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Nanomolar to sub-picomolar affinity measurements of antibody–antigen interactions and protein multimerizations: Fluorescence-assisted high-performance liquid chromatography

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ABSTRACT

Although several techniques exist for the measurement of high-affinity interactions, it is still challenging to determine dissociation constants around or even below 1 pM. During the analysis of several human-derived monoclonal antibodies to adalimumab, we found a clone with a very high affinity that could not be measured using conventional surface plasmon resonance assays. We developed a straightforward and robust method to measure affinities in the nanomolar to sub-picomolar range. The assay is based on separation of bound and free fluorescently labeled antigen using size exclusion chromatography and quantification by in-line fluorescence detection. We describe optimal conditions and procedures that result in a very sensitive assay that can be used to reliably determine ultra-high affinities. Using the method described in this article, a dissociation constant of 0.78 pM could be determined for the anti-adalimumab antibody.

Reliable measurement of affinity constants below 1 nM or even below 1 pM is important for development of potent biopharmaceutical drugs such as therapeutic monoclonal antibodies [1–3]. Few methods exist to reliably determine affinities below 1 pM. Technologies for measuring high-affinity interactions based on biosensor chips offer the possibility to determine dissociation constants based on the on-rate and off-rate [4]. However, for ultra-high-affinity interactions, the off-rates become too slow to be reliably measured [5]. Another way to measure high-affinity interactions is to combine both binding partners A and B in fluid phase and then measure the concentration of, for example, free A via capture to a solid phase to which B is coupled. Quantification can be achieved via a number of immunochemical methods, including enzyme-linked immunosorbent assay (ELISA) and precipitate-enhanced immunoassay (PEIA) [5–8]. In addition, the Kinexa platform is based on this principle, and a few studies have reported sub-picomolar affinities determined using this technique [9–11]. However, these methods require secondary antibodies for detection, which can limit their use in many cases.

Here, we describe a method we developed to determine dissociation constants in the nanomolar to sub-picomolar range using fluorescence-assisted high-performance liquid chromatography (HPLC).[1] We developed this method because the use of common techniques failed to provide a dissociation constant for an ultra-high-affinity human anti-adalimumab monoclonal antibody that was obtained from an adalimumab-treated individual. The method is based on the following principles. To monitor the binding process, one binding partner (A) is fluorescently labeled and incubated with different concentrations of the other binding partner (B). Bound and free A is separated using size exclusion chromatography and detected by an in-line fluorescence detector (Fig. 1A). From the variation of peak heights of either bound or free A with the concentration of B, the dissociation constant can be calculated. The time needed for separation on the column should be sufficiently short compared with the dissociation rate of the A–B complex to ensure minimal disturbance of the equilibrium. For high-affinity interactions (nanomolar to picomolar range), this condition will usually be met. Because bound and free protein is separated based on differences in size, this method can also be used to monitor homo-multimerizations, that is, if A equals B.

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[1] *Abbreviations used:* HPLC, high-performance liquid chromatography; DTT, dithiothreitol; IL-6, interleukin 6; TT, tetanus toxoid; PBS, phosphate-buffered saline; PBS-I, PBS containing intravenous immunoglobulin; ada-Fab-488, adalimumab Fab–DyLight 488; RU, response units; IVIG, intravenous immunoglobulin; SPR, surface plasmon resonance.

