules across multiple capture probes is insignificant. For microparticle systems where these assumptions are reasonable, the model provides an appropriate method for estimating equilibrium dissociation constants of microparticle-based duplex formation.

The particular 22mer oligonucleotides (GC content = 41%) tested in our experimental studies are representative of oligonucleotides used for specific hybridization in real-world applications such as PCR, mutagenesis, probing of nucleic acid blots, diagnostic assays and solid-phase capture procedures. All these methodologies utilize specific oligonucleotides of lengths ~15–30 nt with GC contents from ~40 to 60%. As is the case for most oligonucleotides of this length used in real-world applications, the oligonucleotide pair employed in our experiments does not exhibit a strict two-state transition in solution. Also each partner contains a short hairpin sequence (TCTT ... AAGA) which would be expected to form at low temperature or in high salt. In spite of these deviations of the experimental oligonucleotides from ideality, the simple model of two-state, bimolecular hybridization provided a good fit to experimental particle hybridization data over a range of temperatures and concentrations. We are therefore confident that the method will be useful not only for laboratory investigations of model systems but also during development of commercial assays involving nucleic acid hybridization on microparticles.

This model provides two valuable pieces of information for developing hybridization assays: the $K_d$ value and the surface density of capture probe. Characterizing assay performance with $K_d$ values rather than just signal-to-noise ratios focuses research on the physical–chemical nature of the hybridization reaction. Since many commercial particle hybridization assays are configured for a defined temperature in a particular buffer system, $K_d$ is a convenient single parameter that expresses the expected level of hybridization at equilibrium independent of concentration. The model also provides a straightforward, non-radioactive method for determining the amount of functional capture-probe oligonucleotide on the particle’s surface. Knowing this parameter is essential for evaluating the efficiency of various coating procedures and for determining the range of surface densities yielding acceptable levels of hybridization.

Acceptable ranges for the two parameters can be defined to generate performance characteristics for qualifying lots of particles for hybridization assays. Likewise, with knowledge of these parameters, reaction conditions can be adjusted to obtain equivalent results with differing lots of particles. When the $K_d$
values for particle lots differ, for example, the number of particles required per reaction to ensure hybridization of equivalent amounts of solution-phase target can be determined from our model. In addition, when the target molecule is large, our method can be applied to screen various portions of the sequence for utility as potential capture-probe oligonucleotides based on their Kd values for particle-based hybridization.

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We would like to thank Professor Robert Letsinger and Dr Robert Elghanian (Northwestern University, Evanston, IL) for allowing us to use their spectrophotometer and the computer program Extinct.

REFERENCES