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A B S T R A C T

Biosensors are a very active research field. They have the potential to lead to low-cost, rapid, sensitive, reproducible, and miniaturized bioanalytical devices, which exploit the high binding avidity and selectivity of biospecific binding molecules together with highly sensitive detection principles. Of the optical biosensors, those based on chemical luminescence detection (including chemiluminescence, bioluminescence, electrogenerated chemiluminescence, and thermochemiluminescence) are particularly attractive, due to their high-to-signal ratio and the simplicity of the required measurement equipment.

Several biosensors based on chemical luminescence have been described for quantitative, and in some cases multiplex, analysis of organic molecules (such as hormones, drugs, pollutants), proteins, and nucleic acids. These exploit a variety of miniaturized analytical formats, such as microfluidics, microarrays, paper-based analytical devices, and whole-cell biosensors. Nevertheless, despite the high analytical performances described in the literature, the field of chemical luminescence biosensors has yet to demonstrate commercial success.

This review presents the main recent advances in the field and discusses the approaches, challenges, and open issues, with the aim of stimulating a broader interest in developing chemical luminescence biosensors and improving their commercial exploitation.

1. Introduction

Chemical luminescence-based biosensors can exploit the measurement or imaging of the light emitted by a bio-chemiluminescence (BL, CL), thermochemiluminescence (TCL), or electrogenerated chemiluminescence (ECL) reaction. They offer an interesting and powerful alternative or complementary approach with respect to other optical biosensors, based on light absorption or photoluminescence, and different transduction principles (Roda and Guardigli, 2012).

The main advantage is their potentially high detectability. The photons are produced in the dark by a chemical reaction and are therefore easily and efficiently measurable without any non-specific signal, such as that derived from the photoexcitation source in photoluminescence.

The light emitted by chemical luminescence derives from an exergonic chemical reaction yielding an intermediate in its singlet excited state, which undergoes radiative decay. Much effort has been dedicated to increasing light output yield, which is directly related to the quantum yield of the reaction. Despite rather low quantum yield values (about 0.01 for CL reactions), detectabilities down to attomoles can be reached when these labels are used in immunoassays or gene probe assays. Indeed, in the clinical chemistry field, chemical luminescence labels are most widely used in commercial ultrasensitive immunoassays.

Nevertheless, chemical luminescence biosensors have not significantly evolved from research laboratory prototypes to the marketplace. This is despite the very promising features of chemical luminescence detection techniques and continuous advances in the fields of chemistry, micro/nanotechnologies, nanobiotechnology, molecular biology, and microelectronics. Very few chemical luminescence biosensors are commercially available. The outlook becomes more positive if we consider those biosensors where analyte detection is not achieved in real-time and where all-in-one self-standing devices are not used (Park and Kricka, 2014).

The attraction of biosensor research is the possibility of producing miniaturized, multiplexing biosensors, suitable for use in any environment, and which can give semi-quantitative

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information in a few minutes at a relatively low cost. Point-of-need or point-of-care devices are the most challenging, since they require the minimum use of analytical steps, addition of reagents, and fluid manipulation. The ever growing use of nanotechnology and microfluidics technology could expand the potential of such devices (Marquette and Blum, 2011).

In this review we will critically evaluate the current outlook for chemical luminescence biosensors in terms of analytical performance, format, and light detection systems. In particular the review will be divided into three sections. The first will be about the light detection technologies for measuring luminescent signal, than we will discuss the different chemical-luminescence-based probes and labels focusing on the latest progress in this field. Finally we will report different biosensor format pointing out the pros and cons of the different analytical platforms. We will seek to clarify why, despite their enormous potential, these principles have not been translated into the commercial realm, with the exception of ECL-based biosensors and BL cell-based biosensors. To date, the chemical luminescence methods developed have not been defined as biosensors. But they could merge into this category if the definition of biosensors is extended, as has been observed in many recent publications in the field.

2. Light detection technologies for biosensing

The main requisite of chemical luminescence measurements is the ability to collect as much light as possible to achieve the highest detectability. In contrast to photoluminescence, where the optics geometry is crucial to minimizing the excited light interference, a much-simplified optics can be used. Several technological solutions have been proposed for ultrasensitive chemical luminescence detection in biosensors and bioassays (Fig. 1).

2.1. Photomultiplier tubes

The reference detection system for chemical luminescence is the photomultiplier tube (PMT), which provides the highest sensitivity. Typical PMT devices present a high quantum efficiency in the spectral range between 360 and 670 nm, covering the emission wavelengths of the majority of chemical luminescent probes. Implementation of conventional PMT-based devices in biosensors has been hampered by their high cost, large size, and requirement for a power source. However, recent technological advances, such as the “flat panel PMT” technology developed by Hamamatsu, have opened the way for new opportunities. Flat panel PMTs provide high detection efficiency in a very compact device, including the related electronics, where a number of flat panel PMTs can be closely packed, creating an array of sensors.

2.2. Charge-transfer detectors

The requirement for portable light detectors with high sensitivity has led to the use of alternative light sensors, such as charge-coupled devices (CCD), complementary metal oxide semiconductors (CMOS), and silicon and organic photodiodes.

Portable charge-transfer detectors (CCD and CMOS) have become widespread thanks to their compact size and their ability to image and quantify multiple spots simultaneously on the detection area of the sensor (Lengger et al., 2014; Zangheri et al., 2015a; Zhou et al., 2014b). Modern cooled back-side illuminated CCDs can reach a quantum efficiency (QE) of up to 90%, read-out noise of $\leq 5\ e^-$, dark count rates of $0.001\ e^-/s$, and formats as large as 4096×4096 pixels with size down to $4 \times 4\ \mu m$.

The renewed interest in CMOS derives from their small size, low power consumption, camera-on-a-chip integration, and lower fabrication costs (Singh et al., 2011; Rodrigues and Lapa, 2010). In first generation CMOS, the majority of the pixel area was dedicated to the support transistors, with a limited photon-sensing area (fill factor). Modern back-illuminated CMOS, in which the entire area of each pixel is used for photon capture, offer higher sensitivity, ensuring high signal-to-noise ratio even in low-light conditions. The pixel size is reduced, increasing image resolution and device compactness. Integrated circuit biosensors, using low-power CMOS (total power consumption 3 mW) and optimized to detect low-level BL of bacterial bioreporters, have been reported as real-time, on-line, robust biosensors for environmental monitoring (Bolton et al., 2002). Recently a large area CMOS bio-pixel array was used for multiple CL assays directly addressed by single pixels, enabling a large number of targets to be measured simultaneously (Sandeau et al., 2015).

Imaging with charge-transfer sensors requires simple optics to obtain reasonable resolution and to prevent light cross-talk between adjacent objects. Alternatively, optics-free “contact imaging” configurations can be used, in which the surface where the bioassays take place is in contact with the sensor. For example, a CMOS sensor equipped with RGB color filter array was used for the high-content analysis of single cells assembled directly onto the sensor surface (Tanaka et al., 2010). Contact imaging with CCD sensors can be performed through a fiber optic mosaic faceplate or taper, which provides coherent photon transfer between the two surfaces as well as thermal insulation between the bioassay components and the cooled CCD sensor. Recently, an ultrasensitive portable device for point-of-need CL bioassays was proposed, exploiting a thermoelectrically cooled (double Peltier) CCD camera in contact imaging configuration (Roda et al., 2011a). Exploiting this

Fig. 1. Different platforms for CL detection. (a) The accessories (minicartridge and smartphone adapter) and their integration with the smartphone for point-of-need-analysis based on chemical luminescence detection (Roda et al., 2014b), Copyright 2014, reprinted with permission from American Chemical Society. (b) The MCR3 instrument for microarray analysis (Seidel and Niessner, 2014), Copyright 2014, reprinted with permission from Springer. (c) The a-Si:H photodiodes array (Mirasoli et al., 2014a).

device, a miniaturized multiplex biosensor for parvovirus B19 DNA detection and genotyping in serum samples was developed (Mirasoli et al., 2013). The biosensor showed performances competitive with conventional ELISA. A combination of miniaturized BL whole-cell sensor array and lens-free detection on CCD has been reported for on-site environmental toxicant detection (Tsai et al., 2015).

2.2.1. Color CCD and CMOS

The combination in a single assay of several luminescent probes with different specific emission wavelengths and bandwidths requires spectral resolved light imaging detection. Both CCD and CMOS sensors are available in either monochromatic (with up to 90% QE across the full visible spectrum) or one-shot color versions in which a red, green, and blue filter pattern (Bayer matrix) is placed over the pixel array.

Although the difference in readout technique of CMOS with respect to CCDs has significant implications for sensor capabilities and limitations, today there is no clear line dividing the biosensing applications for which each detector is used.

CMOS imagers for mobile phones are the most widely used image sensor applications in the world. The continuous improvements in the backside-illuminated CMOS (BSI-CMOS) used in smartphone/tablet cameras have led to enhanced image quality, superior functionalities, and a compact size. In addition to the higher pixel numbers (up to 41 MP), newly developed CMOS architecture and multi-lens systems facilitate low noise and high quality image capture even in low light conditions, allowing smartphone cameras to be used for sensitive chemical luminescence detection. Although their sensitivity is still lower than cooled CCDs, smartphones could be suitable for measuring analytes present at medium-abundant concentrations, as was recently shown for CL-based point-of-care bioassays (Fig. 2) (Roda et al., 2014a, 2014b).

By developing simple and compact 3D-printed low-cost smartphone accessories, any mobile device can effectively be turned into a portable mini-luminometer for point-of-care testing, exploiting the additional features of direct signal elaboration (using dedicated applications), data handling and storage, connectivity, and cloud servicing for remote sensing.

