Modeling of homogeneous cloned enzyme donor immunoassay

Sang Il Jeon 1, Xiaoyun Yang, Joseph D. Andrade *

Department of Bioengineering, University of Utah, Salt Lake City, UT 84112-9202, USA

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Abstract

One of the most widely used analytical techniques for sensitive detection of biologically and clinically significant analytes is the immunoassay. In recent years direct immunoprobes allowing label-free detection of the interaction between the antibody and the target analyte have proved their capabilities as fast, simple, and nevertheless highly sensitive methods. Cloned enzyme donor immunoassay (CEDIA) homogeneous assay is based on the bacterial enzyme β-galactosidase, which has been genetically engineered into two inactive fragments, enzyme donor and enzyme acceptor. Reassociation of the fragments in the assay forms active enzyme, which acts on substrate to generate a colored product. A comprehensive kinetic model of CEDIA is developed to aid in understanding this method and to facilitate development of a truly homogeneous version, potentially applicable to a dipstick-type multianalyte point of care analytical device (ChemChip). Although the standard assay involves a two-step process, we also chose to model a single-combined process, which would be simpler to apply in a ChemChip device. From the modeling simulation, we obtain the time courses of the amounts of product and active enzyme, from which the dynamic ranges can be obtained as $10^{-6}$–$10^{-7}$ and $10^{-5}$–$10^{-7}$ M analyte concentration for two-step and single-combined processes under the conditions of the assumed parameters, respectively. A simple one-step immunoassay has the merit of reducing time and cost and has an improved dynamic range.

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Keywords: CEDIA immunoassay; Modeling; Analyte; Enzyme acceptor; Analyte-conjugated enzyme donor; Primary antibody; Secondary antibody; Association and dissociation rate constant; Affinity constant; Dynamic range

Immunoassay has become one of the most widely used analytical techniques for sensitive detection of analytes, such as hormones, drugs, tumor markers, specific proteins, viral antigens, etc. Point of care testing applications have also been developed. Improvements in both antibodies and detection systems have resulted in increased sensitivity of immunoassays. For many years radioactive isotopes were used as labels. However, concerns with regard to safety and disposal resulted in the move toward nonradioactive labels [1]. Fluorescent, luminescent, and enzyme labels are now frequently used in commercially available assays. Of these labels the most commonly used reporter molecule is the enzyme, because it introduces signal amplification through turnover of an appropriate substrate to detectable products [2]. In recent years direct immunoprobes allowing label-free detection of the interaction between the antibody and the target analyte have proved their capabilities as fast, simple, and highly sensitive methods. A major breakthrough in immunoassay technology was the introduction of the homogeneous immunoassay, which did not require a physical separation of the bound and unbound fractions, much simplifying the assay and allowing it to be potentially applicable to a simple, quantitative “dipstick” format.
Cloned enzyme donor immunoassay (CEDIA)\textsuperscript{2} homogeneous assay (Figs. 1–3) allows highly sensitive detection of low-molecular-mass analytes without separation steps. It is based on the bacterial enzyme β-galactosidase, which has been genetically engineered into two inactive fragments, enzyme donor (ED) and enzyme acceptor (EA) [3–5]. Complementation of ED and EA forms an active enzyme. The covalent attachment of analyte or ligand to ED does not affect the ability of EA and ED to form active enzyme. Analyte present in a sample competes for binding to the limited number of antibody sites, making ED–ligand conjugate available for enzyme formation. Thus, the amount of enzyme formed is directly proportional to the analyte concentration in the sample.

We have chosen to develop a comprehensive kinetic model of CEDIA [3,4] to aid our understanding of this method and to facilitate development of a truly homogeneous version potentially applicable to a dipstick-type multianalyte point of care analytical device (ChemChip) [6]. This model simulates all the parameters used in the experiment, modeling the association and dissociation rate constants for each reaction taking place in the CEDIA. The model allows for optimizing conditions in the real experiment, helping determine the detection limit of analyte in the sample, and in estimating dynamic range of the assay. It is understood that the commercial kit likely includes a variety of additives and/or excipients which are not considered in the model to follow.

\textbf{Modeling}

Modeling is based on the Microgenics valproic acid (VPA) CEDIA kit experiment [7] (www.microgenics.com). This commercial CEDIA kit is designed to perform the procedure in two steps.
Step 1. Sample with analyte (valproic acid) is incubated with reagent 1 (Fig. 1), containing anti-analyte (anti-VPA) mouse monoclonal antibody (mAb) and an enzyme acceptor. L, A, and EA in Fig. 1 represent analyte, anti-analyte antibody, and enzyme acceptor, respectively.

Step 2. After incubation of sample with reagent 1 in step 1, reagent 2 (upper part of Fig. 2) is added. Reagent 2 is a liquid mixture containing VPA-conjugated enzyme donor, secondary antibody (goat anti-mouse IgG secondary antibody), and substrate for β-galactosidase, which is also pictured in Fig. 2. L–E, Ab, and S in Fig. 2 represent the VPA-conjugated enzyme donor, secondary antibody, and substrate for β-galactosidase, respectively.

