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REVIEW

The unravelling of metabolic dysfunctions linked to metal-associated diseases by blue native polyacrylamide gel electrophoresis

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Abstract Gel electrophoresis is routinely used to separate and analyse macromolecules in biological systems. Although many of these electrophoretic techniques necessitate the denaturing of the analytes prior to their analysis, blue native polyacrylamide gel electrophoresis (BN-PAGE) permits the investigation of proteins/enzymes and their supramolecular structures such as the metabolon in native form. This attribute renders this analytical tool conducive to deciphering the metabolic perturbations invoked by metal toxicity. In this review, we elaborate on how BN-PAGE has led to the discovery of the dysfunctional metabolic pathways associated with disorders such as Alzheimer's disease, Parkinson's disease, and obesity that have been observed as a consequence of exposure to various metal toxicants.

Keywords Blue native polyacrylamide gel electrophoresis · Metabolic dysfunction · Metal toxicity · Enzymes · Functional proteomics · Diseases

Abbreviations

ACC	Acetyl coenzyme A carboxylase
BN buffer	Blue native buffer
BN-PAGE	Blue native polyacrylamide gel electrophoresis
ETC	Electron transport chain
FAD	Flavin adenine dinucleotide
Fe-S	iron–sulfur
G6PDH	Glucose 6-phosphate dehydrogenase
HK	Hexokinase
ICDH	Isocitrate dehydrogenase
INT	Iodonitrotetrazolium chloride
NEIL	Endonuclease VIII-like
PK	Pyruvate kinase
PMS	Phenazine methosulfate
ROS	Reactive oxygen species
SDS-PAGE	Sodium dodecyl sulfate polyacrylamide gel electrophoresis
TCA	Tricarboxylic acid

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Introduction

One of the major impediments to the advancement of life sciences is the inability to probe biological processes in situ, i.e. as they occur and in a non-invasive manner. Although this goal is partially being attained with the advent of positron emission tomography, functional magnetic resonance imaging and high-resolution confocal microscopes, these techniques are expensive and not readily accessible [1–3]. Furthermore, these procedures cannot delineate the molecular interactions precisely. Blue native polyacrylamide gel electrophoresis (BN-PAGE) provides a potent proteomic tool to study enzymatic activity, protein–protein interactions, metabolons and supramolecular structures as these molecular entities are maintained in a relatively native form during the detection process [4–6]. These features render it a great technique to use in conjunction with the aforementioned in situ analyses.

BN-PAGE is a technique used to separate and characterize microquantities of proteins. What separates BN-PAGE from other electrophoretic techniques is the maintenance of biomolecules in their native conformation, thus keeping enzymatic activity and protein–protein interactions intact

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throughout the process [4]. Protein samples in the form of both total and subcellular fractions can be analysed by BN-PAGE. Differential and density centrifugation often prove to be suitable methods to fractionate the total cellular extract [7, 8]. Isolated soluble and membranous proteins are then diluted in 3× blue native buffer (BN buffer) in order to provide the reductive environment necessary for BN-PAGE. Membranous proteins are, however, treated with mild detergents such as *n*-dodecyl-β-D-maltoside, digitonin and Triton X-100 as these help solubilize membranous proteins. In addition, BN buffer contains ε-aminocaproic acid, a moiety of low ionic strength which provides support for the solubilization of membranous proteins [6]. BN-PAGE uses the anionic dye Coomassie brilliant blue G-250, which imposes a charge shift on proteins and permits the visualization of the migrating biomolecules [9]. Once the dye is associated with these moieties, it prevents the aggregation of membrane-bound proteins, averting the need for a strong detergent in the gel and greatly reducing the risk of denaturation [10]. Prepared proteins are then loaded into acrylamide gradient gels, which is the commonest gel type used in BN-PAGE [11]. The reagents required to compose a single gel include acrylamide, BN buffer, glycerol (for gradient formation), tetramethylethylenediamine and ammonium persulfate. Gradient formers can be applied to produce the desired gradient gel. Once polymerization is complete, water should be removed from the top of the gradient gel and the stacking gel can be placed with a comb. Electrophoresis is performed with the current and voltage limited to 15 mA and 500 V, respectively. Approximately at one third or half of the running distance, the blue cathode buffer—50 mM tricine, 15 mM [bis(2-hydroxyethyl)amino]tris (hydroxymethyl)methane/7.5 mM imidazole, 0.02 % Coomassie brilliant blue G-250, pH 7.0—is replaced with clear cathode or slightly blue cathode buffer—50 mM tricine, 15 mM bis(2-hydroxyethyl)amino]tris(hydroxymethyl)methane, pH 7.0 (slightly blue cathode buffer consists of 0.002 % Coomassie brilliant blue G-250)—in order to provide improved detection of protein bands. Additionally, the lower Coomassie dye content in the gel can improve the native blotting results as Coomassie dyes tend to compete with protein binding to poly(vinylidene difluoride) membranes [6]. Proteins are separated according to their size in the polyacrylamide gels and gradually decelerate as they progress through a gradient gel until they stop at a specific pore size [12]. Cytoplasmic proteins and other proteinaceous biomolecules are also kept in their native forms owing to the interaction of the dye [10]. This allows the supra-structures that are usually held together by weak forces to migrate as a single entity [6] (Fig. 1).

