2013

# Ultrasensitive Analysis of Binding Affinity of HIV Receptor and Neutralizing Antibodies Using Solution-Phase Electrochemiluminescence Assay

Xiao-Hong Nancy Xu
*Old Dominion University*, xhxu@odu.edu

Zhaoyang Wen
*Old Dominion University*

William J. Brownlow
*Old Dominion University*



## Original Publication Citation

Xu, X. H. N., Wen, Z. Y., & Brownlow, W. J. (2013). Ultrasensitive analysis of binding affinity of HIV receptor and neutralizing antibodies using solution-phase electrochemiluminescence assay. *Journal of Electroanalytical Chemistry, 688*, 53-60. doi:10.1016/j.jelechem.2012.08.004

Published in final edited form as:
*J Electroanal Chem (Lausanne Switz)*. 2013 January 1; 688: 53–60. doi:10.1016/j.jelechem.2012.08.004.

# Ultrasensitive Analysis of Binding Affinity of HIV Receptor and Neutralizing Antibody Using Solution-Phase Electrochemiluminescence Assay

**Xiao-Hong Nancy Xu**[*], **Zhaoyang Wen**, and **William J. Brownlow**
Department of Chemistry and Biochemistry, Old Dominion University, Norfolk, VA 23529, USA

## Abstract

Binding of a few ligand molecules with its receptors on cell surface can initiate cellular signaling transduction pathways, and trigger viral infection of host cells. HIV-1 infects host T-cells by binding its viral envelope protein (gp120) with its receptor (a glycoprotein, CD4) on T cells. Primary strategies to prevent and treat HIV infection is to develop therapies (e.g., neutralizing antibodies) that can block specific binding of CD4 with gp120. The infection often leads to the lower counts of CD4 cells, which makes it an effective biomarker to monitor the AIDS progression and treatment. Despite research over decades, quantitative assays for effective measurements of binding affinities of protein-protein (ligand-receptor, antigen-antibody) interactions remains highly sought. Solid-phase electrochemiluminescence (ECL) immunoassay has been commonly used to capture analytes from the solution for analysis, which involves immobilization of antibody on solid surfaces (micron-sized beads), but it cannot quantitatively measure binding affinities of molecular interactions. In this study, we have developed solution-phase ECL assay with a wide dynamic range (0–2 nM) and high sensitivity and specificity for quantitative analysis of CD4 at femtomolar level and their binding affinity with gp120 and monoclonal antibodies (MABs). We found that binding affinities of CD4 with gp120 and MAB (Q4120) are $9.5\times10^{8}$ and $1.2\times10^{9}$ $M^{-1}$, respectively. The results also show that MAB (Q4120) of CD4 can completely block the binding of gp120 with CD4, while MAB (17b) of gp120 can only partially block their interaction. This study demonstrates that the solution-phase ECL assay can be used for ultrasensitive and quantitative analysis of binding affinities of protein-protein interactions in solution for better understating of protein functions and identification of effective therapies to block their interactions.

### Keywords

Electrochemiluminescence; binding constant; binding affinity; HIV receptors; CD4; gp120-CD4; ligand-receptor interaction; neutralizing antibody; protein-protein interaction; ultrasensitive analysis



[*]To whom correspondence should be addressed: xhxu@odu.edu; www.odu.edu/sci/xu/xu.htm; Tel/fax: (757) 683-5698.

## Introduction

Persistent infections of human immunodeficiency virus type 1 (HIV-1) in human leads to immunodeficiency syndrome (AIDS) [1–3]. Specific binding of the HIV envelope glycoprotein (gp120) to a receptor (CD4) on the T cell surface initiates their binding with co-receptors (e.g., CCR5, CXCR) and triggers the entry of the virus into the host T cell, which causes the HIV infection [2–3]. The binding of gp120 with CD4 is the most obvious initial step in HIV infection. Thus, gp120 is among the first targets for design of effective therapy (HIV vaccine) to treat the HIV infection, in which neutralizing antibodies are designed to block the binding of gp120 with CD4 [1, 4]. Unfortunately, efforts to develop HIV vaccines targeting gp120 have been hampered by distinctive chemical and structural properties of gp120 [1, 5–6]. It is difficult for antibodies to access and bind with gp120 because the viral surface shields the gp120 from its binding with neutralizing antibodies, while its loose structure can be easily captured by CD4. These interesting properties underscore the importance of targeting both gp120 and CD4, and quantitative analysis of their binding affinities with prospective antibodies to identify neutralizing antibodies that can effectively block the binding of gp120 with CD4.

HIV infection causes a progressive reduction of CD4 T cells [7]. Thus, CD4 counts (normal blood values: $500–1200\times10^6$/L) have been used as an effective biomarker to monitor the progress of AIDS and efficacy of its treatment. CD4 is also associated with a number of other autoimmune diseases (e.g., vitiligo and type-I diabetes mellitus) [8]. Thus, it is very important to quantitatively analyze CD4 for better understanding of its roles in cellular functions and for effective disease diagnosis and treatment.

Conventional assays for detection of protein (antigen, ligand, and receptor) and study of protein-protein (antigen-antibody, ligand-receptor) interactions include bead-based ECL immunoassay [9–11], enzyme-linked immunosorbent assay (ELISA), fluorescence immunoassay, protein A immunoassay, and radioimmunoassay (RIA). The detection schemes of these assays involve immobilization of a counter part (antibody) of analytes of interest onto solid surfaces to create immunoadsorbents, which then capture the analytes from the solution using molecular recognition via sandwich, competition or direct immunoassay. The solid-phase assays require high amount of the counter part (antibody) of the analytes. It remains a challenge to accurately control and quantitatively characterize the number of molecules and their distribution on the solid surfaces, which makes it difficult to quantitatively measure binding affinity of protein-protein interactions. Furthermore, the solid-phases may create steric effects that can affect molecular recognition and their binding affinities, leading to lower selectivity and sensitivity. Moreover, these assays require separation or washing steps, and thus cannot fulfill real-time measurements of molecular (antigen-antibody, ligand-receptor) interactions. Such limitations demand the development of new solution-phase assays that can study binding affinities of both molecules in solution. Recently, we have achieved study of ligand-receptor and antigen-antibody binding reactions in solution and on single live cells in real time at single-molecule level for better understanding of their functions using photostable single-molecule nanoparticle optical biosensors (SMNOBS) and far-field photostable optical nanoscopy (PHOTON) [12–14].

Unlike fluorescence, chemilumiscence and localized surface plasmon resonance (LSPR), ECL is generated electrochemically by forming reactive species at an electrode [9]. The ECL has many distinct advantages over other detection means (chemluminescence, fluorescence and radioisotopes). They possess: (i) high sensitivity, because ECL does not require any light sources, which reduces scattering light and noise, and hence can achieve low background and high signal-to-noise ratio; (ii) high selectivity and high temporal and spatial resolutions, because ECL occurs at a given electrochemical potential and can be

easily and rapidly controlled; (iii) wide dynamic ranges over six orders of magnitude. (iv) high stability (long life time) and less interference, because ECL tags, such as $Ru(bpy)_3^{2+}$, are small molecules (~1000 Da) and they can be used to label the analytes (proteins, DNA, drug) without affecting their binding affinity, biological activity, solubility, or stability; (v) simple, rapid and easy for automation.

Notably, the intensity of ECL is directly related to the mass and size of ECL active species. These distinctive features enable ECL to quantitatively study DNA intercalation [15–19], antigen-antibody binding affinity and protein-protein interactions for better understanding of their functions and exploring their clinical applications [11, 20–21]. In our previous studies [20–21], we have developed and used solution-phase ECL immunoassay to specifically detect and identify three different forms (free, complex and total) of prostate specific antigens (PSA) with a sensitivity as low as 1.7 pg/mL. Currently, ECL immunoassay is primarily used for detection of analytes (DNA, proteins) for disease diagnosis, rather than study of binding and functions of proteins and discovery of effective therapy.

In this study, we have developed solution-phase ECL assays for quantitative analysis of HIV receptor, its ligand-receptor interactions and their binding affinities with MABs, aiming to study their functions and identify effective therapies (potential neutralizing antibodies) to block the interaction of gp120 with CD4 and to prevent HIV from infecting host T cells.

## Experimental Section

### Chemicals and Materials

MAB (Q4120) of CD4, MAB (17b) of gp120, CD4, gp120, MAB of human IgM (anti-IgM), and MAB of mouse IgG (anti-IgG) were provided by NIH AIDS Reagent Program. BCA (bicinchoninic acid) protein assay reagents (Pierce), bovine serum albumin (BSA) (Pierce), PD-10 Column (Pharmacia Biotech), N-hydroxysuccinimide ester of a ruthenium (II) tris-bipyridine chelate, $Ru(bpy)_3^{2+}$-NSH ester (Tag-NHS ester, IGEN), ECL analyzer assay buffer (PBS buffer containing tripropylamne, TPA, IGEN), cell cleaner buffer (IGEN), and all other chemicals (Sigma) were purchased and used as received.

