QPNC-PAGE: Informative Background

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Abstract: QPNC-PAGE, or quantitative preparative native continuous polyacrylamide gel electrophoresis, is a bioanalytical, high-resolution and highly accurate technique applied in biochemistry and bioinorganic chemistry to separate proteins quantitatively by isoelectric point. This standardized variant of native gel electrophoresis is used by biologists to isolate active or native metalloproteins in biological samples and to resolve properly and improperly folded metal cofactor-containing proteins or protein isoforms in complex protein mixtures[1].

A high reproducibility in gel pore size and a high-yield electroelution of proteins performed by this highly reliable technique strongly correlate with the polymerization time of the acrylamide gel representing an inherent stability constant of the separation system. Accordingly, as "omics" platform for quantitative biomedical and interdisciplinary approaches, QPNC-PAGE may essentially contribute to development of metal-based drugs including protein and non-protein bound metal species[2].

This sophisticated technique further addresses the investigation of the role of environmental contaminants like copper in the etiology of Alzheimer's disease (AD)[3] since inorganic copper[4] that cannot be detoxified completely by the liver, may be a major triggering agent in AD[5].

INTRODUCTION

Proteins perform several functions in living organisms, including catalytic reactions and transport of molecules or ions within the cells, the organs or the whole body. The understanding of the processes in human organisms, which are mainly driven by biochemical reactions and specific protein-cofactor interactions, depends to a great extent on our ability to isolate active proteins in biological samples for more detailed examination of chemical structure and physiological function. This essential information can imply an important indication of a patient's state of health.[6]

As about 30-40% of all known proteins contain one or more metal ion cofactor(s) (e.g., ceruloplasmin, ferritin, amyloid precursor protein (APP), matrix metalloproteinase), especially native metalloproteins have to be isolated, identified and quantified after liquid biopsy. Many of these cofactors (e.g., iron, copper, or zinc) play a key role in vital enzymatic catalytic processes or stabilize globular protein molecules.[7]

Therefore, the outlined high-precision electrophoresis and other native separation techniques (e.g., GPC[1]) are highly relevant as initial step of protein and trace metal speciation analysis, subsequently followed by mass spectrometric and magnetic resonance methods for quantifying and identifying the soluble proteins of interest.[8] The following paper describes the method, applications and principle of QPNC-PAGE.

METHOD

Separation and buffering mechanisms

In gel electrophoresis proteins are normally separated by charge, size, or shape. The aim of isoelectric focusing (IEF), for example, is to separate proteins according to their isoelectric point (pI), thus, according to their charge at different pH values.[9] Here, the same mechanism is accomplished in a commercially available electrophoresis chamber (see **Fig. (1)**) for separating charged biomolecules, for example, superoxide dismutase (SOD)[10] or allergens,[11] at continuous pH conditions and different velocities of migration depending on different isoelectric points. The separated (metal) proteins elute sequentially, starting with the lowest (pI > 2-4) and ending with the highest pI (pI < 10.0) of the solute protein molecules.

Due to the specific properties of the prepared gel and electrophoresis buffer solution which is basic (pH 10) and contains 20 mM Tris-HCl and 1 mM NaN_3, most proteins of a biological system (e.g., *Helicobacter pylori*[12]) are charged negatively in the solution, and will migrate from the cathode to the anode due to the electric field. At the anode, electrochemically-generated hydrogen ions react with Tris molecules to form monovalent Tris ions. The positively charged Tris ions migrate through the gel to the cathode where they neutralise hydroxide ions to form Tris molecules and water. Thus, the Tris-based buffering mechanism causes a constant pH in the buffer system.[13]

Fig. (1). Equipment for analytical electrophoresis: electrophoresis chamber, peristaltic pump, fraction collector, buffer recirculation pump and UV detector (in a refrigerator), power supply (5 W) and recorder (on a table).

