Dually Labeled Biomolecules for Characterizing Biotinylated Species through Competitive Binding Reactions

Yan Xu, Yuli Pan, Lingnuo Li, and Ming Zhou*

ABSTRACT: The capability of biotinylated molecules to bind streptavidin may be a more functional measure of the success of target biotinylation than titration of total bound biotins per molecule. It was demonstrated that the binding capability could be assessed by a competitive assay, in which a biotinylated antibody (BA) (or protein, ligand, receptor etc.) of interest competed with a reference antibody (or a protein) dually labeled with biotin and electrochemiluminescence (ECL) moieties for the binding sites of streptavidin coated on the surface of magnetic beads. Inversely related to the ECL signal, the binding capability of a biotinylated antibody can be reproducibly evaluated by multiple sets of easily acquired data series rather than by a single measurement. This method can be employed in an ordinary laboratory with an automated ECL analyzer or other readout instruments for routine characterization of any biotinylated species, such as proteins, ligands, receptors, and polypeptides.

INTRODUCTION

The strong noncovalent interaction of biotin and avidin or streptavidin has long been employed in many protein and nucleic acid assays and purification methodologies. The rapid formation and high stability in various solvents of a biotin–(strept)avidin complex and the small molecular weight of biotin have established biotin–(strept)avidin chemistry as a platform, specifically, for clinical immunoassays so far developed by many researchers and product manufacturers. In immunoassays involving biotin–(strept)avidin chemistry, an analyte-specific antibody is usually labeled with biotin at its amino sites through amide bond formation. Since both the α-amino groups of N-terminals and a large number of ε- amino groups of lysine residues are readily available for biotinylation, the labeled amino sites are randomly distributed in the Fab and Fc fragments of an antibody.

A question is then raised as to how the batch-to-batch variation can be evaluated for better control of the biotinylation process, especially when the biotinylated species are critical ingredients in clinical reagents. This question has long been addressed by analytical approaches focusing on quantitation of biotin or determination of an average molar conjugation ratio of biotin to protein. No matter what the reporting signal modalities were, these methods were based on replacement of 2-(4'-hydroxyazobenzene)benzoic acid (HABA) by biotin or biotinylated species, competition of (strept)avidin binding sites between biotin and biotinylated species, liquid chromatography, and electrospray ionization mass spectrometry (ESI-MS). In terms of quantifying the bound biotin, these methods give either an average conjugation ratio or a distribution profile of the conjugation ratios. While widely used, the HABA assay does not have sufficient sensitivity and reproducibility. On the other side, although a new commercial product QuantTag claimed a better assay performance in quantitation of bound biotin and ESI-MS provided precise distribution profiles of conjugation ratios, the variation of reaction conditions or manufacturing parameters may change the distribution of the amino sites to which the biotin moieties are attached. The inconsistency in the distribution of the occupied amino sites can certainly cause inconsistent performance of the biotinylated species (e.g., antibodies in immunoassays).

A more reasonable characterization of a biotinylated antibody (BA) is its binding capability after biotinylation of the antibody. In clinical immunoassays, the biotinylated antibodies (BAs) are, in most cases, used for immobilization of immunologically active species (analyte and analytes specific antibodies) on a streptavidin-coated solid state surface. Therefore, the term “binding” is threefold—first, binding to the streptavidin-coated solid-state surface through biotin–streptavidin chemistry; second, binding specifically to the analyte (antigen); and third, binding nonspecifically to other species in a sample. In this work, the authors report a method of characterizing the binding capability or activity of a BA toward streptavidin-coated magnetic beads.

Received: October 6, 2020
Accepted: November 20, 2020
Published: December 11, 2020
This method is based on a competitive biotin–streptavidin reaction and electrochemiluminescence (ECL) detection. A reference antibody (or protein) dually labeled with biotin moieties and ECL luminophores is used in this method. When mixing the dually labeled antibody (DLA) and the BA of interest with streptavidin-coated magnetic beads (MBs), the conjugated biotin moieties on DLA and BA will compete for the binding sites of streptavidin on MBs. At the end of the competitive biotin–streptavidin reaction, both the DLA and the BA of interest are immobilized on the surface of MBs (see Scheme 1).

