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Reviews

Chemiluminescence Platforms in Immunoassay and DNA Analyses

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Chemiluminescent (CL) detection techniques for DNA assays and immunoassays have become very popular in recent years. This review discusses recent advances in those CL assays that have occurred over the last few years. In the monoplex assay section, different classes of CL labels including nanoparticle, DNAzyme, acridinium ester, enzyme and luminol-based CL assays are reviewed concerning the detection of DNAs and proteins. In the multiplex assay section, both spatial resolution and substrate zone-resolved techniques are discussed.

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1 Introduction

CL is the production of electromagnetic radiation by a chemical reaction. The CL intensity is directly proportional to the concentration of a limiting reactant involved in the CL reaction. CL has been exploited for its use in a wide range of applications due to its high sensitivity, wide calibration range, and suitability for miniaturization. Protein and DNA analyses have recently attracted much attention. Many different types of techniques, such as electrochemical, optical, radiochemical, and piezoelectronic methods have been applied for protein and DNA analyses. To provide an overview of CL detection assays for DNAs and proteins, we have summarized their essential techniques. Monoplex and multiplex assays are described, and their current reports are intended to provide an overview of the CL techniques currently employed in numerous laboratories.

2 Monoplex Assay

A monoplex assay aims to measure the presence of a single analyte in any given sample, in which nanoparticle, DNA enzyme, horseradish peroxidase (HRP), alkaline phosphatase (AP) and acridinium ester are commonly used as labels.

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2·1 Nanoparticle-based CL techniques

Nanoparticles offer excellent prospects for DNA detection and immunoassay, owing to their many attractive properties. Compared to existing labels, nanoparticles are more stable and cheaper, and easier to be purified. In addition, they indicate faster binding kinetics with high sensitivity and a high reaction rate for many types of monoplex assays, ranging from immunoassays to DNA detection.

2·1·1 Gold nanoparticles

Colloidal gold labels are ideal in biotechnological systems for several reasons. Firstly, they can be readily prepared in a wide range of sizes, from approximately 2 nm to above 100 nm. Secondly, the biochemical activity of the labeled biomolecules can be retained when colloidal gold is coupled to the biomolecules and, thirdly, colloidal gold labels can be easily visualized as dense structures within biological entities using transmission electron microscopy. As a biological tag, colloidal gold has been employed in several CL detection systems.

2·1·1·1 Gold nanoparticle-based CL immunoassay. The Au^{3+}-luminol reaction, in which 10^{-9} M Au^{3+} ions can be sensitively detected, is a classic CL reaction. Thousands of Au atoms are contained within the gold nanoparticles; for example, 2.3×10^5 Au atoms are theoretically contained in a 20-nm spherical gold particle and, consequently, picomolar detection limits can be attained by employing oxidative gold metal dissolution in acidic solutions. Our group[1,2] reported a sensitive CL immunoassay based on a colloidal gold label after oxidative gold metal dissolution (using 0.01 M HCl–0.5 M NaCl–0.5 mM Br_2), using a simple and sensitive luminol-CL reaction (Fig. 1).

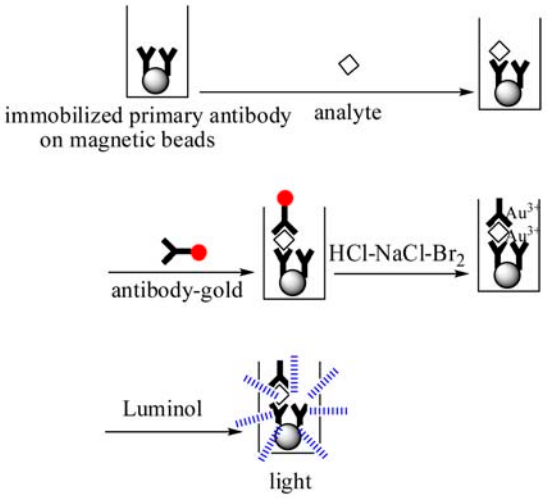

Fig. 1 Schematic representation of the noncompetitive CL immunoassay by using magnetic beads and a colloidal gold label (reproduced from Ref. 1 with permission).

Concentrations as low as 3.1×10^{-12} M human IgG could be determined, which was as sensitive as other immunoassay formats such as colloidal gold-based anodic stripping voltammetry (ASV), colorimetric enzyme-linked immunosorbent assay (ELISA) and time-resolved fluorescence. Hu *et al.*[3] reported a similar method for the detection of the ApxIV antibody of *Actinobacillus pleuropneumoniae* (APP) in pig serum samples. In this study, the authors compared the CL immunoassay with reference standards (IHA and ApxIV-ELISA), and found that the agreement of this CL immunoassay with two other methods was 94.2%. Li *et al.*[4] employed 2% HNO_3–3.4% HCl instead of 0.01 M HCl–0.5 M NaCl–0.5 mM Br_2 for the dissolution of gold nanoparticle, and developed a CL-detection method for an indirect measurement of goat-anti-human IgG with a detection limit of 1.5 ng/mL. To avoid the dissolution of gold under these conditions, Li *et al.*[5] further precipitated silver onto the surface of the gold nanoparticles, and employed an Ag^+-$K_2S_2O_8$-Mn^{2+}-H_3PO_4-luminol CL reaction after the dissolution of silver particles for the determination of IgG.

Nanoparticles have very interesting catalytic properties. A non-stripping CL immunoassay based on gold nanoparticles was recently reported by Wang *et al.*,[6] which was established according to the fact that the irregular gold nanoparticles could greatly enhance the CL intensity of the luminol–H_2O_2 system. Although this protocol avoided any strict stripping procedure, the synthesis of irregular nanoparticles was hard to control, requiring stirring for long periods of time at a controlled temperature of 40°C for 24 h, and the purging of oxygen. Duan *et al.*[7] found that luminol could react with $AgNO_3$ in the presence of normal spherical gold nanoparticles that were 8 – 68 nm in diameter to produce a strong and fast CL at 425 nm. In this reaction, $AgNO_3$ was reduced to Ag atoms by virtue of the catalysis of gold nanoparticles, which occurred on the surface of gold nanoparticles. Meanwhile, luminol was oxidized to the luminol radical, which further reacted with the dissolved oxygen, giving rise to light emission. By using this method, they developed a microplate compatible CL immunoassay for the determination of human IgG with a detection limit of 80 pM.

2·1·1·2 Gold nanoparticle-based CL DNA detection. By employing the gold nanoparticle-dissolution method, our group[8] presented a sensitive gold nanoparticle-based CL detection assay for the analysis of DNA hybridization. The proposed CL protocol was evaluated for a 30-base model DNA sequence, and the detection limit was found to be 0.01 pmol. Further signal amplification was achieved by the assembly of biotinylated colloidal gold onto the surface of streptavidin-coated polystyrene beads. Such amplified CL transduction allowed for the detection of DNA targets down to the 100 amol level.

2·1·2 Other nanoparticles

Compared with gold nanoparticles, Liu *et al.*[9] found that silver nanoparticle probes could be easily dissolved in a dilute HNO_3 solution. Ag^+ released from silver nanopaticles could be sensitively measured using an Ag^+-$K_2S_2O_8$-Mn^{2+}-H_3PO_4-luminol CL reaction. They developed a silver nanoparticle-based CL method for the ultrasensitive detection of DNA hybridization. This combination provided remarkable sensitivity to the CL method in which a large number of Ag^+ were released from each hybrid allowing for the detection of a specific sequence in DNA targets at levels as low as 5 fmol.

