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Microcalorimetric study of the adsorption of PEGylated lysozyme on a strong cation exchange resin

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ABSTRACT

Adsorption of native as well as mono-, di-, and tri-PEGylated lysozyme on Toyopearl Gigacap S-650M in sodium phosphate buffer is studied by isothermal titration calorimetry and by independent adsorption equilibrium measurements at pH 6 and 25 °C. The production and separation of PEGylated lysozyme is discussed. Two different PEG sizes are used (5 kDa and 10 kDa) which leads to six different forms of PEGylated lysozyme which were systematically studied. The sodium chloride concentration is varied according to the elution conditions in the production process. The specific enthalpy of adsorption Δh_p^{ads} is determined from the calorimetric and the adsorption equilibrium data. It was found to be exothermal and constant with increasing adsorber loading for native lysozyme. For all PEGylated forms there is an influence of the adsorber loading on Δh_p^{ads} which is found to become more important with increasing PEGylation degree (total molecular weight of conjugated PEG). At low loadings the adsorption of all PEGylated lysozyme forms is exothermal. With increasing loading the adsorption becomes less exothermal and for the species with higher PEGylation degree also endothermal effects are observed at higher loadings. A thermodynamic analysis is carried out by which the enthalpic and entropic contributions to the binding constants are quantified. The findings are discussed on a molecular level. The results provide insight into the adsorption mechanisms of polymer-modified proteins on chromatographic resins.

1. Introduction

Upon PEGylation one ore more polyethylene glycol (PEG) molecules are covalently bound to a protein. This chemical modification of proteins is of increasing interest for the pharmaceutical industry as the properties of the protein can be modified in order to improve, e.g. in vivo half life, chemical stability, immunogenicity and solubility. PEGylation chemistry and strategies as well as their influence on protein behaviour have been described elsewhere [1–4]. Upon PEGylation usually different forms of PEGylated protein are obtained. They differ in the site to which the PEG is attached as well as in the number of sites that are PEGylated.

The purification task after PEGylation is always a separation of the PEGylated protein from unreacted protein and PEG as well as a separation of different PEGylation products. It has been shown that ion exchange chromatography (IEC) is a promising technique to accomplish this task as PEGylation influences the surface charge of the protein and its binding behaviour [5–7]. However, the design of IEC-based process in downstream processing of proteins is very complex due to the large number of process parameters like type and concentration of salt, temperature, pH, etc. There is a lack of fundamental understanding of the retention mechanisms in ion exchange chromatography which leads to an inefficient process design often based on high throughput (HTS) studies [8]. Much work has been done in the past to overcome this drawback, especially calorimetric techniques were shown to yield results which contribute to a better understanding of the adsorption mechanisms. Dieterle et al. [9] have shown that the adsorption of monoclonal antibodies on cation exchange resins is far from being a strongly exothermal process driven by ionic forces. Similar results were found in other studies on the adsorption energetics of proteins on chromatographic resins especially at high salt concentrations, adsorber loadings and near the isoelectric point of the proteins [10,11]. It has been shown that there also can be strongly endothermal effects in ion exchange chromatography similar to the behaviour found in hydrophobic interaction chromatography [12–14] and that entropic effects must play a major role in the adsorption of proteins on ion exchange resins [15].

In the present study this work is extended to PEGylated proteins. The production process for PEGylated lysozyme was adopted from Moosmann et al. [16] and adjusted to our needs. Lee and Park [17] have shown that only three out of six lysine residues react in a considerable amount (Lys 33, 97, 116). Therefore six different forms of PEGylated lysozyme were obtained: mono-, di-

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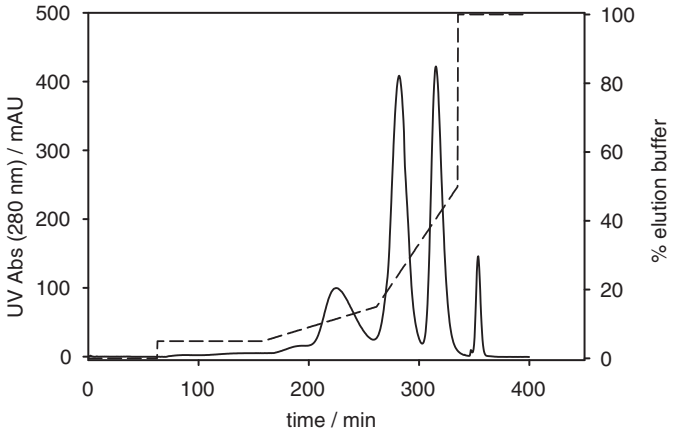

