Antibody-antigen interactions: What is the required time to equilibrium?

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The use of antibodies is widespread in many areas including *in-vivo* and *in-vitro* diagnostics, quantitative analysis in research laboratories and as therapeutic substances. Since the methods for generation of antibodies has improved and regularly results in high-affinity interactions, the standard assays used for quantification of the interaction properties should be revisited because they do not necessarily produce accurate results. Here we show that in several cases, the affinity determination of strongly binding antibodies will be inherently difficult when using standard procedures, due to impractically long incubation times. Real-time kinetic analysis is often the only realistic alternative for affinity determination.

The estimate of how strongly an antibody binds to its antigen is often reported as the affinity, K_D, of the interaction. The affinity can be measured using a variety of assays, most requiring that the reading takes place when equilibrium has occurred. The increased focus on high-affinity antibodies (typically $K_D < 1$ nM) has led to a situation where the time to equilibrium increases vastly. The essence of reading at equilibrium is seldom discussed, and the appropriateness of protocols developed for normal affinity antibodies are often taken for granted. Conducting assays before reaching equilibrium can result in false negative results[1] and the importance of sufficiently long incubation times has been discussed in the context of DNA microarrays[2], immunohistochemistry[3] and drug-receptor interactions[4]. There are however still a wide-spread use of inappropriate protocols where too short incubation times may generate confusing or inaccurate results.

Time to equilibrium (T) depends on three parameters: the association rate (k_a), the dissociation rate (k_d) and the antibody concentration (C), according to the approximate expression[4] $T \approx 3.5/(k_a*C + k_d)$ (s). Figure 1 shows simulations of how four different monovalent interactions with different affinity and kinetic properties would perform in an immunofluorescense assay with different incubation times. Two examples were generated for the affinity 1 nM, one fast on fast off interaction representing an average antibody interaction and one slow on slow off representing a relatively good antibody. The fast interaction would be accurately quantified at incubation times beyond three hours, while as the slow interaction would require over-night incubation to reach equilibrium and produce accurate results. At higher affinities, which are common for therapeutic antibodies and other optimized binding proteins, incubation time exceeding 10 hours is required for reasonable accuracy in the immunofluorescense assay. Incubation time will in particular increase to many hours at slower dissociation rate i.e. k_d less than approximately $10^{-5}s^{-1}$. Even if an assay is interrupted prematurely, the read data will look like a good affinity determination. When using such results to estimate affinity, the

produced values may differ more than two orders of magnitude compared to the true value, as seen in figure 1D.

In practice, the time to equilibrium for therapeutic antibodies may well be longer than 24 hours. Figure 2 shows the temporal progress of pertuzumab binding to the HER2 receptor on SKOV3 cells and cetuximab binding to the EGF receptor on U343 cells as measured using LigandTracer Green[5]. The antibody was titrated in three steps; 0.44 nM, 1.3 nM and finally 4.4 nM, followed by a dissociation measurement. It is clear that even at 4.4 nM, which is greater than K_D in both cases, more than 20 hours are required to reach equilibrium. These two antibodies bind to their targets with high-affinity, albeit not in any extreme manner.

In some cases these errors can be devastating, in particular when comparison of molecular properties should be conducted. In competitive assays where one compound is preincubated, insufficient preincubation time may cause incomplete competition and generate confusing results[1]. Furthermore, when using the concept of microdosing with PET tracers to test substances in man during early phases of drug development, the effects of high affinity and low concentration in relation to time to equilibrium has also to be considered for obtaining relevant results[6]. In other cases, such as immunoassay formats for concentration determination based on standards, short incubation time may not be any serious complication.

It is not always possible to use sufficiently long incubation times. In particular in cell based assays the cells may metabolize the antibody if incubating too long. Thus, the preferred alternative is to follow the interaction in real time, and use the curvature of the binding event for estimation of binding kinetics and affinity. This has been done for protein-protein interactions since the advent of SPR based real-time biosensors[7] and is a robust method for estimating the affinity. SPR based techniques are however usually limited to dissociation rates greater than $10^{-5}s^{-1}$ and cannot be used for cell-based assays. This is now possible with new technology equipment that produce similar real-time binding traces that can be used for kinetic based affinity determinations and for receptor density on cells[5].