Ding *et al.*[10] reported on a method for the determination of short sequences of DNA based on a flow-injection CL system of luminol-H_2O_2-Cu^{2+}. The sensitivity of this method was improved with a preconcentration step of cupric ions using ASV processes. The CL intensity showed a wide linear relationship with the concentration of the target DNA, ranging from 2.0×10^{-12} to 1.0×10^{-10} M, and had a detection limit of 5.5×10^{-13} M for the targeted DNA.

2·2 DNAzyme-based CL techniques

The use of nucleic acids as catalytic labels (DNAzymes) for the amplified analysis of DNA has several advantages, such as lower nonspecific adsorption and fewer analyses. Hence, the preparation of DNA-based enzymes has attracted substantial research interest in the development of novel biocatalysts. A G-rich nucleic acid sequence binds hemin and yields a peroxidase mimicking DNAzyme that generates CL in the presence of H_2O_2 and luminol. Pavlov *et al.*[11,12] employed this DNAzyme as a catalytic label for the analysis of a 36-mer ssDNA target with a 17-mer capture probe immobilized on a Au surface. The detection limit was found to be 1×10^{-9} M. Our group[13] reported a CL method for the determination of 30-mer ssDNA targets by employing the DNAzyme as a label coupled with an efficient magnetic isolation step of the hybrid molecule. The CL intensity was found to be linearly proportional to the targeted DNA amount in a range of 0.02 – 2.0 pmol with a detection limit of 1×10^{-10} M. Li *et al.*[14] introduced a CL method with the DNAzyme for the recognition of thrombin, and for the determination of a specific DNA sequence using capillary electrophoresis. Weizmann *et al.*[15] reported on an autonomous DNA-based machine for the amplified determination of M13 phage ssDNA. As shown in Fig. 2, template 1 consists of three regions: region I is complementary to the primer; region II is complementary to a nucleic acid that, upon hybridization, yields a double strand that binds to a nicking endonuclease, N.BbvC IA; region III is complementary to the synthesized DNAzyme. The polymerase-induced reaction replicates the template and yields a double-stranded domain that associates with N.BbvC IA and results in a nicking of the replicated ssDNA. This cleavage generates a new site for the initiation of replication. Thus, the polymerase completes the replication of the DNAzyme, and the replication at the scission site displaces the previously synthesized DNAzyme. Subsequently, in the presence of hemin, the autonomous synthesis of the G-quadruplex DNAzyme structure is activated. The DNAzyme catalyzes the generation of CL in the presence of luminol-H_2O_2. The application of this DNA machine for the amplified analysis of M13 phage ssDNA was achieved by using a hairpin probe that

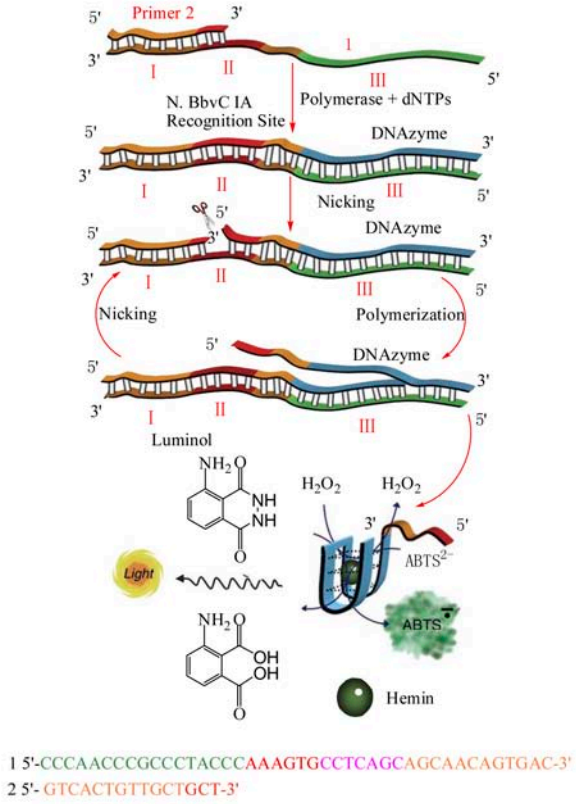

Fig. 2 Primer-induced autonomous synthesis of DNAzyme units on template DNA by using polymerase/dNTPs and a nicking enzyme as the biocatalyst (reproduced from Ref. 15 with permission).

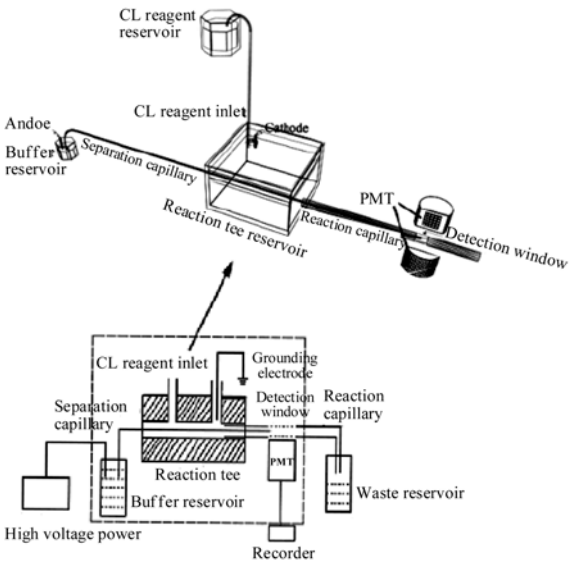

Fig. 3 Scheme of the CE-CL apparatus, which belongs to off-column coaxial flow mode systems. A reaction tee reservoir was made using plexiglass with a baffle in the middle. CL reagents were introduced through a hole in the baffle. The cathode was placed in the same hole. The PMT was fluted in a slot in the inside wall to allow the reaction capillary to go across its light-collection window. A 1-cm detection window was made at a suitable position on the reaction capillary. The detection window was placed just in front of the PMT's light-collection window. The outlet for the separation capillary was also placed before the window, where the analyte and the CL reaction takes place. The whole device was held in a light-tight box to exclude stray light (reproduced from Ref. 21 with permission).

is opened by the analyte DNA. The detection limit was 1×10^{-14} M with a signal-to-noise ratio of 2.8. Willner *et al.*[16] has extended the concept of DNA machines by using nucleic acid functionalized magnetic particles. Magnetic separation of the complexes leads to a low background signal and to a high signal-to-noise ratio of the transduced CL signal. Cheglakov *et al.*[17] designed a PCR-induced generation of DNAzymes for ultrasensitive detection of DNA. As low as 40 molecules of M13 phage DNA in a sample of 50 µL could be sensitively detected.

2·3 Acridinium ester-based CL techniques

Oates *et al.*[18] developed an HPLC one-site immunometric assay for the determination of L-thyroxine. A HPLC column and acridinium ester (AE) for postcolumn CL reaction were used in this method. Following 20 – 45 min incubation with AE-labeled Fab fragments, the CL signal could be obtained within 1.5 min after sample injection. About a 10^{-11} M level of thyroxine could be sensitively detected. Ahn *et al.*[19] reported on an automated CL immunoassay for the detection of 3-phenoxybenzoic acid (3-PBA) in the urine of exposed human populations by using AE as a CL probe. This assay improved the sensitivity with a low IC50 value of 0.1 µg/L for 3-PBA. The ACS: 180 analyzer used in this study could produce up to 130 results per hour. Hence, by employing a fully automated analyzer after a competitive incubation step, a high-throughput assay was developed for population-based human studies.

