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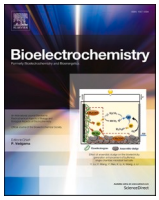

Electrochemiluminescence within veterinary Science: A review

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ABSTRACT

Veterinary science or veterinary medicine is a diverse and significant field. Concerned not only with the diagnosis and treatment of domestic animals and livestock, but it also places focus upon zoonotic diseases, the development and effectiveness of potential vaccines and the possibility of transmission of veterinary medication or viruses into animal food products. Electrochemiluminescence (ECL) is a powerful analytical technique, which despite its significant intrinsic benefits has not seen enormous adoption into the wider analytical chemical community. In contrast, the veterinary science sector has reaped the merit of ECL as far back as the late 90′s and continue to benefit from development of the technique a further three decades later. ECL offers the superb sensitivity, low running costs, rapid results and high reliability required within the veterinary science sector, as such its employment in this area shouldn't be surprising. To this end this article aims to summarise the standing of ECL within the veterinary science field, in an attempt increase the awareness of its successful employment within this area to the electro-analytical and wider analytical chemistry communities. Where it is hope veterinary science will gain recognition as possible end user targets for academic and industrial electrochemical researchers.

1. Introduction

Electrochemiluminescence or electro-generated chemiluminescence (ECL) is beginning to emerge as a leading transduction technique owing to its optimal combination of electrochemical and spectroscopic characteristics [1,2]. ECL measurements are based upon the recording of emitted light following highly energetic electron transfer between two electrochemically generated radicals, forming the electronically excited state. Relaxation of this state, results in the emission of a photon proportional to the concentration of species involved in the redox processes [1–8]. Despite its inception in the 1960′s [9] it is only within the last decade that ECL has been embraced within the general analytical and bio-analytical communities. ECL is endowed with a variety of intrinsic benefits over the traditional analytical approaches namely; higher sensitivity, spatial control, notably lower background noise (owing to the lack of external light source), operational simplicity, significantly reduced instrument footprints (which promote portability and external laboratory use) and a relatively low instrument cost [1–8]. All these elements have only been heightened by the technological advancements of the last decade facilitating the large reduction in instrument size and complexity, ensuing a wider adoption of electrochemical sensors within the biomedical industry and heightened interest of ECL within both the academic and industrial sectors [1,2,5,10,11].

Today ECL is considered a powerful addition to the bio-analytical toolbox with a growing number of bioassays employing the technique witnessed. Extensive reviews have discussed at length the advantages of ECL for such applications and recent research developments provide enhanced confidence in the suitability of the technique for this field [1,4,12–16]. With the number of publications concerned with the use of ECL for bio-analytical monitoring ever expanding, one begins to considered what other fields may or indeed have benefited from these inherent advantages. One field which has significantly benefited from the developments of ECL is that of veterinary science. Yet despite the vast employment of the technique in both a research and commercial settings, to the best of our knowledge no comprehensive review of its use and impacts within this field have been presented to the ECL community.

Veterinary science or veterinary medicine is a medical field specifically focusing on domestic, farm and wild animals. The field is concerned not only with animal diagnostics, care and treatment, but also in the control and prevention of zoonotic diseases; that being diseases which can cross species including transmission to humans, the monitoring of veterinary drug residues within animal food products, alongside other priority areas including development of vaccines, surgical techniques and predictive statistics both with domestic and livestock consequences. In fact, veterinary science was directly responsible for the

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reduction in human exposure to tuberculosis [17], improvement in surgical techniques for hip replacements and organ transplants [18,19], and even the first anti-cancer vaccine which has been developed for the the control of Marek's disease in chickens, an oncogenic alpha-herpesvirus that produces deadly T-cell lymphomas [20–22]. These factors indicate that more collaboration between the veterinary science, biomedical and analytical chemistry fields can produce fruitful findings for both species and thus promotes the desire for continued and improved communication between these sectors. Indeed, it appears that the veterinary field has commercially employed ECL for a number of years using a range of luminophore's, including the traditional ruthenium complexes but also iridium, osmium and rhenium [23]. A fascinating fact given the significant current interest of these alternative metal luminophore's within the ECL community [24–32]. As such this bares the question if ECL specialists could indeed draw upon the findings and developments already in place within the veterinary science sector to expedite their development of new ECL technologies. To this end we hope herein to discuss the use of ECL within the veterinary science field, to highlight it success and begin to form a bridge between the electro-analytical community and those within the veterinary science field, who are actively employing ECL monitoring. In addition to raising awareness of other sectors employing the technique not considered within the traditional electro-analytical scope.

