Novel Electrokinetic Approaches To Improve Purification Processes with Monoclonal Antibodies

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2 Zusammenfassung

Antikörper stellen eine Proteinfamilie mit physiologischen Funktionen in der Immunabwehr dar. Sie besitzen eine gemeinsame Grundstruktur aus jeweils zwei identischen schweren und leichten Aminosäureketten, die dann eine Y-Form bilden. An ihren kurzen Armen können Antigene gebunden werden, die somit neutralisiert und/oder zum weiteren Abbau markiert werden. Der lange Arm enthält Bindungsstellen für Rezeptoren zur Aktivierung weiterer Faktoren der Immunantwort. Diese Funktionen eignen sich zur Bekämpfung von Krankheiten wie Krebs oder Rheumathoider Arthritis. Aktuell sind einundzwanzig Antikörper oder Antikörper-abgeleitete Moleküle zur Therapie solcher Krankheiten zugelassen. Viele weitere befinden sich in klinischen Untersuchungen und täglich werden weitere entwickelt. Antikörper zur gezielten Therapie von Krebs oder chronischen Entzündungskrankheiten werden heute als monoklonale rekombinante Proteine biotechnologisch hergestellt. Sie werden mittels Säugetierzellen produziert und müssen danach aufwändig von bei der Produktion entstehenden Verunreinigungen getrennt werden. Dieser Aufreinigungsprozess wurde durch die Verbesserung der Produktionsraten immer mehr zum Flaschenhals in der Gesamtproduktion. Die vorliegende Arbeit ist Teil eines BMBF geförderten Projektes zur Entwicklung einer Platformtechnologie für die Aufarbeitung monoklonaler Antikörper (mAb) und sollte Verbesserungen von Reinigungsprozessen und deren Entwicklung hervor bringen. Reinigungsprozesse für mAbs beinhalten mehrere Schritte, um den notwendigen hohen Reinheitsgrad zu erreichen. Neben Zentrifugation und Filtration ist die Chromatographie die wesentliche Methode zur Abtrennung von Kontaminationen. Dabei kommen neben der Affinitätschromatographie mittels Protein A hauptsächlich die Ionenaustausch- und Hydrophobe Interaktionschromatographie zum Einsatz. Der Fokus dieser Arbeit wurde auf die letzeren Methoden gelegt, da zum einen die Protein A Chromatographie gut etabliert ist,

aber auch mehrere Nachteile beinhaltet. Die Entwicklung von Reinigungsprozessen ist bis heute ein lang dauernder und auf systematischem Ausprobieren basierender Prozess, da die genauen Mechanismen der Adsorption von Proteinen an stationäre Phasen nicht vollständig geklärt sind. Es wurden im Rahmen dieser Arbeit Methoden entwickelt, die diese langwierigen Entwicklungsstrategien verkürzen und durch weitere Aufklärung der grundlegenden Mechanismen verbessern. Dabei wurden elektrokinetische Methoden wie die Bestimmung von Zetapotentialen von Antikörpern mittels Laserlichtstreuung und elektroakustische Untersuchungen von Salzlösungen und Biochromatographiematerialien eingesetzt.

Mit Hilfe von Zetapotentialmessungen wurde eine Methode zur Beschreibung von Protein – Salz – Interaktionen etabliert. Das Zetapotential von Proteinen ist abhängig von den Pufferbedingungen der Proteinlösung. Der isoionische Punkt (iP) eines Proteins, bei dem keine Nettoladung nach außen vorliegt, verschiebt sich aus Gründen der Elektroneutralität durch Anlagerung von Salzionen. Dabei spielen die Anionen die überwiegende Rolle, denn der isoionische Punkt verschiebt sich mit steigender Salzkonzentration zu sauren pH – Werten. Die Verschiebung des isoionischen Punktes hängt auch vom Salztyp ab, stärker kosmotrope Salze verschieben bei vergleichbarer Konzentration den iP weiter in den sauren Bereich. Der iP kann also für die Beschreibung von Protein – Salz – Wechselwirkungen herangezogen werden. Für die Adsorption von Proteinen an Chromatographiematerialien müssen sowohl an das Protein wie auch an das Gel angelagerte Ionen entfernt werden, da diese sowohl sterisch als auch durch elektrostatische Kräfte die Adsorption behindern können. Die dynamische Bindekapazität von Kationenaustausch – Chromatographiegelen, ein sehr wichtiger Parameter für die Effizienz von Aufarbeitungsprozessen, zeigt ein pH abhängiges Maximum. Dies hängt einerseits mit den Gleichgewichtsbedingungen der Protein- und Gegenionenadsorption an die Gelmatrix und andererseits mit Massentransferphänomenen bei

der Aufnahme der Proteine in das Porensystem der Gele zusammen. Mit abnehmenden pH – Werten steigt die positive Ladung der Proteine und die zur Adsorption nötige elektrostatische Wechselwirkung mit der negativ geladenen Matrix nimmt zu und verbessert dadurch die Kapazität. Bei weiter zunehmender Proteinladung können abstoßende Kräfte zwischen den Antikörpermolekülen die nahe Aneinanderlagerung der mAbs auf der Matrix und deren Massentransfer in das Porensystem der Partikel, das kaum größere Durchmesser hat als die Antikörpergröße, behindern. Die pH – Lage des Kapazitätsmaximums hängt ebenso von der Salzstärke ab. Bei erhöhter Salzkonzentration schirmt das Salz die Ladung der Proteine zunehmend ab, behindert aber auch die Proteinadsorption durch die höhere Gegenionenkonzentration. Dadurch verschiebt sich das Kapazitätsmaximum mit steigender Salzkonzentration ins Saure. Dies gilt wie oben beschrieben auch für den iP. Da Ionen durch sterische Gründe in ähnlichem Ausmaß vom Protein und vom Gel zur Adsorption entfernt werden müssen, verhalten sich die pH – Verschiebungen des iP und des Kapaziätsamaximums ebenfalls ähnlich. Aus diesen Erkenntnissen wurde eine Methode zur Bestimmung der Bedingungen für eine maximale dynamische Bindungskapazität basierend auf Zetapotentialmessungen entwickelt. Dabei werden keine zeit- und materialintensive chromatographische Experimente mehr benötigt, was die Entwicklung von Reinigungsprozessen mit Kationenaustauscherschritten beschleunigt und verbessert.

Über die Untersuchung der Eigenschaften von Salzen mittels Elektroakustik wurden bisher ungeklärte chromatographische Phänomene beleuchtet. Bei der Hydrophoben Interaktioonschromatographie (HIC) wird die Adsorption von Proteinen aus einem Entropiegewinn durch Wasserumlagerung getrieben. Durch hohe Salzkonzentrationen werden sowohl den hydrophoben Bereichen der Proteine als auch der hydrophoben Geloberfläche von HIC Gelen Wassermoleküle entzogen. Durch Zusammenlagerung von hydrophoben Bereichen von Gel und Proteinen teilen diese sich ihre Wasserhüllen und dadurch entsteht ein

Entropiegewinn. Die dynamische Bindekapazität von HIC Gelen hängt ebenfalls vom pH – Wert ab. In der Literatur wird meist der Einfluss des Ladungszustandes der Proteine als Ursache dafür diskutiert. Dabei wird ein Maximum der Bindekapazität am isoelektrischen Punkt (mittels isoelektrischer Fokussierung bestimmt) erwartet, da dort am wenigsten elektrostatische Kräfte vorliegen, die die Adsorption behindern könnten. Es wurde aber von vielen Gegenbeispielen berichtet. Meistens liegen Kapazitätsmaxima in HIC Systemen im neutralen pH – Bereich, auch bei Proteinen deren isoelektrischer Punkt bei stark sauren oder basischen Werten liegt. Bedenkt man die Konzentrationsverhältnisse in solchen Systemen, sollten die Eigenschaften der Salze am stärksten ins Gewicht fallen, da diese in höchster Konzentration im Vergleich zu Proteinen und funktionellen Gruppen der HIC – Gele vorliegen. Durch elektroakustische Untersuchungen wurden pH – abhängige Eigenschaften von (Neutral-)Salzlösungen gefunden. Dabei wurden Maxima in Bezug auf das strukturelle Volumen der Salzionen detektiert. Dies kann zum einen durch eine Vergrößerung der Hydrathülle der Ionen und zum anderen durch Ionenassoziation oder durch eine Kombination beider hervorgerufen werden. Die pH – Lage der maximalen strukturellen Volumen der Ionen hängen von der Salzkonzentration und dem Salztyp ab. Mit steigender Salzkonzentration verschieben sich die Maxima zu basischeren pH – Werten. Sie bewegen sich je nach Salz und Konzentration im Bereich von pH 4,5 – pH 8. Da in HIC – Systemen mit hohen Salzkonzentrationen gearbeitet wird, liegen hier die maximalen strukturellen Ionenvolumen im pH – Bereich von 6 – 8. Wie oben beschrieben, werden in diesem Bereich auch die Kapazitätsmaxima in der HIC gefunden. Der Adsorptionsprozess in der HIC ist ein äußerst komplexer Vorgang, bei dem im Hinblick auf kurze Verweilzeiten und flache Isothermen der Massentransfer in die Gelporen eine wichtige Rolle spielt. Geht man von starken Adsorptionsbedingungen aus, kann der Massentransfer am Poreneingang geblockt sein, was zu niedrigeren Kapazitäten führt. Mit Hilfe der Scaled Particle Theorie kann die Beziehung

zwischen Ionenvolumen und dynamischer Bindekapazität in der HIC erklärt werden. Diese Theorie ist eine statistische Bearbeitung der Nicht-Idealität von Lösungen, die durch weitere gelöste Stoffe verursacht wird. Sie beschreibt die reversible Arbeit, die nötig ist, um eine spherische Kavität um ein einzelnes Protein aufzubauen, das in einer Lösung mit verschiedenen harten spherischen Partikeln vorliegt (Wasser und Salz – Ionen). Der Scaled Particle Theory folgend tritt ein maximaler Protein stabilisierender Effekt auf, wenn gelöste Stoffe (Salzionen) ein Volumen vom 1,8-fachen des Volumens eines Wassermoleküls einnehmen. Ändert sich also das Volumen der Salzionen mit dem pH – Wert, kann der Protein stabilisierende Effekt zunehmen. Dadurch kann eine Porenblockade in der HIC vermindert werden, was wiederum zu einer Erhöhung der dynamischen Bindekapazität führt. Die elektroakustische Untersuchung von Salzlösungen kann somit bei der Suche nach den idealen Bedingungen für die Hydrophobe Interaktionschromatographie genutzt werden.

In laufenden Produktionsprozessen von Biopharmazeutika wie monoklonalen Antikörpern besteht durch die begrenzte Lebenszeit der Gele die Notwendigkeit, dass das Chromatographiematerial erneuert werden muss. Da dabei die Produktidentität der mAbs gewährleistet werden muss, können nur zertifizierte Materialien verwendet werden. In zugehörigen Analysezertifikaten werden einige Parameter der jeweiligen Produktionschargen aufgelistet, und somit die Identität dieser Gelchargen vom Hersteller nachgewiesen. Doch kommt es trotz eingehaltener Spezifikationen zu Abweichungen im chromatographischen Verhalten zwischen den Produktionschargen, die nicht aus den Zertifikaten ersichtlich sind. Daher fordern Regulierungsbehörden sehr ausführliche und aufwändige chromatographische Tests, bevor eine neue Gelcharge zur Produktion eingesetzt werden kann. Mit Hilfe elektroakustischer Untersuchungen wurden verschiedene Chargen mehrerer Chromatographiematerialien untersucht und mit chromatographischen Eigenschaften verglichen. Dabei wurde eine Korrelation zwischen unspezifischen

Proteinbindungseigenschaften der Gele und dem kolloidalen Vibrationsstrom (CVI), der Messgröße in der Elektroakustik, gefunden. Somit können durch die Anwendug der hier entwickelten elektroakustischen Methode zur Charakterisierung von Biochromatographiematerialien die Eigenschaften der Gele bezüglich unspezifischer Proteinbindung schnell beschrieben werden. Damit kann die Zahl der aufwändigen und kostenintensiven chromatographischen Tests verringert werden, die vor dem Einsatz einer neuen Gelcharge in einer laufenden Produktion von monoklonalen Antikörpern durchgeführt werden müssen. Dies führt schließlich zu Zeit- und Kostenersparnis. Möglicherweise kann die Methode auch als ein zusätzlicher Parameter in Analysenzertifikate einbezogen werden. Dazu sollte jedoch die Entwicklung einer stimmigen Theorie zur Erklärung der elektroakustischen Phänomene in dieser Methode vorangetrieben werden. Dies könnte auch zur ihrer Akzeptanz bei Regulierungsbehörden beitragen.

3 Summary

Antibodies are a protein family with important physiological functions in immune defence. They share a common Y-shaped structure of two identical light and heavy amino acid chains. At their short arms antigens are bound and thereby neutralized and become marked for further degradation. The long arm consists of receptor binding sites for activation of further immune defence factors. Because of these functions antibodies are suitable therapeutics for cancer or rheumatoid arthritis. Currently twenty one approved monoclonal antibodies or derived proteins (mAbs) are used to medicate patients with such diseases. Many are tested in clinical trials and new ones are developed every day. Today antibodies for specific therapy of cancer or chronically inflammatory diseases are produced as recombinant monoclonal antibodies. They are expressed by mammalian cell lines followed by complex separation processes to remove production contaminants. Caused by increasing production titer purification became more and more the bottleneck in the whole production processes. The present work is part of a government (Federal Ministry of Education and Research, BMBF) supported project to develop a platform technology for purification of monoclonal antibodies and should improve purification processes and their development. Purification processes for mAbs consist of several steps to reach the required purity. Beneath centrifugation and filtration chromatography is the most important method to separate contaminations. Thereby affinity chromatography via immobilized protein A, ion-exchange (IEX) and hydrophobic interaction chromatography (HIC) are mostly used techniques. This work was focussed on mAb separations using IEX and HIC, because protein A chromatography is well established and the process parameters are well understood, but it contains several disadvantages. Until today the development of purification processes is a long winded trial-and-error approach, because the specific mechanisms of protein adsorption on stationary phases are not fully understood. In context with this work, meth-

ods to accelerate these long winded development strategies were developed facilitated by further improvement of understanding the basic adsorption mechanisms of proteins on chromatographic resins. The new experimental electrokinetic methods introduced are zeta potential determination with proteins via laser light scattering and electro-acoustic investigations of salt solutions and biochromatographic resins.

Zeta potential measurements were used as a experimental tool to characterize protein – salt – interactions. The zeta potential of proteins depends on buffer conditions of the protein solution. The isoionic point (iP) of a protein, where the proteins' net charge is zero, is shifted by bound salt ions caused by electroneutrality. Thereby salt anions play a predominant role, because iP is shifted to more acid pH values with increasing salt concentration. The shift of iP depends on the salt type, too. IP is more shifted by kosmotropic salts than by chaotropic salts at comparable salt concentrations. As follows, the isoionic point can be used to describe protein – salt – interactions. Dynamic binding capacity (DBC) of cation exchange resins is an important efficiency parameter of purification processes and exhibits a pH dependent maximum. This could be explained by the interplay of equilibrium condition of protein and counter ion adsorption on the matrix and by limited mass transfer during protein uptake into the resin pore system. With decreasing pH values protein surface net charge increases and positive charge for electrostatic interaction with the negative charged resin can improve DBC. With further increasing protein charge electrostatic repulsion between mAb molecules can handicap the adsorption on the matrix, because mAbs tend to stay at a certain distance from each other. As a result repulsive forces inhibit the mass transfer into the resin pores, which are barely bigger than the mAbs themselves. The pH position of maximum DBC depends on salt concentration, too. With increasing salt concentration, first, protein charge is more shielded reducing the ion exclusion effect, and second, protein adsorption is depleted by higher counter ion concentration. Therefore the pH position of maximum DBC decreases with higher salt

concentration as shown for the isoionic points above. Ions have to be released for protein adsorption in similar extent from protein and matrix caused by steric reasons. Thus, the pH position of iP and of maximum DBC shift to more acid pH values by increasing salt concentration in a similar extent. Based on these findings a method to determine the conditions for maximum DBC based only on zeta potential measurements was developed. Thereby time and material expensive chromatographic experiments are no longer necessary. This improves and accelerates the development of purification processes for cation exchange chromatography steps with monoclonal antibodies.

An additional result of this work is a correlation of salt solution properties determined by a electro-acoustic method and dynamic binding capacity in hydrophobic interaction chromatography (HIC). In hydrophobic interaction chromatography protein adsorption is mainly driven by entropy gain caused by water rearrangement. Water molecules are withdrawn from hydrophobic patches of proteins and stationary phase by high ion concentrations. By attached hydrophobic sites of proteins and matrix, they join hydration water partially and an entropy gain occurs. It is discussed in literature that the charge state of proteins affects adsorption and DBC in HIC. Thereby a DBC maximum is propagated to be at the isoelectric point (pI, determined by isoelectric focussing) of the protein, because at this point repulsive electrostatic forces are minimized representing a hindrance for adsorption. But counter-examples are reported. Often DBC maxima in HIC exist near neutral pH values, even though the pI of the protein is acidic or basic. Considering concentration relations in HIC systems, salts have the highest concentration regarding protein and functional resin groups and should consequently be the main important parameter. Via electro-acoustic investigations, pH dependent properties of (neutral) salt solutions were found. The structural volume of salt ions exhibits a pH dependent maximum. This can be a result of an increased water shell or ion association. The pH position of maximum structural ion volume depends on salt type

and concentration. With increasing salt concentration the maximum shifts to higher pH values. The pH range of detected maxima is depending on salt type and concentration between pH 4.5 to pH 8. In HIC high salt concentrations are used and ion volume maxima are between pH 6 and 8. As described above, DBC maxima in HIC are found in similar pH ranges. Considering the short residence times and shallow isotherms in HIC mass transfer into resin pores plays a dominant role for DBC. At strong adsorption conditions, resin pores can be blocked resulting in decrease of DBC. With the Scaled Particle theory the relationship between structural ion volume and DBC in HIC can be explained. This theory is a statistical treatment of the non-ideality of solutions caused by cosolutes. It describes the reversible work to create a spherical cavity around a single protein in a solution containing different hard spheres (salt and water ions). Following the Scaled Particle theory a maximum protein stabilizing effect occurs when the cosolute volume reaches 1.8 times the volume of a water molecule. Changing the cosolute volume by altering the pH, the protein stabilizing effect can increase. Therefore pore blocking by too strong adsorption conditions can be diminished resulting in increasing DBC. Thus, the electro-acoustic investigation of salt solutions can help to find ideal conditions in hydrophobic interaction chromatography.

In production processes of biopharmaceuticals like monoclonal antibodies limited resin life time holds the need to replace column packings. Because product identity of mAbs must be guaranteed, only certified chromatographic media can be used. In dedicated certificates of analysis some parameters of the current resin batches are listed and therefore the resin identity is verified by the manufacturer. But in spite of kept specifications differences in chromatographic behaviour of new resin batches occur, which are not included in the certificates. Thus, regulatory authorities require extensive chromatographic tests before a new batch can be used in an ongoing purification process. Via electro-acoustic measurements different batches of several resin types were investigated and compared with chromatographic experi-

ments. Thereby a correlation between unspecific protein binding properties and the colloidal vibration current (CVI), the electro-acoustic measurand, was found. Thus, using the electro-acoustic method developed in this work to characterize biochromatographic media, resin properties regarding unspecific protein binding can be described. As a result the amount of cost and time intensive chromatographic tests to prove new resin batches can be diminished. Possibly this approach can be used as an additional parameter in certificates of analysis. But for this purpose an appropriate theory to explain the found electro-acoustic phenomena should be developed. This could contribute to the acceptance of the method by regulatory authorities.

4 Introduction

4.1 Monoclonal Antibodies

Antibodies, or immunoglobulins (Ig), are a protein family that consists of structurally related glycoproteins. They are produced by B lymphocytes which function as mediators of specific humoral immunity. All antibodies have a common basic structure of two identical light chains and two identical heavy chains (Fig. 1). Each chain consists of a variable domain which differs among different specificities and a constant domain which allows (sub-) classification of antibodies. The variable regions (F_V) of heavy and light chains each contain three separate hyper variable regions forming antigen binding sites and are able to bind antigens. The constant regions of the heavy chains form the constant part of Igs (F_C) can bind to F_C receptors on e.g. eosinophils, mast cells and natural killer cells thereby stimulating their function.

Besides their original function antibodies are also indispensible tools in the laboratory. Radioimmunoassay (RIA), enzyme-linked immunosorbent assay (ELISA), immunoprecipitation and Western blotting are only a small choice of approaches based on antibodies.

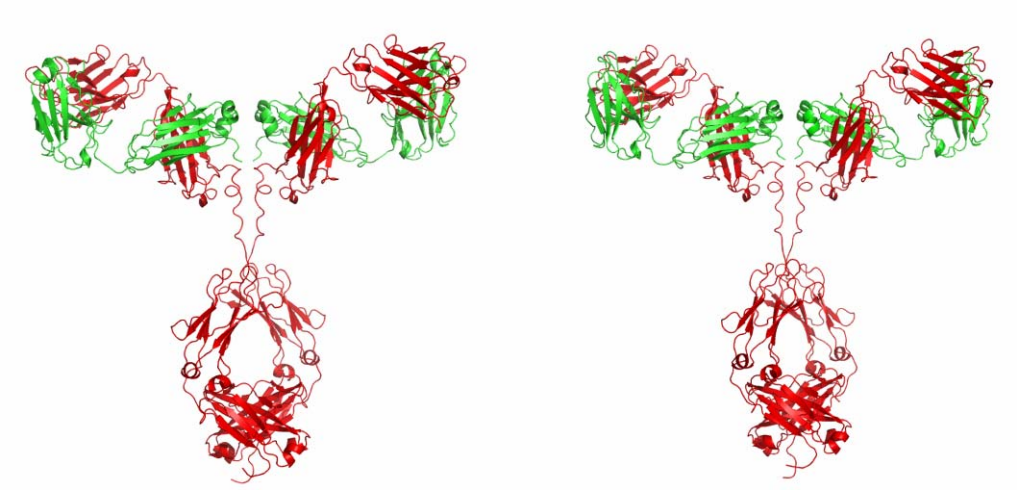

Fig. 1: Computed models of mAb1 (left) and mAb2 (right) used in this work. Light chains green, heavy chains red, picture kindly provided by M. Widmann and A. Steudle, ITB University of Stuttgart.