### Synthesis and Characterization of CD4-tag

The CD4 (1.0 mL, 0.625 mg/mL in 150 mM of PBS buffer, pH 7.8) was mixed with tag-NHS ester (50 μL, 1.5 μg/μL in DMSO) at a challenging molar ratio of tag/CD4 of 5 under vortexing in the dark at room temperature for 60 min. The ruthenylation reaction was stopped by adding glycine (20 μL, 2M) at room temperature for 10 min. The mixture was added into the PD-10 column which had been equilibrated with a PBS buffer (25 mL, 150 mM, pH 7.2) and covered with aluminum foil to protect the tag, $Ru(bpy)_3^{2+}$, from exposure to the light. The samples (CD4, CD4-tag with various labeling ratios, tag) were then separated and eluted from the column by adding the buffer (0.5 mL each) into the column. Each eluate was collected and characterized using both BCA protein assay and UV-vis absorption spectroscopy to determine the concentration of CD4 and tag of each eluate, respectively.

The protein (CD4) concentration was determined using the BCA protein assay with BSA standards, as described by the following [22–23]. A dilution series of BSA (0–15 μM) in the PBS buffer (150 mM PBS buffer, pH 7.2) were prepared and used as standards. The samples eluted from the column (CD4-tag), blank control (the PBS buffer), and standard BSA solutions at 10 μL each were pipetted into each well on a 96-well plate and each sample was sampled in triplicates. A 200 μL of BCA reagent was added into each well. The plate sealed with parafilm and Al-foil was incubated at 37°C for 30 min and then at room temperature for 15 min. Absorbance of each sample in the plate was measured at 562 nm ($BCA_2$-$Cu^+$

generated by the reduction of $Cu^{2+}$ by a protein, CD4 or BSA) using a plate reader (Bio Tek). Plot of absorbance produced by BSA (standards) versus their concentrations was constructed and used as a calibration curve to determine unknown concentrations of CD4 in each eluate using the absorbance ($BCA_2$-$Cu^{+}$) at 562 nm produced by CD4.

The tag concentration of each sample was determined by measuring the absorbance of $Ru(bpy)_3^{2+}$ at 455 nm using a UV-vis spectrometer (Cary 3G Varian). The molar ratio of tag to CD4 for each sample was determined by dividing its tag concentration by its CD4 concentration. The highest molar ratio of one eluate was used for the study.

## ECL Analysis of CD4-tag and its Binding Affinities with gp120 and MAB

A dilution series of CD4-tag (0–1.88 nM) in the assay buffer (PBS buffer with TPA) were prepared and analyzed by an ECL analyzer (Origen Analyzer, IGEN). The analyzer includes an electrochemical flow-cell with working, reference and counter electrodes, a potentiostat, and a single photomultiplier tube (PMT), which was interfaced with a computer [10]. Plot of integrated ECL intensity of each sample acquired by the analyzer versus CD4-tag concentration was determined and used as a calibration curve. ECL intensity of unknown concentrations of CD4-tag solution was acquired using the ECL analyzer, which was then utilized to determine its concentration using the calibration curve.

The mixtures of CD4-tag (1.88 nM) with various molar ratios (R) of gp120, MAB (Q4120), anti-IgM, or anti-IgG to CD4-tag in the PBS buffer containing TPA (1050 μL) were incubated at 4 °C for 3 h until their binding reaction with CD4 reaches binding equilibrium. The mixtures were then analyzed using the ECL analyzer triplicate (350 μL each) at room temperature and in dark. The ECL intensity of each sample was normalized by dividing the intensity of each sample (CD4-tag in the presence of gp120 or MAB, $R > 0$) by the intensity of CD4-tag alone (in the absence of gp120 or MAB, $R = 0$). The normalized ECL intensity of each sample was plotted against the molar ratios (R) to determine binding constant of gp120 or MAB with CD4-tag. The study of binding of anti-IgM or anti-IgG with CD4-tag serves as control experiments.

## ECL Study of Neutralizing Antibodies

The MAB-CD4-tag was prepared by incubating CD4-tag (1.88 nM) with the high ratio ($R_{Q4120/CD4\text{-}tag} = 15$) of its MAB (Q4120) at 4 °C in dark for 3 h to ensure the complete formation of MAB-CD4-tag (none unbound CD4-tag). The MAB-CD4-tag was then incubated with various molar ratios (0–10) of gp120 in the buffer at 4 °C in dark for 3 h and studied using the ECL analyzer. The ECL intensity of each sample was normalized by dividing the intensity of each sample (MAB-CD4-tag with gp120, $R > 0$) by the intensity of MAB-CD4-tag alone (in the absence of gp120, $R = 0$). The normalized ECL intensity of each sample was plotted against the molar ratios of gp120 to MAB-CD4-tag to determine whether gp120 can bind with MAB-CD4-tag, and whether Q4120 can serve as a neutralizing antibody to block the interaction of gp120 with CD4.

Using the similar approaches, the 17b-gp120 complex was prepared by incubating gp120 with the high ratios ($R_{17b/gp120} = 15$) of its MAB (17b) at 4 °C in dark for 3 h to ensure the complete formation of 17b-gp120 (none unbound gp120). The CD4-tag (1.88 nM) was then incubated with various molar ratios (0–10) of 17b-gp120 in the buffer at 4 °C in dark for 3 h and studied using the ECL analyzer. The ECL intensity of each sample was normalized by dividing the intensity of each sample (in the presence of both CD4-tag and 17b-gp120, $R > 0$) by the intensity of CD4-tag alone (in the absence of 17b-gp120, $R = 0$). The normalized ECL intensity of each sample was plotted against the molar ratios of 17b-gp120 to CD4-tag

to determine whether CD4-tag can bind with 17b-gp120 and whether 17b can serve as a neutralizing antibody to block the interaction of gp120 with CD4.

## Results and Discussion

### Synthesis and Characterization of CD4-tag

We conjugated CD4 with $Ru(bpy)_3^{2+}$-NHS ester to prepare CD4-tag via a ruthenylation reaction. The carboxyl group of the tag-NSH was linked with the amine group of CD4 through a peptide bond mediated by 1-ethyl-3-(3-dimethylaminopropyl)-carbodiimide hydrochloride (EDC) and *n*-hydroxysulfosuccinimide (sulfo-NHS), as described in Figure 1. The mixture of CD4-tag, CD4 and tag were separated using a size-exclusive column. The conjugation ratios of the tag with CD4 for each CD4-tag sample were characterized by measuring CD4 and tag concentrations using BCA assay and UV-vis absorption spectroscopy, respectively.

The BCA assay includes two reactions [22–23]: (i) the reduction of $Cu^{2+}$ to $Cu^{+}$ by peptide bonds of protein at 37 °C. (ii) Each $Cu^{+}$ chelated with two BCA molecules to generate a purple-colored complex ($BCA_2$-$Cu^{+}$) that shows intense absorption at 560 nm. The amount of reduced $Cu^{+}$ is proportional to the amount of the protein. Thus, the absorbance of $BCA_2$-$Cu^{+}$ is proportional to the amount of the protein. We measured absorbance of $BCA_2$-$Cu^{+}$ generated by a dilution series of each BSA sample (0–15 μM, protein standards) at 562 nm, which was then plotted against the BSA concentration to construct a calibration curve (Figure 2). Unknown concentration of CD4 in each eluate was determined using the absorbance ($BCA_2$-$Cu^{+}$) produced by CD4 and the calibration curve.

$Ru(bpy)_3^{2+}$ exhibits a distinct absorption at 452 nm with an extinction coefficient ($\varepsilon_{452\ nm}$) of $1.4\times10^4\ M^{-1}\ cm^{-1}$ [24]. Thus, the amount of conjugated $Ru(bpy)_3^{2+}$ in each eluate was determined using the absorbance of $Ru(bpy)_3^{2+}$ at 452 nm measured by UV-vis absorption spectroscopy. The result shows the highest conjugation ratio of the tag with CD4 as 2.5 (2.5 tag molecules per CD4 molecule) in one eluate, while other eluates are primarily tags and CD4. Therefore, we collected this eluate and used it to study binding affinities of CD4 with gp120 and MABs.

$Ru(bpy)_3^{2+}$ can generate ECL at 600 nm in the presence of co-reactors, such as TPA [9–10, 25]. Therefore, labeling CD4 with $Ru(bpy)_3^{2+}$ (CD4-tag) enables us to quantitatively analyze CD4 and study its binding affinities with gp120 and MABs. Notably, $Ru(bpy)_3^{2+}$ has distinctive advantages over other tags (enzymes, radioisotopes), including high chemical stability, low molecular weight, water soluble, and simple labeling approach with versatile labeling ratios.