Fig. 1. Chromatogram for the separation of a 5 kDa PEGylated lysozyme sample. UV absorbance at 280 nm corresponds to the full line, elution buffer composition to the dashed line. Peaks in order of elution: tri-PEG-lysozyme, di-PEG-lysozyme, mono-PEG-lysozyme, native lysozyme.

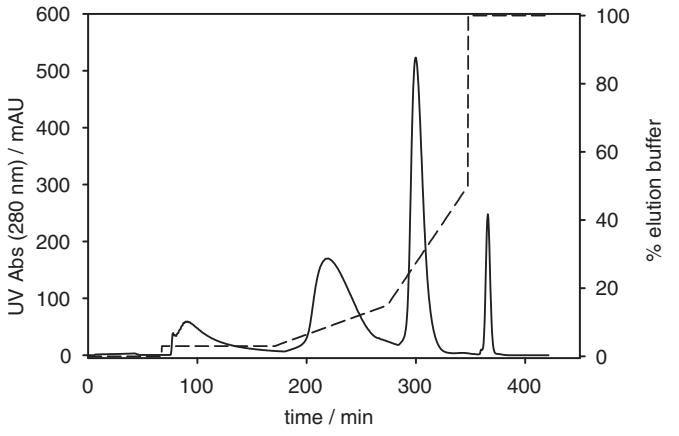

Fig. 2. Chromatogram for 10 kDa PEG-lysozyme. UV absorbance at 280 nm corresponds to the black line, elution buffer to the dashed line. Peaks in order of elution: tri-PEG-lysozyme, di-PEG-lysozyme, mono-PEG-lysozyme, native lysozyme.

and tri-PEGylated lysozyme for 5 and 10 kDa PEG each. Note that each mono- and di-PEGylated form is a mixture of molecules with different PEG attachement points. Further information about PEG binding residues and isoform concentrations can be found in [16]. The adsorption of native lysozyme and these six forms of PEGylated lysozyme on Toyopearl Gigacap S-650M, a strong, polymer-grafted, cation exchange resin, was studied with microcalorimetric and adsorption equilibrium measurements. Note that the adsorption process of which the thermodynamic details were studied, is the same one which was also employed in the present work to separate the PEGylated lysozyme forms. The specific enthalpy of adsorption Δh_p^{ads} of the PEGylated lysozyme forms was determined. A new way to thermodynamically analysing the results is presented which gives further insight in the enthalpic and entropic contributions to the adsorption of the proteins. A molecular picture is discussed which describes the complex adsorption mechanism.

2. Materials and methods

2.1. Chemicals

Hen egg white lysozyme was obtained from Sigma–Aldrich (St. Louis, MO, USA, L6876). Methoxy-PEG-aldehyde was obtained in two average molecular weights (5 and 10 kDa) from NOF Corp. (Tokyo, Japan). The strong cation-exchange resin Toyopearl Giga-Cap S-650M was obtained from Tosoh Bioscience GmbH (Stuttgart, Germany). The salts used for the buffer preparation, namely monosodium phosphate ($NaH_2PO_4 \cdot 2H_2O$), disodium phosphate ($Na_2HPO_4 \cdot 2H_2O$), and sodium chloride (NaCl), were obtained from Carl-Roth GmbH (Karlsruhe, Germany). All other chemicals were of analytical grade and obtained from Carl-Roth GmbH (Karlsruhe, Germany).

2.2. Production of PEGylated lysozyme

The production process for the preparation of PEGylated lysozyme was adopted from Moosmann et al. [16] and optimized for the present equipment. Process analytics were carried out exactly as described in [16].

2.2.1. Reaction

The reaction was carried out in a 25 mM sodium phosphate buffer containing 20 mM $NaCNBH_3$. Initial concentrations were 5 g/l lysoyzme as well as 4 g/l and 8 g/l of 5 kDa and 10 kDa methoxy-PEG-aldehyde, respectively. The reaction was carried out in 500 ml PE bottles in a thermostated shaking bath at 15 °C for 20–48 h. Reaction time was adjusted to the required PEGylation degree [16,17].

