Important Contributions of a New Quantitative Preparative Native Continuous Polyacrylamide Gel Electrophoresis (QPNC-PAGE) Procedure for Elucidating Metal Cofactor Metabolisms in Protein-Misfolding Diseases – A Theory

Bernd Kastenholz*

Institute for Chemistry and Dynamics of the Geosphere, Institute III: Phytosphere, Research Centre Juelich, Juelich 52425, Germany

Abstract: The quantitative analysis of metallochaperone proteins in biofluids (e.g. blood, liquor) may be a major prerequisite for clinical investigations concerning the structure-function relationships of biologically-active metal cofactor-containing chaperones in protein-misfolding diseases (e.g. Alzheimer’s or related diseases). For these purposes, a new state-of-the-art gel electrophoresis [quantitative preparative native continuous polyacrylamide gel electrophoresis procedure (QPNC-PAGE)] combined with biological mass and NMR spectrometries might essentially contribute to provide fundamental insights into the metabolisms of important metal cofactors in biological systems and the proper folding of metallochaperones in conformational diseases.

Keywords: QPNC-PAGE, GPC, NMR, ICP-MS, Quantitative analysis, Biologically-active metallochaperones, Metal cofactor-containing proteins, Native conformation, Chemical stability, Alzheimer’s disease.

INTRODUCTION

In 1906 Richard Willstätter published a paper concerning the elemental composition of chlorophyll present in plants. In this study, he concluded that the biological processes occurring in living organisms (plants and animals) are predominantly determined by the catalytic effects of metals bound to organic compounds [1]. These important results and conclusions have to be treated as a ‘milestone’ in the history of bioinorganic chemistry. For his research work concerning pigments (especially chlorophyll) in the plant kingdom, Willstätter was awarded the Nobel Prize in 1915. Since then, the contributions of this scientific field have led to an improved understanding of the functions of several metal chelates and cofactors (pigments and metal ions), e.g., chlorophyll molecules and chlorophyll-protein complexes in biological organisms [2, 3]. Simultaneously, the area of instrumental analytical chemistry made enormous progress. Modern bioanalytical techniques, such as X-ray crystallography [2, 3], NMR spectroscopy [4], mass spectrometric methods, inductively coupled plasma and matrix-assisted laser desorption ionization mass spectrometries (ICP-MS/MALDI-MS) [5, 6] and atomic spectrometry [6, 7] were developed and applied to elucidate and identify the different metal-containing biomolecules in biological samples. By definition, the entirety of metal and metalloid species within a cell or tissue type is now referred to as the ‘metallome’ of a biological system [5].

Because of the poor separation from the matrix components, attempts by molecular mass spectrometry to identify the metallobiomolecules detected by ICP-MS at the picogram level often failed. High-resolution techniques for the separation of these compounds, for example, gel electrophoresis or capillary high performance liquid chromatography combined with ICP-MS, make ICP-MS an attractive partner of electrospray and MALDI-MS for the investigation of metallobiomolecules in complex biological matrices [5].

SIGNIFICANCE OF METAL COFACTORs

In the literature, it is generally accepted that numerous essential biological functions require metal ions, and most of these involve metalloproteins [5]. For example, it is well-known that many metal cofactor-containing enzymes play an essential role in the syntheses and metabolic functions of genes (RNA and DNA) and proteins in the cell [6]. The metalloproteins correspond to about a third of all structurally characterized proteins in the cell [8] and the vast majority of the metal cofactor-containing proteins function as essential metalloenzymes [9].

The understanding and investigation of the metabolisms of metal cofactors and of the proper folding of cofactor-containing proteins and enzymes in biological organisms may be essential for the diagnosis and therapy of several diseases (e.g. Alzheimer’s or related diseases) caused by protein-misfolding. In these diseases malfunctions of the cellular processes may rely on the structural changes of the native conformations of metallopeptide molecules (amyloid peptides). Furthermore, pathological disruption of metal trafficking inside a cell is probably a major reason for conformational changes in protein-misfolding diseases. Thereby, inactive metallochaperone proteins that protect and guide
metal ions to targets could perhaps play a decisive role [8–22].