At 25 °C Tris buffer has an effective pH range between 7.5 and 9.0. Under the conditions given here (addressing the concentration, buffering mechanism, pH and temperature) the effective pH is shifted in the range of about 10.0 to 10.5. Native buffer systems all have low conductivity and range in pH from 3.8 to 10.2.[14]

Although the pH value (10.00) of the electrophoresis buffer does not correspond to a physiological pH value within a cell or tissue type, the separated ring-shaped protein bands are eluted continuously into a physiological buffer solution (pH 8.00) and isolated in different fractions (see **Fig. (2)**).[15] Provided that irreversible denaturation cannot be demonstrated (by an independent procedure), most protein molecules are stable in aqueous solution, at pH values from 3 to 10 if the temperature is below 50 °C.[16]

As the Joule heat and temperature generated during electrophoresis may exceed 50 °C,[17] and thus, have a negative impact on the stability and migration behavior of proteins, the separation system, including the electrophoresis chamber and a fraction collector, is cooled in a refrigerator at 4 °C. Overheating of the gel is impeded by internal cooling of the gel column and generating a constant power (see **Fig. (1)**).

Gel properties and polymerization time

Best polymerization conditions for acrylamide gels are obtained at 25-30 °C[18] and polymerization seems terminated after 20-30 min of reaction although residual monomers (10-30%) are detected after this time.[19]

The co-polymerization of acrylamide (AA) monomer /N,N'-methylenebisacrylamide cross-linker initiated by ammonium persulfate (APS)/tetramethylethylene-diamine (TEMED) reactions, is most efficient at alkaline pH. During polymerization linear acrylamide chains are created and cross-linked by covalent bonds at a time. If the polymerization is incomplete the proteins of interest could be modified during a run by reaction with unpolymerized monomers of acrylamide, forming covalent acrylamide adduction products that may result in multiple bands.[20] For avoiding resulted artifacts the gel polymerization is conducted at pH 10.00 of the electrophoresis buffer making sure an efficient use of TEMED and APS as catalysts of the polymerization reaction, and concurrently, suppressing a competitive hydrolysis of the produced acrylamide polymer network (see **Fig. (3).**, cf. section Principle).

Additionally, the time of polymerization of a gel may directly affect the peak-elution times of separated metalloproteins in the electropherogram due to the compression and dilatation of the gels and their pores with the longer incubation times (see **Fig. (2)**, cf. subsection Reproducibility and recovery). In order to ensure maximum reproducibility in gel pore size and to obtain a fully polymerized and non-restrictive large pore gel for a PAGE run, the polyacrylamide gel is polymerized for a time period of 69 hr at room temperature (RT). The exothermic heat generated by the polymerization processes is dissipated constantly while the temperature may rise rapidly to over 75 °C in the first minutes, after which it falls slowly.[21] After 69 hr the gel has reached room temperature, and thus, is in its lowest energy state because the chemical reactions and the gelation are terminated. Gelation means that the solvent (water) gets immobilized within the polymer network by means of hydrogen bonds and also van der Waals forces.

As a result, the prepared gel is homogeneous (in terms of homogeneous distribution of cross-links throughout the gel [22]), inherently stable and free of monomers or radicals. Fresh polyacrylamide gels are further hydrophilic, electrically neutral and do not bind proteins.[23] Sieving effects due to gravity-induced compression of the gel can be excluded for the same reasons. In a medium without molecular sieving properties a high-resolution can be expected.[24]

Before an electrophoretic run is started the prepared 4% T (total polymer content (T), [w/v]), 2.67% C (cross-linker concentration (C), [w/w]) gel is pre-run to equilibrate it. It is essentially non-sieving and optimal for electrophoresis of proteins greater than or equal to 200 kDa. Proteins migrate in it more or less on the basis of their free mobility.[25] For these reasons interactions of the gel with the biomolecules are negligibly low, and thus , the proteins separate cleanly and predictably at a polymerization time of 69 hr (see **Fig. (2)**).

The separated metalloproteins including biomolecules ranging from approximately < 1 kDa to greater than 30 kDa (e.g., metal chaperones, prions, metal transport proteins, amyloids, metalloenzymes, metallopeptides, metallothionein, phytochelatins) are not dissociated into apoproteins and metal cofactors.[26]

Reproducibility and recovery

The bioactive structures (native or 3D conformation or shape) of the isolated protein molecules do not undergo any significant conformational changes. Thus, active metal cofactor-containing proteins can be isolated reproducibly in the same fractions after a PAGE run (see **Fig. (2)**). A shifting peak in the respective electropherogram may either indicate that a denatured metalloprotein is available in a complex protein mixture to be separated (cf. Biomedicine) or the standardized time of gel polymerization (69 hr) is not implemented in a PAGE experiment.