After addition of reagent 2, colored product of catalysis by β-galactosidase is monitored as a function of time via absorbance (standard spectrophotometric assay). Fig. 3 briefly describes the generalization of the two-step CEDIA procedure.

Methods

The Scatchard model is the most widely used mathematical approach to the quantitative description of the multiple equilibriums taking place when an antibody binds reversibly to an analyte molecule [4]. The Scatchard model focuses on the individual binding sites of the antibody and applies the law of mass action for each site, defining the affinity constant (association constant) $K_i$ and assuming that the affinity of each particular site for the ligand is not influenced by the extent of occupancy of the other sites (independent and noninteracting binding sites). The reaction between antibody and analyte may be simplistically described:

Antibody + Analyte

$$k_i$$

Binding complex between antibody and analyte.

Here, $k_i$ is the association rate constant and $k_{-i}$ is the dissociation rate constant. The ratio of the two rate constants gives the equilibrium constant $K_i$, which represents the final ratio of bound to unbound analyte and antibody. It is also known as the affinity constant,

$$K_i = \frac{k_i}{k_{-i}} = \frac{[\text{binding complex between antibody and analyte}]}{[\text{antibody}] [\text{analyte}]}$$

Step 1

Step 1 involves antibody (A) reversibly binding to a ligand molecule (L, antigen or analyte; depicted as m in Fig. 1). The antibodies are assumed to have two equivalent binding sites, i.e., they are divalent. The reactions are

$$L + A \rightleftharpoons L : A$$

and

$$L : A + L \rightleftharpoons L : A \cdot L$$

All of the parameters (symbols) and rate constants of step 1 are given in Table 1.

The rates of reaction are represented as four differential equations with four unknown parameters: L, A, L : A, and L : A : L.

Table 1

<table>
<thead>
<tr>
<th>Rate constants and parameters in Step 1 of CEDIA</th>
</tr>
</thead>
<tbody>
<tr>
<td>Rate constants</td>
</tr>
<tr>
<td>$K_i$: affinity constant for reaction (1)</td>
</tr>
<tr>
<td>$k_i$: association rate constant for reaction (1)</td>
</tr>
<tr>
<td>$k_{-i}$: dissociation rate constant for reaction (1)</td>
</tr>
<tr>
<td>$K_2$: affinity constant for reaction (2)</td>
</tr>
<tr>
<td>$k_2$: association rate constant for reaction (2)</td>
</tr>
<tr>
<td>$k_{-2}$: dissociation rate constant for reaction (2)</td>
</tr>
</tbody>
</table>

Parameters (symbols): L (m): analyte in reactions (1) and (2); A ( ): antibody for the analyte in reaction (1); L : A ( ): binding complex between one antibody and one analyte in reactions (1) and (2); L : A : L ( ): binding complex between one antibody and two analytes in reaction (2).
In the standard CEDIA kit [7], first the analyte and antibody are mixed and incubated for a fixed time (step 1), and second the analyte-conjugated enzyme donor (L-E), the second antibody (Ab), and the substrate (S) are added (step 2) (Fig. 2). All of the reacting species participating in the reactions are involved in step 2. The possible interaction and reaction schemes of step 2 are:

1. \[ L + A \xleftrightarrow{K_1} L : A \]
2. \[ L : A + L \xleftrightarrow{K_2} L : A : L \]
3. \[ L - E + A \xleftrightarrow{K_1} E - L : A \]
4. \[ E - L + A \xleftrightarrow{K_1} E : L : A \]
5. \[ L : A + Ab \xleftrightarrow{K_1} Bab \]
6. \[ E - L : A \xleftrightarrow{K_1} E + Ab \]

The rates of reaction are represented as 15 differential equations with 15 unknown parameters:

\[
\frac{d[L]}{dt} = k_{-1}[L : A] - k_1[L][A] + k_2[L : A : L] - k_2[L : A][L].
\]

(3)

\[
\frac{d[A]}{dt} = k_{-1}[L : A] - k_1[L][A].
\]

(4)

\[
\frac{d[L : A]}{dt} = k_1[L][A] - k_{-1}[L : A] + k_2[L : A : L] - k_2[L : A][L].
\]

(5)

\[
\frac{d[L : A : L]}{dt} = k_2[L : A][L] - k_{-2}[L : A : L].
\]

(6)

**Step 2**

All of the parameters (symbols) and rate constants used in reactions (7)–(18) are summarized in Table 2.