The metallochaperones belong to a family of proteins that control the activity of intracellular metal ions and help confine them to vital roles. For example, a yeast protein, Cu chaperone for superoxide dismutase (CCS), was shown to be required for superoxide dismutase activity. In healthy organisms these metallocompounds usually stabilize the native states of numerous proteins. However, in order to function, metallochaperones as well as the corresponding cell proteins and enzymes to be activated have to be folded in a proper manner. Especially metal cofactors serve as a nucleation site that direct polypeptide folding. Thus, they may affect both the mechanism and speed of folding for the rapid achievement of the 3-D conformation of a native protein molecule. The native state of a protein is absolutely necessary to carry out its specific biological function or activity [8–22]. Conclusively, for a deeper understanding of the metabolic behavior of metal cofactors (Fe, Cu, Zn, Mo, a.o.) and for the investigation of the protein folding in vivo, it is urgently demanded to isolate, identify and quantify known and unknown biologically-active metallochaperones in clinical samples (e.g. biofluids) and to determine their structure-function relationships.

According to this approach it is evident that native analytical procedures have to be conducted for isolating bioactive metal chaperone proteins in samples (e.g. whole blood, plasma, serum, urine, fluids excreted by organs and tissues of animals / human beings and cytosols of biopsy materials) from healthy and also diseased organisms. By these procedures it is possible to make a comparison of the measured concentrations of isolated metalloproteins in numerous matrices of healthy organisms with the amounts of the respective metal cofactor-containing proteins detected in clinical matrices of sick people. For example, an altered metal cofactor metabolism may be revealed either by the presence or by lack of certain metallochaperones in biofluids. Additionally, low or high concentrations, respectively, the ratios of peak areas of the detected analytes could serve as indicators of dysfunctional biochemical processes in the cell.

Some exemplary and excellent reviews [23, 24] concerning the association of biomolecules with trace metals in biological fluids are presented by Cornelis, Borguet and De Kimpe.

**ANALYTICAL PREREQUISITES**

Before the structure-function relationships of metallocompounds in a biological system can be determined by using the very important detection systems [2-7] they must be preceded by the various analytical separation steps and prerequisites being discussed here.

An important prerequisite to elucidate and identify bioactive metalloproteins in biological samples is the development of a protein purification procedure adhering to several analytical criterions. As a key requirement, native separation conditions provide for the integrity of native protein samples during analysis. Sampling and storage of biological samples are critical in this regard and they have to be conducted under non-denaturing conditions. In a recently published review, the proper sampling of clinical samples is revealed [25]. In this context some cryocontainers for the native storing and transport of different biological samples may also be an important prerequisite [26–28]. Furthermore, the applied analytical systems must be suitable for separating preparative amounts (mg-range) of complex protein samples because the physiological concentrations of the different metalloproteins in various biofluids may be very small [29, 30].

A new method should be available to the many different biological matrices and the matrix components and contaminants of a biological sample must be separated almost completely before some specific analytes can be subjected to further examinations. Because of the danger of contamination and denaturation [23, 24], sample preparation steps have to be avoided when native metalloproteins are analyzed in biological fluids. Finally, reproducible and quantitative analytical results [23, 24] are imperative required for the structure determinations of the biomacromolecules of interest.

For example, the quantitative analysis of biomolecules is a very important step because the concentrations of the analytes, especially proteins, have to be optimized in order to detect native metalloprotein molecules in aqueous solutions, e.g. by using NMR spectroscopy as detection method. Therefore, preparative analytical systems with continuous elution of the separated compounds [29, 30] should be applied. Furthermore, as a major prerequisite the chemical stability of the native proteins during the purification and separation processes of a complex biological sample has to be checked by an independent method [31]. Only if all the mentioned prerequisites are fulfilled one can assume that the results obtained for the structural determination of specific metal cofactor-containing proteins are accurate and reliable.

Currently, most electrophoretic and chromatographic procedures belong to the technologies of choice in protein purification and separation. Especially gel electrophoresis is one of the most important electrophoretic methods for isolating all kinds of proteins, peptides and enzymes in biological samples. In the past, the analytical criterions required for isolating biologically-active compounds, mentioned above, could not be accomplished thoroughly by some of these procedures [29-32]. By a new approach concerning a preparative native polyacrylamide gel electrophoresis (PAGE) method for isolating native metalloproteins in biological fluids, it is concluded that the above mentioned criterions apply to this method. In the following sections, firstly the properties of a new electrophoretic method are discussed and reviewed in detail. Secondly, it is briefly considered why this procedure might be of further interest for clinical investigations concerning protein-misfolding diseases.

