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Electrochemical sensing of blood proteins for mild traumatic brain injury (mTBI) diagnostics and prognostics: towards a point-of-care application†

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Traumatic Brain Injury (TBI) being one of the principal causes of death and acquired disability in the world imposes a large burden on the global economy. Mild TBI (mTBI) is particularly challenging to assess due to the frequent lack of well-pronounced post-injury symptoms. However, if left untreated mTBI (especially when repetitive) can lead to serious long-term implications such as cognitive and neuropathological disorders. Computer tomography and magnetic resonance imaging commonly used for TBI diagnostics require well-trained personnel, are costly, difficult to adapt for on-site measurements and are not always reliable in identifying small brain lesions. Thus, there is an increasing demand for sensitive point-of-care (POC) testing tools in order to aid mTBI diagnostics and prediction of long-term effects. Biomarker quantification in body fluids is a promising basis for POC measurements, even though establishing a clinically relevant mTBI biomarker panel remains a challenge. Actually, a minimally invasive, rapid and reliable multianalyte detection device would allow the efficient determination of injury biomarker release kinetics and thus support the preclinical evaluation and clinical validation of a proposed biomarker panel for future decentralized *in vitro* diagnostics. In this respect electrochemical biosensors have recently attracted great attention and the present article provides a critical study on the electrochemical protocols suggested in the literature for detection of mTBI-relevant protein biomarkers. The authors give an overview of the

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analytical approaches for transduction element functionalization, review recent technological advances and highlight the key challenges remaining in view of an eventual integration of the proposed concepts into POC diagnostic solutions.

Introduction

Traumatic Brain Injury (TBI) is a leading cause of disability and death in both developed and developing countries.[1] Nearly seventy million people suffer from TBI worldwide every year.[2] In Europe alone 2.5 million people experience TBI each year, 1 million of them are being hospitalized, while 75 000 people die.[1] This results in significant public health implications, given the frequency of head impacts particularly among adolescents and in contact sports.[3] The considerable financial burden that arises from TBI depends on many factors, mainly duration of rehabilitation and long-term patient care, costs of prescriptions, therapies, need for medical equipment, costs of employment loss, *etc.* The overall global economic burden of TBI is estimated at about 400 billion dollars, meaning that 1 out of every 200 dollars generated in the global economy is spent to cover the costs and consequences of TBI.[1]

Diagnosis of TBIs is mainly based on patient's medical history, findings on neurological examination, clinical assessment scales and neuroimaging tools, such as computed tomography (CT) and magnetic resonance imaging (MRI), single-photon emission computed tomography (SPECT), or positron emission tomography (PET) of the brain. Based on the severity of the condition TBIs are typically classified as mild (GCS 13–15), moderate (GCS 9–12) and severe (GCS 3–8), using the Glasgow Coma Scale (GCS) which is an assessment of conscious level of the patient motor, eye, and verbal responses. Mild TBI (mTBI) accounts for 80–90% of all cases and is the most prevalent form of brain injury. Patients with mTBI frequently develop non-specific symptoms, including fatigue, headaches, visual or sleep disturbances, depression, or seizures, which can occur immediately following the injury or after several days or weeks. Occurrence of mTBI, especially repetitive, has been associated with an increased risk of long--term cognitive and neuropathological disorders. Moderate and severe TBIs are easily diagnosed, often evident from patient's history and injury signs or abnormalities detected on the neuroimaging screen. However, diagnosis of mTBI is rather challenging. The GCS can be inaccurate in distinguishing between mild and moderate TBIs and provides a poor prediction of patient outcome (not appropriate for patients with prior neurological conditions). mTBI is typically not associated with any structural changes on brain MRI and is difficult to be assessed by standard diagnostic workup. Furthermore, MRI and CT scans are quite costly and difficult to be made available on-site (undeveloped areas, emergency rooms, battlefield, sport facilities, car accident sites), not to mention the harm of exposure to ionizing radiation during a head CT and difficulties with exposure to strong magnetic fields in patients with metal implants (*e.g.*, pacemaker, heart valve, cochlear implant, *etc.*). Therefore, there is a growing need in additional diagnostic tools for aiding both diagnosis and prognostics in order to enable an accurate, inexpensive and fast triage and decision-making in the treatment of mTBI.[4–8]

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Biomarkers relevant to mTBI

Biomarkers are promising candidates for aiding the identification of mTBI and prognosis. They could be classified as diagnostic (indicating the presence or absence of a specific physiological/pathophysiological state or disease), prognostic (categorizes patients by degree of risk for disease occurrence or progression of a specific aspect of a disease), or predictive (categorizes patients by their likelihood of response to a particular treatment relative to no treatment). A good biomarker should provide good specificity (be uniquely present in the central nervous system and accurately reflect the extent of brain damage), high sensitivity (high abundance in the analyzed fluid and easy/sensitive detection), as well as be of use for estimating the therapeutic efficacy/intervention.[9,10] In the case of mTBI the most promising are brain protein biomarkers that can be safely quantified by analyzing biofluids, such as cerebrospinal fluid (CSF), serum and plasma. mTBI biomarkers are mostly studied in reference to specific injured cell types, including markers of glial cell injury (glial fibrillary acidic protein, GFAP; calcium binding protein B, S100β; myelin basic protein, MBP), axonal and neuronal injury (ubiquitin carboxyl-terminal hydrolase-L1, UCH-L1; neuron-specific enolase, NSE; Tau protein and phosphorylated-Tau [p-Tau]), and due immunological (*e.g.* antibodies) or inflammatory responses (cytokines).[11] Table 1 gives the detailed list of protein biomarkers which have been shown to be relevant to mTBI and mTBI-recovery.[12] An extensive survey of blood biomarkers relevant for head (brain) injuries can be found in the recent review article by Zoe S. Gan *et al.*[12] Peptides and cleavage products were omitted in the present

Table 1 Mild traumatic brain injury (mTBI) protein biomarkers and their clinically relevant concentration ranges[12]