2. ECL applications within livestock care

2.1. Disease detection

The use of ECL within the veterinary science field can be seen as far back as the late 90s, where the technique was employed for animal diagnostics [33]. One of the first examples came from the study of Yamaguchi *et al.* [33] who pursued an ECL based approach for the detection of Borna disease virus (BDV) [33]. BDV is a highly contagious and often fatal viral infection which effects the grey matter of the brain stem, manifesting in the clinical signs shown within Fig. 1 of depression with apathy, somnolence, and stupor [34]. The authors choose to develop a new ECL based immunoassay approach for the monitoring of BDV within a Japanese feral horse population, over the alternative and more widely employed techniques used for the screening of this disease including, immunofluorescence assays (IFA), enzyme-linked immunosorbent assay (ELISA) and Western blots. These far more typical techniques, however encounter a range of problems regarding BDV detection, prompting the authors to investigate their alternative system [33]. Specifically noting that IFA suffers from poor sensitivity coupled with its subjectivity as a consequence of user interpretation. ELISA and Westerns blots are, as identified by the authors, more sensitive and reliable but suffer in regard to their complexity and time-consuming nature alongside their expense [33]. As such, the motivation lay to develop a new approach which offered the required superb sensitivity at a relatively low cost with high reliability, which ECL could theoretically offer. Utilising BDV p40 and p24 recombinant proteins, the authors proposed a ECL immunoassay system where a combination of $[Ru(bpy)_3]^{2+}$ with the anti-horse IgG polyclonal antibody was coated upon magnetic micro-beads [33]. Following incubation and washing, the magnetic beads were then coated onto the working electrode surface for analysis. As is typical for ECL systems, the authors required a reliable cut-off point, below which a sample was regarded as negative for BDV [33]. Utilising this methodology the authors were able to determine that 16 of the 90 feral Japanese horses screened where indeed BDV positive, confirmed with comparison to IFA and Western blot results [33]. Yamaguchi *et al.* [33] methodology was proven as a viable ECL immunoassay system for the identification of BDV within equine and human serum. Utilising the traditional ruthenium luminophore with magnetic micro-beads a sensitive and reliable methodology was demonstrated, offering improved precision, dynamic range and shortened analysis time to that of the traditional assay methods [33]. Such a system being developed in the infancy of the field provided a high degree of confidence toward the future use of ECL within the veterinary community. The authors furthered this assessment through a second successful study concerned with the detection of BDV in both domestic and feral horse as well as rat serum, alongside a third and final publication detailing its continual use over a 4 year period for monitoring of the viral spread within the same feral horse population [35,36]. This provided a significant degree of confidence in the stability and reliability of their developed methodology. Yet its use following these initial publications was not observed, this was in-spite of the increased sensitivity, reliability and precision compared with the traditional IFA, ELISA and Western blot systems. In fact only one subsequent publication employing this ECL immunoassay has since been reported, 16 years following the initial publication, by Torres-Castro *et al.* [37] who utilised the same ECL immunoassay system described by Yamaguchi *et al.* [33] for the detection of BDV antibodies within domestic Mexican horses, for the first time [37]. The reasoning behind this lack of adoption of the ECL technique could be a consequence of the poorer understanding of the technique in general, and limited awareness of the huge benefits it offers. ECL can be considered a somewhat niche technique with its awareness even within the analytical chemical community limited. Although this has begun to improve over recent years Fig. 2.

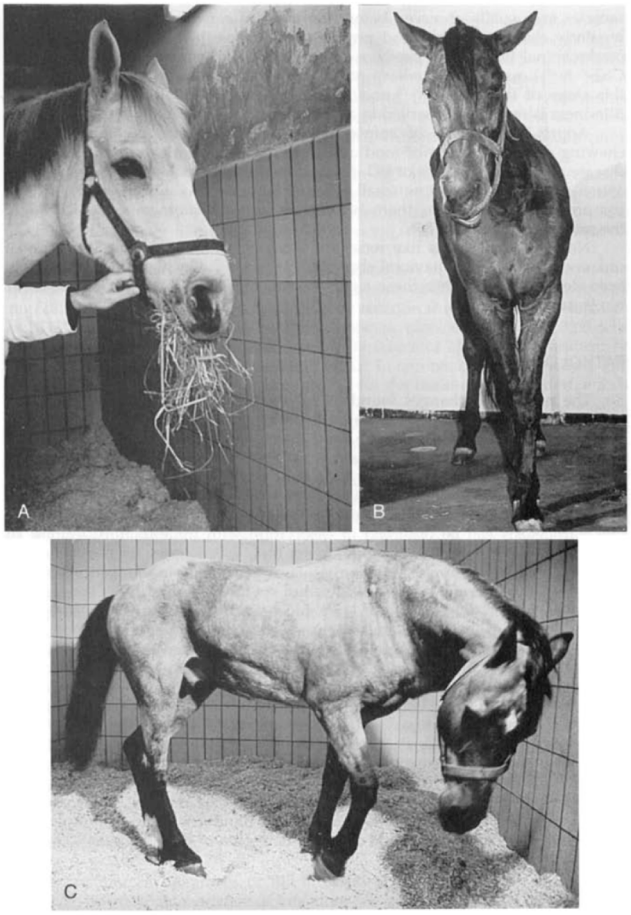

Fig. 1. Clinical manifestations of Borna disease in horses, showing; *A* somnolence and displaying characteristic arrested, eating with chewing movements termed "pipe smoking". *B* disturbances in proprioception (abnormal posture) and paralysis of the facial nerves and *C* neurogenic torticollis and compulsive circular walking. Reproduced from ref.34 with permission from Elsevier, Copyright 2000.

The use of ECL within animal husbandry has however progressed beyond the initial reports of BDV detection for the screening and identification of other animal born diseases of concern. Such as, Foot-and-

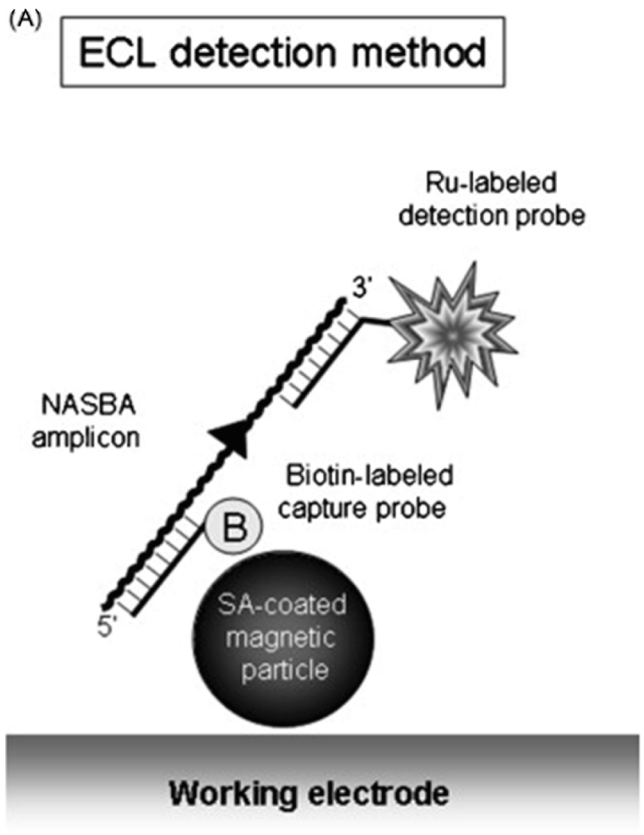

Fig. 2. Schematic of the NASBA-ECL detection principle employed by both Collins *et al.* [41] and Lau *et al.* [42]. The biotin-labelled capture probe is attached to the magnetic bead via biotin-streptavidin interactions. The 3′ end complements the forward primer product, and the ruthenium-labelled detection probe complements the reverse primer. The ECL probe produces luminescence at 620 nm upon application of a potential. Reproduced from ref.42 with permission from Elsevier, Copyright 2008.