2·4 Enzyme-based CL techniques

2·4·1 HRP

HRP has been widely used for capillary electrophoresis (CE), flow injection, and fiber-optic sensors in many fields.

2·4·1·1 HRP in CE. CE has become one of the most powerful and conceptually simple separation techniques for the analysis of complex mixtures. CE immunoassays combine the effective separation power of CE with the ligand specificity of immunoassays, and have been proven to be a powerful technique for the separation and analysis of biological compounds. CE coupled with CL detection has certain advantages in combining the high separation efficiency of CE with the high sensitivity of CL. Hence, CE-CL combined with immunoassay would be a powerful technique for bioanalysis.

Tsukagoshi *et al.*[20] designed a simple and convenient CL detection cell for CE. The cell, to which only a fused-silica capillary and a grounding electrode were inserted, eliminated the need for an optical fiber. Luminol CL could be sensitively detected by using the detection cell with a detection limit of 2.5×10^{-10} M. By employing this new cell, a preliminary immunoassay with HRP as a label was also developed. Wang *et al.*[21] successfully developed a CE-immunoassay method coupled with enhanced CL for the determination of bone morphogenic protein-2 (BMP-2) in rat vascular smooth muscle (Fig. 3). The CL detection was based on the catalysis of HRP and HRP-Ab_2-mAb-BMP-2 complexes for the luminol-H_2O_2 reaction. HRP-Ab_2-mAb-BMP-2 complexes could be separated from free HRP in 3 min, and the detection limit of BMP-2 was 6.2 pM (75 zmol). Ji *et al.*[22] reported on a CE immunoassay coupled with a CL reaction for the determination of clenbuterol (CLB) based on a competitive reaction between HRP-labeled CLB and free CLB. Under the optimal conditions, the linear range for CLB was 5.0 – 40 nM with a detection limit of 1.2 nM. In addition, medroxyprogesterone acetate (MPA), a tumor marker, carbohydrate antigen 15-3 (CA15-3), and a tumor Ag, CA 125 have been detected by the CE immunoassay coupled with a HRP label.[23–25]

The sensitivity of CL makes it very promising for microchip CE-detection systems. Tsukagoshi *et al.*[26] developed a micro total analysis system (μ-TAS) incorporating the CL reaction of isoluminol isothiocyanato-microperoxidase-hydrogen peroxide. The total analysis could be completed within 2 min. Human serum albumin (HSA) and immunosuppressive acidic protein (IAP) were determined in this study, and were found to have a detection limit of 1.0×10^{-7} M.

2·4·1·2 HRP in flow injection. Flow-injection immunoassays were introduced by Ruzicka and Hansen in 1975, and many analytical flow-injection immunoassay techniques have since been developed. However, classical technique of flow-injection immunoassays failed to satisfy the requirements for trace analysis due to its low sensitivity. Hence, the approach of flow-injection CL immunoassay (FICLIA) has been performed by combining sensitive CL analysis with the techniques of flow-injection analysis and immunoassay.

Luo *et al.*[27] developed a FICLIA method for the determination of rabbit IgG with two CL enhancers, *p*-phenylphenol (PPP) and sodium tetraphenylborate (NaTPB). The total analysis time required for one cycle was as short as 8 min, and the detection limit was 0.68 fmol. Wang *et al.*[28] reported a FICLIA method based on a solid-phase immunoassay format for the determination of 17β-estradiol (E_2) by using *p*-iodophenol (PIP) as a CL enhancer. An E_2-OVA immobilized immunoaffinity column was inserted in the flow system to trap any unbound HRP-labeled anti-E_2 antibody after an offline incubation of E_2 with the HRP-labeled anti-E_2 antibody. The CL intensity was linearly related to the concentration of E_2 in the range of 10.0 – 1000.0 ng/mL with a detection limit of 3.0 ng/mL. Lin *et al.*[29] developed a FICLIA method for the determination of carcinoembryonic antigen (CEA). Jain *et al.*[30] modified protein G on to a monolithic porous poly(glycidyl methacrylate-co-trimethylolpropane trimethacrylate) polymer disc placed in a flow cell right in front of a PMT. By employing a HRP-catalyzed CL reaction using luminol-H_2O_2-PIP, 33 ng/L of triazine could be sensitively detected. Surugiu *et al.*[31] developed a FICLIA for the detection of the herbicide 2,4-dichlorophenoxyacetic acid by using imprinted polymer that was modified to the inner capillary wall of a glass capillary tube. The target analyte was labeled with the enzyme tobacco peroxidase and, in a competitive format, the bound fraction of the conjugate was quantified by using a photomultiplier tube or a CCD camera.

Sequential injection analysis (SIA) is also suitable as an analytical method for ELISA procedures, because washing, separation of the bound-free antibody and the addition of reagent solutions can be automated using a computer-controlled syringe pump and a switching valve. Magnetic nanoparticles, as special biomolecule-immobilizing carriers, offer a promising alternative to conventional methodologies. Ruzicka *et al.*[32] proposed a beads injection technique combined with SIA. Soh *et al.*[33] proposed a CL immunoassay using magnetic microbeads in a SIA system equipped with a samarium-cobalt magnet for a highly sensitive and rapid determination of carp vitellogenin (Vg). As shown in Fig. 4, a microchannel flow cell equipped with a CL detector and a magnet was used in this work. The introduction, trapping and release of the magnetic beads in the flow cell were controlled by the magnet and the flow of the carrier solution. In a sandwich-type immunoassay, 2 ng/mL of carp Vg could be sensitively detected. Zhang *et al.*[34] also developed a SIA-CL method for the determination of linear alkylbenzene sulfonates (LAS). In a competitive immunoassay, a 25-ppb level of LAS could be sensitively detected within 15 min.

2·4·1·3 HRP in fiber-optic sensors. Cholera toxin B was covalently immobilized onto the fiber-optic silica surface through a photochemical reaction, as described by Leshem *et al.*[35] With the HPR-labeled secondary antibody used as a marker, the cholera toxin in rabbit serum could be sensitively detected. Salama *et al.*[36] fabricated an optical fiber-based CL immunosensor for the detection of native autoimmune response to GIPC-1. The results of clinical sera tests showed that the fiber-optic immunosensor may serve as a diagnostic tool for screening ovarian and breast cancer at an early stage.

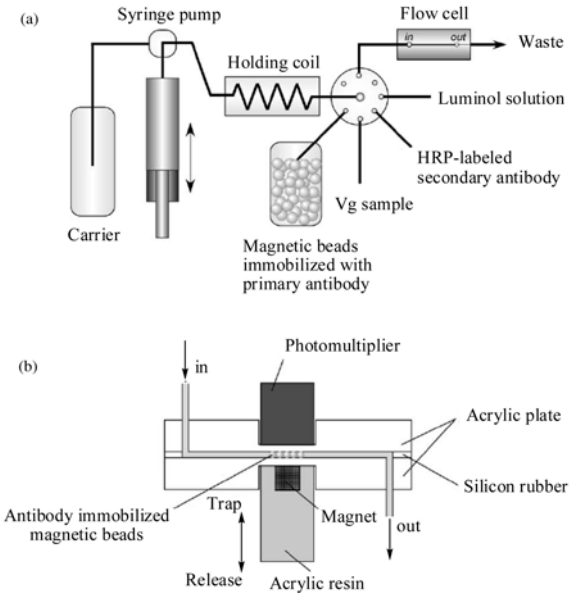

Fig. 4 Sequential injection analysis system (a) and a flow cell (b) (reproduced from Ref. 33 with permission).