Serum antibodies produced in response to an antigen are heterogeneous because multiple epitopes or antigenic sites induce the proliferation and differentiation of a variety of B- cell clones. For laboratory or especially therapeutic use of antibodies heterogeneity causes the disadvantage of possible non specific side reactions. Removal of unwanted antibody species is a time-consuming task involving repeated adsorption techniques. An alternative way to generate pure or monoclonal antibody preparations is the hybridoma technology (Kohler and Milstein 1975). Thereby B- cells of hyperimmunised mice are fused with mouse myeloma cells (cancerous plasma cells). They are able to generate a hybrid cell, called hybridoma, that possesses the immortal properties of the myeloma cell and secrete antibodies. By selection monoclonal antibody (mAb) producing cell lines can be achieved. Using recombinant DNA technology mAbs can be modified in specificity, the backbone can be humanized and the encoding DNA sequence can be transferred into high producing systems for commercial manufacturing. The possibility to change the antibody backbone allows their use as therapeutic agents for human diseases, because they were much less likely to elicit an inappropriate immune response in patients (Jones, Dear et al. 1986). Humira®, the first fully human monoclonal antibody drug, was launched in 2003 as a treatment for rheumatoid arthritis. Today, monoclonal antibodies account for more than twenty percent of all new treatments. These include therapeutic products for asthma, arthritis, psoriasis, breast cancer, leukaemia, and transplant rejection, and much more that are in late-stage clinical trials. The growth of the therapeutic antibody market is projected to more than double to around £16 billion per year by 2010 (Council 2008). Currently tons of mAbs per year are used to medicate patients and in the times of high titer fermentations downstream processing can become the bottleneck in production (Li, Zhou et al. 2005). Thus, improvement of purification processes as well as their development strategies are pressed ahead again by industrial and academic institutions.

4.2 Chromatography

The starting point of liquid chromatography is seen in 1903, when Michael Tswett presented his lecture “On a new category of adsorption phenomena and their application to biochemical analysis”. Three years later his most important paper regarding chromatography was published describing the developed zones on a column by using different eluents (Tswett 1906) in the applications on the chemistry of chlorophyll. He detected two yellow zones (carotinoids) and two green zones of chlorophylls a and b. Caused by his great competitor in chlorophyll research Willstätter, chromatography was forgotten for nearly 25 years. Chromatography was rediscovered and further developed pushed by increasing interest in natural organic chemists. The first commercial instrumentations were presented in 1969 and after that the requirements of pharmaceutical chemistry drove forward the advancement of High performance liquid chromatography (HPLC), which allowed the determination of product quality and presents the state of the art method until now (Engelhardt 2004).

For preparative separation of (bio-) pharmaceutical products chromatography is the prevalent separation tool next to centrifugation and filtration. Most important modes are size exclusion chromatography (SEC), ion exchange chromatography (IEC), hydrophobic interaction chromatography (HIC) and affinity chromatography. In downstream processes with mAbs protein A chromatography became the most dominant capture step, exhibiting high affinity and high purity. Development times for mAb purification is short, because purification parameters are well known (Ishihara, et al. 2005). But protein A chromatography has a number of disadvantages. Elution conditions are at very acid pH values (e.g. pH 3), which can increase loss of active product by aggregation. Aggregates are crucial inducing immunogenic reactions in biopharmaceutical applications. Additionally protein A resins are prone to leach immobilized protein A necessitating further depletion. Moreover protein A resins are the most expensive chromatographic media. All these disadvantages stand behind the efforts to replace a protein

A capture step by alternative methods. As a result approaches involving biomimetic phases come into discussion, which are resins where immobilized low molecular weight affinity ligands show similar properties like protein A. Until now no biomimetic resin is prevailed in purification processes. Mixed mode resins are another group for capturing mAbs under physiological conditions present in fermentation supernatant. On these gels low molecular weight compounds like pyridine or molecules containing other hydrophobic and charged domains are immobilized, which allows protein adsorption based on electrostatic and hydrophobic/entropic interactions. Thereby the purification conditions are less known and the purity is not as good as using protein A (Chen, Tetrault et al. 2008). An additional alternative approach to replace resins with immobilized proteins is the serial operation of ion exchange and hydrophobic interaction chromatography (Ishihara and Yamamotob 2005; Ghosh and Wang 2006; Ishihara, Kadoya et al. 2006), which represent commonly used methods. Today process design involving IEC and HIC is still a trial and error approach and therefore their fully exhausted potential is rarely achieved.

The goal of this work was to improve the development of purification processes with mAbs regarding development time and process efficiency, especially for IEC as primary capture step and HIC for intermediate and polishing step. Additionally an important point for biopharmaceutical industry is to fulfil regulatory requirements. In particular the replacement of column packing material in ongoing purification processes has to be implemented maintaining product identity. Thus, resin batch to batch variations within product specifications have to be considered and potential new batches must be tested in validated performance tests, which are very time and cost expensive. So a further point in this work is to find a possibility to reduce the number of replacing batches to be tested by a preselecting method.

4.2.1 Cation Exchange Chromatography

Ion exchange chromatography is used in fractionation of plasma proteins since 1950s and a standard protocol to purify polyclonal antibodies was established in 1970s. This provided its application for mAbs, too. Due to advances in support media it became the dominant method in the field (Josic and Lim 2001). For purification processes with monoclonal antibodies, mostly basic proteins, cation exchange chromatography (CEX) is advised for primary product capturing. The traditional cation exchange model describes electrostatic interactions between a positive charged protein (pH below its isoelectric point (pI)) and the negative charged resin and therefore pI should be the sole determinant of protein's retention. But experience shows that the minor part of proteins fulfil this model's prediction. Some proteins bind up to one pH unit above their pI on cation exchangers and some do not bind one pH unit above pI on anion exchangers. This indicates that positive and negative domains on protein surfaces are mixed contributing differentially to the respective interactions with the exchanger. This indicates that the traditional model is too general. The distinction between proteins net charge as a whole and the charge characteristics of the actual binding site is necessary (Ishihara, et al. 2005; Dismer and Hubbuch 2007; Dismer, Petzold et al. 2008). These qualifications are important for the selectivity of adsorption. Bound proteins can be eluted by altering pH, by addition of competing ions or small molecule displacers (Rege, Ladiwala et al. 2005; Tugcu and Cramer 2005) or by combinations of both. Each approach changes selectivity, too. At the practical level sodium chloride is used for elution in linear or stepwise increasing concentrations. But there are several parameters also affecting the performance in CEX, namely buffer system and -concentration, column geometry, flow rate and resin properties, which can be mass transfer hindrance for protein uptake into the resin pores by steric or electrostatic effects. In the development of purification processes column geometry or flow rate are mostly given by facility environment and pressure of time, respectively, whereas buffer system and –concentration as

well as the resin of choice often can be elected based on operators experience. Regarding the high amount of mAb to be produced, column capacity becomes more and more important. Next to improved resins promising high dynamic binding capacity (DBC) the quest for chromatographic conditions for ideal exploit of column capacity are the main starting points for efficient processes. To find ideal conditions for high DBC is a time and material expensive approach. Many breakthrough experiments, where a high amount of pure protein is required, have to be performed to find the conditions for maximum DBC, screening different pH values and appropriate buffers. One approach to fulfil these needs is a high throughput screening method, which was established successfully (Wierling, Bogumil et al. 2007), but requires a high instrumental setup. The method for fast determination of conditions for maximum DBC in CEX with mAbs described in this work is based on further protein characterization by electrokinetic measurements and deeper insight into the adsorption process itself, namely the coherence of resin bound cations and protein bound anions, which both are involved in protein adsorption on adsorbers.

4.2.2 Hydrophobic Interaction Chromatography

Since the late 1940s, hydrophobicity is utilized as an adsorption mechanism and HIC evolved to one of the most powerful techniques in preparative biochemistry. For adsorption of proteins on hydrophobic surfaces high salt concentrations are required. Then hydrophobic patches on the protein surface and hydrophobic ligands on the stationary phase partially can share water shell resulting in an entropy gain, because more free bulk water can be used for salt ion hydration. The influence of salt type and salt concentration was extensively discussed by Melander and Horvath (Melander and Horvath 1977). There a linear relationship between salt molality and the log of protein retention factor was shown. The slope was related to the surface tension increment of the used salt. Basically salts with higher surface tension increment result in

higher retention. Additionally regarding Hofmeister's work showing the efficiency of salts to precipitate proteins, the individual salts can be arranged in the Hofmeister series, against which the series of salts regarding surface tension increments is leaned. Hydrophobic interaction is a further aspect of retention in HIC. The adsorption initiated by entropy gain can be stabilized by the interaction of non-polar groups between resin and protein. The power of the attracting force depends on the hydrophobicity of both protein and particle interacting sites. HIC resins are commercially available functionalized with different ligands or ligand densities, from weak hydrophobic ligands like ether or propylenglycol to strong hydrophobic ligands like butyl or octyl high and low substituted. By rule of thumb more hydrophobic ligands and more hydropathy of the proteins result in higher retention. But strong hydrophobic interactions hold the possibility of protein denaturation or irreversible protein unfolding which leads to activity loss and/or diminished protein recovery (Jungbauer, Machold et al. 2005; Tsumoto, Ejima et al. 2007). For the development of purification processes, parameters to consider are similar for CEX and HIC, namely high product purity, DBC and recovery, all resulting in high efficiency. Regarding the relatively strong hydrophobic character of mAbs compared to their bulk contaminants, HIC is a powerful purification step. Though compared to CEX resins, which have DBC up to 100 mg/ml column volume (CV), with HIC resins merely achieve up to 60 mg/ml DBC. This difference is caused by the possibility to graft IEX resins with functionalized polymers increasing the space for protein binding. With HIC resins such grafting was not successful owing to a collapse of the functionalized hydrophobic polymer in the water environment (Muller 2003). Consequently resins were improved altering their porosity to smaller pores and higher surface area leading to enhanced mass transfer rates and higher DBC (Susanto, Herrmann et al. 2007; Chen, Tetrault et al. 2008). Nevertheless finding optimal conditions for high DBC is still a long-winded job. Parameters like salt type and concentration were discussed, but additionally pH plays an important role. Traditionally

at proteins pI the highest retention and DBC is expected due to reduced repulsive electrostatic interactions, but numerous exceptions are reported (Fausnaugh, Kennedy et al. 1984; Fausnaugh, Pfannkoch et al. 1984; Fausnaugh and Regnier 1986). Remembering the present conditions in HIC, some µmol protein, some mmol ligands and some mol salt are the effective concentrations. Most investigated parameters explained above are the stationary phase and the protein, regarding pH even only the effect of protein pI was discussed extensively in literature. But salts and their properties should contribute exceedingly to the performance in HIC caused by their high concentrations. For buffer salts like phosphate and acetate, pH effect is obvious altering charge states and surface tension increment. One goal of this work was to extend the investigation of salt properties, especially for HIC relevant and neutral salts like sodium chloride and sulphate, and their effect on retention and DBC in HIC. Further knowledge of material properties present in HIC should contribute to shorter process development times and more efficient purification steps. Therefore an electrokinetic method, namely electro-acoustic, was applied to investigate salt properties and the results were consulted to enlighten unexpected HIC behaviour of several proteins, which differ in size and pI.

4.3 Electrokinetic Methods

For the characterization of particles and their surfaces often electrokinetic methods are applied. Electrokinetic phenomena are based either on relative motion of substances present in different phases or relative motion of phases in an applied electric field. Mostly electrokinetic phenomena are distinguished in five different effects, namely electrophoresis, electroosmosis, sedimentation potential, streaming potential and electro-acoustic. These effects are listed and further explained in **Tab.** 1.

Tab. 1: Electrokinetic phenomena, their driving forces and resulting effects.

Electrokinetic effect	Driving force	Effect
Electrophoresis	Potential difference	Particle motion solid phase mobile liquid phase static
Electroosmosis	Potential difference	Particle motion liquid phase mobile solid phase static
Sedimentation potential	Particle motion driven by gravity solid phase mobile liquid phase static	Potential difference
Streaming potential	Particle motion driven by pressure gradient liquid phase mobile solid phase static	Potential difference
Electro-acoustic	Particle motion driven by pressure gradient created by sound waves	Potential or current difference

Commercial devices for determination of electroosmosis or sedimentation potential are rarely available and their use in characterization of proteins, salt ions and bigger particles like chromatographic media is not well described or inapplicable. Streaming potential devices are commercially sold but test measurements provided by manufacturers were less promising for resin characterization and not suitable for salt ion or protein investigations. Thus the focus was set on electrophoretic and electro-acoustic methods, which are described in the next sections.

4.3.1 Electrophoretic mobility determination via Laser Doppler velocimetry

In 1842 Christian Doppler described the change in wavelength and frequency of a wave observed by a viewer moving relative to the waves' source. This phenomenon is known as Doppler effect and is described for sound waves as well as for electromagnetic waves like light (Russel 1848). Analyzing the differences in frequency and wavelength, the relative velocity between the wave source and the viewer can be determined. Today the Doppler effect is widely implemented for example in astronomy, submarines (sonar), medical imaging and blood flow measurements. To characterize colloidal systems (Derjaguin, et al. 1941) laser Doppler velocimetry is used to determine the electrophoretic mobility of the colloids in an applied electric field (Stoltz, Janot et al. 1984). In case of protein solutions the proteins have a pH dependent surface net charge. Thus, (counter-) ions bind on the proteins surface creating an electrical double layer (EDL) around the protein. Near the surface in the inner region of the EDL, called the Stern layer, ions are strongly bound, whereas in the outer region ions are less firmly attached. Within this outer region there is a notional boundary. Beyond this boundary ions can be sheared off by moving the particles. So in an applied electric field, charged proteins move and the potential remaining on the EDL is called the zeta potential (Fig. 2).

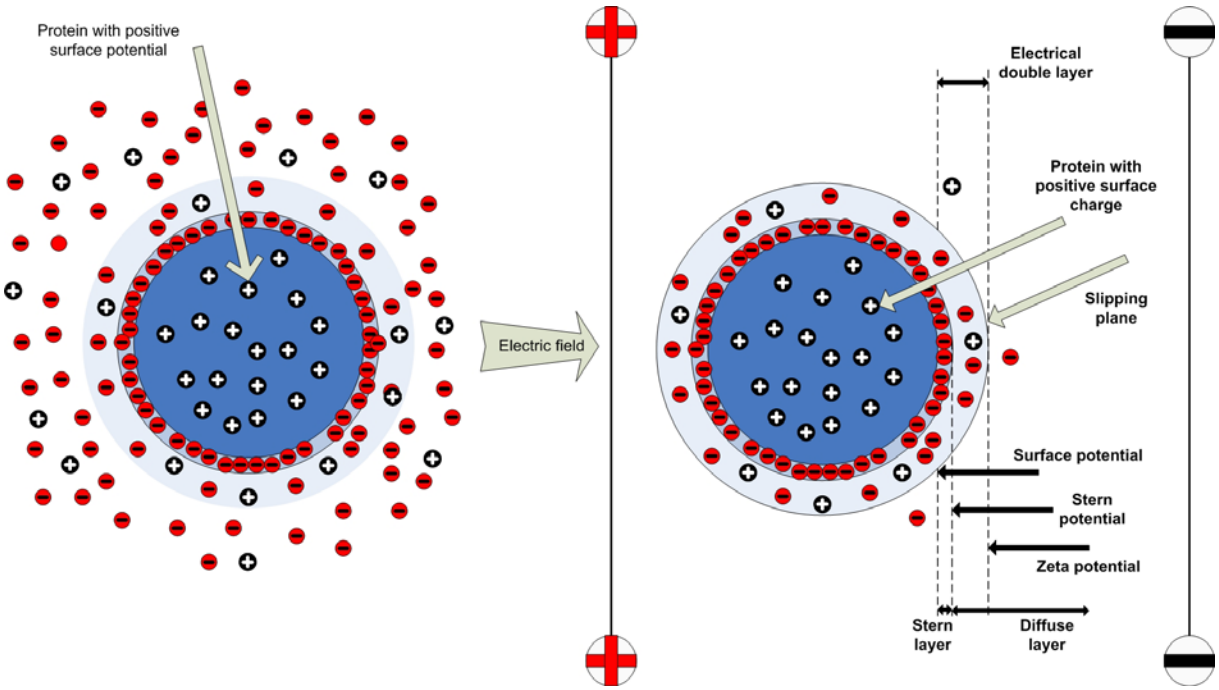

Fig. 2: Scheme of zeta potential description.

Using Smoluchowski's theory the zeta potential of the proteins can be calculated with Eq. (1)

$$U_e = 2\varepsilon k\zeta / 3\eta \qquad (1)$$

where U_e = electrophoretic mobility, ζ = zeta potential of the protein, ε = dielectric constant, η = viscosity of the solution and k = 1.5 (model based constant for salt concentrations higher 1 mM, Smoluchowski 1921 (Saville 1977))

The point of zero zeta potential, called the isoionic point, describes the electro neutral state of the protein when it does not move in an electric field. Because of the involvement of bound ions, the salt or buffer type present affects the zeta potential and the pH of the isoionic point. Approaches of zeta potential measurements regarding biotechnology were the description of charge compensation of ion exchange matrices during lysozyme adsorption or the impact of membrane and protein potential on membrane chromatography performance (Yamamoto,

Hachiya et al. 1992; Bonincontro, et al. 2006). Additionally zeta potential measurements were used to investigate and predict protein (de-) stabilizing conditions as well as to develop a method to determine the conditions for maximum DBC in HIC (Faude 2007). Beneath these applications a method for fast determination of conditions for maximum DBC in CEX with mAbs using zeta potential measurements was developed in this work.

4.3.2 Electro-acoustic

4.3.2.1 Ion vibration potential (IVP)

Another method to investigate ions or charged particles is electro-acoustic. This phenomenon was predicted by Peter Debye in 1933 without the possibility of experimental proofs. It is described as the coupling of electric and acoustic fields for simple electrolyte solutions. In the presence of a ultrasonic sound wave, effective mass or friction coefficient differences of anions and cations cause their different displacements by the pressure gradient. This again brings out charge isolation and leads to an electrical potential between different points in the solution. This potential can be measured and is called ionic vibration potential. The measured value represents the maximum amplitude of the generated potential. Regarding chromatographic behaviour of salts in HIC depending on pH, changes in IVP of salt solutions different concentrations caused by variation of pH should be investigated via electro-acoustic. Parameters contributing to IVP signal are ion mass, ion transfer number, intrinsic ion volumes, ion charge electrostatic contribution, cavity forming volume and structural volume. For low salt concentrations, only intrinsic ion volume and structural volume are contributing to IVP changes with altered pH. But for higher salt concentrations electrostatic contribution and cavity forming volume can have an impact, too, because ion association can occur. Assuming constant ion transport numbers and molecular weights the main parameter causing an IVP change is partial volume change, which is the sum of intrinsic and structural volume. Thus,

IVP is directly proportional to the hydration volume of the ion, shown for anions in Eq. (2) (Muller and Faude 2008).

$$\Delta IVP = \frac{c_m}{e} \rho_m \frac{t^-}{z^-} \Delta \overline{V}_0{}^- \quad (2)$$

where c_m is ultrasound velocity, e the unit charge, ρ_m the solution density, t the ion transport number, z the ion charge and $\overline{V}_0$ the hydration volume of the ion.

The great benefit of electro-acoustic is that its application is possible with salt concentrations up to maximal solubility of the salts which also covers all conditions used in HIC. This allows the comparison of salt IVP changes and chromatographic behaviour in HIC, which can help to elucidate retention or DBC phenomena unexplainable with chromatographic experiments only.

4.3.2.2 Colloid vibration current (CVI)

The IVP theory was further extended to colloidal systems. There ultrasonic sound waves stimulate the suspended colloids to vibrate. Thereby the colloids themselves and their surrounding electrical double layer fluctuate differently. This leads to induced dipoles, which can be measured as a colloid vibration current or potential. The higher the colloid mobility respective its EDL the higher the expected CVI would be. With Eq. (3) and (4) it can be derived, that the CVI for low frequency is a function of density differences of the particle and the solution ρ, volume fraction φ, pressure gradient P and the electrophoretic mobility μ. μ depends on particle electrokinetic potential ζ, medium properties like viscosity η or permittivity ε and a model dependent constant/term C (Dukhin, Shilov et al. 1999):

$$CVI_{\omega \to 0} = \mu \frac{\varphi(\rho_P - \rho_S)}{\rho_S} \nabla P \quad (3)$$

$$\mu = \frac{\varepsilon_0 \varepsilon_m \zeta}{\eta} C \qquad (4)$$

In this work the experimental procedures were adapted to investigate biochromatographic media. Thereby the colloids (resins) were measured no longer in suspension but in sedimented beds. Thus, the measured CVI is not entirely conforming to CVI theory described above. Here a pressure gradient is applied on the resin bed by ultrasound which creates a electrical current/potential. This current can be measured. The mechanism creating this current reminds more to a wet piezoelectric effect than to CVI and is not reported in literature yet. To keep the theoretic demands as simple as possible, in this work only unprocessed data are used and theoretic expansions were left outside. As explained above many parameters contribute to CVI and therefore it is a complex variable. Additionally in this approach the mechanical properties of the investigated materials like porosity, wettability and elasticity contribute to the measured values, too, which can make interpretation difficult. The aim of this part was to find a method to characterize batch to batch variations of chromatographic media to reduce long-winded, time and cost expensive procedures to fulfil authority demands regarding replacement of column packings in ongoing purification processes. The application of electro-acoustic pointed out this potential approach to describe unspecific protein binding properties of different batches with different resin types. The complexity of electro-acoustic holds the potential to investigate additional parameters of biochromatographic materials, which could be helpful for resin characterization and better understanding the protein adsorption mechanism.

5 Results and discussion

5.1 Fast determination of conditions for maximum dynamic binding capacity in cation-exchange chromatography of human monoclonal antibodies

(see also 6.1)

For the purification of monoclonal antibodies cation-exchange chromatography is a commonly used technique (Gagnon 1996). High capacities allow high concentration factors under favourable conditions and matrices are of good chemical stability and show long life times. For development of purification processes the dynamic binding capacity is the most important factor beneath purity for efficient process steps. Nonetheless the quest to find optimal conditions in CEX is still a trial and error approach and therefore time and cost expensive. The first aim of this work was to extent the understanding of the adsorption in CEX and to reduce development times of this process step with mAbs.

5.1.1 Dynamic binding capacity in CEX with mAbs

The chromatographic experiments were performed to show the impact of pH, buffer type and –concentration. DBC showed impressive pH dependent maxima. At neutral pH values DBC was low with tendency to increase to more acid pH values, caused by an increasing positive charge of the protein. This leads to stronger attractive forces between positive charged antibodies and negative charged resin. Further lowering pH values, DBC decreased again. Thereby protein surface net charge increased and repulsive forces between antibody molecules began to affect adsorption. On the one hand repulsive forces can inhibit protein uptake into the resin pore system slowing mass transfer, which leads to a decrease of DBC consider-

ing short residence time. On the other hand mAbs tend to adsorb on certain distances between each other, caused by high surface charge causing electrostatic repulsion. The pH position of the maximum DBC altered with the used buffer system. Used buffer salt were sodium acetate, sodium phosphate and potassium citrate. The position of maximum DBC was nearest neutral pH values with sodium acetate, at more acid pH values with sodium phosphate followed by potassium citrate. This could not be explained totally by different conductivities regarding the buffer salts. The different cations Na^+ and K^+ could partially explain the differences in the pH position of maximum DBC regarding the higher affinity of potassium to the negative resin charges, namely SO_3^-, which made it a stronger competitor for protein adsorption. Thus the buffer type affected DBC directly. Not only the buffer type but also the buffer concentration affected the maximum DBC. With higher buffer concentrations lower DBC could be achieved. This effect was described and modelled with the steric mass action model (SMA) already (Brooks, et al. 1992). The presence of more counter ions displaced the equilibrium constant to disadvantage of protein adsorption. Lowering the pH value protein adsorption and DBC could be improved by higher protein charge. Additionally the pH position of maximum DBC was affected by the buffer concentration. With increasing buffer concentration the pH position for maximum DBC decreased with all tested buffer types and both antibodies. The reason for this effects could be explained by the different buffer anions and is discussed in the next section.