The rates of reaction are represented as 15 differential equations with 15 unknown parameters:

\[
\]

(19)

\[
\frac{d[E - L : A]}{dt} = k_3[L - E][A] - k_{-3}[E - L : A]
+ k_{-4}[E - L : A : L] - k_4[E - L : A][L]
+ k_{-5}[E - L : A : L - E] - k_5[E - L : A][L - E],
\]

(20)

\[
\frac{d[L]}{dt} = k_{-1}[L : A] - k_1[L][A] + k_{-2}[L : A : L] - k_2[L : A][L]
- k_3[L : A][L] - k_{-4}[E - L : A : L]
- k_4[E - L : A][L],
\]

(21)

\[
\frac{d[L - E]}{dt} = k_{-3}[E - L : A] - k_3[L - E][A]
+ k_{-5}[E - L : A : L] - k_5[E - L : A][L - E]
+ k_{-6}[E - L : A : L - E] - k_6[L : A][L - E]
- k_{-10}[E] - k_{10}[L - E][EA],
\]

(22)

\[
\frac{d[A]}{dt} = k_{-1}[L : A] - k_1[L][A] - k_{-3}[E - L : A]
- k_3[L - E][A],
\]

(23)

\[
\frac{d[L : A : L]}{dt} = k_2[L : A][L] + k_{-2}[L : A : L] + k_{-7}[Bab]
- k_7[L : A : L][Ab],
\]

(24)
Table 2
Rate constants and parameters in step 2 of CEDIA

<table>
<thead>
<tr>
<th>Rate constants</th>
<th>Parameters (symbols)</th>
</tr>
</thead>
<tbody>
<tr>
<td>$K_1$: affinity constant for reaction (7)</td>
<td>$k_i$: association rate constant for reaction (7)</td>
</tr>
<tr>
<td>$K_2$: affinity constant for reaction (8)</td>
<td>$k_{i-1}$: dissociation rate constant for reaction (7)</td>
</tr>
<tr>
<td>$K_3$: affinity constant for reaction (9)</td>
<td>$k_{i-2}$: association rate constant for reaction (8)</td>
</tr>
<tr>
<td>$K_4$: affinity constant for reaction (10)</td>
<td>$k_{i-3}$: dissociation rate constant for reaction (8)</td>
</tr>
<tr>
<td>$K_5$: affinity constant for reaction (11)</td>
<td>$k_{i-4}$: association rate constant for reaction (9)</td>
</tr>
<tr>
<td>$K_6$: affinity constant for reaction (12)</td>
<td>$k_{i-5}$: dissociation rate constant for reaction (9)</td>
</tr>
<tr>
<td>$K_7$: affinity constant for reaction (13)</td>
<td>$k_{i-6}$: association rate constant for reaction (10)</td>
</tr>
<tr>
<td>$K_8$: affinity constant for reaction (14)</td>
<td>$k_{i-7}$: dissociation rate constant for reaction (10)</td>
</tr>
<tr>
<td>$K_{10}$: affinity constant for reaction (16)</td>
<td>$k_{i-8}$: association rate constant for reaction (11)</td>
</tr>
<tr>
<td>$K_{11}$: affinity constant for reaction (17)</td>
<td>$k_{i-9}$: dissociation rate constant for reaction (11)</td>
</tr>
<tr>
<td>$K_{12}$: association rate constant for reaction (18)</td>
<td>$k_{i-10}$: association rate constant for reaction (12)</td>
</tr>
</tbody>
</table>

Parameters (symbols). $L$ ( ): analyte in reactions (7), (8) and (10); $A$ ( ): antibody for the analyte in reactions (7) and (9); $L : A$ ( ): binding complex between one antibody and one analyte in reactions (7), (8) and (12); $L : A : L$ ( ): binding complex between one antibody and two analytes in reactions (8) and (13); $L – E$ ( ): analyte-conjugated enzyme donor in reactions (9), (11), (12), and (16); $E – L$ : $A$ ( ): binding complex between analyte-conjugated enzyme donor and antibody in reactions (9)–(11); $E$ : $A$ ( ): enzyme acceotor in reaction (16); $E$ ( ): active enzyme in reaction (16); $Ab$ ( ): secondary antibody in reactions (13)–(15); $Bab$ ( ): binding complex between primary and secondary antibody in reactions (13)–(15); $S$ ( ): substrate in reaction (17); $ES$ ( ): enzyme–substrate complex in reaction (17); $P$ ( ): product in reaction (18); $E – L : A$ ( ): binding complex between one antibody and one analyte and analyte-conjugated enzyme donor in reactions (10), (12) and (14); $E – L : A : L$ ( ): binding complex between one antibody and two analyte-conjugated enzymes in reactions (11) and (15).

\[
+ k_8[Bab] - k_9[E - L : A : L]|Ab|,
\]

(25)

\[
\frac{d[E - L : A : L - E]}{dt} = k_5[E - L : A][L - E] \\
- k_{-5}[E - L : A : L - E] + k_{-9}[Bab] \\
+ k_9[E - L : A : L - E]|Ab|,
\]

(26)

\[
\frac{d[E]}{dt} = k_{-10}[E] - k_{10}[L - E]|EA|,
\]

(27)

\[
\frac{d[E]}{dt} = k_{10}[L - E]|EA| - k_{-10}[E] + k_{-11}[ES] \\
- k_{11}[E]|S| + k_{12}[ES],
\]

(28)
Combined step

Although the standard assay involves a two-step process, we also chose to model a single-combined-step process, which should be simpler to apply in a ChemChip device [6]. All reacting species are assumed to be simultaneously mixed in the same vessel at the same time; this concept is simply pictured in Fig. 4.