### Quantitative Analysis of CD4-tag using ECL

$Ru(bpy)_3^{2+}$ conjugated with CD4 (CD4-tag) in the PBS buffer containing TPA is electrochemically oxidized to generate $Ru(bpy)_3^{3+}$, which reacts with an electrochemically generated co-reactant (TPA•) at an electrode. Electron transfer between these two species creates an excited state of $Ru(bpy)_3^{2+*}$, which emits at 600 nm and returns to its ground state (Figure 3A) as described in Reactions (1–5).

$$CD4 - Ru(bpy)_3^{2+} - e \rightarrow CD4 - Ru(bpy)_3^{3+} \quad (1)$$

$$CH_3CH_2CH_2\ddot{N}Pr_2 - e \rightarrow CH_3CH_2CH_2\dot{N}Pr_2^{+} \quad (2)$$

$$CH_3CH_2CH_2\dot{N}Pr_2^+ - e \rightarrow CH_3CH_2\dot{C}HNPr_2^+ + H^+ \quad (3)$$

$$CD4-Ru(bpy)_3^{3+} + CH_3CH_2\dot{C}HNPr_2^+ \rightarrow CD4-Ru(bpy)_3^{2+*} + CH_3CH_2CHNPr_2^+ \quad (4)$$

$$CD4 - Ru(bpy)_3^{2+*} \rightarrow CD4 - Ru(bpy)_3^{2+} + hv \quad (5)$$

The dilution series of CD4-tag (0–1.88 nM) with the lowest concentration of 47 pM in the PBS buffer containing TPA (350 μL) were analyzed using the ECL analyzer. Plot of ECL intensity of each sample versus CD4-tag concentration shows a high linearity and a large dynamic range (Figure 3B). The small intercept is attributed to the background emission of the buffer containing TPA (blank). The result indicates that the ECL intensity is proportional to the concentration of CD4-tag, and unknown concentration of CD4-tag can be quantitatively analyzed and determined at the femtomolar level (16 fmolars) by measuring their ECL intensity, which was then used to calculate the concentration via the calibration curve (Figure 3B). The result shows that solution-phase ECL assay can achieve the same sensitivity as the solid-phase ECL assay. At such low concentrations, potential adsorption of protein molecules on the electrode surface is minimized. Furthermore, each ECL analysis is completed very rapidly (within a second) and the analyzer cleans the electrode between each analysis, which further avoids the potential adsorption of the protein molecules on the electrode surfaces and ensures the reproducible quantitative analysis of protein concentrations with high precision and accuracy.

As stated above, the CD4 has been used as an effective biomarker for diagnose of a number of autoimmune diseases (AIDS, type I diabetes), and for monitoring of efficacy of their treatment [7–8]. The CD4 (a member of the immunoglobulin superfamily) plays highly significant roles in cellular functions. Thus, new tools and assays for ultrasensitive analysis of CD4 are essential to earlier and effective diagnosis and treatment of diseases, as well as better understanding of disease development.

## Design of Solution-Phase ECL Assays

We have further developed solution-phase ECL assay to study binding affinity of gp120 or MAB with CD4-tag, as described in the following. Binding reaction of gp120 with CD4-tag with one-to-one binding ratio can be described by Eq. (6).

$$gp120 + CD4 - Tag \underset{k-1}{\overset{k_1}{\Longleftrightarrow}} gp120 - CD4 - Tag \quad (6)$$

The binding constant (K) (binding affinity) of gp120-CD4 binding reaction can be described by Eq. (7).

$$K = \frac{k_1}{k_{-1}} = \frac{[gp120 - CD4 - tag]}{[gp120][CD4 - tag]} = \frac{C_b}{(C_{gp120} - C_b)(C_{t,CD4-tag} - C_b)} \quad (7)$$

Where $C_b$, $C_{gp120}$ and $C_{t,\ CD4\text{-}tag}$ represent the equilibrium concentration of gp120-CD4 complex, the total concentration of gp120 and CD4-tag, respectively. We then use molar ratio of gp120 to CD4 (R), molar fractions of bound gp120 ($X_b$) and unbound gp120 ($X_f$) as defined by Eqs. (8)–(12), to express Eq. (7).

$$C_t = C_b + C_f \quad (8)$$

$$X_b = \frac{C_b}{C_t} \quad (9)$$

$$X_f = \frac{C_f}{C_t} \quad (10)$$

$$X_b + X_f = 1 \quad (11)$$

$$R = \frac{C_{gp120}}{C_{t,CD4-tag}} \quad (12)$$

Thus, Eq. (7) can be derived to Eq. (13).

$$K = \frac{X_b}{C_{t,CD4-tag}(R - X_b)(1 - X_b)} \quad (13)$$

Since ECL intensity ($I_{t,f}$) of unbound CD4-tag is proportional to its concentration as described by Eq. (14) and as shown in Figure 3B, ECL intensity ($I_{t,b}$) of CD4-tag titrated with gp120 is the sum of ECL intensity of unbound CD4-tag ($I_f$) and bound CD4-tag (gp120-CD4-tag) ($I_b$), as described by Eq. (15).

$$I_{t,f} = B\Phi_f D_f^{1/2} C_t \quad (14)$$

$$I_{t,b} = I_f + I_b = B\Phi_f D_f^{1/2} C_f + B\Phi_b D_b^{1/2} C_b \quad (15)$$

Where B is a constant that can be acquired from calibration curve of ECL intensity versus CD4-tag concentration (Figure 3B), and $\Phi_f$ and $\Phi_b$ are the luminescence efficiency constant of unbound CD4-tag and bound CD4-tag (gp120-CD4-tag), respectively. $D_f$ and $D_b$ are the diffusion coefficient of unbound CD4-tag and bound CD4-tag (gp120-CD4-tag), respectively.

The normalized ECL intensity ($I_{t,b}/I_{t,f}$) in Eq. (16) is obtained by dividing Eq. (15) by Eq. (14).

$$\frac{I_{t,b}}{I_{t,f}} = 1 - [1 - \sqrt{\frac{D_b}{D_f}}\frac{\Phi_b}{\Phi_f}]X_b \quad (16)$$

If all CD4-tag molecules are bound with gp120, the molar fraction of bound CD4 ($X_b$) is equal to 1. Eq. (16) can then be simplified to Eq. (17).

$$\frac{I_{t,b}}{I_{t,f}} = \sqrt{\frac{D_b}{D_f}}\frac{\Phi_b}{\Phi_f} \quad (17)$$

The diffusion coefficient can be described by Stokes-Einstein equation as shown in Eq. (18), which states that diffusion coefficient of a molecule (particle) is inversely proportional to its size [26–27].

$$D = \frac{kT}{6\pi\eta a} \quad (18)$$

*

Where D, a, k, T, and η represent diffuse coefficient, the diameter of a molecule, Boltzmann constant, temperature and viscosity of solution, respectively. The viscosity (η) is proportional to the square root of molecule weight ($M^{1/2}$) of solvent [26–27]. The gp120-CD4-tag (bound CD4-tag) is larger than unbound CD4-tag. Thus, it has a smaller diffusion coefficient and lower ECL intensity than unbound CD4-tag.

By solving Eq. (13), the normalized ECL intensity as a function of affinity constant (K), molar ratio of gp120 to CD4-tag (R) and ratio of diffusion coefficients of gp120-CD4-tag to unbound CD4-tag can be described by Eq. (19).

$$\frac{I_{t,b}}{I_{t,f}} = 1 - \left[1 - \sqrt{\frac{D_b}{D_f}}\frac{\Phi_b}{\Phi_f}\right]\frac{(1+R+1/KC_t) - \sqrt{(1+R+1/KC_t)^2 - 4R}}{2} \quad (19)$$

Therefore, plot of normalized ECL intensity ($I_{t,b}/I_{t,f}$) versus R (molar ratio of gp120 to CD4-tag) can be used to determine binding constant (affinity) of gp120 with CD4-tag. Notably, similar approaches can be used to determine binding affinity of MAB with CD4-tag or any protein-protein interactions using such solution-phase ECL assay.

## Study of Binding Affinity of gp120 and MAB with CD4-tag

We quantitatively measured the binding constant of gp120 or MAB (Q4120) with CD4-tag using the solution-phase ECL assay. Plot of normalized ECL intensity of CD4-tag incubated with various molar ratios (R) of gp120 to CD4-tag in the PBS buffer containing TPA (Figure 4) shows that ECL intensity decreases as the ratio (R) increases and then remains constant. The result indicates that gp120 binds with CD4-tag to create gp120-CD4-tag binding complexes, which leads to the decrease of ECL intensity, as more gp120 molecules bind with CD4-tag upon the presence of higher amount of gp120 molecules (the molar ratio increases). When all CD4-tag molecules are bound with gp120, the ECL intensity remains unchanged, regardless the increase of gp120 molecules. We determined the binding constant (K) of gp120 with CD4-tag as $9.5\times10^8$ $M^{-1}$ and the binding ratio as one by fitting the experimental data using Eq. (19), as shown in Figure 4.