Biomarker		Physiological concentration[a]	
Abbreviation	Full name	Normal	Mild TBI
BDNF	Brain-derived neurotrophic factor	RG: 15.8–79.8 ng mL^{-1} (ref. 25) MN: 32.7 ng mL^{-1} (ref. 25)	MD: 8.3 ng mL^{-1} (ref. 26)
CRP	C-reactive protein	MN: 2.1 μg mL^{-1} MD: 1.2 μg mL^{-1} (HP) (ref. 27) MN: 1.4 μg mL^{-1} (ref. 28)	Elevated (ref. 29 and 30)
GFAP	Glial fibrillary acidic protein	RG: 0.002–0.049 pg mL^{-1} (ref. 31) MD: 0.004 pg mL^{-1} (ref. 31)	≥0.033 pg mL^{-1} (ref. 31) CO: 22 pg mL^{-1} (ref. 32)
GM-CSF	Granulocyte-macrophage colony-stimulating factor	<145 pg mL^{-1} (ref. 33)	Elevated (ref. 34)[b]
h-FABP	Heart-fatty acidic binding protein	<5.5 ng mL^{-1} (ref. 35) MN: 3.78 ng mL^{-1} (ref. 36)	CO: 2.62 ng mL^{-1} (HS/HP) (ref. 37)
IL-6	Interleukin-6	<5.9 ng L^{-1} (ref. 38)	Elevated (ref. 39)
IL-8	Interleukin-8	5–18 pg mL^{-1} (ref. 40) <62 ng L^{-1} (HP) (ref. 38)	Elevated (ref. 41)
IL-10	Interleukin-10	RG: 4.8–9.8 pg mL^{-1} MN: 7.1 pg mL^{-1} (ref. 42)	Elevated (ref. 43)
MMP-2	Matrix metallo-proteinase-2	MN: 251.4 ng mL^{-1} (HS, PL) (ref. 44)	Elevated (ref. 45 and 46)[c]
MT3	Metallo-thionein	MN: 0.51 ng mL^{-1} (ref. 47)	MN: 0.13 ng mL^{-1} (ref. 47)
NCAM	Neuron cell adhesion molecule (CD56)	MN: 54.82 μg mL^{-1} (ref. 48)	Elevated (ref. 49 and 50)[d]
NFL	Neuro-filament light	MD: 14.5 pg mL^{-1} (ref. 51) 13 pg mL^{-1} (ref. 52) RG: 4.1–23.5 pg mL^{-1} (ref. 53)	RG: 2.6–246.9 pg mL^{-1} (ref. 53) MN: 32.1 pg mL^{-1} MD: 19 pg mL^{-1} (ref. 53)[c]
NGB	Neuroglobin	MN: 10.31 ng mL^{-1} (ref. 54) 14.54 ng mL^{-1} (ref. 55)	MN: 17.58 ng mL^{-1} (mTBI) (ref. 55) 28.76 ng mL^{-1} (sTBI) (ref. 55)
NRGN	Neurogranin	MD: 0.02 ng mL^{-1} (ref. 56)	Elevated (ref. 47 and 56)
NSE	Neuron-specific enolase	5–15 ng mL^{-1} (ref. 57) MN: 7 ng mL^{-1} (ref. 58) MN: 3.5 ng mL^{-1} (ref. 47)	CO: 20 ng mL^{-1} (ref. 59) MN: 14 ng mL^{-1} (mTBI) (ref. 58) 20 ng mL^{-1} (mdTBI) (ref. 58) 32 ng mL^{-1} (sTBI) (ref. 58)
S100β	S100β calcium-binding protein	MD: 50 pg mL^{-1} (HP) (ref. 60) <0.11 pg mL^{-1} (ref. 38)	≥100 pg mL^{-1} (sTBI, HP) (ref. 61) >75 pg mL^{-1} (ref. 62) CO: 0.042 μg L^{-1} (HS/HP) (ref. 37)
T-Tau	Total tau (P- + non-phosphor.)	MN: 86 pg mL^{-1} (ref. 63) RG: 52.2–215 pg mL^{-1} (T-Tau) (ref. 63) MN: 289 pg mL^{-1} (ref. 64)	MN: 188 pg mL^{-1} (ref. 63) RG: 52.2–850 pg mL^{-1} (T-Tau) (ref. 63) Elevated(ref. 65)
UCH-L1	Ubiquitin C-terminal hydrolase	MD: 0.09 ng mL^{-1} (ref. 66) RG: 0.03–0.11 ng mL^{-1} (ref. 66)	≥1 ng mL^{-1} (sTBI, HP) (ref. 61) CO: 327 pg mL^{-1} (ref. 32)
VCAM-1	Vascular cell adhesion protein 1	RG: 449–1103 ng mL^{-1} (ref. 67) MD: 631 ng mL^{-1} (ref. 67)	Lowered (ref. 39 and 68)
Biomarkers without reported EC detection approaches (as of December 2020)			
BMX	Bone marrow tyrosine kinase on Chromosome X	MN: 6.08 pg mL^{-1} (ref. 69)	MN: 7.47 pg mL^{-1} (ref. 69)
CKBB	Creatine kinase B type	<3 pg mL^{-1} (sTBI, HP) (ref. 61), <6 pg mL^{-1} (ref. 70)	>6 pg mL^{-1} (ref. 70), ≥3 pg mL^{-1} (sTBI, HP) (ref. 61)
ICAM-1	Intracellular adhesion molecule-1	MN: 236.9 ng mL^{-1} (ref. 71) RG: 210–306 ng mL^{-1} (ref. 72)	Elevated (ref. 73)
MDA-LDL	Malondialdehyde modified low density Lipoprotein	MDA-LDL-to-LDL-C-ratio: 1.16 LDL-C: MN 1270 μg mL^{-1} (ref. 74)	n/a
NFM	Neurofilament medium	MD: 2.29 ng mL^{-1} (ref. 75) RG: 0.26–8.57 ng mL^{-1} (ref. 75)	RG: 0.21–202.2 ng mL^{-1}, MD: 7.89 ng mL^{-1} (mTBI) (ref. 75) RG: 3.48–45.4 ng mL^{-1}, MD: 13.3 ng mL^{-1} (sTBI) (ref. 75)
Nogo-A	Neurite outgrowth inhibitor protein	MN: 128 ng mL^{-1} (ref. 55)	MN: 220.09 ng mL^{-1} (mTBI), 315.67 ng mL^{-1} (sTBI) (ref. 55)
pNF-H (NF–H)[e]	(Phosphorylated) neurofilament heavy protein	RG: 189.59–634.12 pg mL^{-1} (ref. 76) MN: 311.98 pg mL^{-1} (ref. 76)	Elevated (ref. 77)
E-selectin	E-selectin	MD: 39.6 ng mL^{-1} (ref. 78) RG: 33.2–44 ng mL^{-1} (ref. 78)	Elevated (ref. 79)[d]

Table 1 (*Contd.*)

Biomarker		Physiological concentration[a]	
Abbreviation	Full name	Normal	Mild TBI
SNTF	Calpain-derived αII-spectrin N-terminal fragment	Absent from healthy neurons (ref. 80)	Elevated (ref. 80 and 81)
Ub	Ubiquitin	<100 ng mL^{-1} (ref. 82) MN: 29.6 (fUb), 4.1 ng mL^{-1} (mtUb) (ref. 83) MN: 37.2/126 pg mL^{-1} (fUb) (ref. 84) MN: 3.4/3.86 pg mL^{-1} (mtUb) (ref. 84)	Elevated (ref. 85)

[a] Physiological concentrations are indicated for human serum, unless otherwise specified. Values reported in samples other than blood/serum/plasma (*e.g.*, sweat, urine, muscle-on-tissue *etc.*) are not considered. [b] Based on the results for the protein levels in postmortem cortical tissue, studies conducted for sTBI. [c] Data for uncomplicated mild TBI. [d] Based on animal model. [e] The terms pNF-H and NF–H are used interchangeably in the literature, due to the fact that the NF heavy chain is always phosphorylated.[86]

work, as well as biomarkers related specifically to severe TBI (sTBI) diagnostics and prognostics. Discussion of autoantibodies as potential biomarkers has been put aside here, mainly due to the fact that not sufficient clinical data has been reported yet in order to confirm diagnostic and prognostic values of autoantibodies for mTBI.[13] Nevertheless, it is worth mentioning that some of the autoantibodies have been shown to be relevant *e.g.* for repetitive sub-concussive events (anti-S100β[14,15]) or severe trauma (anti-GFAP[15,16]).

The first applications of mTBI biomarkers in medical practice dates from 2015, when S100β has been included in an algorithm of the Scandinavian guidelines to triage patients with mTBI to CT after TBI[17] (the cost for S100β analysis in Sweden is 21 euro, while the cost of CT scan is 130 euro).[18] Furthermore, in February 2018 the first biomarker core lab blood assay proposed by Banyan Biomarkers has been cleared by the FDA (based on the 2018 ALERT-TBI pivotal trial with 1959 mild-to-moderate TBI patients).[19] The latter relies on a chemiluminescent-based ELISA for measuring the concentrations of two proteins, GFAP and UCH-L1, and has been shown to be able to predict the TBI-positive CT scan with the sensitivity of 97.5% and negative predictive value of 99.6%.[19] Practically the latter means that in more than 33% of the cases the patients being suspected of brain injury can be ruled out prior to CT scan.[19] Following the approval of the test *via de novo* FDA pathway and into the Class II, a new product code has been created to designate the brain assessment tests. Thus, the subsequent (*e.g.*, POC) tests that have the same use will be classified into the same product code and will be reviewed by the 510(k) regulatory pathway. The search for the 'ideal' mTBI biomarkers still faces many challenges, such as insufficient specificity, as well as influence of age, gender, injury severity, pre-existing medical conditions and other individual differences.[12,20,21] TBI biomarker discovery today is mainly focused on detection at very early stages after injury (hyper acute and acute TBI), which will allow for implementation of patient treatments at an earlier time point. For the chronic stages of mTBI, Tau protein and phosphorylated-Tau are under examination as markers of neurodegeneration for *in vivo* detection of neurodegenerative disorders which are possible long-term sequelae of mTBI such as Alzheimer's disease (AD) and chronic traumatic encephalopathy (CTE).[22] Neurogranin could be mentioned here as one of the prospective candidates that can be measured in whole blood samples – researchers aim at evaluating its potential role for avoiding CT overuse in mTBI diagnostics. Myelin Basic Protein (MBP) was also highlighted in the literature as potential negative predictive biomarker for the absence of TBI.[23] The role of the CRP biomarker within mTBI related publications is dual: on one hand, CRP is often employed as a model analyte for method development, on the other hand, despite being an inflammatory non-specific biomarker it has a potential of being part of a future mTBI multi-biomarker panel.