Procedures

The differential equations were numerically integrated using MatLab software, specifying the initial concentrations of each component and estimates of the rate constants. The time course of the materials for various concentrations were obtained.

Study of the kinetics of the CEDIA system allows the derivation of equations which predict the concentrations of reactants and products at any time, even if the system has not yet reached equilibrium. The assumptions of the model are: (1) all of the analytes and antibodies are in the same homogeneous conditions, (2) second-order reversible kinetics are considered for the interaction between analytes and antibodies, (3) analytes are considered monovalent with regard to each antibody, (4) the affinity of each particular site for the analytes is not influenced by the extent of occupancy of other sites (binding should be uniform with no positive or negative allosteric effects), and (5) no nonspecific binding occurs. Although it is impossible for all of these assumptions to be completely met in practice, the law of mass action does provide a useful framework on which to base a theoretical appreciation of the kinetic principles.

Results and discussion

Step 1

The affinity constant consists of association and dissociation rate constants. The knowledge of these rate constants is very important to perform the modeling. The advent of biosensor technology has generated considerable interest in its use to characterize high-affinity interactions between antigens and antibodies [8–12]. Many studies have employed the Biacore instrument (Pharmacia Biosensor, Uppsala, Sweden), in which one reactant flows through a microchannel over the biosensor surface on which the secondary reactant is immobilized to form an affinity matrix, which is detected by surface plasma resonance. The IAysm instrument (Affinity Sensors, Cambridge, UK) has also been employed to study the affinity matrix between a reactant on the biosensor surface forming the base of a stirred cuvette and a flowing secondary reactant; the refractive index change associated with matrix formation is monitored by resonant mirror technology. These investigations have employed expressions developed for the analysis of the association and dissociation kinetics derived from the time course of the biosensor response. Many values of rate constants are generated with these methods and analyzed by pseudo first-order kinetics, based on the assumption of 1:1 stoichiometry and constant concentration of one of the reactants [8–12].

The diffusion-controlled association rate constant is assumed to be $10^6$ or $10^7 \text{M}^{-1} \text{s}^{-1}$ [13]. But, the analyzed values of $k_i$ from experiments are about $10^8 \text{M}^{-1}\text{s}^{-1}$; some authors explain this lower value as being due to the stagnant layer [13]. On the other hand, the dissociation rate constants depend on the binding strengths between reactants (in this case, the antigen and antibody). This affinity constant of the order of $10^7$ order is a typical value for ordinary antigen–antibody systems determined by biosensor technology [14]. We assume this value for the high-affinity case [15]. We adopted the association and dissociation rate constant as $10^5 \text{M}^{-1}\text{s}^{-1}$ and $10^2 \text{s}^{-1}$ for our calculations. Table 3 shows the values of rate constants and parameters used in step 1 of the CEDIA.

The concentration changes of each component with time were calculated by integration of the differential equations under the conditions noted in Table 3. Time 0 is when the antibody is mixed with the ligand, analyte. A plateau is reached at equilibrium. Three cases, depending on the concentration of analyte at a fixed antibody concentration ($10^{-7} \text{M}$), are considered for the first step. The values chosen are appropriate to the CEDIA kit experiment [7]. The zero concentration case, i.e., only antibody is present in the reaction vessel, is shown in Fig. 5A. There is no reaction. Next is the case of low analyte concentration; the analyte and antibody react to form a complex. The complex is formed in a small amount in the presence of low analyte concentration, shown in Fig. 5B. If more analyte is added to react with
antibody, the amount of free antibody is decreased, as the binding complex is produced in considerable amounts; this case is shown in Fig. 5C.

While the amounts of binding complex between one antibody and two analytes (L : A : L) can be greatly increased with time at the higher concentrations of analyte, no variation of it is observed in the lower concentration case. The consumption of antibody or the production of binding complex between analyte and antibody affects the assay. More binding complex means more free analyte-conjugated enzyme donor, which can produce more active enzyme.