Similar to those observed in Figure 4, plot of normalized ECL intensity of CD4-tag incubated with various molar ratios (R) of MAB (Q4120) to CD4-tag in the buffer with TPA (Figure 5) shows that ECL intensity decreases as the molar ratio (R) of MAB/CD4-tag increases and then remains constant. The result indicates that the MAB binds with CD4-tag to form the MAB-CD4-tag complexes, which causes ECL intensity of CD4-tag to decrease. The ECL intensity remains unchanged and independent upon the R when all CD4-tag

molecules are bound with the MAB. Using the same approaches, we determined the binding constant (K) of MAB (Q4120) with CD4-tag as $1.2\times10^9$ $M^{-1}$ and the binding ratio as one by fitting the experimental data using Eq. (19), as presented in Figure 5.

Control experiments are conducted using anti human IgM (anti-IgM, $MAB_{anti\text{-}IgM}$, MAB of human IgM) or anti mouse IgG (anti-IgG, $MAB_{anti\text{-}IgG}$, MAB of mouse IgG) to replace gp120 and MAB (Q4120), and study their binding affinity with CD4-tag using the same approaches. The results in Figure 6 show that normalized ECL intensity of neither $MAB_{anti\text{-}IgM}$ nor $MAB_{anti\text{-}IgG}$ incubated with CD4-tag in the buffer with TPA is dependent upon the molar ratios of the MAB/CD4-tag, which indicates that neither anti-IgM nor anti-IgG binds with CD4-tag.

Taken together, the results demonstrate that the decrease of ECL intensity in Figures 4–5 is attributed to the specific binding of gp120 or MAB (Q4120) with CD4-tag, and the solution-phase ECL assay can be used to determine the binding affinity of protein-protein interactions. Even though the signal changes are small, the binding affinities of protein-protein interactions can be quantitatively measured.

It is worth noting that the solution-phase ECL assay requires much less MAB than the solid-phase assay because the higher amount of MAB is needed to prepare the immobilized MAB on the solid (bead) surfaces. Even though the solid-based ECL assay generates higher signal than the solution-phase assay due to its pre-concentrated (immobilized) MAB on the surface, it cannot be used to quantitatively measure binding affinities of protein-protein interactions (antigen-antibody, ligand-receptor). For the solid-phase ECL assay, only ECL of the binding complexes (gp120-CD4-tag) on the magnetic beads that were captured by the working electrode via magnetic field would have been measured. Thus, the ECL intensity is unrelated to the diffusion coefficients of bound and unbound analytes (CD4-tag), and cannot be used to measure the binding affinities.

## Identification and Characterization of Neutralizing Antibodies

We studied the binding of Q4120-CD4-tag with gp120, and 17b-gp120 with CD4-tag, aiming to determine which MAB can serve as a more effective neutralizing antibody to block the binding of gp120 and CD4.

We first incubated Q4120 with CD4-tag to enable the binding of Q4120-CD4-tag and then measured their binding affinity with gp120. Unlike the study of binding of gp120 with CD4-tag (Figure 4), we found that ECL intensity remains essentially unchanged over the presence of higher amount of gp120, and ECL intensity is nearly independent upon the molar ratio (R) of gp120/Q4120-CD4-tag (Figure 7A). The slight increase of ECL intensity upon the presence of higher concentration of gp120 is most likely attributed to the higher hydrophobicity of the solvent and hence higher quantum yield (QY) of $Ru(bpy)_3^{2+}$ [24, 28]. The result in Figure 7A indicates that gp120 cannot bind with Q4120-CD4-tag. Thus, the MAB of CD4 (Q4120) can effectively block the binding of gp120 with CD4-tag.

Using the similar approaches, we first incubated 17b with gp120 to form the 17b-gp120 binding complexes, and used them to titrate CD4-tag. Unlike the study of binding of gp120 with CD4-tag (Figure 4) or gp120 with Q4120-CD4-tag (Figure 7A), the ECL intensity gradually decreases as the molar ratio (R) of 17b-gp120/CD4-tag increases. The ECL intensity (Figure 7B) decreases much slowly than that of gp120 with CD4-tag (Figure 4), indicating the lower binding affinity of 17b-gp120 with CD4-tag and suggesting that 17b partially blocks the interaction of gp120 with CD4-tag. The large amount of 17b-gp120 is needed to bind with all CD4-tag molecules in the solution in order to create a base-line of the titration curve, which is essential to quantitatively determine the binding affinity using

Eq. (19). Due to the limited amount of 17b, here we qualitatively characterize their binding affinity, which is sufficient to demonstrate the proof-of-concept of the ECL assay for identification of more effective neutralizing antibody to block the binding of gp120 with CD4.

Taken together, the results in Figure 7 show that the MAB (Q4120) of CD4-tag (Figure 7A) is a more effective neutralizing antibody than the MAB (17b) of gp120 (Figure 7B) for blocking the interaction of gp120 with CD4. The results further suggest that targeting of binding sites of CD4 can be even more effective than targeting the binding sites of gp120, which can be attributed to that gp120 rapidly changes its conformation and effectively adapts to its surrounding environments [29]. This study demonstrates that the solution-phase ECL assay can serve as an effective tool to quantitatively identify neutralizing antibodies for blocking the interaction of gp120 with CD4, and to screen effective therapy for preventing HIV infection of host T cells.

## Summary

In summary, we have developed a solution-phase ECL assay and demonstrated that the ECL assay can serve as an effective ultrasensitive and specific assay to quantitatively analyze biomarkers of interest (CD4), to determine binding affinity and binding ratio of protein-protein interactions (ligand-receptor, gp120-CD4; antigen-antibody, MAB-CD4), and to identify neutralizing antibody for potentially blocking of HIV infection of T cells. We found that the ECL assay shows a large dynamic range (0–2 nM) for detection of protein biomarkers (CD4) in solution with high sensitivity (16 fmol). Our results show that the binding affinity of gp120 with CD4-tag in the PBS buffer solution is $9.5\times10^{8}$ $M^{-1}$ with a binding ratio of one; and the binding affinity of MAB (Q4120) with CD4-tag in the solution is $1.2\times10^{9}$ $M^{-1}$ with a binding ratio of one, suggesting that the MAB (Q4120) can potentially neutralize the HIV infection of T cells by blocking the binding of gp120 with CD4. Our study of binding of gp120 with Q4120-CD4-tag and 17b-gp120 with CD4-tag shows that Q4120 can completely block the binding of gp120 with CD4-tag, while 17b can only partially block their binding. The results indicate that Q4120 can serve as a more effective neutralizing antibody than 17b to potentially block the HIV infection of T cells, and suggest that targeting the binding site of CD4 may be more effective than targeting the binding site of gp120 for blocking the entry of HIV into T cells. This study shows that ECL is a powerful tool to detect biomarkers for disease diagnosis, to address fundamental questions related to protein-protein interactions, as well as to identify potential therapies for effective disease treatment.

## Acknowledgments

This work is supported in part by NIH (R21-RR15057; R01GM076440; 3R01GM764401-4S1), Old Dominion Summer Fellowship (Brownlow) and Dominion Scholar Fellowship (Wen). We thank NIH AIDS Reagent Program for providing us with all related proteins and antibodies.