mouth disease (FMD), likely one of the most widely recognised and infectious livestock viruses. FMD, a highly contagious virus, affects cloven-hoofed animals such as cattle, sheep, pigs and goats [38–42]. If not identified and immediately controlled it will spread rapidly, even across species, posing a huge threat to the livestock industry. FMD is a single-stranded RNA virus belonging to the *Picornaviridae* family, and exists as seven genetically distinct serotypes (A, C, O, Asia-1, SAT-1, SAT-2 and SAT-3) [38–40]. Clinically, the disease presents as an acute fever followed by the appearance of blisters within the mouth and feet of the infected animals, hence its name [38–40]. A major concern with regard to FMD lies amongst the asymptomatic cases, or those which have recovered but still carry a viral load, whom then go on to spread the virus undetected [38]. A significant hurdled in the management of FMD outbreaks is the difficultly surrounding the rapid analysis of livestock for the virus, a lack of which can see entire uninfected herds slaughtered as a preventative measure [41]. Identification of FMD commonly involves cell culture for virus isolation, immunoassays for antigen detection or nucleic acid testing via PCR and RT-PCR techniques [40–42]. RT-PCR is by far the most common methodology but is considered slow and cumbersome, given the use of gel electrophoresis for detection. As such, rendering it unsuitable for the monitoring of mass herd outbreaks [40–42]. A potential solution for the monitoring of such potential mass outbreaks was proposed by Collins *et al.* [41] and subsequently Lau *et al.* [42], both of whom proposed an alternative methodology utilising nucleic acid sequence-based amplification (NASBA) coupled with ECL [41,42]. Both author sets use of the NASBA-ECL systems offered a viable solution to the drawbacks of the traditional techniques, including a lack of sensitivity and considerable analysis time, combined with an aptitude for contamination. Collins *et al.* [41] initially reported the use of NASBA-ECL for the discrimination of the different FMD serotypes in 2002, with the complementary study of Lau *et al.* [42] following 6 years later. Their technology employs a single-stranded RNA amplification strategy via three enzymes; reverse-transcriptase, ribonuclease-H and T7 RNA polymerase, a specific set of forward and reverse oligonucleotide primers with two types of detection probe [41,42]. The reverse primer contains a binding sequence for the DNA oligonucleotide probe which is labeled with the ruthenium-based ECL tag, as described within Fig. 2 [41,42].

An important factor identified by both sets of authors was the requirement for the inclusion of an internal reference standard (IRS) [41,42]. An internal standard provides a cut-off or limit, below which any emission is regarded as system intrinsic and won't be identified as FMD. Such a value, assigned here as 0.025xIRS (~30–40,000 A.U), is vital for any data interpretation particularly if a method is to be translated from research laboratory to in-field applications. It is thus imperative that this cut-off is determined with significant confidence. Both authors utilised a commercial IRS to achieve this confidence, while highlighting availability of commercial ECL systems even within the early 2000s [41,42]. As is the case for many ECL systems, the traditional ruthenium luminophore was utilised. Application of an external potential to the sample system will produce luminescence at ~ 620 nm, where intensity is proportional to the number of amplified nucleic acid products [41,42]. Utilisation of the ECL-probe achieved detection limits of the seven different serotypes of FMD at significantly lower levels than the traditional antigen ELISA system, with a reported 1.6–16 fold increase in sensitivity observed [42]. The increased sensitivity noted by Collins *et al.* [41] was further observed in the later publication of Lau *et al.* [42] Here the authors also compared the NASBA-ECL system to real-time RT-PCR and virus isolation to determine the correlation of positives between these different techniques. They found that 87% of samples analysed where in agreement with the RT-PCR, indicating that NASBA-ECL has an equivalent sensitivity to RT-PCR [42]. Of the 140 samples, only 14 positive samples were not identified by the NASBA-ECL system but had been positively assigned via RT-PCR [42]. A further 15 samples were identified via NASBA, which were not detected via traditional virus isolation, but confirmed with corresponding positives via RT-PCR.

The NASBA-ECL system developed bore far reaching consequences, offering the ability too rapidly identified the presence of FMD, the authors could save valuable livestock from slaughter. By providing the ability to identify those animals free from the disease with a serotype specific system, the NASBA-ECL avoids the pitfalls of antibody or virus isolation methods. The specificity of the systems and proven ability to identify isolates initially testing negative, provides a degree of confidence in the use of this rapid ECL system for minimising potential FMD spread, preserving the agricultural economy and meat supply.

Of course since this time the NASBA-ECL system has had further success with reports on the identification of avian influenza [43,44], Newcastle disease [45] and dengue fever [46]. The NASBA-ECL system is as such regarded as a highly sensitive, specific and accurate methodology for the rapid diagnosis of animal viral infections. However, the one significant draw back identified is the cost associated with its use. Although the this is not such a concern within developed countries, in developing countries where access to supplies, suitable laboratory space, equipment and trained personnel are not always available. As such, this does present a notable and unavoidable disadvantage of the system. Continued development of technologies does aim to lower these costs and have in the past decade made significant strides in achieving this particularly since the early 2000's when the majority of the NASBA-ECL research was published.