**QUANTITATIVE PREPARATIVE NATIVE CONTINUOUS PAGE (QPNC-PAGE)**

Gel electrophoresis is well-known as a semi-quantitative procedure so far [29–32]. The former state-of-the-art of a preparative native continuous polyacrylamide gel electrophoresis (PAGE) is exemplary shown in two studies dealing, for example, with the separation of the native high molecular weight beryllium-proteins of human serum under physiological conditions [29]. In the first case the chemical stability...
of the detected Be-proteins after the PAGE trials was not verified by an independent method, e.g., gel chromatography. The detection of some platinum species in plant material by using a preparative native isocapaphoreosis [30] could be due to platinum contaminations from the electrodes of the applied detection system [31]. Therefore, in order to characterize a certain method as quantitative and native, first the stability of the separated compounds has to be investigated and confirmed by an independent procedure [31].

A new state of the art of gel electrophoresis is referred to as quantitative preparative native continuous polyacrylamide gel electrophoresis (QPNC-PAGE) for isolating metal cofactor-containing proteins in biological samples with continuous elution of separated analytes. For example, it could be shown that QPNC-PAGE is a very efficient method for separating quantitative amounts of globular Cd-proteins of a similar molecular mass (≈ 200 kDa) and with different chemical structures in cytosols from different plants. Probably different isomers of the native high molecular mass Cd-proteins could be isolated in biological samples [33-35].

In the former state-of-the-art of a preparative native PAGE the time interval between the sample loading and the arrangement of the proteins in the electrical field added up about 60 min by using isocapaphoreosis. In these investigations it was observed that the Be-bonding in a prealbumin fraction of human serum is dissociated during this time [29]. By using QPNC-PAGE it could be shown that the potential risks of proteolysis and denaturation (change of the native conformation) and/or dissociation of the native metal cofactor-containing proteins in complex biological samples are minimized. These important results can partially be explained by the fact that the protein molecules migrate into the gel without major time lag. All proteins to be separated migrate through the PAGE gel with different migration velocities. Because of an efficient purification and simultaneous separation of complex biological samples, possible interactions, for example, between enzymes, proteins, cofactor-containing proteins and further non-proteinogenic neutral species (e.g., carbohydrates) and smaller molecules can be excluded on using QPNC-PAGE [33-35].

In QPNC-PAGE protein molecules with a molecular mass of about 10 to more than 200 kDa are separated according to their individual isoelectric points (pl). By using this native method, protein molecules that differ in isoelectric point by as little as 0.1 pH units can be isolated. Electrophoretic separations of the biomolecules on a gel column (28 mm I.D.) are conducted in the Model 491 Prep Cell from Biorad. Neither the Joule heat generated during the electrophoretic processes, nor a continuous buffer system (20 mM Tris-HCl, 1 mM NaNO₃, pH 10.00) adversely affect the chemical stability of the native metalloproteins to be separated. At a value of pH 10.00 most proteins of a biological system are negatively charged and they exist as anions. Consequently, during electrophoresis the proteins with values of pl 0–10 migrate to the anode of the Model 491 Prep Cell. At pH 10 of the buffer system the protein molecules with pl values of 10–14 are positively charged and will migrate towards the cathode. In this case the electrodes to the power supply have to be reversed to ensure migration into the gel. As exception, proteins with values of pl 10.0 cannot migrate into the PAGE gel because the net charge of these molecules is zero when using a pH 10.00 electrophoresis buffer [33-35].

Due to the time of polymerization of the polyacrylamide gel (69 hr) there are no active sites present in a fully-polymerized QPNC-PAGE gel. Polymerization of the gel is accompanied by exothermal reactions. Therefore, a temperature gradient was observed at the air/glass column interface above the acrylamide solution. The emerging heat during the polymerization process is dissipated according to the instructions of the Biorad manual. After 69 hr of polymerization at room temperature chemical reactions of the gel were no longer observed and, finally, the gel column (height: 40 mm) was homogeneous, mechanically stable and free of radicals. Higher gels may lead to diffusion of separated ring shaped protein bands [33-35]. In the former state-of-the-art of a preparative native PAGE the physicochemical conditions of the gel column were labile. In this case a complete polymerization was assumed after a time of about 10 hr. The surface of the gel had to be stabilized mechanically by a glass frit [29].

Because of the standard parameters of a new PAGE gel it is evident that the interactive forces between the gel column and the biomolecules to be isolated by QPNC-PAGE are reduced to a minimum. Furthermore, the respective gel structure, characterized by the weight percentage (4 % T) of total monomer including the cross linker (acrylamide/bis 37.5:1) contributes to a minimum sieving effect of the bioanalytes during electrophoresis. For those reasons the conditions of this PAGE system are non-restrictive [33-35].