5.1.2 Zeta potential measurements with mAbs

Zeta potential of proteins depends on pH. At acid pH values the zeta potential is positive changing to negative values with increasing pH. The isoionic point, the point of neutral zeta potential, indicates the electro neutral state where the protein does not move in an electric field. In pure water, the proteins are unaffected by ions and the isoionic points are about one

pH unit above the isoelectric points determined by isoelectric focussing. With increasing buffer concentration the isoionic points of the mAbs shift to more acid pH values in all tested buffer systems. This indicated protein bound negative charged buffer anions compensating positive protein surface charges. Therefore surface net charge decreases and more acid pH values (more H^+ ions) are required to reach the isoionic point. The downshift of the isoionic points was different regarding buffer type. Citrate had caused the greatest shift (5.5 pH units), followed by phosphate and acetate, 3.5 and 1.5 pH units respectively. This series reflects the valences of the ions and it matches the Hofmeister series, describing the efficiency of salts to precipitate proteins. Thus, the higher the ion charge and the higher its kosmotropic effect, the greater the extent of the downshift of the isoionic points. The molecular mechanism how the Hofmeister effect works is not entirely clear. On the one hand salts showing higher kosmotropic effect have higher Jones-Dole-B-coefficients, representing higher surface tension increments. The pH position of the isoionic point is independent from the surface tension increment, because viscosity and surface tension can not alter static states, which is the case at the isoionic point. On the other hand it was shown that kosmotropic ions have a greater tendency to interact with proteins directly (Chen, et al. 2003). This confirms the assumption that buffer anions can bind to protein surfaces and affect the pH position of the isoionic points. The effect that higher buffer concentrations lead to greater downshifts of isoionic points could also be explained by direct protein ion interaction. Following Le Chatelier's principle, the more buffer anions are present, the more buffer anions bind on the protein surface and more acid pH values are required to compensate the additional negative charges and the lower are the isoionic points. Thus protein – salt – interactions can be investigated via zeta potential measurements.

5.1.3 Correlation between DBC and zeta potential

In the last two sections results of dynamic binding capacity and zeta potential with two mAbs were worked out. These results are joined together in this paragraph. Buffer type and – concentration have the potential to lower the pH position of maximum DBCs as well as of the isoionic points. Comparing the extent of these downshifts for the tested buffer systems, - concentrations and mAbs, a strong correlation was observed. Both downshifts have the same extent. Using pure buffers, the ratio of buffer cations and - anions is nearly one – by – one. Thus, the buffer cations can affect the pH position of maximum DBC in the same extent than the buffer anions the pH position of the isoionic points. For protein adsorption resin bound cations have to be dislocated as well as protein bound anions. This explains the similar behaviour of the buffer type and – concentration on the pH position of maximum DBC and isoionic points. With higher buffer concentrations the downshift of the pH position of maximum DBC and isoionic point drift apart in the same extent for all buffers and resins. This could be caused by smaller ion binding capacity of the protein compared to the resin. Considering these phenomena, a method to determine the conditions for maximum DBC in CEX with mAbs and without any chromatographic experiments could be deduced and based only on zeta potential measurements shown in Eq. (5):

$$pH_{b,M,\max DBC} = pH_{isionicpo\,\mathrm{int},b,M} \pm (I_{b,r} + 3.5M) \qquad (5)$$

where $pH_{b,\,M,\,max.\,DBC}$ is the pH value for maximum DBC and $pH_{isoionic\ point,\,b,\,M}$ is the isoionic point in the chosen buffer type b and molarity M (M). The sign of the operator depends on the buffer type as well as $I_{b,\,r}$, representing the intercept value for buffer b and resin r, as listed in Tab. 2.

Tab. 2: Intercept values $I_{b, r}$ for the tested buffer types and resins for the calculation of the pH value for maximum DBC in CEX with mAbs.

		EMD SE Hicap	EMD SO_3^-	SP Sepharose XL	Toyopearl Prototype S
Buffer type	Sign		$I_{b, r}$		
Sodium acetate	-	1.2	1.2	1.7	1.7
Sodium phosphate	-	0.5	0.5	1.0	1.0
Potassium citrate	+	1.2	1.2	1.7	1.7

For the sodium salts the pH position of maximum DBC runs lower than of the isoionic points. For potassium citrate it is contrariwise, so the summand has to be added (sign +). The $I_{b, r}$ values listed in Tab. 2 show same values for the EMD gels, which have only different spacers between base matrix and functional groups. The Toyopearl is based on the same matrix but grafting and spacer chemistry are not known. The Sepharose gel is completely different in base matrix and of different grafting types than the EMD resins. Thus, the resin chemistry seems to have an effect on the pH position of maximum DBC. EMD resins show their DBC maximum at more neutral pH values than the other two resin types.

5.1.4 Conclusions

In this part the correlation between maximum DBC and zeta potential was worked out related to CEX with mAbs. Using the developed method to determine conditions for maximum DBC time, material and manpower could be saved in the development of purification processes. There efficiency and development time became more and more important driven by the high

amount of used mAbs for medication and new developed mAbs, which all have to be purified. To summarize the procedure it is shown in three (a-c) steps:

(a) Starting with the mAb of interest, one of the tested buffer types and the buffer concentration have to be chosen.

(b) Then the isoionic point has to be measured. If the isoionic point shifts with increasing buffer concentrations up to 100 mM, specific protein ion interactions are negligible

(c) The final step is the calculation of the needed pH value for maximum DBC using the determined isoionic point and the $I_{b,r}$ value regarding to the used buffer and resin (Tab. 2) using Eq. (3).

Using this new approach the use of chromatographic experiments for determination of maximum DBC can be reduced to one, which is still needed to estimate the value of maximum DBC. The long-winded search by chromatographic experiments could be replaced by fast zeta potential measurements using only small amounts of protein. Additionally the electrokinetic data could be used to draw conclusions regarding protein stability (Derjaguin, et al. 1941; Faude 2007). To establish a general rule, the data body has to be extended with more mAbs, buffers and resins to verify the method.

5.2 Investigation of salt properties with electro-acoustic measurements and their effect on dynamic binding capacity in hydrophobic interaction chromatography

(see also 6.2)

Beneath cation-exchange chromatography hydrophobic interaction chromatography is a powerful technique to purify monoclonal antibodies. The great benefit of this method is its application to separate antibody aggregates. The adsorption mechanism in HIC is less understood

compared to CEX and additionally the parameter salt type is more important caused by the extreme high salt concentrations used. Thus, the next aim of this work was to investigate salt properties extensively and their effect on dynamic binding capacity in HIC.

5.2.1 IVP of salts

The IVP of all salts show a concentration dependency. With increasing salt concentration IVP increases reaching a plateau at higher values than 0.1 M. Comparing different chloride salts, IVP shows direct proportionality to the molecular mass of the cations. IVP increases with increasing cation molecular mass from Li^+ to Na^+, K^+ and Cs^+ showing the highest values. This series matches the cation arrangement in the Hofmeister series, showing Li^+ as most and Cs^+ as least protein stabilizing ion. The comparison of different sodium salts shows differences in IVP, too. Their IVP signals do not hit the Hofmeister series totally. IVP decreases in the order of acetate > chloride = citrate > iodide > sulphate > carbonate = dihydrogenphosphate > bromide > perchlorate. This also does not reflect differences in molecular mass of the anions. Acetate, chloride and citrate have similar IVP signals but have great differences in molecular weight. Excluding iodide and chloride, accordingly to the Hofmeister series structure making ions like sulphate and citrate show high IVP and structure breaking ions like perchlorate show low IVP. This is according to IVP theory, an increase in partial molar anion volume increases IVP. Including all measured data into the discussion, an alternative hypothesis can be involved that ions only affect the first few hydration layers and ions neither enhance nor weaken the hydrogen bond network in bulk solutions. This was shown for short time scales by femtosecond pump spectroscopy (Omta, Kropman et al. 2003). If this holds true for IVP, the values might reflect ion specific water – ion or ion – ion interactions and can not be classified like Hofmeister interactions. Additionally in the work of Hofmeister and following works the pH effect was mostly neglected. This contributes to the differences be-

tween IVP and Hofmeister orderings, because IVP is a function of pH. IVP titration experiments with salts showed a pH dependent maximum with up to 20% higher values than the starting values. The pH position of this maximum was proven by a minimum in conductivity at the same pH, whereby conductivity measurements were near the detection limit for as little as 50 mM salt concentration and IVP values were quiet significant up to 5 M salt. The complimentary behaviour of IVP and conductivity is explained in the theories for salt solutions. Conductivities reflect the sum of ion mobility properties which are inverse to the size of the ions in solution (IVP). IVP maxima should be a result of increasing partial molar ion volume which can be caused by an increasing water shell (structural volume) or ion association. Maxima appeared with all tested salts at different pH and depend on the salt concentration. The pH position of IVP maxima increases with increasing salt concentration. The pH range where IVP maxima were detected is between pH 4.5 and 6 for low salt concentrations and between 6 and 8 for high salt concentrations.

5.2.2 Correlation of electro-acoustic measurements with dynamic binding capacity in HIC

Dynamic binding capacities of the Toyopearl Butyl-650M HIC resin were estimated under different salt and pH conditions with lysozyme, bovine serum albumin and a humanized monoclonal antibody. With most salts DBC shows maxima or at least plateaus in the range of pH 6.5 to pH 8. This phenomenon was unexpected because protein isoelectric points are quiet different (BSA pI = 4.7, lysozyme pI = 11.6 and mAb pI = 7.5 to 8.5). It is reported in literature that protein precipitation occurs first near protein pI, which often was transferred to retention in HIC. But the adsorption process under high loading conditions on a HIC resin differs to precipitation in solution. Comparing the pH positions of IVP and DBC maxima a correlation was observed. In Tab 3 the maximum deviations of IVP and DBC maxima is shown for

mostly used salts in HIC. They were calculated for each anion as the arithmetic mean for all three proteins and except for citrate the deviations are in the range of experimental resolution.

Tab. 3: Deviation from experimental maximum IVP values to the point of maximum DBC in HIC

Salt	ΔpH fro IVP_{max} to DBC_{max}
Chloride	0.5
Sulphate	0.5
Acetate	0.25
Citrate	1.33

At a first glance maximum in DBC and salt solution seem unlikely to be related. But the adsorption process is a sum of interactions between surfaces of resin and protein and mass transfer of protein into the resin pores. For high capacities the transport into the pores contributes to a great extent to DBC values considering short residence times. Considering strong protein ligand interaction, mass transfer could be handicapped by protein blocked pores. The scaled particle theory can help to enlighten the relation of DBC and salt properties. This theory is s statistical treatment considering the nonideality of a solution given by a cosolute. It describes the reversible work, which is necessary to create a spherical cavity for a single protein molecule in a solution containing different hard sphere species (water and salt ions) (Lebowitz 1965). Following the scaled particle theory a maximum protein stabilizing effect occurs if cosolutes achieve 1.8 times the volume of water molecules. Sulphate has a more stabilizing effect than chloride (Arakawa and Timasheff 1984). This is in accordance with the scaled particle theory, because sulphate has an ion radius of 300 pm and chloride one of 195 pm. Comparing with water, which has an ion radius of 155 pm, sulphate radius is closer to 1.8

times water radius than chloride. Changing the cosolute sizes by altered water shell or ion association, the stabilizing effect can be de- or increased. To apply this effect on DBC in HIC, more stabilized proteins can enter the resin pores less handicapped by blocked pores resulting in higher DBC values. Regarding the big ion size of citrate (double of sulphate), the most protein stabilizing effect and DBC maximum with this ion must be above the IVP maximum, where the ion size is smaller again. Maximum DBC with citrate can not be under the IVP maximum because citrate is not fully deprotonated there and can not find its complete expression to provide protein adsorption. This can explain the deviation of more than one pH unit for citrate regarding DBC and IVP maximum.

5.2.3 Conclusion

The higher degree of solvation or ion association detected by electro-acoustic measurements in the pH range of pH 6 to pH 8 can be the reason for DBC maxima of HIC resins in this pH range described by Chen (Chen, Tetrault et al. 2008), too. The benefit of this electro-acoustic approach could be the estimation of salt ion sizes and therefore it can help to find conditions for protein stabilization and high DBC in HIC. Potentially this approach can be extended to predict optimal conditions with salt mixtures, too.

5.3 A novel approach for detection of biochromatography resin batch to batch variations

(See also 6.3)

In ongoing purification processes of biopharmaceuticals, the resin packings of chromatographic columns have to be replaced after certain process cycles when their life time is reached. Therefore batch to batch variations, which are not included in certificates of analysis, can become crucial if they alter product quality or yield significantly. Product identity has to

be proven with replaced column packings, which requires extensive pre – testing of potential new resin batches. The reduction of pre – tests would save time, material and manpower, which always are critical parameters for pharmaceutical companies. Hence, the aim of the work in this section was to find a fast method for resin characterization allowing a preselection of potential new resin batches for ongoing purification processes.

Here several batches of different resin types were investigated via electro-acoustic and chromatography. Correlations could be used to show the real life significance of electro-acoustic detected differences between resin batches.

5.3.1 Electro-acoustic

Several batches of three different sulfo – functionalized resins of different manufacturers and batches of C4 – silica resin were investigated via electro-acoustic. Differences between base materials (sepharose, methacrylate, silica) and functionalization chemistry regarding the spacer arm between base particle and functional group raised the use of different solvents to achieve reproducible results. Additionally the required resin sample volume alters regarding different resin base materials as shown in Tab. 4.

Tab. 4: Experimental sets for electro-acoustic measurements

	Methacrylate MA	Methacrylate MB	Methacrylate MG	Sepharose	Silica
Solvent	ethanol	water	water	water	acetone
Volume fraction (resin/solvent)	26 % (v/v)	26 % (v/v)	26 % (v/v)	52 % (v/v)	20 % (w/v)

Reliable results were achieved with sulfo – methacrylate resin of manufacturer A (MA) using ethanol only. Methacrylate MB, MG and sepharose (Se) resins were successfully measured in water. The Silica particles required acetone as solvent because the wettability was not sufficient with water or ethanol. Irregular results with other solvents can be explained by particle whirling, which can be observed with regarding solvents and particles using a microscope. The different particle behaviour in different solvents could be caused by variable base materials and/or spacer arm chemistries regarding length and structure, which are not published exactly by the manufacturers and therefore can not be interpreted. Furthermore the required volume fraction needed for measurements increases from silica over methacrylate to sepharose reflecting decreasing particle rigidness and increasing particle size. Assuming that denser packing by smaller and harder particles attenuate soundwaves more, lower sediment would be required to decouple sediment height from CVI signal and reach signal saturation. The resulted CVI signals of the different resins and batches showed significant variations for all tested resin types. . The highest difference was detected for the MA (30 %), followed by Si (20 %), MG (19 %), MB (18 %) and Se (17 %) resins. However, caused by the small data body an evaluation of the resin reproducibility might not be representative.

5.3.2 Chromatography

The sulfo – resins and batches shown above were also investigated by lysozyme pulses under isocratic high salt conditions bringing out the actual retention factors. These conditions were totally unusual for ion exchange resins (sulfo – resins) but allow to mark out unspecific protein binding properties and residual hydrophobicity. With this approach, batch to batch variations were detected for all resin types, too. Differences in retention factor with Se resins were up to 11 %, with MA up to 13 %, MB up to 15 % and with MG resins up to 17 %. As ex-

plained above, the assessment of resin reproducibility could not be done caused by the small data body.

Silica resin batches were evaluated using three performance parameters, namely life time, bed quality and bulk properties. These parameters were kindly provided by an industrial cooperation partner. Out of these data a performance score was established containing ten to thirty (thirty = best) points regarding the three parameters. The four investigated silica batches differ in all three categories, resulting in performance scores of 90, 70, 40 and 30, which leads to differences process costs (life time), resolution potential (bed quality) and binding capacity (bulk properties like specific resin volume).

5.3.3 Correlation between electro-acoustic measurements and resin properties

Batch to batch variances were worked out with electro-acoustic and chromatographic approaches and joined in this section. Methacrylate MA batches show increasing retention factors with decreasing CVI values. These resins were measured in ethanol. Residual carboxy groups on the particle surface left by synthesis contribute to CVI signal beneath the sulfo-groups. The more of these extra charged groups the higher is the CVI signal and the lower is the residual hydrophobicity of the resin caused by extra hydrophilic (charged carboxy) groups.

Methacrylate MB, MG and sepharose (Se) resins were measured in water. These resin types show increasing retention factors with increasing CVI values. It is reported that on water - hydrophobic interphases hydroxyl ions adsorb spontaneously. Therefore OH^- ions can adsorb at hydrophobic patches on the MB, MG and sepharose particle surface, which could be the aliphatic spacer arm between base material and functional sulfo-group. Thus, not only

charged SO_3^- but also charged OH^- ions contribute to CVI signals and indicate more hydrophobic areas/patches which cause higher retention factors.

Silica resins show increasing CVI values with increasing performance scores. In this case the main parameter for the correlation could be the wettability of the very hydrophobic C4 functionalized particles. Lacking signal transmitting medium, the CVI signal could be diminished. In Chromatography incompletely moistened particles can cause difficulties in column packing, which can lead to uneven beds, peak broadening, high specific resin volume and enhanced fouling tendency. The balance between possibly residual silanol groups and C4 ligand density can alter the wettability of the particles and therefore the packing performance. Additionally the wettability problem could be even enhanced by altering the packing solvent from nonpolar to more polar solvents.

5.3.4 Conclusions

In this part of the work differences between resin batches were worked out for several resin types by classic chromatographic and novel electro-acoustic methods. The electro-acoustic method is fast, requires low amounts of material and does not depend on column packing quality. Additionally it is more sensitive than the chromatographic approach. So it could be a preselecting tool to reduce time and cost expensive chromatographic tests to guarantee product identity replacing column packing material in ongoing purification processes. Nevertheless, the small data body has to be extended for efficiency proof of the described method. Furthermore the relevance of the electro-acoustic results depends on the particular separation problem/process and should be proven as the case arises. The plurality of material properties contributing to CVI signal and the lack of an appropriate theory can cause difficulties in interpretation but holds the potential to cover a wide range of applications.

6 Publications

6.1 Fast determination of conditions for maximum dynamic binding capacity in cation-exchange chromatography of human monoclonal antibodies

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6.1.1 Abstract

Dynamic binding capacity (DBC) measurements of cation-exchange resins were performed with two human monoclonal antibodies. DBC showed a pH dependent maximum, which was shifted to lower pH values with increasing buffer concentrations and increasing salting-out effect of the buffer anion according to the Hofmeister series. As this downshift correlates well with zeta potential values, a measurement of the latter allows the determination of the pH value for maximum DBC under a given set of conditions. Thus, the use of zeta potential values can accelerate the purification process development and helps to understand the protein adsorption mechanism.

Keywords: Cation-exchange chromatography; human monoclonal antibodies; dynamic binding capacity; Hofmeister series; zeta potential; purification process development

6.1.2 Introduction

For the purification of human monoclonal antibodies (hmAbs) cation-exchange chromatography (CEX) is a widely used and powerful procedure [1, 2, 3]. High capacities allow high concentration factors under mild conditions, preserving product quality. Available matrices have a good chemical stability and a long lifetime [4]. CEX is based on the discriminative adsorption of positively charged proteins on negatively charged porous chromatographic media. Performance depends on the proteins themselves, the matrix and conditions like buffer type, ionic strength and pH value. Often buffer type and ionic strength are fixed for instance in capture steps or due to relevant guidelines. The pH value plays an important role and has to be optimized in each single case, because it directly affects the surface net charge of the respective protein. Such parameters determine the adsorption constants and capacity of the CEX media.

Due to the growing demands for production of high amounts of therapeutic human monoclonal antibodies the dynamic binding capacity is of utmost importance for reasons of efficient purification processes. The complex adsorption mechanism was investigated and modelled in different approaches [5, 6, 7, 8, 9, 10, 11, 12] to better understand and even to predict adsorption behaviour. Parameters used in these works were protein surface net charge, chromatographic media, mass transfer, pH value and ionic strength, whereby they focused on cations, either neglecting salt anions or describing negligible anion effects. Hearn et al. [13] divided adsorption into five subprocesses: (a) exclusion of water molecules or ions surrounding the protein, removing the electrical double layer (EDL); (b) exclusion of water molecules or ions surrounding the resin; (c) direct interactions between the protein and the resin; (d) structural rearrangement of the protein upon adsorption; and (e) structural rearrangement of the excluded water molecules and ions in the bulk solvent.

According to point (a) we investigated the zeta potential for the description of the interactions between proteins and salt/buffer ions. Proteins dissolved in a buffer have a pH dependent surface net charge, so (counter-) ions bind to the surface and an electrical double layer is formed around the protein. The inner region of the liquid layer, called the Stern layer, consists of strongly bound ions, whereas in an outer region the ions are less firmly attached. Within this outer region there is a notional boundary. In an applied electric field, the protein moves and the ions located outside of this boundary are sheared off. This boundary is called the slipping plane or the surface of hydrodynamic shear. The potential that exists at this boundary is known as the zeta potential. Thus the zeta potential is a dynamic value suitable to moving proteins in chromatographic systems, where protein solution flows and the protein transport into the resin pores is partially electrophoretically driven [14]. Zeta potential values are also used to gather information regarding the potential stability of colloidal systems. If all particles in a system have the same high zeta potential, they tend to repel each other and flocculation should be minimal according to the DLVO theory [15]. In connection to chromatography the zeta potential was used to describe charge compensation of ion exchange matrices by lysozyme adsorption so far [16, 17]. It was used to investigate the relation between protein surface net charge and membrane adsorption, even its computation was performed [18, 19], but both approaches neglect protein bound salt ions.

In the present work protein DBC values with hmAbs in cation-exchange chromatography were determined at different pH values, buffer types and buffer concentrations. The buffers used are proper for a pH range from 3.5 to 7.5 and should be therefore usable for CEX with most hmAbs. These experiments pointed out the existence of a pH dependent maximum in DBC [18, 20]. For the efficiency of purification processes the DBC is one of the central points beneath selectivity. The correlation between the pH position of maximum DBC and the points of zero zeta potential, the isoionic points, punctuate the impact of protein bound ions in the

protein adsorption mechanism, because the zeta potential of proteins involves protein bound salt ions. Accordingly, the use of the zeta potential may be helpful to accelerate the development of purification processes.