2.3. Thin-film photosensors

The integration of relatively inexpensive thin-film photosensors directly in the analytical chip is another notable technological advance, which could reduce costs, electrical power consumption, and memory storage space. Several possibilities have

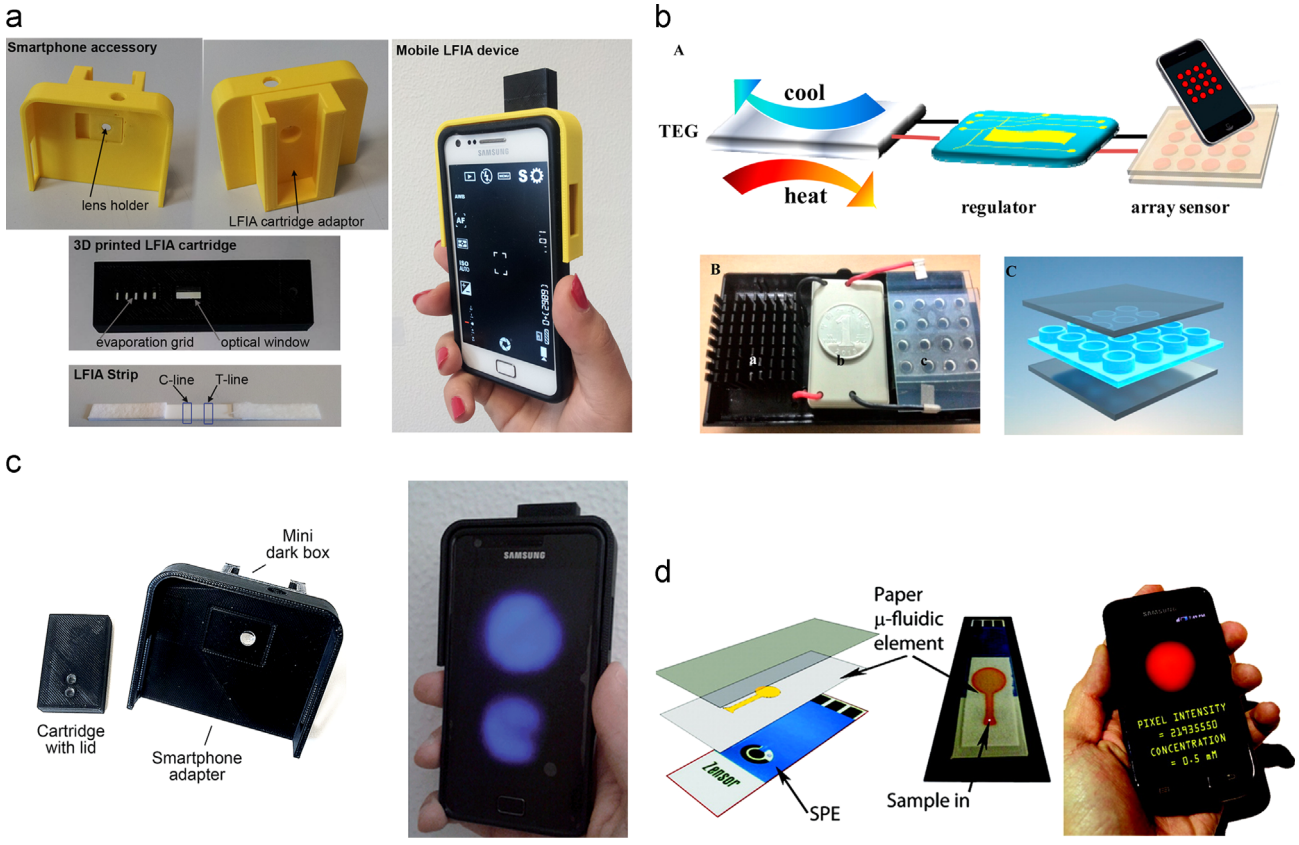

Fig. 2. Smartphone-based chemical luminescence detection. (a) (left) 3D-printed smartphone accessories (ABS) for salivary cortisol detection. The smartphone adapter comprises a plano-convex lens holder and a cartridge, housing the LFIA strip with control- (C-line) and test-line (T-line). On the right, the integrated cortisol LFIA smartphone-based device running Camera FV-5 lite application for CL signal acquisition. (Zangheri et al., 2015b), Copyright 2015, reprinted with permission from Elsevier; (b) (top) Design of the thermo-powered high-throughput visual ECL sensor. When a heat source and a cold source exist, the TEG generates electricity. Voltage regulator allows steady voltage output. The anodic current then flows to a transparent working electrode, while the cathode current flows to a reference ITO electrode. The assay can be directly monitored by the naked eye semiquantitatively or by smartphones quantitatively. (bottom left) Top view of the sensor: an aluminum heat sink, a voltage regulator module, the array electrode. (bottom right) Schematic diagram of the array electrode, which is fabricated with two pieces of ITO glass and a PDMS frame containing 16 cylinder-shaped microwells (Hao, 2013), Copyright 2013, reprinted with permission from American Chemical Society; (c) (left) The cartridge-lid assembly of the mini dark box and smartphone adapter, developed for detecting lactate in oral fluid and sweat. (right) Images obtained by analyzing lactate standard solutions in artificial oral fluid. (Roda et al., 2014a) Copyright 2014, reprinted with permission from Royal Society of Chemistry; (d) Schematic representation of a device for ECL detection in μPAD sensors reported by Delaney et al. (2011). A drop of sample is introduced through a small aperture in the plastic at the base of the channel. When the detection zone is fully wetted, the sensor is placed close to the lens of the camera phone, a potential of 1.25 V is applied, and the resulting emission is captured and analyzed (Delaney et al., 2011), Copyright 2011, reprinted with permission from American Chemical Society.

been described, including amorphous silicon thin-film photodiodes (Caputo et al., 2013; Pereira et al., 2009), organic photodiodes (Wang et al., 2009; Wojciechowski et al., 2009), carbon nanotubes coated with photovoltaic polymers (Shim and Ahn, 2012), and metal–semiconductor–metal photodetectors (Lin et al., 2009). By optimizing chip design, sensor architecture, and readout electronics to maximize photon collection efficiency, analytical performances comparable with CCD have been achieved in a portable integrated device (Mirasoli et al., 2014a). In addition, arrayed photosensors with custom geometries can be easily produced for multiplexed bioassays.

Organic photodiodes (OPD) have recently emerged as an alternative to silicon-based photodetectors (Wojciechowski et al., 2009). These sensors are inexpensive and can be produced to fit customer-specific requirements and in a flexible format by integrating OPDs into polymer films (Nau et al., 2015). Ring-shaped OPDs were integrated into microfluidic channels for CL immunoassays for pathogen detection, demonstrating good performance (Pires and Dong, 2014).

Organic photodiodes have also proved useful as an efficient way of increasing the sensitivity and resolution of conventional silicon CMOS image sensors, by superposing OPD onto a CMOS circuit which doubles the light-input surface area of each pixel (Lim et al., 2015). Indeed, a newly reported design concept demonstrated the possibility of tuning the spectral response of organic photodiodes, allowing selective light detection (Armin et al., 2015).

Single photon avalanche photodiodes (SPAD) contain a silicon photon counting element. They offer very high gain and low dark count, allowing low cost and small detection systems to be developed (Daniel et al., 2008). Recently a highly sensitive CL aptasensor for the detection of circulating protein biomarkers, based on SPAD, was reported (Pasquardini et al., 2015).

3. Chemical-luminescence-based probes and labels

3.1. Chemiluminescence

In CL, the chemical reaction responsible for photon emission is simply triggered by mixing the reagents. This phenomenon has been exploited in a variety of bioanalytical formats including microtiter plate (96- and 384-well), microarrays, microfluidics, paper-based devices, and in vitro microscopy imaging (Marquette et al., 2012; Seidel and Niessner, 2014; Mirasoli et al., 2014b). Chemiluminescence labels can categorized as direct chemical or enzyme-based.

3.1.1. Direct CL labeling

The use of direct labeling with isoluminol or acridinium esters (typically aromatic esters of 10-methylacridinium-9-carboxylic acid) has been reported. Several examples are commercially available for the development of CL immunoassays (CLIA). The light is simply triggered upon addition of a catalyst or alkaline hydrogen peroxide for isoluminol and acridinium salts, respectively, reaching LOD values down to nanomolar concentrations. Despite the potentially high detectability of the signal, characterized by a relatively high signal-to-noise ratio, the main limitation of this approach is the poor quantum yield of the CL reaction, which does not exceed 0.01.

Acridinium salts exhibit a higher CL quantum yield in aqueous media with respect to luminol derivatives. However, in aqueous basic or neutral media, most acridinium salts are in equilibrium with their pseudo-base, which can react only slowly with hydrogen peroxide, strongly reducing the CL reaction yield (Osman et al., 2000).

3.1.2. Enzyme as a label

The use of enzymes as labels is generally preferred over direct labeling. This is because, in the presence of an excess of a suitable CL substrate, the enzyme turnover allows a high amplification factor, in the range of 10^4–10^5 (Roda et al., 2005). Due to its high enzyme turnover number and stability, horseradish peroxidase (HRP) is the most frequently used enzyme label in binding assays. The enzyme catalyzes the luminol oxidation reaction in the presence of a peroxide, leading to the formation of aminophtalate in a singlet excited state. This then decays, releasing a photon in the blue region (428 nm) (Creton and Jaffe, 2001). To increase the signal intensity and obtain a steady-state emission, different enhancers can be added, which act as electron transfer mediators. These enhancers include substituted phenols, substituted boronic acids, indophenols, and *N*-alkyl phenothiazines (Zomer, 2011). Recently, 4-dialkylaminopyridine, a nucleophilic acylation catalyst, showed a potent effect on light signal intensity (Marzocchi et al., 2008).

In addition to luminol, acridinium derivatives can also serve as HRP substrates. In particular, acridan derivatives can be converted by HRP into acridinium intermediates, which rapidly react with hydrogen peroxide, preventing the formation of the low efficient pseudo-base acridinium derivative (Osman et al., 2000). These substrates are commercially available and a large number of applications have been published and patented.

1,2-Dioxetanes are another class of molecules suitable for CL detection. A variety of compounds of this class have been studied and it is now well known that the presence of suitable substituents on the dioxetane ring can yield to efficient CL labels that can be chemically or enzymatically triggered. Commercially available substrates have been produced for enzymes such as alkaline phosphatase or β-galactosidase when these are used as CL labels.

3.1.3. Metal-enhanced chemiluminescence

It has been observed that photons resulting from a CL reaction, produced in close proximity to nanoparticles or thin films of a resonant metal, can induce the surface plasmons in the metal. New strategies have been developed to increase the detectability of CL reactions by exploiting this observation (Aslan and Geddes, 2009). These include metal-enhanced CL (MEC) techniques, which provide signal amplification, and surface plasmon coupled CL, in which photons are emitted in preferential directions. The authors also showed that the efficiency of photon collection can be further increased using the microwave-triggered MEC technique, in which low-power microwaves are exploited to accelerate CL detection and thus enable the collection of a larger number of photons per time unit (Previte et al., 2007).