Following electrophoresis, the separated proteins can be studied using a variety of techniques aimed at deciphering their molecular characteristics. Activity staining is a common technique used to examine the state of enzymes as well as their localization in gel. Additional analyses, such as digestion and protein sequencing, can be applied to confirm the identity of a particular protein in gel. Incubation of the gel in a reaction buffer consisting of substrates, cofactors and the required enzymes for linked reactions results in the detection of the immobilized proteins. In these instances, the specificity of the substrate and cofactor is the key determinant of the enzyme being probed. In the case of a dehydrogenase, the reduction of a cofactor such as $NAD(P)^+$, flavin adenine dinucleotide (FAD) or flavin mononucleotide is coupled to the reduction of phenazine methosulfate (PMS), which is in turn coupled to the reduction of iodonitrotetrazolium chloride (INT), producing a red formazan precipitate where the enzyme is immobilized in the gel (Fig. 2) [13]. This preliminary analysis of enzymatic activity can be confirmed using negative controls, selective inhibitors for the

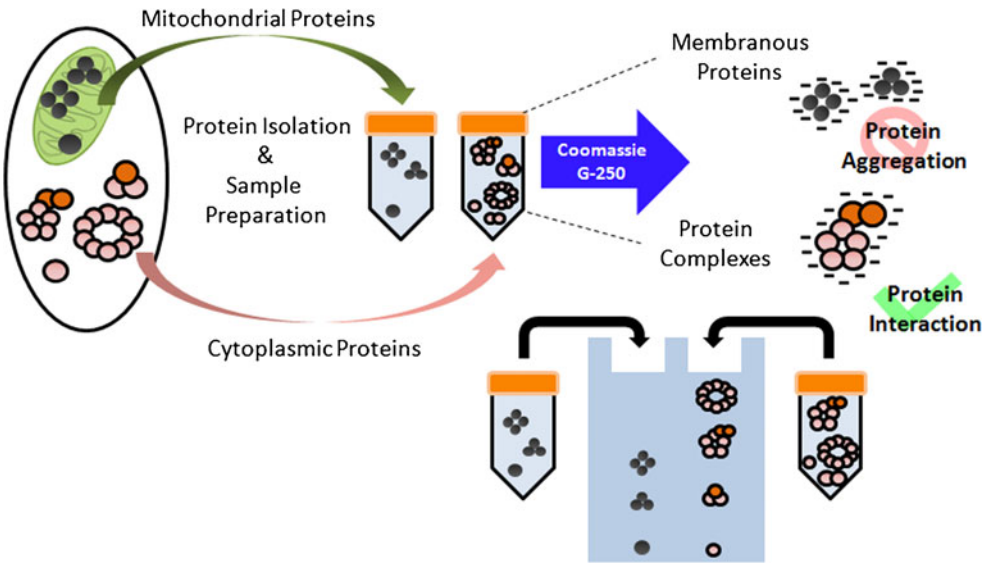

Fig. 1 A schematic depiction of blue native polyacrylamide gel electrophoretic process. The dye Coomassie brilliant blue G-250 prevents protein aggregation in membranous proteins without disrupting protein–protein interactions. Proteins subjected to BN-PAGE are then separated according to their size, while maintaining their native conformations

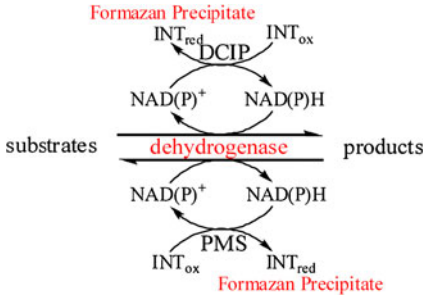

Fig. 2 The in-gel method to detect dehydrogenases. Reduction of iodonitrotetrazolium chloride (*INT*) produces a formazan precipitate at the location of the enzyme in the gel. *DCIP* 2,6-dichloroindophenol, *PMS* phenazine methosulfate

target of interest or by excising the band and observing the substrate consumption via analytical techniques such as high-performance liquid chromatography. It is the ability to excise bands for further studies that gives added value to this analytical tool. Two-dimensional gels, protein sequencing, visualization by crystallization and electron microscopy, immunoblotting and protein–protein interaction studies are some of the techniques that can be used following BN-PAGE (Fig. 3). The ability of BN-PAGE to function as a source of semipurified protein for any further proteomic studies is also a great advantage [14].

Many enzymes in central metabolism can be studied using BN-PAGE (Table 1). Glycolytic enzymes such as hexokinase (HK; EC 2.7.1.1), phosphofructokinase, pyruvate kinase (PK; EC 2.7.1.40) and lactate dehydrogenase (LDH; EC 1.1.1.27), tricarboxylic acid (TCA) cycle enzymes such as isocitrate dehydrogenase (ICDH; EC 1.1.1.42), α-ketoglutarate dehydrogenase, succinate dehydrogenase (EC 1.3.5.1) and malate dehydrogenase, and the oxidative phosphorylation complexes I, II, IV and V can be probed readily depending on the quantity of sample available, thus providing a quick view of central energy metabolism in any given system being studied (Fig. 4). The sample quantity can be as low as 10 μg of total protein from cell-free extracts. As BN-PAGE permits the study of energy metabolism and other metabolic pathways, this technique can be employed to monitor the modulation of such networks by a variety of environmental toxins, such as metals [15–17].