6.1.3 Material and methods

The experiments reported here studied zeta potential and the maximum dynamic binding capacity of two human monoclonal antibodies on a variety of commercial cation-exchange matrices, namely Fractogel EMD SO_3^-, Fractogel EMD SE Hicap (both from Merck, Darmstadt, Germany), SP Sepharose XL (GE Healthcare, Chalfont St Giles, UK) and an experimental Toyopearl Prototype S (Tosoh Biosciences, Tokyo, Japan). Antibodies were provided by Boehringer Ingelheim (Biberach, Germany) at purities greater 99.99 %, as detected by ELISA. All chemicals were obtained from Carl Roth (Karlsruhe, Germany). Solutions were filtered through Whatman filter with a pore size of 0.22 microns. All experiments were done at room temperature (25 °C).

6.1.3.1 Chromatography

Chromatographic experiments were performed on an Äkta Purifier System (GE Healthcare) with Omnifit (msscientific, Berlin, Germany) columns of 10 mm I.D. and 20 mm bed length. The flow rate was 300 cm/h and the sample antibody concentration was 2 g/l. The resins were equilibrated with 10 column volumes (CVs) of the loading buffer. Breakthrough points were determined at 1 % leakage relative to sample UV absorption at 280 nm. Dynamic binding capacities were calculated by Eq. (1),

$$DBC = (V_P - V_0)c_P/V_R \quad (1)$$

where V_P = volume of protein loaded at 1% breakthrough point, V_0 = void volume of the system, c_P = antibody concentration and V_R = volume of packed resin. DBC values were determined for both antibodies in sodium phosphate, sodium acetate and potassium citrate buffers at several pH values and buffer concentrations for each resin. The standard deviation for capacities was determined to be 5% out of threefold experiments. The resins were regenerated with 4 CVs of 2.0 M NaCl, 0.5 M NaOH, 0.1 M HCl and stored in 20 % ethanol.

6.1.3.2 Zeta potential

For the measurement of the zeta potential the electrophoretic mobilities of the proteins were determined by laser Doppler velocimetry using a Zetasizer ZS Nano (Malvern, Malvern, UK) and dedicated disposable cells. The zeta potential was calculated by Henry's Eq. (2),

$$U_e = 2\varepsilon k\zeta / 3\eta \qquad (2)$$

where U_e = electrophoretic mobility, ζ = zeta potential of the protein, ε = dielectric constant, η = viscosity of the solution and k = 1.5 (model based constant for salt concentrations higher 1 mM, Smoluchowski 1921).

For sample preparation the hmAbs were brought up to the appropriate buffer by centrifugal ultrafiltration (Vivaspin, Sartorius, Göttingen, Germany), the protein was adjusted to 0.5 g/l and to the respective pH value. Buffer solutions (sodium chloride, sodium acetate, sodium phosphate, potassium citrate) were prepared at the most acid pH value needed and then titrated to the other pH values with the appropriate base to minimize conductivity. No further salts were added in order to prevent side effects. Isoionic points were calculated by linear regression of the sign change surrounding values. Each measurement was repeated threefold and average values are reported. The standard deviation for zeta potential values was 10 %.

6.1.4 Results and discussion

6.1.4.1 Dynamic binding capacity

The aim of the chromatographic experiments was to study the impact of pH value, buffer type and concentration on the adsorption of human monoclonal antibodies, representing rather large proteins, on cation-exchange matrices in dynamic systems. For Fractogel EMD SE Hi-cap, a strong and grafted cation-exchanger, the dynamic binding capacities of the hmAbs show a pH dependent maximum (Fig. 1).

The increase of binding capacity from neutral pH values to the values of DBC maximum is caused by the more positive charge of hmab at lower pH values. This results in stronger attractive forces between positively charged antibodies and the negatively charged matrix. At more acid pH values the DBC decreases. Under these conditions, the antibody charge is so high, that repulsive forces between antibody molecules affect the adsorption on the matrix. The pore sizes of commonly used grafted resins are about 10 nm [5, 21]. Thus, repulsive forces between antibody molecules of 5 to 10 nm size [22] can inhibit the protein uptake into the chromatographic particles by ion exclusion [2, 20]. This leads to a slower transport which causes decreasing DBC considering short residence times. Furthermore the adsorption on the matrix at high protein charges could be handicapped, because the proteins tend to stay at a certain distance from each other [18, 23].

The pH position of the DBC maxima changes with the used buffer type. However, although the conductivity of citrate buffers is higher compared to phosphate or acetate buffers, the pH values of the respective DBC maxima for each buffer type were similar (Table 1), due to higher conductivities at higher pH values. So this extent of pH displacements of DBC maxima by variation of the buffer cannot be explained completely by the variation of conductivity [20]. It can be deduced, therefore, that the buffer type affects DBC. The buffer cations, Na^+ or

K^+, affect the adsorption of protein on cation-exchangers to a different extent. Equilibrated resins contain bound counter ions. Described by the SMA model [9, 10] the ion exchanger must release adsorbed counter ions for protein adsorption. Due to the model the nature of the salts can alter the equilibrium constant [11]. K^+ ions are large compared to Na^+ ions and will orientate less water molecules leaving the resin. Thus, for proteins K^+ is a stronger competitor than Na^+ in order to adsorb on the resin. This could explain partially the necessary higher surface net charge of the hmab for maximum DBC in potassium citrate buffer achieved with lower pH values and the lower amount of maximum DBC as well.

Table 1 Conductivities (mS/cm) of the used buffers (50 mM) at relevant pH values. The conductivities at the pH values of maximum DBC are similar.

pH	Conductivities (mS/cm)		
	Sodium acetate	Sodium phosphate	Potassium citrate
4,5	2,1	3,4	5,9
5,0	3,2	3,5	6,7
5,5	3,5	3,7	7,8
6,0	3,6	3,9	

The influence of the buffer concentration on maximum DBC is shown in Fig. 2. An increase in buffer concentration leads to a reduction of DBC. The existence of more cations displaces the adsorption equilibrium constant [10, 11, 24, 25] to the disadvantage of protein adsorption. Lower pH values counteract this by higher protein surface net charge and therewith the DBC can be improved. Exceeding the maximum, the DBC decrease is caused by mechanisms explained above. So the pH position of maximum DBC decreases with an increase of the buffer concentration. This behaviour is common for all tested buffer types and concentrations.

The impact of buffer anions was discussed by some groups [12, 13, 26, 27] and this work discusses this topic in the next subsection.

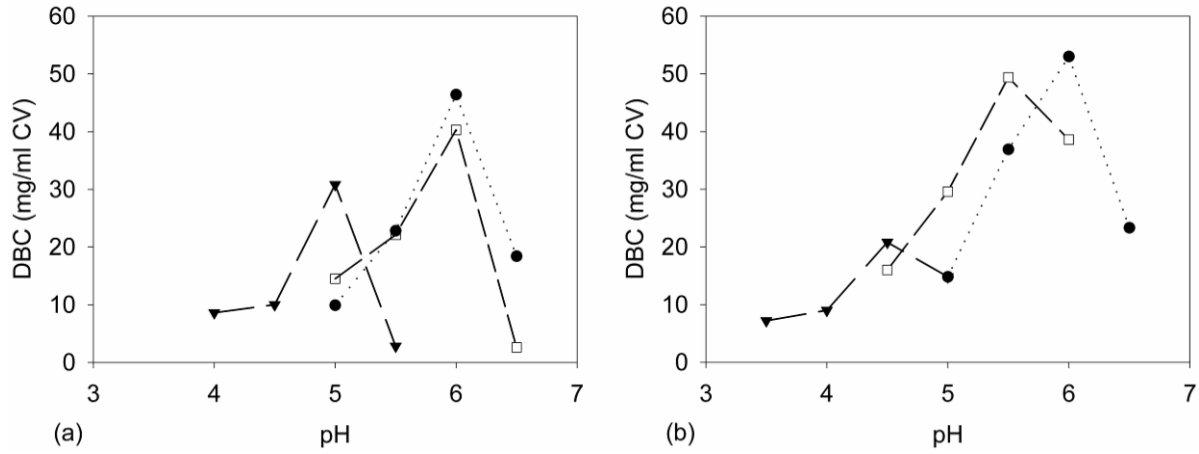

Fig. 1. DBC of Fractogel EMD SE Hicap for (a) hmAb1 and (b) hmAb2 in different 50 mM buffers, displaying pH dependent DBC maxima (connecting lines used as eye guiding lines). Furthermore the pH position of maximum DBC depends on the buffer system. (●) sodium acetate, (□) sodium phosphate, (▼) potassium citrate.

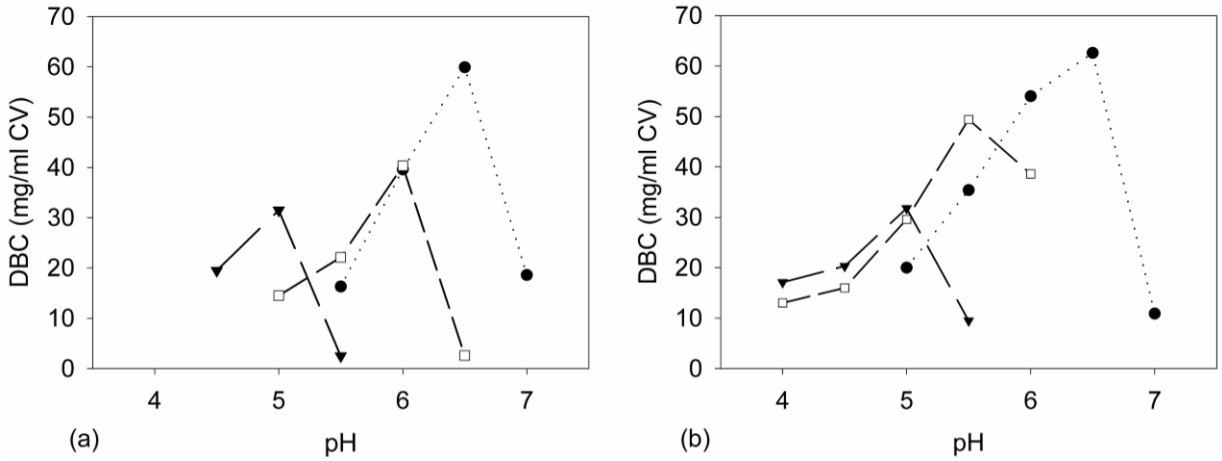

Fig. 2. DBC of Fractogel EMD SE of (a) hmAb1 and (b) hmAb2 at different sodium phosphate concentrations (connecting lines used as eye guiding lines). The pH position of maximum DBC decreases with increasing buffer concentration. (●) 20 mM, (□) 50 mM, (▼) 100 mM.

6.1.4.2 Zeta potential

Fig. 3 shows the zeta potential titration curves of hmab1 and hmab2 and their respective dependency on the pH values. With an increase of the pH value for all buffer concentrations

used, the zeta potential decreases, moving from the positive into the negative range similar as observed for protein titration curves [16, 18]. The isoionic point, the point of zero zeta potential, indicates the electro neutral state of the moving protein. Unexpectedly, the isoionic point is not identical with the isoelectric point (pH 8.5 for hmab1, pH 8.0 for hmab2) as determined by isoelectric focussing, but is rather located at more basic pH values (9.3 for hmab1 and 8.4 for hmab2). In pure water the dissolved protein is unaffected by ions, except those deriving from dissociated water. The shift from pH 8.5 to 9.3 (hmab1) is unlikely caused by adsorbed protons due to the high pH values. Adsorbed hydroxide anions, on the other side, would lower the isoionic point. However, buffer ions clearly affect the zeta potential of the protein. With increasing buffer concentration the isoionic points shifts to lower pH values and in parallel the slope of the zeta potential titration curves increases. Ions like phosphate can bind to the antibody [28, 29] and with increased concentrations more phosphate binds following Le Châtelier's principle. Bound phosphate ions then compensate positive charges of the protein. Therefore the surface net charge decreases and more acid conditions are needed to reach the isoionic point. Further this indicates a negligible binding of monovalent cations to the proteins [26, 28]. The slope of the zeta potential titration curves increases at higher ionic strength. This could be explained regarding Eq. (2). Higher ionic strength increases the permittivity of the solution, therewith the modulus of the zeta potential decreases whereas the slope of the titration curves increases. This behaviour can be observed with both hmAbs and in all tested buffers, as exemplary shown for sodium phosphate buffer (Fig. 3).

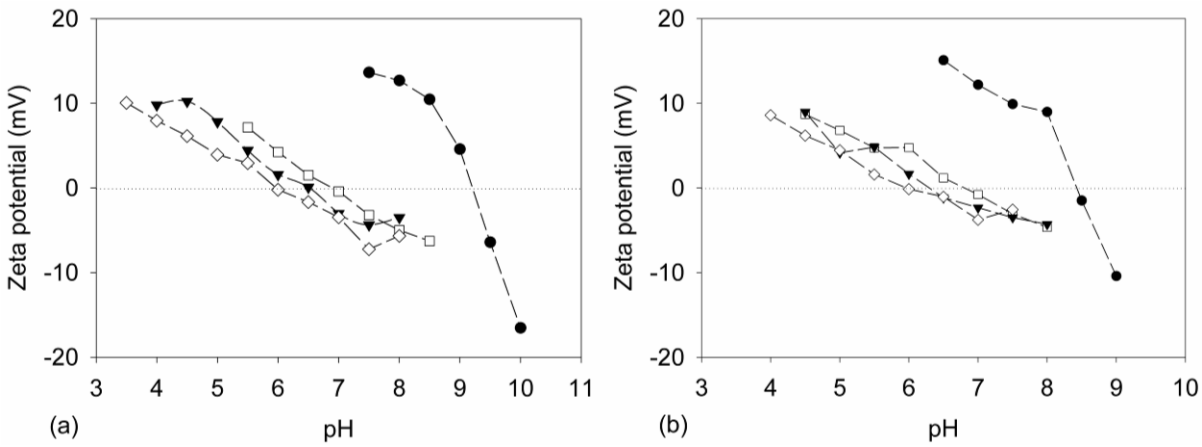

Fig. 3. Zeta potential titration curves of (a) hmAb1 and (b) hmAb2 at different sodium phosphate concentrations (connecting lines used as eye guiding lines). The zeta potential modulus and pH positions of the isoionic points decrease with increasing phosphate concentrations. (●)0 mM, (□) 20 mM, (▼) 50 mM, (◊) 100mM.

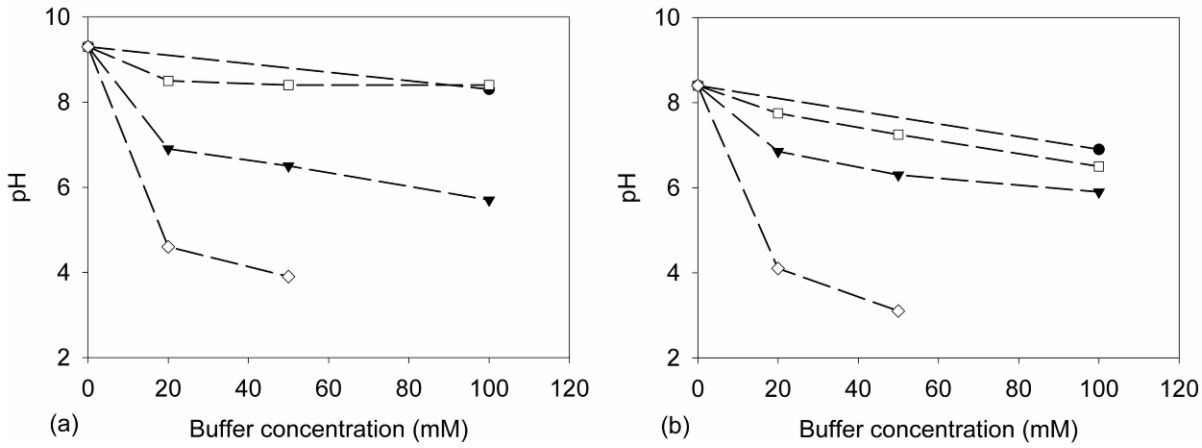

Fig. 4. Incrementally decrease of isoionic points of (a) hmAb1 and (b) hmAb2 with increase of kosmotropic character of the buffer anions (connecting lines used as eye guiding lines). (●) sodium chloride, (□) sodium acetate, (▼) sodium phosphate, (◊) potassium citrate.

The pH position of the isoionic points of both hmAbs are shown in Fig. 4. For potassium citrate the value at 100 mM could not be determined due to protein aggregation at such low pH values. The ability of citrate to shift isoionic points by several pH units is already reported for inorganic colloids [30, 31]. The values in 100 mM sodium chloride are non buffer references.

The decrease of the pH positions of the isoionic points depends on the buffer type. The extent of the downshift of the isoionic points with increasing buffer concentrations forms a series, citrate with greatest and chloride with least impact, listed as citrate > phosphate > acetate > chloride. On the one hand, this series reflects the valences of the ions. Chloride has a single negative charge, acetate up to one, phosphate and citrate carry up to three negative charges, dependent on the pH value. Thus, it is obvious that citrate could affect the zeta potential more strongly compared to chloride because one bound citrate can compensate more charges of the protein. For hmab1 the isoionic point was determined at pH 4.6 in 20 mM citrate buffer and at 6.9 in 20 mM phosphate buffer. For both anions these pH values were near their pK_{a2} values, so nearly the same concentrations of one- and twofold charged forms are present. Taking this into consideration, the ion valence is not the single important factor. On the other hand, the series above hits the Hofmeister series [32, 33, 34] for salts affecting the solubility of proteins, reflecting the Jones-Dole *B*-coefficients [35] and polarisability of ions [36]. The more the present ions shift the isoionic points of hmAbs to lower pH values, the higher is their salting-out effect according to the Hofmeister series. The molecular mechanisms determining the Hofmeister series are not entirely clear, but two effects are described. First, salting-out ions increase water surface tension and therefore the hydrophobic interactions. Second, the direct interaction of salting-in ions and proteins stabilize protein structure. The border from salting-in to salting-out ions and their effects is floating. With reference to the first point, the surface tension does not modify the point of zero zeta potential (Eq. 2). However, the correlation of the Hofmeister series and the extent of the downshift of isoionic points can be explained by the second point, direct binding of ions to proteins. Supposing that ions with higher salting-out effect, higher kosmotropic character, can be bound in a higher amount by proteins than ions with lower salting-out effect, more positive charges of the protein can be compensated and therefore the isoionic point shifts to a lower pH value. A correlation between the molar

surface tension increment of salt and the tendencies of ions for direct binding to proteins was found by Chen et al. [37] substantiating this assumption. In their work a salt coefficient was described, which refers to the number of salt molecules participating in the dehydration of proteins. This coefficient also is considered as the affinity of the salt to bind to proteins and correlates to molar surface tension increments. Thus, salts with higher molar surface tension increments will bind to proteins in higher amounts and vice versa. The Hofmeister series and the molar surface tension increment series do not match perfectly. This could be explained by protein specific interactions with salts, whereby the establishment of general rules like the Hofmeister series is warped [27, 38, 39]. Though for salts used in this work both series conform to our results. Merely the effect of sodium acetate on hmab1 shows an unexpected shape, which also adverts to specific ion-protein interaction diminishing acetate binding. The curve shapes in Fig. 4 resemble to exponential decay curves. This indicates a reduced increment of ion binding to the hmAbs by constant increase of the salt concentration implying that saturation [33] could occur. Unfortunately zeta potential measurements at such high conductivities can not be done, because the voltage has to be low to prevent high currents and thereby the electrophoretic mobility is too low for reliable measurements. But for all that it would be interesting to determine the ion saturation point of a protein, it is conceivable that there the protein starts to precipitate.

6.1.4.3 Correlation between dynamic binding capacity and zeta potential

In the last sections we worked out results about zeta potentials and dynamic binding capacities in CEX of human monoclonal antibodies. These results are joined in this chapter. Buffer anions like acetate, phosphate and citrate have the potential to affect the pH position of maximum DBC as well as buffer cations. Fig. 5 shows the downshift of the pH position of

maximum DBC and isoionic point of both hmAbs caused by an increase in buffer concentration.

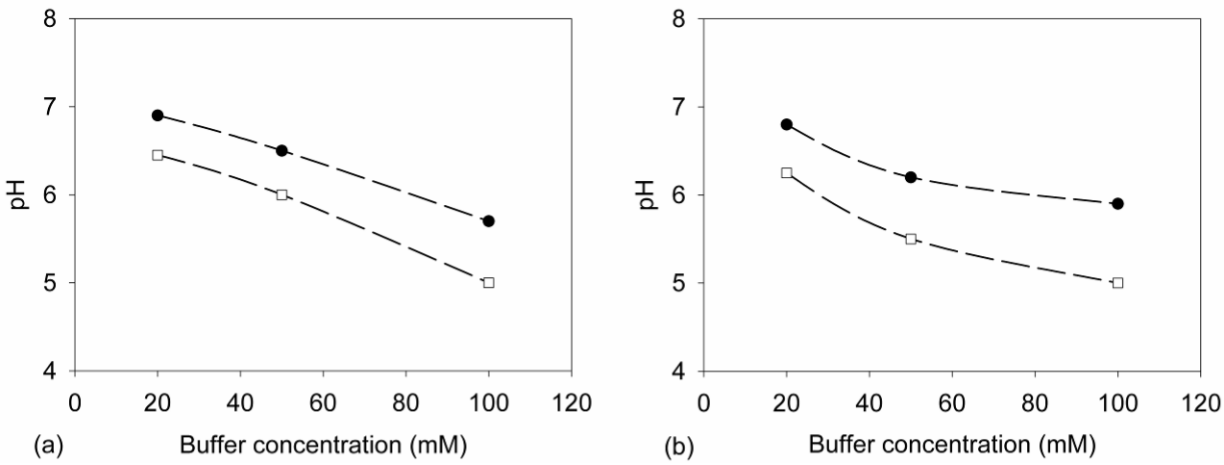

Fig. 5. Correlation between maximum DBC and isoionic points of (a) hmAb1 and (b) hmAb2 (connecting lines used as eye guiding lines). The difference between the curves behaves similar for both antibodies in phosphate buffer regarding to Fractogel EMD SE Hicap. (●) isoionic points, (□) maximum DBC.

The shape of the curves depends on the protein. For hmab1 both curves show a nearly linear decrease, for hmab2 the decrease of both curves flattens. As described above higher protein net charge compensates higher buffer cation concentration for maximum DBC and the protein bound buffer anions for isoionic points as well. The usage of pure buffers comprises nearly a one to one ratio of buffer anions and cations. Thus, the buffer anions can affect the pH position of isoionic points in the same dimension as the buffer cations the pH position of maximum DBC. Due to subprocesses in chromatography with proteins [13, 24, 26, 40, 41] ions bound at the resin have to be dislocated as well as ions bound to the protein and/or hamper the adsorption process [41, 42]. This explains the similar decrease of both values by increasing buffer concentrations, but the curves drift apart. A reason for this could be that the ion binding capacity of the protein would be reached at lower buffer concentrations than the ion binding capacity of the resin.