Instead of silica surfaces, the fiber-optic electro-conductive surface was obtained by the deposition of a thin layer of indium tin oxide (ITO), on which monomers of pyrrolebenzophenone were electro-polymerized. The photochemically modified fiber optics was tested as an immunosensor for the detection of the anti-E_2 protein antibody.[37] In addition, the inactivated Ebola virus was immobilized to the tip of an optical fiber by using a photoelectrochemical method.[38] Finally, by using the HPR-H_2O_2-luminol CL reaction, the fiber-optic immunosensor could sensitively detect a low titer of 1:960000 and 1:1000000 for subtypes in Zaire and Sudan, respectively. Herrmann *et al.*[39] reported on a CL anti-West Nile virus (WNV) IgG optical fiber immunoassay based on the immobilization of inactivated virions onto a fiber glass surface previously functionalized by using argon-phase silanization. Compared with colorimetric ELISA and CL-ELISA, the optical fiber ELISA showed a much lower detection limit.

2·4·1·4 Other application based on HRP label. Surugiu *et al.*[40] developed a highly sensitive CL-ELISA for the determination of 2,4-dichlorophenoxyacetic acid, based on disposable thin glass capillaries. The monoclonal antibodies were immobilized onto the inner face of glass capillaries, where covalently attached activated carboxymethyl dextran of different sizes had been attached. In a competitive detection model, 2 pg/mL of 2,4-dichlorophenoxyacetic acid could be sensitively detected using HRP as a label. Fumonisin B1 (FB1) in food samples can also be determined using an ELISA-CL method, as described by Quan *et al.*[41] Roda *et al.*[42] developed a sandwich-type ELISA-CL method for the detection of Cry1Ab protein in maize samples following a simple extraction procedure. With HRP as a label, the detection limit of the CL immunoassay

using 96-well and 384-well microtiter plates were 3 and 5 pg/mL, respectively. Aslan *et al.*[43] discovered that CL reactions could be accelerated with low-power microwaves. Based on this fact, Previte *et al.*[44] developed microwave triggered metal-enhanced CL (MT-MEC) method for protein detection. With the MT-MEC approach, 150 fmol of target protein could be detected in less than 1 min. Stratis-Cullum *et al.*[45] reported an intensified biochip system for the detection of *Bacillus globigii* spores. The biochip detection system consisted of an intensifier and an integrated circuit-photosensing array chip. The CL light originating from the sample chambers could be amplified prior to detection by each one of the 16 elements of the photosensing chip. Hence, as low as 1×10^5 *Bacillus globigii* spores could be detected by using this biochip system.

Interferon alpha (α-IFN) in human serum was determined based on ELISA with a TCPO-H_2O_2-glyoxaline-HRP CL reaction.[46] Because of the high-quantity yield, 0.8 pg/mL of α-IFN could be sensitively detected. Based on the TCPO-HRP CL reaction, recombinant human interleukin 6 (rHu IL-6) and β-human chorionic gonadotropin (β-HCG) were also determined with a detection limit of 0.5 pg/mL and 3 mIU/mL, respectively.[47]

Sattler *et al.*[48] designed a CL microplate assay with the Fpg-based 4D assay for the detection of oxidative base damage on DNA. Weizmann *et al.*[49] demonstrated that the rotation of the nucleic acid-functionalized magnetic particles together with the naphthoquinone-modified magnetic particles yielded an enhanced CL in the presence of HRP bioconjugates and luminol. The enhanced CL and amplification of rotating magnetic particles led to a sensitive detection of M13 phage DNA with a detection limit of 1×10^{-14} M. Meanwhile, Patolsky *et al.*[50] presented a magnetically amplified DNA analysis (MADA) of M13 phage DNA and of single-base mismatches (Fig. 5). With the amplification of polymerase-induced incorporation of labels using thermally controlled replication-cycles and rotating the magnetic particles, as low as 8.3×10^{-18} M of M13 phage DNA could be sensitively detected. Wu *et al.*[51] proposed a CL assay, which made use of differences in the thermal stability between perfectly and imperfectly matched hybrids, for the identification of cytochrome P450 1A1 (CYP1A1) polymophisms. Ambretti *et al.*[52] developed a miniaturized polymerase-chain reaction (PCR) CL-EIA based on a 384-well microtiter plate for the detection and typing of oncogenic high and low-risk human papillomavirus (HPV) in genital lesions. The digoxigenin-labeled PCR product was hybridized with biotin-labeled oligoprobes immobilized on the streptavidin-coated wells of a 384-well microtiter plate, and then quantified by means of a HRP-labeled anti-digoxigenin antibody. The method provided semi-quantitative information on the viral load, with a limit of detection of 10 – 50 DNA copies for HPV.

2·4·2 AP

2·4·2·1 AP in immunoassay. Tanaka *et al.*[53] prepared bacterial magnetic particle (BMP) complexes of protein A, and found that they were quite monodisperse after binding of the antibody. Based on a fully automated sandwich immunoassay, human insulin could be sensitively detected by employing these antibody-protein A-BMP complexes and with AP-conjugated secondary antibodies. Matsunaga *et al.*[54] assembled nano-magnetic beads onto polystyrene microbeads, named "Beads on Beads". Approximately 2000 magnetic particles could be uniformly assembled on a single microbead without aggregation. Furthermore, as low as 1.48 ng/mL of prostate-specific antigens could be sensitively detected in a fully automated detection system. Progesterone in human serum was determined by using CL-EIA with the highly sensitive AMPPD-AP reaction by Ren *et al.*[55]

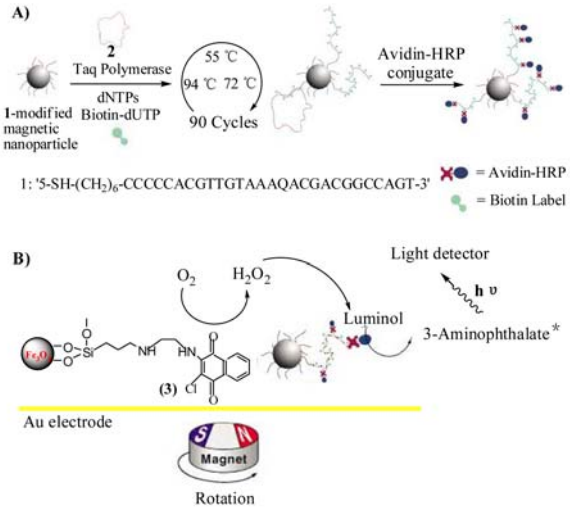

Fig. 5 Amplified detection of M13 phage DNA by multilabeled rotating magnetic particles: A) labeling of the nucleic acid replica on the particles with biotin units by using thermal cycles, B) generation of amplified CL upon rotation of the functionalized magnetic particles on the electrode surfaces (reproduced from Ref. 50 with permission).