3.1.4. Artificial pseudo-enzyme labels

In recent years, a variety of synthetic molecules and materials with catalytic properties have been proposed for the development of enzyme-free biosensors. These molecules and materials display higher thermostability and are easily obtained by chemical synthesis. Catalytic DNA molecules (DNAzymes) with peroxidase-like activity have been proposed as alternatives to HRP using the same CL cocktails for detection. To provide signal amplification, Au NPs have been used as a carrier for numerous DNAzyme molecules, increasing the signal intensity. DNAzymes offer stability and ease of fabrication. They also offer the possibility of producing functional DNAs in which the DNAzyme sequence is combined with an analyte-specific DNA sequence (e.g. an aptamer or an oligonucleotide sequence complementary to the target DNA), to provide functional DNAs, in which peroxidase-like activity is enabled only upon binding to the analyte.

In addition, signal amplification technologies have been

developed based on metal or semiconductor nanoparticles (NPs) (Qi and Li, 2011; Park et al., 2015; Huang et al., 2014; He and Cui, 2012; Hong et al., 2013; Yu and He, 2015; Li et al., 2014b). In particular, metal NPs have been proposed as labels in bioassays. In most cases, they act as catalysts of the CL reactions (nanozymes), but in some cases they may act as reductant, energy acceptor, or nanosized reaction platform (Li et al., 2014b). Most of the applications concern AuNPs, but AgNPs, PtNPs, PdNP, Au/Ag alloy NPs, and Ag/Pd alloy NPs have also been used, taking advantage of their low cost, high stability, and catalytic properties. Indeed, metal NP-initiated CL presents good stability and a low background with respect to conventional CL reactions in solution.

3.2. Bioluminescence

Bioluminescence (BL) is basically a CL reaction occurring in living organisms and involving enzymes, generally called luciferases, or photoproteins. The peculiar photo-physical property of BL, with respect to CL, is that the light emission derives from an enzyme-singlet excited state product complex. The main peculiarity of BL reactions is the high quantum yield emission efficiency (i.e. 44% for *Photinus pyralis* luciferase) (Niwa et al., 2010). It is much higher than any CL reaction, allowing ultrasensitive detection down to attomole level in diverse bioanalytical applications (Thouand and Marks, 2014). Several BL genes have been isolated and cloned from different luminescent marine and terrestrial organisms. The origin and emission properties of commonly used BL proteins are summarized in Table 1.

Of all the BL proteins, beetle luciferases are the most exploited in high-throughput assays and in vivo applications. In particular, firefly luciferase catalyzes the formation of luciferyl-adenylate (LH_2-AMP) from D-luciferin (LH_2) and ATP. LH_2-AMP is converted through a multi-step oxidative process to excited-state oxyluciferin, the light-emitting product. The decomposition of these intermediates generates electronically singlet state products that decay, emitting a photon at 550–570 nm. The yellow–green color of firefly BL changes to red (610 nm) by decreasing the pH value or increasing the temperature, while those of click beetles and railroad worms do not depend on the reaction conditions (White et al., 1971).

The requirement for ATP as a substrate has been widely exploited to develop BL assays for ATP quantification. This well-established approach, pioneered about 50 years ago, has been continuously "revisited" and updated (Chappelle and Levin, 1968; Borghei and Hall, 2014). Since the BL signal is proportional to the ATP amount and thus microbial number, BL ATP bioassays are a powerful analytical tool for enumerating microbial cells or cell viability in different biological samples, in both benchtop and portable configurations. They thus fulfill the classic "biosensor" definition. Recent technical and instrumental advances have allowed detection of 1 attomole of ATP or the amount contained in a single bacterial cell (Satoh et al., 2004). A number of portable devices and ready-to-use kits for hygiene monitoring are commercially available, including the Milliflex® Rapid Testing (Merck-Millipore), DeltaTox® II (Modern Water Inc.), Clean-Trace™ (3M™), and many others.

The bacterial BL reaction arises from proteins, LuxA and LuxB, which catalyze the oxidation of a long-chain fatty aldehyde in the presence of reduced riboflavin phosphate ($FMNH_2$) and oxygen. This reaction has also been exploited in biosensors (Close et al., 2009). In living species, other proteins, encoded by lux operon, LuxC, LuxD, and LuxE, are also involved in the reaction, where they regenerate the aldehyde substrate.

Secreted luciferases (20 kDa) from the copepod *Metridia longa* (MetLuc) and *Gaussia princeps* (Gluc) catalyze the oxidation of the substrate coelenterazine in a reaction that emits light at 470–490 nm and does not require ATP. Nevertheless, the auto-oxidation and low stability of coelenterazine leads to high background signals. More recently, the Nanoluc luciferase (19kDa), cloned from the deep sea shrimp *Oplophorus gracilirostris,* has been extensively engineered, in combination with a novel imidazopyrazinone substrate (furimazine), to create a more efficient BL system. This system produces a glow-type luminescence at 465 nm (signal half-life >2 h) with a specific activity $\sim$150-fold greater than that of firefly luciferase (Hall et al., 2012).

The apoprotein of aequorin and obelin binds to coelenterazine and molecular oxygen to form a stable photoprotein complex. The addition of calcium ions produces a conformational change, leading to the oxidation of the chromophore with the release and blue light (470 nm) in a flash-type kinetic. Aequorin variants showing increased intensity, slower BL decay, or improved thermostability were obtained by in vitro evolution of the wild-type apoprotein (Rowe et al., 2008; Qu et al., 2014). Thanks to their calcium-controlled light emission, these photoproteins have been widely used for intracellular calcium imaging and for homogeneous binding assays.

There are several luciferase variants with improved emission properties, such as slower emission decay, increased intensity, and red-shifted emission wavelength. These can be used for different bioanalytical applications, even in multiplexed format. BL multiplexing could be achieved via different strategies, such as spectral resolution of luciferases emitting at different wavelengths or chemical resolution with luciferases requiring different substrates.

The detection of multiple targets in the same bioassay can be further expanded using a combination of the above-mentioned strategies or, in the case of cellular biosensors, by using intracellular and secreted luciferases.

Bioluminescence color changes of luciferase have been widely

Table 1
Commercially available BL proteins commonly used in BL-based biosensors.

Luciferase (species)	Length (aa)	Size (KDa)	BL λmax (nm)	Substrate (ATP requirement)	Notes
Fluc *(P. pyralis)*	550	61	557	D-luciferin (Yes)	pH- and temperature-sensitive BL emission
CBG99 *(P. plagiophthalamus)*	542	64	537	D-luciferin (Yes)	pH-insensitive
CBR *(P. plagiophthalamus)*	542	64	613	D-luciferin (Yes)	Red-shifted emission mutant
Eluc *(P. termitilluminans)*	543	61	538	D-luciferin (Yes)	pH-insensitive
LitRE *(L. italica)*	548	61	610	D-luciferin (Yes)	Red-shifted emission mutant
Rluc *(R. reniformis)*	312	36	475	Coelenterazine (No)	
GLuc *(G. princeps)* ***Gaussia-Dura***	185	19.9	482	Coelenterazine (No)	Secreted in mammalian cells Flash-type kinetic Glow-type kinetic
MetLuc *(M. longa)*	220	24	470	Coelenterazine (No)	
Nanoluc *(O. gracilirostris)*	171	19.1	465	Furimazine (No)	Secreted in mammalian cells Highly stable
Cluc *(C.noctiluca)*	555	61	463	Cypridina Luciferin (Vargulin)	Highly stable enzyme
Lucia (Synthetic enzyme)	211	23	460	Coelenterazine (No)	Secreted Extended flash (5 minutes)

investigated and several models proposed (Hosseinkhani, 2011). In addition to mutagenesis of the enzyme, the chemical structure of the BL substrate can be modified to tune the emission color or enhance enzyme specificity. Amino-luciferin derivatives showed red-shifted emission (596 nm) and higher luciferase selectivity over the natural D-luciferin substrate (Mofford et al., 2014).

Caged luciferins, in which 6′-hydroxy is conjugated with different chemical moieties, were used as functional substrates to monitor enzyme activity and cellular processes, and for imaging of bioactive small molecules (Li et al., 2013a).

In addition to native coelenterazine, several derivatives have been synthesized (i.e. cp, f, h, hcp, i, n and others) (Zhao et al., 2004) to tune the BL properties of luciferases and photoproteins or to create functional substrates (Lindberg et al., 2013).

3.3. Electrogenerated chemiluminescence

Electrogenerated chemiluminescence is an electrochemical process in which molecules undergo electron transfer reactions at an electrode surface, leading to a singlet excited photon emission state. The process is controlled by the applied potential, allowing precise control over the time and position of the emission, so photon collection efficiency can be optimized. In addition, varying the electrode potential can finely tune the specificity of the ECL reaction. This technique offers a very challenging opportunity to develop biosensors in miniaturized format and characterized by a very high signal-to-noise ratio, since the emitted light is generated by a different physical orthogonal principle.

Several ECL biosensors have been described, taking advantage of technological advances in the field of miniaturized electrodes and novel materials. A wide variety of ECL labels have been reported over the years, based essentially on inorganic complexes and organic molecules (Pyatia and Richter, 2007). However, the vast majority of ECL biosensors employ the well-known tris(2,2′-bipyridine) ruthenium (II) complex, $Ru(bpy)_3^{2+}$ (Zhou et al., 2014c). This is because of its high ECL quantum yield, its solubility in both aqueous and organic media, and its ability to undergo a reversible one-electron transfer reaction. This label was extensively used in association with a tertiary amine (generally tripropylamine, TPA) in the so-called co-reactant system, in which $Ru(bpy)_3^{2+}$ is oxidized at the electrode surface and then receives an electron from the radical intermediate of the co-reactant (also formed at the electrode surface) to produce an excited state. Under these conditions, one molecule of the $Ru(bpy)_3^{2+}$ label can produce many photons, with the final result being enormous amplification of the probe detection. One of the main drawbacks is the necessity of a high voltage (usually around -1 V). This is close to the hydrolysis potential of water (-1, 23 V) and often causes electrode surface passivation. In addition, the use of a co-reactant adds one more reagent delivery step to the analytical procedure. So unlike photoluminescence or TCL, the system is not reagentless.

3.3.1. Nanomaterials in ECL

Recently, analytical performances of ECL biosensors have been improved by nanomaterials (NMs) (Pei et al., 2013), which have been used as containers to load a great number of ECL labels, energy acceptors to quench ECL emission in developing signal-off biosensors, labels, electrocatalysts, and electrode materials.