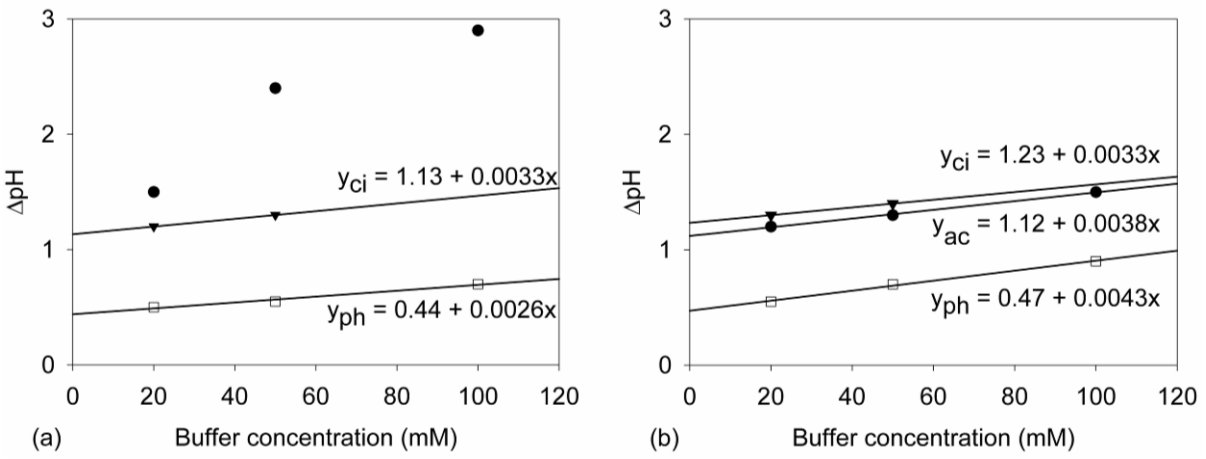

Fig. 6. Differences Δ pH between the curves of the pH positions of isoionic points and maximum DBC for all three buffers showing similar slopes and intercepts of linear regression lines for (a) hmAb1 and (b) hmAb2 with each salts. (●) sodium acetate, (□) sodium phosphate, (▼) potassium citrate.

Fig. 6 shows the increment of the pH value differences between maximum DBC and isoionic points (Eq. 3),

$$\Delta_{pH\ b,\ M} = \left| \text{pH (isoionic point} - \text{max. DBC)}_{\ b,\ M} \right| \qquad (3)$$

where $\Delta_{pH\ b,\ M}$ is the absolute value of the pH difference according to the buffer type b and molarity M (M). With increasing buffer concentrations the differences behave similarly for all salts and hmAbs, as indicated by the slopes of the linear regressions (average: 3.5 pH units/M), except the values for hmAb1 in sodium acetate due to the reason listed above. Moreover the intercepts of the regression lines of hmAb1 comply with those of hmAb2 according to the buffer. Considering this phenomenon the correlation of maximum DBC and isoionic points can be used for the determination of the pH value for maximum dynamic binding capacity in cation-exchange chromatography. Due to Eq. (4), the calculation of the pH value for maximum DBC in CEX with hmAbs is possible without any chromatographic experiments based only on zeta potential measurements,

$$pH_{b, M, max.\ DBC} = pH_{isoionic\ point, b, M} \pm (I_{b, r} + 3.5\ M) \quad (4)$$

where $pH_{b, M, max.\ DBC}$ is the pH value for maximum DBC and $pH_{isoionic\ point, b, M}$ is the isoionic point in the chosen buffer type b and molarity M (M). The sign of the operator depends on the buffer type as well as $I_{b, r}$, the intercept value for buffer b and resin r, listed in Table 2.

Table 2: Intercept values $I_{b,r}$ for the tested buffer types and resins for the calculation of the pH value for maximum DBC in CIEC with hmAbs.

		EMD SE Hicap	EMD SO_3^-	SP Sepharose XL	Toyopearl Prototype S
Buffer type	Sign	$I_{b,r}$			
Sodium acetate	-	1.2	1.2	1.7	1.7
Sodium phosphate	-	0.5	0.5	1.0	1.0
Potassium citrate	+	1.2	1.2	1.7	1.7

The pH position values of maximum DBC run lower than these of the isoionic points for sodium phosphate (Fig. 5) and sodium acetate buffer, merely using potassium citrate buffer it is contrariwise (data not shown). According to this with potassium citrate buffer the summand has to be added. Table 2 also contains the $I_{b,r}$ values for the calculations according to other tested strong cation-exchange resins. For both Fractogel EMD resins the $I_{b,r}$ values are equal, these matrices differ only in the type of the spacer, Isobutyl- for SO3- and Ethyl- for Hicap, respectively. The Toyopearl Prototype S resin is made of the same base matrix, but the grafting type and spacers are unknown. SP Sepharose XL is completely different in the base matrix and the grafting type. The impact of these differences between the resins could explain the variability of the respective $I_{b,r}$ values, whereas the modified spacers of the Fractogels seem to have less effect to the pH position of maximum DBC.

6.1.5 Conclusions

In this work the correlation between maximum dynamic binding capacity and zeta potential was described related to cation-exchange chromatography with human monoclonal antibodies. With this new approach of protein zeta potential measurements in the development of purification processes for hmAbs, time, material and manpower for chromatographic experiments can be saved. Currently tons of antibodies per year were used to medicate cancer and rheumatism patients. For these high amounts of needed product the efficiency and with it the capacity in purification processes becomes more and more important. In addition, new hmAbs are permanently developed which have to be purified. The development of individually tailored and optimized purification processes could be accelerated using the method described in this work. The procedure is summarized in three steps (a – c) showing how to use the zeta potential for determination of the pH value for maximum DBC in CEX:

Starting with the hmAb of interest, one of the tested buffer types and the buffer concentration have to be chosen.

Then the isoionic point has to be measured. If the isoionic point shifts with increasing buffer concentrations up to 100 mM, specific protein ion interactions are negligible (hmAb1 and sodium acetate is the example to show the opposite).

The final step is the calculation of the needed pH value for maximum DBC using the determined isoionic point and the $I_{b,r}$ value regarding to the used buffer and resin (Table 2) using Eq. (4).

The application of this zeta potential approach could improve the purification process development and therefore help to fulfil the sprouting requirements in the production of human monoclonal antibodies. Until now the search for optimal conditions in CEX is a trial and error approach. Experienced operators start 1.5 or 2.0 pH units below the isoelectric point of their protein of interest. A deviation of only 0.5 pH units could cost up to 70% of DBC, so several

experiments have to be done. The extent of necessary chromatographic runs for optimization can be minimized to one set of parameters, resulting in the conditions for maximum DBC for this given set of parameters. Additionally to the practical aspects of this work, the impact of protein bound anions in CEX was pointed out using zeta potential measurements, which can improve the further understanding of the adsorption mechanism. Admittedly with the zeta potential application the conditions for maximum DBC can be determined, but not the value of the maximum DBC, and the application is only based on two hmAbs. To establish a general rule, it has to be verified with more antibodies. Additionally, it could be extended to more buffer types, buffer compositions and resins.

Acknowledgements

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6.2 Investigation of salt properties with electro-acoustic measurements and their effect on dynamic binding capacity in hydrophobic interaction chromatography

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6.2.1 Abstract

The pH dependence in hydrophobic interaction chromatography (HIC) is usually discussed exclusively in terms of protein dependence and there are no clear defined trends. Many of the deviations from an ideal solution are caused solely by the high salt concentration, as protein concentration is usually negligible. So pH dependency in hydrophobic interaction chromatography could also be the result of pH dependent changes of ion properties from the salt solution. The possibility that pH dependent ion hydration or ion association in highly concentrated salt solutions may influence the dynamic protein binding capacity onto HIC resins was investigated. In buffer solutions commonly used in HIC e.g. sodium chloride, ammonium sulphate and sodium citrate pH dependent maxima in the electro-acoustic signals were found. These

maxima are related to an increase of the ion sizes by hydration or ion association. At low ionic strength the maxima are in the range between 4.5 and 6 and they increased in concentrated electrolyte solutions to values between 6 and 8. The range of these maxima is in the same region as dynamic protein binding capacity maxima often observed in HIC. For a qualitative interpretation of this phenomenon of increased protein stabilization by volume exclusion effect extended scaling theory can be used. This theory predicts a maximum of protein stabilization if the ratio of salt ion diameter to water is 1.8. According to the hypothesis raised here, if the pH dependent ratio of salt ion diameter to water approaches this value the transport of the protein in the pore system is less restricted and an increase in binding capacity can be produced.

Keywords: PH dependency of HIC; Dynamic binding capacity in HIC; Ion association ion hydration in HIC; Extended scaling theory in HIC; Electro-acoustic measurements for salt characterization in HIC

6.2.2 Introduction

In contrast to ion exchange chromatography (IEX) the mechanism of hydrophobic adsorption is less clear. The main reason is the higher number of the essential driving parameters influencing the binding capacity and selectivity [1]. The salt used in hydrophobic interaction chromatography (HIC) can modulate the selectivity and the binding capacity. In practical applications, sodium chloride, ammonium sulphate, sodium citrate or sodium acetate, are frequently used. Potassium and sodium sulphates and most of the phosphate salts are rarely used because of their limited solubility at room temperature [2]. The influence of salt type on protein solubility and protein retention for hydrophobic chromatography has been extensively discussed by Melander and Horvarth [3] and Regnier and Fausnaugh [4]. A linear relationship

was derived at high salt concentrations for the log of capacity factor k to the salt molality. The slope can be related to the surface tension increment σ from the individual salt and to the outer hydrophobic contact area Ω of the protein:

$$\ln k = \ln k_0 + \Omega \cdot \sigma \cdot m \qquad (1)$$

The surface tension increments of the various salts are arranged in a series which is similar to the Hofmeister series. A possible arrangement of cations and anions in the Hofmeister series is shown in Fig. 1 [5,6].

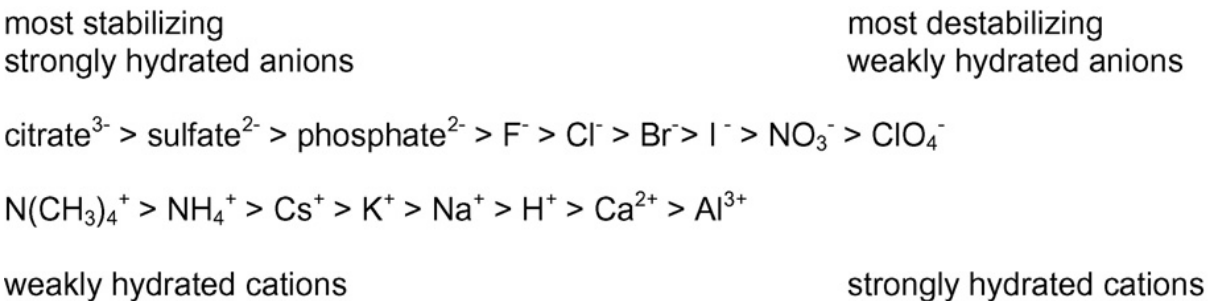


Fig. 1. Hofmeister series.

Fig. 1 demonstrates that, the "structure making" multivalent ions like sulphate and phosphate can be found at the left-hand side and the "structure breaking" ions like perchlorate are at the very right-hand side of the Hofmeister series. In hydrophobic interaction chromatography the retention of proteins did also not always follow the Hofmeister series As described by Fausnaugh and Regnier [4, 33] is the effect of salt composition on protein retention is a very complex phenomenon and is not so easy as it seems owing to Eq. (1). The surface tension of the solution, any specific interactions between the protein and the salt ions which may possibly change the protein structure, and the hydration of the protein should be included. The presence of a hydrophilic amino acid or changes in the charge can also influence the protein retention. This leads to the question of how the retention is related to the protein p*I*? Usually a high

retention at the p*I* value is expected because of the aggregation tendency of the protein. However Fausnaugh, Kennedy and Regnier [33] did not found an obvious relationship between retention and isoelectric points for different proteins. The slightly higher retention of five proteins at pH 4 was discussed as the result of partial denaturation of the proteins and the optimum resolution of the proteins was predicted, by them, to be at pH 7–8. The retention of the proteins in a linear sodium sulphate gradient as the function of pH is nonlinear and parabolic with double maxima for one group in the pH range from 4 to 5.5 and the other in the range from 6 to 7.5. Moreover equation 1 was derived for low loadings, over the linear part of the isotherm, and can be applied for analytical separations with percentage loadings of the full capacity. In process chromatography up to 70% of full saturation capacity is used. Instead of increasing the retention of a protein to a chromatographic phase, here the maximization of the specific dynamic binding capacity is of interest. An enhancement of few percents is enormously beneficial. The reported binding capacities of HIC resins are in the range below60 mg/ml. This is usually lower than the binding capacity for ion exchanger resins which typically in the range of more than 300 mg/ml [7]. Comparing the adsorption process for HIC and IEX is it possible to achieve the same magnitude of binding capacity and on which parameters does it depend? In IEX the process of adsorption can be modelled as an exchange of adsorbed salt ions with protein ions in solution. The exchange is the result of different electrostatic potentials of the protein surface to the surface of the resin. Usually the adsorption is accomplished at low ionic strength and the elution is performed with increasing salt concentration to displace the bound protein [8]. Mass transfer resistance for the solute decreasing the dynamic binding capacities, is mainly the result of pore and surface diffusion processes and this is dependent from the resin surface modification [9, 10]. Polymeric surface modifications exhibit the ionic ligands in a three dimensional arrangement and this guarantees effective utilization of the pore volume and a fast mass transfer. The most successful way to increase the binding

capacity in IEX is consequently through surface modification changes [31]. The hydrophobic interaction adsorption process for proteins is different [5]. The proteins are adsorbed at high salt concentrations. Usually, the process involves as the displacement of ordered water from the ligand and the proteins, which in turn, results in an entropy gain. If the salt ions are kosmotropic (structure makers) they will displace water from the protein. As a result of this process, the water is excluded from the protein hydration layer and is trapped in the bulk solution by hydration of the salt ions. Consequently, hydrophobic patches are then exposed on the protein surface and these interact with other exposed hydrophobic patches on other protein molecules or on solid surfaces such as the chromatographic stationary phases. Thus, it is a competition between protein aggregation in solution and protein adsorption on the HIC resin surface. At high column loadings the aggregation and repulsion by the hydration layers between the proteins might influence the mass transfer into the pore system. A low solubility or high tendency for aggregation will therefore not necessarily result in high dynamic binding capacities on the resin [16]. Hydrophobic interactions are attractive forces whereas the hydration forces are repulsive. Changes in the hydration layers of the involved components: proteins, salts and resins are thus the main factors influencing the binding capacities for HIC resins. Due to the low concentration of the proteins and the ligand (0.01–0.03 mol/l) at the resin surface compared to the concentration of the added salt (>1 mol/l) the main reason for deviation from ideal solution in a HIC column is the salt [11]. In IEX the compound with highest concentration is the available local ionic group density at the surface of the resin. For monovalent ions it is usually in the range of 0.1–0.3 mol/l. This may be the reason why the change in ligand arrangement so successfully increases the binding capacity. The change of the surface modification for HIC resin to graft type or polymeric modifications was not successful because the polymeric network collapsed at high salt concentration which resulted in a lower accessibility to the ligands which is in strong contrast to the IEX adsorption process. Consequently an in-

crease in dynamic binding capacities can only be achieved for HIC media at a given ligand and density by choosing the correct salt, salt concentration, temperature and most often overlooked the optimal pH value. This is why the characterization of salt solutions properties as function of concentration, pH and temperature can be an important part of the description of the whole adsorption process. One method that can be used for the characterization of charged particles in solution is electro-acoustic measurement. Electro-acoustic measurement is currently used for the physicochemical investigations of colloid solutions from food, paint, ink and other materials [14]. Previously partial molar volumes for single ions were determined by this method [13]. Almost forgotten for more than twenty years we re-discovered electro-acoustic measurements as a sensitive tool for detecting small changes in hydration of ions in concentrated salt solutions. We used the experimental data to try to explain phenomena encountered in HIC which cannot be completely solved by chromatographic evaluation alone.

6.2.3 Theory

6.2.3.1 Ionic vibration potential from simple salts

The basics of IVP theory have been described in [24, 32] and will be repeated here and extended to salt solutions. Electro-acoustic phenomenon, first predicted for electrolytes by Debye in 1933 [12] is a result of coupling between acoustic and electric fields. Debye realized that in the presence of a longitudinal sound wave, differences in the effective mass or friction coefficient between anions and cations resulted in different displacements. In turn, this difference in displacement would create an alternating electric potential between points in the solution. This phenomenon is measurable and can yield useful information about the properties of the ions. Zana andYeager [13] summarized the result in a review. The effect of IVP can be described as the sum of the following forces: electric forces, friction forces, relaxation, electrophoretic forces, diffusion and pressure [14]. The contribution of the electrophoretic forces,

relaxation and diffusion terms are in the case of smaller ions negligible. The final expression for a simple binary electrolyte is

$$\frac{IVP}{U_m} = \frac{c_m}{N_A e}\left[\frac{t^+ W^+}{z^+} - \frac{t^- W^-}{z^-}\right] \frac{1}{\left[\frac{\omega^2}{\omega_{MW}^2} + 1\right]^{\frac{1}{2}}} \tag{2}$$

were ω_{MW} is the Maxwell–Wagner frequency. At this frequency, the vibration of the ionic double layer can no longer follow the changes of the ultrasound field. The Maxwell–Wagner frequency is defined as

$$\omega_{MW} = \frac{K_m}{\varepsilon_0 \varepsilon_m} \tag{3}$$

$W_{\pm}$ is the apparent mass of an ion and can be described by the difference of the intrinsic masses of the ions m and partial molar volumes V_0 multiplied by the solution densities:

$$W^{\pm} = N_A\left(m^{\pm} - \rho_m \cdot \overline{V}_0^{\pm}\right) \tag{4}$$

with

$$t^{\pm} = \frac{\Lambda_{\pm}^0}{\Lambda_+^0 + \Lambda_-^0} \tag{5}$$

Originally Eq. (4) was derived for low salt concentrations [12] butYamaguchi [36] confirmed the application of this theory also to concentrated electrolyte solutions. The IVP depends on the concentration of the electrolyte because of changes in the Maxwell–Wagner frequency and this is proportional to the medium conductivity K_m. According to Zana and Yeager [13] only those solvent molecules whose volume, due to the electrostriction differ from the bulk contribute to the apparent molar mass. Solvent molecules that are part of the solvation shell, without appreciable electrostriction, or difference in packing, contribute equally in mass and volume of the ions and their contributions cancel each other. To calculate the IVP Eq. (2) can be rewritten as to

$$\frac{IVP}{U_m} = \frac{c_m}{e} \cdot \left(\frac{t^+}{z^+} \cdot m^+ - \frac{t^-}{z^-} \cdot m^- \right) - \frac{c_m}{e} \cdot \rho_m \cdot \left(\frac{t^+}{z^+} \cdot \overline{V}_0^+ - \frac{t^-}{z^-} \cdot \overline{V}_0^- \right) \quad (6)$$

with $\omega << \omega_{MW}$.

The IVP of salt solution is so the difference between the delta of the molecular masses from the ions and the differences between the partial molar volumes of the individual ions multiplied by the ratio of transfer numbers of the charges. The conductivities of salt solution are complementary to the IVP values because in this case the signal is the sum of the ion mobilities which are inverse proportional to the ion radius in solution [26]. The individual partial molar ion volume is the sum of the molar structural volume of the ion, the intrinsic volume, electrostatic volume contribution and cavity forming volume [15]:

$$\overline{V}_0^{\pm} = V_{\text{int}} + \Delta V_{str} \quad (7)$$

with

$$V_{\text{int}} = 2.52 r^3 \text{ in cm}^3 \text{ mol}^{-1} \text{ and r in A} \quad (8)$$

The IVP from Eq. (6) is a function of several parameters such as the ion mass, ion transfer number, intrinsic ion volumes, ion charge electrostatic contribution, cavity forming volume and the structural volume. According to the method from Stokes and Robinson the electrostatic contribution and cavity forming volume is neglected which may be not correct in higher concentrated salt solutions because ion association is possible [35]. The structural molar volume of an ion seems to be the most important component for the interaction with a protein or a resin surface which can be considered as the volume of the fully hydrated ion. For ab initio calculation of the hydration shell volume of the individual ions the IVP values, the density data as function of concentration and the ion sizes are necessary [13].An alternative method is the use of CsCl as calibration solution to calculate, the unknown value u_m, the maximum amplitude of the IVP, in Eq. (6) [27]. It is discussed in literature that structural molar volumes

can be used as an arrangement criteria to group ions into the Hofmeister series, but the structural volumes are not as indicative as the viscosity *B*-coefficients or the structural entropies [17].

Table 1: Molecular weight, radius, partial molar volume $\bar{V}^{\pm 0}$ and structural volume V_{Str} for selected relevant ions used in hydrophobic interaction chromatography (from Zhao [17]).

Ion	Molecular weight (g/mol)	$r^{\pm}$	$\bar{V}_0^{\pm}$ (cm^3/mol)	V_{Str} (cm^3/mol) (calculated by Eq. (7)) [17]
Li^+	6.94	69	-9.5	-10.3
$NH4^+$	18	148	12.4	4.2
Na^+	22.99	102	-6.7	-9.4
K^+	39.1	138	3.5	-3.1
Cs^+	132.91	170	15.8	3.4
Cl^-	35.45	181	23.3	8.3
I^-	126.9	220	41.7	2
NO_3^-	62	179	34.5	20
PO_4^{3-}	94.97	238	-14.1	-48.1
HPO_4^{2-}	95.97	238	18.7	-15.3
$H_2PO_4^-$	96.97	238	34.6	0.6
SO_4^{2-}	96.1	240	25	-9.9
CH_3COO^-	59.03	190	46.2	28.9
$Citrate^{3-}$	189.6	500	57	-258

Partial molar volumes, structural volumes and radii for some selected ions are summarized in Table 1. Usually the structural volumes of kosmotropic ions have relatively lower values (usually negative) while chaotropic ions have larger positive values. The structural volumes of cations as Li^+, Na^+, K^+ and Cs^+ are following the Hofmeister series (see Fig. 1) with Li^+ as the most stabilizing ion and Cs^+ as the least stabilizing ion. For the anions there are indeed some similarities to the Hofmeister series but they do not follow it totally. The structural volume of sulphate is greater than that of phosphate. The exception is the value of acetate. At 28.9 cm^3/mol it cannot distinguish the kosmotropy of this large organic ion. Knowing the absolute values of the structural volume is not necessary useful for chromatographic application. It is of much more interest to optimize the binding conditions for a given salt-buffer system by changing it's concentration or the pH and monitoring the effect by IVP. In Hofmeisters work and a many subsequent papers, the pH dependency of positioning ions in the Hofmeister series was not considered [29]. Today there are some physico-chemical methods available which can be used to measure the dependency. The decrease in surface tension increments, for example, of multi charged ion species as PO_4^{3-}, HPO_4^{2-} and $H_2PO_4^-$ is a well-known phenomenon and assessed as the result of different solvation degrees [3]. But the dependency of IVP on the pH value of neutral salt (e.g. NaCl) was never investigated and therefore, the potential that there might be a relationship between hydration of ions and the pH was not previously considered. A change in pH of neutral salts can be easily induced by adding small amounts of acid or base. The subsequent change in solvation can then be directly calculated from the Eq. (6) under the assumption that the molecular weights and the equivalent conductivities remain constant. If we assume the volume change is mainly contributed by the anions because only this results in an increase of the IVP value, the following equation for the relationship between IVP and change in partial molar volume can be derived:

$$\frac{IVP}{U_m}_{Maximum} - \frac{IVP}{U_m}_{Start} = \frac{c_m}{e} \cdot \rho_m \cdot \frac{t^-}{z^-} \cdot \left(\overline{V}_0^-{}_{Maximum} - \overline{V}_0^-{}_{Start} \right) \quad (9)$$

In Eq. (9) the partial molar volume is the sum of the intrinsic volume and the structural volume and the change in IVP is directly proportional to the change in hydration volume from the anion and can be estimated.

