Synthesis, labeling and bioanalytical applications of a tris(2,2′-bipyridyl)ruthenium(II)-based electrochemiluminescence probe

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Assays using probes labeled with electrochemiluminescent moieties are extremely powerful analytical tools that are used in fields such as medical diagnostics, environmental analysis and food safety monitoring, in which sensitive, reliable and reproducible detection of biomolecules is a requirement. The most efficient electrochemiluminescence (ECL) reaction to date is based on tris(2,2′-bipyridyl)ruthenium(II) ($Ru(bpy)_3^{2+}$) with tripropylamine (TPrA) as the co-reactant. Here we present a detailed protocol for preparing $Ru(bpy)_3^{2+}$ probes and their bioanalytical applications. This protocol includes (i) the synthesis of a biologically active $Ru(bpy)_3^{2+}$-N-hydroxysuccinimide (NHS) ester, (ii) its covalent labeling with both antibodies and DNA probes and (iii) the detection and quantification of ECL in a microfluidic system with a paramagnetic microbead solid support. In our magnetic bead–based ECL system, two probes are required: a capture probe (labeled with biotin to be captured by a streptavidin-coated magnetic bead) and a detector probe (labeled with $Ru(bpy)_3^{2+}$). The complex consisting of the analyte, the capture probe, the detector probe and the magnetic bead is brought into contact with the electrode by using a magnetic field. The $Ru(bpy)_3^{2+}$ reacts with TPrA in solution to generate the ECL signal. The full protocol, including the synthesis and labeling of the bioactive $Ru(bpy)_3^{2+}$, requires 5–6 d to complete. ECL immunoassays or nucleic acid tests only require 1.5–2 h, including the sample preparation time.

INTRODUCTION

Overview of $Ru(bpy)_3^{2+}$ ECL

Analytical methods using light emission, such as photoluminescence, bioluminescence, chemiluminescence and ECL, are the most commonly used bioanalytical techniques[1–7]. Of these light-emitting technologies, ECL was only discovered in the mid-1960s, and thus it has a relatively short history[8]. The transition metal compound $Ru(bpy)_3^{2+}$ is one of the most commonly used ECL probes. The electrochemical and ECL properties of $Ru(bpy)_3^{2+}$ were discovered and studied by Bard and colleagues[9,10]. Early research into $Ru(bpy)_3^{2+}$ ECL focused primarily on the nonaqueous phase or aqueous phases containing an oxalic acid or persulfate co-reactant[11,12]. An interesting breakthrough occurred in the early 1990s, when Leland and Powell discovered an effective ECL system using $Ru(bpy)_3^{2+}$ and TPrA as reactants that can operate in aqueous solutions[13].

The $Ru(bpy)_3^{2+}$/TPrA ECL reaction mechanism has been studied extensively. However, the exact reaction mechanism is still debated owing to its complexity. The general consensus is that the ECL reaction follows an 'oxidative-reduction' model[14]. The reaction equation is as follows:

$$\text{Tripropylamine} \rightarrow \text{Tripropylamine}\bullet + e- \quad (1)$$

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

$$Ru(bpy)_3^{3+} + \text{Tripropylamine}\bullet \rightarrow [Ru(bpy)_3^{2+}]* + \text{products} \quad (3)$$

$$[Ru(bpy)_3^{2+}]* \rightarrow Ru(bpy)_3^{2+} + h\nu \quad (4)$$

The ECL reaction can be summarized by two oxidation reactions on the working electrode surface. Briefly, TPrA is oxidized on the surface of the working electrode. A cation radical of TPrA ($TPrA^+$) forms and rapidly loses a proton to yield a reducing TPrA radical (equation 1). Simultaneously, $Ru(bpy)_3^{2+}$ is oxidized at the same electrode and forms the strongly oxidizing $Ru(bpy)_3^{3+}$ (equation 2). The TPrA radical reduces the $Ru(bpy)_3^{3+}$ to form an excited $Ru(bpy)_3^{2+}$ (equation 3). The excited-state $Ru(bpy)_3^{2+}$ decays through a normal fluorescence mechanism, emitting a photon in ~620 nm (equation 4). The resulting $Ru(bpy)_3^{2+}$ can be reused in subsequent reaction cycles. Therefore, this process is, in principle, a perpetual reaction in the presence of excess TPrA.

$Ru(bpy)_3^{2+}$/TPrA systems react effectively and emit light stably in aqueous medium at room temperature (~25 °C) and at the proper pH range in the presence of dissolved oxygen and other impurities. In addition, the emission intensity of ECL is proportional to the $Ru(bpy)_3^{2+}$ concentration in the presence of excess TPrA, which enables quantitative bioanalyses[15–22]. The $Ru(bpy)_3^{2+}$/TPrA ECL reaction can be achieved by using conventional three-electrode electrochemical devices. Platinum, gold or a glassy carbon material can be used as the working electrode, with platinum and Ag/AgCl as the counter and reference electrodes, respectively[23]. Emitted photons can be measured by using a photodetector such as a photomultiplier tube (PMT), photodiode or charge-coupled device camera.

$Ru(bpy)_3^{2+}$ can function as a reporter similarly to the radiolabeled or fluorescent probe by coupling reactive groups (e.g., amino groups) in a biomolecule (e.g., proteins, peptides, ligands and synthetic oligonucleotides) to the pyridine group in $Ru(bpy)_3^{2+}$. For example, antibodies can be labeled by covalently linking the amino group in lysine to a $Ru(bpy)_3^{2+}$-NHS ester[24,25]. A nucleic acid–based ECL reporter can be obtained by coupling an amino group prepared via nucleic acid synthesis to a

$Ru(bpy)_3^{2+}$-NHS ester[26,27]. Owing to their high sensitivity, simple device construction and biolabeling versatility, $Ru(bpy)_3^{2+}$/TPrA ECL systems show great potential for biological, clinical and environmental applications[28–33].

Magnetic bead–based ECL technique

The $Ru(bpy)_3^{2+}$/TPrA ECL reactions occur on the electrode surface. A prerequisite for a high ECL efficiency is the simultaneous reaction of both $Ru(bpy)_3^{2+}$ and TPrA on the electrode surface. In solution-phase ECL assays, $Ru(bpy)_3^{2+}$ and TPrA contact the electrode via free diffusion; thus, the process is inefficient. Surface-phase ECL assays, which immobilize the probe on the electrode surface, are more effective. Thus, many methods have been developed to affix biological molecules to an electrode surface. The most straightforward process is via hydrophobic interactions or electrostatic bonds. However, such adsorptions are unstable and susceptible to disruption by changes in the pH and ionic strength of the liquid phase. The probe may also be affixed to the electrode surface via a streptavidin self-assembled monolayer, which is a more stable and specific electrode surface modification. However, the pretreatment and reuse of such electrodes are cumbersome and time-consuming.

A better option is to use paramagnetic microbeads as the carrier for solid-phase ECL measurements[24,34–40]. Microbead surfaces can be coated with streptavidin. Biotinylated biological probes (antibodies, nucleic acids, peptides and so on) can then be selectively captured via a biotin-streptavidin linkage. The binding of streptavidin to biotin is quick, reliable and strong ($K_d = 10^{-15}$); this system also reduces the sample preparation time from tens of hours (for thiol- or phosphothioate-mediated systems) to several minutes. The placement of a magnetic field below the working electrode allows the paramagnetic microbeads to capture the biomolecules and enrich the sample. ECL detection can be achieved by measuring the photonics emitted from the $Ru(bpy)_3^{2+}$ labeled in the detection antibody or the nucleic acid probe. Magnetic bead–based ECL is very promising because the electrodes can be reused by simply washing them from the surface. As a result, the detection costs are greatly reduced for this rapid detection process. In current magnetic bead–based ECL assay, a capture probe labeled with biotin and a detector probe labeled with $Ru(bpy)_3^{2+}$ are required. Detection of the target results in the formation of a sandwich complex, which can be captured by a streptavidin-coated magnetic bead. The magnetic bead-sandwich complex is brought into contact with the electrode by using a magnetic field. The $Ru(bpy)_3^{2+}$ reacts with TPrA in solution to generate the ECL signal.

Applications and target audience

Owing to their relatively low instrument cost, high sensitivity and automation potential, magnetic bead–based ECL analyses have been successfully developed for basic life science research and clinical applications. For medical diagnostic applications, ECL is more sensitive than classical ELISAs[41,42]. In addition, an automated ECL immunoassay has been commercially developed by Roche. Several ECL immunoassay test reagents have been developed as clinical tumor markers as a means to measure hormones and biochemicals. Recently, our group and others have developed a large number of versatile magnetic bead–based ECL assays for bioanalytical applications such as tumor biomarker detection[36], aptamer-based assay development[43], gene sensing[44,45], enzymatic activity measurement[46], toxin measurement[34], heavy metal ion detection[47] and biological threat agent detection[48]. As more laboratories conduct ECL studies, ECL bioanalyses will become increasingly easy and popular.