The choice of body fluid for mTBI biomarker detection is one of the key aspects to be considered. CSF is attractive because it is in contact with the neural interstitial fluid and detection of CSF biomarkers should reflect neural tissue injury.[24] On the other hand, the disadvantage is the requirement for lumbar puncture, which is an invasive procedure and unlikely combinable with a decentralized POC diagnostic application.[23] As most of the *in vitro* diagnostic (IVD) assays are approved for use with blood samples the scope of this review will be limited to those (whole blood, serum, plasma). However, it must be noted that mTBI biomarker detection and quantification using blood sampling is still challenging. Once the neural tissue has been injured, the mTBI biomarkers need to pass through a biophysical barrier into the bloodstream. Many biomarkers that have excellent specificity for mTBI may not be present in blood in sufficiently high concentrations to be detectable using currently available assays. Detection of biomarkers in the peripheral blood is limited by the clearance from blood by liver or kidney, proteolytic degradation, and their permeability through the blood brain barrier (depending on the molecular size and charge). Due to above mentioned facts the concentrations and kinetic profiles of mTBI biomarkers in blood are quite difficult to determine.[23] While concentrations of some biomarkers continue to rise within days or even weeks, many biomarkers peak early and decline within a few hours after the injury, depending on the molecular and cellular origin and the

release mechanism.[87,88] Moreover, it must be noted that plasma and serum often contain different amount of proteins and the concentration of the proteins is strongly affected by the blood pre-treatment procedure (anti-clotting factors, clotting reagents).[89–91] Some studies indicate that serum samples are not recommended for quantifying certain biomarkers (*e.g.* some small proteins and peptides) and plasma is very much preferred in general cases.[89,92] However, for many biomarkers it has been shown that the serum protein concentrations do correlate with plasma concentrations[90] and thus both serum and plasma are being used for biomarker detection. The choice of the blood fraction to be analyzed may have important implications and depends on the target analyte. In some cases, it is quite straightforward. For example, in the case of the BDNF protein which is known to be bound by the platelets in blood, total concentration can be given by analyzing the serum sample, while free (circulating) BDNF can be detected by analyzing the plasma sample. Currently, the detection of mTBI-relevant biomarkers in body fluids is mostly performed using: (1) clinical analyzers in core/centralized labs that run high-throughput immunoassays (*e.g.*, 96-well plate based) predominantly with fluorogenic, chemiluminescent and colorimetric readout modalities, and (2) biosensor-based approaches described in research literature that employ either electrochemical (EC) or spectroscopic detection principles. The Abbott i-STAT is a rare example of an EC-based (portable) *in vitro* POC diagnostic device for protein quantification (*e.g.* cardiac troponin I in blood and plasma).[93] To the best of our knowledge, all other EC-based POC (portable) diagnostic applications target biomarkers other than proteins, such as *e.g.* the 'game-changing' continuous glucose sensing based on enzymatic amperometric detection. EC measurement of large protein biomarkers appears more challenging due to issues such as nonspecific adsorption of biological fluid, very low abundance of most protein biomarkers, requirement of extremely good specificity due to various interferences of other biomolecules present in physiological samples.[94]

In the last years, there has been a significant number of publications focusing on the EC sensing techniques for protein detection and quantification. The interest in EC techniques for mTBI research field accounts for the following facts: unlike spectroscopic methods, EC measurements are not affected by sample turbidity, colour, quenching, or interference from absorbing and fluorescent compounds commonly found in biological fluids. EC techniques are easily adaptable to relatively cheap mass production and miniaturization to circuit board levels with low power consumption.[95] The low fabrication costs, along with potential high sensitivity, fast response time, small sample volume requirements, low cost of operation, possibility of miniaturization and integration for multianalyte detection have made EC biosensors an attractive tool for mTBI biomarker detection, especially from the point-of-view of possible realization of a POC device for concussion diagnostics.

Table 2 provides a proposal for a target product profile (TPP) of an mTBI POC diagnostic device with presumed key product requirements specifications of the future system. In the next sections we will give an overview of the various sensor designs and types of electrochemical biosensors reported in the literature for detection of mTBI relevant proteins and in the Conclusions and future outlook section we will discuss how they respond to the TPP and their potential implementation into the POC concept.

Design of electrochemical sensors for detection of protein biomarkers relevant to mTBI

This section of the review provides the details on various (bio) sensor designs and components reported in the literature for detection of 19 mTBI-relevant biomarkers, based on the survey that includes 127 publications published until December 2020. The literature search related to NSE, IL-6, IL-8 and CRP biomarkers has been limited to the last three years (from January 2018 up to December 2020), mainly due to a large number of strategies reported in the past decade, as well as already available reviews summarizing EC strategies for the detection of these specific targets (*e.g.* CRP;[101–103] IL-6;[104] CRP, IL-6 and IL-8;[105] NSE[106]).

EC (bio)sensor configuration typically comprises two main parts: (i) immobilized recognition element providing the selectivity to the target analyte (T) and (ii) the EC transducer serving as a converter of the biorecognition event to an electronic signal.[107,108]

The most common transducers reported for mTBI-related biosensors include gold electrodes (disk, microelectrodes, films and interdigitated electrodes[109,110]), carbon-based (glassy carbon, carbon paste, carbon nanotubes (CNTs)[111]) and graphene-based materials. Other approaches have been reported employing conductive polymer (CP) nanowires (NWs), such as polypyrrole (PPy) NWs[112,113] or silica-NWs.[114,115] Metal oxide substrates such as hafnium oxide (HfO_2),[116,117] aluminium oxide (Al_2O_3),[118] indium tin oxide (ITO)[119–121] have been incorporated, more rarely platinum[122,123] and molybdenum.[124]

Regarding the transducer surface modification, researchers often rely on gold nanoparticles (AuNPs) deposition on various substrates to benefit from easily addressable gold-sulphur chemistry and the signal amplification due to the accelerated electron transfer between the electrode and a redox moiety/mediator.[125] Gold-sulphur chemistry is often explored with self-assembled monolayers (SAMs) based on various thiol derivatives (*e.g.*, MPA, MHDA, MUA) employed to immobilize bioreceptors on gold electrode surface. The key issue limiting SAM practical applications remains however the overall stability of the monolayer film.[126,127] CNTs have also found their application for transducer modification in various composites,[128,129] such as AuPd-multiwall carbon nanotubes (MWCNTs) for the detection of the NSE biomarker,[128] CNTs with Nafion and glutaraldehyde for the detection of S100β,[130] MWCNTs incorporated into molecularly imprinted polymer (MIP) layer for the detection of GFAP[131] and MWCNTs with reduced graphene oxide (rGRO) and chitosan for detection of tau-441 protein.[132] Nanostructured conducting polymers (CPs) such as PPy-NWs functionalized *via* diazonium coupling reaction[113] or (nano)composite layer (*e.g.*

Table 2 Target Product Profile (TPP[96,97]) as desirable design input for the development of a POC diagnostic device for mTBI

	Presumed key product requirements specifications of a future system for POC diagnostics and prognostics of mTBI. The number of biomarkers necessary to achieve sufficient diagnostic specificity is an assumption based on recent and ongoing clinical studies.[21,98–100]
Diagnostic sensitivity ≥ 95%	Number of biomarkers detected (multiplex multivariate analysis) ≥3 (∼5–8)
Diagnostic specificity ≥ 75%	Capillary whole blood (finger prick) sample volume ≤50 μL
Intra-assay %CV precision ≤10%	Linear range (*i.e.*, upper limit of quantification, ULOQ relative to LDL) ×50 above concentration cut-off value for specific biomarker, *e.g.*, ≥ 1.1 ng mL^{-1} for GFAP
Inter-assay %CV precision ≤15%	Time-to-results ≤10 min
Reagent shelf life ≥6 months	Hands-on-time ≤5 min

Lower Detection Limit (LDL) 1/10 of the cutoff (CO) value to distinguish mTBI from physiological concentration for specific biomarker, *e.g.*, ≤2.2 pg mL^{-1} for GFAP

with AuNPs[133,134] or graphene[135]) based on polyaniline (PANI), poly(3,4-ethylenedioxythiophene) (PEDOT) or 2,2:5,2-terthiophene-3-(*p*-benzoic acid) (pTTBA) (electropolymerized onto AuNPs[136]) present also an attractive platform for mTBI biosensor fabrication. Furthermore, transducer (bio)functionalization was also achieved *via* composites based on ionic liquids, *e.g.* with TiO_2 mesocrystal nanoarchitectures,[137] MOF architectures[138] or ZnO/porous carbon composite.[139] Furthermore, EC sensing immunoassay-based strategies relevant for mTBI often employ magnetic beads (MBs) in order to amplify the signal and increase sensitivity, reduce matrix effects and for multiplexing purposes.[105,140–142]

Recognition elements

The survey of publications relevant to mTBI biomarkers indicates that antibodies are used as recognition elements in most cases (in 86 out of 127 publications). However, there is an increasing trend towards the application of synthetic recognition structures. For example, aptamers have been employed for detection of CRP,[112,125,143–146] IL-6,[147] NSE,[134] Tau proteins,[123,148,149] and UCH-L1 biomarkers.[123] The number of aptamers available for different target proteins is much lower than that of antibodies and a systematic screening technology is required in order to discover novel molecules for different biosensing applications.[150–152] Other major issues with aptamer-based sensing are the time-dependent and poorly predictable alteration of folded aptamer structure in complex media and sensitivity of aptamer molecules towards degradation catalyzed by ribonucleases. To the best of our knowledge, this biosensing technology has not made it to the market yet, though several companies have been reported to work on the solutions for EC aptamer-based diagnostic devices.[153,154] 'Molecular containers' like cucurbit[*n*]urils (CB[*n*]) have been shown to be promising candidates as recognition elements[155–157] and have been explored for detection of MMP-2 biomarker.[158] MIPs were also employed in the context of mTBI-relevant biomarkers for the detection of GFAP[131] and NSE.[159–161] Wang *et al.*[159] suggested an ionic liquid with a pyrrole moiety and NSE as a template to fabricate MIP by electrochemical deposition in aqueous phase without any harsh polymerization initiators. Such sensors had a detection limit of 2.6 pg mL^{-1} (below cutoff value of NSE biomarker of 20 ng mL^{-1},[59] Table 1). Another interesting strategy for detection of NSE biomarker using AuNPs decorated with epitope-mediated hybrid MIPs has been developed by Pirzada *et al.*[161] who reported an ultrasensitive detection of NSE in human serum with LDL of 25 pg mL^{-1}.