These early studies indicated that the benefit of ECL based detection strategies toward the detection of infectious diseases were gaining traction within the field of veterinary science. Only confirmed through the continued employment of ECL with the field with further articles

published during 2020 and 2021. Avian leukosis viruses (ALVs), are a group of oncogenic retroviruses which lead to the growth of neoplasms (both begin and malignant) within poultry, namely chickens [47,48]. There are 10 subgroups of ALVs classified from A to J. Sub-group J is considered the most virulent and as such most damaging to commercial farming and the wider economy [47,48]. Since its discovery sub-group J has resulted in significant economic losses to the world wide poultry industry, resulting from its strong pathogenicity and high infection and consequently transmission rate [47,48]. To date, no effective vaccines to specifically eradicate ALV-J have been successfully developed, hence it remains necessary to actively monitor and rapidly identify effected poultry to prevent viral spread, protecting the healthy birds. Several detection strategies have been employed for the detection of ALV-J including PCR and ELISA, however these methods are impeded by the aforementioned limitations namely cost and time to perform. Once again the benefits offered by ECL to circumventive these limitations at low cost appears obvious. As such, the resultant employment of ECL for the detection of ALV-J is not a surprise. Several methods have been used for the development of ALV-J ECL biosensors with a variety of different constructs used to achieve maximum signal amplification from the $[Ru(bpy)_3]^{2+}$ luminophore. In 2018 Liu *et al.* [49] proposed the use of hollow MnO_2 nano-spheres, to encapsulate the cationic $[Ru(bpy)_3]^{2+}$ luminophore within, with the exterior surface functionalised with poly (diallyldimethylammonium chloride) (PDDA), poly (acrylic acid) (PAA)

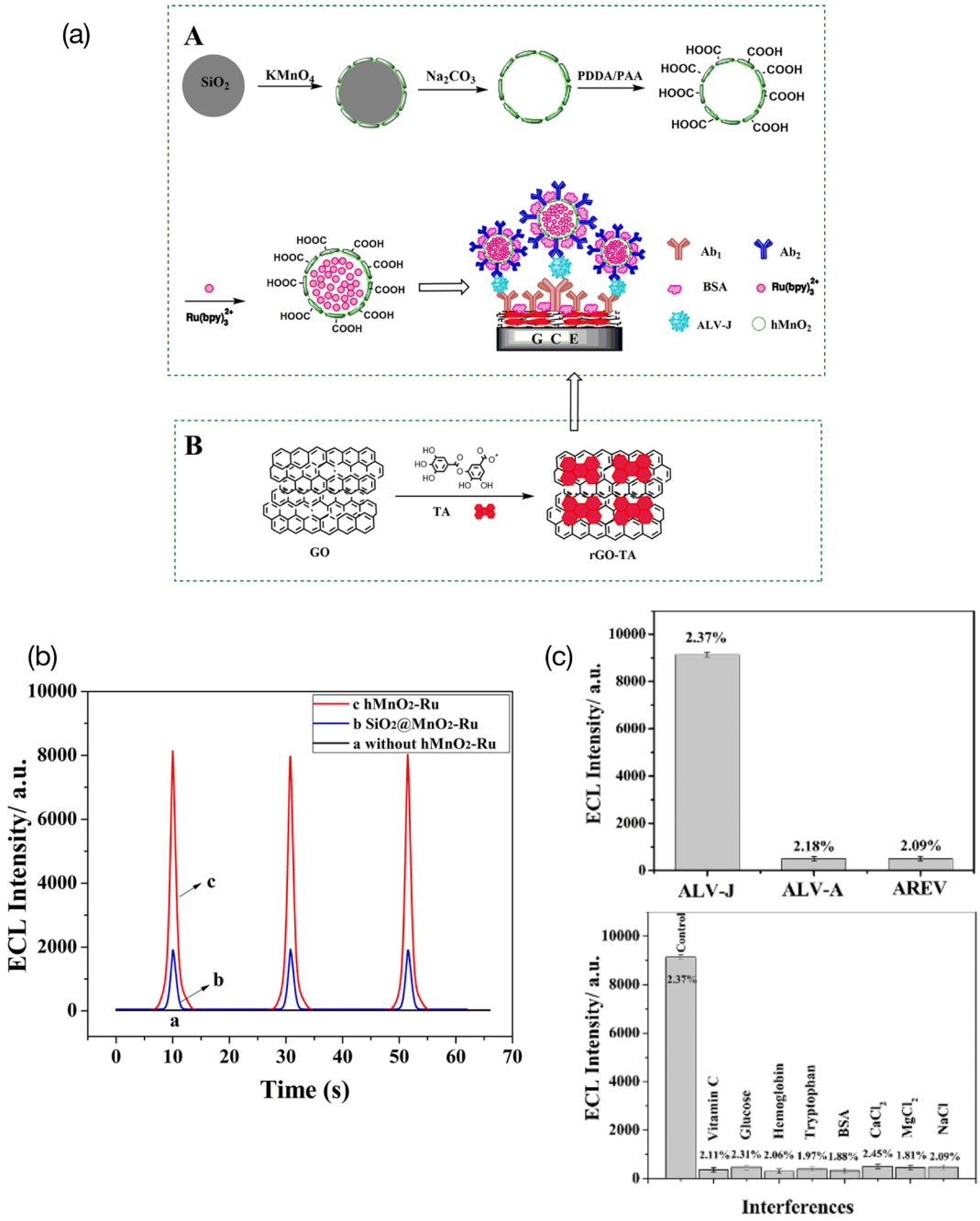

Fig. 3. (a) Schematic detailing the fabrication of the $hMnO_2$-PDDA/PAA-Ab_2 and the sandwich-type immunosensor (A) and the rGO-TA (B). (b) ECL intensity comparison of the sandwich assay without $hMnO_2$-Ru (black), with SiO_2@MnO_2-PDDA/PAA-Ru (blue) rather than the hollow nano-spheres and finally with the $hMnO_2$-PDDA/PAA-Ru (red) highlighting the amplification seen through inclusion of the hollow MnO_2 nano-spheres. (c) ECL intensities recorded using the developed sensor from a variety of relevant interferents highlighting superb specificity. Reproduced from ref. 49 with permission from Elsevier, copyright 2018. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

and the secondary antibody [49]. The glassy carbon (GC) working electrode was modified with reduced graphene oxide, functionalised with tannic acid forming covalent bonds to the primary antibody, thus immobilising it upon the electrode surface [49]. The entire process of the authors sensor fabrication is depicted within Fig. 3 (a) and operates on the principle of a sandwich type electrochemical assay, where ECL emission is based upon the well established TPA mechanism [49]. The inclusion of their hollow MnO_2 nano-spheres lead the authors to observe a significant increase in signal intensity gifting a high degree of sensitivity toward ALV-J [49]. Confirmation of the signal amplification strategy was a direct result of the hollow MnO_2 nano-spheres, as demonstrated via the typical TPA system with an ~ 4-fold signal increase witnessed, shown within Fig. 3 (b) [49]. Utilisation of their unique fabrication strategy achieve a sensitivity down to $10^{1.71}$ $TCID_{50}$/mL [49], with remarkable specificity with no signal amplification noted for a number of interferents including ALV subgroup A or avian reticuloendotheliosis virus (see Fig. 3 (c)) [49]. Despite their obvious signal amplification strategy however, the sensitivity achieved by the authors was comparable to the traditional methods [49].

