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Influence of pH on adsorption of human immunoglobulin gamma, human serum albumin and horse myoglobin by commercial chromatographic materials designed for downstream processing of monoclonal antibodies

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Abstract

Static adsorption capacity was measured for three different proteins: human immunoglobulin gamma, human serum albumin and horse skeletal muscle myoglobin. Eight commercial chromatographic materials were tested: FractoGel EMD SE HiCap (M), SP Sepharose Fast Flow, S Ceramic HyperD F, MabSelect, rProtein A Sepharose Fast Flow, Poros 50A High Capacity, ProSep –vA High Capacity and MEP HyperCel. The adsorption capacity was measured at pH values of 4.0, 5.0, 6.0, 7.0 and 8.0.

Keywords: adsorption capacity, chromatography, horse myoglobin, human immunoglobulin gamma, human serum albumin

Introduction

Chromatographic methods are an important component of each downstream processing of monoclonal antibodies (Roque 2004). Numerous different chromatography modes might be employed – affinity, ion-exchange, or hydrophobic interactions. In all of these processes, the interaction between stationary phase and solute molecules is modified *in situ* by properties of the mobile phase such as its pH, ionic strength and presence of other compounds. These properties have an influence on the adsorption capacity and selectivity.

The influence of pH on the adsorption capacity of cation exchangers is well understood. The decrease of pH value results in an increase of the concentration of H_3O^+ cations, which, on one hand, compete with charged solute molecules for negatively charged

adsorption sites and, on the other hand, have an impact on the total charge of solute molecules. The second mentioned effect might be approximately predicted from protein's amino acid sequence (Fig.1.). The value of pH also influences the protein binding on affinity and hydrophobic charge-induction chromatographic materials since the binding is in both cases based partially on polar interactions. The interactions are however far more complex and strongly depend on sterical issues, e.g. protein conformation. Presently, there are no reliable computational means to predict and quantify such interactions and direct measurements remain the only credible method.

In this work, the influence of pH on the adsorption capacity was studied. Human immunoglobulin gamma (IgG) was used as an antibody of interest and human serum albumin (HSA) and horse skeletal muscle myoglobin (MYO) were selected to represent common protein impurities. Three of tested chromatographic materials were strong cation exchangers (FractoGel, SP Sepharose and S Ceramic HyperD F), four were affinity materials functionalized by recombinant protein A (MabSelect, rProtein A Sepharose, Poros 50A and ProSep) and one material contained a hydrophobic charge induction ligand 4-mercapto-ethyl-pyridine (MEP HyperCel).

Materials and Methods

Chromatographic materials used and their respective producers were as follows: FractoGel EMD SE HiCap (M) (Merck, Darmstadt, Germany), SP Sepharose Fast Flow (Amersham Biosciences, Uppsala, Sweden), S Ceramic HyperD F (BioSepra, Cergy-Saint-Christophe, France), MabSelect (Amersham Biosciences, Uppsala, Sweden), rProtein A Sepharose Fast Flow (Amersham Biosciences, Uppsala, Sweden), Poros 50A High Capacity (Applied Biosystems, Foster City, CA, USA), ProSep –vA High Capacity (Millipore, Bedford, MA, USA) and MEP HyperCel (BioSepra, Cergy-Saint-Christophe, France). Essential information about tested chromatographic media is summarized in Tab.1.

All used proteins were of a high purity grade. The source of polyclonal human IgG was product Gammanorm (Octapharma, Stockholm, Sweden), whose protein content comprises of 95% IgG (59% IgG_1, 36% IgG_2, 4.9% IgG_3 and 0.5% IgG_4). HSA and MYO were purchased in lyophilized form from Sigma, St. Louis, MO, USA (HSA 99% purity, MYO 95-100% purity).