Quantum dots (QDs) are the most widely used NMs, owing to their high surface-to-volume ratio for bio-conjugation, broad excitation spectra, narrow and size-tunable emission spectra, high resistance towards photobleaching, very high emission quantum yields, and applicability both as enhancer and quencher of ECL emission. QDs have enabled excellent analytical performances in terms of detectability. But they have great disadvantages, such as their cytotoxicity and the need for functionalization to become hydro-compatible (Derfus et al., 2004). Carbon dots (CDs) could be a valuable alternative (Esteves da Silva and Gonçalves, 2011), as they are inert, readily water soluble, non-toxic, easily labeled, and cheap. Nevertheless, their use in ECL applications is still limited.

Among the materials used to design ECL sensors, carbon nitride nanosheets (CNNS) have been reported as a cathodic ECL emitter in the presence of dissolved oxygen to produce an endogenous coreactant H_2O_2 on electrode surface (Feng et al., 2015).

Silica and metal nanoparticles (NPs) also appear particularly suited to ECL immunosensor development, with the former used as nano-containers and the latter as electrocatalysts and/or carriers for ECL labels (Pei et al., 2013; Rampazzo et al., 2012).

Concerning electrode NMs, the most frequently used in ECL are graphene and CNTs, which exhibit great conductivity and enhanced electron transfer features. However, some applications of TiO_2 nanotubes have been reported (Tian et al., 2012).

3.4. Thermochemiluminescence

Thermochemiluminescence (TCL) is the emission of photons as a consequence of the thermolysis of a suitable molecule, usually a 1,2-dioxetane derivative, which leads to an excited singlet state product. Adamantylidene adamantane 1,2-dioxetane derivatives were first proposed in the 1980s as labels in immunoassays (FATIMA technology) (Hummelen et al., 1986, 1988; Luider et al., 1990). The high temperature needed to decompose the molecules (up to 200–250 °C) and the low quantum yield ($\phi_F = 5.2 \times 10^{-3}$) limited their use even when a fluorescent energy acceptor was used. To further amplify the detection system, the TCL label was included in a complex with a protein, in an attempt to increase the label/molecule ratio of the probe. Despite poor analytical performance, these pioneering works demonstrated that, in addition to sharing the advantage of high signal-to-noise ratios with the other chemical luminescence techniques, TCL has the unique feature of allowing reagentless detection. No addition of reagents is required to trigger light emission from the TCL label, which requires only heat. For these reasons, TCL was abandoned, but in recent years, research has resumed to try to overcome its limitations.

In particular, new TCL labels were synthesized to lower the triggering temperature, maintain room temperature stability, and increase the detectability of the labels. A new family of acridine-containing 1,2-dioxetane derivatives (Roda et al., 2012; Di Fusco et al., 2013) was reported, which showed triggering temperatures in the range of 80–100 °C and improved fluorescence quantum yields of the excited product generated upon thermolysis (ϕ_F = 0.1–0.5) (Fig. 3a) (Di Fusco et al., 2014). In addition, although it was shown that this family of molecules can be used as TCL labels without needing a fluorescence energy acceptor, further improvements were achieved by incorporating these TCL molecules into organically modified silica nanoparticles (ORMOSIL NPs) together with a fluorescence energy acceptor. The resulting label had amplified TCL properties, containing up to 1000 molecules of 1,2-dioxetane derivative. These doped ORMOSIL NPs were functionalized with biotin, exploiting the amino groups on their surface. They were successfully used as TCL labels to detect streptavidin in a non-competitive heterogeneous immunoassay, using a portable cooled CCD-based device for TCL imaging measurements, which includes a battery powered mini-heater and an ultrasensitive sensor (Di Fusco et al., 2015). The TCL-based immunoassays using NPs present LODs comparable to those obtained using conventional HRP-catalyzed CL detection.

4. Biosensor format

The first CL–BL based biosensors used an analyte-specific enzyme coupled with one or more “indicating” enzymes, terminating

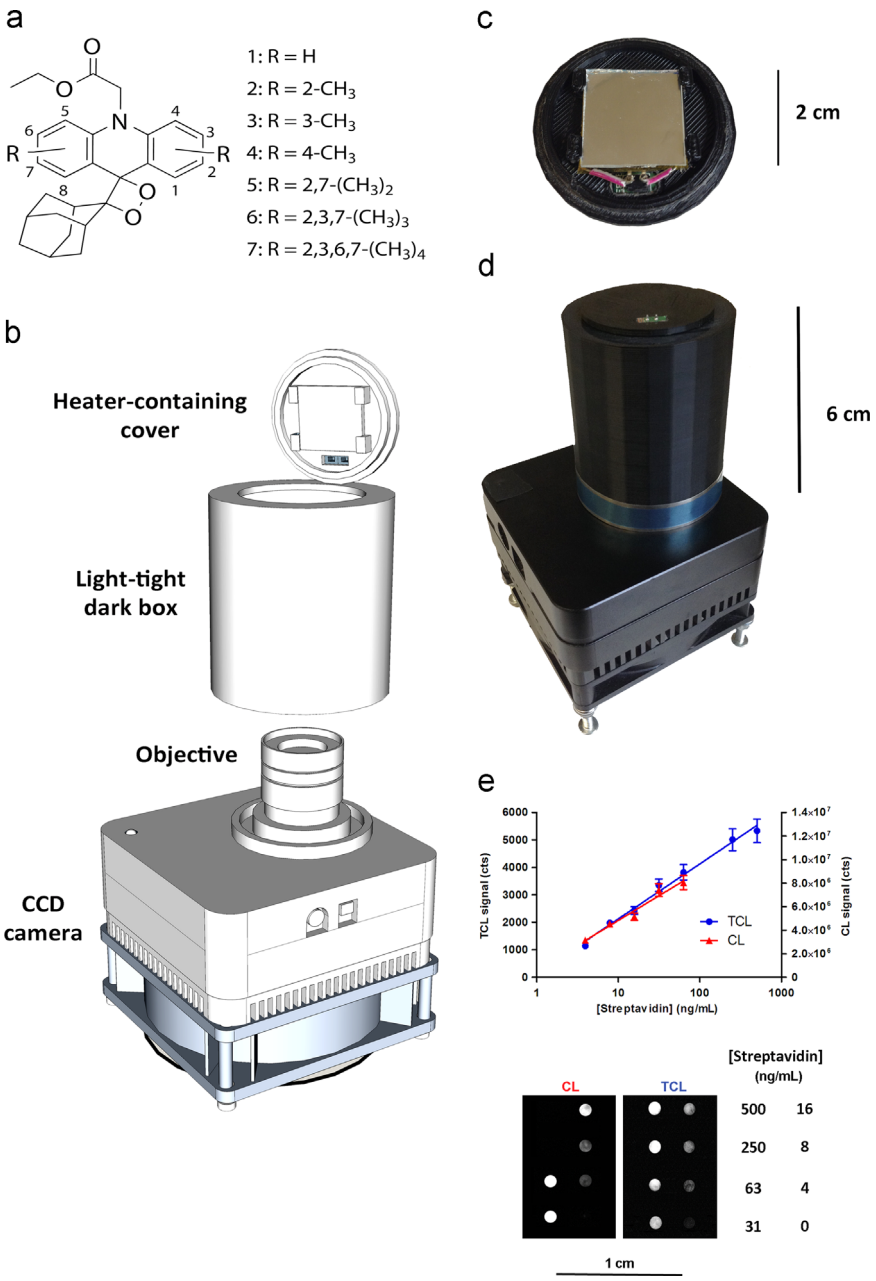

Fig. 3. Thermochemiluminescence labels and measurements. (a) Chemical structures of the new family of TCL acridine-containing 1,2-dioxetane derivatives. (b) Scheme of the portable cooled CCD-based device for TCL measurement. (c) Battery powered mini-heater. (d) The CCD camera/dark-box/heater-containing cover assembly. (e) Comparison of the calibration curves and emissions obtained for the immunoassay of streptavidin using TCL and CL imaging detection (Di Fusco et al., 2015), Copyright 2015, reprinted with permission from Springer.

with a CL or BL emission (Blum et al., 1989).

The most popular CL systems used the luminol/HRP system to measure hydrogen peroxide produced by any oxidase enzyme (e.g. glucose oxidase, ethanol oxidase, cholesterol oxidase). They also exploited the possibility of using oxidases as intermediate reactions in longer cascades (e.g. acetylcholinesterase/choline oxidase/peroxidase enzyme system) (Rauch et al., 1997). The bacteria luciferase coupled with NAD-FMN oxidoreductase has been extensively used to measure any NAD(P)/NAD(P)H-dependent dehydrogenase enzymes or their substrates, including glucose, lactate, ethanol, and bile acids. An air-segmented flow system, with the enzymes (co)immobilized on the inner wall of a nylon tube, has been developed and applied to many analytes (Roda et al., 1988).

A wide range of enzyme immobilization strategies have been proposed, which can improve enzyme stability and activity, orientation, and loading, thus increasing biosensor sensitivity, selectivity, and rapidity (Sassolas et al., 2012; Leca-Bouvier et al., 2014). The literature contains many examples of applications. However, these systems have not succeeded commercially and have been surpassed in both publication numbers and commercial exploitation by electrochemical enzyme biosensors. The main limitations of CL/BL enzyme biosensors are that these assays lack robustness and have different pH requirements for the enzyme reactions in the cascade.

Researchers have also reported homogenous bioluminescence resonance energy transfer (BRET)-based biosensors in a microfluidic format for quantifying chemical or biological analytes (Le et al., 2014; Wu et al., 2014).