**Scheme 1. Dually Labeled Antibody (DLA) and a Biotinylated Antibody (BA) Competing for the Binding Sites of Streptavidin on the Surface of a Magnetic Bead (MB)**

When brought into an ECL measurement cell built in an automated ECL immunoassay analyzer, the ECL moieties, which are attached to the DLA immobilized on the surface of MBs, will generate an ECL signal under conditions described generally in a number of review papers and specifically in a recent book chapter. In a competitive immunoassay, a stronger ECL signal implies a weaker binding capability of the BA of interest. This case: a stronger ECL signal implies a higher quantity of the DLA and a lower quantity of BA on the surface of MBs. However, as previously discussed, the binding capability rather than the actual quantity, should be associated with the ECL intensity in this case: a stronger ECL signal implies a weaker binding capability of the BA of interest.

In this work, a mouse immunoglobulin G (IgG) was used as the reference for the proof-of-concept experiments. To validate the method and to expand the selection of references, bovine serum albumin (BSA), as a more robust protein, was used as the dually labeled reference. The NHS esters of tris(2,2′-bipyridine)ruthenium(II), i.e., Ru(bpy)$_3^{2+}$ and a long-chain biotin were used as the labeling reagents (Scheme 2).

**RESULTS AND DISCUSSION**

A procalcitonin (PCT) antibody was chosen for biotinylation assessment in this work. The biotinylated PCT antibody (BA-PCT) has been used in our previous study on PCT sandwich immunoassay. PCT is a highly specific biomarker with 116 amino acids for the diagnosis of clinically relevant bacterial infections and sepsis. Three [PCT]/[biotin] challenge ratios of 1:5, 1:10, and 1:20 were set for biotinylation. Bearing biotin moieties, DLA-IgG and BA-PCT both can get anchored on the surface of streptavidin-coated MBs through a biotin–streptavidin reaction. However, in an ECL measurement cell, only ruthenylated DLA-IgG anchored on the surface of MBs can generate an ECL signal.

Before the competitive assay with both DLA-IgG and BA-PCT was carried out, the ECL property of DLA-IgG was first studied alone under a noncompetitive condition, i.e., in the absence of BA-PCT.

To choose an appropriate quantity of MBs, the MB suspension was diluted from its original concentration of 10 mg/mL to 0.075, 0.15, 0.35, 0.5, and 0.75 mg/mL. Each MB suspension (32 μL) was mixed with 100 μL of DLA-IgG of varied concentrations and 68 μL of a phosphate-buffered saline (PBS) diluent. After 9 min of incubation, an aliquot of 150 μL of the reaction mixture was aspirated into the ECL flow cell for measurement. As anticipated, the ECL intensity increased with the DLA-IgG concentration in the range of 1.5–750 ng/mL. However, ECL did not always increase with the quantity of the MB and a saturation-like behavior was observed when the MB concentration was higher than 0.5 μg/mL. At a higher concentration of DLA-IgG, ECL intensities decreased after reaching the maxima at 0.5 mg/mL (corresponding to a quantity of 16 μg of MB in 200 μL of the reaction mixture).

### Scheme 2. NHS Esters of a Ruthenium(II) Complex, Ru(bpy)$_3^{2+}$-NHS, and a PEG-Spaced Biotin (NHS-PEG4-Biotin) Used as Labeling Reagents in This Work

![Image of Scheme 2](https://dx.doi.org/10.1021/acsomega.0c04877)
volume of 100 μL of DLA-IgG for the concentrations of 1.5, 150, 500, 600, and 750 ng/mL corresponds to the quantities of 0.15, 15, 50, 60, and 75 ng of DLA-IgG, respectively. Therefore, for the combinations of lower concentration MBs and a higher concentration of DLA-IgG, the surface of the MBs might be saturated and the unbound DLA-IgG did not contribute to the signal generation. Theoretically, six concentration combinations (in the two green circles of Figure 1) fall into this category.