Tab.1. Properties of chromatographic media

Material	Type	Ligand	Matrix	Shape and size
MabSelect	affinity	recombinant protein A	crosslinked agarose	beads, average 85 μm
rProtein A Sepharose FF	affinity	recombinant protein A	crosslinked agarose	beads, average 90 μm
Poros 50A HiCap	affinity	recombinant protein A	crosslinked poly(styrene-divinylbenzene)	beads, average 50 μm
ProSep –vA HiCap	affinity	protein A	controlled-pore glass	irregular, average 75 μm
FractoGel EMD SE HiCap	cation exchange	sulphoethyl	crosslinked polymethacrylate	beads, average 65 μm
SP Sepharose FF	cation exchange	sulphopropyl	crosslinked agarose	beads, average 90 μm
S Ceramic HyperD F	cation exchange	sulphonate	hydrogel polymerized inside ceramic particle	beads, average 50 μm
MEP HyperCel	hydrophobic charge induction	4-mercapto-ethyl-pyridine	crosslinked porous cellulose	beads, average 90 μm

The buffers were prepared using Millipore-grade water and filtered through 0.45 μm filter. All used chemicals were of p.a. grade, purchased from MikroChem, Pezinok, Slovakia (acetic acid, 99%; sodium acetate, anhydrous; potassium dihydrogen phosphate, anhydrous; di-sodium hydrogen phosphate, dodecahydrate). An acetate buffer was used for the measurements at pH 4.0 and 5.0. The measurements at pH 6.0, 7.0 and 8.0 were performed in a phosphate buffer. The concentration of salts in each prepared buffer was 50 mM. The buffers were used for equilibration of chromatographic materials as well as for preparation of bulk protein solutions.

The adsorption capacity of each protein from one-component protein solutions was measured by a static method. A storage solution (usually containing ethanol) was washed out

from samples of chromatographic materials with redistilled water and then with a buffer of specific pH. The particles were repeatedly re-suspended in respective buffer and left overnight to equilibrate. Before use, each material was rinsed several times with an excess of buffer. Inter-particle liquid was removed from the slurry by suction on a glass frit.

Precise amounts of wet chromatographic materials (10 to 100 mg) were transferred into a set of plastic micro-vials. Consequently, a specific amount of standard protein solution was added into each vial using a micropipette. The volume of protein solution was 1.3 mL and the concentration of protein varied up to 3 g/L depending on a theoretical estimation for each adsorbent and protein. The vials were hermetically sealed and fixed into the shaker GFL 1083 (Gesellschaft für Labortechnik, Burgwedel, Germany) in a horizontal position. Equilibrium conditions were reached at ambient temperature after 24 hours.

Afterwards, a liquid sample was taken from each vial. To remove particles, the samples were filtered through a low protein binding membrane Durapore (PVDF, 0.22 μm) (Millipore, Bedford, MA, USA). The protein concentration was determined by spectrophotometric measurement using a diode array detector set at 280 nm (Agilent, Palo Alto, CA, USA). The calibration curves with a correlation coefficient of over 99.9% were obtained using original bulk protein solutions of known concentration and their multi-fold dilutions. A unique calibration curve was measured for each pH.

The static adsorption capacity was determined from the decrease of protein concentration in the bulk liquid using the material balance equation (1).

$$q = \frac{\left(c_0 - c^*\right)V}{m_a} \tag{1}$$

q adsorption capacity, [mg/g]
c_0 initial protein concentration in the bulk solution, [mg/mL]
c^* equilibrium protein concentration in the bulk solution, [mg/mL]
V volume of bulk protein solution, [mL]
m_a weight of wet adsorbent, [g]

Results and Discussion

The total charge of examined proteins was calculated as a function of pH by the method outlined in (Lehninger 1982). The calculation was based on amino acid sequences of

IgG_1 (Padlan 1994), HSA and MYO (Protein Data Bank, http://www.pdb.org). Obtained values are approximate, since the method assumes sterically unhindered ionizability of all polar residues.

The adsorption capacity of a specific protein by a specific material is a function of several process conditions. The most significant ones are the pH, ionic strength of the solution and temperature. In this study, the pH and ionic strength were precisely controlled and all experiments were simultaneously conducted at the same ambient temperature. A single point of adsorption isotherm (with respect to equilibrium protein concentration) was measured.