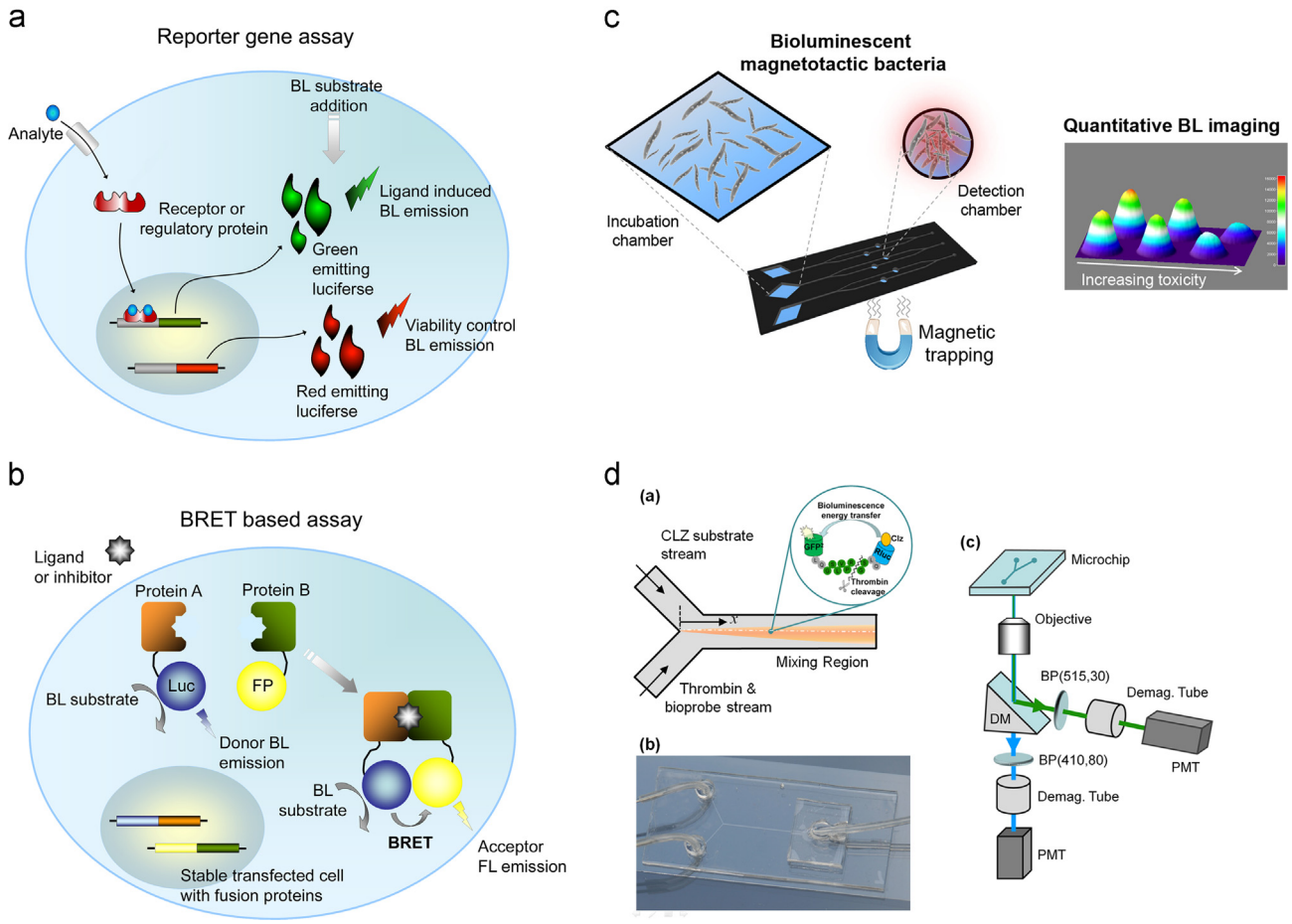

Fig. 4. a) Schematic drawing of a dual reporter cell biosensor expressing green- and red-emitting luciferases. One reporter is analyte-dependent, i.e., when the analyte enters the cell it binds to a specific receptor/regulatory protein and interacts with a promoter sequence, driving the expression of the reporter protein. The second luciferase can be used as viability control to monitor nonspecific effects. (b) Schematic view of BRET-based biosensor, after interaction with analyte the bioluminescent donor, on addition of BL substrate, can transfer its energy to a fluorescent acceptor which in turns emits light (Michelini et al., 2010), Copyright 2010, reprinted with permission from Springer; (c) Genetically engineered bioluminescent magnetotactic bacteria integrated into a microfluidic analytical device to create a portable toxicity detection system (Roda et al., 2013), Copyright 2013, reprinted with permission from Royal Society of Chemistry; (d) BRET based microfluidic assay for thrombin based on donor and acceptor proteins linked by a thrombin target peptide (Wu et al., 2014), Copyright 2014, reprinted with permission from Public library of Science (PLOS) (a) Schematic view of the microchannel network (b) picture of the device, (c) Schematic of the optoelectronic detection apparatus.

4.1. *Bioluminescence whole-cell biosensors*

Bioluminescence detection has been widely exploited in cell-based and in vitro assays and for in vivo imaging, but has found little application in portable biosensor systems. There have been many advances in this field (i.e. new luciferases with improved properties and chemically modified substrates), particularly in implementing whole-cell bioreporters into portable analytical devices. But there are few commercially available devices for on-field analysis.

Whole-cell biosensors do not always fulfill all the ideal biosensor characteristics, such as robustness, long-term stability, high sensitivity, and selectivity (Merulla et al., 2013; Park et al., 2013).

The majority of BL whole-cell biosensors are based on reporter gene technology, relying on cells that have been genetically engineered to express an analyte-specific receptor or regulatory protein, whose activation ultimately leads to the amplified expression of the selected luciferase gene. The light emission can be quantified with a suitable detector (luminometer, CCD, CMOS) upon addition of the BL substrate. It is proportional to the activation level of the target receptor/pathway.

Using live cells as biosensors has two main advantages. Firstly, they are versatile (cells can be engineered to express biorecognition elements for several classes of analytes). Secondly, they can measure the bioavailable fraction of a given analyte (i.e. the fraction able to enter into live cells and activate specific response pathways) rather than its total concentration, providing highly predictive information about the real “biological activity” of a sample.

Several BL whole-cell bioreporters have been developed based on bacteria, yeast, or mammalian cell lines. The choice of the “sensing cell” is usually determined by balancing the advantages of microbial and mammalian cells against each other. Microbial cells offer specificity and robustness (pure receptor activation, no crosstalk between signalling pathways). Mammalian cells offer sensitivity/predictivity, since the presence of different metabolizing enzymes offers insight into how a sample may behave in vivo. Indeed, the analytical performance of a BL whole-cell biosensor largely depends on the availability of an optimized BL reporter.

One of the fastest and most sensitive whole-cell biosensors is the CANARY sensor (Cellular Analysis and Notification of Antigen Risks and Yields). It is based on engineered B cells expressing the photoprotein aequorin together with membrane-bound pathogen-specific antibodies. Upon binding of the pathogen, the increase of intracellular calcium ions triggers aequorin to emit light within seconds. The possibility of performing multiplex assays within a miniaturized chip could be particularly suitable for biological defense applications (Rider et al., 2003).

Several regulatory systems have been exploited including those for heavy metal resistance (for developing heavy metal responsive biosensors), for organic compound degradation (to obtain organic compound sensors), and for cellular stress responses (to obtain general toxicity sensors) (Banerjee and Bhunia, 2010; Raut et al., 2012; Bereza-Malcolm et al., 2014; Cerminati et al., 2015).

An emblematic example is CALUX® (Chemical Activated Luciferase gene eXpression), which is the laboratory assay most frequently used to detect endocrine-disrupting chemicals and dioxin-like compounds. CALUX® bioassays rely on the firefly luciferase gene under the control of human receptors such as aryl hydrocarbon, estrogen, and androgen receptors, and have shown high sensitivity (e.g. LOD for 17-β-estradiol 0.2 ng L^{-1}) (Sonneveld et al., 2005).

One of the main limitation of reporter gene-based bioassays is the long assay time (several hours) which is required for the expression of the BL reporter. In order to speed up the biosensor response, alternative approaches based on BRET and luciferase complementation strategies have emerged (Robinson et al., 2014). A smart strategy to monitor the signal transduction of GPCRs, in real-time (few minutes), have been developed and commercialized by Promega (GloSensor™ Technology).

Most notably, these whole-cell sensing systems can be easily adapted to develop high-throughput screening (HTS) assays in 96- and 384-well microplates. Despite their HTS suitability, their implementation into "true biosensors" is not straightforward and several technical challenges must be overcome (Michelini et al., 2010).

The current challenge is to move from traditional benchtop "cell-based assays" to real "whole-cell biosensors" where bioreporters are integrated into portable biosensing devices (Fig. 4). One of the major limitations is their poor robustness. This is because living cells are not as stable as chemical reagents or antibodies, and cannot be stored for long periods of time without losing viability and responsiveness. Another critical issue is the nonspecific and out-of-control factors influencing cells' metabolism and viability due to sample constituents, including temperature and pH. An internal viability control using spectral resolution of luciferases emitting at different wavelengths has been proposed (Cevenini et al., 2013).

To minimize the matrix effect, BL-engineered magnetotactic bacteria (MTB) have been proposed. MTB can orient according to geomagnetic field lines and are thus attracted by magnets. The possibility of controlling and moving the biosensing cells creates new possibilities, since the cells could be easily navigatedtowards and concentrated in specific detection chambers, where the sample matrix is removed and the bacteria are washed (Roda et al., 2013).

Several strategies have been proposed to keep whole-cell bioreporters alive. These include immobilization into biocompatible polymeric matrices, cell encapsulation, or even sporulation. We recently demonstrated that yeast biosensors could be immobilized "entrapped" into polymeric matrix and stored for up to one month without significant viability loss, then used as ready-to-use cell-cartridges suitable for on-field detection of endocrine-disrupting compounds (Roda et al., 2011b). Lee et al. (2005) developed a cell array comprising 20 immobilized recombinant BL bacteria in which the lux gene was regulated by different promoters using CCD as detector. Spores have also been proposed as an alternative to cell immobilizations (Knecht et al., 2011). They can be stored at room temperature for long periods of time without losing responsiveness, which is a considerable advantage. However, they must be "awakened" (germinated) before use to produce viable and metabolically active cells, requiring incubation at 37 °C for several hours. One group has proposed a self-organized 3D sol–gel biomatrix architecture to keep cells alive even in harsh conditions, including short wave UV radiation (Ponamoreva et al., 2015). One of the most promising approaches to keeping cell biosensors viable for long periods of time is the use of cell encapsulation technology based on alginate hydrogels, which are highly biocompatible. This technology's potential applications range from biosensors to cell therapy (Kim et al., 2014).

Very recently, a promising whole-cell array, named LumiChip, has been reported. It harbors a 16-member sensor array, which allowed detection of water pollutants within 15–45 minutes (Tsai et al., 2015).

4.2. Static systems (single spot, tube, dot, array)

Polystyrene microtiter-well plates are the most popular format for carrying out immunoassays because it is not necessary to chemically modify the solid support (Chen et al., 2011, 2012; Wang et al., 2012a, 2011, 2012b; Liu and Li, 2011; Yu et al., 2014; Sabouri et al., 2014; Boro et al., 2011; Gao et al., 2014). For example, Sabouri et al. (2014) described a very simple approach to detecting the hepatitis B surface antigen (HBsAg) using an Au-NPs-based immunosensor. The capture antibody Ab1 was immobilized in polystyrene wells. After the capture of HBsAg, the sandwich complex was formed by adding Ab2 co-immobilized on Au NPs labeled with luminol. The CL signal was produced by adding H_2O_2 as an oxidant and Au^{3+} as an efficient catalyst, obtaining a very low detection limit of 14 pg mL^{-1}. Yu et al. (2014) described a similar approach, using Ag NPs labeled with luminol to detect chloramphenicol (CHL) down to 10 ng mL^{-1} in foodstuffs.