It is indicated that the electrical migration behavior of the native metalloproteins to be separated strongly correlates with the properties of the gel and buffer used in QPNC-PAGE. This means, for example, that the electrophoretic resistance of the gel (friction between gel and biomolecules) in this system is apparently minimized and the migration velocity of the biomacromolecules during electrophoresis is maximized. The separated protein bands are not broadened because the ion activity of the electrophoresis buffer in this system is sufficient for the current flow. Furthermore, by an internal cooling circuit within the Model 491 Prep Cell, temperature gradients along the gel column are avoided. As a result, the resolution of the detected metalloprotein peaks in an electropherogram is optimized. Conclusively, the electrophoretic separations of metalloproteins by using QPNC-PAGE have to be regarded as quantitative and native [33-35].

Coupled off-line to gel permeation chromatography (GPC), QPNC-PAGE and GPC present a combined procedure with complementary analytical properties that provide for the biological activity of the chemical species to be separated. In GPC the biomolecules are eluted according to the molecular mass of globular proteins, for example. By using a combination of GPC and QPNC-PAGE metalloprotein monomers as well as polymers can be highly purified from a biological sample. QPNC-PAGE is proposed to be a universal high-resolution method for separating quantitative amounts of metal cofactors (< 1 ng/mL) in biological matrices. The most important cofactors to be isolated by this electrophoretic method are, e.g., Fe, Zn, Cu, Cd, Pd, and Mo. QPNC-PAGE is a very efficient method for isolating cova-
lently and especially non-covalently bound cofactors [33-35].

The integrity of metal protein-binding is maintained during the electrophoretic separation of these metallocompounds by using QPNC-PAGE. Exemplary, it was proved that preparative native gel permeation chromatography is a suitable method to confirm the stability of isolated metalloproteins, e.g., Cd-proteins (≈ 200 kDa) in plant cytosols. By a combination of GPC off-line coupled with QPNC-PAGE nearly all contaminants of a biological sample are excluded from further analytical examinations. Due to the separation principles of GPC and QPNC-PAGE specific metalloproteins may be isolated and highly purified from complex protein samples without applying concentration steps, such as freeze-drying or ultrafiltration of the samples. For those reasons, subsequent determinations of the chemical structures of purest native metalloenzymes and/or globular metalloproteins in aqueous PAGE fractions can be conducted by using, for example, NMR spectroscopy [33-35].

Nuclear magnetic resonance (NMR) is an important tool for high-resolution structural studies of proteins. It demands high protein concentrations and high purity [36]. However, it is also possible to obtain NMR spectra at sub-micromolar protein concentrations or at even lower concentrations of analytes [37]. In (Fig. 1) the proposed analytical processing including the sampling, separation and detection steps with regard to various kinds of biofluids is clarified. Thereby, all analytical steps, except the ICP-MS as metal detector, are conducted under non-denaturing conditions. The respective parameters of the GPC and QPNC-PAGE methods as well as some results obtained by this procedure are listed in literature [33-35].

By comparing the separation conditions of a former state-of the-art [29, 30] and the new state-of the-art of a prepara-
tive native continuous PAGE [33-35] the following conclusions can be drawn. The pH-values of the electrophoresis buffers (leading buffer) being used for separating metalloproteins in plant material (pH 8.0) [30] or blood samples (pH 7.4) [29] do not result in quantitative results for the analytes in the respective samples. Applying a value of pH 10.00 of the electrophoresis buffer, the results obtained by a new PAGE procedure are quantitative for the analyzed metalloproteins present in plant cytosols. However, these results could only be obtained in combination with the specific properties of a fully-polymerized PAGE gel and the applied parameters of a new QPNC-PAGE procedure as already described in literature [33-35].

In QPNC-PAGE the proposed pH conditions of the electrophoresis buffer may not necessarily correspond with the physiological pH conditions of a complex biological sample. However, in order to isolate native metalloproteins from these samples, pH 10.00 of the electrophoresis buffer was found to be an optimal pH-value for purification and quantitative separation of metalloproteins in biofluids. There are some reasons why the metal proteins to be separated are not denatured under the pH 10.00 conditions of this PAGE method [33-35].

It is a well-known fact that metalloproteins may be dissociated under certain pH conditions. For example, the Be- binding in a pre-albumin fraction of blood samples is dissociated at pH 8.9 of the terminating buffer by using preparative isoelectrofocusing [29]. In contrast to this method, the protein samples to be separated by QPNC-PAGE are mixed with glycerol before loading a sample on a new PAGE gel. By this procedure the biological sample is compressed and protected against influences of the electrophoresis buffer. Glycerol itself does not change the physiological pH-value of biofluids. Therefore, it is concluded that the physiological pH conditions of a complex biological sample remain stable for some time after layering this sample beneath the upper electrophoresis buffer in the Model 491 Prep Cell. The native metalloproteins are not dissociated into cofactors and apoproteins under these conditions [33-35].