Since the largest quantity of DLA-IgG in these experiments was 75 ng (100 μL of 750 ng/mL DLA-IgG), far smaller than the binding capacities of the MB with concentrations of 0.35 mg/mL (binding capacity 112 ng IgG), 0.5 mg/mL (binding capacity 160 ng IgG), and 0.75 mg/mL (binding capacity 240 ng IgG), the weakened ECL for the MB of 0.75 mg/mL is an interesting phenomenon. This can be explained by the reduced accessibility of the coreactant tri-n-propylamine to an electrochemical electrode surface or interface. It is well known that in a Ru(bpy)₃²⁺/tri-n-propylamine ECL system, the emission occurs during the course of the concomitant electrochemical oxidation of Ru(bpy)₃²⁺ and tri-n-propylamine. Although multiple reaction pathways have been proposed to explain the ECL generation, the circulation of ruthenium complexes, i.e., Ru(bpy)₃²⁺ → Ru(bpy)₃³⁺ or Ru(bpy)₃⁺ → Ru(bpy)₃²⁺ → Ru(bpy)₃³⁺, and the crucial role of the electrochemical oxidation of tri-n-propylamine have been commonly accepted. Because of the circulation of ruthenium complexes, the supply of tri-n-propylamine is critical to the efficient ECL. However, the employment of MB of higher concentration results in higher electrode occupancy or a smaller bare surface area of the working electrode and, consequently, the reduced accessibility of tri-n-propylamine to the working electrode.

To build up efficient competition between DLA-IgG and BA-PCT, 30 μL of the 0.35 mg/mL MB (binding capacity 105 ng of IgG), 70 μL of DLA-IgG (35 and 52.5 ng for 500 and 750 ng/mL, respectively), and 50 μL of BA-PCT were used at different concentrations (250–1000 ng for the concentration range of 5–20 μg/mL). The combined total quantities of DLA-IgG and BA-PCT were 3–10 times larger than the binding capacity of 105 ng of the antibody for the added MB, allowing their competitive binding to the streptavidin on the surface of MB. Again, the total reaction volume (200 μL) was kept constant by the addition of 50 μL of PBS to each reaction mixture. In the absence of BA-PCT, 100 μL of PBS was added to the reaction system.

As is visible from the multiple data sets in Figure 2, ECL intensities decreased with the increasing quantity of the added BA-PCT, indicating that the competition between BA-PCT and DLA-IgG for MB surface streptavidin reduced the surface concentration of DLA-IgG. Consequently, the number of Ru(bpy)₃²⁺ luminophores, which were attached to DLA-IgG on the MB surface, and the ECL intensity were also decreased. The competition depends on the conjugation ratios [IgG]/[biotin] and [PCT]/[biotin] and the concentration ratio of [DLA-IgG]/[BA-PCT] as well. An optimal combination of parameters could be determined by trial and error.

Being a reference reagent in this proof-of-concept work, DLA-IgG had a challenge ratio of 1:5 for both [IgG]/[biotin] and [IgG]/[Ru(bpy)₃²⁺]. The challenge ratios for [PCT]/[biotin] were set to be 1:5, 1:10, and 1:20. Although the actual conjugation ratios were unknown, the data series in Figure 2 explicitly proved that the binding capability of BA-PCT varied largely with the challenge ratios. Therefore, it can be extrapolated that a minor variation of the biotinylation process could cause a detectable ECL change in the competitive assays. Although DLA-IgG was used as a reference in this work, it must be understood that it was not solely or specifically chosen for BA-PCT. It can serve the same purpose for other biotinylated species. Using an automated ECL immunoassay analyzer, multiple sets of data series from competitive assays with different reagent concentrations and volume ratios can be conveniently acquired and compared in a time-saving manner. There might be a concern regarding the consumption of biotinylated species prepared for laboratory use rather than for industrial use. In Figure 2, the quantities of 0.25–1.0 μg of BA-PCT were used for each data point, and they can be much smaller after assay optimization.

One of the reviewers of this article has raised a question as to how the reference DLA-IgG could be reproducibly prepared and well characterized. The complexity of preparing a reference material depends on the target use and frequent preparation of the reference reagent should be avoided. For
quality control within a laboratory, a routine practice is to divide a batch of reference into appropriate aliquot parts, which are then kept frozen at low temperature until use. The characterization of DLA-IgG can be actually carried out under a noncompetitive condition, as already described in the experiments of Figure 1. If there is any further concern regarding the batch-to-batch variation of the reference DLA-IgG, usual quantification methods for IgG (e.g., BCA assay), biotin (e.g., HABA assay), and Ru(bpy)₃²⁺ (e.g., absorption at 454 nm) can be employed.