A lower deviation of the standardized polymerization time (< 69 hr) stands for incomplete polymerization, and thus, for inherent instability due to gel softening during the cross-linking of polymers as the material reaches swelling equilibrium,[27] whereas exceeding this time limit (> 69 hr) is an indicator of gel aging.[28] The phenomenon of gel aging is closely connected to long-term viscosity decrease of aqueous polyacrylamide solutions[29] and increased swelling of hydrogels.[30] The electroelution and subsequent detection of the analysed protein is not reproducible due to sieving effects (see **Figs. (2, 3)**).

Under standard conditions (69 hr), metalloproteins with different molecular mass ranges and isoelectric points have been recovered repeatedly in biologically active form at a quantitative yield of more than 95%.[31] By preparative sodium dodecyl sulfate (SDS) polyacrylamide gel electrophoresis standard proteins (cytochrome c, aldolase, ovalbumin and bovine serum albumin) with molecular masses of 14-66 kDa can be recovered with an average yield of about 73,6%.[32] Preparative isotachophoresis (ITP) is applied for isolating palladium-containing proteins with molecular masses of 362 kDa (recovery: 67%) and 158 kDa (recovery: 97%).[33]

Quantification and identification

Physiological concentrations (ppb-range) of Fe, Cu, Zn, Ni, Mo, Pd, Co, Mn, Pt, Cr, Cd and other metal cofactors can be identified and absolutely quantified in an aliquot of a fraction by inductively coupled plasma mass spectrometry (ICP-MS)[34] or total reflection X-ray fluorescence (TXRF)[35], for example. In case of ICP-MS the structural information of the associated metallobiomolecules[36] is irreversibly lost due to ionization of the sample with plasma.[37] Another established high sensitive detection method for the determination of (trace) elements is graphite furnace atomic absorption spectrometry (GF-AAS) (see **Fig. (2)**).[38]

Because of high purity and optimized concentration of the separated metalloproteins, for example, therapeutic recombinant plant-made pharmaceuticals such as copper chaperone for superoxide dismutase (CCS) from medicinal plants, in a few specific PAGE fractions, the related structures of these analytes can be elucidated by using solution NMR spectroscopy under non-denaturing conditions.[39]

Alternatively, cryo-electron microscopy can be applied for the structural elucidation of proteins purified to homogeneity.

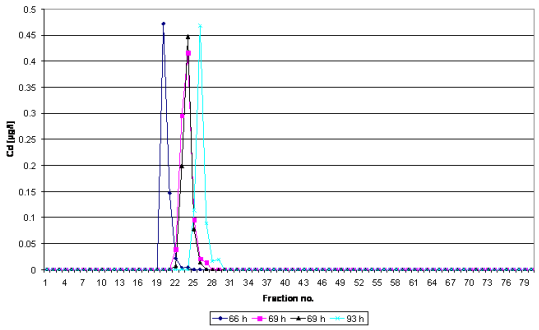

Fig. (2). Electropherogram showing four PAGE runs of a native chromatographically prepurified high molecular weight cadmium protein (MW≈200 kDa) as a function of time of polymerization of the gel (4% T, 2.67% C). Detection method for Cd cofactors (in µg/L): GF-AAS[15].

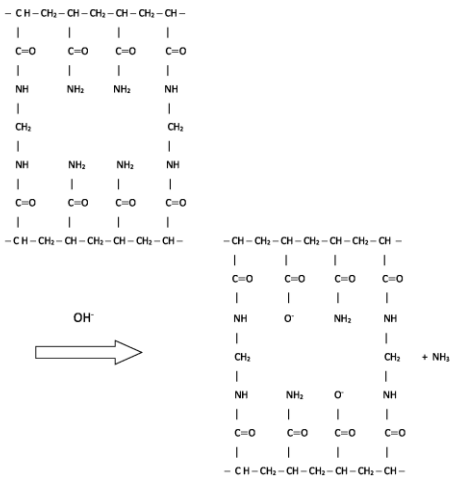

Fig. (3). Hydrolysis of a polymeric network structure at basic pH: the carboxamide groups react with H_2O leaving ionized carboxyls. The modified acrylamide gels swell as they suck up buffer ions and water to neutralize the carboxyls. Thus, the gel properties are changed.