Further developments by Zhou *et al.* [48] seen a further MnO_2 construct for the detection of AVL-J. Similar to Liu *et al.* [49], the authors employed a sandwich style immunoassay with the $[Ru(bpy)_3]^{2+}$ ECL label [48]. Here rather than the hollow shell nano-spheres, flower-like nano-clusters of MnO_2 were employed. These flower-like 3D clusters were then functionalised with palladium nanoparticles (PdNP) via $–NH_2$ binding from polyethyleneimine (PEI) inclusion [48]. The $[Ru(bpy)_3]^{2+}$ label was introduced and finally the secondary anti-bodies, completing the detection probe. Here, the working electrodes were modified via AuNP, gifting high stability and biocompatibility, with the primary antibodies bound to the AuNP directly via the $–NH_2$ groups upon the gold surface [48]. By utilising both AuNP and MnO_2 the authors focused their sensor development on components of high biocompatibility, tuneable morphology and environmentally friendly elements [48]. These are key focus areas, which have likely lead to the upsurge of these materials in recent years; with applications including biosensors, energy storage, catalysis and molecular imaging [48]. For ECL bioassays they also boost significantly high surface areas allowing for high loading of the signal molecules, in this case the ruthenium luminophore, gifting significant enhancement effects and achieving the desired sensitivities for ECL systems to offer viable alternative technologies for virus detection [48].

In contrast to the system of Liu *et al.* [49] where the TPA system was utilised, here another common ECL co-reactant was used, $K_2S_2O_4$. Unlike the TPA system, persulfate works via the reductive-oxidation mechanism, where the $[Ru(bpy)_3]^{2+}$ label is first reduced rather than oxidised via the application of negative potentials [48]. The ECL mechanism identified the PdNP as the key component for the production of the luminescence enhancement effect [48]. The impact of the enhancement was studied in the same manner as that of the hollow nano-spheres where the the sandwich assay was tested with and without the addition of all the sensor components [48]. Once again enhancement was observed at ~ 4-fold intensity increase cf. the absence of the PdNP, with remarkable specificity toward the AVL-J subgroup virus also demonstrated [48]. Inclusion of the PdNP into the MnO_2 3D nano-clusetrs alongside the AuNP electrode modification lead to an increase in sensitivity, more so than the hollow nano-sphere graphite system used by Liu *et al.* [49] two years prior, with a detection limit of $10^{1.90}$ $TCID_{50}$/mL ~ 1.5 times greater [48].

Both sets of authors demonstrated the ability to utilise different constructs and components involving MnO_2 species to significantly enhance the intensity of luminescence from the traditional ruthenium luminophore. Both author sets utilised different electrode modification strategies, co-reactants and probe constructions but achieved similar enhancement effects with both reporting an ~ 4-fold increase. Not only was the ability to detect AVL-J shown with comparable sensitivity to current ELISA methods at lower cost and time expense but with superb specificity with differentiation achieved even between subgroups of the virus.

All these developments highlight the success of ECL for the detection of a number of viruses of concern within the animal husbandry sector. With ECL based systems dating back to the late 90′s and research continuing almost 4 decades later a precedent exists in the veterinary science field of a degree of confidence in the utilisation of the technique. Such an embrace of ECL has not translated across to the wider analytical chemical community, where it is most often considered a small niche area. Consequently, consideration of the successes demonstrated within the field of animal virology must be acknowledged, and translation of the same theories and methodologies across to human virology begun. However, virus detection isn't the only area where ECL has proven successful within the veterinary science sector other areas which have adopted the technique include animal welfare and the identification of trace residues of veterinary drugs into animal-based products has also been observed.

2.2. Identification of veterinary medicine within animal products

Veterinary pharmaceutical drugs such as antibiotics, antiparasitics, and hormonal compounds are routinely used in animal agriculture to treat and prevent disease or improve feed efficiency. Although these are essential for animal welfare, the ability of residues of the parent molecule or metabolites of such compounds to persist in animal-derived products poses significant food safety and environmental risk [50,51]. In response to these threats, strict national and regional legislation has been imposed on maximum residue limits (MRL); that being the maximum concentration of residues allowed in or on food items produced from livestock [52]. Ergo, the need to screen for these residues has arisen, a sentiment which has been echoed by the European Commission in Regulation (EU) No 37/2010. The screening methodologies adopted for this should at the very least be able to detect the drug of interest at the maximum residue limit. Other desirable features include rapid detection, simple operation, and applicability to a wide range of food types and biological fluids. For these reasons, ECL-based sensors have been investigated for their performance as residue detection systems.

Hitherto, antibiotics have been the focal point of residue detection via ECL, most likely due to the ongoing antibiotic resistance crisis, one of the most concerning global public health issues. The ESKAPE group, comprised of *Enterococcus faecium, Staphylococcus aureus, Klebsiella pneumoniae, Acinetobacter baumannii, Pseudomonas aeruginosa,* and *Escherichia coli* have become the most life-threatening multidrug-resistant and virulent pathogens [53]. All six ESKAPE bacterium are found in livestock and so antimicrobial agents are frequently used for their treatment within veterinary medicine [54]. Kanamycin (KAN) is a broad-spectrum antibiotic that when used in animal husbandry often persists in produce. In Great Britain, the Veterinary Medicines Directorate (VMD) has imposed strict MRL's on KAN in various target tissues. The detection of KAN in milk has been shown with the use of an ECL aptasensor [55]. In this work by Cheng *et al.* [55], a conventional three-electrode system with a platinum working electrode was developed that used silver nanoparticles and KAN aptamers to increase the specificity and sensitivity of the sensor [55]. Analytical performance of the fabricated aptasensor was initially investigated in 0.01 M PBS. Various relevant concentrations of KAN were detected via ECL, a calibration study revealed a linear correlation between the decrease of ECL signal and the decrease in concentration of the target drug (Fig. 4) [55]. A limit of detection (LoD) was estimated at 0.06 ng/mL, which when compared to alternative analytical techniques for the detection of KAN displayed superior sensitivity [55]. In order to verify the robustness of the sensor, numerous parameters were examined. The sensor displayed a good degree of stability with the detectable ECL signal decreasing by only 4.52% with four weeks of storage [55]. The addition of five common interferent antibiotics produced no ECL response, showing the aptasensor was highly selective for KAN. Following this, the study aimed to apply the