In conclusion, the search for high-affinity antibodies implies that the basic requirements for the assays used to quantify antibody-antigen interactions should be revised. Particular focus should be put on ensuring that the interaction is given sufficient time to reach equilibrium, where it is required. Alternative assays that do not require equilibrium exist, and are feasible alternatives for accurate quantification of high-affinity interactions.

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Online methods

Monoclonal antibody trastuzumab (purified from Herceptin®, Roche AB, Stockholm, Sweden) was labeled with fluorescein isothiocyanate (FITC, Thermo Fisher Scientific Inc., Waltham, MA, USA) according to the manufacturers protocol. Monoclonal antibody cetuximab (purified from Erbitux®, Merck KGaA, Darmstadt, Germany) was labeled with Alexa Fluor ® 488 (Invitrogen, Carlsbad, CA, USA) according to the manufacturers protocol. The human ovarian carcinoma cell line SKOV3 (HTB-77, ATCC, Rocksville, MD, USA) known to express HER2 and the human glioma cell line U343MGaCl2:6 (denoted U343) known to express EGFR were used for interaction analysis. The cells were grown at 37ºC in incubator with humidified atmosphere and 5 % CO_2 in Ham´s F10 cell culture medium (Biochrom AG, Berlin, Germany) supplemented with 10 % fetal calf serum (FCS, Sigma, St Louis, MO, USA), PEST (penicillin 100 IU/ml and streptomycin 100 µg/ml, Biochrom AG, Berlin, Germany) and L-glutamin (2 nM). The cells were seeded on a small local area of a petri dish (Nuclon™, dish size 100x20, NUNC A/S, Roskilde, Denmark) as described previously [Björke and Andersson, 2005] . LigandTracer® Green was used to monitor the interaction of [trastuzumab-FITC] – HER2 on SKOV3 cells and [cetuximab-AlexaFluor488] – EGFR on U343 cells, essentially as described previously [Björke and Andersson, 2005]. The antibody concentration was titrated during continuous monitoring of the antibody-antigen interaction: First 0.44 nM during three hours, then 1.3 nM during four hours, and finally 4.4 nM during 17 hours. After the incubation with antibody, the cell dish was emptied and measured during four hours with fresh cell-culture medium (devoid of antibody) to show antibody dissociation.

Figure Legends

Figure 1. Theoretical binding assay results for four different interactants at five different incubation times. The legend in section A applies to all four sections in the figure. A: K_D=1 nM, k_a=10^5 $M^{-1}s^{-1}$, k_d=10^{-4} s^{-1}, B: K_D=1 nM, k_a=10^4 $M^{-1}s^{-1}$, k_d=10^{-5} s^{-1}, C: K_D=0.1 nM, k_a=10^5 $M^{-1}s^{-1}$, k_d=10^{-5} s^{-1}, D: K_D=30 pM, k_a=10^5 $M^{-1}s^{-1}$, k_d=3×10^{-6} s^{-1}.