It should be noted that non-specific binding (NSB), one of the most frequently encountered problems when designing affinity-based (bio)sensors is particularly important in the context of mTBI, as some of the potential biomarkers are present in blood in very low concentrations (*e.g.*, GFAP cutoff 22 pg mL^{-1},[32] or UCH-L1 cutoff 327 pg mL^{-1},[32] Table 1). The overview and the discussion of the advantages and disadvantages of the most applied strategies to prevent the NSB including the immobilization of 'blocking' proteins and chemical approaches is given in ESI Section SI-3.†

Types of electrochemical (bio)sensors for detection of protein biomarkers relevant to mTBI

Potentiometric (bio)sensors

Both ion-selective electrodes (ISE) and non-ISE formats have been proposed for mTBI related protein biosensing.[162] The ISE formats are commonly based on registering pH changes resulting from the catalytic reaction. For example, Liang *et al.*[163] proposed an electrochemical immunoassay on a handheld pH meter using glucose oxidase-loaded liposomes (GOx-LS) for signal amplification. A sandwich immunocomplex was composed of a microplate coated with capture antibodies, NSE biomarker as antigen, and detection antibodies labelled with GOx-LS, employed to oxidise glucose into gluconic acid and hydrogen peroxide, leading to a pH change recorded with a pH meter. The authors explored the usage of liposomes with strong encapsulation ability for loading of natural GOx enzyme and for enhancing the catalytic efficiency after the antibody–antigen reaction. This strategy showed an improvement compared to

Table 3 Selected electrochemical strategies for detection of blood protein biomarkers relevant to mTBI. The complete list of publications is available in the ESI SI-1

Technique	Biomarker/publication year	Transducer	Surface modification	Label	Analysis time[a]	Sample	Lower detection limit[b]	Range[c]
Potentiometry	NSE/2019 (ref. 163)	pH electrode	Ab_1/T/Ab_2-GOx-LS	GOx-LS	~45 min	Buffer Serum	8.9 pg mL^{-1} <0.5[b] ng mL^{-1}	0.01–100 ng mL^{-1} (pH *vs.* lg C_T)
Conductometry	CRP/2018 (ref. 112)	CuPT-PPy nanowiremesh	NIPAAm-AM/Apt/CRP polymer	Label-free	~20 min	Buffer Serum	9.03 × 10^{-17} g mL^{-1} <70[b] ng mL^{-1}	Non-linear up to *ca.* 10^{-8} M
Conductometry (RPS)	CRP/2019 (ref. 145)	Nanocarriers: SPBs	Peptide-Apt/non-binding DNA/T	Label-free	~90 min	Buffer	n/a	*ca.* 0.5–2.5 μM
SWV/EIS	CRP/2019 (ref. 165)	Au arrays	MPA/(EDC + NHS)/Ab/T	Label-free	~30 min	Buffer Serum	2.25 fg mL^{-1} (SWV) 3 fg mL^{-1} (EIS) 4.5 fg mL^{-1} (SWV)	5–220 fg mL^{-1} 7–215 fg mL^{-1} 12–166 fg mL^{-1}
EIS	VCAM-1/2017 (ref. 166)	Au microelectrode	DTSP/Ab/T or DTSP/Ab_1/T/Ab_2	Label-free	~15 min	Buffer Urine	8 fg mL^{-1} <500[b] μg mL^{-1}	8 fg mL^{-1}- 800 pg mL^{-1} (*vs.* lg C_T)
EIS	IL-6/2018 (ref. 113)	PPy-NWs layer	PPyPAC/(EDC + NHS)/Ab/T	Label-free	~30 min	Buffer	0.36 pg mL^{-1}	1–50 pg mL^{-1}
EIS	MMP-2/2015 (ref. 167)	Au	Pept-SH (target-induced cleavage)	Label-free	~60 min	Buffer	0.5 pg mL^{-1}	0.1–400 ng mL^{-1} (non-linear)
Capacitive (impedance derived)	CRP/2020 (ref. 169)	Graphene nanoplate SPE	PANI-PA/Ab/T	Label-free	~10 min	Buffer	0.5 μg mL^{-1}	0.25–2 μg mL^{-1}
DPV	MMP-2/2016 (ref. 140)	GCE	MBs/(EDC + NHS)/Pept-SH/AuNPs-DNA_1	MeB-DNA_2+Exo III	~180 min	Buffer Serum	0.15 pg mL^{-1} <0.1[b] ng mL^{-1}	0.5 pg mL^{-1}-50 ng mL^{-1} (*vs.* lg C_T)
DPV	Tau/2018 (ref. 173)	Au DE	MPA/(EDC + NHS)/Ab/T/AuNPs–SH–Apt	Label-free	~45 min	Buffer Serum	0.42 pM <1.5[b] pM	0.5–100 pM
DPV	IL-8/2020 (ref. 200)	ITO	β-Ag_2MoO_4/(EDC + NHS)/Ab/T	Label-free	~10 min	Buffer	90 pg mL^{-1}	1 fg mL^{-1}-40 ng mL^{-1} (two linear ranges)
CPA	CRP/2018 (ref. 174)	Carbon SPE	AuNPs/L-cysteine/(EDC + NHS)/Ab/T	Label-free	~30 min	Buffer Serum	17 ng mL^{-1} <0.932[b] mg L^{-1}	0.05–23.6 μg mL^{-1}
CPA	BDNF/2018 (ref. 136)	Carbon SPE	AuNPs/pTTBA/(EDC + NHS)/Ab_1/T/Ab_2/(EDC + NHS)/TBO-pTTBPA/AuNPs/carbon SPE#2	TBO	~20 min	Buffer Serum	0.015 ng mL^{-1} <0.1[b] ng mL^{-1}	0.004–0.6 ng mL^{-1}
CPA	Tau/2020 (ref. 178)	Dual SPCE	pABA/(EDC + NHSS)/3D-Au-PAMAM/GA/Ab_1/T/Ab_2-HRP	Ab_2/HRP	~120 min	Buffer Plasma	2.3 pg mL^{-1} (~pg mL^{-1})[b]	8–5000 pg mL^{-1}
SWV	h-FABP/2012 (ref. 179)	GCE	GRONRs/(EDC + NHS)/Ab_1/T/Ab_2/GA/TiP–Zn^{2+}-probe	TiP–Zn^{2+}-probe	~120 min	Buffer Serum	3 fg mL^{-1} <1.7[b] μg mL^{-1}	0.05 pg mL^{-1} –50 ng mL^{-1} (*vs.* lg C_T)
SWV	IL-8/2019 (ref. 201)	Carbon SPE	PEI-AuNPs/GA/Ab_1/T/PEI-AuNPs-Ab_2-Ag^+	PEI-AuNPs-Ab_2-Ag^+	~80 min	Buffer Serum	1 fg mL^{-1} <2.5[b] pg mL^{-1}	0.5–100 pg mL^{-1} 2.5–50 pg mL^{-1}
ASV	h-FABP/2017 (ref. 180)	GCE	CD-GS/Ab_1/T/Ab_2-ZnO-MWCNTs/CdS	ZnO-MWCNT/CdS	~120 min	Buffer Serum	0.3 fg mL^{-1} <5[b] pg mL^{-1}	1.3 fg mL^{-1}-130 ng mL^{-1} (*vs.* lg C_T)
FED (OFET)	GFAP/2017 (ref. 186)	Si/SiO_2/pentacene/Au	(PS-MA + PEG)/Ab/T	Label-free	~30 min	Buffer	1 ng mL^{-1}	0.5–100 ng mL^{-1}

Table 3 (*Contd.*)