2·4·2·2 AP used in DNA analysis. Chen *et al.*[56] developed a CL dot-blot hybridization assay for the determination of Epstein-Barr virus (EBV) DNA by using AP as a label. Approximately 4 pg of purified EBV-DNA target could be detected. Based on single base extension (SBE), magnetic separation and CL, Kakihara *et al.*[57] developed a method for typing single nucleotide polymorphisms (SNPs), named MagSNiPer. SBE was performed with a biotinylated primer, whose 3′-terminus was contiguous to the SNP site with a tag-labeled ddNTP. Then, the primers were captured by magnetic-coated beads with streptavidin and reacted with an AP-labeled anti-tag antibody. After removal of excess conjugates by magnetic separation, SNP typing was performed by measuring the CL. Using MagSNiPer, as little as 10^{-17} mol of template DNA could be performed by SNP typing. Bonvicini *et al.*[58] reported a peptide nucleic acid (PNA)-based *in situ* hybridization assay with CL detection for ultrasensitive localization and the quantitative detection of parvovirus B19 nucleic acids in single infected cells at various times post-infection. Li *et al.*[59] developed a CL method for the sensitive detection of DNA hybridization and SNPs with target-primed rolling circle amplification (RCA). With the RCA-based method, as low as 3.6 pM target DNA could be detected. Wild-type DNA and the one-base mutant DNA could be differentiated with high selectivity through this RCA reaction.

The molecular beacon, first developed by Tyagi and Kramer[60] in 1996 is one of the most important molecules that possess a single-stranded oligonucleotide with a stem-loop structure, in which the 5′ and 3′ ends are self-complementary, bringing a fluorophore and a quencher into close proximity. Bockisch *et al.*[61] designed a signal-on sensor for the detection of DNA targets utilizing stem-loop structured oligodeoxynucleotide probes. The principle is depicted in Fig. 6. Upon target hybridization, the stem-loop structure is broken up and the probe switches to a linear conformation, thereby restoring the accessibility of the label. Employing AP as a label, as low as 8 fmol of target DNA could be detected.

2·4·3 Other kinds of enzymes

The cloned enzyme-donor immunoassay is known as a homogeneous immunoassay that employs complementation

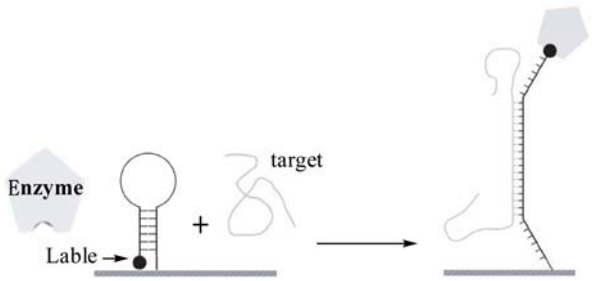

Fig. 6 Principle of the enzymatic conformational switch system (reproduced from Ref. 61with permission).

within the β-galactosidase system. Enzyme donor (ED) and enzyme acceptor (EA) are two inactive fragments that are genetically engineered from β-galactosidase. The association of the ED and EA fragments results in the formation of an active enzyme, which reacts with the substrate to generate a CL signal. Yang *et al.*[62] proposed three methods for the determination of valproic acid. They study a bioluminescent system by employing D-luciferin-*O*-β-galactopyranoside as a substrate, because of the short assay time, small volumes of the sample and reagents, simple handling, and relatively low expense.

Sakharov *et al.*[63] developed a sandwich CL-ELISA for the determination of mouse IgG using soybean peroxidase (SbP) as a label. By comparing the parameters of the immunoassays, they found that the assay with SbP conjugate was more sensitive, and its linear range was a broader.

2·5 Luminol as direct CL label

Lu *et al.*[64] reported a luminol-enhanced CL reaction between basic aqueous H_2O_2 and acetonitrile. The maximum CL peak was reached after approximately 1.5 min in the presence of 25% acetonitrile and as low as 16 fmol isoluminol-labeled streptavidin could be easily detected. Based on this fact, Lau *et al.*[65] coupled this new glow-type reaction to CL imaging for the detection of protein and DNA on a microplate and membrane. By employing isoluminol as a label, 10000-fold dilution of biotinylated IgG and 20 ng of DNA could be easily visualized on the microplate and membrane, respectively.

Luminol was heavily immobilized on digoxin by employing BSA as a carrier. Based on the luminol-BSA-digoxin conjugate, Qi *et al.*[66] proposed two immunoassay formats, direct homogeneous immunoassay and competitive immunoassay, to determine anti-digoxin antibody and digoxin, respectively. The detection limit of digoxin was 2.8×10^{-10} g/mL. Zhang *et al.*[67] synthesized luminol- or isoluminol-containing dextran T500 CL probes. The synthesized CL compounds were used as CL-labeling macromolecular probes for the sensitive CL imaging of a cytochrome P450 (CYP) protein on a poly(vinylidene difluoride) (PVDF) membrane. At least 190 fmol of CYP3A4 on a PVDF membrane could be selectively detected.

2·6 Electrochemiluminescence (ECL)

ECL is a mean for converting electrochemical energy into radioactive energy at the surface of an electrode *via* an applied potential. Luminescent signals can be obtained from the excited states of an ECL luminophore during the electrochemical reaction. Because of the high sensitivity, wide dynamic range, and selectivity, ECL methods have been widely used in bioanalytical systems.

2·6·1 Tris(2,2-bipyridyl)ruthenium(II) ($Ru(bpy)_3^{2+}$) labels

Among many organic and inorganic ECL systems, ECL based on an inorganic compound, $Ru(bpy)_3^{2+}$ has received considerable attention.

2·6·1·1 $Ru(bpy)_3^{2+}$ in immunoassay. By using $Ru(bpy)_3^{2+}$ as a label and tripropylamine (TPA) as a coreactant, Miao *et al.*[68] presented an ECL method for the determination of C-reactive protein (CRP) by immobilizations on Au electrodes. Zhang *et al.*[69] developed a homogeneous ECL immunoassay for the determination of an anti-digoxin antibody and digoxin hapten by employing $Ru(bpy)_2$(dcbpy)NHS as an ECL label and BSA as a carrier protein. For further signal amplification, Miao *et al.*[70] utilized polystyrene beads (PSB) as the carrier of a large number of $Ru(bpy)_3^{2+}$. The sensitivity for the analyte CRP was about 100-fold improved compared to the former method. Furthermore, Zhan *et al.*[71] prepared biotin-modified liposomes containing $Ru(bpy)_3^{2+}$ as an ECL tag using a lipid extrusion method, and developed a sandwich-type immunoassay for the determination of human CRP. The procedure is depicted in Fig. 7. The detection limit for human CRP was 100 ng/mL. Egashira *et al.*[72] also fabricated an immunoliposome-encapsulating Ru-complex with two aminobutyl moieties to detect the presence of the hemagglutinin molecule, which plays an important role in influenza virus infection. Under the optimum conditions, the hemagglutinin molecules could be determined in a concentration range from 3×10^{-13} to 4×10^{-11} g/mL. Wang *et al.*[73,74] prepared $Ru(bpy)_3^{2+}$ doped silica nanoparticles. With a core-shell structure, $Ru(bpy)_3^{2+}$ molecules incorporated into a silica matrix, could be prevented from entering the surrounding environment and the ECL intensity could be enhanced due to an increase in the number of $Ru(bpy)_3^{2+}$ doped per nanoparticle. By employing the $Ru(bpy)_3^{2+}$-doped silica nanoparticle as a label, a sensitive ECL assay was developed for the detection of thrombin based on target protein-induced strand displacement of the DNA probe. The detection limit was down to 1.0 fmol, since silica nanoparticles containing a large number of $Ru(bpy)_3^{2+}$ molecules were labeled on the DNA probe.