**Step 2**

The analyte-conjugated enzyme donor, L–E (●), is added to the mixture of the first step of the CEDIA kit method; antibody binds to the analyte and analyte-conjugated enzyme donor as L : A : L (▽), E–L : A : L (♦), and E–L : A : L–E (△). Here, we have a sequential immunoassay where one of the two competing components (i.e., the analyte and analyte-conjugated enzyme donor) reaches the antibody first to initiate the interaction that culminates in the equilibrium state (reactions 7,9). When the analyte-conjugated enzyme donor and antibody are present together in the sample, the analyte-conjugated enzyme donor is capable of binding, in a competitive fashion, either to antibody or to the enzyme acceptor (reactions 9,16). The active enzyme is involved in the conversion of substrate into product (signal); the active enzyme is produced by the binding of free analyte-conjugated enzyme donor and enzyme acceptor (reaction(16)). As the amount of analyte is increased, it binds to antibody, leaving more free analyte-conjugated enzyme to combine with the enzyme acceptor and produce more active enzyme. The secondary antibody, Ab, function is to bind to the primary antibody (reactions(13)–(15)) and improve the sensitivity of the analysis by inhibiting complementation [4]. The coupling of antibody to the ligand (analyte)-conjugated enzyme donor (reaction(9)) slows the rate of complementation of enzyme acceptor (EA) (Σ) and analyte-conjugated enzyme donor (L–E) (●) (reaction (16)).
Such coupling, however, does not completely prevent complementation. If the secondary antibody (Ab) is coupled to the primary antibody-ligand-conjugated enzyme donor, it enhances steric interference of the primary antibody and may completely prevent complementation by that enzyme-donor population which is bound. The reactions (17) and (18) constitute the catalyzed enzyme reaction.

From the interaction of analyte and antibody of step 1, we can calculate \([L], [A], [L : A], \text{and } [L : A : L]\) at the equilibrium condition. With these values and the added concentration of analyte-conjugated enzyme donor, enzyme acceptor, substrate, and secondary antibody as an initial condition, the concentrations of each constituent with time can be calculated by solution of the differential equations. The enzyme acceptor is originally inserted in step 1, but it remains in an unbound form and participates in an interaction of step 2. The values of the affinity constants and the accompanied rate constants of reactions (7) through (18) (Table 4) are based on the considerations for ordinary antigen–antibody systems; a 10\(^7\) affinity constant is used for all antibody–antigen systems (reactions (7)–(15)). The rate constants of formation of active enzyme from the binding of free analyte-conjugated enzyme donor and enzyme acceptor (reaction (16)) are estimated from the folding and association of β-galactosidase [16], neglecting the detailed dimer–tetramer reaction mechanism. The rate constants of catalyzed enzyme reactions (reactions (17) and (18)) are also roughly estimated from information of ordinary Michaelis–Menten treatment for several enzymes [17].

The initial concentrations of each species are \([A] = 1.0 \times 10^{-7} \text{M, } [L–E] = 1.0 \times 10^{-8} \text{M, } [EA] = 1.0 \times 10^{-6} \text{M, } [Ab] = 1.0 \times 10^{-5} \text{M, and } [S] = 1.0 \times 10^{-4} \text{M for each analyte concentration. Table 4 summarizes the rate constants and parameters in step 2 of the CEDIA. The results obtained (only the concentrations of active enzyme and the final product are depicted) are given in Fig. 6, using the values suggested by Table 4. Fig. 6 has three pictures corresponding to the amounts of analytes. The shape of the time curves are similar, but their magnitudes are different. E and P produced are dependent on the added amounts of analytes, and the equilibration time becomes longer for higher amounts of analytes (Fig. 6C), which can be seen from the time course of E. If we add more analyte to the reagents, more time is needed to achieve equilibrium. The light signal increases steeply until the experimental condition reaches equilibrium (Fig. 6C).

The final product P is directly related to the light absorbance signal (accumulated amounts of product), which corresponds to the experimental signal. The calculated time courses at several analyte concentrations ([L] = 0, \(1 \times 10^{-8}\), \(10^{-7}\), \(10^{-6}\), and \(10^{-5}\) M) are shown in Fig. 7A as a function of analyte concentration. The amount of P increases with time. Reactions (17) and (18) are enzyme-catalyzed reactions and the final colored product (P) is entirely dependent on the amounts of active enzyme (E).

Many immunoassays employ chemiluminescence as the measured signal [4]. We are interested in modifying CEDIA to utilize chemiluminescence output. Chemiluminescence intensity is directly proportional to the enzyme activity (photons do not accumulate, as do chromophores in conventional CEDIA systems). Therefore, it is important to know the calculated amount of active enzyme and its variation with the change of concentrations of any other species. To illustrate this phenomenon, the time courses of the active enzyme concentrations at several analyte concentrations are given in Fig. 7B. The active enzyme concentration reaches equilibrium within 200 s at lower analyte concentrations and continues to increase at higher analyte concentrations. Fig. 7 is a kind of immunoassay dose–response curve. The response curve shows two groups, i.e., lower concentrations of analytes and higher concentrations of analytes. The discrimination of responses for the change of analyte concentrations in ranges of low concentration of analyte is slight; i.e., it is very difficult to discriminate the responses with the change of analyte concentration.