Advantages, limitations and adaptations

ECL has the following bioanalytical advantages over other techniques such as chemiluminescence, fluorescence and radioactive probes: (i) because ECL does not require an excitation source, as does fluorescence, it is not subject to interference from light scattering, which improves the sensitivity and selectivity; (ii) ECL reactions have a high temporal and spatial resolution because their light emissions can be controlled via the electrochemical potential; (iii) ECL measurements are simple, rapid (a few seconds) and easily automated; (iv) the $Ru(bpy)_3^{2+}$ probe is a small molecule (~1,000 Da) that is easily conjugated to biomolecules, which causes minimal disturbance to immune activity (for antibody probes), ensures good solubility and stability (it can be stored for >1 year), and represents a marked improvement over chemiluminescence and radioisotope probes; (v) ECL detection covers a dynamic range of approximately six orders of magnitude; and (vi) ECL instruments are relatively simple and inexpensive.

Great success has been achieved in the use of ECL for *in vitro* molecular diagnostics; however, its use for *in vivo* analyses has been limited because the reaction only occurs on the electrode surface and requires certain potentials, a stable buffer environment and co-reactants. In addition, multicolored ECL reporters are limited; thus, unlike fluorescence techniques, multiplex detection is not easily achieved unless the adaptation of an array electrode, where the carbon work electrode is coated with capture probes to immobilize different analytes in a sample, or the employment of microwell array (Meso Scale Discovery, http://www.mesoscale.com) for imaging individually ECL moieties-encoded microbeads, is used[49]. Moreover, the transition metal compound tris (2,2′-bipyridyl) ruthenium (II) ($Ru(bpy)_3^{2+}$), or its analogs, such as $Ir(ppy)_3$ and $Os(phen)_2(dppene)^{2+}$, are usually used as the ECL moieties. However, the safety of these ECL moieties upon human exposure cannot be demonstrated. The potential toxicity of both $Ru(bpy)_3^{2+}$ and its co-reactant TPrA are key drawbacks of the protocol. Thus, more environmentally friendly ECL systems should be explored. Recently, 2-(dibutylamino)ethanol has been shown to be much less toxic and less volatile than TPrA, but with higher efficiency[50].

Experimental design

The ECL equipment. The magnetic bead–based ECL measurement system was developed in-house. **Figure 1** shows a schematic of the self-built ECL system and includes basic components such as the pumps, electrochemical workstation, PMT, computer and electrochemical flow cell. The core of the electrochemical flow cell includes three electrode systems: a platinum working electrode, a platinum counter electrode and an Ag/AgCl reference electrode. The working and counter electrodes are responsible for initiating the ECL reaction whenever a voltage is applied by the electrochemical workstation. The reference electrode provides a reference for the voltage applied by the working electrode. The pump pulls the sample, ECL buffer or cleaning solution through the flow cell via a fluid path. A permanent neodymium-iron-boron

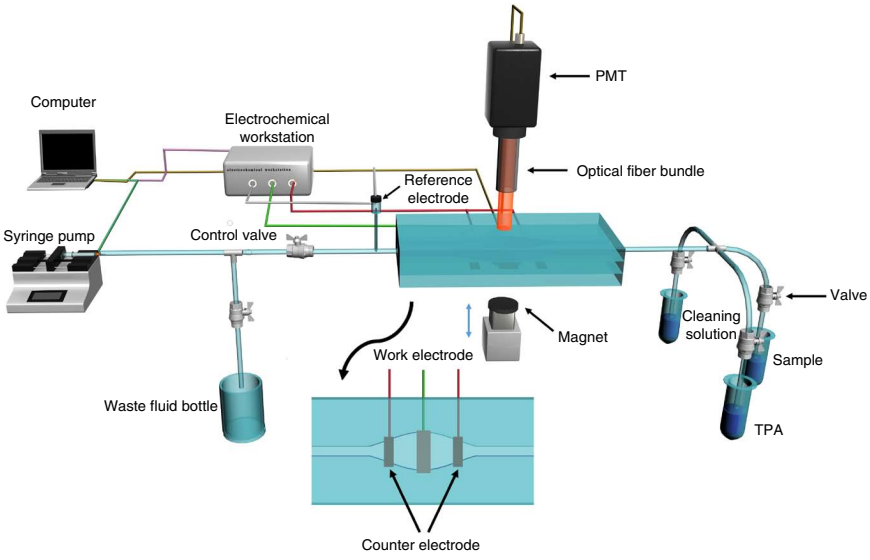

Figure 1 | Magnetic bead–based ECL measurement system. The magnetic beads complex with the sample in a vial and are pulled into the electrochemical cell by a syringe pump with only the sample valve opened and a magnetic field applied to capture the complexes. After introducing the sample, the sample valve closes and the ECL buffer valve opens. The ECL buffer is pulled into the electrochemical cell, which consists of a platinum work electrode, a platinum counter electrode, and a Ag/AgCl reference electrode. Excess probes and the unwanted sample matrix are washed away using the ECL buffer. The electrochemical workstation creates a potential for the ECL reaction. PMT measures the photons generated by the ECL reaction by using an optical fiber linker to reduce the magnetic field effect. After the ECL reaction, a cleaning solution is pulled through the electrochemical cell to clean the electrode. Afterward, the other valves are closed and the waste valve is opened. A syringe pump pushes the waste solution into the waste fluid bottle. TPA, tripropylamine.

alloy magnet sits beneath the working electrode to capture the magnetic beads. The emitted light is measured by a single photon counting module, which consists of a PMT, high voltage power, signal preamplifier and integrated amplifier screening. To avoid magnetic interference, the PMT uses an optical fiber to detect photons and maintain distance from the magnetic field. The signal from the PMT was amplified and measured. We counted transistor-transistor logic (TTL) pulses every second for 30 s using a multifunction acquisition card (PCL-836, Advantech) controlled by LabVIEW software, and we used a personal computer for signal collection and data analysis processes. Preferably, the work electrodes are made of platinum. However, despite the present work using a platinum working electrode, gold can also be used as the working electrode to obtain similar ECL efficiencies as platinum[51,52].

Preparation of Ru(bpy)$_3^{2+}$-NHS ester. Rutheniumbis(2,2′-bipyridine) (2,2′-bipyridine-4,4′-dicarboxylic acid) bis(hexafluorophosphate) ([Ru(bpy)$_2$(dcbpy)(PF$_6$)$_2$]) was obtained by refluxing *cis*-dichlorobis(2,2′-bipyridine)ruthenium(II) with a slight excess of the corresponding 2,2′-bipyridine-4,4′-dicarboxylic acid in an ethanol/water mixture[53,54] (**Fig. 2**).

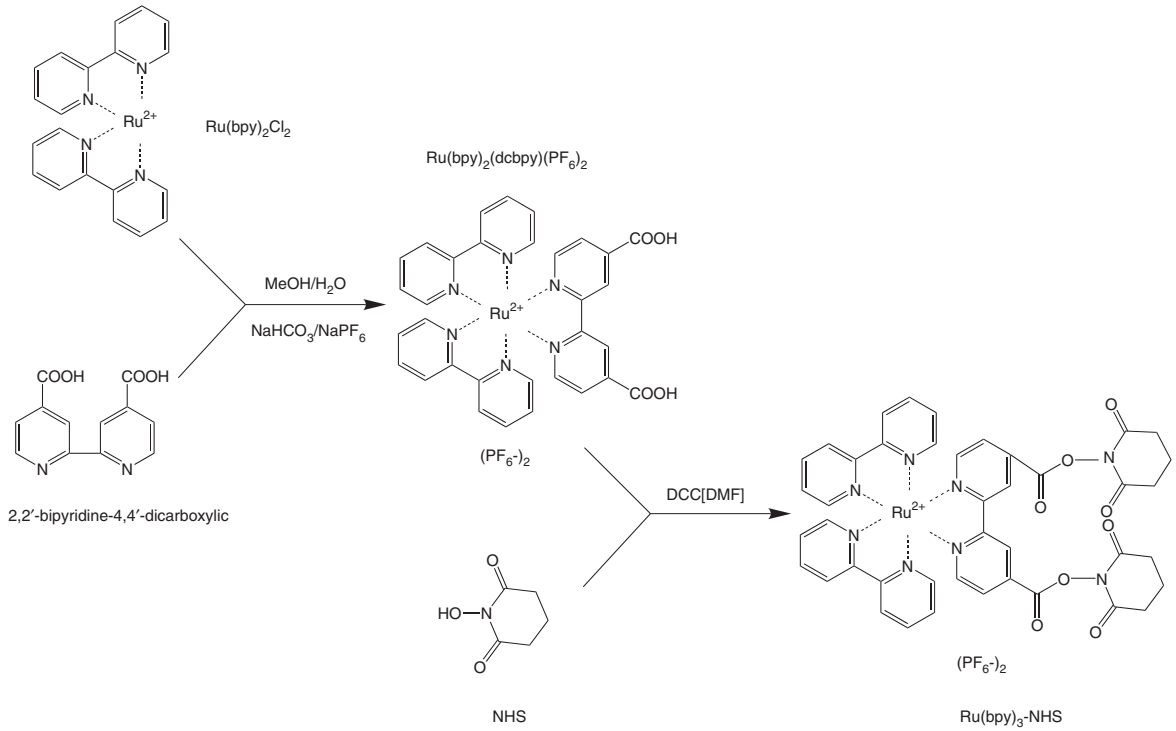

Figure 2 | Synthetic routes to Ru(bpy)$_3^{2+}$-NHS.