The amount of adsorbed protein, as a function of equilibrium protein concentration in the bulk liquid, usually has a form describable by Langmuir equation. The term "adsorption capacity" represents upper limiting value of adsorbed protein amount, which is not a function of equilibrium protein concentration in the solution. The points of adsorption isotherm are close to this value at higher equilibrium concentrations; usually above 1 g/L. Thus, the experimental setup was designed in the way to measure a point of the isotherm located close to the adsorption capacity – to provide a value that would be inter-comparable among individual resins.

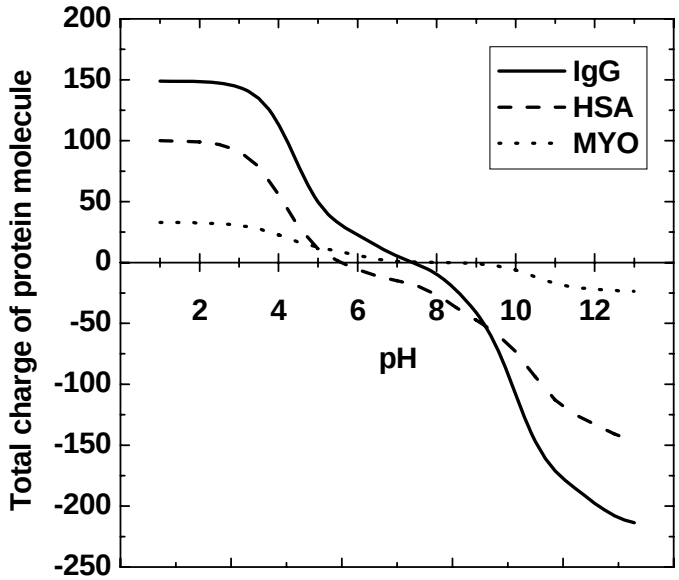

Fig.1. Charge of investigated protein molecules as a function of pH

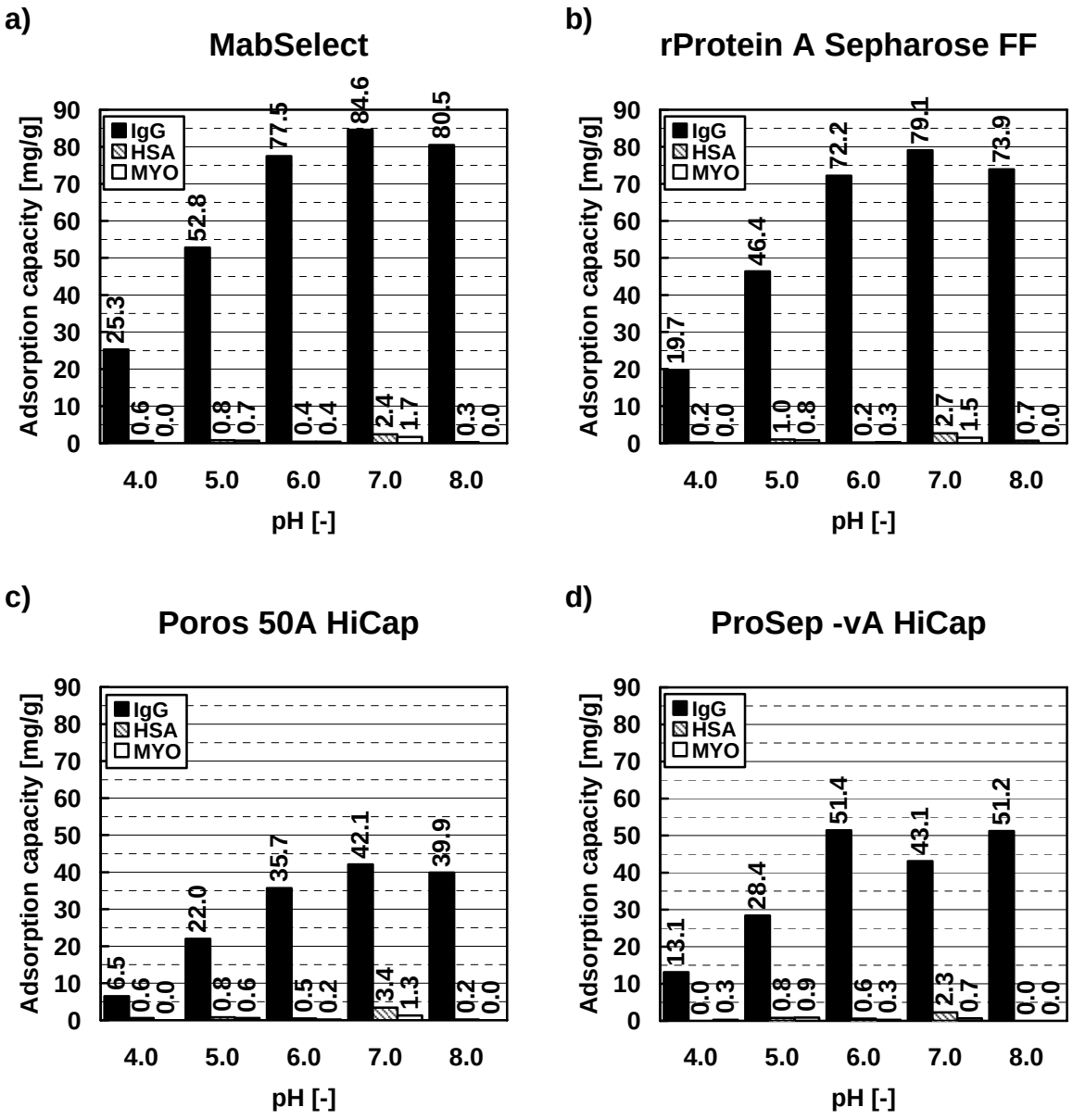

Fig.2. Adsorption capacity of affinity materials as a function of pH

The study was intended especially to detect sudden sharp changes of adsorption capacity with pH (ion-exchangers) and to determine overall trend of adsorption capacity as a function of pH (affinity and hydrophobic resins). This information would help to narrow the range of pH for optimal process conditions and for further experiments with multicomponent protein mixtures to determine the capacity/selectivity ratio. Another important aspect was to prove whether theoretically calculated protein charges (Fig. 1) correlate well with the experimentally determined adsorption capacity of ion exchangers. The results are presented in form of charts accompanied by numeric values (Fig. 2 – 4).