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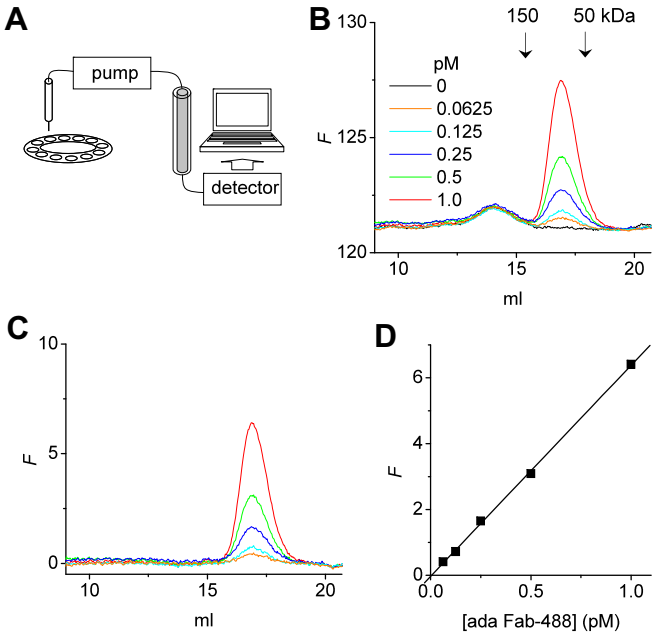

Fig.1. Detection of fluorescently labeled protein using HPLC with in-line fluorescence detection. (A) Flow chart of experimental setup. (B) Fluorescence elution profiles of serial 2-fold dilutions of adalimumab Fab fragments labeled with DyLight-488 (ada Fab-488; 1, 0.5, 0.25, 0.125, 0.0625, and 0 pM). The additional peak at approximately 150 kDa arises from the 0.1-mg/ml IVIG in the samples. (C) Fluorescence elution profiles with background trace (i.e., 0 pM ada Fab-488) subtracted. The peak maxima are plotted in panel D and depend linearly on the concentration of ada Fab-488 ($r^2 = 0.9996$).

Materials and methods

Materials

Anti-adalimumab antibodies were obtained from B cells of an adalimumab-treated rheumatoid arthritis patient with an anti-adalimumab response as described before [12]. Fab′ fragments of adalimumab (Abbott, Abbott Park, IL, USA) were prepared by reducing pepsin-generated F(ab′)$_2$ fragments with 10 mM dithiothreitol (DTT), followed by alkylation with iodoacetamide as described before [12]. Interleukin 6 (IL-6) was obtained from Sanquin (Amsterdam, The Netherlands). Tetanus toxoid (TT) was obtained from RIVM (Bilthoven, The Netherlands) and purified by size exclusion chromatography to remove aggregated and fragmented material. Adalimumab Fab, IgG4 (natalizumab, Tysabri, Biogen Idec), TT, and IL-6 were fluorescently labeled with DyLight 488 amine reactive dye (Pierce/Thermo Scientific) according to the instructions of the manufacturer. Unreacted dye was removed by repeated dilution/concentration using Amicon Centriprep centrifugal filter devices (Millipore, Billerica, MA, USA) until no more dye could be detected in the filtrate. The average degree of labeling (D) was 1, 5, 3, and 1, respectively, calculated as $D = A_{493}/(\varepsilon_l \times c_p)$, with $c_p = (A_{280} - A_{493} \times CF)/\varepsilon_p$, where c_p is the molar concentration of the protein, A_{280} and A_{493} are the absorptions at 280 and 493 nm, respectively, CF is a correction factor (A_{280}/A_{493} for label only; 0.144), ε_p is the molar extinction coefficient for the protein (70,000, 210,000, 186,000, and 10,000 M^{-1} cm^{-1}, respectively), and ε_l is the extinction coefficient for DyLight 488 (70,000 M^{-1} cm^{-1}). The purity of the fluoresescently labeled proteins was checked by size exclusion chromatography.