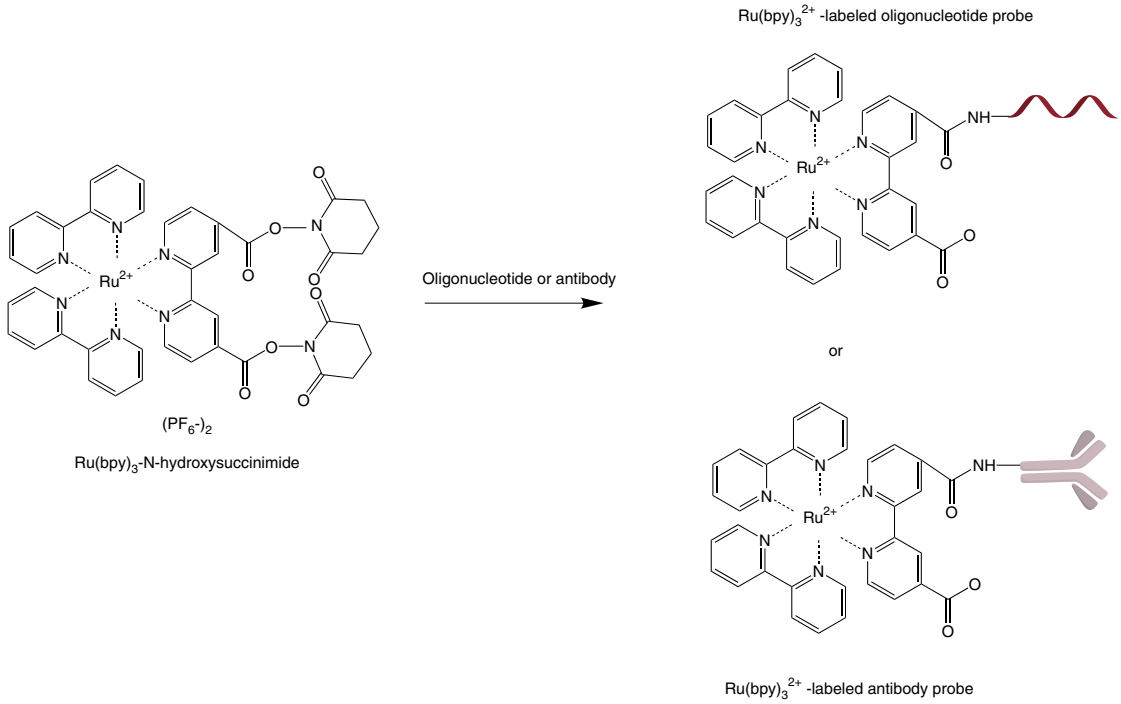

Figure 3 | Synthetic route for $Ru(bpy)_3^{2+}$-labeled antibody and $Ru(bpy)_3^{2+}$-labeled oligonucleotide probes.

After this reaction, the solution is cooled and acidified to precipitate out any unreacted 2,2′-bipyridine-4,4′-dicarboxylic acid. The complete removal of the unreacted 2,2′-bipyridine-4,4′-dicarboxylic acid by filtration is crucial because the 2,2′-bipyridine-4,4′-dicarboxylic acid can participate in the subsequent reactions. Adding an excess of sodium hexafluorophosphate ($NaPF_6$) in water to the resultant filtrate isolates the ruthenium complexes as PF_6 salts. Cooling the solution during this process is crucial for the complete precipitation of the products. We recommend using a centrifuge to collect the $Ru(bpy)_2(dcbpy)(PF_6)_2$ product with satisfactory yields and purity without recrystallization.

$Ru(bpy)_2(dcbpy)(PF_6)_2$ can be activated by reacting with NHS and *N,N*′-dicyclohexylcarbodiimide (DCC) in an organic solvent. This reaction requires an anhydrous organic solvent (e.g., *N,N*′-dimethyl formamide (DMF), DMSO) to prevent the hydrolysis of the NHS ester. The resultant bioactive ruthenium complexes are stable for months under the appropriate storage conditions. Our ruthenium complexes were stored at 4 °C and protected from both humidity and light for at least 1 year without substantially affecting the stability and activity.

Biolabeling of $Ru(bpy)_3^{2+}$-NHS ester. The $Ru(bpy)_3^{2+}$-NHS ester reacts efficiently with primary amines in proteins (e.g., the side chain of lysine) or modified amines in the 3′ or 5′ or the intermediate terminus of an oligonucleotide via a nucleophilic attack to form a stable amide bond and release the NHS (**Fig. 3**; refs. 25,55).The conjugation reaction is favored under slightly alkaline conditions (pH 7.4–9), owing to the deprotonation of the amino group. Notably, coupling buffers that contain primary amines, such as tris buffer, must not be used. In addition, if an antibody is dissolved in a buffer containing a carrier protein or sodium azide, the buffer must be exchanged before labeling to prevent the NHS ester from reacting with the carrier protein or azide. To label nucleic acids, the amino-modified products should be of HPLC grade to prevent the produced salt from interfering. The coupling ratio for the $Ru(bpy)_3^{2+}$-NHS ester and corresponding antibody or oligonucleotide should be determined. Usually, a high concentration of the $Ru(bpy)_3^{2+}$-NHS ester (100 μM–1 mM level for the final concentration) is recommended for the labeling reaction to minimize the effect that hydrolysis has on the NHS ester. Furthermore, the antibody (>100 μg ml^{-1}) and oligonucleotide (>10 μM) concentration should also be controlled. We recommend a 10–20:1 molar ratio for antibody and oligonucleotide labeling.

General considerations for ECL measurements: detection strategy. In this protocol, we synthesize and label a $Ru(bpy)_3^{2+}$-based ECL probe and detail a magnetic bead–based, sandwich-type ECL assay by using carcino-embryonic antigen (CEA) and the *HBsAg* gene (referred to here as the pre-S gene) of hepatitis B virus (HBV) as the model antigen and nucleic acid target, respectively (**Fig. 4**). However, with a few adaptations, this program could also be used as a general detection strategy template for other ECL protocols.

For example, small antigen molecules can be detected via a competitive immunoassay. Nucleic acid detection can be amplified by PCR by using biotin and $Ru(bpy)_3^{2+}$-labeled primers to form biotinylated PCR products that can be captured by the streptavidin-modified magnetic beads. We recently designed an *in situ*, bead-based PCR-ECL detection technology to amplify ruthenium probes on the bead surface, which further simplifies the ECL nucleic acid detection procedures[39]. In addition, highly sensitive nucleic acid detection can be achieved by using probes labeled with biotin and ruthenium via isothermal amplification technologies such as nucleic acid–based amplification (NASBA), rolling-circle DNA amplification (RCA) and so on[45,56].

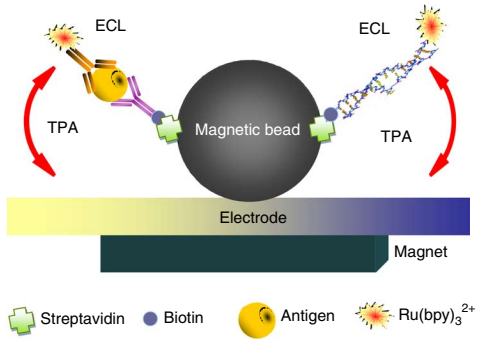

Figure 4 | Schematic of the sandwich immunoassay and nucleic acid assays. The antibody or nucleic acid probes are labeled with biotin and $Ru(bpy)_3^{2+}$. The presence of the target antigen or nucleic acid forms a sandwich complex that is captured on the magnetic beads via the streptavidin-biotin linkage. The ECL reaction occurs once tripropylamine is introduced in solution and a potential is applied.

ECL buffer. The composition of the ECL buffer greatly influences the ECL efficiency. ECL buffer is usually constituted of phosphate component and some additive agents, such as surfactants and preservatives. Some nonionic surfactants, such as Triton X-100 and Tween-20, have been proven to enhance $Ru(bpy)_3^{2+}$/TPrA ECL efficiency. The effect of surfactants on the $Ru(bpy)_3^{2+}$/TPrA ECL efficiency has been extensively studied[57,58]. The TPrA solubility generally increases with the assistance of nonionic surfactants. Moreover, nonionic surfactants tend to stick to the electrode surface, which increases the hydrophobicity of the electrode surface. The luminous efficiency of the $Ru(bpy)_3^{2+}$/TPrA system primarily depends on the direct oxidation of the co-reactants on the electrode surface, and changes in surface hydrophobicity of the work electrode increase the TPrA oxidation rate, which increases the $Ru(bpy)_3^{2+}$/TPrA oxidation-reduction reaction rate and thus the emission intensity. This ECL efficiency enhancement by nonionic surfactants is stable and reproducible. For example, the ECL efficiency can be increased (>10-fold) by adding up to 0.5 mM Tween 20 to the ECL buffer.