Figure 2. Two therapeutic antibodies interacting with their targets on living cells, monitored in real-time using LigandTracer Green. Red curve shows [trastuzumab-FITC] – HER2 on SKOV3 cells and black curve shows [cetuximab-AlexaFluor488] – EGFR on U343 cells.
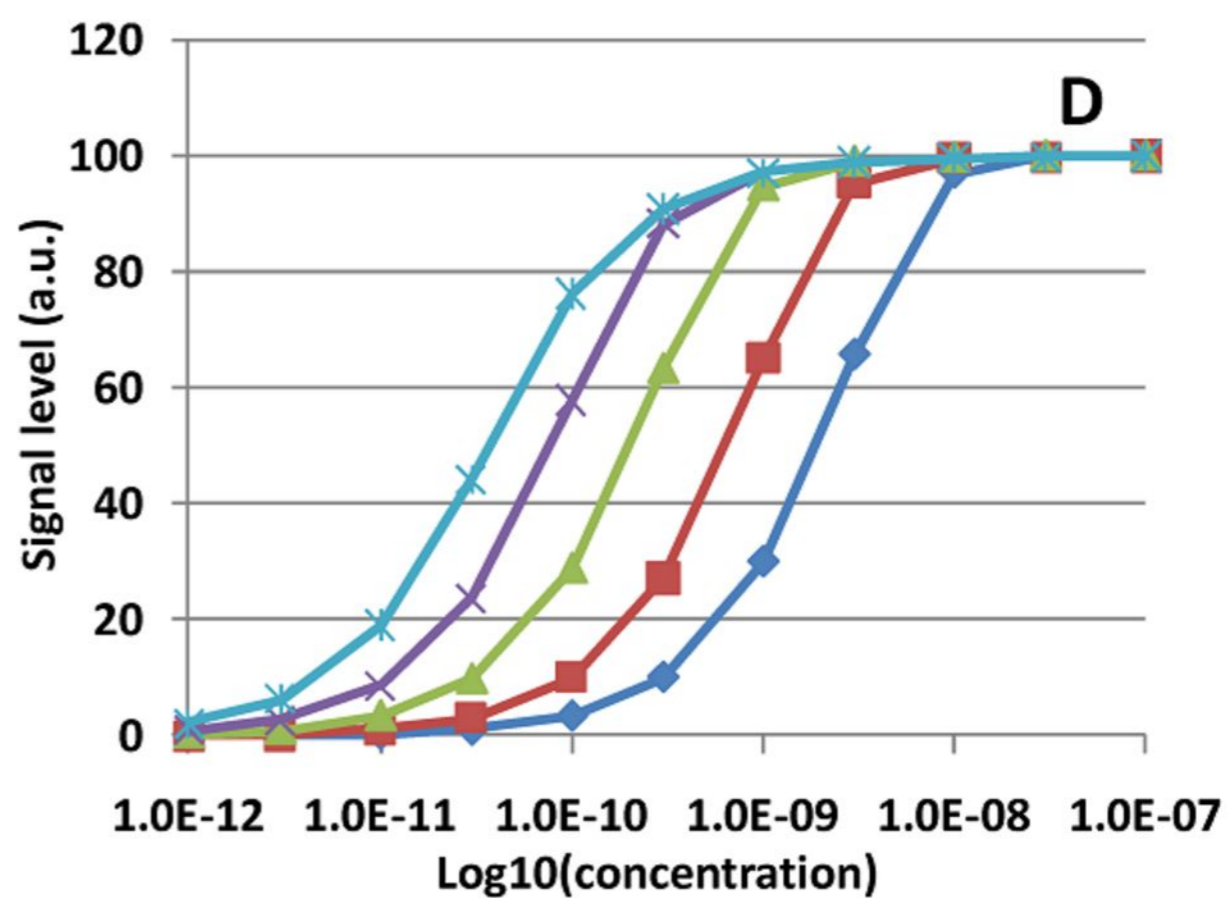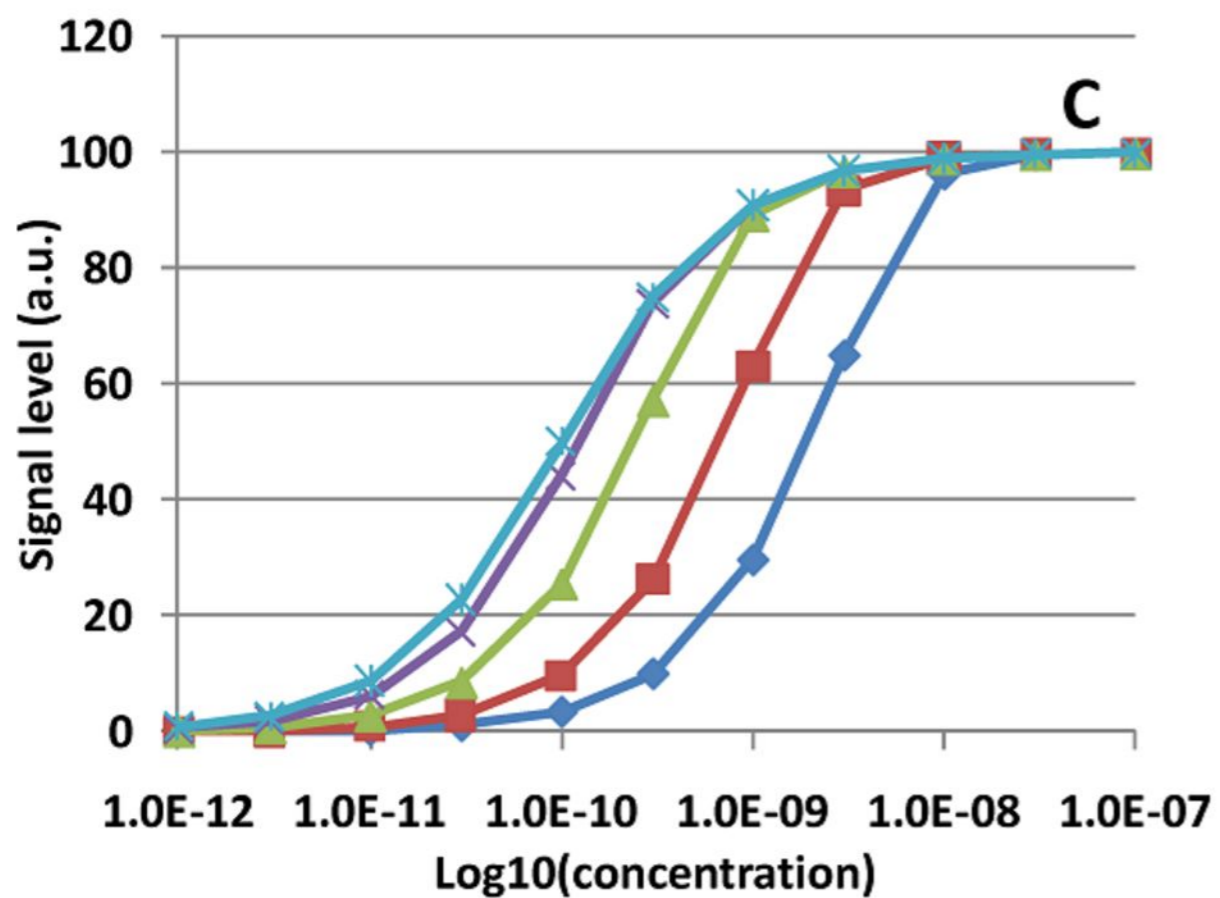