The storage stability of the DLA-IgG might be another issue for application. Because both biotin and Ru(bpy)₃²⁺ moieties are highly stable, IgG could be an instability factor and can be replaced by a more robust macromolecule in case where the long-term stability cannot be guaranteed.

Therefore, for validation of the method, a standard grade BSA was chosen as the reference reagent. The dually labeled BSA reference was first characterized under a noncompetitive condition (30 μL of MB at 0.35 mg/mL and 70 μL of dually labeled BSA 1 μg/mL). The average over five successive ECL intensity measurements (8500558, 8661187, 8724283, 8690344, and 8554250) was 8626124 with a coefficient of variation of 1.1%. The BA-PCT validation samples from three lots were diluted to 1.25, 2.5, 5.0, 10.0, 15.0, and 20 μg/mL. Three replicate measurements were performed for each concentration of the sample from the same lot. The three ECL measurements were then plotted against the BA-PCT concentration to generate three curves for each lot. It can be seen from Figure S2 that the three curves within each lot overlap very well.

To determine any difference among the three lots, the average of three measurements of a lot were plotted, as shown in Figure 3. The two overlapping curves of lot 2 and lot 3 indicate that the two lots of biotinylated PCT have the same biotin−streptavidin binding capability and can be considered to be the same. On the other hand, the slightly deviating curve of lot 1 indicates a slightly stronger biotin−streptavidin competition and, thus, a higher degree of biotinylation of BA-PCT in lot 1.

It must be understood that whether or not the three lots are acceptable from the quality control point of view depends on the setup of a standard, which is based on the specific needs of a researcher or manufacturer. Nevertheless, the validation experiments and aforementioned proof-of-concept experiments establish the applicability of the method.

It is worth noting that the ECL detection is a highly sensitive technique, but the high sensitivity or very low limit of detection methodology is not a prerequisite for this type of application because the targeted analyte is at a high concentration range (μg/mL) in the purposefully prepared samples. Nevertheless, combined with some newly developed signal modes (e.g., the mass spectrometric signal), this method provides a more functional characterization of biotinylated species than conventional quantitation of bound biotin by single measurement data and can be used generally for evaluating the biotinylation degree of any species.

Another reviewer of this article has advocated for a broader application area of the method for all ligand−receptor, protein−protein, and protein−antibody interactions and encouraged the authors to claim the use of the dually labeled internal standard as a means to correct the error and add quality control to many types of binding assays. In view of the extremely low equilibrium dissociation constant (10⁻¹⁵ M) for a biotin−(strept avidin complex and the usually multilabelling situation of the target species, the extended applications could be considered with caution.

### CONCLUSIONS

In summary, using a reference IgG or BSA dually labeled with biotin and electrochemiluminescent moieties and through a competitive biotin−streptavidin binding reaction and ECL signal detection, the binding capability of a biotinylated antibody toward streptavidin on the magnetic beads can be assessed. Such a binding capability assessment provides a more functional characterization of biotinylated species than a single quantitative measurement of the conjugation ratio. With an automated ECL analyzer, this method proved to be convenient and reliable for evaluating and, consequently, controlling batch-to-batch variation in immunoassay development and the manufacturing process of biotinylated reagents. Certainly, it can also be employed in any other situations in which the biotinylated species need to be functionally evaluated. Beyond the biotin−streptavidin binding reaction, the method used in this work could find broader application in general ligand−receptor reactions.

### EXPERIMENTAL SECTION

**Chemicals and Materials.** The ruthenium(II) complex-labeled Ru(bpy)₃²⁺-NHS was received from Rubipy Scientific Inc. The amine-reactive biotinylation reagent NHS-PEG₄-biotin (EZ-Link, MW 588.67) and the streptavidin-coated magnetic beads (Dynabeads M-280) were received from Thermo Fisher Scientific. The size exclusion purification column (PD-10) was supplied by GE Healthcare. The phosphate buffer solutions ProCell (0.18 mol/L N-tripropylamino, pH = 6.8) and CleanCell (1 wt % KOH, pH = 13) were obtained from Roche Diagnostics. BSA (Standard Grade) and mouse IgG were purchased from Jackson ImmunoResearch Laboratories, Inc. and Proliant Biologicals, respectively. The PCT antibodies were received from two suppliers, i.e., the PCT (JG07) for proof-of-concept experiments was obtained from Kitgen Biotech Co., Ltd., and the PCT (19B2) for method validation was obtained from CNPair Biotech Co., Ltd.
**Instruments.** Electrochemiluminescence detection was carried out with an ECL analyzer (ProScientia 2020 from Accucise Diagnostics Inc.), which is a user-reconfigurable automated system specifically designed for scientific research involving the detection of an ECL signal generated in a flow cell. The detection unit and the working procedure of the instrument are the same as that of the Roche Diagnostics’s cobas e411 clinical immunoassay analyzer.16