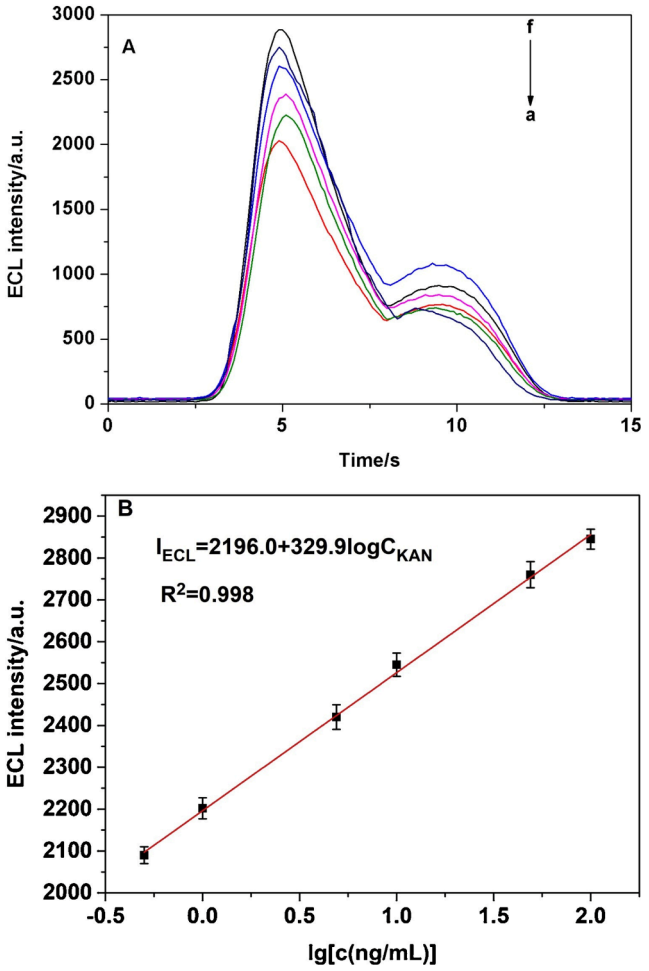

Fig. 4. A) ECL response of the aptasensor for various kanamycin concentrations in 0.01 M PBS: a) 0.5 ng/mL, b) 1.0 ng/mL, c) 5.0 ng/mL, d) 10.0 ng/mL, e) 50.0 ng/mL, f) 100.0 ng/mL. **B)** Calibration curve of the aptasensor for the detection of Kanamycin. Taken with permission from ref. [55].

fabricated aptasensor to spiked shop-bought milk samples. Two milk samples spiked with KAN generated reproducible ECL signals at 1.0 ng/mL and 5.0 ng/mL, well below the VMD's MRL of 100 μg/kg [55]. While the study showed the ability of KAN to be detected in spiked milk samples, it failed to show its use as a quantifying methodology in non-spiked samples.

One of the major pitfalls of ECL is the wide array of analytes that are able to enhance or inhibit the signal produced by $[Ru(bpy)_3]^{2+}$, this can be particularly problematic when analysing complex livestock-derived samples which may contain numerous electrochemically active species including antibiotics. A novel sensor that implements capillary electrophoresis (CE) has been developed to address this problem [56]. CE is a highly effective separation technique that, when coupled with ECL, can greatly enhance its selectivity [57]. The aforementioned study by Long *et al.* [57] utilised this coupling for the analysis of chlortetracycline (CTC), ampicillin (APM), and saraflocaxin (SAR). The method proved highly sensitive in PBS with the LoD for CTC, APM and SAR being 0.017 μg/mL, 0.018 μg/mL and 0.0013 μg/mL, respectively [57]. The sensor not only exhibited a high degree of reproducibility with a relative standard deviation (RSD) of $< 2.6\%$ but also good repeatability over a period of five days (RSD $< 2.3\%$). The ECL responses of 5 milk samples were compared to those of blank samples, the milk samples spiked with the antibiotics, and a standard solution of the antibiotics. Each sample was found to contain at least one antibiotic with CTC, APM, and SAR all producing a distinct and separate ECL signal. Correlating non-spiked and spiked signals were used to quantify the amount of antibiotic present in the samples, with concentrations ranging from 0.1 to 0.3476 μg/mL [57]. It was purported that the ECL behaviour of the antibiotics was attributed to amine functional groups present within the molecules, thus it can be assumed that this methodology could potentially be applicable to other amine-containing antibiotics.