6.2.4 Materials and methods

Toyopearl Butyl-650M was from Tosoh Bioscience GmbH. A humanized monoclonal antibody was kindly provided by Boehringer Ingelheim (Biberach, Germany) at a purity of greater 99%, as detected by ELISA. All salts and the proteins lysozyme and BSA were obtained from Sigma-Aldrich (Taufkirchen, Germany). Distilled water was purified through a Millipore system (Simplicity 185) with conductivity <0.054 _S cm-1. Solutions were prepared using Millipore water and filtered through Whatman filter grade 4. All experiments were performed at room temperature (25 ◦C).

6.2.4.1 Electro-acoustic measurements

The electro-acoustic measurements were performed using a DT 300 instrument from Dispersions Technology (Bedford Hills, NY, USA) at a default frequency of 3MHz in the most precise measurement mode. A detailed description can be found in reference [14]. The instrument was calibrated daily using the provided silica ludox calibration solution (Quantachrome, Odelzhausen, Germany). In the experiments 100 ml stirred salt solutions were measured. For titration experiments the instrument included titration unit was assembled with the salt suitable acid or base of appropriate concentration, e.g. 1 M HCl, or NaOH for titration of NaCl solutions and 1 M sulphuric acid or NaOH for titration of Na_2SO_4 solutions. The pH was adjusted to the most acid value with the appropriate acid for upward titrations or the most basic

value for downward titration with the appropriate base. The relative standard deviation of electro-acoustic measurements was tested to be 5%.

6.2.4.2 Chromatography

All binding capacity determination experiments were performed with a 1 mg/ml protein solution adjusted to 80% of the precipitation salt concentration value which was determined by turbidity measurements at 595 nm in 96 well plates. Chromatographic experiments were done on a Äkta Explorer System (GE Healthcare, Chalfont St Giles, UK) with Omnifit (MSScientific, Berlin, Germany) columns of 107mm bed length and 6.6mm i.d. for lysozyme and BSA (flow rate 150 cm/h), with columns of 20mm bed length and 10mm i.d. for monoclonal antibody (flow rate 75 cm/h). The resin was equilibrated with 10 column volumes (CVs) of loading buffer. Breakthrough points were determined at 10% leakage relative to the sample UV adsorption at 280 nm. Dynamic binding capacities were calculated by Eq. (10):

$$DBC = (V_P - V_0)c_P/V_R \quad (10)$$

The relative standard deviation was determined to be 5% (threefold measurements). The resin was regenerated with 5 CVs of water, 0.5M NaOH, 0.1M sodium phosphate buffer pH 7 and stored in 20% aqueous ethanol.

6.2.5 Results and discussion

6.2.5.1 IVP for different salts as a function of the concentration

The magnitudes of the IVP's for caesium chloride, potassium chloride, sodium chloride and lithium chloride are shown in Fig. 2. The signal is highest for caesium chloride with more then 2,000,000 units. The signal for all salts is increases from low values at low salt concentration to reach a plateau at concentrations above 0.1 M. This can be explained by the following: The IVP/*u*m value in Eq. (2) depends on the electrolyte concen tration through the *K*m

term which is proportional to C. When C tends toward 0, *K*m → 0 and IVP/*u*m → 0. As C is increased the C-dependent term tends toward unity and IVP/*u*m becomes independent of C. This concentration dependence can be interpreted in terms of the internal loading of the effect by the intrinsic capacitance of the solution leading to the "plateau effect" [13].

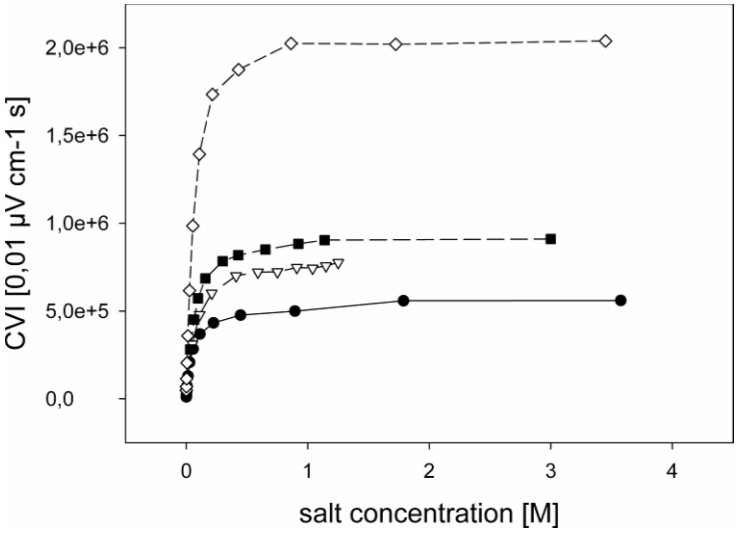


Fig. 2. IVP for chloride salts with different cations as a function of concentration (connecting lines used as eye guiding lines): (◊) Cs, (■) K, (▽) Na, (●) Li.

A clear relationship between differences in molecular masses and the magnitude of the ultra-sound signal exist for the alkaline chloride salts. The magnitude of the electro-acoustic signal increases with increasing molecular masses for the cations from Li^+ to Cs^+ (6.9–132.9 g/mol). Using the values from Table 1 and the ion conductivities from Marcus [30] the IVP/*u*m values were calculated with Eq. (6). The IVP/*u*m of 1,400,000 for CsCl at a concentration of 0.1 M was taken as the standard value. The data are summarized in Table 2. The calculated values are a factor of 2 below the experimental values. The reason for this may be that no absolute calibration was performed, including the determination of *u*m the amplitude of the alternating IVP current as described by Zana.

Table 2: IVP/u_m values measured and calculated by Eq. (6) for alkali halide salts.

Salt	IVP (measured at 0.75 M salt concentration)	IVP (calculated with Eq. (6))
CsCl	1,400,000	Standard
KCl	686,000	312,000
NaCl	311,000	216,000
LiCl	136,000	57,000

Formally the cations can be arranged in a Hofmeister series according to their IVP values with Lithium as the most stabilizing cation and Caesium the least stabilizing ion as shown in Fig. 1.

The IVP values for sodium salts of different anions are shown in Fig. 3. The magnitudes can be arranged in following order: NaAcetate >NaCl = NaCitrate > NaI > Na2SO4 >Na2CO3 =Na2HPO4 = NaH2PO4 > NaBr > NaClO4.

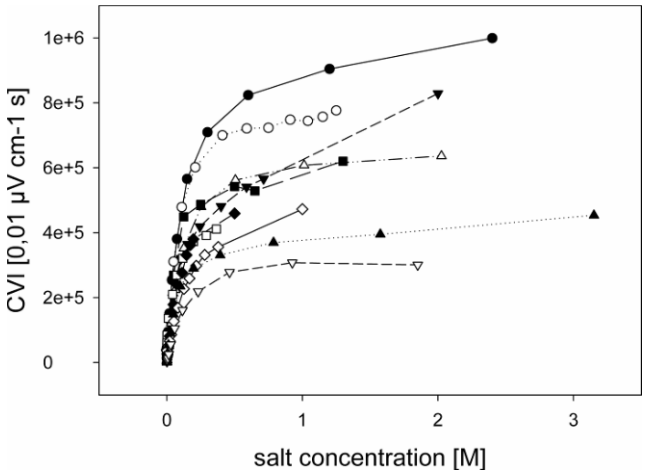


Fig. 3. IVP of sodium salts with different anions as a function of concentration (connecting lines used as eye guiding lines): (●) acetate, (○) chloride, (▼) citrate, (Δ) iodide, (■) sulphate, (□) carbonate, (♦) phosphate dibasic, (◊) phosphate monobasic, (▲) bromide, (▽) perchlorate.

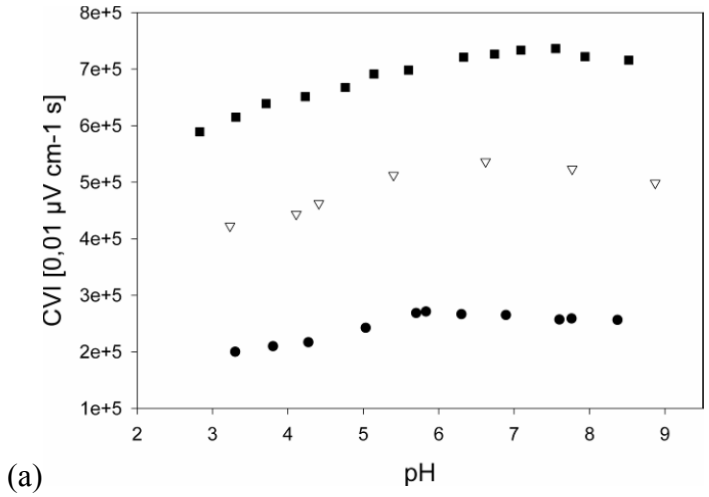
This does not completely follow the Hofmeister series and cannot be explained by mass differences of the anions only, because acetate, chloride and citrate have similar signal magnitudes. They are however at the opposite end in the Hofmeister or lyotrophic series and differ in their molecular weights (Citrate 189 g/mol and Chloride 35 g/mol) but if NaCl and NaI are excluded from the experimental series as outliers the similarity to the Hofmeister series is unambiguously, with the most stabilizing ions such as acetate, citrate and sulphate with the highest IVP values and the most destabilizing ions such as perchlorate with the lowest values. This is in accordance with the IVP calculated by Eq. (6): An increase in partial molar anion volume increases the IVP. The value for acetate seems to be excessively too high as already discussed. As an alternative hypotheses it can discussed the possibility that the classification of ions in “structure makers” and “structure breakers” according to the Hofmeister series is not existing. It has been shown that ions only affect the first few hydration shells. Ions neither enhance nor weaken the hydrogen bond network, at least over the time scale experienced by femtosecond pump spectroscopy [37]. If these results hold true for the IVP also, the magnitudes of the measured values might reflecting the specific interactions water-ion and ion-ion only but can not be classified in a Hofmeister series.

The curve progression of IVP versus salt concentration is not fixed and depends on the experimental conditions. It was found that the IVP value of salt solutions is a function of the pH value. This will be described in the next chapter.

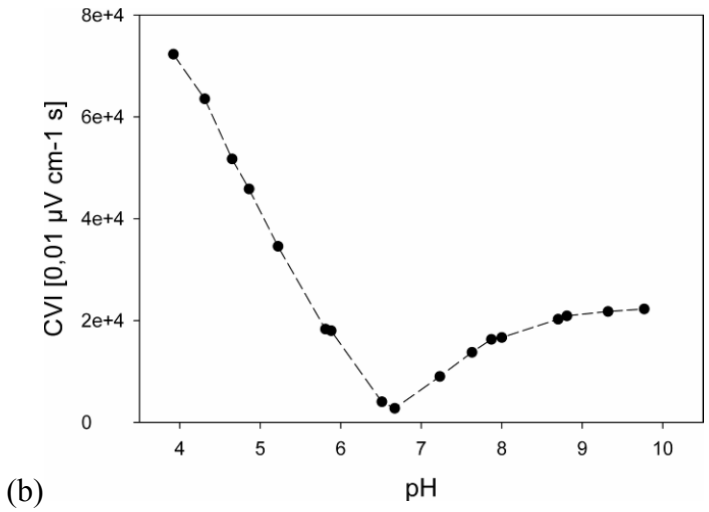
6.2.5.2 Changes in the electro-acoustic signal as a function of the pH are changes in the solvation shells of the ions or are caused by ion association

As discussed in the introduction small variances in a given salt system, that do not change the molecular weight of the ions and marginally alter the conductivity can be easily monitored by IVP value and used to follow changes in partial molar ion volume.

As described in the experimental section neutral salts solutions like sodium chloride and sodium sulphate are only slightly different to the neutral point if they are freshly prepared using carbon dioxide free water. Small amount of acid or base will thus cause a large change in pH value. For example 16 µmol NaOH was required to titrate a 5 M NaCl sample from pH 4 to 9. In Fig. 4a the change in the measured IVP for 50 mM NaCl, 150 mM and 5 M NaCl titrated with 1 M NaOH are shown. For the 50 mM NaCl solution at pH 6 a maxima of about 20% from the starting value is observed. This disappears at higher pH values. The maximum is also observed by back titration with HCl. To exclude the possibility that the maximum is the result of the changed total salt concentration by added NaOH only, a sample of high purified water was titrated with the same amount of sodium hydroxide as used for the salt solutions. The resulting IVP–pH curve (Fig. 4b) has a minimum at pH 6 instead of the maximum found for the salt containing solutions. If the minimum of the blank titration is added to value of the salt solution the overall increase is 25% above the starting value.



(a)



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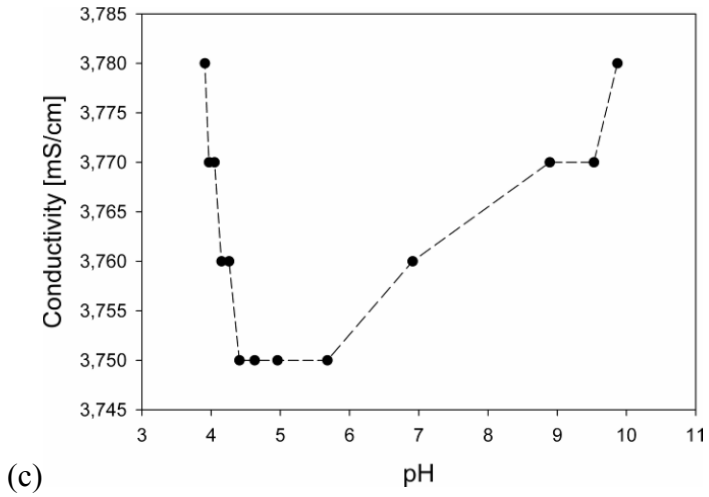


(c)

Fig. 4. (a) Changes in IVP of NaCl as a function of pH are caused by changes in the solvation shells of the ions or ion associates . (●) 50 mM, (▽) 150 mM, (■) 5 M. (b) IVP signal for a blank titration as function from pH of high purified water with 14 µm NaOH. (c) Changes in conductivity of 50 mM NaCl as a function of pH are complementary to the according IVP changes (connecting lines used as eye guiding lines).

In Fig. 4c the conductivity as a function of pH for a 50 mM NaCl solution is shown. As previously discussed in the introduction is the minimum of the function complimentary located at the same value were the maximum of IVP was found. But in contrast to the large change in IVP (25%) the change in conductivity is only 30 µS/cm (<1%). The reason for the maximum in IVP is not clear but considering Eq. (9) it has to be the result of an increase in partial molar volume of the anion. A change in partial molar volume could either be the result of increasing the solvation shell (structural volume) or possibly is the formation of ion associates at higher salt concentrations. Following the classification from Braunstein [28] ion associates in salt solutions are found at concentrations higher than 50 mM. This was also discussed by Zana [17]. He observed stronger deviations in calculated partial molar volumes for electro-acoustic measurement results for anions than for cations especially for the multi charged ions and explained the phenomenon as ion association [34].

In Fig. 4 it is shown that the IVP maxima not only occurs in low concentrated solution but is also present in highly concentrated salt solutions. The change in the partial molar volume for the Cl^- ion can be estimated by using Eq. (6). With the values $\overline{V}_{0\ Maximum}^{-}$ = 0.5 mV cm s^{-1}, $\overline{V}_{0\ Maximum}^{-}$ = 0.4 mV cm s^{-1} and c_m =1 M the ratio of both partial molar volumes can be estimated to:

$$\frac{\overline{V}_{0\ Maximum}^{-}}{\overline{V}_{0\ Start}^{-}} \approx 1.25 \qquad (11)$$

The partial molar volume of chloride $V_{0\ \mathrm{Start}}^{-}$ = 23.3 cm^3/mol and the increase by the pH change is 5.825 cm^3/mol. The structural volume for chloride, which is the water shell, is 83 cm^3/mol [17]. As the intrinsic ion volume does not change the water shell volume is increased by more then 70%.

Using 5 M NaCl solution the maximum is moved to pH 7.5. The position of the maximum value is therefore concentration dependent. It can be elevated by 1.5 pH units by simply increasing the salt concentration from 50 mM to 5 M.

The shift in pH cannot be explained by a change in the activity coefficients of the salt solution. In concentrated electrolyte solution the function of the activity coefficient from concentration has often parabolic shape and the activity coefficients for 50 mM NaCl and 5 M NaCl are comparable with a value of approximated 0.8 [18]. The assumption that the maxima is the result of the ubiquitously soluble carbon dioxide [25] in the solution can also be excluded because of the above described titration of pure water which shows a minimum instead of an maximum in salt solutions (Fig. 4a).

We found maxima in the tested electrolyte solution but at different pH values for each solution. According to our findings forming a maximum of solvation or association may be an intrinsic property of salt solutions. At lower electrolyte concentrations (50–150 mM) the maxima are all positioned in the range from 4.5 to 6. The maxima shift for the salts K_3Citrate, Na_2SO_4, NaCl, NaAcetate, Na_3PO_4 are summarized in Fig. 5. For Na_2SO_4 the maximum is shifted to pH 6 and for citrate to pH 7 whereas sodium chloride has a maximum shift to pH 7.5. The shifts are dependent on the salt itself and on the salt concentration but with the exception of phosphate all are in the pH range from 6 to 8. The largest changes were observed for phosphate and citrate salts.

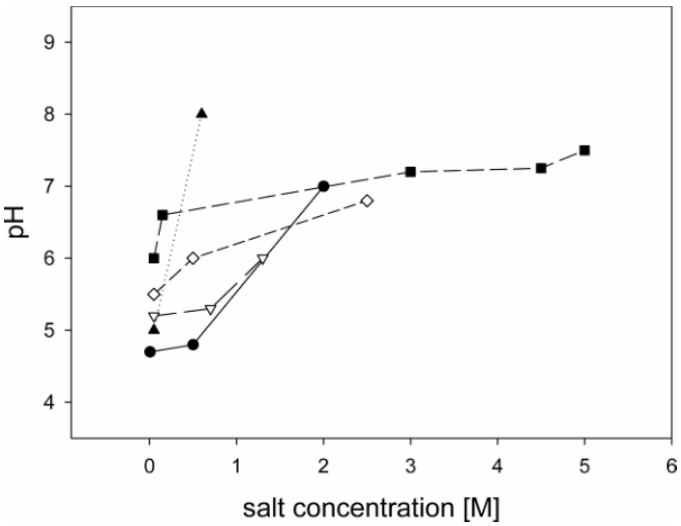


Fig. 5. The pH shift of IVP maxima of the salts as a function of concentration (connecting lines used as eye guiding lines): (●) sodium citrate, (▽) sodium sulphate, (■) sodium chloride, (◊) sodium acetate, (▲) sodium phosphate.

6.2.5.3 Correlation of Electro-Acoustic Measurements with Dynamic Protein Binding Capacities in Hydrophobic Interaction Chromatography

The dynamic protein binding capacities determined with the Toyopearl Butyl-650M HIC resin for hen egg white lysozyme, bovine serum albumin and a humanized antibody at level 80% away from their precipitation limit (shown in Figs. 6–8) for different salts do have maxima or plateau values in the range from 6.5 to 8. An exception is sodium acetate were we observed one minimum in this range. In some cases, especially lysozyme at pH 4.5 no dynamic binding values could determine because of precipitation. In Fig. 9(1)–(3) the maximum protein binding capacities of lysozyme, BSA and mAb using sodium chloride, ammonium sulphate, sodium acetate, sodium citrate and sodium phosphate salts are plotted as function of the pH and compared with the corresponding IVP maxima. There is a correlation between the position of IVP maxima and maximum binding capacity in the pH range from 6 to 8. The maximum deviations for the pH of the different salts and proteins are summarized in Table 3.

They were calculated for each anion as the arithmetic mean for all three proteins. The largest deviation with 1.33 pH units was observed for citrate.

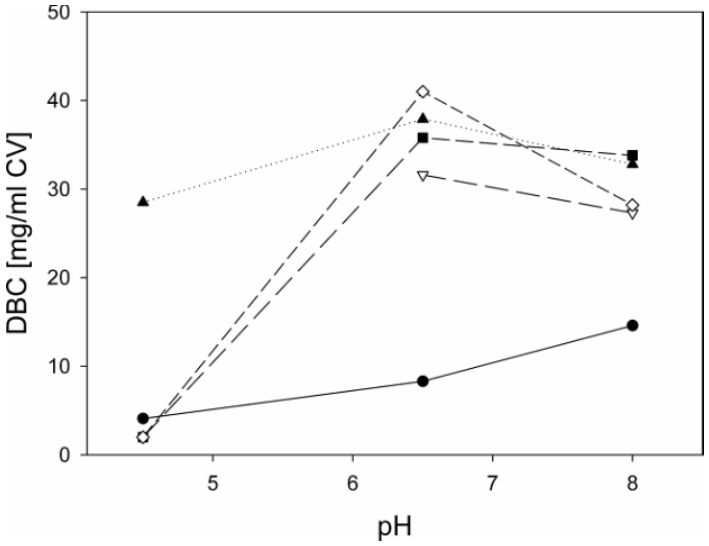


Fig. 6. Dynamic binding capacity (DBC) of Toyopearl Butyl-650M for lysozyme with different salts have maxima in the pH range from 6 to 8. (●) 1,1 M sodium phosphate, (▽) 4,5 M sodium chloride, (■) 1,1 M sodium citrate, (◊) 3 M sodium acetate, (◊) 2 M ammonium sulphate.

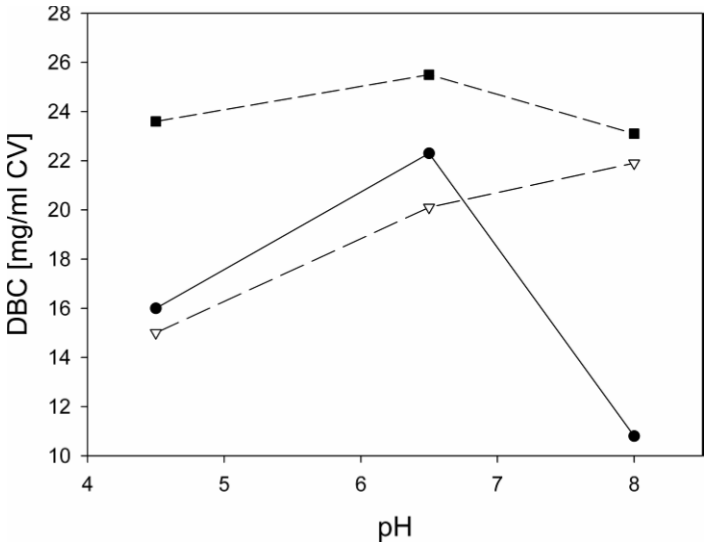


Fig. 7. DBC of Toyopearl Butyl-650M for BSA with different salts have maxima in the pH range from 6 to 8. (●) 4,5 M sodium chloride, (▽) 1,1 M sodium citrate, (■) 2 M ammonium sulphate.