Electrode cleaning. Assessing the electrode performance before its use for ECL measurements is important. The electrode cleanliness markedly affects the ECL efficiency and reproducibility. A stable electrode state is a prerequisite for a reproducible analysis. Specifically, the analysis of biological samples in a complex matrix, such as serum, other bodily fluids and nucleic acid amplification products, requires such electrodes. The detection process causes proteins or nucleic acids to adsorb onto the electrode, which contaminates the electrode surface. Adapting conventional work electrode cleaning procedures, such as polishing, to this detection procedure is difficult owing to the electrode being within the flow detection cell. Before running the assay, performing an alternative cleaning procedure to remove any contamination and oxidation layers from the work electrode surface is highly recommended. This procedure involves flushing the electrode with dilute acid or alkali solutions (200 mM NaOH or H_2SO_4) containing 0.5% (vol/vol) detergent (Triton X-100 or NP 40), followed by an electrochemical pretreatment. This electrochemical pretreatment varies according to the electrode material. For a platinum work electrode, a clean surface can be ensured by repeatedly switching the potential between 1.5 V and −0.5 V while cleaning the washing solution.

Magnetic beads. The size and number of beads used with the ECL assay are also key to the measurement performance. Theoretically, smaller bead sizes have larger surface volume area ratios, higher reaction efficiencies and reduced light-shielding effects. However, beads that are too small thus produce too weak a magnetic force to be effectively captured by the electrode surface, which can reduce the ECL signal and decrease the sensitivity. Usually, beads that are 1–4 μm in diameter can be used.

The number of magnetic beads used is key to the detection efficiency of the assay[59]. Having more magnetic beads captures more targets; however, excess beads are physically prevented from reaching the PMT for ECL. The quantity of magnetic beads used should be optimized before performing a bioassay. Individually optimizing the bead quantity of each application by titration is highly recommended. Generally, the ratio of biotin-binding sites on the magnetic beads to the biotinylated antibodies or nucleic acids should be 10:1. Data for the binding sites can be acquired from the product supplier.

Probe. When adopting this detection protocol to other antigens and/or nucleic acid targets, it is important to optimize the performance via the systematic variation of probe concentrations. At low probe concentrations, the signal often reaches saturation at high antigen concentrations, which decreases the slope of the dynamic range. In contrast, having excessive biotinylated and ruthenium-labeled probes can saturate the binding sites of the magnetic beads and yield a high background signal. Finally, one must carefully select the best capture/detection antibody pair with counseling from the supplier.

Quantification of the analytes. The quantitative analysis of an unknown sample requires a calibration curve. To subtract the background ECL intensity, a blank sample (without the target) should be run. The calibration curve should also have at least five points, or the sample conditions should match the expected values. Each sample and standard should be assayed in triplicate. The signal of the blank ± 3 s.d. is widely used to evaluate the assay sensitivity.

MATERIALS

REAGENTS

- $Ru(bpy)_3Cl_2$,Tris(2,2′-bipyridyl)dichlororuthenium(II) hexahydrate (99.95%; Sigma-Aldrich, cat. no. 544981) **! CAUTION** Use a respirator while handling this material and store it in a drying cabinet.
- $Ru(bpy)_2Cl_2$,*cis*-dichlorobis (2,2′-bipyridine)ruthenium(II) (97%; Sigma-Aldrich, cat. no. 733202) **! CAUTION** Use a respirator while handling this material and store it in a drying cabinet.
- 2,2′-Bipyridine-4,4′-dicarboxylic acid (98%; Sigma-Aldrich, cat. no. 550566) **! CAUTION** Use a respirator while handling this material.
- Sodium hexafluorophosphate ($NaPF_6$; 99%; Alfa Aesar, cat. no. 13064)
- Methanol (CH_3OH; >99.5%; AR, Tianjin Damao Chemical Work)
- Sodium bicarbonate ($NaHCO_3$; >98%; Sangon Biotech, cat. no. ST0873)
- Boric acid (H_3BO_3), AR, (Gguangzhou chemical reagent factory)
- Sodium hydroxide (NaOH; Gguangzhou chemical reagent factory)

! CAUTION NaOH can cause burns. Wear gloves and protective clothing while handling this material.
- Hydrochloric acid (HCl; ~37%; Gguangzhou chemical reagent factory)
! CAUTION HCl can cause burns. Wear gloves and protective clothing while handling this material.
- Sulfuric acid (H_2SO_4; Gguangzhou chemical reagent factory)
! CAUTION H_2SO_4 can cause burns. Wear gloves and protective clothing while handling this material.
- Tween 20, (Sangon Biotech, cat. no. T0777)
- Triton X-100, (Sangon Biotech, cat. no. T0694)
- Tris base (≥99.8%; Sigma-Aldrich, cat. no. 252859)
- Dynabeads M-280 Streptavidin (Invitrogen)
- Tripropylamine (TPrA; >98%; Sigma-Aldrich, cat. no. 143979)
- *N*,*N*′-Dicyclohexylcarbodiimide (DCC; ≥99.0%; Sigma-Aldrich, cat. no. 36650)
- *N*-Hydroxysuccinimide (NHS; 98%; Sigma-Aldrich, cat. no.130672)
- *N*,*N*′-Dimethyl formamide (DMF; >99.5%; Sangon Biotech, cat. no. D0464)
- Glycine solution, 2 M (Sigma)
- Ethanol (95% and 70%, vol/vol)
- Sodium azide (NaN_3; Sigma-Aldrich, cat. no. 438456) **! CAUTION** Store it in a cool, dry secondary container protected from acids and heavy metals.
! CAUTION NaN_3 is flammable, explosive, highly corrosive and highly toxic by inhalation, ingestion or on contact with both skin and eyes.
- Sodium phosphate, monobasic (NaH_2PO_4; >99%; Sangon Biotech)
- Sodium phosphate, dibasic (Na_2HPO_4; >99%; Sangon Biotech)
- Dipotassium hydrogen phosphate (K_2HPO_4; >99%; Sangon Biotech)
- Potassium dihydrogen phosphate (KH_2PO_4; >99%; Sangon Biotech)
- Sodium chloride (NaCl; >99%; Sangon Biotech)
- Silicon oil (Sangon Biotech)
- BSA, Molecular Biology Grade (New England Biolabs)
- Pierce bicinchoninic acid (BCA) protein assay kit (Pierce, cat. no. 23227)
- Zeba Spin desalting columns and devices, 40-kDA MWCO (Pierce, cat. no. 87766)
- NHS-PEG_4-biotin no-weigh format (Pierce, cat. no. 21329)

EQUIPMENT
- CHI 660C electrochemical workstation (CH Instruments)
- Reference electrode (Ag/AgCl) (CH Instruments)
- Spectrophotometer (Eppendorf BioPhotometer)
- PerkinElmer LS55 luminescence spectrometer
- PerkinElmer Lambda 35 UV-visible spectrometer
- Syringe pump (KD Scientific)
- Single-photon counting module (PMT, MP-962, PerkinElmer)
- Heat collection-constant temperature type magnetic stirrer
- Thermomixer comfort (Eppendorf)
- Centrifuge 5415R (Eppendorf)
- Vacuum freeze Dryer (IlShin Lab)
- Vacuum filter apparatus
- Ultrasonic bath

Box 1 | Labeling an antibody with biotin to form the capture antibody
● TIMING 3–5 h

Preparation of a biotinylated capture antibody is based on the formation of a stable amide bond between NHS-PEG_4-biotin and the amino group of the lysine in the antibody. This is similar to the labeling of detection antibody and $Ru(bpy)_3^{2+}$-NHS. In brief, carry out the following steps:

1. Remove the anti-CEA antibody from the refrigerator, collect it via centrifugation (2 min at 5,000*g* at 4 °C) and equilibrate it to room temperature.

▲ **CRITICAL STEP** If an antibody is dissolved in a buffer containing a carrier protein or NaN_3, it must be exchanged before labeling to avoid a reaction between the NHS ester and the carrier protein or azide.

2. Dilute the antibodies to 1 mg ml^{-1} in PBS free of carrier proteins and azide.

▲ **CRITICAL STEP** The dilution and reaction buffers should be weakly alkaline and free of both amines and preservatives.

3. Add 170 µl of water to 2 mg of NHS-PEG_4-biotin to prepare a 20 mM NHS-PEG_4-biotin solution.

! CAUTION NHS-PEG_4-biotin dissolved in water cannot be stored for later use. To reduce costs, a multiple antibody labeling reaction can be executed simultaneously.

4. Add NHS-PEG_4-biotin reagent to the antibody solution in a 20:1 biotin:antibody molar ratio.
5. Shake the solutions for 1 h at room temperature by using a small vortexer.
6. Terminate the reactions by adding 20 µl of 2 M glycine and incubate them for 15 min at room temperature.
7. Pass the antibodies through a Zeba spin desalting column (40K MWCO) before equilibrating with PBS to remove any unreacted labeling reagent.
8. Store the biotinylated antibody at 4 °C by using PBS containing 0.1% (wt/vol) BSA.