Fluorescence-assisted HPLC

A: Serial 2-fold dilutions of ada-Fab-488 (0.0625–1 pM) were made in phosphate-buffered saline (PBS: 10 mM phosphate buffer [pH 7.4] and 140 mM NaCl) containing 0.1 mg/ml intravenous immunoglobulin (PBS-I, Nanogam, Sanquin).
B: Serial 4-fold dilutions of anti-adalimumab monoclonal antibody (0.015–1000 ng/ml, 0.2–13,000 pM binding sites) were incubated with a fixed concentration of 31 pg/ml (0.625 pM) or pg/ml (1.25 pM) of adalimumab Fab–DyLight 488 (ada-Fab-488) in PBS-I. Samples were equilibrated for up to 4 days at 20 °C before analysis.
C: Serial 4-fold dilutions of anti-IL-6.16 (0.05–50 ng/ml, 0.67–667 pM binding sites, Sanquin) were incubated with 2 pM IL-6-488 in PBS-I containing 0.1% Tween 20 and incubated for 24 h at 20 °C before analysis.
D: Serial 4-fold dilutions of a mouse monoclonal anti-TT (0.05–50 ng/ml, 0.67–667 pM binding sites, clone 16E12, Sanquin) were incubated with 1 pM TT-488 in PBS containing 0.1% Tween 20 and incubated for 24 h at 20 °C before analysis.
E: IgG4 (natalizumab) and IgG4-488 were reduced with 1 mM DTT (60 min, 37 °C) and alkylated with 5 mM iodoacetamide. This procedure results in complete reduction of the interchain disulfide bonds [13]. Serial 4-fold dilutions of reduced/alkylated IgG4 (0.025–25 μg/ml, 0.33–333 nM half-molecules) were incubated with 10 ng/ml reduced/alkylated IgG4-488 in PBS containing 0.1 mg/ml human serum albumin (Sanquin) and incubated at 37 °C for 90 min before analysis. Albumin results in a relatively high background signal and can be used only as carrier protein to monitor lower affinity interactions (i.e., using $\sim$ > 0.3 nM DyLight 488).

Samples (A–D: 1000 μl; E: 50 μl) were applied using a thermostatted autosampler (20 °C) to a Superdex 200 HR 10/300 column (24 ml, GE Healthcare, Uppsala, Sweden), which was connected to an ÄKTAexplorer HPLC system (GE Healthcare) and eluted at 0.5 ml/min. Elution profiles were monitored by measuring the fluorescence (excitation/emission: 488/525 nm) with a Prominence RF-20Axs in-line fluorescence detector (Shimadzu, Kyoto, Japan). The elution buffer was PBS (A, B, and E) or PBS containing 0.1% Tween 20 (C and D). Molecular weight estimations were based on the elution profiles of the monomeric and dimeric IgG fractions of intravenous immunoglobulin, which were monitored by measuring the absorbance at 214 nm.

To calculate dissociation constants, background-corrected fluorescence intensities (F) corresponding to the peak maxima of either bound or free labeled protein were plotted against the concentration of antibody x (molar concentration of the number of Fab arms/half-molecules), and a 1:1 binding model ($F = F_0 + \Delta F \times x/[K_d + x]$, where F_0 is the fluorescence at zero concentration, ΔF is the fluorescence at saturating concentrations minus F_0, and K_d is the dissociation constant) or a homodimerization model ($F = F_0 + 0.25 \times \Delta F \times [K_d + 4x - (K_d^2 + 8x \times K_d)^{0.5}]/x$) was fitted to the data using Microcal Origin 7.0 software. Standard errors were calculated from duplicate or triplicate values of the K_d as determined in independent experiments.

SPR measurements

Surface plasmon resonance (SPR) measurements were performed using a Biacore 3000 instrument (Biacore, Breda, The Netherlands) at 25 °C. Mouse anti-human IgG (MH16-1, Sanquin) was immobilized at a concentration of 10 μg/ml in 10 mM sodium acetate (pH 5.0) on a CM5 sensor chip using *N*-hydrosuccinimide/1-ethyl-3-(3-dimethylaminopropyl)carbodiimide hydrochloride (NHS/EDC) at a flow rate of 5 μl/min. Anti-adalimumab monoclonal antibodies were dissolved at 10 μg/ml in Biacore buffer (10 mM Hepes [pH 7.4] containing 3.4 mM ethylenediaminetetraacetic acid [EDTA], 0.15 mM NaCl, and 0.005% Tween 20) and passed through the cells at 20 μl/min, yielding approximately