Immunoassays can also be performed simply in solution (pseudo-homogeneous immunoassay) using labeled antibodies. This approach carries the problem of washing the excess of unreacted labeled species. However, different strategies to overcome this have been explored. One possibility is to use magnetic nano-/micro-particles labeled with an antibody, so each analytical step is conducted without washing. Finally, the generated immunocomplex for a given analyte is removed from the solution, using a magnet, to be deposited in the measuring area for CL (Qin et al., 2012; Fu et al., 2012; Zhang et al., 2013) or ECL detection (Wang et al., 2012a; Ge et al., 2013; Gan et al., 2013; Zhou et al., 2013; Zhu et al., 2012; Liao et al., 2014). Gan et al. (2013) described an ECL immunoassay for carbohydrate antigen 19-9 (CA 19-9) in serum. The proposed method used Ab1 labeled magnetic nanoparticles as capture antibodies, and graphene/CdTe QDs as signal amplifiers. The immunocomplex resulting from a competitive immunoassay was magnetically purified, re-dispersed in buffer and added dropwise to the surface of a screen-printed carbon electrode. With this format, the authors obtained a very wide detection range of 0.005–100 pg mL^{-1}, and a detection limit of 2 fg mL^{-1}. Liao et al. (2014) described an enzyme-free ECL biosensor for the detection of microRNA in tumor cells and tissues, exploiting DNA circuits as novel amplification method. Target microRNA was incubated with hairpin probes (H1 conjugated with $Ru(bpy)_3{}^{2+}$ and H2 conjugated with biotin) and enzyme-free amplification was obtained in 1 h. The amplification products were captured by streptavidin magnetic beads, magnetically separated, redissolved in buffer, and finally ECL detected. In this manner, the authors reached a sensitivity of 10 fmol and good specificity.

Another strategy for performing immunoassays in solution is to exploit the CL resonance energy transfer (CRET) phenomenon to quench the CL signal deriving from a labeled antibody after the recognition of the analyte (Lee et al., 2012; Huang and Ren, 2011). Huang and Ren (2011) described an immunoassay for AFP, which exploits this principle. They used Ab1 labeled with Au NPs and Ab2 labeled with HRP. In the absence of AFP, a strong CL signal derived from the reaction between luminol and H_2O_2. But in the presence of the analyte, the immunocomplex AuNPs-Ab1/AFP/Ab2-HRP was

formed and the CL signal was quenched by long-range CRET from luminol to Au NPs.

Other solid supports used in chemical luminescence-based immunoassays include glass (Yang et al., 2014; Seidel and Niessner, 2013; Roda et al., 2011a) and paper, on which biorecognition elements are chemically immobilized to develop single spot or microarray biosensors (Seidel and Niessner, 2014).

4.3. Flow-assisted systems

Microfluidic-based devices are a promising platform for the development of biosensors. This is because they enable the parallel analysis of multiple analytes in a small volume of sample, and can integrate multiple functionalities for processing samples and for generating and acquiring signals (Manz and Becker, 1998). The main advantage offered by microfluidics is the possibility of shortening analysis time thanks to the increased surface-to-volume ratio, the proximity of the analytes to the boundary layer (Rossier et al., 2000), and the rapid replenishment of depleted analytes in the boundary layer (Goldstein et al., 1999).

Moreover, a miniaturized microchannel dimension reduces consumption of samples and reagents, and offers automated integration with other functions, such as valves, pumps, mixers, and detectors (Bange et al., 2005; Lin et al., 2010). These advantages could allow POC goals to be achieved. There have been several microfluidic-based analytical platforms reported, which exploit chemical luminescence detection and which are very different from each other in terms of complexity and assay type.

4.3.1. Micro flow injection analysis

Flow injection analysis (FIA) in microchip format (micro flow injection, μFI) is based on online sample treatment and chemical detection. FIAs are characterized by reliability, rapidity, and robustness. Their miniaturization offers advantages such as low reagent consumption and reduced analysis times. The downscaling of flow-assisted systems is represented by two different approaches: lab-on-valve (LOV) (for a sequential injection based on high- precision syringe pump) and micro-machined lab-on-chip (LOC) characterized by the design of a fixed architecture on a single chip (Miró and Hansen, 2007; He et al., 2005; Guan et al., 2006). The LOV system has been used to facilitate real time detection. Sensitivity can be increased for low kinetic reactions using the stopped-flow technique. The LOC system involves low sample volumes, ranging from nano- to picoliters, allowing single molecule detection techniques (Roda et al., 2011c). Although the configuration of the flow cell in the LOV unit is ideal for combining with CL, just a few examples have been reported in the literature (Miró and Hansen, 2012). Infact, efficient and rapid reagent mixing is crucial for obtaining high sensitivity, especially when using flash-type CL reactions that last just a few seconds. Yang et al. (2006) reported the CL detection of tetracycline residues in milk, where tailor-made units coupled to a Z-type flow-through cell were used for the in situ generation of the oxidizing reagent from solid reagents at peripheral ports of the LOV. These methods have poor selectivity, due to interferences in the biological matrix, which can affect the measurement. This limitation can be overcome using on-line analyte extraction procedures integrated into microfluidic devices (Mirasoli et al., 2014b). Several examples have used flow injection techniques with chromatography, capillary electrophoresis, and multisyringe chromatography (Suda et al., 1993).

Among the separative flow-assisted techniques coupled to CL detection, field-flow fractionation (FFF) is an emerging tool. (Giddings, 1993; Schimpf et al., 2000; Roda et al., 2009a). This technique allows compounds of different shapes and sizes to be separated with total maintenance of native properties during the assay. It also allows integrated flow systems to be realized. Ultrasensitive CL detection can be combined with FFF to separate and quantify free and bead-immobilized antibodies, or to separate different cells exhibiting BL signals. This has already been reported as a promising format for developing highly sensitive, rapid, simple, and multiplexed biosensor assays (Melucci et al., 2003; Melucci et al., 2004).

A competitive immunoassay for chloramphenicol (CAF) was described. Antibody-coated microspheres were employed as a solid phase and CAF-HRP as a tracer, and samples were introduced in the FFF-CL system used for incubation, separation and analyte quantification. Free and bound tracer fractions were indeed separated during the FFF run and measured by online CL detection after a post-column flow injection of CL substrate. Moreover, the use of differently-sized microbeads coated with antibodies specific for different analytes could be adopted to develop a multianalyte immunoassay (Roda et al., 2006).

FFF devices, with a bio-functionalized wall for modulating the fractionation process, were also coupled with imaging CL detection to develop microfluidic integrated devices with high versatility and the ability to perform multiplexed assays in a short time (Roda et al., 2009b).

Further studies have been devoted to miniaturizing the instrumental set-up to obtain portable biosensor devices based on the FFF format (Casolari et al., 2013).

4.4. Microfluidic systems

To perform multiplex and rapid assays in a point-of-care setting, one of the most promising strategies is the production of capture probe (e.g., antibody) microarrays, coupled with microfluidics to deliver samples and reagents (Seidel and Niessner, 2014). The Munich Chip Reader (MCR3) has been designed as a portable stand-alone device suitable for performing fully automated flow-based CL microarray assays. This device was used for a number of multiplex immunoassays (Wutz et al., 2011; Oswald et al., 2013) and nucleic acid detection assays (Lengger et al., 2014) using regenerable chips. Using the CCD-based contact imaging approach, a miniaturized multiplex biosensor for parvovirus B19 genotyping was developed, enabling the multiplex detection of DNA from three Parvovirus B19 genotypes down to 80 pM in serum samples (Mirasoli et al., 2013).

4.4.1. Paper-based microfluidic

Another emerging platform for portable biosensors are the paper-based methods. One of the simplest microfluidic approaches is to exploit capillary forces (Zimmermann et al., 2005). Using porous materials, it is possible to generate a controlled volume flow governed by capillary action without needing external forces, like pumps or syringes, achieving the POC goal. Capillary-driven flow requires no peripheral equipment. Liquids can also flow in opposition to external forces like gravity, making it ideal for portable immunodiagnostic tests (Apple et al., 1999; Cheng et al., 2010). Moreover, diagnostic devices based on paper require minimal infrastructure and inexpensive raw materials. The device can be prototyped rapidly since paper is very easy to handle (Mace and Deraney, 2014). Luminescence paper-based devices are an interesting and growing field of research, since they couple the simplicity of the luminescence detection principle with the ease of use of the paper-based devices. One of the main paper-based formats is the lateral flow immunoassay (LFIA) (Fig. 5). Since conventional colorimetric LFIA only yields semiquantitative information with a limited sensitivity, various strategies have been pursued to enhance analytical performance, including the implementation of CL detection using enzyme labels (e.g. HRP) (Cho et al., 2009; Mirasoli et al., 2012a). A compact immunosensor,

