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Rapid affinity measurement of protein–protein interactions in a microfluidic platform

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ABSTRACT

A rapid screening method has been developed to determine binding affinities for protein–ligand interactions using the Gyrolab workstation, a commercial microfluidic platform developed to accurately and precisely quantify proteins in solution. This method was particularly suited for assessing the high-affinity interactions that have become typical of therapeutic antibody–antigen systems. Five different commercially available antibodies that bind digoxin and a digoxin–bovine serum albumin (BSA) conjugate with high affinity were rigorously evaluated by this method and by the more conventional kinetic exclusion assay (KinExA) method. Binding parameter values obtained using Gyrolab were similar to those recovered from KinExA. However, the total experimental time for 20 binding affinity titrations, with each titration covering 12 data points in duplicate, took approximately 4 h by the Gyrolab method, which reduced the experimental duration by more than 10-fold when compared with the KinExA method. This rapid binding analysis method has significant applications in the screening and affinity ranking selection of antibodies from a very large pool of candidates spanning a wide range of binding affinities from the low pM to μM range.

Assessing the intrinsic affinities for interacting protein–protein species, such as the receptor/ligand systems and their modulation by therapeutic proteins, is a fundamentally important component to discovering and developing effective therapeutic antibodies. For example, during the early stages of a therapeutic antibody discovery program, hundreds of clones of anti-target antibodies are characterized during the various steps of hybridoma generation, affinity maturation, and other engineering approaches. The selection of the short list of final candidates among hundreds of samples is a tedious and difficult task. It requires triaging and ranking through screening using quantitative selection criteria that often are biased around intrinsic affinity for target ligand. Affinity assessment is also the integral feature of proper reagent selection in developing robust quantitative analytical assays such as enzyme-linked immunosorbent assay (ELISA)[1] methods [1]. In addition, a standard approach to monitoring the apparent affinity or activity of reagents used in quantitative assays can provide a simple means of assessing reagent quality and assay characteristics during method validation, storage, and downstream applications [2]. Currently, the most common techniques employed to assess affinity are kinetic exclusion assay (KinExA), surface plasmon resonance (SPR) such as BIACORE, fluorescence-activated cell sorting (FACS), isothermal titration microcalorimetry (ITC), fluorescence methods, and radioimmunoassay methods. The common limitations of these techniques are low throughput, lack of sensitivity at the high binding affinity range, and labor-intensiveness. The microfluidic technology is emerging as a powerful analytical tool for a wide variety of applications [3–6]. The use of microfluidics scales down the bioanalytical processes, resulting in significantly reduced analysis time and reagent consumption. The Gyrolab workstation is a commercial microfluidic instrument that is increasingly employed for bioanalytical measurements of antibodies and proteins by ligand binding assays [7]. Our goal was to exploit the advantage of this microfluidic platform to develop an effective method for binding affinity measurements that will address the limitations of the current methods.

In this study, we demonstrate the capability of the Gyrolab workstation to measure binding affinity of protein–ligand interactions and as a rapid affinity ranking method. The binding affinities of

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[1] *Abbreviations used:* ELISA, enzyme-linked immunosorbent assay; KinExA, kinetic exclusion assay; SPR, surface plasmon resonance; FACS, fluorescence-activated cell sorting; ITC, isothermal titration microcalorimetry; BSA, bovine serum albumin; DMF, dimethylformamide; LC, long chain; PBS, phosphate-buffered saline; CD, compact disc; NHS, *N*-hydroxysuccinimidyl ester; LIF, laser-induced fluorescence; PMT, photomultiplier tube; BSA–Dig–biotin, biotinylated BSA–digoxin; IgG, immunoglobulin G; a.u., arbitrary units.

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anti-digoxin antibodies against digoxin and bovine serum albumin (BSA)-conjugated digoxin (BSA–digoxin) were measured using Gyrolab and KinExA. Gyrolab was used to conduct simultaneous binding analysis experiments of a wide range of antibody concentrations in the ligand titrations. This allowed a fast determination of antibody–antigen stoichiometry and apparent binding affinity. In addition, the well-established KinExA method for assessing solution binding isotherms was used as a reference method for benchmarking for Gyrolab.

Materials and methods

Materials

Sheep anti-digoxin polyclonal antibody and mouse anti-digoxin monoclonal antibody (clone 1.17.256) were purchased from Roche Diagnostics (Indianapolis, IN, USA). Mouse anti-digoxin monoclonal antibody (clone DI-22), digoxin, BSA–digoxin, potassium phosphate, Tween 20, and sodium chloride were purchased from Sigma (St. Louis, MO, USA). Mouse anti-digoxin monoclonal antibodies MA1-10809 and MA1-10811, dimethylformamide (DMF), *N*-hydroxysulfosuccinimide ester–LC–LC–biotin, and 96-well plates were purchased from Thermo Fisher (Rockford, IL, USA). Dulbecco's phosphate-buffered saline (PBS) and *N*-hydroxysulfosuccinimide ester Alexa Fluor 647 were obtained from Invitrogen (Carlsbad, CA, USA). Goat anti-mouse and rabbit anti-sheep antibodies were acquired from Jackson ImmunoResearch Laboratories (West Grove, PA, USA). GammaBind G Sepharose was obtained from GE Healthcare (Piscataway, NJ, USA). I-Block assay buffer was prepared at Amgen (Thousand Oaks, CA, USA). Bioaffy 200 compact discs (CDs) were purchased from GYROS U.S. (Warren, NJ, USA).