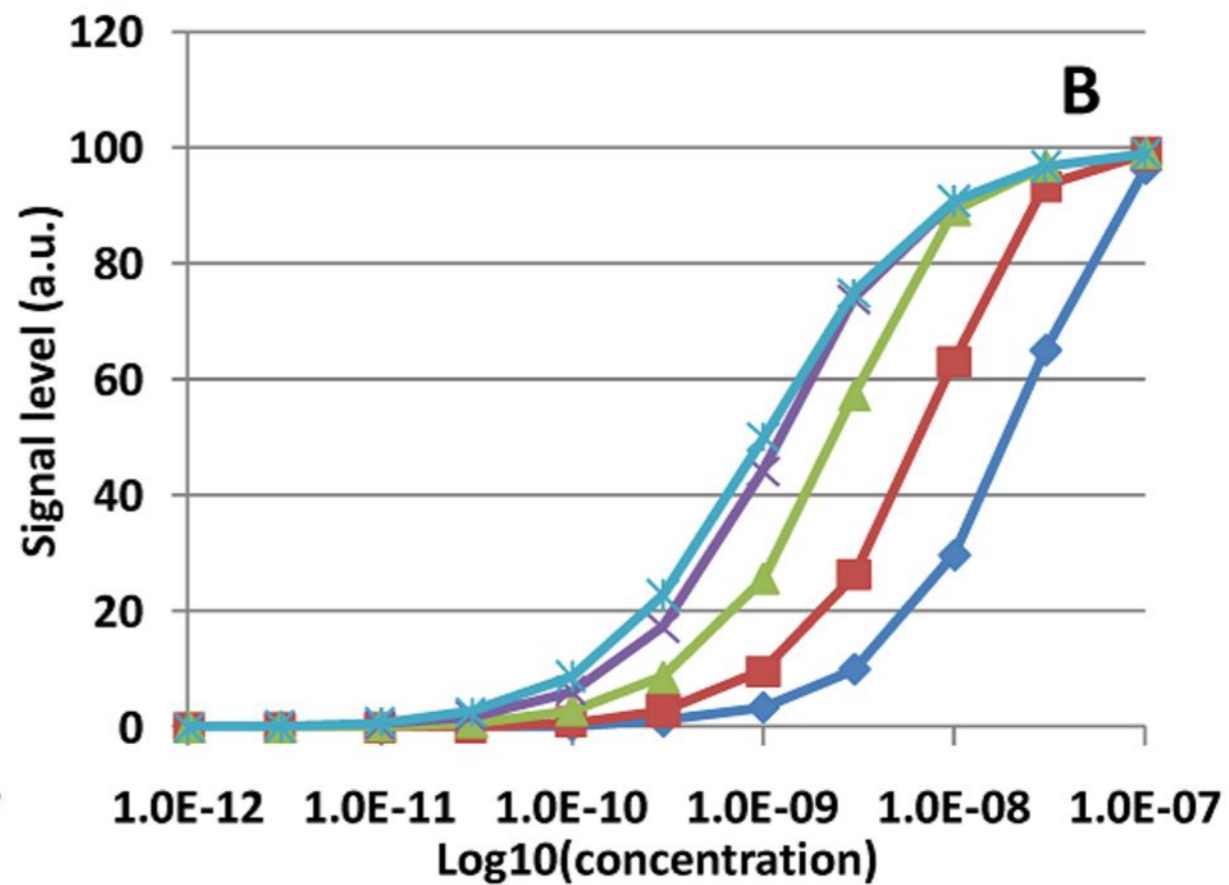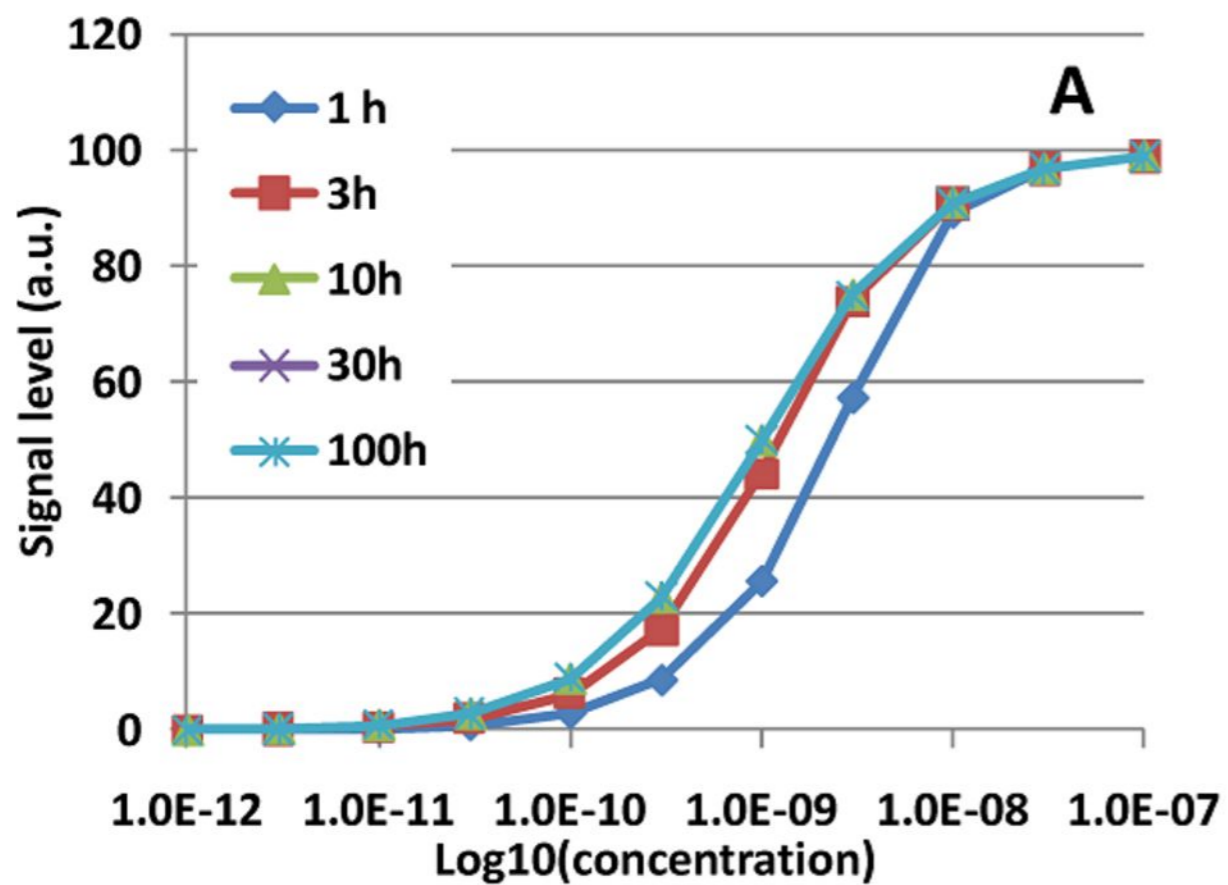
Therapeutic antibodies interacting with their antigens on living cells