■ **PAUSE POINT** The $Ru(bpy)_3^{2+}$-labeled antibody can be stored at 4 °C for at least 1 month before use.

9. Remove the anti-CEA antibody from the refrigerator, collect it via centrifugation (2 min at 5,000*g* at 4 °C) and equilibrate it to room temperature.

▲ **CRITICAL STEP** If an antibody is dissolved in a buffer containing a carrier protein or NaN_3, it must be exchanged before labeling to avoid a reaction between the NHS ester and the carrier protein or azide.

10. Dilute the antibodies to 1 mg ml^{-1} in PBS free of carrier proteins and azide.

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11. Add 170 µl of water to 2 mg of NHS-PEG_4-biotin to prepare a 20 mM NHS-PEG_4-biotin solution.

! CAUTION NHS-PEG_4-biotin dissolved in water cannot be stored for later use. To reduce costs, a multiple antibody labeling reaction can be executed simultaneously.

12. Add NHS-PEG_4-biotin reagent to the antibody solution in a 20:1 biotin:antibody molar ratio.
13. Shake the solutions for 1 h at room temperature by using a small vortexer.
14. Terminate the reactions by adding 20 µl of 2 M glycine and incubate them for 15 min at room temperature.
15. Pass the antibodies through a Zeba spin desalting column (40 kDa MWCO) before equilibrating with PBS to remove any unreacted labeling reagent.
16. Store the biotinylated antibody at 4 °C in PBS containing 0.1% (wt/vol) BSA.

■ **PAUSE POINT** The $Ru(bpy)_3^{2+}$-labeled antibody can be stored at 4 °C for at least 1 month before use.

• LabVIEW software (LabVIEW Core, National Instruments)
• ELGA ultrapurified water system (ELGA)
• Round-bottom flask
• Filter
• Aluminum foil
• Three-neck flask
• Glass plate
• Glass funnel
• Glass rod
• Filter paper

REAGENT SETUP

▲ **CRITICAL** In our laboratory, all solutions in the listed in Reagent Setup were prepared by using ultrapurified water (18 MΩ cm) from an ELGA LabWater system.

Capture and detection antibodies The anti-CEA monoclonal capture antibody and detection antibody, in 0.01 M PBS, pH 7.4 (>90%), used as the example in this procedure, were purchased from Zhengzhou Biocell Biotechnology. Generally, it is best to choose the antibody pairs that can perform well in ELISA for use in ECL detection. The capture antibody should be labeled with biotin. A procedure for doing this is described in **Box 1**.

Oligonucleotide probes The following synthetic oligonucleotide probes (HPLC purified) were used for the nucleic acid assays for specifically detecting the pre-S gene of HBV, and they are referenced from the literature[60]. Capture probe: 5′-biotin-TTTTTGGAGCACCCACGTGTCCTGGCC-3′; signal probe: 5′-GCTCAGTTTACTAGTGCCATTT-NH_2-3′; synthesized target derived from the pre-S gene of HBV: 5′-ACTAGTAAACTGAGCA TACTGGCCAGGACACGTGGGTGC-3′. Both capture and signal probes should have a length of about 20 bp to ensure specificity. About three T bases should be added as the linker at the label item to reduce the steric effect. The target should be made up in water, and a dilution series covering, for example, a concentration range of 1 pM to 10 nM should be prepared. This can be stored at −20 °C for up to 1 month.

Protein antigen standard Human CEA antigen (50 ng ml^{-1}) (used as a standard for the example experiment) is obtained from Roche Diagnostics. The antigen should be made up in PBS buffer, and a dilution series covering, for example, a concentration range of 0.1–100 ng ml^{-1} should be prepared. This can be stored at −20 °C for up to 1 month.

$NaPF_6$ solution Dissolve 5 g of $NaPF_6$ in 25 ml of H_2O immediately before use.

PBS buffer (pH 7.4), calcium and magnesium free Dissolve 8 g of NaCl, 0.2 g of KCl, 1.42 g of Na_2HPO_4 and 0.27 g of KH_2PO_4 in 1 liter of distilled H_2O, and then adjust the pH to 7.4 with 1 M HCl. Sterilize the buffer via vacuum filtration through a 0.22-μm filter. Store PBS buffer at room temperature for up to 1 month.

PBS containing 0.1% (wt/vol) BSA Add 10 mg of BSA to 10 ml of PBS buffer. Store it at 4 °C for up to 1 week. For long-term storage, keep the solution in 0.025% (wt/vol) NaN_3.

Sodium borate buffer, 0.1 M Dissolve 6.18 g of H_3BO_3 in 900 ml of water. Adjust the pH to 8.5 with 1 M NaOH and fill it to 1 liter with water. Sterilize the buffer via vacuum filtration with a 0.22-μm filter. This can be stored at −20 °C for up to 1 month.

Hybridization buffer, 2× (pH 7.0) Dissolve 17.52 g of NaCl and 8.8 g of sodium citrate in distilled water and adjust the pH to 7.0 with HCl before diluting to 1,000 ml with water. Sterilize the buffer via filtration. Store the buffer at room temperature for up to 1 month.

ECL buffer (100 mM potassium-phosphate buffer, 100 mM TPrA and 0.1% (vol/vol) Tween 20, pH 8.0) Dissolve 0.816 g of K_2HPO_4 and 16.73 g of KH_2PO_4 in water, add 19 ml of TPrA and 1 ml of Tween 20 and dilute the mixture to 1 liter with water. Store the buffer at 4 °C for up to 2 weeks. For long-term storage, store the solution containing 0.025% (wt/vol) NaN_3.

! **CAUTION** TPrA is readily absorbed through skin. Wear suitable gloves and eye and skin protection.

ECL washing solution (0.2 M NaOH, 0.5% Triton X-100) Dissolve 8 g of NaOH in H_2O, add 5 ml of Triton X-100 and dilute it to 1 liter with water. Store the solution at room temperature for up to 1 month.

PROCEDURE

Synthesis of $Ru(bpy)_2(dcbpy)(PF_6)_2$

1| Soak a 200-ml three-neck flask, magnetic stir bar, condenser, glass plate, glass funnel and glass rod in aqua regia (3:1 concentrated HCl:HNO_3) for 30 min, clean them in deionized water via ultrasonication, rinse copiously with deionized water and dry them in an oven at 100 °C.

! **CAUTION** Aqua regia can cause burns. Be extremely careful and perform this experiment in a fume hood.

2| Assemble a reflux apparatus by using a three-neck round-bottomed flask, reflux condenser, Tygon tubing and rubber septa.

3| Add silicone oil to the heat collection-constant temperature type magnetic stirrer and place the flask in the silicone oil.

! **CAUTION** Be sure to keep two-thirds of the three-neck flask immersed in the silicone oil but not in contact with the hot plate.

4| Add 0.2 g of *cis*-dichlorobis(2,2′-bipyridine)ruthenium(II), 0.15 g of 2,2′-bipyridine-4,4′-dicarboxylic acid, 0.2 g of sodium bicarbonate, 32 ml of CH_3OH and 8 ml of deionized water to the bottom of the three-neck bottle.

! **CAUTION** These reagents should be added carefully to the bottom of the three-neck flask. Do not add the reagents to the flask walls.

▲ **CRITICAL STEP** To acquire a high yield, the 2,2′-bipyridine-4,4′-dicarboxylic acid should be present in slight excess relative to the *cis*-dichlorobis(2,2′-bipyridine)ruthenium(II).

5| Connect the condenser to the middle neck of the flask and the stopper to the other two necks. Heat the silicone oil to 80 °C.

6| Sluggishly reflux the solution for 10 h. Ensure that water is flowing through the reflux condenser. During this period, the color of the solution will turn from purple-brown to a yellowish-red.

? **TROUBLESHOOTING**

7| Adjust the pH of the solution to 4.4 with concentrated H_2SO_4, and then cool it in an ice bath for 2 h.
! CAUTION Keep the solution in a dark room.
▲ CRITICAL STEP Carefully adjust the pH to 4.4, as the excess nonreacted 2,2′-bipyridine-4,4′-dicarboxylic acid precipitates at this pH range.
? TROUBLESHOOTING

8| Assemble the vacuum filtration apparatus and place coarse filter paper on the funnel.

9| Filter the cool solution mixture and transfer the fluid into a clean glass bottle.
! CAUTION Draught the solution by using a glass rod. Replace the filter paper if it is blocked.

10| Wash the residues caught by the filter paper with 4–6 ml of CH_3OH. Combine the filtrates after all of the solids have been removed.

11| Add 12.5 ml of a $NaPF_6$ solution (2.5 g $NaPF_6$ in 12.5 ml H_2O) to the filtrate.

12| Stir the solution for 5 min before cooling it in an ice bath for 2 h. Observe the formation of a maroon precipitate.
? TROUBLESHOOTING

13| Transfer the solution and precipitate to centrifuge tubes (15 ml), and collect the solution in the bottom of the tube via centrifugation (5,000*g*, 5 min, 4 °C).

14| Carefully remove the supernatant and freeze-dry the maroon precipitate to obtain the evaporated crystal products.