Technique	Biomarker/publication year	Transducer	Surface modification	Label	Analysis time[a]	Sample	Lower detection limit[b]	Range[c]
FED (GFET)	Tau/2020 (ref. 189)	Si/SiO_2	APMES/rGRO/PBASE/Ab/T	Label-free	~20 min	Buffer Plasma	n/a 1 pg mL^{-1} (HP)	100 fg mL^{-1}-10 ng mL^{-1} (HP, *vs.* lg C_T)
FED (FEED)	S100β/2018 (ref. 130)	Carbon SPE	SWCNTs-Nafion-GA/Ab_1/T/HRP-Ab_2	HRP	~100 min	HS	10 fg mL^{-1}	10 fg mL^{-1}-10 ng mL^{-1}
PEC	MMP-2/2020 (ref. 195)	ITO	Fe_3O_4@SiO_2/(EDC + NHS)/Ab_1/T/Ab_2/TiO_2-AgNPs	TiO_2–Ag NPs/Ab_2	~120 min	Buffer Serum	0.34 fg mL^{-1} <350[b] pg mL^{-1}	1 fg mL^{-1}–100 pg mL^{-1} (*vs.* lg C_T)
PEC	NFL/2020 (ref. 196)	Pt NWs on FTO (biocathode)	(MUA + MCH)/Ab, photoanode: FTO/$BiVO_4$–FeOOH	Label-free	~60 min	Buffer Plasma	38.2 fg mL^{-1} n/a	0.1–1000 pg mL^{-1} (*vs.* lg C_T)

[a] The total analysis time (unless clearly indicated in the original publication) is an approximate value estimated by the authors of this review article based on the sum of the duration of individual analytical steps reported in the original publications. For more detailed information please consult ESI Table SI-1. The analysis time serves only an indicative purpose and does not take into account the potential for shortening upon further method optimization. [b] Lowest reported LDL using EC detection methods; '<*x*' corresponds to the lowest concentration analyzed within the working range of the sensor (employing standard addition method and/or a reference material/method for validation, with a decent recovery), actual LDL being possibly lower than the indicated value. [c] Linear *vs.* target concentration (C_T), if not stated otherwise (*e.g.*, *vs.* lg C_T). The upper limit of the range indicated often presents the maximum concentration explored but not the upper detection limit. Please consult original papers for details. See Abbreviations. Complete list of all published electrochemical strategies (with details on biosensor configuration) for the detection of blood protein biomarkers relevant to mTBI is available in ESI SI-1.

the traditional GOx labelling, enabling low-cost detection of NSE biomarker with the detection limit of 8.9 pg mL^{-1} (cutoff for mTBI 20 ng mL^{-1})[59] and within the dynamic linear range of 0.01–100 ng mL^{-1}. However, in addition to the multi-step reagent-demanding nature of the proposed sandwich assay, the storage stability is reported to be relatively short owing to the usage of easy-broken liposomes.[163]

Potentiometric sensors based on surface molecular imprinting have been suggested for protein biomarker detection, however to our knowledge not to mTBI specific protein analytes.[164]

Conductometric (bio)sensors

Very few researchers have adopted conductometric strategies for detection of mTBI-related biomarkers (Table 3 and Table SI-1†). Lin *et al.* have suggested a CRP-biosensor using conductive polypyrrole nanowire mesh architecture uniformly dispersed within a polymeric matrix with molecularly imprinted cavities hosting immobilized aptamers. Serum CRP levels were analyzed through monitoring the conductance change caused by the polymeric network shrinkage upon target binding, providing ultrasensitive detection of CRP in blood samples from melanoma patients (LDL 9×10^{-14} mg mL^{-1} in a wide dynamic range up to approximately 10^{-3} mg mL^{-1}).[112] Carbonaro *et al.*[122] proposed an interesting multianalyte label-free approach for detection of GM-CSF (and G-CSF) biomarker based on resistive pulse sensing (RPS) and multiple artificial pores integrated on a chip. The sensor was detecting the size change of latex colloids upon specific antigen–antibody binding on the colloid surface. Another approach based on resistive RPS was proposed by Maugi *et al.*[145] who developed a strategy combining RPS with nanocarriers for detection of CRP biomarker. The surface of a nanocarrier was coated with a mixture of peptide aptamer and a non-binding DNA. The target binding to the aptamer creates a corona around the carrier, shielding the phosphate groups of the DNA and resulting in a change in electrophoretic mobility of the nanocarrier. The advantage of this approach lies in its universality as it can be easily adapted to other proteins without the need for chemical modifications. On the other side, the main limitation is the limit of detection (sub-micromolar level).

Capacitive and impedimetric (bio)sensors

Most of the impedimetric/capacitive detection strategies reported for mTBI biomarker detection are based on the faradaic model and capture immunoassay format where the electron-transfer resistance is changed due to interaction between immobilized (capture) antibody and the target molecule. For example, Vilian *et al.*[165] have reported on a gold wire array grown on polycarbonate substrate and functionalized with anti-CRP *via* self-assembly of 3-mercaptopropionic acid (MPA) for the electron-mediated detection of CRP using both EIS-based and voltammetric techniques (see Fig. 1). The authors reported a LDL of 3 fg mL^{-1} with EIS with the linear dynamic range of 7 to 215 fg mL^{-1}. This strategy exhibited high selectivity against various potentially interfering species (dopamine, lysine, uric

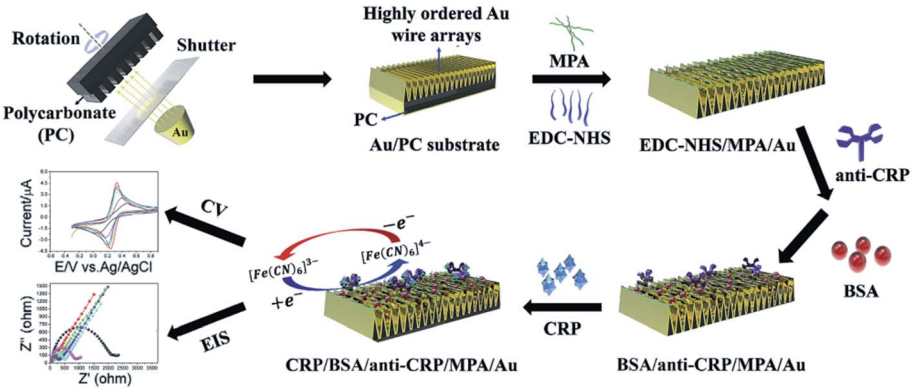

Fig. 1 EIS- and CV-based detection of CRP, reprinted from Vilian *et al.*,[165] Copyright (2019), with permission from Elsevier.

acid, glucose, *etc.*) and was capable of directly probing trace amounts of the target CRP in human serum.

A non-faradaic impedimetric strategy has been suggested by Selvam *et al.*[166] for detection of VCAM-1 biomarker. The approach is based on the immobilization of capture antibodies on gold microelectrodes resulting in the formation of a charged electrical double layer (EDL). Binding of the target to the antibody results in highly specific capacitance changes, while the authors have also observed an improvement of the overall signal upon optional addition of a second (detection) antibody to the immobilized immunoassay, resulting in further accumulation or perturbation of charges in the capacitive EDL. The strategy exhibits potential utility for POC applications with an LDL of 8 fg mL^{-1} and a dynamic range of 8 fg mL^{-1} to 800 pg mL^{-1}.[166] Another non-faradaic approach has been developed by Garcia-Cruz *et al.*[113] who have reported on the fabrication of PPy-NWs using innovative nanocontact printing, allowing for low-cost fabrication of electrodes with highly controllable architecture (see Fig. 2). The impedimetric immunosensor has been designed by immobilizing IL-6 antibodies *via* diazonium coupling reaction and carbodiimide crosslinker on the PPy-NWs printed using controlled chemical polymerization with an LDL of 0.36 pg mL^{-1} and a linear range of 1–50 pg mL^{-1}.[113] Some other strategies exploit more peculiar transducer substrates for immobilizing an antibody, such as *e.g.* molybdenum (*via* cross-linking with EDC and NHS, employed for detection of CRP)[124] or HfO_2 (*via* self-assembly, detection of IL-10).[116]

Impedimetric detection of mTBI-relevant enzymatic proteins like matrix metalloproteinases (MMPs) is addressed *via* approaches resting upon a cleavage event of a peptide specific to MMPs after injection of target-containing solution.[167]

Detection of non-enzymatic proteins (which is the vast majority of target proteins for mTBI) can be as well accomplished *via* peptide-supported aptasensing (*e.g.* gold electrode fuctionalization with a ferrocene-tagged peptide, followed by cross-linking with the aptamer[143]) or by anchoring the recognition molecule (*e.g.* capture antibody) onto a redox active composite (*e.g.* graphene oxide and zwitterionic monomer based composite incorporated into a 11-ferrocenyl-undecanethiol monolayer[168]).