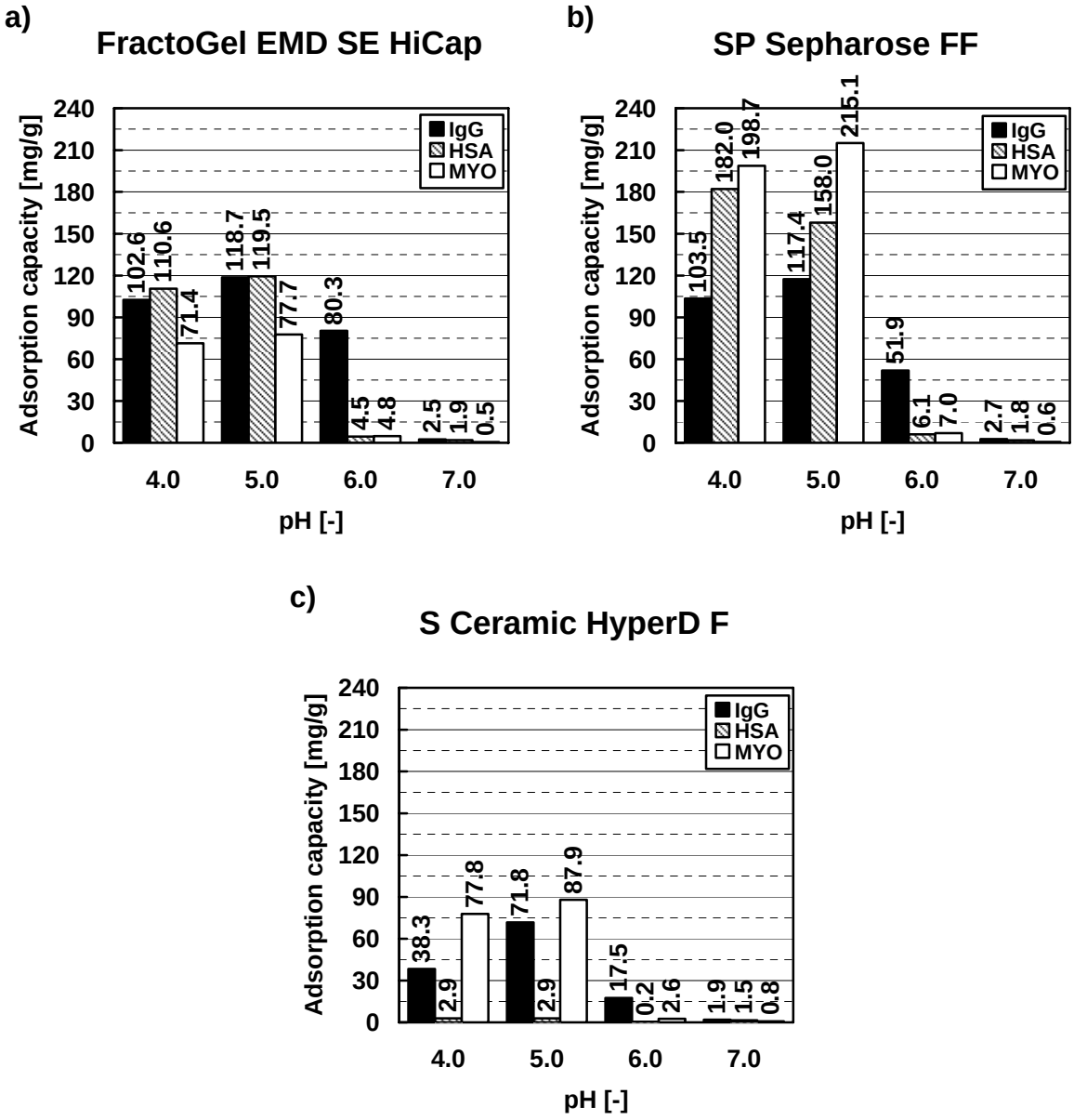

Fig.3. Adsorption capacity of ion-exchanger materials as a function of pH

The adsorption capacity is displayed in units of milligram adsorbed protein per gram of wet resin. This is pertinent to experimental method, in which the weight of wet resin samples was directly measured. At such small scale (10 to 100 mg samples), it is easier and more precise to determine the weight instead of volume. However, the conversion of adsorption capacity value to more conventional units, e.g. mg/mL, is quite straightforward. All the resins had very similar apparent density in the wet state, ranging from 1.02 to 1.06 g/mL (except for ProSep made of porous glass, which had apparent density of 1.3 g/mL) (Gramblička, unpublished).

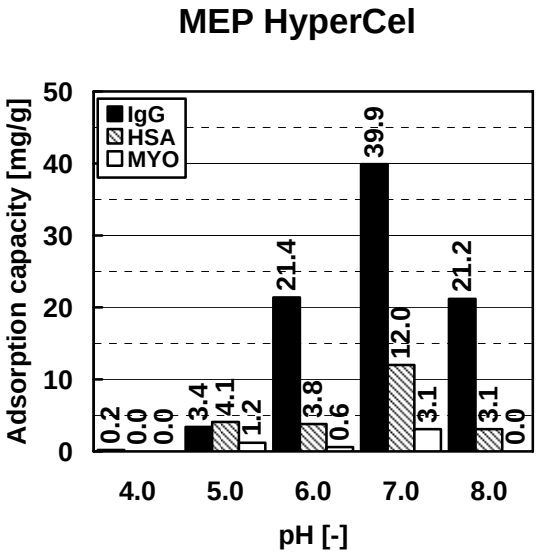

Fig.4. Adsorption capacity of hydrophobic charge-induction material as a function of pH