Metals in biology and metal toxicity

Metals are important constituents of all living organisms as they are involved in a variety of functions. Approximately 30 metals are known to be essential, and nearly half of all known enzymes require metals for their proper functioning [18]. In particular, biochemical reactions are most commonly aided by metals as there are a limited number of functional groups available in amino acids for chemical modifications. The presence of metals also allows the movement of electrons within redox-cycling metals rather than the organic compounds, in order to reduce the toxic effect

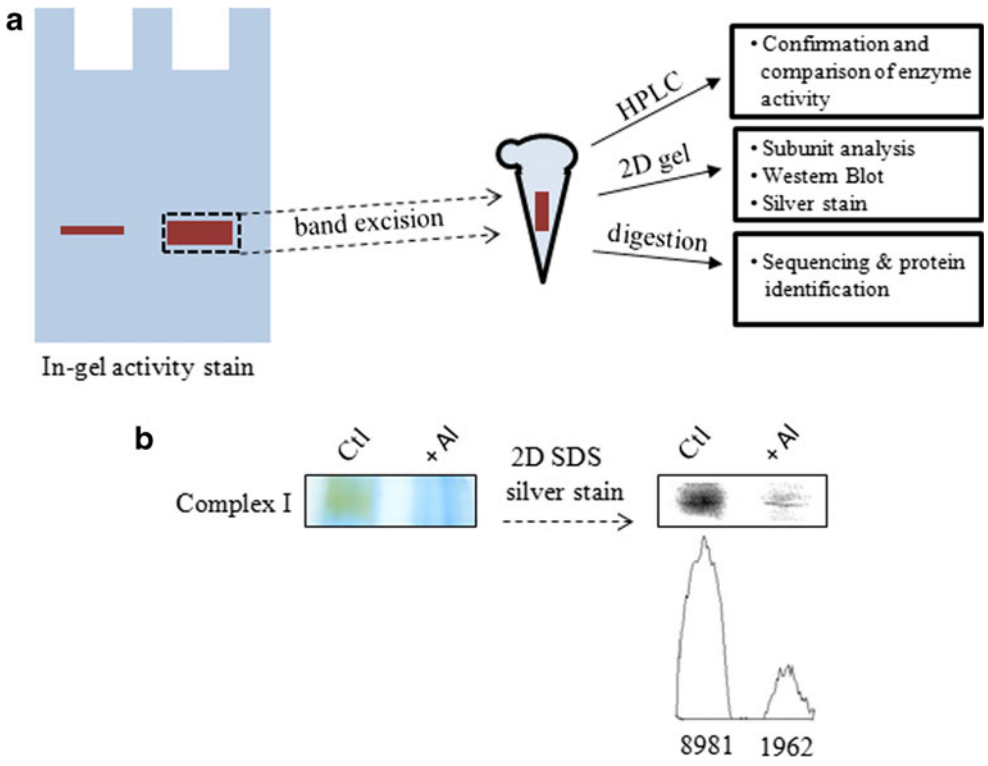

Fig. 3 BN-PAGE: an analytical tool with multiple applications. **a** Excision of the enzyme following BN-PAGE permits a number of follow-up studies. **b** Representative gel showing activity of complex I, which was excised and subjected to sodium dodecyl sulfate (*SDS*) polyacrylamide gel electrophoresis and silver staining. Densitometry with ImageJ for Windows was used to compare band intensity. *Ctl* control, *Al* 500 μM aluminium citrate. (Adapted from [45])

Table 1 Enzymes which can be monitored via blue native polyacrylamide gel electrophoresis *CoA* coenzyme A

Tricarboxylic acid cycle	Oxidation phosphorylation	Glycolysis
Aconitase [62]	Complex I [4, 6]	Glyceraldehyde 3-phosphate dehydrogenase [64]
NAD/NADP-dependent isocitrate dehydrogenase [25, 26]	Complex II [4, 6, 26]	Pyruvate kinase [37]
α-Ketoglutarate dehydrogenase [26]	Complex III [4, 6]	Pyruvate phosphate dikinase [37]
Fumarase [62, 63]	Complex IV [4, 6]	Lipogenesis
Malate dehydrogenase [62]	Complex V [4, 6]	Glycerol 3-phosphate dehydrogenase [40]
Pyruvate dehydrogenase [5]	Gluconeogenesis	Acetyl-CoA carboxylase [40]
Malic enzyme [32]	Pyruvate carboxylase [63]	ATP-citrate lyase [40]
	Phosphoenolpyruvate carboxykinase [63]	

derived from highly reactive electrons. Iron and copper are well-known examples of redox-active metals which can potentially be toxic to organisms; thus, these metals are tightly regulated in all biological systems. For example, iron uptake, transport and storage is regulated by proteins such as ferrireductase and the ferroxidase hephaestin, which control its redox state, divalent metal transporter 1 and ferroportin, which specifically transport iron, and transferrin, which keeps iron bound in serum. Iron uptake is regulated by signalling processes and hormones such as hepcidin which can affect iron uptake and localization in response to iron levels. Even though the movement is limited to Fe(II) and Fe(III), these processes are tightly regulated and their perturbation can cause detrimental effects [19–22].

Metal pathogenesis is essentially derived from the direct interactions of metals with thiol, histidyl and carboxyl groups on proteins, generation of oxidative stress by redox-active metals such as iron and copper and displacement of essential cations, thus nullifying the catalytic capabilities of enzymes. These perturbations can change the