## References

1. Kwong PD, Wyatt R, Robinson J, Sweet RW, Sodroski J, Hendrickson WA. Structure of an HIV gp120 envelope glycoprotein in complex with the CD4 receptor and a neutralizing human antibody. Nature. 1998; 393:648–659. [PubMed: 9641677]
2. Cicala C, Arthos J, Fauci AS. HIV-1 envelope, integrins and co-receptor use in mucosal transmission of HIV. J. Transl. Med. 2011; 9(Suppl 1):S2. [PubMed: 21284901]
3. Lusso P. HIV and the chemokine system: 10 years later. EMBO J. 2006; 25:447–456. [PubMed: 16437164]

4. Cludts I, Meager A, Thorpe R, Wadhwa M. Detection of neutralizing interleukin-17 antibodies in autoimmune polyendocrinopathy syndrome-1 (APS-1) patients using a novel non-cell based electrochemiluminescence assay. Cytokine. 2010; 50:129–137. [PubMed: 20116277]
5. Mascola JR, Montefiori DC. The role of antibodies in HIV vaccines. Annu. Rev. Immunol. 2010; 28:413–444. [PubMed: 20192810]
6. Montefiori DC, Mascola JR. Neutralizing antibodies against HIV-1: can we elicit them with vaccines and how much do we need? Curr. Opin. HIV AIDS. 2009; 4:347-5. [PubMed: 20048696]
7. Bofill M, Janossy G, Lee CA, MacDonald-Burns D, Phillips AN, Sabin C, Timms A, Johnson MA, Kernoff PB. Laboratory control values for CD4 and CD8 T lymphocytes. Implications for HIV-1 diagnosis. Clin. exp. Immunol. 1992; 88:243–252. [PubMed: 1349272]
8. Zamani M, Tabatabaiefar MA, Mosayyebi S, Mashaghi A, Mansouri P. Possible association of the CD4 gene polymorphism with vitiligo in an Iranian population. Clin. Exp. Dermatol. 2010; 35:521–524. [PubMed: 19843086]
9. Bard, AJ. Introduction. In: Bard, AJ., editor. Electrogenerated Chemiluminescence. New York: Marcel Dekker; 2004. p. 1-22.
10. Blackburn G, Shah HP, Kenten J, Leland J, Kamin R, Link J, Peterman J, Powell M, Shah A, Talley D, Tyagi S, Wilkins E, Wu T, Massey R. Electrochemiluminescence detection for development of immunoassays and DNA probe assays for clinical diagnostics. Clin. Chem. 1991; 37:1534–1539. [PubMed: 1716534]
11. Debad, JD.; Glezer, EN.; Leland, JK.; Sigal, GB.; Wohlstadter, J. Clinical and Biological Applications of ECL. In: Bard, AJ., editor. Electrogenerated Chemiluminescence. New York: Marcel Dekker; 2004. p. 359-396.
12. Huang T, Browning LM, Xu X-HN. Far-field photostable optical nanoscopy (PHOTON) for real-time super-resolution single-molarcular imaging of signaling pathways of single live cells. Nanoscale. 2012; 4:2797–2812. [PubMed: 22331098]
13. Huang T, Nallathamby PD, Xu X-HN. Photostable single-molarcule nanoparticle optical biosensors for real-time sensing of single cytokine molarcules and their binding reactions. J. Am. Chem. Soc. 2008; 130:17095–17105. [PubMed: 19053435]
14. Huang T, Xu X-HN. Multicolored nanometer-resolution mapping of single protein-ligand binding complex using far-field photostable optical nanoscopy (PHOTON). Nanoscale. 2011; 3:3567–3572. [PubMed: 21633732]
15. Xu X-H, Bard AJ. Immobilization and hybridization of ss-DNA on an aluminum (III) alkanebisphosphonate thin film with electrogenerated chemiluminescent detection. J. Am. Chem. Soc. 1995; 117:2627–2631.
16. Xu, X-H.; Bard, AJ. Biosensor for and Method of Electrogenerated Chemiluminescent Detection of Nucleic Acid Adsorbed to a Solid Surface. World Intellectual Property Organization No. WO9606946A; Australian Patent No. 703344 (Ed). 1996.
17. Xu X-H, Yang H-C, Mallouk TE, Bard AJ. Immobilization of DNA on an aluminum (III) alkanebisphosphonate thin film with electrogenerated chemiluminescent detection. J. Am. Chem. Soc. 1994; 116:8386–8387.
18. Carter MT, Bard AJ. Electrochemical investigations of the interaction of metal chelates with DNA. 3. Electrogenerated chemiluminescent investigation of the interaction of tris-(1,10-phenanthroline)ruthenium(II) with DNA. Bioconjug. Chem. 1990; 1:257–263. [PubMed: 2096918]
19. Rodriguez M, Bard AJ. Electrochemical studies of the interaction of metal chelates with DNA 4. Voltammetric and electrogenerated chemiluminescent studies of the interaction of tris(2,2'-bipyridine)osmium(II) with DNA. Anal Chem. 1990:2658–2662. [PubMed: 2096730]
20. Xu X-HN, Jeffers RB, Gao J, Logan B. Novel solution-phase immunoassays for molarcular analysis of tumor markers. Analyst. 2001; 126:1285–1292. [PubMed: 11534594]
21. Xu, X-HN.; Zu, Y-B. Electrochemiluminescence Detection in Bioanalysis. In: Xu, X-HN., editor. New Frontiers in Ultrasensitive Bioanalysis: Advanced Analytical Chemistry Applications in Nanobiotechnology, Single Molarcule Detection, and Single Cell Analysis. New Jersey: Wiley; 2007. p. 235–168

22. Smith PK, Krohn RI, Hermanson GT, Mallia AK, Gartner FH, Provenzano MD, Fujimoto EK, Goeke NM, Olson BJ, Klenk DC. Measurement of protein using bicinchoninic acid. Anal. Biochem. 1985; 150:76–85. [PubMed: 3843705]

23. Tuszynski GP, Murphy A. Spectrophotometric quantitation of anchorage-dependent cell numbers using the bicinchoninic acid protein assay reagent. Anal. Biochem. 1990; 184:189–191. [PubMed: 2321754]

24. Roundhill, DM. Photochemistry and Photophysics of Metal Complexes in Modern Inorganic Chemistry. Springer; 1994.

25. Bard, AJ.; Debad, J.; Leland, J.; Signal, G.; Wilbur, J.; Wohlstadter, J. Analytical Applications of Electrogenerated Chemiluminescence. In: Meyers, RA., editor. Encyclopedia of Analytical Chemistry: Instrumentation and Applications. New York: Wiley; 2000. p. 9842-9848.

26. Atkins, PW. Phys. Chem. San Francisco: Freeman; 1982. p. 823-905.

27. Bard, AJ.; Faulkner, LR. Electrochemical Methods Fundamentals and Applications. New York: Wiley; 1980. p. 488-510.

28. Cosa G, Focsaneanu K-S, McLean JRN, McNamee JP, Scaiano JC. Photophysical properties of fluorescent DNA-dyes bound to single- and double-stranded DNA in aqueous buffered solution. Photochemistry and Photobiology. 2001; 73:585–599. [PubMed: 11421063]

29. Kwong PD, Doyle ML, Casper DJ, Cicala C, Leavitt SA, Majeed S, Steenbeke TD, Venturi M, Chaiken I, Fung M, Katinger H, Parren PW, Robinson J, Van Ryk D, Wang L, Burton DR, Freire E, Wyatt R, Sodroski J, Hendrickson WA, Arthos J. HIV-1 evades antibody-mediated neutralization through conformational masking of receptor-binding sites. Nature. 2002; 420:678–682. [PubMed: 12478295]

## Research Highlights

- We develop a solution-phase ECL assay for quantitative analysis of biomarkers.
- We use the assay to study binding affinities and binding ratios of HIV receptors.
- We use the assay to identify neutralizing antibodies for blocking of HIV infection.
- We show that the assay can be used to study binding and functions of proteins.
- ECL is a powerful tool to detect biomarkers for disease diagnosis and therapy.

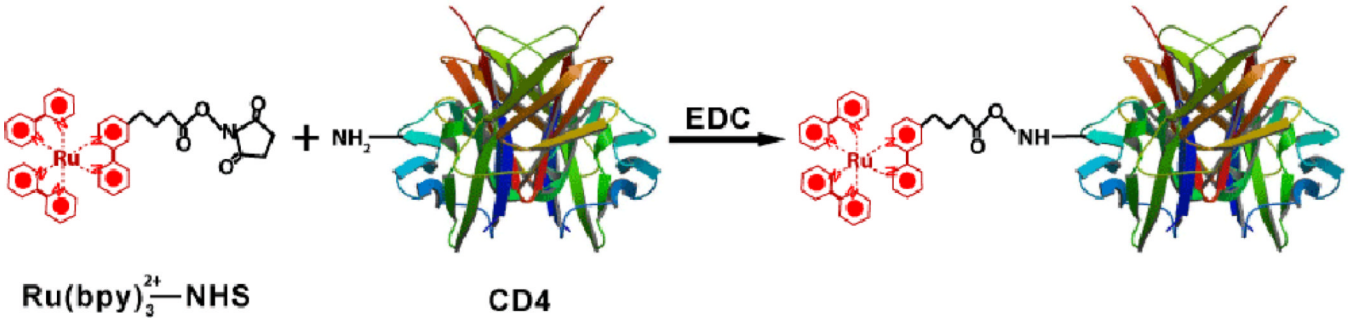


**Figure 1.**

A synthetic reaction of conjugation of a protein (CD4) with $Ru(bpy)_3^{2+}$ to produce CD4-$Ru(bpy)_3^{2+}$ (CD4-tag) by linking the carboxyl group of $Ru(bpy)_3^{2+}$-NSH with the amine group of CD4 via a peptide bond mediated by EDC and sulfo-NHS.