**Dually Labeled IgG and BSA.** The dually labeled mouse IgG (DLA-IgG) was prepared in two steps. In the first step, IgG was labeled with Ru(bpy)₃⁺⁻⁺⁴-NHS ester at a challenge ratio of [IgG]/[Ru(bpy)₃] equal to 1:5. Two milligrams (13.3 nmol) of mouse IgG was dissolved in 1 mL of 0.01 M PBS (pH 7.4) to form a 13.3 μmol/L solution. To this solution, 67 μL of 1 mmol/L Ru(bpy)₃⁺⁻⁺⁴-NHS dimethylformamide solution was added. The mixture was incubated for 1 h followed by separation and purification with a PD-10 column. The concentration of the ruthenylated IgG was determined to be 8.1 μmol/L by the BCA method, while the labeled Ru(bpy)₃⁺⁻⁺⁴ unit was 21.1 μmol/L determined by its absorption at the wavelength of 454 nm. The conjugation ratio of [IgG] to [Ru(bpy)₃] was then 2.6. In the second step, 2 mg of NHS-PEG₄-biotin was dissolved in 1 mL of PBS buffer to form a solution of 3.4 mmol/L of NHS-PEG₄-biotin for labeling ruthenylated IgG with biotin. For a challenge ratio of 1:5, an aliquot of 8.9 μL of 3.4 mM of NHS-PEG₄-biotin was added to 750 μL of ruthenylated IgG (8.1 μmol/L). The dually labeled mouse IgG (DLA-IgG) was then separated and purified with another PD-10 column, and the DLA-IgG concentration was determined to be 6 μmol/L (0.9 mg/mL). Finally, 50 μg of BSA was added to the DLA-IgG PBS solution, filtered with a 0.2 μm filter, and stored at 4 °C. The as-prepared DLA-IgG was diluted with PBS to different concentrations to be used as competition references for assessing binding capability of a biotinylated antibody of interest.

Labeling BSA was conducted in a one-step protocol, by which BSA was mixed concomitantly with both labeling reagents, i.e., Ru(bpy)₃⁺⁻⁺⁴-NHS and NHS-PEG₄-biotin, at a challenge ratio same as the DLA-IgG (i.e., 1:5:5). The conjugation ratio of [BSA] to [Ru(bpy)₃] was determined to be 2.2. The as-prepared dually labeled BSA was diluted with PBS to different concentrations for use as competition references for assessing binding capability of a biotinylated antibody of interest.

**Biotinylation of PCT Antibody.** The procedure for labeling a PCT antibody with NHS-PEG₄-biotin has been previously described.17 Three challenge ratios of 1:5, 1:10, and 1:20 for [PCT]/[biotin] were chosen for the biotinylation of the PCT antibody. To each 2 mL of citrate buffer saline (CBS) (pH 9.5) containing 2.0 mg of the desalted PCT antibody (JG07), respectively, an aliquot of 19.5, 39, and 78 μL of 3.4 mmol/L of NHS-PEG₄-biotin solution was added. Each mixture was incubated for 1 h followed by the dialysis (Spectrumlabs’ Spectra/Per 2 Dialysis membrane with MWCO 12–14 kD) of total 20 h in PBS buffers. They were then concentrated to 1 mL with Pierce protein concentrators (MWCO 10 kD). The final concentrations of three biotinylated PCT antibody (BA-PCT) PBS solutions were determined to be 1.73, 1.69, and 1.76 mg/mL by a BCA assay method.

For validation, PCT (1B2) was labeled with NHS-PEG₄-biotin at a challenge ratio of 1:5. Three batches of BA-PCT were prepared under the same conditions and eventually diluted to different concentrations.

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