A very recent approach by Baradoke *et al.*[169] for the first time employed a surface-confined redox active polymer (*i.e.* phytic acid-doped PANI film, see Fig. 3) as a support for reagentless redox capacitive (impedance-derived) sensing of CRP. In this strategy the CRP-sensitive surface has been obtained *via* glutaraldehyde cross-linking of amine functionalities in the PANI film with the antibody. The construction of the sensory interface by electropolymerization allowed for tuning the surface coverage and capacitive properties of the polymers, which could be used to modulate the assay selectivity, fouling, and sensitivity (LDL 0.5 μg mL^{-1}).

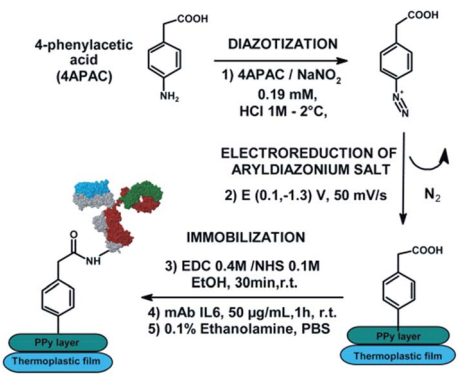

Fig. 2 Non-faradaic EIS-based detection of IL-6, reprinted from Garcia-Cruz *et al.*,[113] Copyright (2018), with permission from Elsevier.

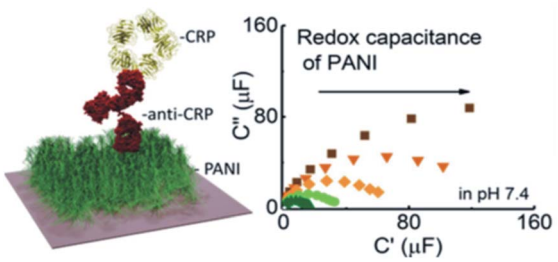

Fig. 3 EIS-derived capacitance-based detection of CRP, reprinted with permission from Baradoke *et al.*[169] Copyright (2020) American Chemical Society.

Capacitive and impedimetric measurements represent an attractive platform for practical applications not only because of decreased measurement times and costs due to absence of labelling step, but as well due to enabling continuous real-time sensing which is rarely possible with label-based EC assays.[170,171] Their potential for multianalyte mTBI diagnostics was demonstrated by Cardinell *et al.*[172] (detection of GFAP, NSE, S100β and tumor necrosis factor-α), who have characterized mTBI biomarkers in purified solutions (LDL 2–5 pg mL^{-1}) and then verified the detection approach in spiked rat whole blood and plasma solutions (LDL of 14–67 pg mL^{-1} in 90% whole blood).

Amperometric/voltammetric (bio)sensors

Several approaches have been shown to be applicable for detection of mTBI biomarkers with relatively high clinically relevant concentration ranges. Very few label-free strategies have been reported, *e.g.* Shui *et al.*[173] have developed an aptamer-antibody sandwich assay by using a tau antibody and an aptamer specific to tau-381 as the recognition element and cysteamine-stabilized gold nanoparticles for signal amplification (see Fig. 4). Detection of tau-381 in buffer and human serum was accomplished using differential pulse voltammetry (DPV) in the presence of $[Fe(CN)_6]^{3-/4-}$ with an LDL of 0.42 pM and <1.5 pM (*ca.* 17 and 60 pg mL^{-1}), respectively, within the linear range from 0.5 to 100 pM. Another label-free approach has been proposed by Thangamuthu *et al.*[174] who employed a simple constant potential amperometry (CPA)-based capture assay for detection of CRP using an antibody-functionalized AuNPs modified carbon screen-printed electrode (SPE). The measurement relies on the decrease of the oxidation current in the presence of redox mediator ($[Fe(CN)_6]^{3-/4-}$) and the authors reported an LDL of 17 ng mL^{-1} in the range of 0.05–23.6 μg mL^{-1}.

Most amperometric workflows are based on either a labelled competitive assay (*e.g.* using free and alkaline phosphatase (ALP)-labelled target molecules for detection of GM-CSF biomarker[175]) or, more commonly, sandwich assay (*e.g.* horseradish peroxidase (HRP)-labelled microfluidic bead-based enzyme-linked immunosorbent assay for detection of IL-6 [176] or Tau proteins[177,178]). A peculiar dual-probe sandwich-like assay with a single incubation step has been suggested by

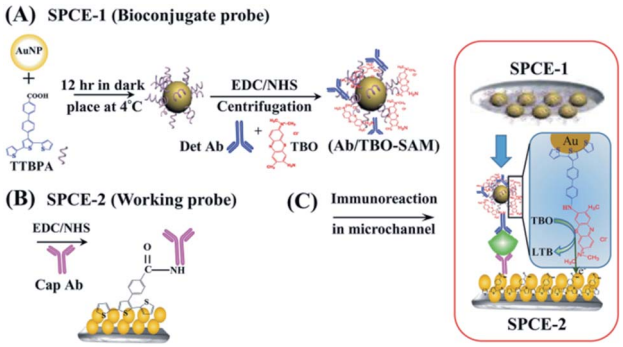

Fig. 5 CPA-based detection of BDNF, reprinted from Akhtar *et al.*,[136] Copyright (2018), with permission of Elsevier.

Akhtar *et al.*[136] for relatively rapid (*ca.* 20 min) BDNF biomarker detection in the extracellular matrix of neuronal cells (see Fig. 5). In this approach two independently prepared carbon SPE-based probes were placed in front of each other to form a microfluidic channel for the sample solution. The working probe (B) was fabricated by modifying a carbon SPE by covalently attaching capture antibodies to the layer of AuNPs–pTTBA (CP) composite. The bioconjugate probe (A) was prepared from second carbon SPE, modified by drop casting the bioconjugate particles composed of conducting polymer self-assembled onto AuNPs and functionalized with detection antibodies and toluidine blue O (TBO). The method allowed for the detection of BDNF concentrations as low as 100 pg mL^{-1} (the median serum concentration for mTBI is 8.3 ng mL^{-1},[26] decreased compared to healthy physiological range, see Table 1) spiked in undiluted human serum using CPA.[136] The bioconjugate attachment being already available on the bioconjugate probe that can be fabricated in advance, the proposed strategy allows for a single incubation step with the target analyte and thus is more attractive for the realization of POC diagnostic methods, compared to conventional time-consuming sandwich-based approaches.

Feng *et al.*[179] have reported on a duplexed sandwich immunoassay for simultaneous detection of h-FABP and troponin I using titanium phosphate nanospheres functionalized with Zn^{2+} and Cd^{2+} (respectively) as labels (see Fig. 6). The proposed strategy employs graphene oxide nanoribbons (GRONRs) as

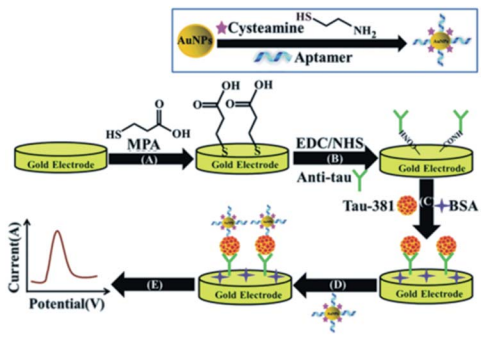

Fig. 4 DPV-based detection of tau-381, reprinted from Shui *et al.*[173] Copyright (2018) The Royal Society of Chemistry.

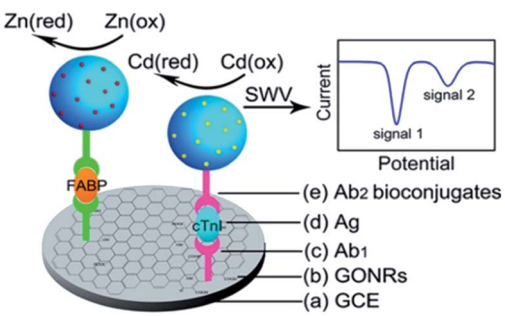

Fig. 6 SWV-based detection of h-FABP, reprinted with permission from Feng *et al.*[179] Copyright (2012) American Chemical Society.

a substrate for capture antibody immobilization and enables direct detection of metal ions in the bioconjugates using SWV without acid dissolution and preconcentration (stripping) steps.[179] The reported assay allowed for detection of *ca.* 1.7 μg mL^{-1} of h-FABP in undiluted serum (cutoff for mTBI 2.6 ng mL^{-1},[37] Table 1), clinically relevant lower target amounts have been quantified in buffer only. Despite of avoiding stripping steps, the protocol still requires more than 100 minutes turn-around time due to incubation steps with both the target and the label-carrying probe.