2·6·1·2 $Ru(bpy)_3^{2+}$ in DNA detection. Miao *et al.*[68] developed an ECL detection method with TPA as a coreactant for the determination of a 23-mer ssDNA target. In order to improve the detection sensitivity, they developed an ultrasensitive ECL-detection method for the determination of the target DNA by employing PSB as a carrier of the ECL labels of $Ru(bpy)_3$-$[B(C_6F_5)_4]_2$.[75] The integrated ECL intensity was found to be linearly proportional to the concentration of target DNA in a range of 1.0 fmol to 10 nmol under the optimized conditions. A very low concentration of target DNA, 1.0 fmol, could be detected and distinguished from 2-bp-mismatch-ssDNA and non-complementary ssDNA. By employing $Ru(bpy)_2$-(dcbpy)NHS as an ECL label and gold nanoparticle as a carrier, Wang *et al.*[76] developed an ECL method for the detection of DNA hybridization of a 12-mer target DNA with a 18-mer DNA probe based on gold nanoparticles carrying multiple ECL probes. The ECL intensity was linearly related to the concentration of target DNA in the range of 1.0×10^{-11} to 1.0×10^{-8} M, and the detection limit was 5.0×10^{-12} M. Chang *et al.*[77] combined $Ru(bpy)_3^{2+}$-doped silica nanoparticles as DNA tags with an ECL method for the detection a 24-mer ssDNA target by amplifying the ECL signal through boosting up the amount of $Ru(bpy)_3^{2+}$ labeled onto DNA probes. Li *et al.*[78] developed an ECL detection method for the determination of a 42-mer ssDNA target by loading an ssDNA and ruthenium complex on the surface of single-walled carbon-nanotubes (SWNT). The ECL intensity was linearly related to the concentration of the target DNA in the range from 2.4×10^{-14} to 1.7×10^{-12} M with a detection limit of 9.0×10^{-15} M. Zhou

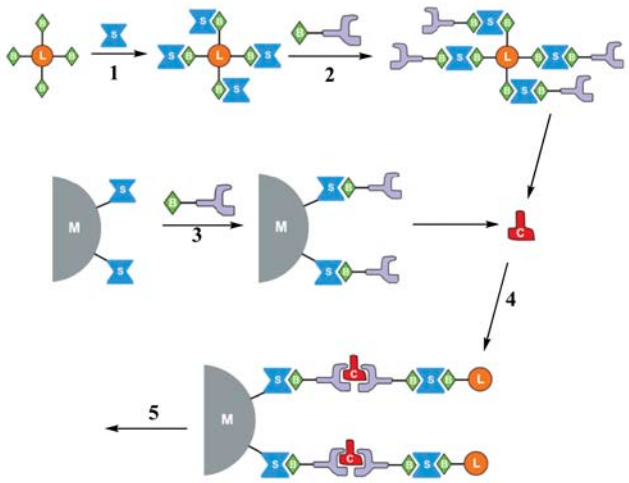

Fig. 7 Immunoassay of human C-reactive protein. Species and materials involved: B, biotin; L, liposomes; S, streptavidin; C, C-reactive protein (analyte); and M, magnetic microbeads. Experimental steps: (1) coating of streptavidin on liposome surface; (2) coupling biotinylated anti-human CRP to liposomes; (3) decoration of streptavidin-coated magnetic beads with biotinylated anti-human CRP; (4) formation of anti CRP (liposomes)-CRP-anti CRP (magnetic beads) sandwich structure; (5) quantification of CRP by detecting ECL signal based on $Ru(bpy)_3^{2+}$ and tri-*n*-propylamine (reproduced from Ref. 71 with permission).

et al.[79] developed a telomerase activity assay that combined the modified telomere repeat-amplification protocol (TRAP) with a magnetic beads-based ECL-detection system. The ECL intensity was linear over the range of 1-1000 HeLa cell equivalents. Zhang *et al.*[80] developed a highly selective ECL biosensor using hairpin DNA as a recognition element. The ECL intensity of the DNA biosensor generated a switch-off mode, which decreased with an increase in the concentration of target DNA, and a detection limit of 9×10^{-11} M complementary target DNA was achieved. Single-mismatched target ssDNA was effectively discriminated from complementary target ssDNA.

2·6·2 Other ECL labels

Isothiocyanate derivatives of several Tb(III) chelates can be used as efficient ECL labels in bioaffinity assays. Ala-Kleme *et al.*[81] developed a sandwich-type immunometric heterogeneous ECL determination of hCRP based on the detection of Tb(III) chelate labels by cathodic pulse polarization of disposable, thin insulating film-coated silicon electrodes. Helin *et al.*[82] introduced an ECL immunoassay for a human thyroid-stimulating hormone (hTSH) on oxide-coated *n*-silicon electrodes by utilizing the Tb(III) chelate as a label. The calibration curve of the immunometric hTSH assay was found to be linear over a wide range of hTSH concentration, and the detection limit of the hTSH was below 4 pM.

A derivative of luminol, ABEI is a favorable ECL marker for bioanalytical detection, since there is no significant reduction in the luminescence activity when it is labeled to biological substances. Qi *et al.*[83] developed a homogeneous ECL immunoassay for the determination of hIgG using ABEI at gold nanoparticles modified with a paraffin-impregnated graphite electrode (PIGE). The integral ECL intensity was linearly related to the concentration of hIgG antigen from 3.0×10^{-11} to 1.0×10^{-9} g/mL with a detection limit of 1×10^{-11} g/mL. Yang *et al.*[84] reported on an ECL detection system for DNA hybridization of a 24-mer ssDNA target with a 24-mer probe DNA by employing ABEI as a marker. The intensity of the ECL was linearly related to the concentration of the complementary sequence in the range of 9.6×10^{-11} – 9.6×10^{-8} M with a detection limit of 3.0×10^{-11} M.

CdS nanocrystals could be electrochemically reduced during a potential scan, and reacted with the coreactant, $S_2O_8^{2-}$, to generate strong ECL in aqueous solution. Based on the ECL, Jie *et al.*[85] fabricated an ECL biosensor by a self-assembly technique for the determination of low-density lipoprotein.