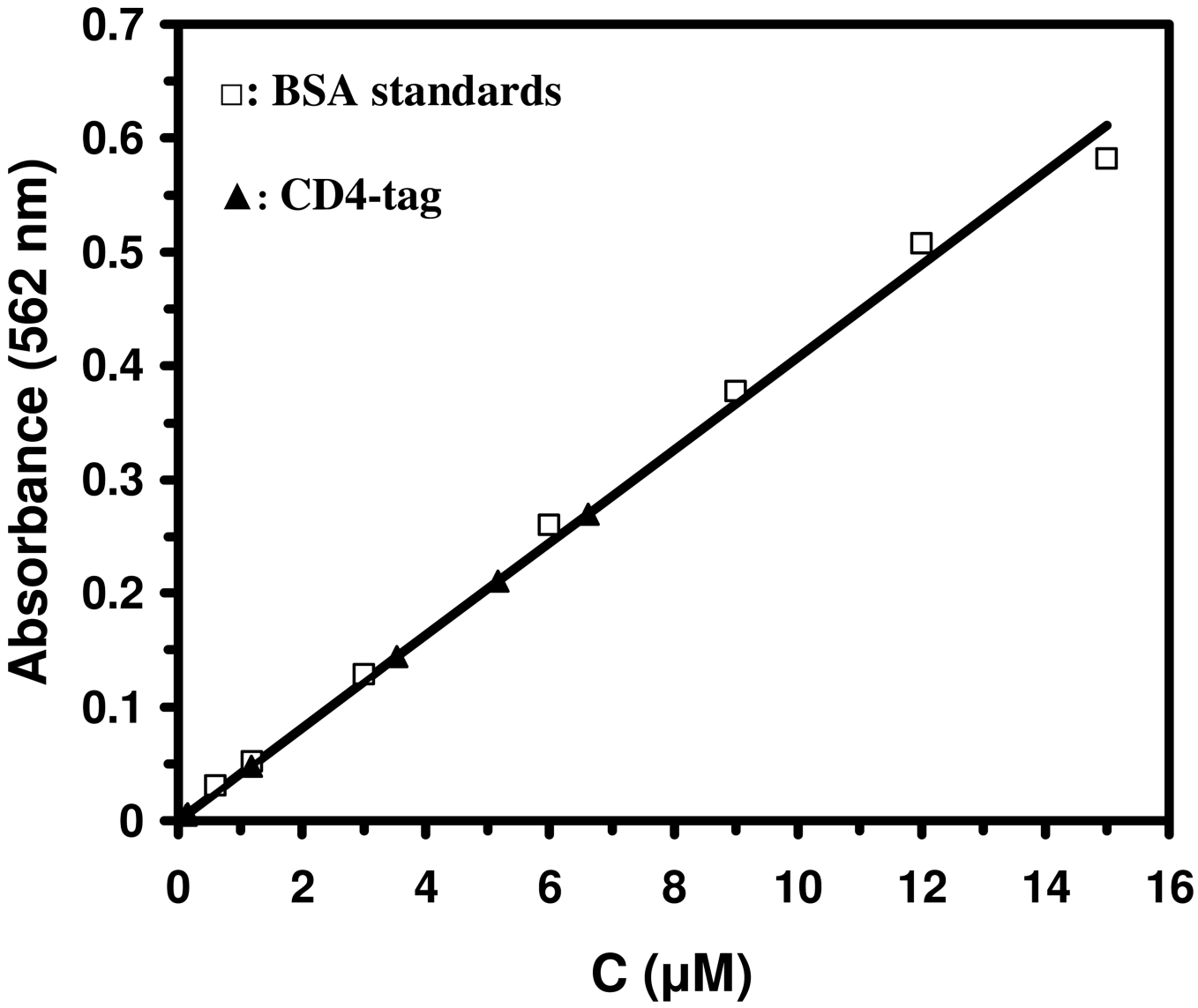


**Figure 2.**

Characterization of CD4 concentration of CD4-tag samples using BCA assay. Plot of absorbance (□) of $BCA_2$-$Cu^+$ at 562 nm generated by standard BSA samples versus their concentrations (0–15 μM) shows a high linearity with a slope of 0.041 $\mu M^{-1}$ and linear regression (R) of 0.99, which serves as a calibration curve to determine CD4 concentration of each fraction of CD4-tag eluted from a separation column. Absorbance of $BCA_2$-$Cu^+$ created by CD4 at 562 nm (▲) is measured and its concentration is determined using the calibration curve. Points are experimental data and the line is created by fitting the data using the linear regression and least squares analysis.

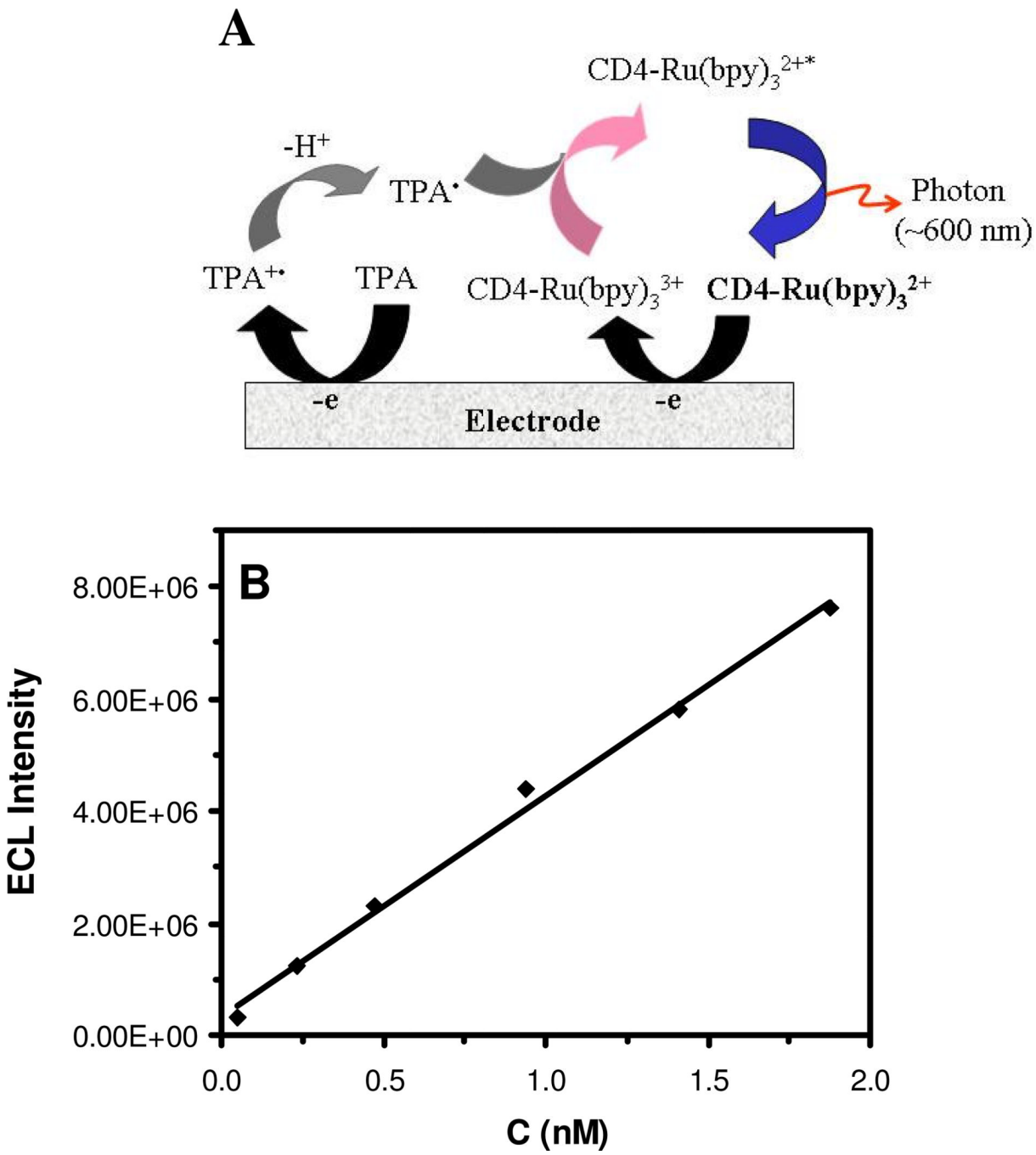


**Figure 3.**

Solution-phase ECL assay for quantitative analysis of concentration of CD4-tag. **(A)** The ECL mechanism: ECL of CD4-Ru(bpy)$_3^{2+}$ in the PBS buffer containing tripropylamine (TPA) is generated upon the electron transfer between two electrochemically oxidized products, Ru(bpy)$_3^{3+}$ and TPA·, which regenerates CD4-Ru(bpy)$_3^{2+}$ and creates an amplification detection scheme that leads to high detection sensitivity. **(B)** Plot of ECL intensity of CD4-tag versus its concentrations (0.047–1.88 nM) show a linear calibration curve with a large dynamic range, a slope of $4\times10^6$ nM$^{-1}$ and a liner regression of 0.99. The result indicates that the ECL intensity of CD4-tag is proportional to its concentration and

one can determine unknown concentrations of CD4-tag in solution using the ECL assay. Points are experimental data and the line is produced by fitting the data using linear regression and least squares analysis.