15| Weigh the crystal solid to quantify the collected product and determine the yield.
■ PAUSE POINT $Ru(bpy)_2(dcbpy)(PF_6)_2$ can be stored in an anhydrous environment for months before use.

Synthesis of $Ru(bpy)_3^{2+}$-NHS

16| Dissolve 0.23 g of DCC and 0.12 g of NHS in 2 ml of DMF with stirring, and then cool it in an ice/water bath.
? TROUBLESHOOTING

17| Add 0.19 g of $Ru(bpy)_2(dcbpy)(PF_6)_2$ (prepared in Step 15) to the mixture. Cool the solution to 0 °C in an ice/water bath. Stir the reaction mixture at 0 °C for 30 min and at room temperature for 5 h.
▲ CRITICAL STEP Ensure that all of the reactants are anhydrous.

18| Discard any insoluble material via centrifugation (5,000*g*, 5 min, 4 °C).

19| Collect the supernatant in a brown glass bottle. Keep the $Ru(bpy)_3^{2+}$-NHS in a cold, dry and photophobic environment.
! CAUTION $Ru(bpy)_3^{2+}$-NHS is moisture-sensitive. For long-term storage, use separate packages to avoid excessive exposure to air and moisture caused by repeated opening.
■ PAUSE POINT $Ru(bpy)_3^{2+}$-NHS can be stored at –20 °C for 1 year.

20| Take 10 µl of the synthesized $Ru(bpy)_3^{2+}$-NHS and add 490 µl of water to dilute it 50-fold. Determine the $Ru(bpy)_3^{2+}$-NHS concentration according to the Beer's Law by measuring the absorbance at 455 nm:

$$A = \varepsilon CL$$

Where ε is the molecular absorption coefficient ($\varepsilon 455$ = 13,700 M^{-1} cm^{-1} for $Ru(bpy)_3^{2+}$-NHS), C is the analyte concentration, L is the travel length of light through the sample and A is the absorbance of the sample.
Calculate the concentration of the bio-active $Ru(bpy)_3^{2+}$-NHS according to Beer's Law.

21| For further characterization, add 490 µl of water to 10 µl of the synthesized $Ru(bpy)_3^{2+}$-NHS to dilute it 50-fold, and then measure the emission spectra.

22| Measure the absorbance and emission spectra of $Ru(bpy)_3^{2+}$. Compare the absorbance and emission spectra of $Ru(bpy)_3^{2+}$ with the synthesized $Ru(bpy)_3^{2+}$-NHS.
▲ CRITICAL STEP Note the several-nanometer red shift of the plasmon peak due to changes in the local refractive index after coupling to the NHS group.

23| You can now either label an antibody (option A) or a nucleic acid (option B) with $Ru(bpy)_3^{2+}$-NHS. This would be the detection antibody or detection nucleic acid, respectively.

(A) Labeling the antibody with $Ru(bpy)_3^{2+}$-NHS

(i) Remove the anti-CEA antibody from the refrigerator, collect it by centrifugation (2 min at 5,000*g*, 4 °C) and equilibrate it to room temperature.
▲ **CRITICAL STEP** If the antibody is dissolved in a buffer containing a carrier protein or NaN_3, it must be exchanged before labeling to avoid a reaction between the NHS ester and the carrier protein or azide.

(ii) Dilute the antibodies to 1 mg ml^{-1} in PBS free of carrier proteins and azides.
▲ **CRITICAL STEP** The dilution and reaction buffer should be weakly alkaline and free of both amines and preservatives.

(iii) Dilute the $Ru(bpy)_3^{2+}$-NHS reagent (prepared in Step 19) in DMF to 2 mM immediately before use.
! **CAUTION** To avoid moisture condensation, equilibrate the reagent to room temperature before opening.

(iv) Add the $Ru(bpy)_3^{2+}$-NHS reagent to the antibody solution (the molecular weight of the IgG antibody is ~150 kDa) to yield a 20:1 ruthenium:antibody molar ratio.
▲ **CRITICAL STEP** The DMF contents should not exceed 10% of the total reaction volume.
? **TROUBLESHOOTING**

(v) Wrap the tube with aluminum foil and shake the solution for 1 h at room temperature with a small vortexer.

(vi) Terminate the reactions by adding 20 µl of 2 M glycine and incubate them for 15 min at room temperature.

(vii) Pass the antibodies through a Zeba spin desalting column (40 kDa MWCO) before equilibrating with the PBS to remove any nonreacted $Ru(bpy)_3^{2+}$-NHS.

(viii) Collect and pool the $Ru(bpy)_3^{2+}$-labeled anti-CEA antibody fractions.

(ix) Determine the $Ru(bpy)_3^{2+}$ label concentration by measuring the absorbance at 455 nm (ε = 13,700 M^{-1} cm^{-1}).
? **TROUBLESHOOTING**

(x) Determine the antibody concentration by using a BCA protein assay kit.
▲ **CRITICAL STEP** The $Ru(bpy)_3^{2+}$ label will affect the OD_{280} absorbance readings. Therefore, it is not recommended to calculate the labeled protein concentration by measuring the OD_{280} absorbance.

(xi) Concentrate the antibodies by using a spin-filtration system as needed.

(xii) The final concentrations and labeling ratios should be calculated according to the following formula:

$$[\text{Label ratio}] = A_{\text{antibody absorption}} \times \text{dilution factor} / 13{,}700\,M^{-1}\,cm^{-1} \times C_{\text{antibody concentration}}$$

(xiii) Store the $Ru(bpy)_3^{2+}$-labeled antibody at 4 °C in PBS containing 0.1% (wt/vol) BSA.
■ **PAUSE POINT** The $Ru(bpy)_3^{2+}$-labeled antibody can be stored at 4 °C for at least 1 month before use.

(B) Labeling an oligonucleotide probe with $Ru(bpy)_3^{2+}$-NHS

(i) Centrifuge a six (or three)-carbon amino terminal oligonucleotide probe (8,000*g*, 5 min, 4 °C) and collect the powdered DNA stuck to the microcentrifuge tube wall.
! **CAUTION** Amine-modified oligonucleotides are usually ordered from commercial companies. Typically, HPLC-grade products can be used for direct labeling reactions. For coarse materials, an ethanol precipitation is recommended.

(ii) Gently open the lid and add 0.1 M sodium borate buffer (pH 8.5) to obtain an oligonucleotide concentration of 50 µM, and then immediately close the lid.
▲ **CRITICAL STEP** Ensure that the buffer provides mildly basic conditions and that it is free of interfering compounds, especially amines such as triethylamine, Tris and ammonium salts.

(iii) Gently shake the oligonucleotide solution for 3–5 min, mix it well and collect the material in the bottom of the tube after centrifugation (5,000*g*, 3 min, 4 °C).

(iv) Immediately before use, dilute the $Ru(bpy)_3^{2+}$-NHS reagent (prepared in Step 19) in DMF to 10 mM.

(v) Add the $Ru(bpy)_3^{2+}$-NHS reagent to the oligonucleotide solution in a 20:1 ruthenium:oligonucleotide molar ratio.

(vi) Wrap the tube with aluminum foil and place it on a shaker oscillating at low speeds for at least 6 h or overnight at room temperature in the dark.
! **CAUTION** Gently vortex or tap the tube twice during the first 2 h to ensure that the reaction remains well mixed. Do not mix it violently. The reaction mixture may stick to the wall of the tube.

(vii) Add 20 µl of 2 M NaCl and 500 µl of cold absolute ethanol to the reaction tube.

(viii) Place the solution in an ultralow-temperature refrigerator (–80 °C) for 20 min or at –20 °C for 30 min.
? **TROUBLESHOOTING**

(ix) Centrifuge the solution for 20 min at 12,000*g* for 4 °C.

(x) Carefully remove the supernatant and rinse the pellet with 1 ml of cold 70% (vol/vol) ethanol.

(xi) Repeat Steps 8 and 9 three times.

(xii) Air-dry the pellet.
▲ **CRITICAL STEP** Do not dry the pellet completely; otherwise, it will be difficult to re-dissolve.

(xiii) Re-dissolve the probe by using PBS or dH_2O. Mix well and remove any solid via centrifugation (5,000*g*, 3 min, 4 °C).
! CAUTION The oligonucleotide probe can be labeled effectively (>80%) under these conditions. To obtain higher-purity probes, we recommend obtaining the probes from commercial companies or further purifying them by HPLC or gel electrophoresis using established methods.
? TROUBLESHOOTING

(xiv) Dilute a stock oligonucleotide probe solution with water, measure its absorbance at 260 nm and calculate the concentration using the formula below:

$$[\text{Concentration, in } \mu M] = (A_{\text{antibody absorption}} \times \text{dilution factor} \times 33{,}000) / \text{MW of oligonucleotide probe}$$

(xv) Store the probes separately at –20 °C to avoid repeated freeze-thaw cycles.
■ PAUSE POINT The $Ru(bpy)_3^{2+}$-labeled oligonucleotide probe can be stored before use at –20 °C for at least 3 months and at –80 °C for 1 year.

ECL assays

24| If you made a protein probe (Step 23A), detect the protein as detailed in option A. If you made a nucleic acid probe (Step 23B), use it as described in option B.