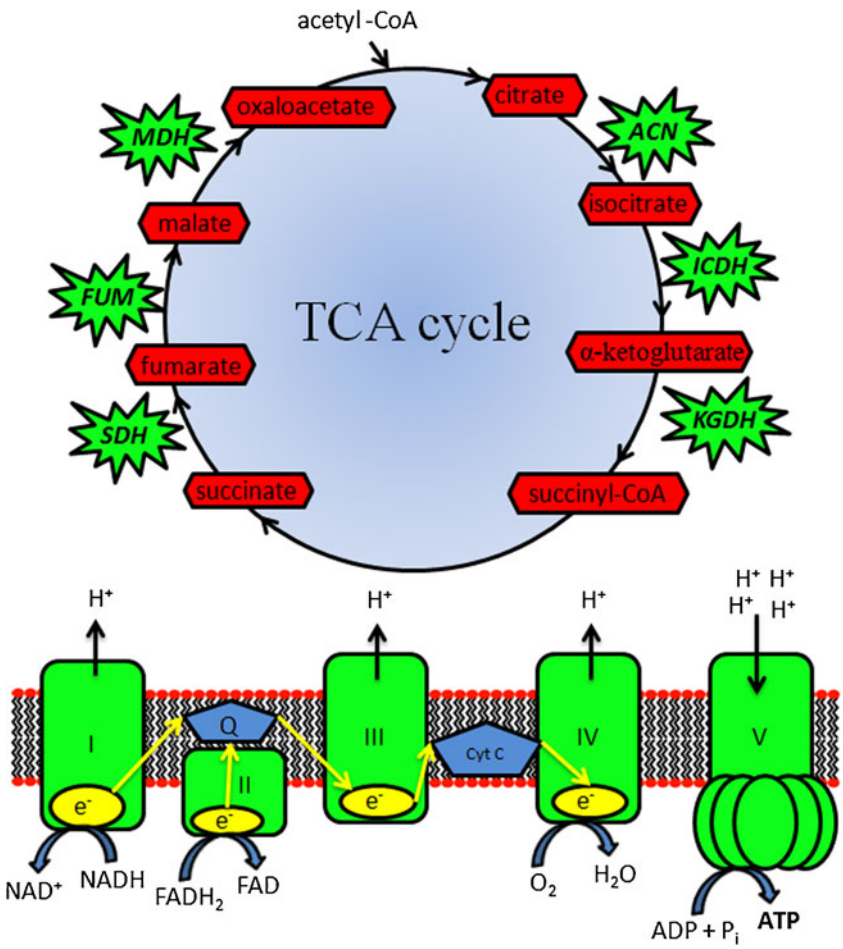

Fig. 4 The tricarboxylic acid (*TCA*) cycle and oxidative phosphorylation. Enzymes which can be detected via BN-PAGE are in *green*. *ACN* aconitase, *FAD* flavin adenine dinucleotide, *FUM* fumarate hydratase, *ICDH* isocitrate dehydrogenase, *KGDH* α-ketoglutarate dehydrogenase, *MDH* malate dehydrogenase, P_i inorganic phosphate, *SDH* succinate dehydrogenase,

cellular redox state, with major implications for energy metabolism, intermolecular and intracellular signalling and gene expression [23–25] (Fig. 5). Metal imbalances due to nutrition, pollution and genetic defects are known to be an important cause of numerous diseases. Particularly, anthropogenic activities tend to result in an increased amount of bioavailable metals in the environment. Thus, metal pollution is known to have a direct correlation with diseases. Numerous disorders, such as obesity, neurological diseases and metabolic abnormalities, are associated with metal toxins (Table 2). Although a variety of approaches may be used to shed light on the molecular details underlying metal-associated diseases, BN-PAGE provides a unique tool to probe enzymatic processes and protein interactions in a native environment [4, 12, 14, 15].

Table 2 Disorders which may stem from exposure to metal toxicants

Diseases	Metals	References
Hypersensitivity	Ni	Roediger and Weninge [54]
		Heiss et al. [55]
Alzheimer's disease	Cu, Zn, Pb, Al	Zheng et al. [58]
		Bucossi et al. [59]
		Wu et al. [60]
		Pithadia and Lim [39]
		Rondeau et al. [61]
		Ribes et al. [62]
Parkinson's disease	Zn, Fe	Barnham and Bush [63]
		Altamura and Muckenthaler [64]
		Viles [40]
		Rose et al. [65]
Obesity	Pb, Hg, As	Padilla et al. [66], Hyman [67]

Monitoring metal-induced metabolic dysfunction in ATP synthesis using BN-PAGE

Dysfunctional metabolism is a common event occurring in many known diseases. This also holds true for metal toxicity. In particular, the ability of metals to interact with proteins places a great burden on the metabolic networks in biological systems. Among the many different types of metabolic pathways, the energy-generating networks appear to be heavily influenced by metal imbalances. Metals exert their toxic effects mainly through the displacement of cations in the enzymes, interfering with electron-rich centres and generating reactive oxygen species (ROS). As iron is an important component of most if not all energy-producing machinery, this system succumbs to metal stress [26, 27].

The redox-active metal iron is an important moiety in numerous enzymes. This metal is known to act as an electron acceptor and donor, thus contributing significantly to biological processes. The stability of iron is maintained by locking this moiety up in iron–sulfur (Fe-S) clusters and haem proteins. Most frequently, the Fe-S clusters are arranged in the forms Fe_2S_2, Fe_3S_4 and Fe_4S_4. Aconitase (EC 3.2.1.3) is a primary example of an Fe-S-cluster-containing enzyme in energy metabolism. This gate-keeping enzyme of the TCA cycle is sensitive to oxidative stress as the Fe-S cluster of this protein is readily dissociated in an oxidative environment, thus limiting carbon flow and the electron transport chain (ETC.) from producing additional