Anodic stripping voltammetry (ASV) has been employed for detection of h-FABP,[180] IL-8,[111,181] MMP-2 [182] and NSE.[183] Cyclic voltammetry (CV) has not been applied in many strategies. Exceptionally, Ramgir *et al.* have reported on sensitive IL-10 detection using silica-NWs by employing ALP-labelled sandwich assay with the *p*-nitrophenyl phosphate added as a substrate for detection.[114]

Field-effect based biosensing devices (Bio-FEDs)

FEDs have a potential in the field of POC diagnostic device development due to their ability to provide instantaneous (possibly real-time) label-free measurements using very small sample volumes, low production cost, high density integration and miniaturization.[184,185] Various designs of FET-based sensing devices have been proposed in the literature for detection of mTBI-related biomarkers. Song *et al.* have developed and organic field effect transistor (OFET)-based biosensor with extended solution gate architecture for label-free detection of GFAP biomarker[186] *via* a strategy for overcoming Debye screening length limitations. The latter has been achieved by mixing the bioreceptor layers with different molecular weight PEGs, which has been previously shown to increase the 'effective Debye screening length' for a given ionic strength.[186,187] Hao *et al.* reported on a sensitive and fast (10 min) detection of IL-6 using a graphene-based field-effect transistor (GFET) with the graphene surface covalently functionalized with a negatively charged aptamer undergoing conformational changes upon target binding,[188] while Park *et al.* used reduced graphene oxide FET for detection of T-Tau.[189]

A peculiar strategy based on field-effect enzymatic detection (FEED) reported by Mathew *et al.* has adopted gating voltage for signal amplification for ultrasensitive detection of S100β.[130] In this approach (see Fig. 7), a sandwich assay with an HRP-labelled detection antibody has been realized on a working electrode (WE) using a conventional screen-printed three-electrode cell. An insulated copper wire wound around the WE served as the gating electrode. To achieve voltage-controlled amplification, the WE has been connected to the gating electrode *via* a DC power supply to apply the gating voltage (V_G) yielding an electric field at the WE/solution interface and thus resulting in changes of interfacial charge distribution.[130] The proposed technique enabled LDL as low as 10 fg mL^{-1} in serum which is sufficient for S100β quantification (cutoff for mTBI 42 pg mL^{-1},[37] Table 1).[130] Importantly, this is one of very few publications on FED-based architectures reporting its successful application in undiluted serum.

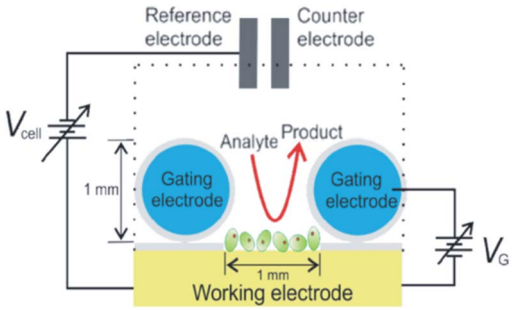

Fig. 7 Field effect enzymatic detection of S100β using an insulated copper wire as a gating electrode for signal amplification. Reprinted by permission from Springer Nature, Molecular Diagnosis & Therapy, Mathew *et al.*,[130] copyright (2018).

However, as of today, there are still challenges to be overcome for the FEDs to make their way into medical diagnostics. One of the major issues is obtaining well-defined recognition element structures (reproducible and easily manufacturable solid phase/solution interface)[190,191] and overcoming Debye screening in order to provide for direct measurements in undiluted clinical samples with high ionic strength (*e.g.* blood, serum).[190] Passivation in aqueous media is also of great concern (hydrophobicity of passivation materials greatly affects both the stability and overall performance of the sensor[191]), as well as the problem of noise for nanoscale-devices originating from variations in interfacial charge.[191]

Photoelectrochemical (PEC) (bio)sensing

The PEC sensor performance relies on photoactive materials that produce photocurrent upon absorbing photons and engage in redox reaction at the WE surface *via* different transduction mechanisms: formation of electrons/holes, introduction of photoactive species, steric hindrance, *in situ* induction of light, or resonance energy transfer.[146,192,193] PEC-based (bio)sensing, although presenting a promising novel analytical method for biomarker detection, is yet at a very early stage for practical application. Nevertheless, a few examples of PEC sensing strategies have been suggested for detection of mTBI-related biomarkers such as MMP-2,[194,195] NFL,[196] NSE,[197] Tau proteins,[149,198] and CRP.[146,199] Further discussion of PEC affinity-based detection principles, types of photoactive species and signal transduction mechanisms is outside the scope of this work and can be found *e.g.* in a recent detailed review by Victorious *et al.*[193]

Conclusions and future outlook

The aim of this manuscript has been to give a detailed overview of EC approaches developed and used for detection of blood protein biomarkers previously shown to be relevant for diagnostics and prognostics of mTBI with the possible application towards POC testing.[12] The relevance of a POC diagnostic mTBI biomarker test cannot be overstated. The optimal approach would likely comprise early biomarker detection (on site of an accident and immediately after injury) with follow-up

measurements at various intervals (*e.g.*, in the ambulance, upon arrival at the hospital and pre- or post-diagnostic imaging), allowing for the observation of injury characteristic biomarker increases and decreases. Instead of a single time point measurement, data on the early acute phase trend could be useful both as a diagnostic determinant and as an indicator of injury progression.[87]

As briefly highlighted with an excerpt of a target product profile for a POC diagnostic device for mTBI (see Table 2), the following requirements should be considered when developing a POC biosensor for clinical applications: (i) high sensitivity and specificity to the target analytes; (ii) good reproducibility, reliability and stability of sensor's readings; (iii) short analysis time; (iv) low sample consumption; (v) low cost of production; (vi) portability; (vii) environment-friendly disposable design; (viii) user-friendliness (ease of operation); (ix) preferably requiring no sample pre-treatment, else a pre-treatment as simple as possible; (x) preferably reagent-free, else with small reagent consumption.

The key advantages of (bio)sensors for *in vitro* POC diagnostics include relatively short analysis time and low reagent consumption (Fig. 8). In respect to the sensor fabrication technology, most of the sensor elements are easily miniaturizable, have low fabrication costs and could be readily integrated into a POC platform. Transducer elements are typically manufactured using microfabrication technology convenient for mass fabrication process, while the electrode functionalization with the recognition elements (*e.g.*, antibodies) is considerably more challenging even before the scale-up of the production process.

Although EC sensors for measurement of non-protein metabolites in whole blood such as glucose, lactate, uric acid, cholesterol, blood gases and electrolytes are commercially available and used routinely for POC diagnostic applications, the development of robust, accurate and highly reproducible strategies remains a challenge for protein biomarkers, especially those relevant to mTBI. The challenge for the POC test development is the significant variation in mTBI biomarker kinetics and the fact that some biomarkers may be more suited to cross the blood brain barrier than others, raising the need for ultrasensitive detection strategies. Detections limits as low as a few fg mL^{-1} have been reported for the EC detection of certain biomarkers (*e.g.* 1 pg mL^{-1} for GFAP;[202,203] 0.3 fg mL^{-1} for h-FABP;[180] 0.32 fg mL^{-1} for IL-6 [137]). As discussed in this article and summarized in Table 3 and SI-1,† various EC sensor designs and analytical approaches have been reported in the literature for detection of 19 mTBI-relevant blood proteins (total 127 publications). However, most of the EC approaches developed so far for detection of mTBI related proteins either suggest serum/blood dilution or have not been tested with clinical samples.[204] In the published cases where these performances are claimed to be determined in serum or plasma matrix the question comes up as to what extent these high-sensitivity results are reproducible with multiple biosensor lots manufactured and a statistically relevant number of clinical samples. Moreover, some approaches require a preconcentration step; additional dilution is often required for FET-based biosensing which is limited by high ionic strength of biological fluids due to Debye-screening length, as well as for strategies with short dynamic ranges. In conclusion, the realization of reproducible, sensitive but highly scalable transducer–electrolyte interfaces remains a big challenge. The ongoing studies worldwide are currently aimed at improving the test performance by increasing the sensitivity, specificity, turnaround time and decreasing the costs.

While most designs exploit the antibody–antigen interaction, smaller biorecognition molecules such as DNA/RNA or peptide aptamers, MIPs and other specific synthetic receptors have a promising potential for improving the performance of EC sensors in terms of high specificity and sensitivity, inexpensive and readily scalable cell-free chemical synthesis and low batch-to-batch variability.[153,154,205]

The lack of stability, enough long shelf-life (often required to exceed 6 months) and deterioration of the analytical performance of the transducer functionalized with biological recognition elements over time could become a serious issue in product development and envisioned commercialization. In fact, POC diagnostic devices for various analytes are known to often underperform in terms of accuracy and precision compared to central laboratory instruments, so a multiplex diagnostic test for mTBI protein biomarkers is likely going to encounter a cumulation of challenges to meet. On the other hand, from the viewpoint of signal processing, multiplexing (*i.e.*, support of multi-biomarker analysis) is more straightforward with EC techniques.