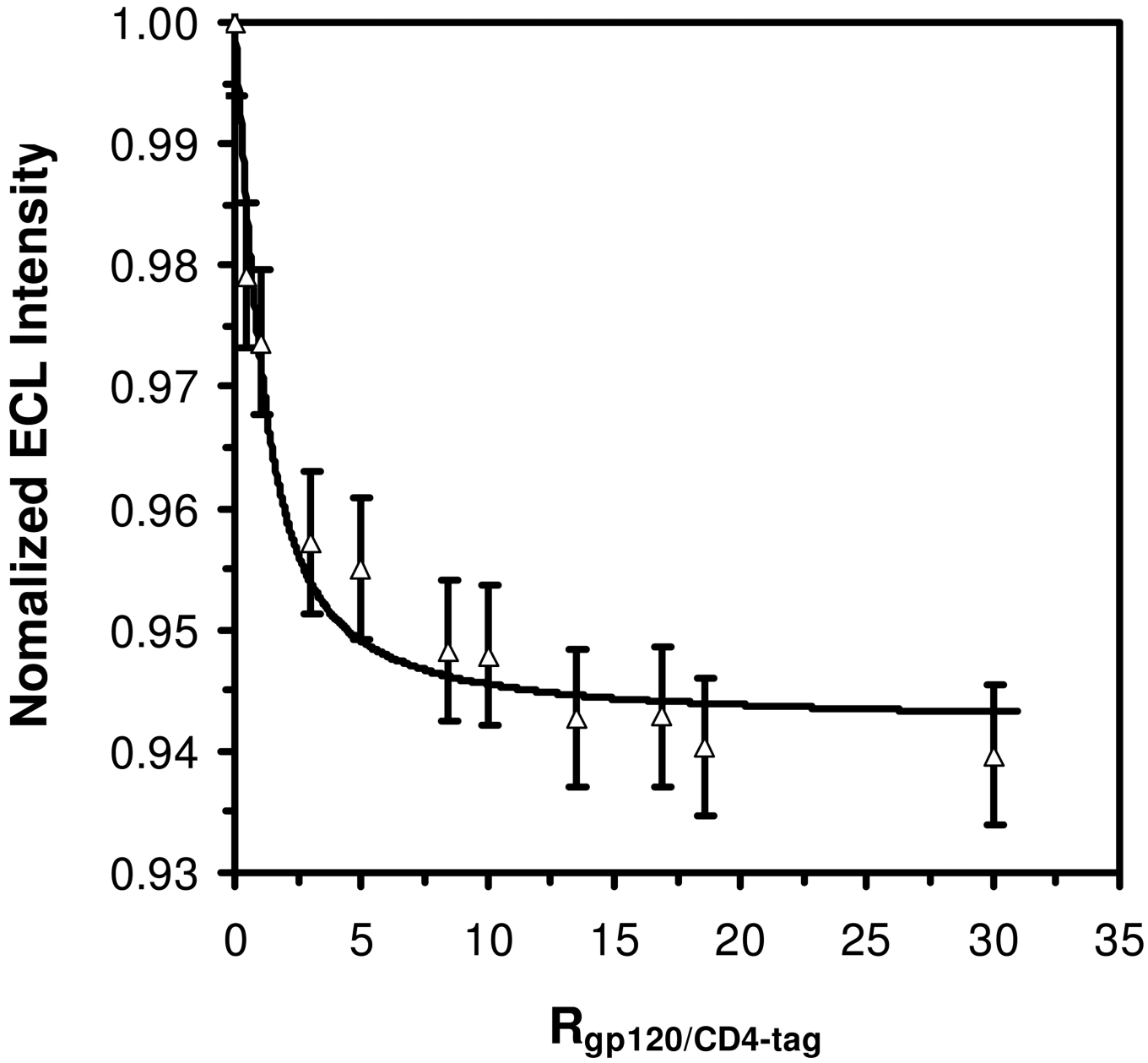


**Figure 4.**

Study of the binding constant of ligand-receptor (gp120-CD4) using the solution-phase ECL assay. Plot of normalized ECL intensity of CD4-tag (1.88 nM) versus the molar ratios (R) of gp120 to CD4-tag shows that the ECL intensity decreases and then remains unchanged, as the ratios increase, indicating the binding of gp120 with CD4 occurs and then reaches binding equilibrium upon the presence of sufficient amount of gp120. The experimental data (points) fitted by Eq. (19) shows the binding constant ($K_{gp120\text{-}CD4}$) of $9.5\times10^{8}$ $M^{-1}$ with a binding ratio of one.

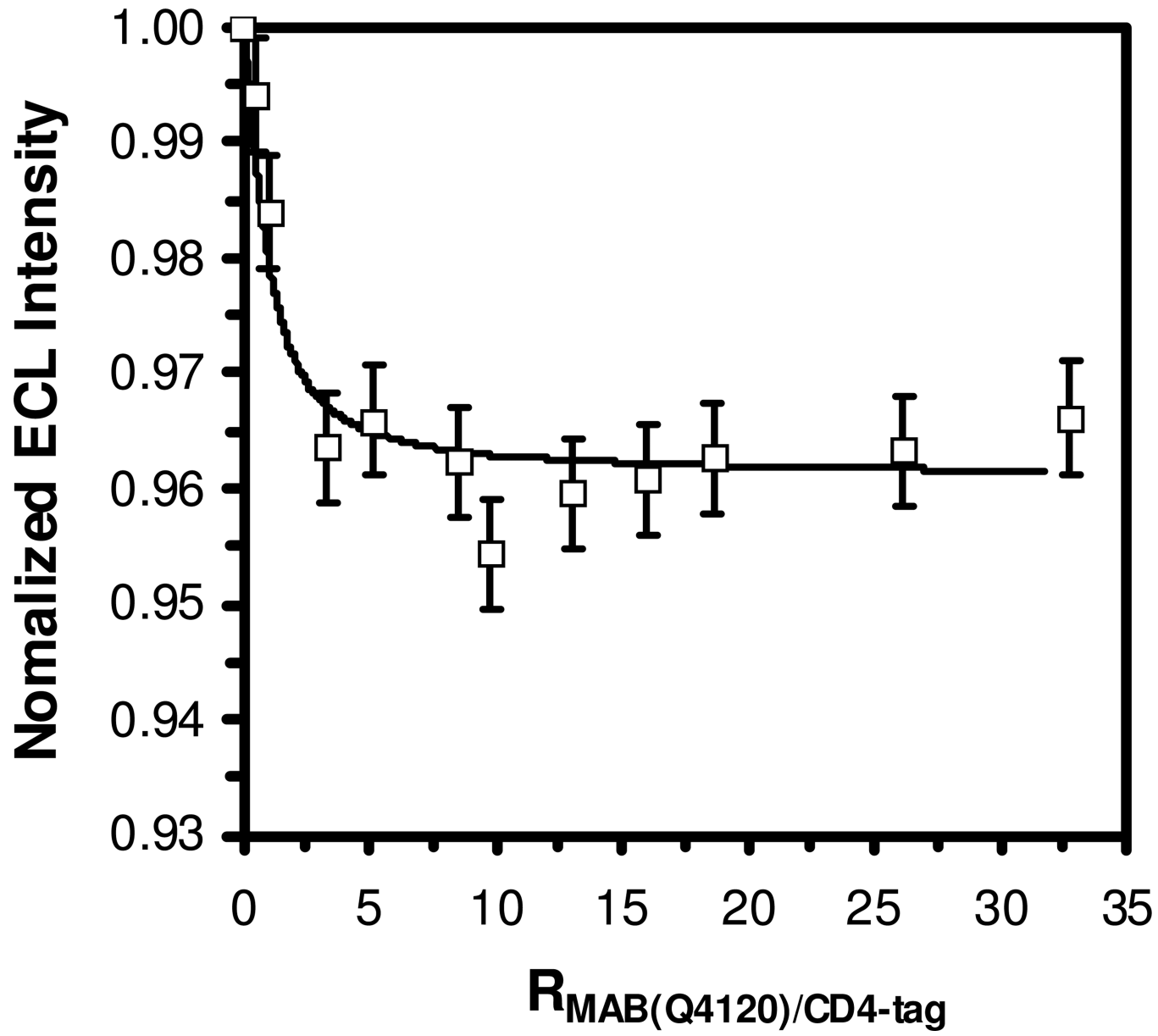


**Figure 5.**

Characterization of the binding constant of antigen-antibody (Q4120-CD4) using the solution-phase ECL assay. Plot of normalized ECL intensity of CD4-tag (1.88 nM) versus the molar ratios (R) of MAB (Q4120)/ CD4-tag shows that the ECL intensity decreases and then remains unchanged, as the ratios increase, which indicates the binding of Q4120 with CD4 occurs and the binding reaction reaches binding equilibrium upon the presence of sufficient amount of Q4120. The experimental data (points) fitted by Eq. (19) shows the binding constant ($K_{Q4120\text{-}CD4}$) of $1.2\times10^{9}$ $M^{-1}$ with a binding ratio of one.