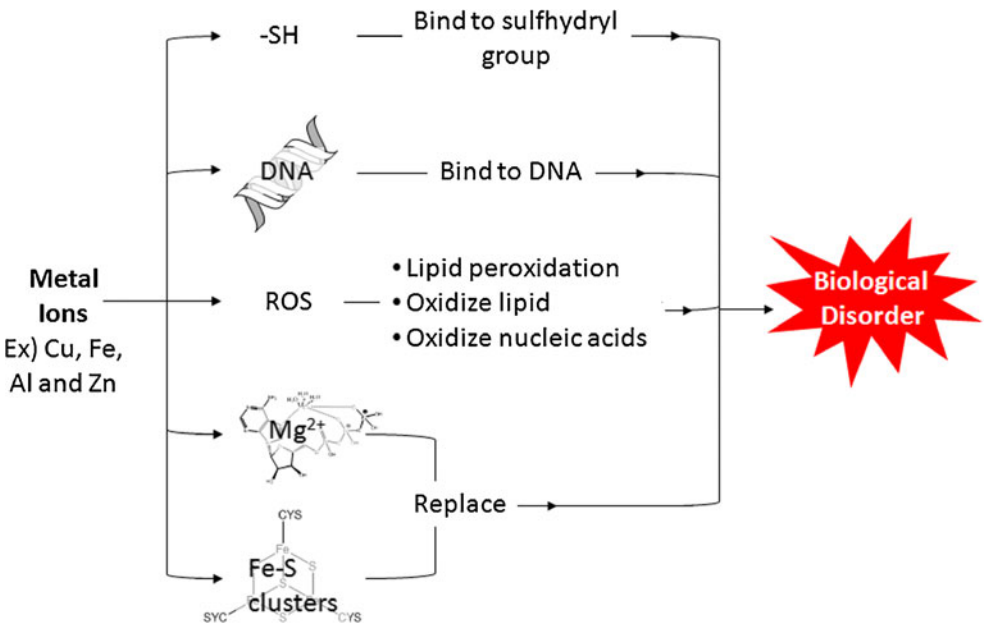

Fig. 5 Toxicopathology of metal ions. The latter can bind to sulfhydryl groups and DNA. Additionally, they may directly or indirectly produce reactive oxygen species (*ROS*) and replace Mg^{2+} and metal cofactors in proteins

ROS. In mammalian systems, aconitase also plays a non-catalytic role by acting as a regulatory protein that controls the stability and translation of messenger RNAs which govern iron and energy homeostasis. Metals such as aluminium readily target Fe-S clusters, thus releasing iron, an event that helps promote an oxidative environment. Succinate dehydrogenase and fumarate hydratase [EC 4.2.1.2] are also Fe-S-cluster-containing enzymes belonging to the TCA cycle. ETC. enzymes such as complex I (EC 1.6.5.3), complex III (EC 1.10.2.2) and complex IV (EC 1.9.3.1) also harbour iron in the form of either Fe-S clusters or haems. Hence, metals such as aluminium, gallium, and zinc are known to severely affect these energy-synthesizing pathways [28–30].

BN-PAGE provides an ideal tool to monitor these enzymes involved in ATP production. Indeed, using human astrocytes and hepatocytes, Lemire et al. [31] applied BN-PAGE to evaluate these enzymes in an aluminium-rich environment. BN-PAGE was particularly efficient in identifying the aluminium-induced downregulation of ATP production, as there were numerous enzymes that were shown to be negatively affected, such as complex II and complex IV. Although complex IV was monitored with the aid of diaminobenzidine, complex I was followed by the oxidation of NADH in the presence of INT. The activity of complex II was observed by incubating the gel in the presence of succinate, FAD^+ and INT. The fate of dehydrogenases operative in the TCA cycle can be detected by running these mitochondrial proteins on a 4-16 % polyacrylamide gel followed by incubation of the gel in appropriate substrates. The precipitated formazan appears on the gel at the site of the immobilized enzyme; α-ketoglutarate dehydrogenase, NAD^+-ICDH and malate dehydrogenase are known to be downregulated in the presence of aluminium and zinc [29, 30, 32]. Hence, the perturbation of the TCA cycle and complexes of oxidative phosphorylation can be easily followed by BN-PAGE. Indeed, mitochondrial dysfunction in Alzheimer's disease can be readily monitored using BN-PAGE. In cognitive-deficient 3xTg-AD mice, carnosine was used to alleviate the disruption in energy metabolism, potentially through the chelation of Zn^{2+}, an essential micronutrient which can engender toxic effects at high concentrations [33]. By probing the ETC. complexes, BN-PAGE was effective in illustrating mitochondrial dysfunction in 3xTg-AD mice and the improvement of mitochondrial energy metabolism following dietary supplementation of carnosine. There was a restoration in the activity of complexes I, II and IV in the presence of carnosine [34]. In another study involving 3xTg-AD mice, BN-PAGE activity assay was applied to evaluate the hippocampus, cerebral cortex and cerebellum regions of the mice in order to assess the activity of complexes I, II and IV under abnormal conditions. The impact of mercury on complex V was observed by the precipitation of phosphate in gel after electrophoresis. In this instance, a marked decrease in the activity of this enzyme triggered by metal toxicity was observed [33].

Nucleoside diphosphate kinase (EC 2.7.4.6), an enzyme involved in maintaining the equilibrium of different nucleoside triphosphates, is also affected by metal stress and can be studied by BN-PAGE [35]. The formation of ATP from GTP and ADP can be monitored by HK and glucose 6-phosphate dehydrogenase (G6PDH; EC 1.1.1.49). In this instance, the phosphorylation of glucose by HK occurs only when ATP is generated from the activity of nucleoside diphosphate kinase. The interaction of glucose 6-phosphate with G6PDH and $NADP^+$ is followed by the precipitation of INT in presence of PMS [36] (Fig. 6). The ability to separate enzymes in their active form by BN-PAGE affords a quick and efficient technology to monitor the status of the oxygen-dependent energy-generating machinery in the presence or absence of metal toxins. Clearly, the ability of aluminium, zinc and mercury to impede this ATP-producing network may have implications for a variety of disorders [23, 37–40]. This analytical tool provides interesting insights into the biochemical pathways underlying diseases stemming from metal exposure.