APPLICATIONS

Improperly folded metal proteins, for example, CCS or Cu/Zn-superoxide dismutase (SOD1) present in brain, blood or other clinical samples, are indicative of neurodegenerative diseases like Alzheimer's disease or Amyotrophic Lateral Sclerosis (ALS).[40] Active CCS or SOD molecules contribute to intracellular homeostatic control of essential metal ions (e.g., $Cu^{1+/2+}$, Zn^{2+}, $Fe^{2+/3+}$, Mn^{2+}, Ni^{3+}) in organisms, and thus, these biomolecules can balance pro-oxidative and antioxidative processes in the cytoplasm. Otherwise, free (loosely bound) transition metal ions take part in Fenton-like reactions in which deleterious hydroxyl radical is formed, which unrestrained would be destructive to proteins.[41]

The loss of (active) CCS increases the amyloid-β production in neurons which, in turn, is a major pathological hallmark of AD.[42] Therefore, copper chaperone for superoxide dismutase is proposed to be one of the most promising biomarkers of Cu toxicity in these cureless diseases.[43] CCS should be analysed primarily in blood because a meta-analysis of serum data showed that AD patients have higher levels of serum Cu than healthy controls.[44]

Selenium is another important trace element associated with glutathione peroxidase which is involved in processes of redox-regulation and oxidative stress response.[45] On the other hand, the intake of selenium is counteracted by its poor bioavailability in the soils of industrialized areas.[46] Furthermore, toxic effects of selenium species may occur at lower concentrations than previously believed.[47]

QPNC-PAGE is applied in the field of molecular biology to purify enzymes and recombinant proteins of microbial strains.[48] The thermostability and activity of enzymes expressed by thermophilic bacteria is genetically encoded. These biomolecules can be used as research reagents and as catalysts for industrial processes.[49]

PRINCIPLE

History

In the 20th century it was generally accepted that APS/TEMED-initiated reactions should be allowed to proceed for 5-15 min[50] to approximately 30 min[51] to ensure maximum reproducibility in gel pore size of PAGE gels. In another review it is recommended to allow the gel to polymerize overnight at room temperature.[52] Longer incubation times (16 hr to 2 wk) to finish the three-dimensional matrix (network) formation might not have any essential effects on the protein separation process.[53] Westermeier annotates that due to silent polymerization the gels might be placed in a refrigerator shortly after (visible) polymerization.[54] In the cold room, however, the (exothermic) reaction is not completed within a reasonable period of time. In a technical note ("Acrylamide Polymerization - A Practical Approach") Bio-Rad claims that polymerization may be largely complete after about 90 min at room temperature.

Now here is the question to answer: Is there only an approximate polymerization time in the range of minutes to hours, or is there reliable experimental data indicating the general validity of a polymerization time for any PAGE gels?

Progressively hydrolyzing into polyacrylic acid (PAA) and ammonia (NH_3) polyacrylamide gels were further considered as inherently unstable with respect to polymer resistance to alkaline media (see **Fig. (3)**). However, this hypothesis cannot be generalized.

As acrylamide starts to hydrolyze at pH around 10[55] the hydrolysis rate of aqueous solutions of polyacrylamide is at a maximum at pH 4, and at a minimum at pH 10.[56] Hydrolysis of the acrylamide gels polymerized for 69 hr could not be observed here (see **Fig. (2)**). After overnight polymerization, however, only a semi-quantitative analysis of low amounts of the cofactor of interest could be performed by native PAGE with AAS detection due to inherently unstable gels[57] For these reasons, the dynamic relationship between the polymerization time on the one hand, and gel stability (incomplete polymerization, hydrolysis) on the other hand, had to be investigated addressing the absolute quantification of specific protein molecules:

In 2001 a new basic principle of gel electrophoresis was discovered at the Forschungszentrum Jülich, applied for patent in 2003 and subsequently issued in 2014.[58] This invention provided the first conclusive evidence that the time of polymerization of a polyacrylamide gel directly affects the result of protein purification because the separation properties implying the mechanical and chemical stability of a gel and its pores are determined by this parameter.