Impedimetric measurements, along with FET-based devices, constitute the main platform for rapid label-free and potentially reagent-free detection of mTBI biomarkers. Nevertheless, the issue of non-specific binding remains challenging for most if not all biosensing strategies and especially for label-free assays since the latter do not discriminate between the signal caused by specific *versus* non-specific interactions.[170,206,207] Despite the inherent advantages offered by label-free techniques, labelled assays continue to be an important direction in the development of sensing strategies providing the benefits of improved

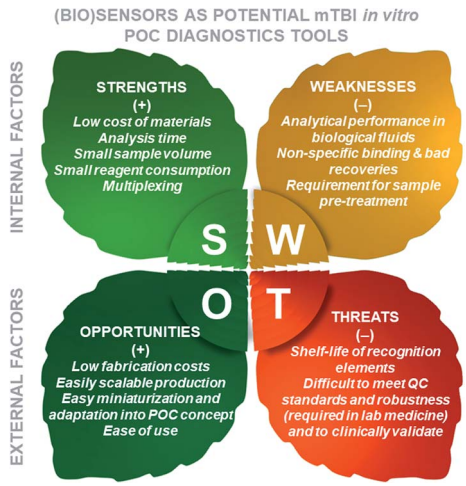

Fig. 8 SWOT analysis of (bio)sensors as potential mTBI *in vitro* POC diagnostic tools.

selectivity and significantly increased sensitivity using various signal amplification approaches, such as *e.g.* functionalized nanoparticles. One of the drawbacks for the integration into clinical analysis is the long time required to complete the assay. The latter neither makes them attractive for the development of POC diagnostic applications nor advantageous compared to well-established analytical solutions in laboratory medicine.

Therefore, despite the large and diverse pool of developed EC (bio)sensor designs, for the eventual integration of the proposed concepts into mTBI POC diagnostic device solutions much work still must be accomplished requiring a close collaboration of the researchers in the field of biochemistry with material scientists and nanotechnologists, engineers, and clinicians. Importantly, the development of easy-to-use and affordable tools for detection of specific biomarkers and biomarker panels, could aid not only the diagnostics of well-established disease biomarkers, but as well in the process of evaluation and identification of prognostic value of currently investigated biomarkers and in the establishment of an 'ideal' biomarker panel for TBI diagnostics. The interdisciplinary synergy seems to be necessary to overcome the barrier between rapidly progressing academic research and real-life medical diagnostic applications.

A big step toward the first US commercial POC diagnostic test for mTBI was made by Abbott Diagnostics, recently. Following a non-exclusive license agreement with Banyan Biomarkers in 2019, Abbott received FDA 510(k) clearance for the first rapid handheld blood test for concussions in January 2021. The test runs on the i-STAT™ Alinity™ POC device and it measures amperometrically the UCH-L1 and GFAP biomarkers. The UCH-L1 biomarker complements GFAP as each result is produced by a different type of cell and measures distinctive molecular events.[208] The results are given 15 min after the plasma sample is inserted in the cartridge. Building on this initial clearance, Abbott is also working on a test that would use whole blood on i-STAT device.[209] Further developments, optimization and additional prospective studies are required to assure sufficient diagnostic specificity and sensitivity in evaluating concussions in patients with mTBI (*i.e.* multiplex panel extension with additional biomarkers or biomarker types like inflammation proteins and brain damage proteins). As other companies, such as NanoDx™[210] that uses an ultrasensitive nanowire technology to resistively measure the biomarkers S100β and GFAP, are following on Abbott's heels, electrochemical POC sensing of blood proteins for mTBI may at last experience a much needed push forward.

Author contributions

The manuscript was written through contributions of all authors.

Abbreviations

Ab	Antibody
AD	Alzheimer's disease
ALP	Alkaline phosphatase
AM	Acrylamide
Apt	(Oligonucleotide) aptamer for the target (T)
APMES	3-(Ethoxydimethylsilyl)propylamine
ASV	Anodic stripping voltammetry
AuNPs	Gold nanoparticles
BDNF	Brain-derived neurotrophic factor
bio-FED(s)	Field-effect based biosensing devices
CD-GS	β-Cyclodextrin-graphene sheets
CMOS	Complimentary metal-oxide semiconductor
CNTs	Carbon nanotubes
CO	Cutoff
CP	Conductive polymer
CPA	Constant potential amperometry
CS	Chitosan
CSF	Cerebrospinal fluid
CT	Computer tomography
CV	Cyclic voltammetry
DA	Dopamine
DE	Disk electrode
DPV	Differential pulse voltammetry
DTSP	Dithiobis (succinimidyl propionate)
EC	Electrochemical
EDC	1-Ethyl-3-(3-dimethylaminopropyl)carbodiimide
EDL	Electric double layer
EIS	Electrochemical impedance spectroscopy
Exo III	Exonuclease III
Fc	Ferrocene
FDA	Food and Drug Administration
FED	Field-effect based detection (voltage controlled current amplification)
FEED	Field-effect enzymatic detection
FET	Field-effect transistor
FTO	Fluorine doped tin oxide
GA	Glutaraldehyde
GCE	Glassy carbon electrode
GCS	Glasgow Coma Scale
GFAP	Glial fibrillary acidic protein
GFET	Graphene-based field-effect transistor
GM-CSF	Granulocyte-macrophage colony-stimulating factor
GOx	Glucose oxidase
GOx-LS	Glucose oxidase-loaded liposomes
GR	Graphene
GRO	Graphene oxide
GRONRs	Graphene oxide nanoribbons
h-FABP	Heart-fatty acidic binding protein
HP	Human plasma
HRP	Horseradish peroxidase
HS	Human serum
ID	Interdigitated
IDE	Interdigitated electrode
IL	Ionic liquid
IL-6	Interleukin-6
IL-8	Interleukin-8
IL-10	Interleukin 10
ISE(s)	Ion-selective electrode(s)
ISFET	Ion-sensitive field-effect transistor
ITO	Indium tin oxide

IVD	*In vitro* diagnostics
LDL	Lower detection limit
LFA	Lateral flow assay
MBP	Myelin basic protein
MBs	Magnetic beads
MD	Median
mdTBI	Moderate TBI
MeB	Methylene blue
MHDA	Mercaptohexadecanoic acid
MIP	Molecularly imprinted polymer
MMP(s)	Matrix metalloproteinases
MN	Mean
MOF	Metal–organic framework
MPA	3-Mercaptopropionic acid
MRI	Magnetic resonance imaging
MT3	Metallothionein 3
mTBI	mild TBI
MUA	11-Mercaptoundecanoic acid
MuxT	Multiple protein biomarker targets detected within the same immunoassay
MWCNTs	Multiwalled carbon nanotubes
NCAM	Neuron cell adhesion molecule (CD56);
NFL	Neuro-filament light
NGB	Neuroglobin
NHS	*N*-Hydroxysuccinimide
NHSS	Hydroxysulfosuccinimide
NRGN	Neurogranin
NSB	Non-specific binding
NSE	Neuron-specific enolase
NWs	Nanowires
OFET	Organic field-effect transistor
pABA	*p*-Aminobenzoic acid
PAMAM	Poly(amido)amine
PANI	Polyaniline
PBASE	1-Pyrenebutyric acid *N*-hydroxysuccinimide ester
PEC	Photoelectrochemical (detection)
PEG	Polyethylene glycol
PEI	Poly(ethyleneimine)
Pept-SH	Thiolated peptide
PET	Positron emission tomography
POC	Point-of-care
PPy	Polypyrrole
PPy-NWs	Polypyrrole-nanowires
PPyPAC	Polypyrrole electrodes modified by electrodeposition of diazonium salts using 4-aminophenylacetic acid (4APAC)
PS–MA	Polystyrene-*co*-methacrylic acid
*p*TTBA	(2,2:5,2-Terthiophene-3-(*p*-benzoic acid))
*p*TTBPA	4′-([2,2′:5′,2″-Terthiophen]-3′-yl)-[1,1′-biphenyl]-4-carboxylic acid
RG	Range
rGRO	Reduced graphene oxide
RPS	Resistive pulse sensing
RT	Room temperature
S100β	Calcium binding protein B
SAM(s)	Self-assembled monolayer(s)
SH	Thiol group
SH-Apt	Thiolated aptamer for the target (T)
SI	Supplementary information
SL	Single layer
SPBs	Superparamagnetic beads
SPE	Screen-printed electrode
sTBI	Severe TBI
Stv	Streptavidin
SWCNTs	Single wall carbon nanotubes
SWV	Square wave voltammetry
T	Target
TBI	Traumatic brain injury
TBO	Toluidine blue O
TPP	Target product profile
T-Tau	Total Tau (phosphorylated and non-phosphorylated)
UCH-L1	Ubiquitin carboxyl-terminal hydrolase-L1
undil.	Undiluted
VCAM-1	Vascular cell adhesion protein 1
WE	Working electrode

Conflicts of interest

There are no conflicts to declare.

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