BN-PAGE: metal-linked dysfunction in anaerobiosis and glycolytic ATP production

This non-denaturing electrophoretic technique is also ideal for investigating enzyme systems responsible for ATP formation in the absence of oxygen. Since aluminium, zinc and other metals affect the ETC. in most organisms, energy production

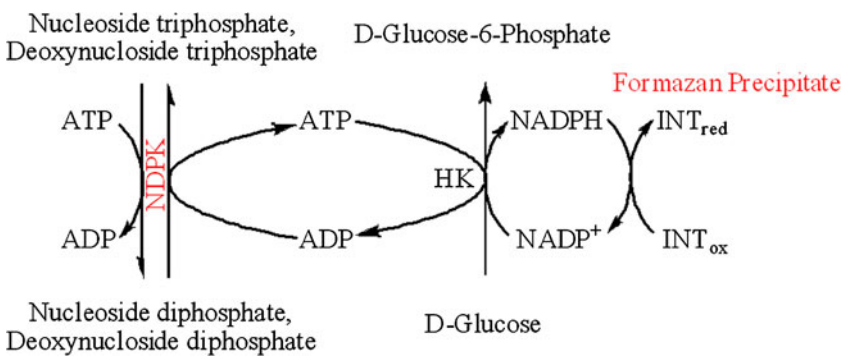

Fig. 6 The enzyme-coupled method to detect enzymes in gel. Here, hexokinase (*HK*) was used as a coupling enzyme to produce formazan precipitate at the site of nucleoside diphosphate kinase (*NDPK*) in gel. (Adapted from [35])

is shifted to glycolysis, where ATP is generated by substrate-level phosphorylation. PK and HK, two enzymes that are upregulated during aluminium stress, can be monitored by BN-PAGE. The PK-mediated production of pyruvate from phosphoenolpyruvate and ADP can be followed in the presence of lactate dehydrogenase, NADH and 2,6-dichloroindophenol [41]. In the presence of glucose and ATP, HK generates glucose 6-phosphate, which can be detected via the addition of G6PDH, $NADP^+$, PMS and INT. These enzymes were indeed found to be upregulated in hepatocytes and astrocytes subjected to aluminium toxicity. However, in astrocytes, ATP storage was deemed ineffective as creatine kinase (EC 2.7.3.2), an enzyme that plays a critical role in the stabilization of high-energy phosphate, was downregulated [31]. This enzyme can also be visualized by BN-PAGE with the aid of G6PDH. The dysfunction in ATP production resulted in globular-shaped astrocytes, an event which may contribute to neurological disorders. Indeed, changes in astrocyte morphology have previously been linked to the neuropathogenesis of Alzheimer's disease [42–44].

Use of BN-PAGE in detecting dyslipidaemia triggered by metal toxicity

The use of this electrophoretic technique is not only limited to studying mitochondrial or glycolytic energy production. Other complex metabolic pathways involving numerous enzymes can also be evaluated. Lipid metabolism is among the many networks that can be monitored by BN-PAGE. Although a link between dyslipidaemia and metal toxicity has been suggested, the molecular details on how this dysfunction occurs are not fully understood. In a recent study [45], lipid accumulation in the form of very low density lipoprotein was demonstrated in human hepatocytes undergoing aluminium stress. With use of BN-PAGE, enzymes such as acetyl coenzyme A carboxylase (ACC; EC 6.4.1.2), the NADPH producing enzymes G6PDH and $NADP^+$-ICDH and glycerol 3-phosphate dehydrogenase (EC 1.1.1.8) that facilitate enhanced lipogenesis were found to be upregulated (Fig. 7). Whereas ACC provides malonyl coenzyme A, a key determinant of lipogenesis, the NADPH generated by G6PDH and $NADP^+$-ICDH allows the eventual condensation of malonyl coenzyme A and acetyl coenzyme A into fatty acids. Glycerol 3-phosphate dehydrogenase provides the glycerol backbone of the triglyceride, a major constituent of very low density lipoprotein [45]. The dehydrogenases are detected by INT and PMS, with the appropriate substrates. The detection of activity of ACC relies on the inorganic phosphate that is generated following the formation of acetyl coenzyme A from acetate, coenzyme A and ATP. The inorganic phosphate reacts with ammonium phosphomolybdate, $(NH_4)_3[PO_4(MoO_3)_{12}]$, which can be visualized with the aid of triethylamine [46].