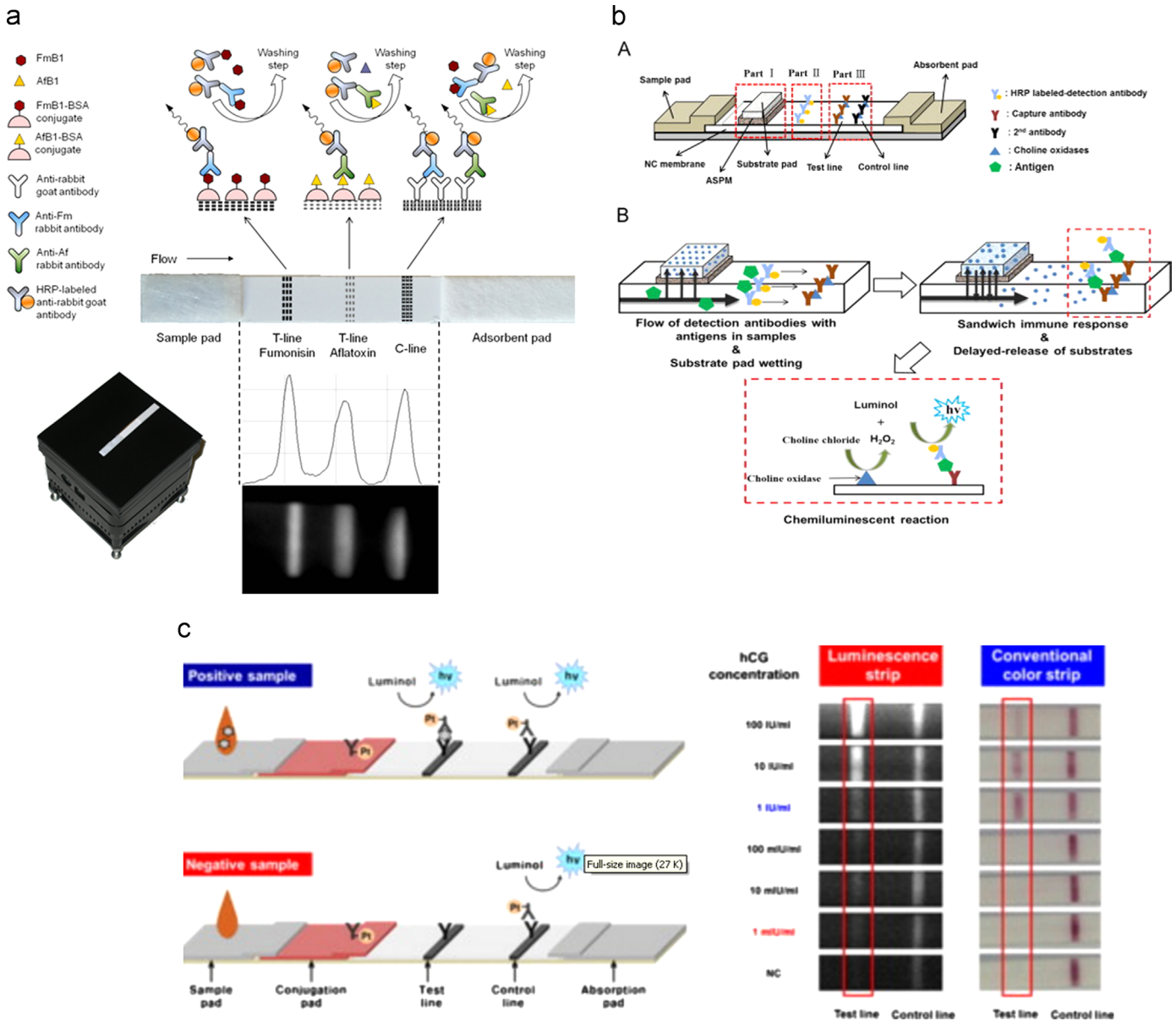

Fig. 5. Schematic representation of assays developed based on LFIA, exploiting CL detection. (a) (Top) The multiplex CL-LFIA assay on the nitrocellulose strip for the simultaneous quantification of Fumonisin type B and Aflatoxin B1. (Bottom) CCD-based device in a contact imaging detection format for acquiring CL signals from LFIA strips and the CL image with its intensity profile. (Zangheri et al., 2015a), Copyright 2015, reprinted with permission from Royal Society of Chemistry. (b) The structure and over all reaction process of the CL LFIA-based EIA system that does not require additional steps (such as mechanical fluidic control, washing, or injecting) and based on a delayed-release effect of CL substrates (luminol enhancer and hydrogen peroxide generator) by an asymmetric polysulfone membrane (ASPM) (Joung et al., 2014), Copyright 2014, reprinted with permission from Elsevier. (c) LFIA with CL signal band using Pt nanoparticles. (left) Configuration of a strip test for detecting hCG using a CL reaction of Pt nanoparticles. (right) Comparison between the sandwich assay results of hCG using anti-hCG antibodies conjugated with Pt nanoparticles and the conventional assay with gold nanoparticles (Park et al., 2015), Copyright 2015, reprinted with permission from Elsevier.

exploiting CCD-based lensless contact imaging detection, was developed to detect type-B fumonisins in maize samples, providing a useful tool for rapid on-field quantitative analysis (Mirasoli et al., 2012b). This biosensor has recently been used for multiplex and ultrasensitive on-site quantification of aflatoxin B1 and type B-fumonisins in maize samples (Zangheri et al., 2015a). Recently, CL-LFIA was successfully coupled with smartphone-camera-based photon detection for the quantitative sensitive detection of salivary cortisol (Zangheri et al., 2015b).

Despite the advantages offered by chemical luminescence biosensors, there are critical problems. First, there are integration issues. CL reagent delivery and signal detection are most often performed with external systems, which have to be coupled to the reaction chip. To overcome the problem of CL substrate addition, Joung et al. (2014) designed a new LFIA strip to sequentially control the immunoassay, the delayed release of substrates with pH change, the hydrogen peroxide generation, and the luminol reaction. The key concept relies on a delayed-release effect of cl substrates (luminol enhancer and hydrogen peroxide generator) by an asymmetric polysulfone membrane (ASPM).

As the chemical luminescence signal is not stable over time, the light-emitting species are subject to diffusion phenomena in solution, causing a loss in resolution (Cheek et al., 2001). Furthermore, when enzyme labels are used, care must be taken to keep enzyme activity unchanged, considering temperature and buffer reaction (Ramachandran et al., 2014). Finally, the biological sample constituent can have unwanted effects on the CL chemical reaction, by enhancing or inhibiting the light-producing reaction.

In terms of ECL detection, to the best of our knowledge there are only a few examples of ECL microfluidic paper-based analytical devices (or μ-PADs). Delaney et al. (2011) presented the first ECL-μPAD paper-based microfluidic sensors using a mobile camera phone as an ECL detector. Using inkjet-printed paper, fluidic substrates, and screen-printed electrodes, they constructed very low-cost, disposable ECL sensors which may be read with a mobile camera phone.

Another paper-based technology is "origami", an approach that involves layering or folding patterned paper into a 3D stack (3D μPAD), and then using a pre-fabricated clamp (or lamination) to hold the stack together. Just a few works have been published about CL origami based on the typical luminol–H_2O_2 CL system. Ge et al. (2012a) reported a 3D origami-based CL immunodevice in which the luminol–H_2O_2 CL reaction is catalyzed by Ag nanoparticles, for the simultaneous detection of four tumor markers. The origami system has greater diffusion in combination with the ECL detection. Ge et al. (2012b) proposed a 3D μPAD ECL immunosensor in which it is possible to distribute a sample from a single entry point on several testing regions, while avoiding cross-talk of one electrode signal to a neighboring one (Fig. 6a). Based on this 3D μPAD ECL immunosensor, the same research group has presented other similar applications (Yan et al., 2012; Wang et al., 2013a). Several origami ECL biosensors have recently been developed using different materials as working electrodes. Wang et al. described a graphene-modified porous Au-paper electrode for DNA hybridization (Wang et al., 2013b), while Li et al. (2013b) proposed a 3D origami multiplex ECL device based on an Ag-NPs-modified paper working electrode (Ag-PWE) and multi-labeled nanoporous gold-carbon nanospheres (NPG–CNSs) as labels. The same research group (Li et al., 2014a) presented another 3D μPAD ECL immunosensor for CEA using a nanoporous gold-chitosan-modified paper working electrode (NGC-PWE) and graphene QDs (GQDs) functionalized Au–Pt NPs (Fig. 6b). μPADs are currently the most cost-effective approach for developing portable and disposable devices. They could be further improved using TCL detection, which requires only the implementation of μPAD with a miniaturized heater without the need to add reagents.

5. Preanalytical step

5.1. On-line sample treatment and cleanup

Despite the fast development of new biosensors with different analytical formats, on-line preanalytical steps, which include sample clean up, analyte enrichment, and matrix treatment, remain an open issue. To improve selectivity, separation and