2.2.2. Chromatographic separation

Preparative purification was carried out on an Äkta Purifier 100 UPC System (GE Healthcare, Uppsala, Sweden) equipped with an Omnifit glas column (25 mm × 400 mm, Bio-Chem Fluidics, Cambridge, UK) packed with Toyopearl Gigacap S-650M (Bed height 30 cm, CV = 150 ml). A multiple gradient chromatographic step was carried out from 0 to 500 mM NaCl. The gradients and corresponding chromatograms are shown in Figs. 1 and 2, respectively.

After chromatographic purification the pooled fractions were ultrafiltrated with ultrapure water to remove remaining salt using Vivaflow 50 membranes (Sartorius Stedim Biotech GmbH, Göttingen, Germany) with a MWCO of 10 kDa. Then the concentrated samples were freeze-dried for storage using a Lyovac GT2 Lyophilizator (Amsco Finn-Aqua GmbH, Huerth, Germany).

2.3. Adsorption equilibrium isotherms

The equilibrium adsorption experiments were carried out in 1.5 ml microtubes. First, the desired volume of adsorber (20–80 μl) was filled into the microtube using a MediaScout ResiQuot device (Atoll GmbH, Weingarten, Germany). Then protein solution with the desired concentration was added to a total volume of 1 ml. The tubes were stirred with mini-magnetic propellers for 3 h at 25 °C. Preliminary experiments showed that equilibrium is always reached within that time. After equilibration and filtration with 0.22 μm syringe filters the protein concentration in the liquid phase c_p was determined by UV Absorption at 280 nm with a UV-photometer (PerkinElmer, Waltham, MA, USA). The UV extinction coefficients of all PEGylated forms of lysozyme were calculated from the theoretical molecular weights under the assumption that PEG does not influence UV-absorption of lysozyme and are shown in Table 1.

Table 1
UV extinction coefficients at 280 nm for lysozyme and PEGylated lysozyme isoforms.

Protein	Theoretical M_W (kDa)	E_{280} (ml g^{-1} cm^{-1})
Lysozyme	14.3	2.50
Mono-PEG-5	19.3	1.85
Di-PEG-5	24.3	1.47
Tri-PEG-5	29.3	1.22
Mono-PEG-10	24.3	1.47
Di-PEG-10	34.3	1.04
Tri-PEG-10	44.3	0.81

Table 2
Parameters of the colloidal model for the adsorption equilibrium isotherms of PEGylated and native lysozyme in 25 mM PO_4 pH 6, 25 °C. Note that for the thermodynamic analysis of native lysozyme without sodium chloride a linear fit of the steep initial part of the isotherm has been used with $K_p^{ads} = 9153$.

		Colloidal			
	c_{NaCl} (mM)	K_p^{ads} (–)	β (–)	γ (–)	NRMSD (10^{-2})
Lysoyzme	0	4.11E+06	6.21	22.78	25.0
	100	9.83E+02	101.12	71.30	3.8
	200	6.83E+01	0.57	18.40	1.4
Mono-PEG-lysoyzme-5 kDa	0	3.87E+05	8.73	24.32	2.9
	50	2.80E+01	101.35	55.28	0.2
	100	5.62E+00	1.85	15.21	1.6
Di-PEG-lysoyzme-5 kDa	0	3.24E+04	2.340	7.51	3.0
	25	8.55E+01	21.86	23.28	0.7
	50	5.79E+01	0.70	1.24	0.3
Tri-PEG-lysoyzme-5 kDa	0	4.94E+03	1.18	5E−6	4.7
	10	1.44E+03	1.28	1E−6	3.5
	20	9.22E+01	0.78	4E−5	3.6
Mono-PEG-lysoyzme-10 kDa	0	3.50E+04	1.79	5.46	3.2
	25	7.76E+02	1.59	5.46	0.5
	50	5.76E+01	0.92	2.81	1.0
Di-PEG-lysoyzme-10 kDa	0	2.99E+03	1.33	3E−5	5.0
	10	3.36E+02	1.11	6E−6	3.1
	20	3.60E+01	0.69	9E−7	4.1
Tri-PEG-lysoyzme-10 kDa	0	5.37E+01	1.14	4E−5	7.2
	5	4.98E+00	153.14	15.60	6.3
	10	2.80E+02	3.55	6E−5	2.0