<table>
<thead>
<tr>
<th>Rate constants</th>
<th>Parameters</th>
</tr>
</thead>
<tbody>
<tr>
<td>(k_1 = 10^5 \text{M}^{-1} \text{s}^{-1}) and (k_{-1} = 10^{-2} \text{s}^{-1}) ((K_{1} = 1.0 \times 10^0))</td>
<td><strong>Step 1</strong></td>
</tr>
<tr>
<td>(k_2 = 10^5 \text{M}^{-1} \text{s}^{-1}) and (k_{-2} = 10^{-2} \text{s}^{-1}) ((K_{2} = 1.0 \times 10^0))</td>
<td>([L] = 0 \text{ and } [A] = 10^{-7} \text{M})</td>
</tr>
<tr>
<td>(k_3 = 10^5 \text{M}^{-1} \text{s}^{-1}) and (k_{-3} = 10^{-2} \text{s}^{-1}) ((K_{3} = 1.0 \times 10^0))</td>
<td>(\rightarrow [L] = 0, [A] = 10^{-7}, [L : A] = 0, \text{ and } [L : A : L] = 0 \text{M})</td>
</tr>
<tr>
<td>(k_4 = 10^5 \text{M}^{-1} \text{s}^{-1}) and (k_{-4} = 10^{-2} \text{s}^{-1}) ((K_{4} = 1.0 \times 10^0))</td>
<td>([L] = 10^{-8} \text{ and } [A] = 10^{-7} \text{M})</td>
</tr>
<tr>
<td>(k_5 = 10^5 \text{M}^{-1} \text{s}^{-1}) and (k_{-5} = 10^{-2} \text{s}^{-1}) ((K_{5} = 1.0 \times 10^0))</td>
<td>(\rightarrow [L] = 5.10 \times 10^{-9}, [A] = 9.51 \times 10^{-9}, [L : A] = 4.85 \times 10^{-9}, \text{ and } [L : A : L] = 2.43 \times 10^{-11} \text{M})</td>
</tr>
<tr>
<td>(k_6 = 10^5 \text{M}^{-1} \text{s}^{-1}) and (k_{-6} = 10^{-2} \text{s}^{-1}) ((K_{6} = 1.0 \times 10^0))</td>
<td>([L] = 10^{-6} \text{ and } [A] = 10^{-7} \text{M})</td>
</tr>
<tr>
<td>(k_7 = 10^5 \text{M}^{-1} \text{s}^{-1}) and (k_{-7} = 10^{-2} \text{s}^{-1}) ((K_{7} = 1.0 \times 10^0))</td>
<td>(\rightarrow [L] = 8.62 \times 10^{-7}, [A] = 5.86 \times 10^{-9}, [L : A] = 5.06 \times 10^{-9}, \text{ and } [L : A : L] = 4.35 \times 10^{-13} \text{M})</td>
</tr>
<tr>
<td>(k_8 = 10^5 \text{M}^{-1} \text{s}^{-1}) and (k_{-8} = 10^{-2} \text{s}^{-1}) ((K_{8} = 1.0 \times 10^0))</td>
<td><strong>Step 2</strong></td>
</tr>
<tr>
<td>(k_9 = 10^5 \text{M}^{-1} \text{s}^{-1}) and (k_{-9} = 10^{-2} \text{s}^{-1}) ((K_{9} = 1.0 \times 10^0))</td>
<td>([L–E] = 1.0 \times 10^{-8} \text{M})</td>
</tr>
<tr>
<td>(k_{10} = 10^5 \text{M}^{-1} \text{s}^{-1}) and (k_{-10} = 10^{-2} \text{s}^{-1}) ((K_{10} = 1.0 \times 10^0)) [13]</td>
<td>([EA] = 1.0 \times 10^{-5} \text{M})</td>
</tr>
<tr>
<td>(k_{11} = 10^5 \text{M}^{-1} \text{s}^{-1}) and (k_{-11} = 1 \text{s}^{-1}) ((K_{11} = 1.0 \times 10^0)) [14]</td>
<td>([Ab] = 1.0 \times 10^{-7} \text{M})</td>
</tr>
<tr>
<td>(k_{12} = 0.1 \text{M}^{-1} \text{s}^{-1}) [14]</td>
<td>([S] = 1.0 \times 10^{-4} \text{M})</td>
</tr>
</tbody>
</table>
Fig. 6. Time courses of production of E (reaction (16)) and P (reaction (18)) assuming divalent antibody for three analyte concentrations: (A) [L] = 0 M (blank), (B) [L] = 10^{-8} M (low concentration), and (C) [L] = 10^{-6} M (high concentration). The calculated molar concentrations of final product, P (reaction (18)), and active enzyme, E (reaction (16)), are shown on the y axes. The initial concentrations of L, A, L : A, and L : A : L for three cases are obtained from the calculations of step 1 and are given in Table 4. The concentrations of the other species and the rate constants used are given in Table 4.