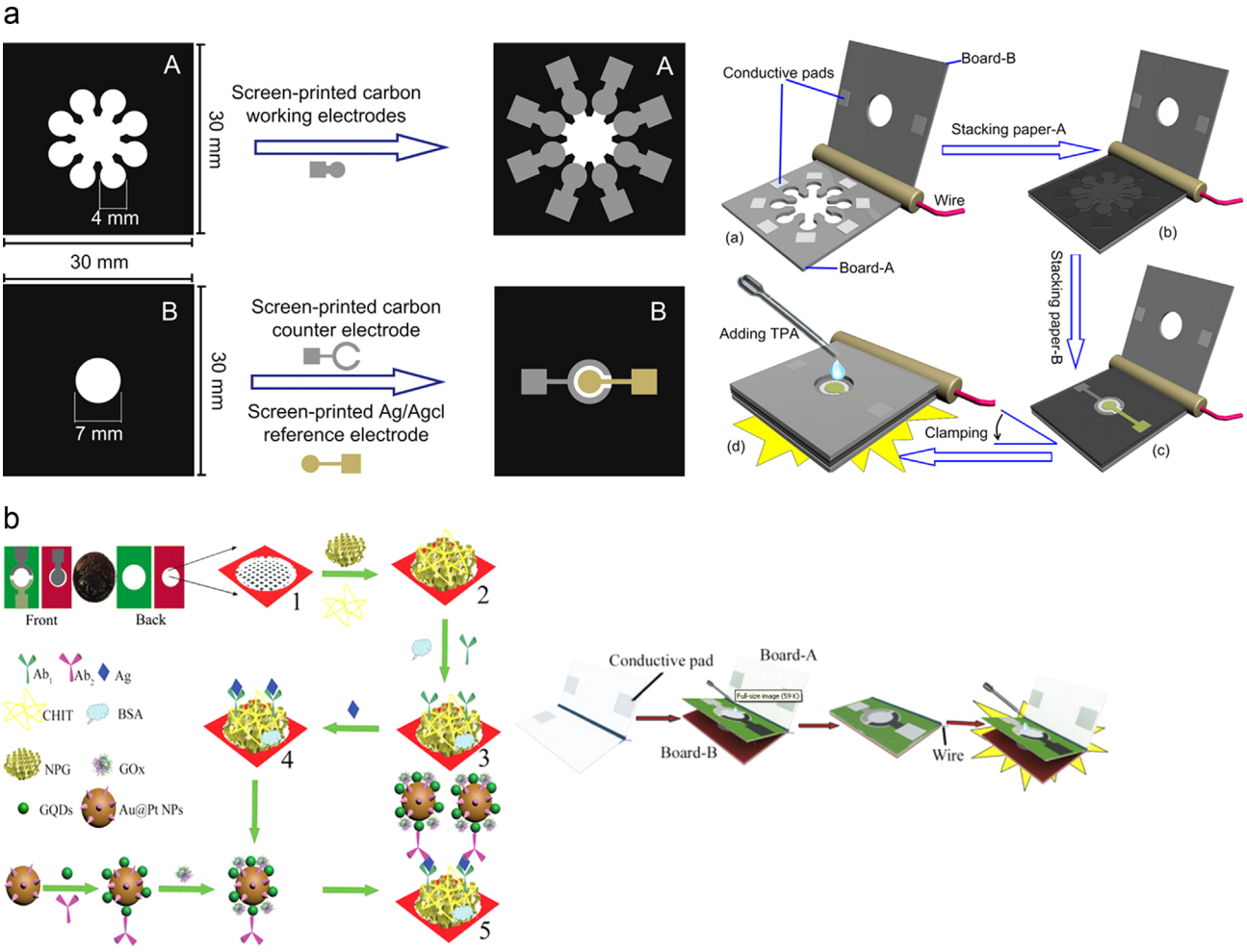

Fig. 6. Schematic representation of different 3D origami-based devices. (a) The schematic representation of the manufacturing process for a simple homemade 3D paper-based ECL device. Paper sheets were firstly patterned in bulk using a wax printer, then electrodes were screen-printed on sheet-A and sheet-B respectively in bulk. Finally, sheet-A and sheet-B were cut to paper-A and paper-B with the same size (30.0 mm × 30.0 mm) (Ge et al., 2012b), Copyright 2012, reprinted with permission from Elsevier. (b) The schematic representation of the fabrication procedure for the immunosensor for CEA using nanoporous gold-chitosan-modified paper as working electrode (NGC-PWE) and graphene QDs (GQDs) functionalized Au–Pt NPs. It is integrated with a transparent device-holder. Glucose addition triggers the ECL reaction (Li et al., 2014), Copyright 2012, reprinted with permission from Elsevier.

purification procedures have been integrated into microfluidic devices, which use structural materials that can on-line extract the target analyte (Browne et al., 2011; Thongchai et al., 2010; Lin et al., 2011). Currently, microchip-based analytical systems mostly involve capillary electrophoresis (microchip capillary electrophoresis; MCE) as a separation technique (Janasek et al., 2006). The miniaturization obtained by MCE systems adds positive features, such as portability and minute consumption of the sample and reagents. MCE with chemical luminescence detection (either CL or ECL) has been exploited for different applications (Mangru and Harrison, 1998; Liu et al., 2003; Hashimoto et al., 2000; Tsukagoshi et al., 2000). For example, Tsukagoshi et al. reported a novel concept for a μ-TAS, incorporating CL detection and an immunoassay. They used isoluminol isothiocyanato (ILITC) as a CL reagent, microperoxidase as a catalyst, and hydrogen peroxide as an oxidant. The system was applied to human serum to detect cancer markers (Tsukagoshi et al., 2005).

FFF systems have also been exploited. An FFF-CL system was used for a new non-competitive immunoassay to detect pathogenic bacteria in enriched human fecal samples. The method combines the high sensitivity of CL detection with the FFF ability to efficiently separate the bound bacterium-antibody complexes from the free antibodies. (Magliulo et al., 2007). A compact FFF-CL system was developed as a new bioassay format for blood samples.

As proof of principle, the direct on-line analysis of plasma alkaline phosphatase activity, a biomarker of obstructive liver diseases and bone disorders, was developed, starting from whole blood samples. The FFF-CL POCT system gives quantitative results on blood samples from control subjects and patients with low sample volume (0.5 μL), low reagent consumption, short analysis time (10 min), high reproducibility, and a linear range of 50–1400 IU L^{-1}(Casolari et al., 2013).

5.2. Chemical luminescence-based biosensors: analytical performance comparison

Chemiluminescence detection has been successfully used in conventional microtiter-plate-based immunoassays (CLIA) using HRP and AP as enzyme labels or direct isoluminol or acridinum ester labels.

CLIA and ECLIA are the most frequently used methods in clinical chemistry automated laboratories, fully replacing the use of radioisotopes (RIA) and photoluminescence labels.

Fully automated analyzers based on Biochip Array Technology (BAT, Randox, Seidel and Niessner, 2014) and Liaison (Diasorin spa Vercelli Italy) based on paramagnetic bead solid phase and isoluminol label are used in routine assays for clinical, veterinary, and forensic chemistry.

ECL is used in one of the most successful automated immunoassay (ECLIA) stations in the clinical chemistry field. The Cobas e 601 Analyzer combines ECL and magnetic particles to achieve highly sensitive and selective detection (Roche Diagnostics).

CLIA and ECLIA are the most frequently used methods in clinical chemistry automated laboratories, fully replacing the use of radioisotopes (RIA) and photoluminescence labels (FIA).

The high detectability combined with a simple transduction electronics technology make these systems particularly suitable for miniaturization and biosensing development. Nevertheless, just a few prototypes have been commercialized, despite many published reports describing different biosensors using new materials, nanotechnology, and detection devices.

Most of these prototypes are relatively new and we expect some technologies to be commercialized soon, particularly in the field of POCT, where sensitive and multiplexed approaches are required to measure several analytes simultaneously (Zangheri et al., 2015a; Mirasoli et al., 2013; Oswald et al., 2013).

The use of a photon emission phenomenon generated by a chemical reaction offers undoubted advantages over other optical technologies, which are based on light absorption measurements or photoexcitation. The continuous technological improvements in photon detectors create more challenges for such systems. That is because the devices are very small, requiring simple electronics and related technology. This is the case, for example, when using the photocamera of the newer smartphones and tablets as a detector (Roda et al., 2014a; Roda et al., 2014b; Zangheri et al., 2015b; Delaney et al., 2011; Hao, 2013).

Additional aspects in favor of the expected growth of these biosensors in the possibility of operating in a reagentless mode. This is true for TCL (Roda et al., 2012; Di Fusco et al., 2013) and, to some extent, for ECL techniques (Liao et al., 2013; Zhao et al., 2004a, 2014b; Zhou et al., 2014a). Under these conditions, the system is further simplified, requiring only the addition of the sample and the trigger of the luminescence by heat or electricity. We also expect future growth based on using nanotechnology to prepare new grapheme-based electrodes, nanotubes, fast and ultraminiaturized heating systems, and functionalized nanoparticles including CL or TCL molecules as amplified labels (Roda et al., 2012; Di Fusco et al., 2013).

Since so many parameters affect sensitivity precision and overall analytical performance, it is difficult to draw comparisons with other biosensing technologies, particularly with electrochemical biosensors and photoluminescence- or absorbance-based biosensors.

In Table 2, we collect the data obtained by different biosensing technologies when quantitatively measuring H_2O_2. This analyte is involved in all the enzyme oxidases (glucose oxidase, peroxidase, lactate oxidase) that have been measured with the biosensors described above.

The lowest LOD was obtained by CL, down to pmol L^{-1}. An electrochemical biosensor exhibited similar LOD, albeit slightly higher. A higher LOD was obtained using ECL and fluorescence or colorimetric detection.

Although this sample is not fully representative, it demonstrates that the high detectability achieved in a small volume of sample and reactants is one of the most appealing properties of some chemical luminescence biosensors.

The specificity and selectivity of these biosensors are mainly determined by the recognition element, which could be an enzyme (catalytic) or antibody/nucleic acid (affinity). This is true for all the biosensors, independent of the format. Moreover the transduction when a label is used should only be related to the biospecific interaction. It should be without interferences caused by variations in the recognition reaction, such as variation in the

Table 2
Comparison of LOD for the determination of H_2O_2 using different detection techniques.

Method	LOD	Reference
CL	0.025 nM	(Rubtsova et al., 1998)
	0.3 nM	(Pontén et al., 1995)
	0.4 nM	(Yuan and Shiller, 1999)
ECL	0.1 μM	(Zou and Ju, 2004)
	0.2 μM	(Guo et al., 2009)
	0.1 μM	(Cui et al., 2007)
BL	0.5 μM	(Van de Bittner et al., 2013)
	2.5 μM	(Van de Bittner et al., 2010)
Fluorescence	50 nM	OxiSelect™ Hydrogen Peroxide Assay Kit
Colorimetric	1 μM	OxiSelect™ Hydrogen Peroxide Assay Kit
Electrochemistry	0.4 nM	(Salimi et al., 2007)
	1.0 nM	(Santhosh et al., 2006)
	7.5 nM	(You et al., 2003)

pH or interference by sample constituents.

6. Conclusions

A critical review of the data and literature suggests the potential of chemical luminescence to compete with other more commonly used transduction principles based on electrochemistry or fluorescence. Miniaturized systems, using immobilized reagents on new materials and with nanoscale technology, have helped improve the analytical performance of these biosensors.

The main focus of chemical luminescence biosensors is the ultrasensitive detection and the portable point-of-need format using affinity-based recognition elements spanning from antibodies, nucleic acid to imprinting polymer and aptamers.

The main advantage compared to other systems is the simplified instrumentation for measuring the emitted light. The main contribution is the improved CCD and CMOS sensitivity. This is thanks to the improved size of the detection CCD area and the reduction in instrumental noise, achieved with the back-illuminated format. Using cooled CCD with a Peltier principle drastically reduces thermal noise, while retaining a device that is small, portable, and battery-powered. Despite advances in PMT technology and the use of an array of PMT, they have been almost replaced by CCDs. Not only can CCDs measure the light like a PMT, they can also image the emitting area, obtaining a 2D image adequate for multiplexing and independent information in one measurement run.

New smartphones combine improved camera sensitivity with the connectivity of a cellphone. Integrating CL biosensors into a smartphone will allow researchers to exploit these improvements to create low cost solutions to analytical challenges.

These improvements have been paralleled by the exponential increase in photodiode sensitivity. This creates new opportunities to further integrate the biological element with the detection system. We recently designed a miniaturized array where each spot is addressed by a photosensor. Thus, the results on multiple spots are obtained by each photosensor instead of by 2D imaging with a CCD. The system is more miniaturized and all the devices could be disposable. But this advantage must be balanced against the fact that, despite their simplicity, the electronics must be highly calibrated to ensure a similar response from all the photosensors used.

Constructing a biosensor is relatively straight forward. However, if the prototypes are to be commercialized, multidisciplinary teams are required, which include specialists in electronics, mechanical design, and biotechnology.

These new biosensor formats could revolutionize the way in which analytical information is retrieved. This will allow the detailed study and automatic control of certain processes, mainly in the fields of clinical analysis, fermentation, food technology, and wastewater treatment. A suitable panel of biomarkers and an administered drug could be simultaneously monitored, in real time, with a minimally invasive approach. This would allow the dose of a given drug and its pharmacological effect to be precisely controlled, increasing the potential of personalized medicine and companion diagnostics.

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