Although the quality, quantity and mixing ratio of TEMED, APS and AA/Bis-AA^2 as well as the ambient temperature are the most important factors to initiate and impel the polymerization reaction of PAGE gels, it is evident that the time of polymerization is the limiting factor in these physico-chemical processes to form an inherently stable network of polymers.[59]

Different polymerization times (66, 69, 72, 93 hr) of acrylamide gels showed that the peak-elution times of the separated metalloproteins in the electropherogram may vary considerably. On the other hand, it was proved that the reliability of the results obtained by gel electrophoresis can be optimized by strict adherence to a standardized time of gel polymerization (69 hr) (see **Fig. (2)**).

This optimization process of one selected and severely underestimated parameter, namely polymerization time, paved the way to "quantitative native PAGE" implying a high reproducibility in gel pore size and a high-yield electroelution of proteins in biological samples.[60]

Systematic investigations of the hydrogel stability over time reveal significant changes in gel structure, namely the porosity, by day 3 (72 hr) after (visible) polymerization of 10% T, 15% T, and 20% T gels (under acidic conditions).[61] This study from 2016 is supported by another systematic investigation on gel aging from 1984, indicating that low concentrations of carboxyls are detectable in gel after three days of acrylamide polymerization.[62]
Said results are in excellent agreement with that the aging of fresh polyacrylamide gels begins after 69 hr cross-linking and gelation time bringing a shift of the peak-elution times of proteins due to gel swelling induced by hydrolysis of carboxamide groups into carboxylate anions (see **Figs. (2, 3))**. Consequently, the polymerization time of 69 hr is a pH-independent stability constant in the pH range of about 4 (acrylamide/aqueous solution) to 10 (acrylamide/buffer solution) for acrylamide gels with total monomer concentrations in the range of 4 to 20% T.

Persons

First publications from 2007/2009 concerning the medical applications of this technique were edited or co-authored by the Münsteran human geneticist Prof. em. Jürgen Horst and the well-known American scientist and internationally recognized expert in the fields of DNA sequence analysis and protein electrophoresis David E. Garfin (see **Fig. (4)**).
Beginning in the late 1960s D.E.G. used paper electrophoresis to sequence oligonucleotides prepared from tobacco mosaic virus ribonucleic acid.[63] In the 1970s and the early 1980s Dr. Garfin became one of the most important investigators and early pioneers in the field of prion research (scrapie) in the team of the later Nobel Prize winner Stanley B. Prusiner at the UCSF[3].[64]

Fig. (4). David Garfin at the start of his historic talk presenting his *Reminiscences About Electrophoresis* on the occasion of the 2013 AES Electrophoresis Society Awards Session in San Francisco.

In addition to his pioneering work on one- and two-dimensional gel electrophoresis in the following decades at Bio-Rad Laboratories (Hercules, USA) as Proteomics Applications Manager[65] he became co-editor of the *Handbook of Isoelectric Focusing and Proteomics* (2005)[66] and co-authored a worth reading chapter on bioseparation methods in the *Kirk-Othmer Encyclopedia of Chemical Technology* (2007).[67]
Furthermore, Garfin is author of several breakthrough articles, some of which are cited here and which may substantially complement and confirm the breakthrough method presented here. For significant contributions to electrophoresis in both the engineering and biology communities Dave Garfin received the 2013 AES Electrophoresis Society Career Award in San Francisco (see **Fig. (4)**).[68]