Having started the electrophoretic separation of a protein sample the analytes migrate into the gel. The constituents of the electrophoresis buffer (20 mM Tris-HCl, 1mM NaNO₃, pH 10.00), especially the OH⁻ ions present in a homogeneous gel, do not interact with the metal proteins to be isolated because the same ions are responsible for the current flow during the electrophoretic processes on using QPNC-PAGE. The separated bioanalytes are continuously eluted in an elution chamber by using a physiological eluent (e.g. 20 mM Tris-HCl, 1mM NaNO₃, pH 8.00) and thus, cannot interact with the lower electrophoresis buffer. Conclusively, the value of pH 10.00 of the continuous electrophoresis buffer does not adversely affect the metal protein-binding of biological samples at any time of the electrophoretic processes [33-35].

During a QPNC-PAGE run the voltage and current are variable parameters ranging from 450 to 1000 V and 11 to 5 mA. The products of the respective voltages and currents are constant at 5 VA. This means that the electrical energy spent per unit of time is constant. Because of an increasing voltage during the electrophoretic separations of biomolecules the field strength E [V/m] between the electrodes of the electrophoresis chamber is highly variable. As an effect of both homogeneous gel and electrical field as well as a high capacity of the electrophoresis buffer, the velocity of each separated protein band is approximately constant. This is a very important prerequisite for the reproducibility of the results obtained for a certain biological matrix to be investigated [33-35].

**THESIS**

The following theoretical considerations could perhaps explain why QPNC-PAGE might be a future method for elucidating metal cofactor metabolisms in protein-misfolding diseases.

If the native structure of a metal protein is denatured by cofactor or protein dissociation, or, if the native conformation of a biomacromolecule is altered due to changed parameters in the cellular environment (e.g. pH) of an organism, consequently, the physicochemical properties of the folded metal cofactor-containing proteins or metalloenzymes, such as redox activities, isoelectric point (pI), molecular mass, hydrodynamic radii and other parameters are modified. In these cases, the biological activities of enzymes or metal binding transport proteins, e.g., metallochaperone molecules may be inhibited, for example by the release of metal ions.

In QPNC-PAGE and GPC denatured metalloproteins show a completely different elution behavior compared to the native state of a protein molecule. In the case of cofactor dissociation, for example, metal cofactors cannot be detected in PAGE or GPC fractions by using element-specific detection methods (e.g., ICP-MS). Furthermore, the conformational changes of native metalloproteins result in a different isoelectric point of the denatured protein compared to the pI-value of the native state of a protein molecule. Therefore, globular and denatured metalloproteins of the same chemical composition are eluted in completely different PAGE fractions. For these reasons, QPNC-PAGE and GPC may contribute to medical examinations concerning the proper folding and intactness of metalloproteins and to studies about the activities of metalloenzymes in aqueous solutions of numerous clinical matrices. QPNC-PAGE may also be a very efficient method for investigating the interactions between metal cofactors and apoenzymes or apoproteins in biological organisms or model systems.

**CONCLUSIONS AND OUTLOOK**

In this report the contributions of a new state-of-the-art of gel electrophoresis ([quantitative preparative native continuous polyacrylamide gel electrophoresis (QPNC-PAGE)] combined with complementary gel chromatography are postulated to provide deeper insights into the metabolisms of metal cofactor-containing chaperones in protein-misfolding diseases (e.g., Alzheimer’s disease). Contrary to the previous state-of-the-art of gel electrophoresis which is well-known as a semi-quantitative method, e.g., preparative native isoelectrofocusing, QPNC-PAGE is proposed to be a highly efficient method for isolating quantitative amounts of biologically-active metalloproteins in complex biological samples. The quantitative and native analysis of these analytes is one...
of the major prerequisites for further investigations concerning the structure-function relationships of native metal cofactor-containing chaperones in protein-misfolding diseases by using ICP-MS and NMR spectroscopy.

For these reasons, a combined procedure consisting of high-resolution QPNC-PAGE, GPC, ICP-MS and NMR, might essentially contribute to medical examinations concerning the proper folding of native metallochaperones and to clinical studies about the biological activities of metalloenzymes in aqueous solutions of numerous human and animal matrices. For example, metallochaperones in blood samples may be quantified by using this procedure.

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REFERENCES