(A) ECL assay for protein detection ● TIMING 1.5–2 h

(i) Dilute the biotin-labeled monoclonal capture antibody and $Ru(bpy)_3^{2+}$-labeled monoclonal signal antibody to 50 μg ml^{-1} by using PBS buffer. (Note: the molecular weight of the IgG antibody is ~150 kDa.)

(ii) Dilute the streptavidin-coated magnetic beads (2.8 μm in diameter) to 1 mg ml^{-1} with the ECL buffer. Wash these beads twice with the ECL buffer. Re-disperse them into twice the original buffer volume.

(iii) Add 15 μl of the biotinylated antibody solution (50 μg ml^{-1}), 15 μl of the $Ru(bpy)_3^{2+}$-labeled antibody (50 μg ml^{-1}), 10 μl of the target antigen (or control) and 10 μl of PBS buffer to a microcentrifuge tube.
▲ CRITICAL STEP The probe concentration is important for successfully implementing the assay. The higher the probe concentration, the more targets captured but the greater the nonspecific binding. When substantial nonspecific binding is observed, one can decrease the probe concentration to reduce the background signal.

(iv) Incubate the resultant mixture at 37 °C for 30 min on an orbital shaker to form the antibody-antigen-antibody sandwich complexes.

(v) Transfer the antibody-antigen complex solution to a 1.5-ml Eppendorf tube, and then add 20 μl of 1 mg ml^{-1} streptavidin-coated magnetic beads (2.8 μm in diameter) to bind the biotin. Incubate the solution mixture at 37 °C for 10 min.
! CAUTION Ensure that the magnetic beads do not settle to the bottom during incubation.
▲ CRITICAL STEP The quantity of the magnetic beads is important for the detection efficiency of the assay. The ratio of biotin-binding sites on the magnetic beads to the biotinylated antibodies should be 5:1. Optimizing the bead concentration for each individual application is highly recommended. Data on the binding sites can be obtained from the product supplier.

(vi) Pull 50 μl of the captured magnetic mixtures into the fluidic ECL detection, and then pipette the ECL buffer for 10 s (at a ratio of 50 μl s^{-1}) to remove any excess probes and the sample matrix.
▲ CRITICAL STEP Avoid air bubbles while pipetting.
▲ CRITICAL STEP Ensure the removal of all unbound probes to reduce the background signal.

(vii) Hold the solution in the electrochemical cell and apply a 1.2-V potential to excite the ECL reaction.

(viii) Record the ECL signal for 30 s.
? TROUBLESHOOTING

(ix) After the measurement, retract the magnet and purge the sample cell by using a washing buffer. Release the magnetically responsive particles, and push the solution out of the detection cell.

(B) ECL assay for nucleic acid detection

(i) Dilute the biotin-labeled captured probe and the $Ru(bpy)_3^{2+}$-labeled signal probe to 1 μM with deionized water.

(ii) Dilute the streptavidin-coated magnetic beads to 1 mg ml^{-1} with ECL buffer. Wash the streptavidin-coated magnetic beads twice with the ECL buffer. Re-disperse the beads by using twice the original buffer volume.

(iii) Add 10 μl of the captured biotin-labeled probe solution (1 μM), 10 μl of the $Ru(bpy)_3^{2+}$-labeled signal probe (1 μM), 10 μl of the target DNA (or control sample) and 30 μl of a 2× hybridization buffer to a microcentrifuge tube.
▲ CRITICAL STEP The probe concentration is important for successfully implementing the assay. The higher the probe concentration, the more targets captured but the greater the nonspecific binding. When substantial nonspecific binding is observed, the probe concentration can be decreased to reduce the background signal.

(iv) Incubate the resultant solution at 70 °C for 10 min and then at 50 °C for 30 min by using a thermal cycler.

(v) Transfer the solution to a 1.5-ml microcentrifuge tube and add 20 μl of the 1 mg ml^{-1} streptavidin-coated magnetic beads (2.8 μm in diameter) to the sandwich hybridization complexes to bind the biotin.

▲ CRITICAL STEP The quantity of the magnetic beads is important for the detection efficiency of the assay. The ratio of biotin-binding sites on the magnetic beads to the biotinylated antibodies should be ≥5:1. Optimizing the bead concentration for each individual application is highly recommended. Data on the binding sites can be obtained from the product supplier.

(vi) Pull 50 µl of the captured magnetic mixtures into the fluidic ECL detection, and then pipette the ECL buffer for 10 s (at a ratio of 50 µl s^{-1}) to remove any excess probes and the sample matrix.

▲ CRITICAL STEP Avoid air bubbles while pipetting.

▲ CRITICAL STEP Ensure the removal of all unbound probes to reduce the background signal.

(vii) Hold the solution in the electrochemical cell and apply a 1.2-V potential to excite the ECL reaction.

(viii) Record the ECL signal for 30 s.

(ix) After the measurement, retract the magnet and purge the sample cell with a cleaning solution. Release the magnetically responsive particle; push the solution out of the detection cell.

? TROUBLESHOOTING

Troubleshooting advice can be found in **Table 1**.

TABLE 1 | Troubleshooting table.

Step	Problem	Possible reason	Solution
6	The reaction volume decreases substantially	The temperature is above 80 °C	Control the temperature
		The flask is not sealed tightly enough	Check the septa and glass stoppers to ensure the system is closed
7	The solution remains clear and no precipitate is observed	The pH was not adjusted to the appropriate value	Re-adjust the pH
		Deliquescence of the reaction reagents	If the problem persists, replace the reagents
12	The solution remains clear and no precipitate forms	Deliquescence of the $NaPF_6$	Use fresh $NaPF_6$ solution
		The solution did not cool completely	Ensure that the solution is sufficiently cooled in an ice bath
16	DCC does not dissolve in the DMF	Deliquesced DCC will not dissolve in DMF	Replace with a new reagent
23A(iv)	Precipitation of the antibody	Excessively high labeling ratios	Reduce the $Ru(bpy)_3^{2+}$-NHS concentration
23A(ix)	No valid measurement of absorption at 455 nm	The $Ru(bpy)_3^{2+}$-NHS–labeled antibody concentration is too low	Adjust the dilution factor of the $Ru(bpy)_3^{2+}$-NHS–labeled antibody
23B(viii)	No obvious DNA precipitation	The solution did not cool completely	Prolong the freezing time
23B(xiii)	DNA pellet does not dissolve	The DNA pellet was dried for too long before elution	DNA pellets that are too dry will require a longer time to dissolve completely. The DNA solution can be given more time to re-dissolve; alternatively, increase the volume of the elution buffer. If the problem is not solved, relabel the DNA probe
24A(viii)	High background signal even at low target concentrations	The signal probe concentration is too high	Optimize the signal probe concentration
			Maintain the detection cell as a completely closed well
		Ambient light is too strong	Keep the testing room dark
	Weak signal even at high target concentrations	The signal probe concentration is too high	Optimize the signal probe concentration and sample flowing rate
		An inappropriate sample flow rate was used	Avoid pulling the sample too quickly because a rapid sample flow through the working electrode may prevent the sample from being effectively captured by the electrode surface
	Large ECL emission fluctuations and poor reproducibility	Work electrode surface is contaminated	Clean the work electrode with the washing buffer and conduct further electrochemical pre-treatments of the electrode

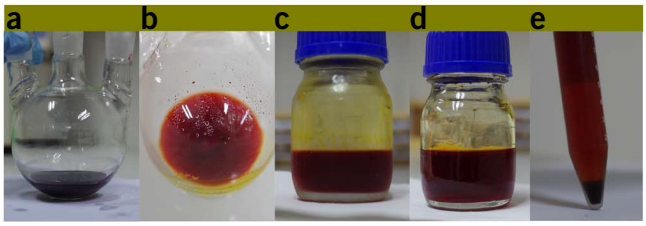


Figure 5 | The reaction in progress. (**a**) The *cis*-dichlorobis(2,2′-bipyridine) ruthenium(II), 2,2′-bipyridine-4,4′-dicarboxylic acid, CH_3OH, $NaHCO_3$ and water were mixed. (**b**) The solution was refluxed for 10 h, and it turned from purple to deep red. (**c**) The pH of the solution was adjusted to 4.4 with concentrated H_2SO_4, and the nonreacted 2,2′-bipyridine-4,4′-dicarboxylic acid precipitated out and created a muddy appearance. (**d**) After filtering the resultant precipitate, the solution seems limpid. (**e**) A metathesis with $NaPF_6$ in a cool ice bath precipitates a maroon product.

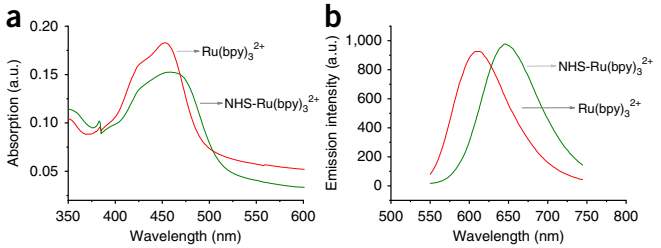


Figure 6 | Spectral characterization of the $Ru(bpy)_3^{2+}$-NHS ester. (**a**,**b**) Absorption (**a**) and emission (**b**) spectra of $Ru(bpy)_3^{2+}$ and the synthesized $Ru(bpy)_3^{2+}$-NHS ester in water at room temperature. a.u., arbitrary units.