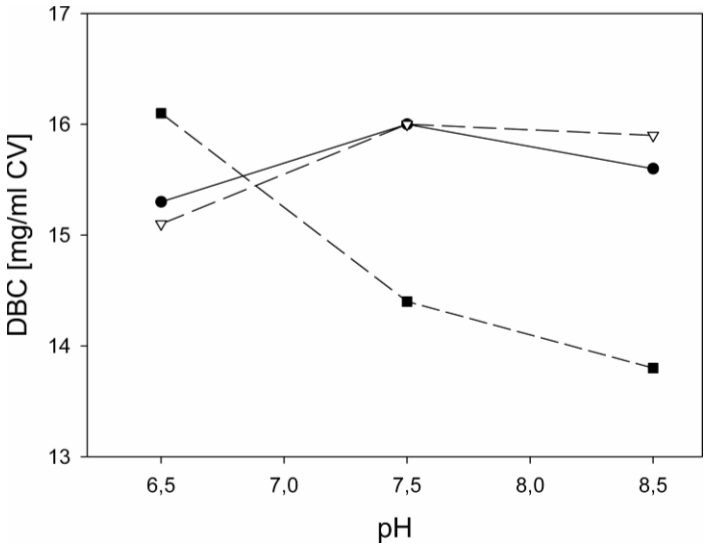
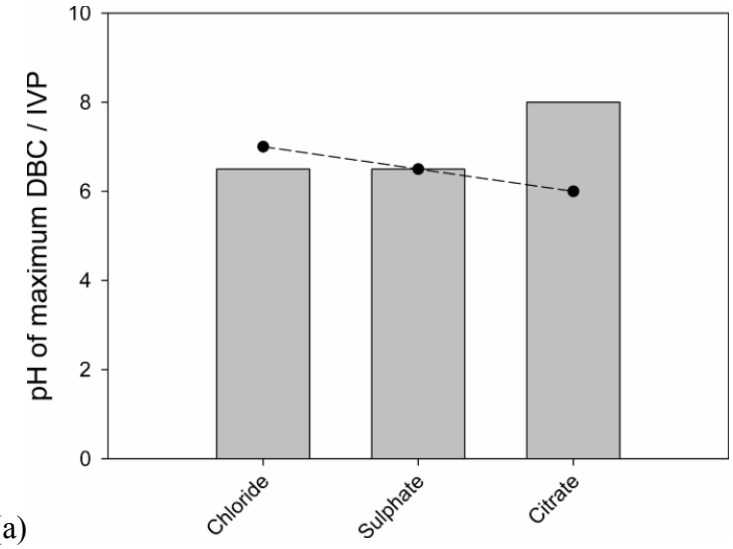
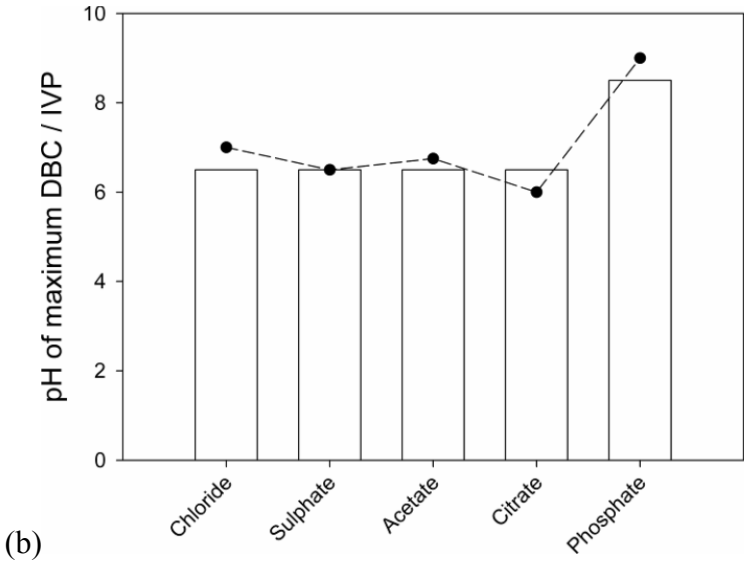


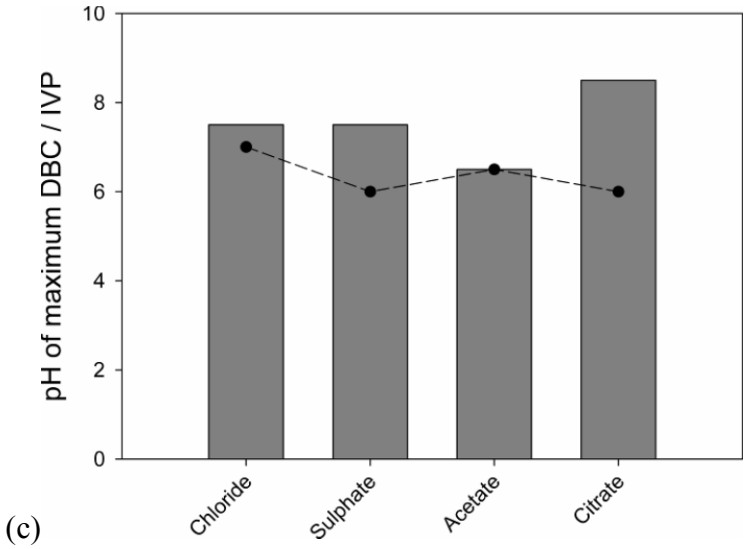
Fig. 8. DBC of Toyopearl Butyl-650M for monoclonal antibody with different salts have maxima in the pH range from 6 to 8. (●) 2 M sodium chloride, (▽) 0,7 M sodium sulphate, (■) 2,2 sodium acetate.



(a)



(b)



(c)

Fig. 9. (a) Overlay of the pH position of maximum DBC (white bars) and maximum IVP (●) for different salts and lysozyme. (b) Overlay of the pH position of maximum DBC (grey bars) and maximum IVP (●) for different salts and BSA. (c) Overlay of the pH position of maximum DBC (dark grey bars) and maximum IVP (●) for different salts and mAb (connecting line used as eye guiding line).

The maxima in dynamic protein binding capacities in the same range are somewhat unexpected because the isoelectric points for the proteins are very different (BSA p*I* = 4.7, lysozyme p*I* = 11.6 and the mAb p*I* = 7.5–8.5). This is not in accordance to the pH dependency from precipitation in solution were a minimum in solubility is observed at the isoelectric points. However, the process of adsorption under high loading conditions on a HIC resin is different than the precipitation process.

At first glance the existence of a correlation between the observed pH maxima in salt solution determined by electro-acoustic measurement, and the dynamic protein adsorption capacities for HIC resins seem unlikely to be related, but the adsorption of a protein to a porous resin is a complex process comprising the interaction between protein and ligand surface and transport in the resin pores [5]. Qualitatively an explanation of the phenomenon can be as follows:

At high loadings the transport of a protein into the pores of a HIC resin is an important step in comparison to the simple equilibrium approach like precipitation for utilizing the binding capacity. In case that the protein interacts too strongly with the ligand the dynamic binding capacity is decreased as the blocked pores slow mass transfer [16].

As described previously the maximum of the electro-acoustic signal corresponds to an increase in the size of the electrolyte ions. These results demonstrate that a possible interpretation or theory must also include the changed size of the ions.

Table 3: Deviation from experimental maximum IVP values to the point of maximum binding capacity in HIC

Salt	Delta pH for IVPmax to binding capacitymax
Chloride	0.5
Sulphate	0.5
Acetate	0.25
Citrate	1.33

6.2.6 Scaled particle theory

Scaled particle theory [19] is a statistical treatment that considers solution nonideality (protein and water) arising from the presence of a cosolute. Using this treatment an obvious advantage of scaled particle theory is the relative ease with which the analysis of thermodynamic non-ideality may be extended to incorporate a range of solute or cosolute concentrations for which description of the nonideality in terms of nearest-neighbour interactions ceases to be an adequate approximation.

Scaled particle theory describes the reversible work $g(r_i)$ necessary to create a spherical cavity of radius r_2 for a single protein molecule in a solution containing different hard-sphere species [11]. The first one can be water with hydrated radius r_1 and the other a co solute with radius r_3 in this case a hydrated anion. An anion is not the same as uncharged glycerol or glycine. At high salt concentrations used in HIC the electrostatic interactions are screened out. The extension of the Scaled Particle Theory in concentrated electrolyte solutions is according to Stillinger possible if ion pairing is included [20]. As already pointed out the pH dependent maxima in the electro-acoustic signals in concentrated electrolyte solutions can thus also be the result

of ion pairing and not a simple increase of the hydration shell from the anions. The counter cation size is for simplification to be considered as the same size as a water molecule. The reversible work g to form a protein cavity in an aqueous salt solution can be described as follows:

$$\frac{g(r_2,\Phi)}{kT} = -\ln(1-S_3) + \left[\frac{6\cdot S_2}{(1-S_3)}\right]\cdot r_2 + \left[\frac{12\cdot S_1}{(1-S_3)} + \frac{18\cdot S_2^2}{(1-S_3)^2}\right]\cdot r_2^2 + \left[\frac{8\cdot S_0}{(1-S_3)} + \frac{24\cdot S_1\cdot S_2}{(1-S_3)^2} + \frac{24\cdot S_2^3}{(1-S_3)^3}\right]\cdot r_2^3 \tag{11}$$

$$S_0 = \frac{1}{8\cdot r^3}\left[\left(\frac{r_3}{r_1}\right)^3\cdot\Phi_1 + \Phi_3\right]$$

$$S_1 = \frac{1}{4\cdot r^3}\left[\left(\frac{r_3}{r_1}\right)^3\cdot\Phi_1 + \Phi_3\right] \tag{12}$$

$$S_2 = \frac{1}{2\cdot r_3}\left[\left(\frac{r_3}{r_1}\right)\cdot\Phi_1 + \Phi_3\right]$$

$$S_3 = \Phi_1 + \Phi_3$$

$$\Phi_i = \frac{4}{3}\cdot\pi\cdot r_i^3\cdot d_i$$

were d_i is the number density and the remainders is the volume of the solute or cosolute. The stabilizing effect depends on the size of protein. As the size of the protein increases the gain in stabilization work is increasing quadratically which can be derived from Eq. (11). In the scaled particle theory the stabilizing effect disappear as the size of the cosolutes approach the size of a water molecule. On the other hand the stabilization effect also disappears for large cosolutes because their concentration approaches zero. In contrast to simple excluded volume theory the scaled particle theory predicts a maximum in protein stabilization if the size of the cosolute is 1.8 times the volume of a water molecule.

The 1.8 ratio value can be exemplified explained by comparing the equivalent radius for a water molecule and sulphate and chloride anions. The hydrated radii were determined from Kiriukhin by size exclusion chromatography [21]:

Anion	radius of hydrated ion (nm)
Sulphate	0.3
Chloride	0.195
Water	0.155

The ratio for the anions to the water molecule size is in between 1 and 2. Sulphate has a more stabilizing effect than chloride and indeed its ratio is more to 1.8 than chloride which is 1.2. This is in accordance with the extended scaling theory. If the sizes of the ions are changed (by additional water adsorption or by ion association) to match the stabilizing size better the increasing binding capacity in the pH range from 6 to 8 could be explained because the protein is stabilized and can enter the inner pore space of the resin easier. Therefore the pH deviation of maximum DBC and maximum IVP of citrate could be caused by the great size of the citrate ion, which matches the protein stabilizing size worse by increasing its size by the phenomena described above. This means the pH of maximum $DBC_{citrate}$ must be above the maximum $IVP_{citrate}$ (according to the results) and not below it because there the ions are not completely deprotonated, and the ion size is not too big.

This hypothesis that pH effects excluded volume changes are rather responsible for increased protein stabilization than specific ion specific effects is supported by results from pressure perturbation calorimetry measurements. Batchelor [22] determined the temperature dependence on solute's partial isothermal compressibility for "structure maker" and "structure

breaker". Contrary to widely held expectations no correlation between a solute's impact on water structure and its effect on protein stability in solution was found.

6.2.7 Conclusions

The higher degree of solvation or ion association in concentrated electrolyte solutions detected by electro-acoustic measurement in pH range from 6 to 8 can be a reason for an unexpected higher dynamic binding capacity of proteins to HIC resins around these pH-values. Maximum binding capacities for MAb in citrate buffer solutions in this pH range were also described by Chen [23]. The profit of the electro-acoustic method for future experiments could be the determination of the size of any beneficial anions at different pH values and salt concentrations. This could allow the prediction of most stabilizing conditions for proteins regarding to salt types and therefore the conditions for maximum DBC in HIC. Possibly this approach could be extended to predict the optimum conditions for salt mixtures, too.

Nomenclature

c_m	ultrasound velocity (m/s)
c_P	protein concentration (mg/ml)
C	salt concentration (M)
d_i	number density
DBC	dynamic binding capacity (mg/ml)
e	unit charge (C)
g	free enthalpy (kJ mol^{-1})
IVP	ionic vibration potential (µV)
k	capacity factor of the solute

k_0 capacity factor with pure water as the eluant

k_m media conductivity (Ω^{-1} cm^{-1})

m molality (mol kg^{-1})

$m_{\pm}$ intrinsic ion mass (kg)

N_A Avogadro number (mol^{-1})

r_i radius of protein, water and cosolute (m)

$t_{\pm}$ transport numbers of cations or anion

u_m oscillation velocity amplitude (cm/s)

V_{cav} cavity forming volume (cm^3 mol^{-1})

V_e volume change by electrostatic contribution (cm^3 mol^{-1})

V_{int} intrinsic volume of the ion (cm^3 mol^{-1})

V_{Str} structural volume (cm^3 mol^{-1})

V_P elution volume (ml)

V_R volume of packed bed (ml)

V_0 void volume (ml)

$\overline{V}_0^{\pm}$ partial molar ion volume (cm^3 mol^{-1})

z ion charge

Greek letters

Φ_i fraction of solution volume occupied by a species I (cm^3)

ε_0 dielectric permittivity in vacuum(C V^{-1} m^{-1})

ε_M relative dielectric permittivity of the liquid(-)

ρ_m density of the solution (g/cm^3)

$\Lambda^0_{\pm}$ limiting ion conductance (Ω^{-1} mol^{-1} cm^2)

σ incremental surface tension (N m^{-1} mol^{-1})

Ω protein surface are (m^{-2})

ω radian frequency of acoustic field (s^{-1})

ω_{MW} Maxwell-Wagner frequency (s^{-1})

6.2.8 References

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6.3 A novel approach for detection of biochromatography resin batch to batch variations

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6.3.1 Abstract

Electro-acoustic and chromatographic measurements were performed with several batches of five different resin types for biochromatography. Remarkable batch to batch variations were brought to light, which were not included in certificates of analysis and could be crucial transferring purification processes as well as replacing column packings in ongoing production of biopharmaceuticals. The latter requires extensive tests of each new gel batch to fulfil regulatory authority demands. The electro-acoustic approach can be a fast preselecting tool identifying most eligible batches and reducing time and cost expensive tests.

Keywords: electro-acoustic; biochromatography; resin characterization; batch to batch variation; unspecific protein binding

6.3.2 Introduction

Today's biopharmaceutical pipeline contains many candidates for clinical studies. After upstream production chromatographic approaches are the main steps in purification processes, which cover capturing, middle purification including virus and DNA removal, and polishing steps. For early stages (Phase I and II) often an initial standardized platform process is usually applied for purification to satisfy the material requirements in short period of time in spite of

improvable productivity and robustness. Only for candidates proven to be safe for patients in early stage studies, processes are further optimized in order to increase robustness and cost of goods for commercial manufacturing [1]. Thereby high validation requirements have to be considered to fulfil regulatory authority demands. In ongoing manufacturing processes chromatographic resins have to be replaced in assessed time caused by their limited life time. Resin manufacturers provide media with certificates of analysis containing specifications like exclusion limit, particle size distribution, pressure drop, ligand density, standard protein elution volumes and adsorption capacities. But keeping product specifications could be not enough to guarantee identity of biopharmaceuticals. Resin properties which are difficult to estimate or are not included in manufacturers specifications hold the risk of product variations. Such resin properties could be mass transfer hindrance [2-4], unspecific binding tendency or hydrophobicity, pore size distribution and mechanical properties [5-9] and can vary from batch to batch. To include batch to batch variations in process validation, each new batch has to be proven explicitly in time and cost expensive tests [10, 11]. This work shows a possible approach to preselect new chromatography media batches to reduce the amount of such tests. Therefore electro-acoustic investigations were performed with several batches of five different resin types. The results show significant batch to batch variations and correlations to chromatographic behaviour of the gels.

6.3.3 Electro-acoustic theory

Electro-acoustic phenomenon was first described by Debye [12] for salts. He described it as a coupling between acoustic and electric fields. Differences in effective mass or friction coefficient between cations and anions result in different displacements in a present longitudinal sound wave. This displacement creates an electrical potential, which can be measured as ionic vibration potential. This theory was further extended to colloids. There a induced electrical

current or potential is explained as a difference in fluctuation of the colloid and its surrounding electrical double layer [13]. This difference changes the colloid character to a dipole, which is the reason for an alternating colloid vibration current (CVI). The more mobile the colloid is respectively to the electrical double layer, the higher the expected current would be. With Eq. (1) and (2) it can be derived, that the CVI for low frequency is a function of density differences of the particle and the solution ρ, volume fraction φ, pressure gradient P and the electrophoretic mobility μ. μ depends on particle electrokinetic potential ζ, medium properties like viscosity η or permittivity ε and a model dependent constant/term C [14].

$$CVI_{\omega \to 0} = \mu \frac{\varphi(\rho_P - \rho_S)}{\rho_S} \nabla P \tag{1}$$

$$\mu = \frac{\varepsilon_0 \varepsilon_m \zeta}{\eta} C \tag{2}$$

To adapt electro-acoustic to measure biochromatographic media, the experimental setup was refined from measurements in suspension to measurements in settled beds. Thus, the classic electro-acoustic theory does not describe the experimental reality exactly. Here particles of a sedimented bed are exposed to a pressure gradient caused by ultrasound, which creates the potential/current to be measured. The mechanism creating this current reminds more to a piezoelectric effect and is not reported in literature yet. Additionally in this approach the mechanical properties of the resin particles like porosity, wettability and elasticity could contribute to the achieved signals in higher extent than expected by CVI theory. Therefore, to keep the theoretic demands as simple as possible, in this work only unprocessed data are used and theoretic expansions [14-18] are left outside.

6.3.4 Material and Methods

6.3.4.1 Resins and reagents

Four sulfo-functionalized cation-exchange resins and a C4 functionalized reversed phase resin were investigated and further described in Tab. 1.

Tab. 1: Resin types and description of investigated biochromatographic media.

Resin notation	MA	Se	MB	MG	Si
Particle size (µm)	40 - 90	45 - 165	50	40 - 90	30
Resin type	classic	classic	classic	grafted	classic
Functional group	SO_3^-	SO_3^-	SO_3^-	SO_3^-	$(CH2)_3CH_3$
Base matrix	methacrylate	sepharose	methacrylate	methacrylate	silica

Sulfo-resins were purchased by the manufacturers, which are not named to maintain scientific neutrality. Silica resins were kindly provided by an industrial cooperation partner. All resins were produced and sold within product specifications of the manufacturers. Sodium chloride, sodium phosphate, ethanol (96 %, 1 % methylethylketone), acetone (99, 8) and lysozyme (hen egg white) were purchased from Carl Roth GmbH & Co. KG (Karlsruhe, Germany). Blue Dextran used as unretained solute was purchased from Sigma-Aldrich (Taufkirchen, Germany). Solutions were prepared using double distilled water and were filtered through Whatman filter with a pore size of 0.22 microns. The Silica Ludox standard solution (ASTM standard silica particles with 30 nm mean particle size) was provided by Quantachrome (Odelzhausen, Germany). All experiments were done at room temperature (25 °C).

6.3.4.2 Electro-acoustic measurements

The electro-acoustic measurements were performed using the DT 300 instrument from Dispersions Technology (Bedford Hills, NY, USA) at a default frequency in the fast measurement mode. A detailed description can be found elsewhere [19, 20]. The instrument was calibrated each day using the supplied Silica Ludox calibration solution. Sulfo- resins were washed with 10 bed volumes water, 5 bed volumes 0,1 M sodium phosphate buffer pH 7, 10 bed volumes water and 10 bed volumes organic solvent if required. Silica resins were suspended and washed with 10 bed volumes particular organic solvent. In the experiments 5 ml slurry was applied on the probe. Measurements were started after sedimentation to achieve higher CVI amplitudes as possible in suspension Eq. (1). The relative standard deviation was determined out of threefold measurement to be < 3 %.

6.3.4.3 Chromatography

To prepare the different adsorbents, the resins were washed with 10 bed volumes of 5 M NaCl solution prior to packing. The suspension was adjusted to be approximately 50 % and filled in a 30 ml reservoir connected to a stainless steel column (250 mm bed length x 4.6 mm ID) which then was flow packed with the same liquid at a flow rate of 600 cm/h.

After equilibration with 50 mM sodium phosphate buffer pH 7 containing 4 M NaCl, pulses of 10 µl of 1 g/l lysozyme were applied and retention times were monitored under isocratic conditions at a flow rate of 300 cm/h using a Spectra Systems P4000 HPLC system. Solute retention factor k' was calculated using Eq. (3):

$$k' = \frac{t_R - t_0}{t_0} \qquad (3)$$

where t_R is the retention time of the solute of interest and t_0 is the time for unretained solute to pass through the column. The relative standard deviation was determined out of threefold measurement to be < 2 %.

For the evaluation of the silica resins a performance score system was established. The performance score is based on three parameters namely resin life time, bed quality and bulk properties like specific volume. Points regarding these parameters were applied to the silica resins in the range from ten to thirty with thirty as best. The sum of points results in the performance score.

6.3.5 Results and Discussion

6.3.5.1 Electro-acoustic

The aim of the electro-acoustic experiments was to find an alternative and faster method for the characterization of small differences between the chromatographic properties of resins for biochromatography, which were not included in certificates of analysis. The CVI of several batches of five different materials was measured in different liquids.

The effect of different slurry liquids on the CVI measurements is shown in Fig. 1. For the MA material measurements the most reproducible results were achieved with ethanol, for MB, MG and Se gels with water, with a relative standard deviation of < 3 %. Not reproducible results for some solvents can be explained by particle whirling, which with a microscope can be observed with resin particles in regarding liquids. This could lead to irregular sedimentation after application of the sample on the probe. Within sulfo-resins different liquids were used. The effect described above could be caused by the base material and/or the spacer arm (length or structure).