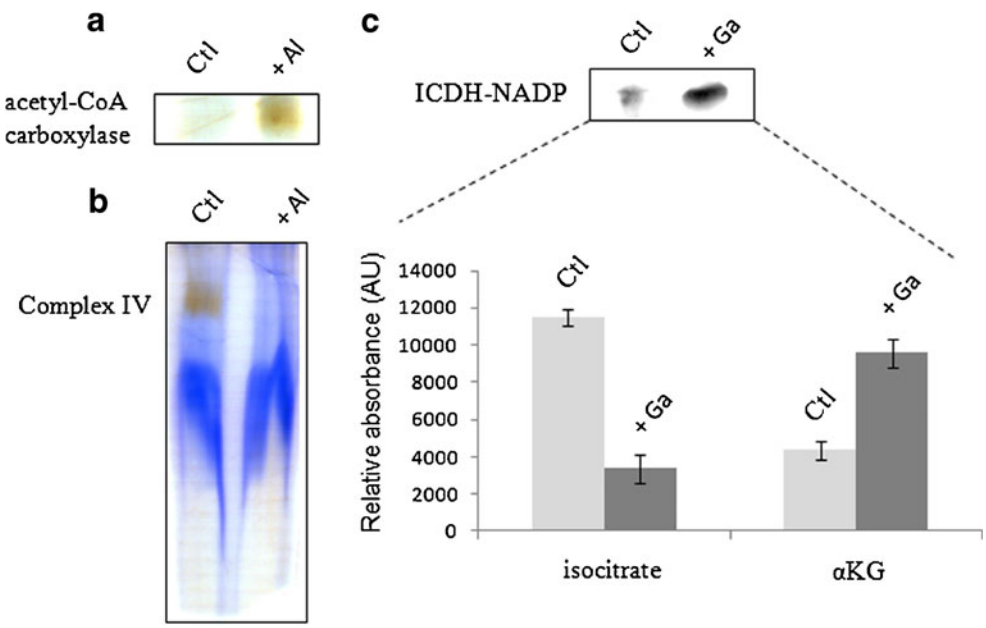

Fig. 7 Various applications of BN-PAGE. **a** Acetyl coenzyme A (*CoA*) carboxylase is detected in gel via the coupling of phosphate precipitation to ammonium molybdate. *Ctl* control, *Al* 500 μM aluminium citrate. **b** Complex IV is detected in gel via the precipitation of 3,3-diaminobenzidine oxides. *Ctl* control, *Al* 100 μM aluminium lactate. **c** Bands corresponding to NADP-ICDH were excised and incubated in reaction mixture containing 2 mM isocitrate and 0.1 mM NADP for 15 min. Consumption of isocitrate and formation of α-ketoglutarate (*αKG*) were increased in the gallium-stressed cells, corresponding to the increase of activity in gel. *Ctl* control, *Ga* 1 mM gallium citrate. (**a**, **b** Adapted from [45]; **b** adapted from [28])

BN-PAGE and β-oxidation of fatty acids

β-oxidation of fatty acids occurs essentially in the mitochondrion. Cytoplasmic fatty acids are transported into this organelle by L-carnitine [30]. Aluminium, zinc and other metals that result in oxidative stress can disrupt L-carnitine production. As mitochondria are severely affected during metal toxicity, the production of this non-essential amino acid also suffers a marked decrease in production. Biosynthesis of L-carnitine is a multistep process requiring the presence of numerous enzymes involved in the conversion of α-ketoglutarate and trimethyllysine into 3-hydroxy-N^6-trimethyllysine and then into L-carnitine. The enzymes γ-butyrobetaine dioxygenase (EC 1.14.11.1) and betaine aldehyde dehydrogenase (EC 1.2.1.8) are intermediates in L-carnitine synthesis [47]. Betaine aldehyde dehydrogenase can be readily measured by a conventional BN-PAGE activity stain technique since the reaction produces NAD^+, which can then be used in the electron transfer cascade to promote formazan precipitate formation. However, only INT is required to measure the γ-butyrobetaine dioxygenase activity as ascorbate can also act as an electron donor in the system [30].

This perturbation in the synthesis of L-carnitine also helps divert α-ketoglutarate towards the detoxification of ROS, as ketoacids are known to be antioxidants [30]. α-Ketoglutarate can nullify ROS, with the concomitant formation of succinate, a dicarboxylic acid that mediates the stabilization of hypoxia inducible factor 1 α, a promoter of anaerobiosis. This two-pronged approach helps decrease oxidative stress and generates ATP in an oxygen-independent fashion [30]. Hence, BN-PAGE is key in deciphering these enzymatic activities and in elucidating how metal toxicity results in decreased mitochondrial energy production and increased lipogenesis. The lack of L-carnitine production in the presence of metal toxins restricts the translocation of fatty acids in the mitochondria and promotes lipogenesis at the expense of lipolysis. These conditions are hallmarks of disorders such as obesity [48, 49]. This dysfunctional energy formation is further exacerbated by the downregulation of cytoplasmic creatine kinase in astrocytes [31].

Structural analyses of proteins in metal toxicity with the aid of BN-PAGE

BN-PAGE is frequently used to monitor the activity of enzymes in a system; however, this analytical method can also be invoked to examine the structural features of proteins. For instance, association among enzymes referred to as a metabolon and proteins with multiple subunits can be readily analysed. Although immunoprecipitation, tandem affinity purification and fluorescence resonance energy transfer/bioluminescence resonance energy transfer can provide structural information on proteins, these methods are labour-intensive and necessitate multiple manipulations. Multidimensional electrophoresis involving first-dimension BN-PAGE coupled with second-dimension sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) do not necessitate the use of expensive equipment and can generate critical information about the number of subunits constituting protein complexes and their molecular masses. The proteins are first separated electrophoretically in their native conformation and the separation of subunits can be achieved through SDS-PAGE. The advantage of the 2D BN-PAGE/SDS-PAGE technique is that it affords the analysis of protein complexes and allows the visualization of protein–protein interaction in gel. Much like 1D BN-PAGE, 2D BN-PAGE/SDS-PAGE can also be subjected to variety of postelectrophoretic assays that provide additional information on these biomolecules. Immunoblot and mass-spectrometric studies of the proteins subjected to 2D BN-PAGE/SDS-PAGE may help elucidate the precise constituents of the proteins.