2·7 Bioluminescence

Ca^{2+}-regulated photoproteins of luminous marine coelenterates, such as aequorin and obelin, are stable complexes of apophotoprotein and peroxycoelenterazine. Upon Ca^{2+} addition, the proteins undergo conformational changes caused by coelenterazine-intramolecular oxidation, resulting in blue light emission. The application of the Ca^{2+}-regulated photoproteins aequorin and obelin as labels in enzyme-linked immunoassays has been shown to have many advantages. Mirasoli *et al.*[86] developed a competitive immunoassay for the determination of cortisol using a recombinant mutant of aequorin as a label. Cortisol can be detected down to 0.1 ng in 100 µL of a saliva sample using this assay, without any sample pretreatment. Furthermore, an aequorin-DNA conjugate was prepared by using either homobifunctional or heterobifunctional cross-linking reagents by Glynou *et al.*[87] By comparing the two conjugations, they found that the conjugates prepared with the heterobifunctional-reagent protocol showed about 2-times higher signal/black ratios than those prepared using the homobifunctional cross-linker. By using the prepared conjugates, a rapid bioluminometric hybridization assay was developed, and the analytical range extended from 2 to 2000 pmol/L of the target DNA. Mavropoulou *et al.*[88] developed a microtiter well-based bioluminometric hybridization-assay for the quantification of genetically modified organisms, known as high-throughput double quantitative competitive PCR. The hybrids were determined by using streptavidin conjugated to the photoprotein aequorin. The overall procedure, including PCR and hybridization assay, was complete in 2.5 h. The limit of quantification for the 35S promoter was 24 copies. Recombinant obelin, derived from the luminous marine hydroid *Obelia longissima*, was used as a label for the determination of thyroid hormones, namely human thyrotropin (hTSH) and two forms of thyroxine (T_4) by Frank *et al.*[89] The hepatitis B virus (HbsAg) and the bacteria *Escherichia coli* and *Shigella sonnei* lipopolysaccharide (LPS) were also determined using obelin as a label.[90]

Luciferases are inactivated upon conjugation to DNA probes or antibodies. To avoid inactivation problems, Verhaegen *et al.*[91] designed an overexpression system that produced *in vivo* biotinylated Gaussia luciferase (GLuc). The purified GLuc could be detected down to 1 amol with a signal-to-background ratio of 2. By using the Gluc as a label, a microtiter well-based DNA hybridization assay was developed for the determination of a 233-bp target DNA generated by RT-PCR of prostate-specific antigen mRNA. The analytical range extended from 1.6 to 800 pmol/L of target DNA.

2·8 Label-free

There are potential advantages, in terms of simplicity and speed for detecting the hybridization step directly without using any labels, and thus numerous applications for the label-free detection of ssDNA and protein have started to emerge recently.

2·8·1 DNA determination

3,4,5-Trimethoxyphenylglyoxal (TMPG) is a special CL reagent. TMPG reacted with the guanine nucleobases of DNA strands to form an unstable CL intermediate for the generation

of light.[92] Telomeres are specific DNA structures at the ends of chromosomes, which consist of TTAGGG repeat-units in vertebrate. Hence, our group[93] developed a label-free detection of telomeres based on a CL measurement of guanine bases in nucleic acids with an efficient magnetic isolation of the hybrid. To further improve the sensitivity, our group[94] also utilized dT_{20}-terminated magnetic beads and an A_{20}-modified capture DNA probe for the label-free CL detection of telomeres. Under the optimized conditions, as low as 5×10^{-9} mol/L target DNA could be sensitively detected.

2·8·2 Protein determination

Telomerase is a potential cancer marker. Tonooka *et al.*[95] developed a telomerase assay based on the TMPG reaction. They employed partially mismatched primers in order to prevent the formation of the short length products. The final PCR products could be easily isolated from the excess primers and dNTP in the reaction mixture by an ultra-filtration membrane. The isolated final PCR products were simply and rapidly detected by the TMPG reaction. The CL intensity was linear for the telomerase activity in 100 to 10000 cells.

Nuclear factor-κB (NF-κB), a sequence-specific DNA-binding protein, is a ubiquitous redox-sensitive transcription factor that responds to pro-inflammation caused by cytokines and oxidative stress. Tonooka *et al.*[96] developed a facile method for the quantitative and sensitive determination of NF-κB by the TMPG-CL reaction. As shown in Fig. 8, the bound and unbound dsDNAs were readily separated with a centrifugal filter that was able to remove molecular sizes lower than 100000 Da. The bound dsDNA, which was dissociated with sodium dodecyl sulfate (SDS), was filtrated. The amount of the DNA in the filtrate was then detected by the CL reaction with TMPG, and as low as 5 nM NF-κB could be detected.

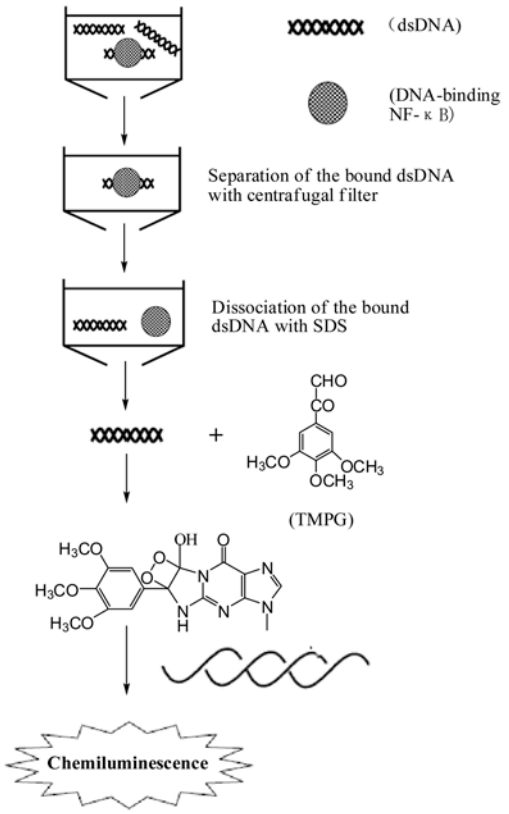

Fig. 8 Schematic protocol for the assay of NF-κB DNA-binding by means of the CL reaction with TMPG (reproduced from Ref. 96 with permission).

3 Multiplex Assay

Despite all of the unique advantages and the plethora of applications, most current CL DNA-detection assays and CL immunoassays are only able to detect a single target DNA or protein. The challenge currently lies in the development of new biotechnologies that can permit the simultaneous determination of multiple DNA or protein targets within a single sample. Two dominant modes have been adopted to realize the goal of multiplex analysis. The first mode is the spatial resolution of different reaction areas using a universal label. The second mode is the multilabel mode performed using different labels.

3·1 Spatial resolution

3·1·1 Poly(*N*-isopropylacrylamide) (PNIP)-based multiplex analysis

PNIP is known to aggregate and precipitate out of water when the temperature is raised above the lower critical solution temperature (LCST) of 31°C; thus, it can be separated from the supernatant by centrifugation. In addition, magnetic beads can also be separated from PNIP by magnetic force, since the temperature is lower than LCST. By using PNIP and magnetic beads as carriers, our group[97] developed a sequential dual protein immunoassay with HRP catalyzed CL detection. There was no cross-reaction, and the detection limits were 1.5 and 2.0 ng/mL for IgG and IgA, respectively.