Biomedicine

Garfin, Horst, Nagel, and other researchers from the Forschungszentrum Jülich anticipated that the above-mentioned approach might be implemented in the therapy and diagnosis of several protein-misfolding diseases: on the one hand, the copper chaperone for superoxide dismutase (CCS) may serve as a fluid-based biomarker for Cu toxicity in neurodegenerative diseases (cf. section Applications). On the other hand, copper chaperones,[69] and smaller non-protein bound metal species (e.g., Cu orotate,[70] Li chloride[71]) are indicated as lead compounds for the etiological treatment of Alzheimer's disease.[72]
The mis-localization (dyshomeostasis) of metal ions (in particular Cu[73]) in the cell is most likely responsible for the onset and progression of sporadic and genetic forms of Alzheimer's disease and other dementias. The above-mentioned compounds may pass the blood brain barrier and trigger a metal-mediated signaling cascade of biochemical reactions that may restore and maintain biometal homeostasis in order to preserve the neuronal function in the brain of AD patients.[74]
As cellular responses to these processes the production of amyloid-β peptides and oxidation are normalized and neuritic plaques in Alzheimer brains are degraded by upregulation of the proteasome and other molecular mechanisms.[75] In particular, specific protein-protein and protein-cofactor interactions (e.g., the "copper interactome") play a crucial role then.[76] The relative biochemical impact of an applied metal-based drug is primarily depending on its dose, bioavailability, trace metal binding form (chemical form), and accuracy of quantitative measurement.[77]
According to the Hofmeister series salt effects could be one approach for the treatment of Alzheimer's disease by using certain salts for dissolving protein aggregates or inhibiting amyloid formation.[78] Adding salts to complex protein mixtures, however, may induce a shift of isoelectric points, and thus, affect protein structure (shape) and activity. In this context, one study found that kosmotropic salts (e.g., ammonium sulfate) that were used for the precipitation of native proteins in solution, subsequently, may result in denatured high molecular mass metalloproteins in the same solution by using ICP-MS detection.[79]

Ions which promote aggregation or prefractionation in the proteome analysis, simultaneously may cause denaturation of the native conformation of the proteins of interest. In opposite case, chaotropic salts force globular proteins to unfold.[80] For these reasons, restoring and maintaining the physiological states of aggregated or unfolded (metal) proteins by applying Hofmeister salts in the therapy of Alzheimer's disease is very unlikely to happen. For example, Li chloride caused a reduction in protein synthesis, and hence, the level of amyloid-β peptides, however, this species may also generate severe side effects induced by long-term, high-dose lithium.[81] Curcumin is one of the most promising therapeutic agents for inflammation, cystic fibrosis, Alzheimer's disease, and cancer because these biomolecules scavenge radicals and maintain levels of (active) antioxidant enzymes (e.g., SOD1)[82] in the presence of copper. Curcumin is almost free from side effects, however, limited for application due to its poor bioavailability.[83] Transgenic plants grown on plant phenotyping platforms to be used for high-throughput screening analysis, may help to provide bioactive therapeutic (metal) proteins as a major basis for pharmacological efficiency (high specificity, well known mechanisms of action, with few side effects) in conformational diseases.[84]

CONCLUSIONS

High protein yield and purity are the bottleneck of the quantitative protein analysis in biological samples. QPNC-PAGE is a unique method and the initial step that opens the bottleneck of native protein isolation in complex protein mixtures. This analytical technique is based on a new principle and a new constant of acrylamide gel electrophoresis implying the accurate control of gel pore size and stability by the time of polymerization of acrylamide. Polyacrylamide gels are inherently stable at 69 hr of polymerization time, bringing a paradigm shift in gel electrophoresis.

The electroelution and subsequent detection of native proteins in reproducible and quantitative amounts are major hallmarks of reliable electrophoretic separation systems. As both the low and high molecular weight metal proteins (< 1 to ≥ 200 kDa) and their respective structures can be analysed in a single run, a combined procedure of solution NMR, QPNC-PAGE, and ICP-MS may be the key for the diagnosis and therapy of several protein-misfolding diseases (AD, ALS, a.o.) related to dyshomeostasis of biometal metabolism in the human brain. Native recombinant proteins from transgenic plants, albeit, possess the pharmacological potential to restore and maintain the homeostasis of trace transition biometal ions (e.g., Cu^{1+}, Zn^{2+}) in physiological and pathophysiological conditions, and thus, contribute as lead molecules to the mechanism of autophagy.[85]

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Dedicated to Claudia and Noah Kastenholz

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Abbreviations
[1]GPC: Gel Permeation Chromatography
[2]Bis-AA: N,N'-methylenebisacrylamide
[3]UCSF: University of California, San Francisco

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