Overall, there has been little progress in the implementation of ECL for the detection of residues derived from drugs other than antibiotics. Nevertheless, one useful application has been found in the identification of the steroid-type β_2-agonist clenbuterol (CLB) in swine urine [58]. Traditionally used for the treatment of chronic breathing disorders, CLB has been reported to be illegally abused within livestock due to its competence in reducing fat and promoting lean muscle growth [59]. As a consequence of its highly potent and heat-resistant nature, CLB residues that persist in animal-related food products have led to outbreaks of severe food poisoning in varying populations [60–62]. Therefore, the monitoring of CLB has gained precedence in an attempt to protect public health. Typically, this involves the detection of CLB residues in animal urine [63–66]. The ECL-based sensor developed by Zongyun *et al.* [58] was investigated for its effectiveness with the utilisation of a competitive immunoassay format [58]. The sensor consisted of a three-electrode setup with a gold nanoparticle doped chitosan composite film on the working glassy carbon electrode for antigen immobilisation. A $[Ru(bpy)_3]^{2+}$-labeled CLB antibody was implemented as the tracer and 0.10 M TPA as the co-reactant [58]. In PBS the ECL signals obtained decreased with an increase in CLB concentration, as expected due to the competitive immunoassay methodology adopted for this sensor (Fig. 5). A subsequent calibration study showed a linear detection range between 0.010 and 1.0 ng/mL with an estimated LoD of 0.0050 ng/mL, illustrating its superior degree of sensitivity when compared to alternative techniques [58]. Moreover, the sensor boasted a good level of reproducibility with low RSD values of under 4.9% for differing concentrations of the drugs. The CLB antibody was found to considerably cross-react with another structurally analogous β_2-agonist, salbutamol, the residues of which have been identified to persist in animal waste [67]. Although the interference of salbutamol indicated potential specificity limitations, the authors of this study argued that these results highlighted the possibility of simultaneously screening for multiple β_2-agonists in samples. 10 swine urine samples spiked with 0.040 ng/mL, 0.20 ng/mL, and 0.50 ng/mL of CLB were investigated to determine the sensor functionality in real-world applications [58]. Compared to ELISA, the ECL-based sensor showed similar CLB recovery rates ranging

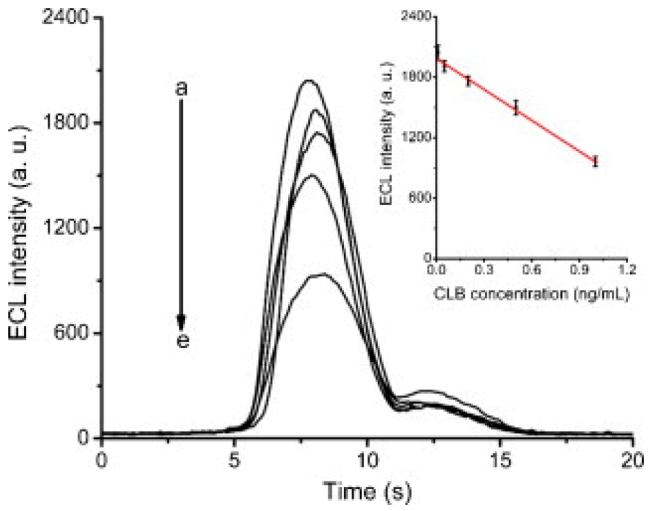

Fig. 5. ECL response of sensor for a) 0.010, b) 0.050, c) 0.20, d) 0.50, and e) 1.0 ng/mL of CLB. The inset displays the correlating calibration study for each concentration where each point represents the average of 5 measurements. Reproduced from ref. [58] with permission from Elsevier, copyright 2012.

between 78 and 118%. Despite this, the ELISA method was unable to detect the 0.040 ng/mL sample due to a LoD of 0.10 ng/mL [58]. Thus, regardless of interference issues, it was concluded that ECL outperformed ELISA for the detection of CLB due to its higher degree of sensitivity, low cost and accuracy.

The fast and accurate screening of drug residues in animal-derived produce and biological fluids, which is imperative for food safety, has been accomplished with the use of ECL. While these sensors have displayed superior sensitivities to alternative techniques, some issues with selectivity remain that should be addressed further in future research. Be that as it may, the ability of ECL to detect residues well below the MRL demonstrates its competence as a screening methodology.

3. ECL applications within domestic animals

ECL within veterinary science is not only limited to applications within animal husbandry; in fact, this powerful analytical tool has also been harnessed for diagnostic purposes in domesticated companion animals. ECL-based immunoassays (ECLIA) developed for human testing have shown effectiveness in other species and therefore have been adopted in the veterinary field as an accurate mode of disease detection and monitoring [68]. These immunosensors most frequently employ 'sandwich'-type immune complexes, such as that shown in Fig. 6A [69]. Generally, the mechanism is initiated by the immobilisation of a capture antibody (Ab1) on the surface of the working electrode, which binds the target antigens (Ag) of interest. Completion of the immunocomplex is then achieved by adding a secondary detector antibody labelled with an electrochemiluminophore (Ab2). ECL is produced in the presence of a co-reactant and is equivalent to the number of antigens present within the sample.

One area that has shown the applicability of this methodology within veterinary medicine is viral disease antibody detection. The determination of viral infections within domestic species plays a crucial role in the transmission of zoonotic diseases and is also of great importance to global public health, as more than 60% of infectious diseases in domestic and wild animals are shared with humans [70]. This is the case for SARS-Cov-2, a virus that emerged in 2019 from bat coronaviruses, the causative agent of a global pandemic that has resulted in devastating health-related and economic consequences [71]. Natale *et al.* [68] have shown the use of a one-step double antigen sandwich ECL assay for the detection of IgG antibodies against the spike protein of the SARS-Cov-2 receptor-binding domain in a symptomatic cat [68]. Serological tests taken 14- and 31-days after the first presentation of symptoms showed a substantial increase in virus-neutralising antibody production from 47.20 U/mL to 1598 U/mL [68]. These results confirmed previous exposure to SARS-Cov-2 and also provided evidence of protection against reinfection by the virus. Although it is highly unlikely that cats play a role in human infection of SARS-Cov-2, reverse zoonosis is certainly possible and with ever-emerging viral mutations interspecies transmission may arise. Consequently, the ability to accurately monitor the disease in pets is imperative, not only to ensure the safeguarding of animal health but also to evade an increase in pet abandonment.