Recently, our group developed another label-free CL platform for the detection of three DNA targets, by taking good advantage of the unique properties of three water-soluble capture DNA immobilizing carriers, namely magnetic beads, polystyrene beads and PNIP.[98] Magnetic beads and polystyrene beads can be separated from PNIP by centrifugation at a lower temperature than LCST, and then magnetic beads can be simply separated from polystyrene beads by magnetic force. The principle is depicted in Fig. 9. Three targets could be simultaneously determined in a single sample without any obvious cross reaction, and the detection limit was 10 – 15 fmol for each target. To further improve the sensitivity, we also designed another HRP-label-based multiplexed assays for the simultaneous detection and quantification of three DNA targets associated with the breast cancer gene (BRCA1). Streptavidin-HRP polymer, which has a high number of HRP units, was used in this work, and thus the detection sensitivity was significantly higher than that in the previous label-free systems.[99]

3·1·2 Channel-resolved approach

Fu *et al.*[100] fabricated a flow-through two-channel immunosensing system using a polyethersulfone membrane as a support for the immobilization of α-fetoprotein (AFP) and carcinoembryonic antigen (CEA)-capture antibodies. With the aid of an optical shutter, the CL signals from the two channels were sequentially collected to detect the levels of AFP and CEA. The whole assay procedure could be completed within 32 min, and the detection limit were 1.5 and 0.25 ng/mL for AFP and CEA, respectively. By using paramagnetic microspheres (PMs) instead of a polyethersulfone memberane, they developed another channel resolved strategy. AFP, CEA and carcinoma antigen 125 (CA 125) as model analytes, were firstly incubated in mixtures of capture antibodies-immobilized PMs, and corresponding AP-labeled antibodies being stirred and pumped into three parallel detection channels, the PMs were simultaneously captured by a magnet, and the CL signals from the three channels were then sequentially collected with the aid

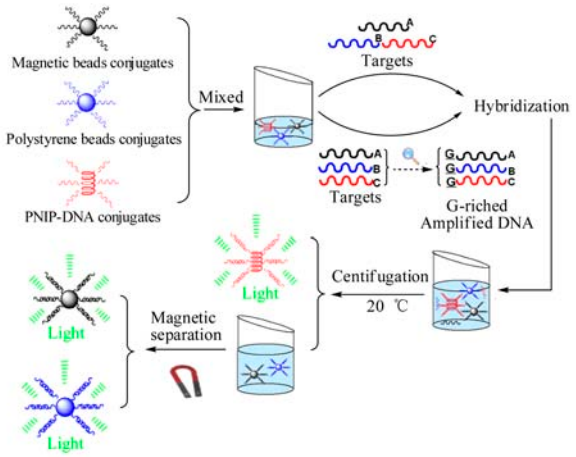

Fig. 9 Multi-target label-free CL detection protocol based on different carrier vehicles (reproduced from Ref. 98 with permission).

of optical shutters to perform quantitative detection.[101] AFP, CEA and CA 125 could be rapidly assayed in the ranges of 1.0 – 40 μg/L, 0.20 – 30 μg/L and 1.0 – 50 kU/L with detection limits of 0.60 μg/L, 0.080 μg/L and 0.70 kU/L, respectively.

3·1·3 Microfluidic biochip

Microarray technologies enable the integration of a large number of different antibodies within one test. Knecht *et al.*[102] presented a parallel affinity sensor array (PASA) for the rapid automated analysis of 10 antibiotics in milk, by immobilizing protein conjugates of the haptens on disposable chips. The CL signal was recorded with a sensitive CCD camera. All liquid handling and sample processing were fully automated, and one analysis was carried out in milk within less than 5 min. Heyries *et al.*[103] developed a fully automated and integrated microfluidic device for allergy agnosis. Three different proteins (β-lactoglobulin, peanut lectin and human IgG) were immobilized *via* a macromolecules of polydimethylsiloxane elastomer, and used as a capturing agent for the detection of specific antibodies. Under the optimized conditions, only 6-min of incubation time was needed for the detection of three specific antibodies at the pmol level.

3·2 Substrate zone-resolved technique

Laios *et al.*[104] discovered that firefly and Renilla luciferase cDNAs could be transcribed and translated simultaneously and independently over a broad range of concentrations in the same expression mixture. Based on this fact, they developed an expression hybridization assay that combines firefly-luciferase DNA with Renilla-luciferase DNA as labels for the simultaneous determination of two target DNA sequences. Fu *et al.*[105] designed a strategy for a substrate zone-resolved multianalyte-immunoassay for the detection of CA125 and CEA by employing HRP and AP as labels, respectively. Under the optimal conditions, CA 125 and CEA could be assayed in the ranges of 5.0 – 100 units/mL and 1.0 – 120 ng/mL, respectively. The whole assay process could be completed in 35 min. Based on the channel-resolved and substrate zone resolved-multiplex immunoassay, Fu *et at.*[106] proposed a two-dimensional resolution system for the determinations of CA 125, CA 153, CA 199 and CEA. The principle is depicted in Fig. 10. CA 125, CA 153, CA 199, and CEA could be assayed in the ranges of 0.50 – 80, 2.0 – 100, and 5.0 – 150 U/mL and 1.0 – 70 ng/mL with lower limits of detection of 0.15, 0.80, and 2.0 U/mL and 0.65 ng/mL, respectively. The whole assay process, including regeneration of the device, could be completed in 37 min. Elenis *et al.*[107]

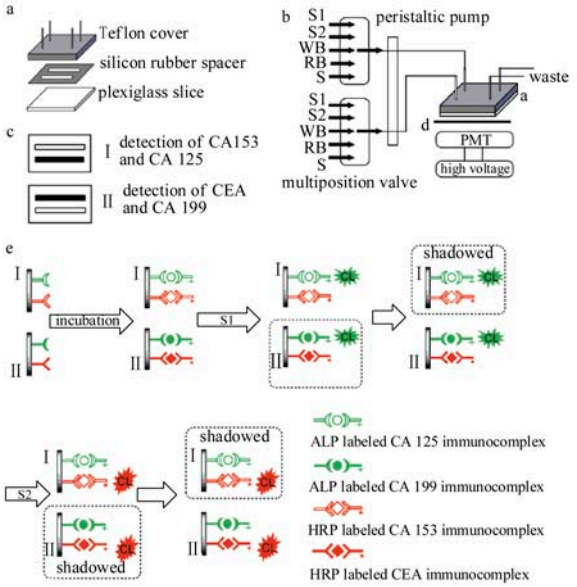

Fig. 10 Scheme of the channel and substrate zone two-dimensional resolution system for multiplex immunoassay of a tumor marker panel: (a) flow cell; (b) flow-through system; (c) transect of flow cell for immunoassay; (d) optical shutter; (e) near-simultaneous multianalyte immunoassay procedure. (S1) ALP substrate, (S2) HRP substrate, (WB) wash buffer, (RB) regeneration buffer, and (S) mixture of sample and tracer antibodies (reproduced from Ref. 106 with permission).

developed a quadruple-analyte CL hybridization assay for simultaneous quantification of four nucleic acid sequence using aequorin, galactosidase, HRP and AP as labels. The four CL reactions were triggered sequentially. The detection limits of the four targets ranged from 0.35 to 0.65 fmol and the entire analysis could be completed within 75 min.

4 Prospects

The studies described in the current review demonstrate that the combination of immunoassay and DNA analysis with CL detection is extremely sensitive and highly efficient. CL methods have a wide range of applications in the areas of clinical diagnostics, forensic chemistry, environmental investigations, pharmaceutical studies, and in biological warfare-agent detections. In order to improve the detection sensitivity, several methods have been developed. However, there are also some drawbacks, such as unsatisfactory reproducibility and tedious preparative steps. Hence, further efforts are required to improve the reproducibility and in simplifying of the procedure, whilst also improving the detection sensitivities. In addition, it is also necessary to synthesize and develop new CL reagents, and novel CL reactions will improve the sensitivity of CL detection.

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