Fig. 7. Time courses of the production of P (reaction (18), (A)) and E (reaction (16), (B)) assuming divalent antibody for several analyte concentrations. The calculated molar concentrations of final product P (reaction (18)) and active enzyme E (reaction (16)), are shown on the y axes. The initial concentrations of L, A, L : A, and L : A : L for five different analyte concentrations are obtained from the calculations of step 1. The concentrations of the other species and the rate constants used are given in Table 4.
at any reaction time, so it has necessarily lower sensitivity. The responses in high concentration ranges of analytes \((10^{-5} \text{ and } 10^{-6} \text{ M})\) can be differentiated slightly. The reason for the difference of discrimination at low and high analyte concentrations is that we have 12 equations describing step 2 of the CEDIA. L and L–E compete for A. As the concentration of L increases, more L : A will be produced and the concentration of A will be necessarily reduced (reaction (7)). If the concentration of A is reduced, the amount of L–E will be reduced slightly (reaction (9)) and this effects the final concentration of active enzyme (reaction (16)); i.e., the concentration of active enzyme increases with the increase in L. But the reaction (12) will complicate the above reactions. More amounts of L : A produced with L will bind to L–E (reaction (12)), so the concentration of L–E decreases profoundly with the increase in L. In the case of very low analyte concentrations such as L = 0, 10^{-8}, and 10^{-7} \text{ M} under the conditions of our assumed parameters, the model cannot be simulated as accurately as the high analyte concentrations. The dynamic range is the range in concentrations which can be distinguished and is not necessarily linear. Appropriate calibration is always used [18,19]. Concentration between \(10^{-6} \text{ and } 10^{-7} \text{ M} \text{ (Fig. 7)} \) can be readily detected—this range is indeed linear. In this case, the dynamic range might be estimated as \(10^{-6}–10^{-7} \text{ M} \). If one requires an extended dynamic range, it can be \(10^{-5}–10^{-7} \text{ M} \), although the coverage over this wider range is not completely linear.

The binding affinities between antibody and antigen were assumed to be the same. Secondary antibody is added to improve the sensitivity of CEDIA analysis by binding to the primary antibody [7], forming a complex. The binding affinities between them may be different from the binding between antigen and antibody. If we assume stronger binding affinities between primary and secondary antibodies, the calculation results representing responses are further improved (although the values are slightly lowered), which is shown in Fig. 8 assuming the affinity constant for reactions (13)–(15) to be \(1.0 \times 10^8 \). Only the association rate constant is 10 times increased \( (k_7 = k_8 = k_9 = 1.0 \times 10^6 \text{ M}^{-1} \text{ s}^{-1}) \), resulting in a 10 times increase in affinity constant. One can see the dynamic range of \(10^{-5}–10^{-8} \text{ M} \text{ from Fig. 8. If the assumed parameters and rate constants are further revised, the responses may be further improved.} \)

We assumed that the antibody has a divalent character and can bind up to two analytes. In the above calculations, the same rate constant values between analyte and antibody are used, regardless of the kind of analytes. The affinity constants of \(10^7 \text{ (association rate constant of } 10^5 \text{ and dissociation rate constant of } 10^{-2} \) \) are used for the reactions between any kinds of analyte (L and L–E) and antibody (A). Assuming the affinity constant of \(10^8 \text{ (association rate constant of } 10^6 \text{ and dissociation rate constant of } 10^{-2} \) between primary and secondary antibodies will expand the dynamic range. Thus the choice and nature of Ab is obviously critical to the analysis and the analytical behavior.

**Combined step**

In a one-step CEDIA, all of the reactants (L, A, L–E, S, Ab, and EA) are mixed simultaneously in the same vessel; the primary incubation step between analyte and antibody is omitted, reducing the time and cost (Fig. 4). Such a one-step assay would be simpler and easier to apply in our developing multianalyte ChemChip device [6]. The calculated results are similar to that of the two-step CEDIA method and are shown in Fig. 9. The results in the ranges of higher concentrations of analyte give better discrimination of responses than the two-step method. The distinction of the responses in the lower range is also not clear. The calculated rates of product formation vs analyte concentration show that the range of \(10^{-5}–10^{-7} \text{ M} \text{ analyte concentration is simply linear from the view of calibration. A dynamic range of } 10^{-5}–10^{-7} \text{ M} \text{ analyte concentration is clearly shown, which means that a one-step assay actually re-}

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**Fig. 8.** Time courses of the production of P (reaction (18), (A)) and E (reaction (16), (B)) assuming divalent antibody for several analyte concentrations and stronger binding affinities between primary and secondary antibodies \((K_7 = K_8 = K_9 = 1.0 \times 10^6 \text{ for reactions (13)–(15))} \). The calculated molar concentrations of final product P (reaction (18)) and active enzyme E (reaction (16)) are shown on the y axes. The initial concentrations of L, A, L : A, and L : A : L for five different analyte concentrations are obtained from the calculations of step 1. The concentrations of the other species and the rate constants used are given in Table 4 except for the reactions (13)–(15).
Table 5