Thus, the value of adsorption capacity in mg/mL differs from the numeric value of capacity expressed in mg/g at most by 6%, except for ProSep – in this case, the value of adsorption capacity in units of mg/mL is by 30% higher than the value of capacity expressed in mg/g. The adsorption capacity expressed in milligram adsorbed protein per milliliter of bed volume (of chromatographic material packed in a column) necessitates the value of bed void fraction. This parameter depends on packing procedure.

The results show that all protein-A affinity media exhibited the same trends of adsorption capacity as a function of pH. The adsorption capacity of impurity proteins (HSA and MYO) was negligible in the whole pH range; its value was usually within the experimental error of the method (about 1 mg/g). The only slight increase was observed at pH 7.0, where adsorption capacity of HSA exceeded the value of 2 mg/g and MYO the value of 1 mg/g. These results confirm a high selectivity of affinity adsorbents for IgG. The dependence of IgG adsorption capacity on pH exhibited an optimum, lying within the range of pH values 6.0 to 8.0, close to pH 7.0. A neutral pH thus seems to be optimal for separations employing tested affinity media. Softer affinity materials (MabSelect, rProtA Sepharose FF – both based on agarose matrix) exhibited a higher adsorption capacity for IgG than rigid affinity materials, whose capacity was by 30 to 50% lower.

Cation exchangers, as a group, also displayed similar qualitative behaviour with respect to adsorption capacity as a function of pH. This may be attributed especially to the fact that all examined materials were functionalized by similar strong ionic group – the sulphonate.

However, each material contained a different ligand spacer – e.g. ethyl or propyl. At lower pH values, up to the value of 5.0, all three tested proteins had a high positive net charge (Fig.1.), thus they were binding well and the selectivity with respect to IgG was low. The only exception was binding of HSA on S Ceramic HyperD F. The adsorption of this protein on resin was very low throughout the whole tested pH range. A breakpoint occurred between pH values of 5.0 and 6.0. In this range, the adsorption capacity of HSA and MYO steeply dropped to very low values, while chromatographic materials retained a reasonably high capacity for IgG. The highest adsorption capacity as well as the selectivity for IgG at pH 6.0 was attained by FractoGel EMD SE HiCap. This behaviour complies well with the predicted net charge of proteins (Fig.1.). The isoelectric point of HSA lies below a pH value of 6.0 and MYO has a very small positive net charge at pH 6.0. On the other hand, IgG retains a significant positive charge at pH 6.0 and its predicted isoelectric point lies between pH 7.0 and 8.0. The measurements explicitly confirmed that optimal conditions of separation are located within a pH range from 5.0 to about 6.5. The values of pH over 7.0 are unusable for the separation of any tested protein since their adsorption capacity was negligible.

MEP Hypercel, the only tested resin with hydrophobic charge induction functionality, exhibited an unique behaviour. The interactions of this type of ligand are complicated to predict, since they consist of complex combination of hydrophobic and polar interactions. At lower pH values (4.0 to 5.0), the adsorption capacity of all tested proteins was too small. At pH values of 6.0, 7.0 and 8.0, the selectivities of IgG vs HSA and IgG vs MYO remained similar, while the absolute values of adsorption capacities went through an optimum. It is located near the pH value of 7.0, which is thus the most reasonable one for separation.

Conclusion

Experiments confirmed that the employed method is time- and cost-effective for screening purposes. Static adsorption capacity might be used as an indicator for discrimination of optimal process conditions for separation and for preliminary comparison of performance of multiple considered materials. The results show that the static adsorption capacity of ion exchangers correlates reasonably with predicted protein net charges. Obtained results could be especially used to narrow the pH range for further multicomponent adsorption-desorption experiments.

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