Another infectious disease that has seen a high prevalence in cats is Borna disease, a neurological affliction caused by BDV. BDV causes so-called staggering disease resulting in clinical signs of ataxia, behavioural changes, and a loss in postural reaction [72]. In a study including 487 domestic cats from various East Asian countries, Horri *et al.* [73] developed an ECLIA sandwich methodology to detect two anti-BDV antibodies [73]. Improved sensitivity was achieved by utilisation of magnetic micro beads, a tool frequently implemented in electrochemical immunosensors to improve surface area and thus increase the probability of capture antibody and antigen binding [33,74,75]. The serological screening system measured antibodies against BDV p24 and p40 recombinant proteins, these were detected in 3.1%, 3.8%, and 2.0% of the domestic cats included within the study from Japan, the Philippines and Indonesia, respectively [73]. Anti-BDV antibodies were found more frequently in older cats (>6 years old), and in almost all cases the p24 antibody was more prevalent than the p40 antibody. Interestingly, the authors noted that serum taken from 5 cats with known neurological conditions tested negative for the anti-BDV antibodies, these results were attributed to the various clinical manifestations of BDV infection. The preliminary nature of this study highlights the gap currently present in the understanding of the implications of p24 and p40 antibodies on BDV infection in domestic cats but provides an accurate and sensitive methodology with which to detect them.

ECL-based sandwich immunoassays have also found application in the prediction and diagnosis of noncommunicable diseases within animals. Canine cognitive dysfunction syndrome (CCDS), frequently paralleled to Alzheimer's disease in humans, is one of the most common noncommunicable diseases encountered in ageing dogs. Various studies have been conducted in an attempt to estimate the prevalence of CCDS in dogs with fluctuating results of 12–68% in cohorts aged between 8 years and 19 years and 8 months [76–79]. It is, however, widely believed that this value could be much higher [80]. The first port of call for veterinarians when diagnosing CCDS normally involves a questionnaire that relies on the recording of physical behavioural observations made by the owner. While these can be useful tools, they often suffer from poor accuracy due to their objective nature, and as a result, alternative methodologies such as biomarker screening are preferred. A study by Herman *et al.* [81] has shown the ability of ECL to screen for

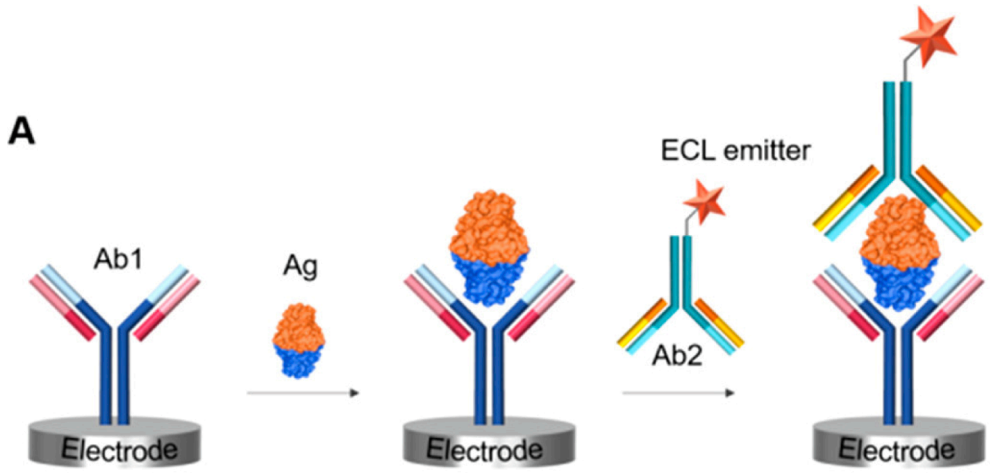

Fig. 6. Schematic representation of A) a conventional sandwich ECLIA. Adapted with permission from Ref. [69]. Copyright 2019 American Chemical Society; permission conveyed through Copyright Clearance Centre, Inc.

biomarkers associated with CCDS in a population of laboratory beagle dogs [81]. One of the neuropathological hallmarks of CCDS is the accumulation of peptide-based amyloid-β (Aβ) plaques in the cerebrum [82]. It is believed that the decline of $A\beta_{42}$ in cerebrospinal fluid (CSF) can be used as a predictive biomarker for the deposition of Aβ in the brain, and therefore as a reliable diagnostic methodology for the onset of CCDS [83]. In this work, a Meso Scale Discovery multi-array assay with a SULFO-TAG label was used to quantify $A\beta_{42}$ in CSF sampled from the lateral ventricle of the dogs over a period of 2 years. This methodology showed a high level of sensitivity with a limit of detection of 4.57 pg/mL [81]. These results were used to separate the dogs into two categories, those with low levels of $A\beta_{42}$ and those with high levels. The cognitive performance of both groups was evaluated by the use of numerous standard cognition tests based on memory, planning, and decision making. High levels of $A\beta_{42}$ were associated with significant learning impairments regardless of age, implicating its role in early disease progression [81]. The level of peptides sAβPPα and sAβPPβ, associated with the cleavage of amyloid precursor protein (the cleavage of which also results in $A\beta_{42}$) were also measured by use of a Meso Scale Discovery assay, these were both quantified to a limit of 1 ng/mL. High and low levels of the peptides corresponded with high and low levels of $A\beta_{42}$ in CSF, respectively, confirming the likelihood of amyloid precursor protein cleavage and thus the probability of Aβ plaque formation [81].

While the diagnostic capabilities of ECL for domestic animal species have been shown in this review, its use in the veterinary field is still in its infancy and thus there is much scope for additional research and investigation. Not only is the high degree of sensitivity of this analytical technique attractive, but its ease of use, low costs, and portability make it particularly well suited to the veterinary clinic.

4. Conclusions

The employment of ECL within the veterinary science sector is far beyond its use within the general analytical chemistry field. Indeed, veterinary researchers are not only employing ECL for research and development aspects but also within commercial settings. Its applications are varied with usage in both livestock and domestic animals, with consideration also given to the potential human impact through contamination of animal-based food products. We have summarised here, in brief, some of these applications in order to provide a wide scope which we hope will provoke further interest into the veterinary sciences. Indeed, the electro-analytical community do not often consider the veterinary fields when assessing potential outcomes of developmental progress regarding electrochemical techniques. ECL in particularly has been embraced by the veterinary sciences, whether it be for disease detection, health monitoring or antibiotic residue identification. A number of successful immunosensors or sandwich type assays have been reported with success in both ideal and complex matrices, a key concern among the electro-analytical community. It is hoped that such successes will only stand to improve the wider adoption of ECL and strength its standing as a powerful analytical tool.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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