Numerous proteins rely on essential metals as these are key participants in electron movement, structural maintenance and stabilization [50–52]. Hence, a perturbation of this relationship has severe implications in biological processes. Metal toxins are known to compromise protein integrity. Diseases such as Alzheimer's disease and Parkinson's disease are partly due to the interference of metals with cerebral proteins. The replacement of the essential metal cofactors by exogenous metals creates structural changes in proteins that can be followed by BN-PAGE. This electrophoretic technology provides valuable information on how protein structure can be affected by metal toxicity. BN-PAGE was also used to follow the fate of the human DNA glycoylases endonuclease VIII-like (NEIL) 1, NEIL 2 and 8-oxoguanine glycosylase in the presence of Cu(II) and Fe(III). It appears that copper and iron can inhibit NEIL-mediated repair of oxidized base damage. The electrophoretic technique was able to illustrate that copper and iron binding produced a small amount of NEIL 2 dimer formation, which could potentially participate in NEIL 2 inhibition [53].

The presence of nickel in jewellery and nickel-containing biomaterials triggers an allergic response referred to as contact hypersensitivity. It is postulated that nickel-interacting proteins in human B cells may mediate this allergic reaction [54]. With the aid of BN-PAGE/SDS-PAGE, it was possible to visualize the high molecular mass complexes of different proteins that bind nickel and initiate an immunologic cascade [55].

Although the maintenance of protein structure in relatively native forms is a significant advantage for BN-PAGE, this technique has some inherent limitations. The use of a proper detergent is a particular restraint for BN-PAGE, as the proteins must be treated minimally prior to loading the gel.

Detergents such as *n*-dodecyl-β-D-maltoside, digitonin and Triton X-100, owing to their mild nature, are commonly used to solubilize membranous proteins [4]. However, the concentration of these detergents must be carefully optimized depending on the samples to produce the maximum resolution in BN-PAGE. There are numerous factors that can influence protein solubilization and separation, including the choice of sample buffer, salt concentration, temperature and forces applied [56]. These must all be controlled for prior to and during electrophoresis. The resolution of 2D BN-PAGE/SDS-PAGE is also an issue when compared with other gel-based proteomic techniques. Proteins of a molecular mass less than 100 kDa are particularly difficult to visualize in BN-PAGE owing to the abundance of proteins in this size range. Although the acrylamide gradient could be modified to increase the resolution, it is still difficult to adequately increase the separation between these proteins [4].

False-positive detection due to artefacts is another drawback of using BN-PAGE to analyse protein–protein interactions. These artefacts are generally caused by non-physiological/artificial protein aggregation during sample preparation procedures. However, it seems that there is lack of studies to determine the margin of error created by these artefacts [57]. Nonetheless, it is essential to recognize that there are possibilities of detecting false interactions. Furthermore, the disruption of weak or transient protein interactions is inevitable upon exposure to detergent. Thus, the current techniques for analysing protein–protein interaction using BN-PAGE should be used in tandem with analogous technologies to produce impartial results. Since the initial application of BN-PAGE in 1991 by Schägger and von Jagow [10], there have been attempts to optimize the efficiency of this technique. Clear native polyacrylamide gel electrophoresis is one such example. The dye Coomassie brilliant blue G-250 used in BN-PAGE can interfere with some analyses as it applies an overall negative charge to the protein. Clear native polyacrylamide gel electrophoresis does not use Coomassie brilliant blue G-250, rendering it a milder approach and thus maximizing the preservation of protein complexes. However, this technique falls short on resolution in comparison with BN-PAGE.

Conclusion and future implications

BN-PAGE is a powerful technique which allows the separation of native protein complexes with high resolution. The uniqueness of BN-PAGE is derived from the absence of a strong detergent in the sample and electrophoretic gel preparation. Also, Coomassie brilliant blue G-250, which surrounds even extremely hydrophobic and many hydrophilic proteins, provides a net negative charge around the protein surface. These two attributes enable this analytical technique to separate proteins according to their size, while maintaining native conformation and enzymatic activity. Once separated, these proteins can be subjected to further examination by a variety of other procedures: 1D BN-PAGE can be used for enzymatic activity assays, immunoblotting, high-performance liquid chromatography, mass spectrometry, 2D BN-PAGE and 2D SDS-PAGE. Furthermore, multidimensional polyacrylamide gel electrophoresis with 1D BN-PAGE can provide high resolution and detailed information regarding protein complexes and their subunits. This unique electrophoretic technique can be effectively used to study how metal toxins interact with living systems. The metabolic networks that are affected by aluminium, zinc and other metals can be deciphered. These metals severely impede energy production via oxidative phosphorylation, an event that may contribute to a variety of human disorders.

Indeed, it is now recognized that metals from the environment can participate in the pathogenesis of numerous diseases. Thus, there has been a significant increase in interest in the mechanisms by which these toxins affect biological systems. As the metals are known to exert their damage via the disruption of proteins, BN-PAGE provides a cost-effective method of delineating these molecular details. In addition to enzymatic activity, the structural features of proteins can also be explored by BN-PAGE, in conjunction with other analyses, and their contribution to diseases can be unravelled. In particular, the ability of BN-PAGE to separate highly hydrophobic proteins provides an opportunity to visualize these proteins in gel. However, the relatively low resolution of the technique implies that BN-PAGE results should be followed up by complementary analyses in order to solidify the results obtained. The easiness of this protocol combined with its cost-effectiveness allows BN-PAGE to be applied in most laboratories. In conjunction with other techniques such as Western blotting, mass spectrometry and inductively coupled plasma mass spectrometry, BN-PAGE can help provide the molecular attributes of macromolecules, metalloproteins and other proteins in relatively native form.

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