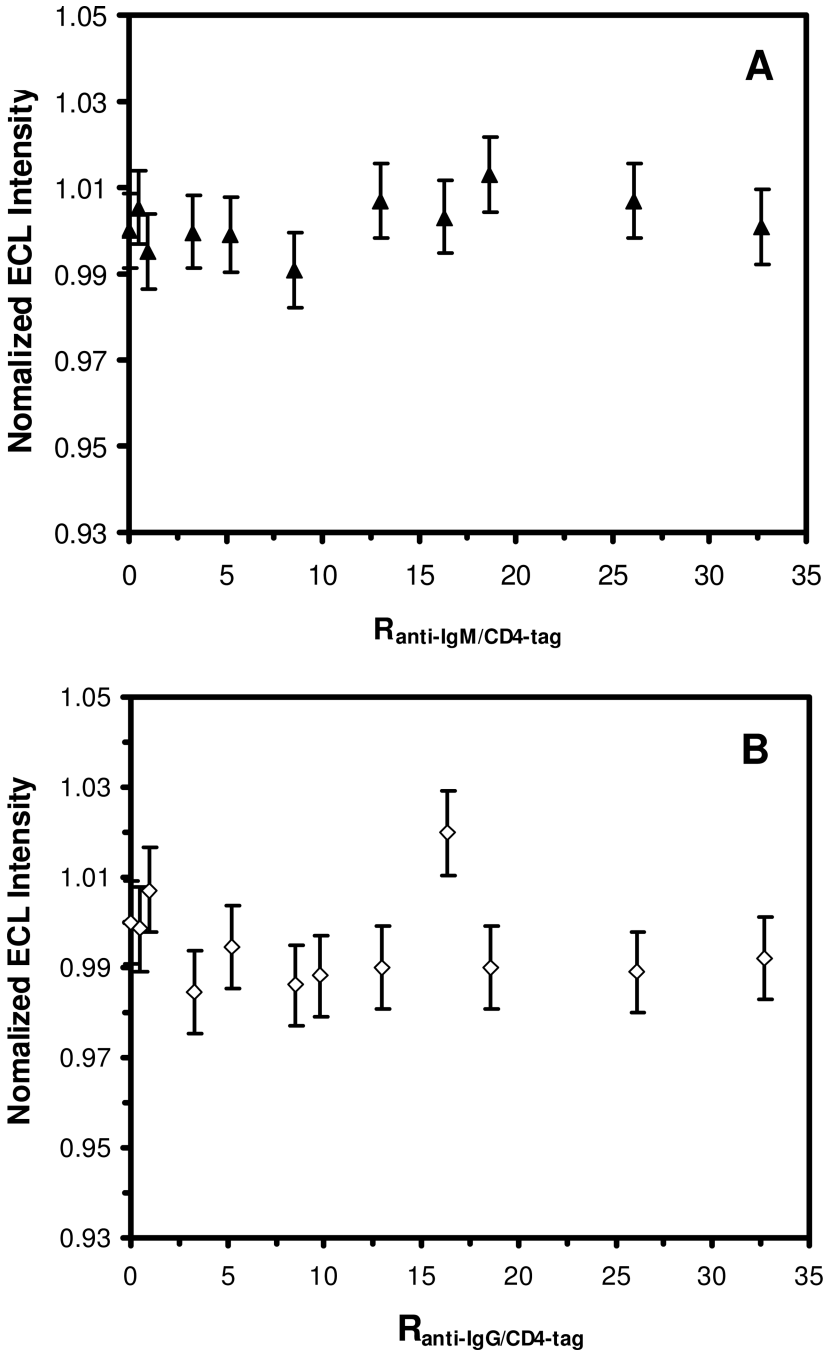


**Figure 6.**

Control experiments for characterization of specificity of the solution-phase ECL assay. Plots of normalized ECL intensity of CD4-tag (1.88 nM) versus the molar ratios (R) of MAB: **(A)** anti-IgM and **(B)** anti-IgG, to CD4-tag show that the ECL intensity remains essentially unchanged as the ratios increase, indicating that neither anti-IgM nor anti-IgG binds with CD4-tag.

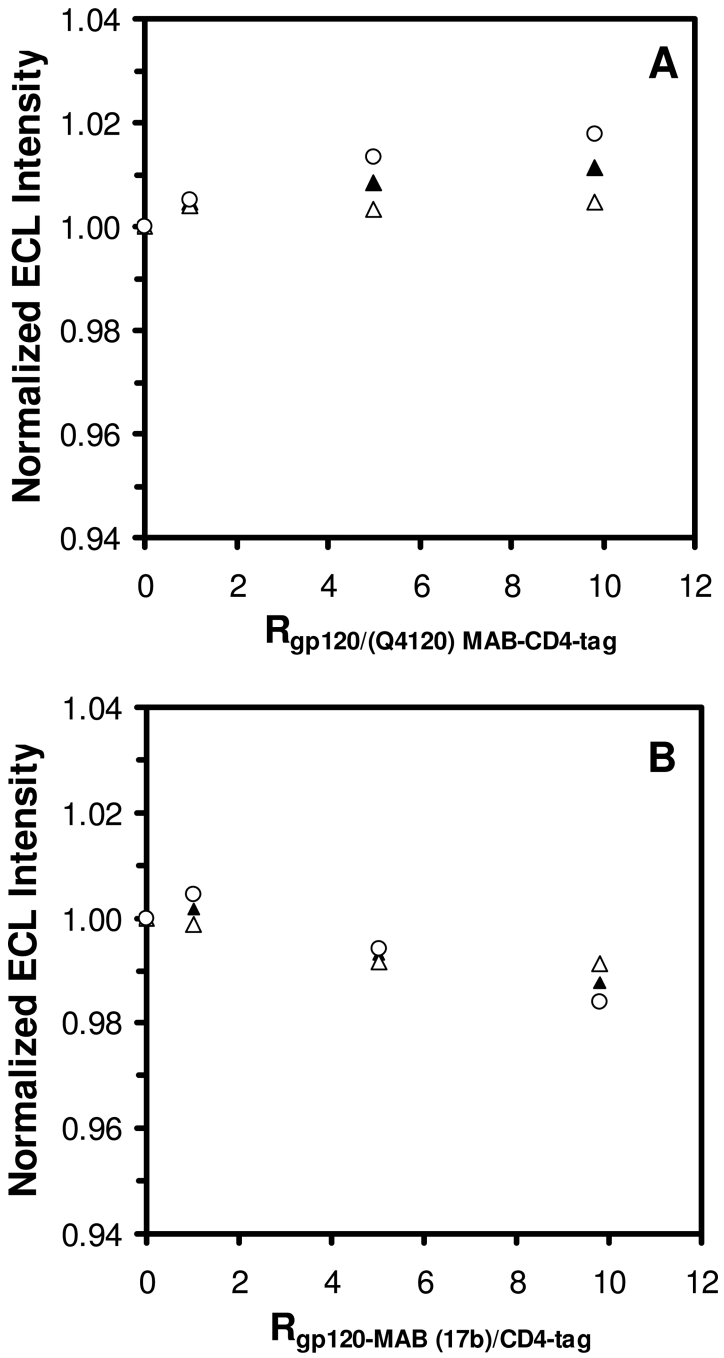


**Figure 7.**

Identification and characterization of neutralizing antibodies for blocking the binding of gp120 with CD4 using the solution-phase ECL assay. **(A)** Plot of normalized ECL intensity of Q4120-CD4-tag versus the molar ratios (R) of gp120/Q4120-CD4-tag shows that the ECL intensity remains nearly unchanged, indicating that Q4120 (MAB of CD4) completely blocks the binding of gp120 with CD4-tag, and can potentially serve as a neutralizing antibody. **(B)** Plot of normalized ECL intensity of CD4-tag versus the molar ratios (R) of 17b-gp120/CD4-tag shows that the ECL intensity decreases slightly and slowly as the ratio increases, which indicates that 17b (MAB of gp120) only partially blocks the binding of

gp120 with CD4-tag, and may not be as effective as Q4120 to block the binding of gp120 with CD4. The symbols (▲), (○) and (△) represent average and standard deviations of ECL intensity of 6 representative measurements at each R, respectively.