Rate constants used to derive Fig. 9

<table>
<thead>
<tr>
<th>Rate constants</th>
<th>Parameters</th>
</tr>
</thead>
<tbody>
<tr>
<td>$k_1 = 10^3 \text{M}^{-1}\text{s}^{-1}$ and $k_{-1} = 10^{-2}\text{s}^{-1}$ ($K_1 = 1.0 \times 10^5$)</td>
<td>[L] = 0 M</td>
</tr>
<tr>
<td>$k_2 = 10^4 \text{M}^{-1}\text{s}^{-1}$ and $k_{-2} = 10^{-2}\text{s}^{-1}$ ($K_2 = 1.0 \times 10^5$)</td>
<td>[L] = $10^{-8}$ M</td>
</tr>
<tr>
<td>$k_3 = 10^5 \text{M}^{-1}\text{s}^{-1}$ and $k_{-3} = 10^{-2}\text{s}^{-1}$ ($K_3 = 1.0 \times 10^5$)</td>
<td>[L] = $10^{-7}$ M</td>
</tr>
<tr>
<td>$k_4 = 10^6 \text{M}^{-1}\text{s}^{-1}$ and $k_{-4} = 10^{-2}\text{s}^{-1}$ ($K_4 = 1.0 \times 10^5$)</td>
<td>[L] = $10^{-6}$ M</td>
</tr>
<tr>
<td>$k_5 = 10^7 \text{M}^{-1}\text{s}^{-1}$ and $k_{-5} = 10^{-2}\text{s}^{-1}$ ($K_5 = 1.0 \times 10^5$)</td>
<td>[L] = $10^{-5}$ M</td>
</tr>
<tr>
<td>$k_6 = 10^8 \text{M}^{-1}\text{s}^{-1}$ and $k_{-6} = 10^{-2}\text{s}^{-1}$ ($K_6 = 1.0 \times 10^5$)</td>
<td>[A] = $1.0 \times 10^{-7}$ M</td>
</tr>
<tr>
<td>$k_7 = 10^9 \text{M}^{-1}\text{s}^{-1}$ and $k_{-7} = 10^{-2}\text{s}^{-1}$ ($K_7 = 1.0 \times 10^5$)</td>
<td>[L–E] = $1.0 \times 10^{-8}$ M</td>
</tr>
<tr>
<td>$k_8 = 10^{10} \text{M}^{-1}\text{s}^{-1}$ and $k_{-8} = 10^{-2}\text{s}^{-1}$ ($K_8 = 1.0 \times 10^5$)</td>
<td>[E</td>
</tr>
<tr>
<td>$k_9 = 10^{11} \text{M}^{-1}\text{s}^{-1}$ and $k_{-9} = 10^{-2}\text{s}^{-1}$ ($K_9 = 1.0 \times 10^5$)</td>
<td>[Ab] = $1.0 \times 10^{-7}$ M</td>
</tr>
<tr>
<td>$k_{10} = 10^{12} \text{M}^{-1}\text{s}^{-1}$ and $k_{-10} = 10^{-2}\text{s}^{-1}$ ($K_{10} = 1.0 \times 10^5$) [13]</td>
<td>[S] = $1.0 \times 10^{-4}$ M</td>
</tr>
<tr>
<td>$k_{11} = 10^{13} \text{M}^{-1}\text{s}^{-1}$ and $k_{-11} = 1\text{s}^{-1}$ ($K_{11} = 1.0 \times 10^5$) [14]</td>
<td></td>
</tr>
<tr>
<td>$k_{12} = 0.1 \text{M}^{-1}\text{s}^{-1}$ [14]</td>
<td></td>
</tr>
</tbody>
</table>

Some results in a wider measurable dynamic range than the two-step assay. The calculated rate of product formation vs. analyte concentration shows that the range of $10^{-5}$ to $10^{-7}$ M is simply linear from the view of calibration. Table 5 lists the rate constants and parameters used to obtain Fig. 9. This situation must be more fully considered and tested.

Chemiluminescent assay methods are simple, inexpensive, and generally more sensitive than standard spectrophotometric assays [19] and can be applied to a one-step CEDIA, with greater simplicity and reduced time and cost [20]. To accomplish a luminescent read-out for our ChemChip purposes, it is necessary to replace the substrate, currently present in the Reagent 2 of Fig. 2, with a chemiluminescent substrate. This application is now underway in our laboratory. Chemiluminescent assay uses a chemiluminescent substrate, all the other reactants in the CEDIA are unchanged. So the results should be same as the color substrate modeled in the paper.

Conclusion

We obtained time course curves of the major reactants and products using a model of CEDIA by integration of the relevant differential kinetic equations. We conclude the following: (1) The obtained time course curve of the production of P (Fig. 7A) can be compared with the light absorption signal of the CEDIA kit experiment [15]. The E time course curve (Fig. 7B) is being tested with a modified chemiluminescent assay method [15]. (2) Using assumed parameters, the analyte concentration response curve can be obtained. In a two-step case, a dynamic range of $10^{-6}$ to $10^{-7}$ M is obtained. If we assume stronger binding affinities between primary and secondary antibodies, the dynamic range can be expanded ($10^{-5}$ to $10^{-8}$ M). (3) Simulation of a one-step CEDIA produces Fig. 9, a dynamic range of $10^{-5}$ to $10^{-7}$ M analyte concentration. (4) From this theoretical consideration, a simple one-step immunoassay has the merit of potentially reducing time and cost and has an improved dynamic range.

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References