The CVI signal height trends to be higher for measurements in water than in ethanol or acetone. This trend can be founded on the decrease in permittivity of water to ethanol and acetone, Eq. (2).

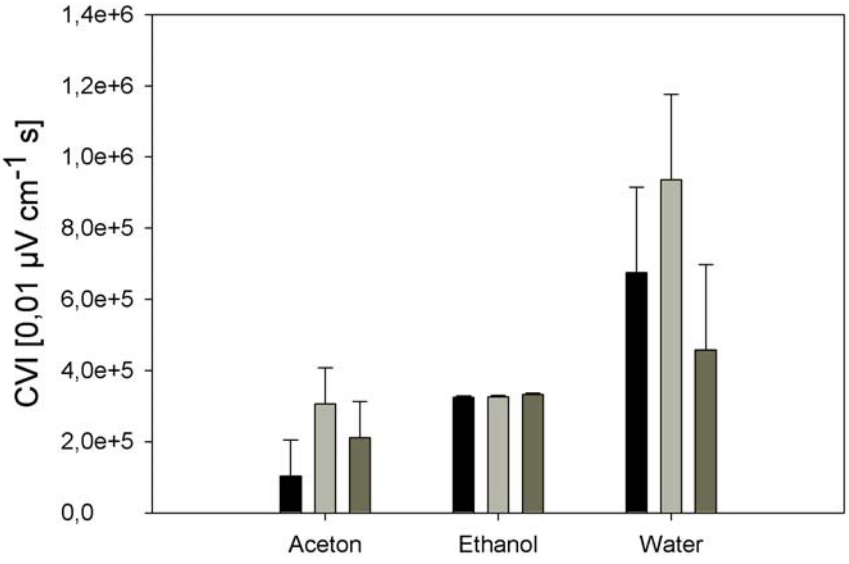

Fig. 1: CVI of threefold measurement (black, grey, dark grey) of one methacrylate resin in acetone, ethanol and water. The signal was reproducible in ethanol only.

Silica C4 particles could be worked on in organic media only, because wettability in water was not sufficient. Furthermore experiments with different volume fractions were performed to estimate their effect. The resin amount was reduced as far as possible to safe sample but maintaining reproducible results. The used experimental sets for the five resin types are listed in Tab. 3. The required volume fraction increases from silica resins to methacrylates and sepharoses. This fact could not be expected but the softness and the size of the resins increase in the same order [5, 21]. Assuming that softer and/or bigger particles attenuate the applied sonic waves less [22], higher sediment would be needed to decouple CVI signal from sediment thickness and reach signal saturation. By the missing data about the resin densities, it is not possible to explain this effect to the density differences affecting CVI, Eq. (2).

Tab. 2: Experimental sets for electro-acoustic measurements.

	Methacrylate MA	Methacrylate MB	Methacrylate MG	Sepharose	Silica
Solvent	ethanol	water	water	water	acetone
Volume fraction (resin/solvent)	26 % (v/v)	26 % (v/v)	26 % (v/v)	52 % (v/v)	20 % (w/v)

The results of the electro-acoustic measurements are summarized in Fig. 2. The CVI values were normalized to the highest value of each resin type. Significant batch to batch variances are pointed out for all tested resin types by the electro-acoustic method. The highest difference was detected for the MA (30 %), followed by Si (20 %), MG (19 %), MB (18 %) and Se (17 %) resins. Considering the small data body, the evaluation of these deviations could be not representative.

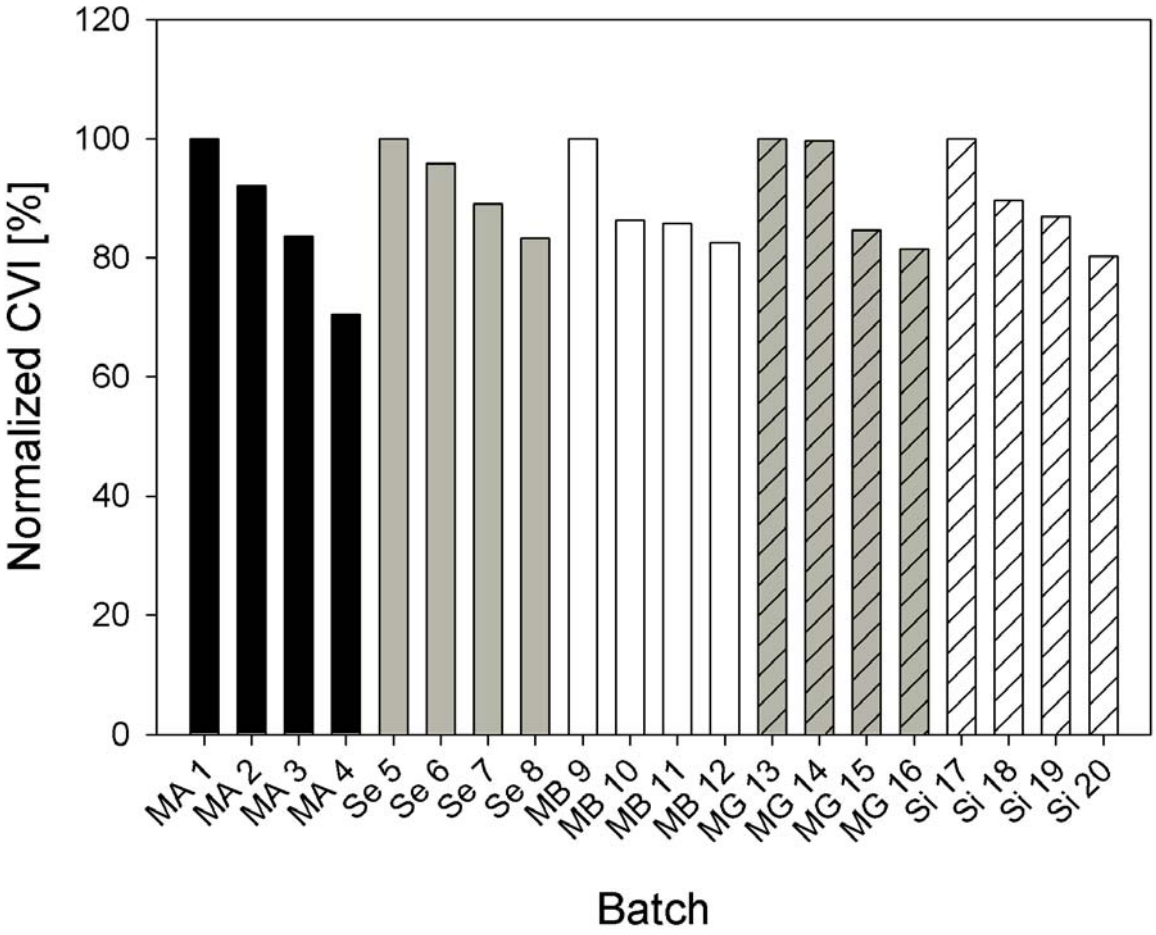

Fig. 2. CVI values of investigated resin batches, normalised to highest value of each resin type, show significant differences.

6.3.6 Chromatography

Chromatographic approaches were developed to show practical relevance of the detected batch to batch variances. Sulfo-functionalized gels were tested for retention factors of lysozyme pulses under isocratic high salt conditions. This experiments screen out the designated electrostatic protein – matrix interactions and mark residual hydrophobic or unspecific properties [8] of the tested ion exchange matrices [23]. Results of the chromatographic experiments with methacrylate and sepharose resins are shown Fig. 3. The retention factor values were normalized to the highest value of each resin type. Differences in retention factor

with Se resins were up to 11 %, with MA up to 13 %, MB up to 15 % and with MG resins up to 17 %. Again considering the small data body, assessment of resin reproducibility could not be done. Nevertheless significant variations between different resin batches regarding unspecific binding properties are shown. Higher amounts of unspecific bound protein hold the risk of less product recovery, worse purity caused by altered resolution factors and product loss by peak displacement. Peak areas in the pulse experiments show less significance as retention factors but trends are the same.

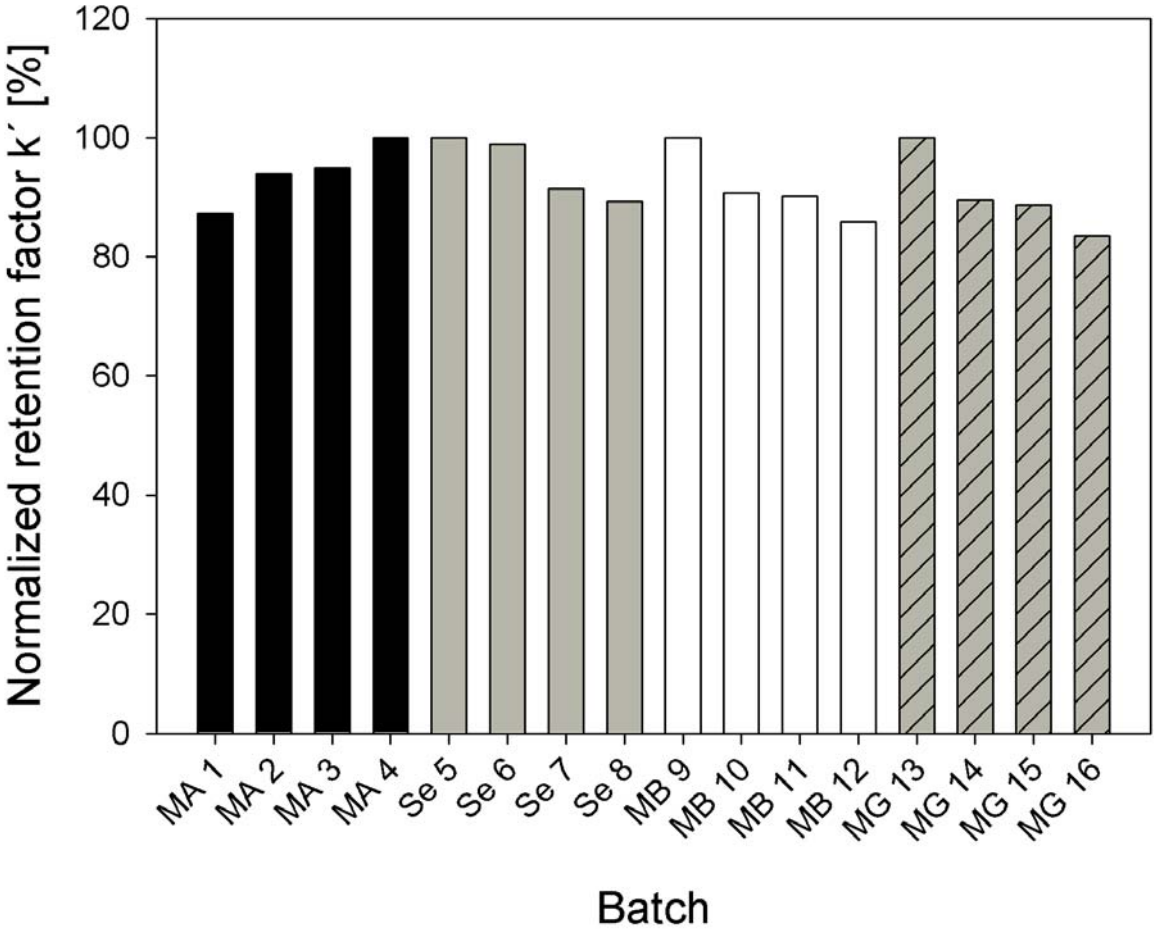

Fig. 3. Normalized retention factors of lysozyme under high salt conditions on methacrylate (black and white) and sepharose (grey) resins show significant differences.

Silica resin batches were evaluated regarding packing and life time properties. These data were kindly provided by an industrial cooperation partner. Silica resin batches differ in their

chromatographic behaviour and performance scores were established and listed in Tab. 3. Batches differ in all three quality positions. Long resin life time could reduce process costs, appropriate bed quality is required to use up resolution potential and constant bulk properties like specific resin volume is required for unchanging binding capacity when replacing the column packing material.

Tab. 3: Performance scores of silica resins.

	Life time	Bed quality	Bulk properties	Performance score
Batch 13	30	30	30	90
Batch 14	10	30	30	70
Batch 15	10	20	10	40
Batch 16	10	10	10	30

6.3.7 Correlations between electro-acoustic measurements and chromatographic resin properties

In the preceding chapters batch to batch variances were worked out for methacrylate, sepharose and silica resins with electro-acoustic and chromatographic approaches. These results are joined in this section. Both chromatographic and electro-acoustic method pointed out differences between sulfo-functionalized gels. Fig. 4 shows CVI values correlated with lysozyme retention factors for the tested sulfo-functionalized materials. Batches are ordered from highest to lowest CVI value. MA batches show lower CVI values with stronger retention. Fig. 4 also shows CVI values and lysozyme retention data regarding Se, MB and MG gels whereas the higher the CVI values the higher the lysozyme retention. On the first moment this data

seem to be contradictory. Considering that sepharoses and methacrylate MB and MG resins were measured in water and methacrylate MA resins in ethanol, this phenomenon can be explained as follows. At water – hydrophobic patch interphase hydroxyl ions adsorb spontaneously [24]. Therefore hydroxyl ions can adsorb at the hydrophobic patches on the sepharose, MB and MG beads, which could be the aliphatic spacer arm between base material and functional group [25]. In this case not only charged sulfo-groups but also adsorbed ions contribute to higher CVI values indicating more hydrophobic areas/patches, which cause stronger retention of lysozyme at high salt conditions. The case is different for MA particles, which were measured in ethanol. There no water – hydrophobic interphase existed eliminating the impact of hydroxyl ions. Additionally hydrolysed ester groups of carboxy type left by synthesis can contribute to CVI. Thus the more residual potentially charged carboxy groups on methacrylate particle surface the higher CVI could be measured and the lower is the expected hydrophobicity of the surface, which is shown by lower lysozyme retention. Additionally the detected differences between batches regarding appropriate resin types were higher with the electro-acoustic method than with the chromatographic method. Therefore electro-acoustic approach is faster, independent of column packing quality, requires less material and is more sensitive than classic chromatographic experiments.

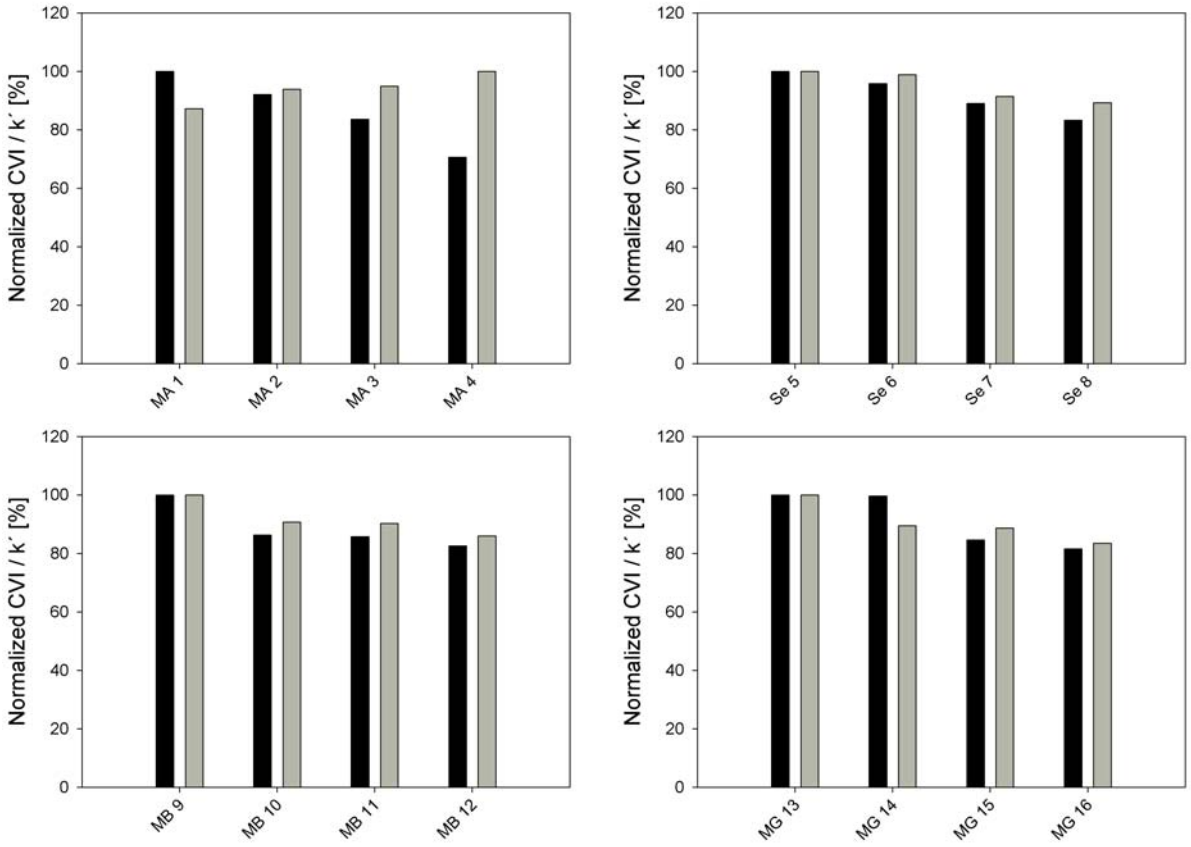

Fig. 4. Correlation between normalized electro-acoustic values (black) and lysozyme retention factors (grey) for MA, Se, MB and MG resins. MA batches (measured in ethanol) with lower CVI values show higher lysozyme retention. Se, MB and MG (measured in water) batches with higher CVI values show higher lysozyme retention.

The correlation between CVI values and performance scores is drawn in Fig. 5. Batches with good chromatographic performance show high CVI values and vice versa. In this case the main effect for high CVI values could be wettability of the particles. Incompletely moistened gels can cause difficulties in packing process leading to uneven beds, band broadening, high specific resin volume, which causes lower binding capacity, and enhanced fouling tendency. Regarding to CVI measurement incompletely wetted particles partially lack signal transmitting medium, which could be a reason for low CVI values. The balance between C4 substituted particle surface patches and possibly residual silanol groups can alter particle wettability and therefore packing performance in used solvent systems.

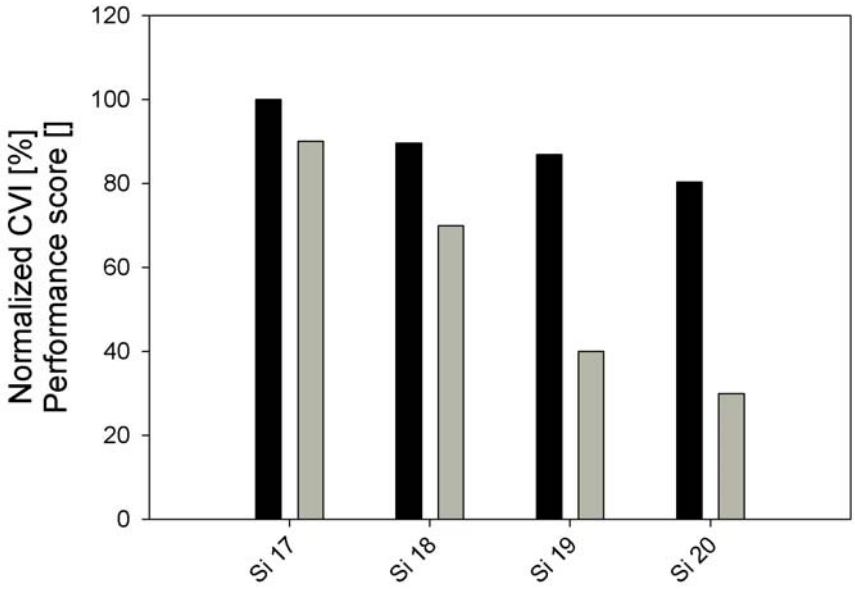

Fig. 5. Correlation between electro-acoustic values (black) and packing score (grey) for silica particles. Batches with higher CVI values show better material properties.

6.3.8 Conclusions

In this work correlations between electro-acoustic measurements of biochromatographic media and their chromatographic properties were pointed out. The electro-acoustic method is fast, requires low amounts of material and does not depend on column packing quality. Additionally it is more sensitive than the chromatographic approach. The electro-acoustic method allows detection of even small batch to batch variances possibly appearing during the manufacturing processes within product specifications [6] but not included in certificates of analysis. Tendency of altered unspecific binding, product recovery and purity, life time and stability of different resin batches can cause issues to guarantee product identity after replacing column packing. Therefore new batches have to be checked in time and cost consuming tests accepted by regulatory authorities. The electro-acoustic approach described in this work could be used for preselecting samples of the resin manufacturers to reduce the quantity of all-embracing acceptance tests. However, the small data body has to be extended to prove the

efficiency of the described method. Its relevance indeed depends on the particular separation process and should be reconsidered as the case arises. In addition CVI depends on a plurality of material properties like porosity, particle size, pH value or temperature. Therefore on the one hand it could be difficult to interpret results regarding specific material parameters but on the other hand it holds the potential to cover a wide range of applications. Furthermore electro-acoustic measurements potentially could be used to investigate resin life time for determination of time point to replace column packings. . Additionally other resin types functionalized with different ligands could be investigated to extent the application range of the method. Furthermore the development of a theory describing this special experimental set in electro-acoustic should be forwarded for better interpretation ability of results.

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9 Erklärung

Ich erkläre hiermit, dass ich die vorliegende Arbeit ohne unzulässiger Hilfe Dritter und ohne Benutzung anderer als der angegebenen Hilfsmittel angefertigt habe; die aus fremden Quellen direkt oder indirekt übernommenen Gedanken sind als solche kenntlich gemacht.

Stuttgart,

Unterschrift

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International Publications

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July 2007	Journal of Chromatography A: Investigation of Salt Properties with Electro-acoustic Measurements and their Effect on Dynamic Binding Capacity in Hydrophobic Interaction Chromatography (accepted)
June 2007	Lecture, European Bioperspectives, Cologne: Fast determination of conditions for maximal binding capacity in chromatography with human monoclonal antibodies.
Apr 2007	Journal of Chromatography A: Fast determination of conditions for maximum dynamic capacity in cation-exchange chromatography of human monoclonal antibodies.
Oct 2006	Lecture, ISPPP, Innsbruck: Zeta potential of human monoclonal antibodies to determine the conditions for maximum dynamic capacity in cation-exchange chromatography.

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June 2005 – present	Purification of monoclonal antibodies, establishment of in-process control, development of strategies for resin characterization

Curriculum vitae

Work Experience

Jan 2004 – Apr 2004	BASF AG, Germany, Internship Project: Assay – Development for high-throughput-screening, herbicides, plant enzyme preparation
July 2003 – Sept 2003	DIARECT AG, Germany, Internship Project: Expression, purification and characterization of antigens (*E. coli* and Sf9 – Insect cells), ELISA –Development
Mar 1999 – Mar 2001	Clinic of Villingen-Schwenningen, Germany, health care (25 %)
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Education

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