Reagents

The goat anti-mouse and rabbit anti-sheep antibodies were conjugated to Alexa Fluor 647 using the standard protocol (Invitrogen, Eugene, OR, USA) by reaction of primary amine with *N*-hydroxysuccinimidyl ester (NHS). BSA–digoxin was conjugated to biotin through the primary amine/NHS reaction using the same procedure. The mouse anti-digoxin clone DI-22 in ascites fluid was purified by protein G columns.

Instrumentation

A Gyrolab workstation (GYROS US Inc., Warren, NJ, USA), an automated microfluidic system with laser-induced fluorescence (LIF) detection, was used according to the vendor's standard protocol for protein quantitation. The immune complexes of the binding pairs (ligand and free analyte) were formed in nanocolumns packed with biotinylated antigen-coated streptavidin beads (10–30 μm) with a 15-nl bed volume inside the Bioaffy 200 CD. The immune complexes formed in the nanocolumns were detected by excitation at 635 nm and emission at 668 nm. The fluorescence values were measured for each sample at three different fixed photomultiplier tube (PMT) settings of 1%, 5%, and 25% representing the signal amplification levels of each setting. The raw florescence values at these PMT settings could be used for each data set as long as the readouts were within the linear dynamic range of PMT output. Up to five CDs can be housed in the Gyrolab workstation to produce up to 560 data points per run.

A KinExA 3200 instrument (Sapidyne Instruments, Boise, ID, USA) was also used according to the vendor's standard protocol [8,9]. The free analyte was captured on a microbead coated with binding partner to the molecule of interest. The captured analyte was detected by the secondary antibody conjugated to Alexa Fluor 647 (similar to the Gyrolab method), and the fluorescent emission was measured by the LIF detection.

Affinity measurement

Gyrolab workstation

The binding interactions of the anti-digoxin antibodies to digoxin or BSA–digoxin were measured at eight different antibody concentrations, and at each antibody concentration with 12 different antigen (digoxin or BSA–digoxin) concentrations in I-Block buffer with 5% BSA, and ranged from 8 nM to 31 pM by 2-fold serial dilution. Anti-digoxin antibodies were prepared at 2000-, 1000-, 500-, 250-, 124-, 64-, 32-, and 16-pM concentrations. A stock solution of 10 mM digoxin was prepared in DMF. It was diluted 1:1000 in the I-Block buffer with 5% BSA to make a 10-μM stock solution. Then, from the 10-μM stock solution, standards at a concentration range of 40,000 to 0.6 pM were made quantitatively by serial dilution in I-Block buffer with 5% BSA. To the 96-well plate, 10 μl each of the anti-digoxin antibody and digoxin (or BSA–digoxin) standards was added. The plates were sealed with aluminum foil plate sealers and incubated at room temperature with gentle shaking (~200 rpm) for approximately 48 h for the binding reaction to reach equilibrium. Then, 50 μg/ml of the capture reagent, biotinylated BSA–digoxin (BSA-Dig-biotin) was loaded onto the nano-streptavidin columns, converting them to BSA–digoxin columns. After washing the columns, 200 nl of the preincubated reaction mixture was passed through by centrifugal force. The free anti-digoxin antibody in the reaction mixture bound to the BSA–digoxin nanocolumns, and the bound antibodies were washed away. The nanocolumns were then passed with 50 nM detection antibody, goat anti-mouse (or rabbit anti-sheep) immunoglobulin G (IgG) conjugated to Alexa Fluor 647, which bound to the captured antibodies on the column. The bound detection antibodies were excited with laser, and the emitted fluorescence signals were measured.

KinExA

The anti-digoxin antibodies at two concentrations from 1 nM to 5 pM were mixed with either digoxin or BSA–digoxin at 11 different concentrations in the range of 100 nM to 1.6 pM and allowed to equilibrate for 40 to 72 h (for 5–100 pM) or for 15 h (for 1 nM) at room temperature. These preincubated reaction mixtures were then passed over the BSA–Dig–biotin-coated beads. The free antibodies of the reaction mixture were captured by the beads, and the bead-bound antibodies were quantified using Alexa Fluor 647-labeled rabbit anti-sheep antibody or goat anti-mouse antibody [8,9].

Data analysis

The fluorescent signal values in the Gyrolab and KinExA experiments correlated to the free concentrations of antibody binding sites and were plotted as a function of ligand concentration to produce a binding isotherm and analyzed. We used a form of Adair's equation for simple binding that accounted for the total concentrations of all species and the corresponding equilibrium binding constant as a function of the free fraction of antibody binding sites [10–13]:

$$\frac{[\mathrm{Ab}]}{[\mathrm{Ab}]_0} = \frac{([\mathrm{Ab}]_0 - [\mathrm{Ag}]_0 - K_\mathrm{d}) + \sqrt{([\mathrm{Ab}]_0 - [\mathrm{Ag}]_0 - K_\mathrm{d}^2) + 4[\mathrm{Ab}]_0 K_\mathrm{d}}}{2[\mathrm{Ab}]_0} \quad (1)$$

where [Ab] is the concentration of free antibody binding sites, $[\mathrm{Ab}]_0$ and $[\mathrm{Ag}]_0$ are the total molar concentrations of the antibody binding sites and antigen, respectively, and K_d is the equilibrium dissociation binding constant. Because the raw untransformed fluorescence