● TIMING

Reagent preparation: 1.5–2 h
Steps 1–3: ~2 h
Steps 4–6: ~11 h, overnight
Steps 7–15: ~6 h
Steps 16–22, $Ru(bpy)_3^{2+}$-NHS synthesis: ~10 h
Step 23A, labeling of the signal antibody with $Ru(bpy)_3^{2+}$-NHS: 7–10 h
Step 23B, labeling of the oligonucleotide probe with $Ru(bpy)_3^{2+}$-NHS: 12 h or overnight
Step 24A, protein assay: 1.5–2 h
Step 24B, nucleic acid assay: 1.5–2 h
Box 1, labeling of the capture antibody with biotin: 3–5 h

ANTICIPATED RESULTS

The carboxylated $Ru(bpy)_3^{2+}$ is synthesized from the reaction of *cis*-dichlorobis(2,2′-bipyridine)ruthenium(II) and 2,2′-bipyridine-4,4′-dicarboxylic acid. $NaHCO_3$ is added during this reaction to promote the solubility of the 2,2′-bipyridine-4,4′-dicarboxylic acid in the $MeOH/H_2O$ solution, as shown in **Figure 5**.

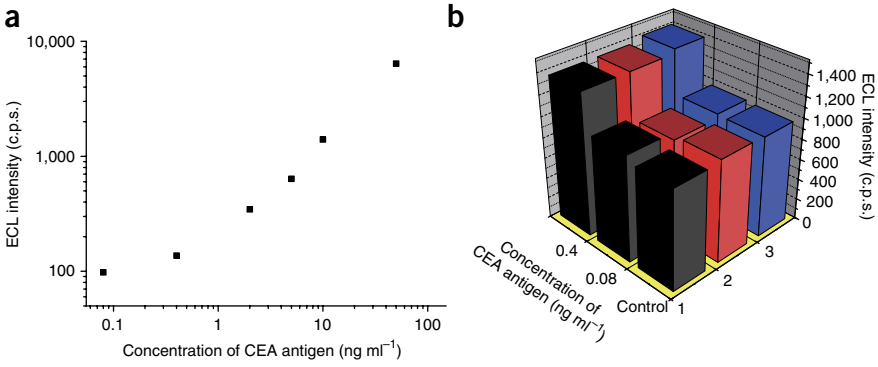
Before completing the reaction, the *cis*-dichlorobis(2,2′-bipyridine)ruthenium(II) and 2,2′-bipyridine-4,4′-dicarboxylic acid mixture looks purple (**Fig. 5a**). The solution appears red after refluxing in $MeOH/H_2O$ (**Fig. 5b**). The unreacted 2,2′-bipyridine-4,4′-dicarboxylic acid can be precipitated out by adjusting the pH to 4.4 (**Fig. 5c**). Filtering this precipitate leaves the product as a clear solution (**Fig. 5d**). The filtrate was treated with $NaPF_6$ to obtain $[Ru(bpy)_2(dcbpy)(PF_6)_2]$ as a red precipitate (**Fig. 5e**). The overall yield is ~70–80%, as shown in **Figure 2**.

$[Ru(bpy)_2(dcbpy)(PF_6)_2]$ is characterized by electrospray ionization mass spectrometry (ESI-MS) and ^1H NMR as shown in **Supplementary Figures 1** and **2**. ESI-MS ion clusters at m/z 329.2 $\{M - PF_6^-\}^{2+}$.

^1H NMR (400 MHz, DMSO) δ 9.25 (s, 2H), 8.84 (dd, J = 8.1, 3.5 Hz, 4H), 8.18 (q, J = 7.5 Hz, 4H), 7.87 (dd, J = 13.6, 5.7 Hz, 4H), 7.73 (dd, J = 12.8, 5.4 Hz, 4H), 7.53 (dt, J = 18.6, 6.5 Hz, 4H).

To ensure that the $Ru(bpy)_3^{2+}$ is biomodifiable, NHS is attached to the synthesized $Ru(bpy)_2(dcbpy)(PF_6)_2$ via a dehydration with DCC. The resultant product, $Ru(bpy)_3^{2+}$-NHS, yields absorption and emission spectra comparable to $Ru(bpy)_3^{2+}$.

Figure 7 | Assay performance of protein detection by current ECL technique. (**a**) Typical results for the ECL detection of a CEA antigen by using a sandwich model with varying concentrations (0.08, 0.4, 2, 5, 10 and 50 ng ml^{-1}). The dynamic quantification range of the current assay is 0.4 to 50 ng ml^{-1}. Note that the upper limit may be extended to higher antigen concentrations; however, such values are not measured in this experiment. (**b**) The limit of detection (LOD) is determined from the control measurement and the lowest antigen concentrations. Each sample is measured three times in parallel. The LOD of the CEA antigen according to the 3σ method is 0.4 ng ml^{-1}.



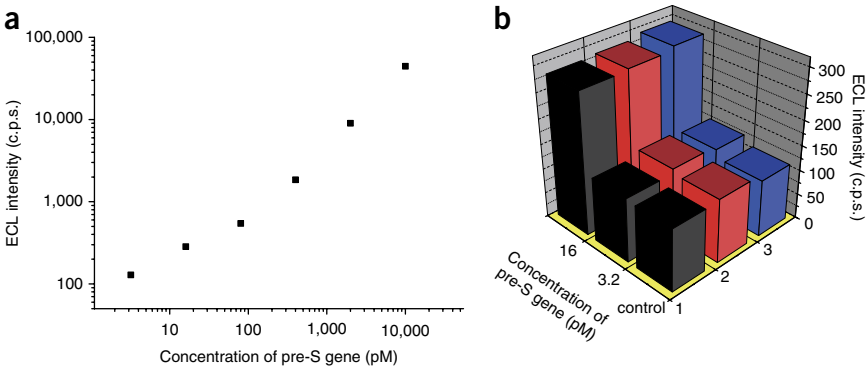


Figure 8 | Assay performance of nucleic acid detection by the current ECL technique. (**a**) Typical results for the ECL detection of pre-S gene via a sandwich hybridization at varying concentrations (3.2, 16, 80 and 400 pM, and 2 and 10 nM). The dynamic quantification range of this assay is 16 pM–10 nM. (**b**) The LOD is determined on the basis of the control measurement and the lowest nucleic acid concentrations. Each sample is measured three times in parallel. The LOD of the pre-S gene according to the 3σ method is 16 pM.

The absorption and emission spectra of $Ru(bpy)_3^{2+}$-NHS in aqueous solution are shown in **Figure 6**. Compared with $Ru(bpy)_3^{2+}$, the absorption maximum of $Ru(bpy)_3^{2+}$-NHS shifted from 453 to 458 nm and the emission maximum shifted from 613 to 645 nm. Note the red shift of the plasmon peak due to changes in the local refractive index and anisotropy after adding the carboxy and subsequently the NHS groups to the symmetrical bpy ligands.

Note that it is possible for two NHS molecules to attach to $Ru(bpy)_3^{2+}$, and therefore two nucleic acids probes might attach to a single molecule (it is almost impossible for two labeling antibodies to bind a single $Ru(bpy)_3^{2+}$ molecule owing to their far greater molecular weight). However, in our experiments, only a single oligonucleotide probe is attached to each $Ru(bpy)_3^{2+}$ molecule. The LC-MS chromatogram shows that uniform products were obtained. This completely 1:1 labeling ratio was the basis for subsequent quantitative measurements (**Supplementary Fig. 3**).

The developed procedure was demonstrated by using a CEA antigen. Sample data from the CEA antigen detection are shown in **Figure 7**. A weak ECL signal was detected in the control (water). The analytical sensitivity of the developed ECL magnetic beads was calculated based on the signal of the blank ± 3 s.d. The magnetic bead–based ECL method was able to detect 0.4 ng ml^{-1} of CEA, which corresponds to ~2.5 pM of CEA (estimated MW of 150,000). The bioanalysis applications using the current ECL assay were extended to include nucleic acids with sandwich-based hybridization. Examples of detecting the HBV pre-S gene with the current ECL protocol are provided in **Figure 8**. **Figure 8** shows the typical dependence of the ECL intensities on the varying target DNA concentration. The detection limit for DNA was estimated to be 16 pM (three times the signal-to-noise ratio). We noted that a higher sensitivity was acquired for protein detection, which may be due to multiple $Ru(bpy)_3^{2+}$ molecules binding to a single signal antibody, which amplifies the signal intensity.

Note: Any Supplementary Information and Source Data files are available in the online version of the paper.

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AUTHOR CONTRIBUTIONS X.Z. and D.X. conceived and designed the study, supervised the work and wrote the manuscript. X.Z., Y.L., Z.M., D.Z. and W.L. conducted the experiments and the data analysis. X.Z., W.L. and H.L. designed and prepared all figures.

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