2.4. Isothermal Titration Calorimetry

The microcalorimetric measurements were carried out with an ITC4200 microcalorimeter (Calorimetry Science Corp., Lindon, NY, USA) as previously described in [9]. The 1 ml sample ampoule was filled with the desired amount of adsorbent (20–80 μL) using a MediaScout ResiQuot device (Atoll GmbH, Weingarten, Germany). Then the resin was suspended in 800 μl of degassed buffer solution. The content was continuously stirred at 430 rpm with a propeller stirrer. Preliminary experiments showed that all resin particles are suspended in solution at this stirring rate. After typically 1 h thermal equilibrium was reached and the degassed protein solution was titrated into the cell stepwise. A 250 μl Hamilton glass syringe (Hamilton Bonaduz AG, Bonaduz, Switzerland) fitted with a 800 mm stainless steel needle was used for the injections. The injections were driven by a computer controlled KDS200 syringe pump (KDScientific, Holliston, MA, USA). Twenty-five 10 μl injections were done with a 30 min interval between two injections. The temperature was 25 °C.The accuracy of the temperature is better than 0.1 °C, fluctuations are below 0.01 °C.

3. Theory

3.1. Equilibrium models

In order to calculate the mass of protein adsorbed after each injection of the ITC experiments the experimental results for the equilibrium isotherms have to be correlated with a model. Note that this model does not have to be physically justified and any suitable equation could have been used for the correlation. Nevertheless, a model from literature was applied which is used for the correlation of results from protein adsorption equilibrium experiments.

3.1.1. Colloidal model

The colloidal model was originally developed by Oberholzer and Lenhoff [18] and modified for fitting experimental results by Chang and Lenhoff [19]:

$$c_p = \frac{q_p}{K_p^{ads}} \cdot \exp\left[\beta\sqrt{q_p/q_0} \cdot \exp\frac{-\gamma}{\sqrt{q_p/q_0}}\right] \quad (1)$$

K_p^{ads} is the equilibrium constant which is equal to the initial slope of the equilibrium isotherm (see Appendix in supplementary material). β and γ are lumped fitting parameters [19] which are kept dimensionless by the introduction of $q_0 = 1\ mg\ ml^{-1}$.

3.2. Thermodynamic analysis

It is common to analyze binding and adsorption phenomena in terms of equilibrium constants. The equilibrium constant represents the difference between the chemical potential of the protein in the liquid and in the adsorbed phase reference states (for a derivation of the following equations see Appendix in supplementary material).

$$K_p^{ads} = \exp\left[-\frac{\mu_p^{ref'} - \mu_p^{ref''}}{RT}\right] \quad (2)$$

Thus, the difference of the molar Gibbs energies in the reference states $\Delta g_p^{ads\,ref}$ can be calculated from the equilibrium constant.

$$\Delta g_p^{ads\,ref} = -RT \ln K_p^{ads} \quad (3)$$

The molar Gibbs energy can be split up in the contributions of entropy and enthalpy.

$$\Delta g_p^{ads\,ref} = \Delta h_p^{ads\,ref} - T \cdot \Delta s_p^{ads\,ref} \quad (4)$$

These two contributions give insight into the mechanisms occurring upon adsorption. If the enthalpy contribution $\Delta h_p^{ads\,ref}$ is dominant it is common to refer to an "enthalpy-driven" process. The term "entropy-driven" is used if the entropy contribution $T \cdot \Delta s_p^{ads\,ref}$ is dominant. It can be shown that the specific enthalpy of adsorption in the reference state $\Delta h_p^{ads\,ref}$ is equal to the specific enthalpy of adsorption Δh_p^{ads} in infinite dilution. The latter can be determined from the experimental ITC data by extrapolation. Furthermore, $\Delta g_p^{ads\,ref}$ can be determined from the initial slope of the experimental equilibrium isotherm.

$$K_p^{ads} = \lim_{c_p \to 0}\left(\frac{c_p}{q_p}\right) \quad (5)$$

Hence, the entropy contribution can be calculated from Eq. (4). Until now, this analysis has only been carried out for infinite dilution

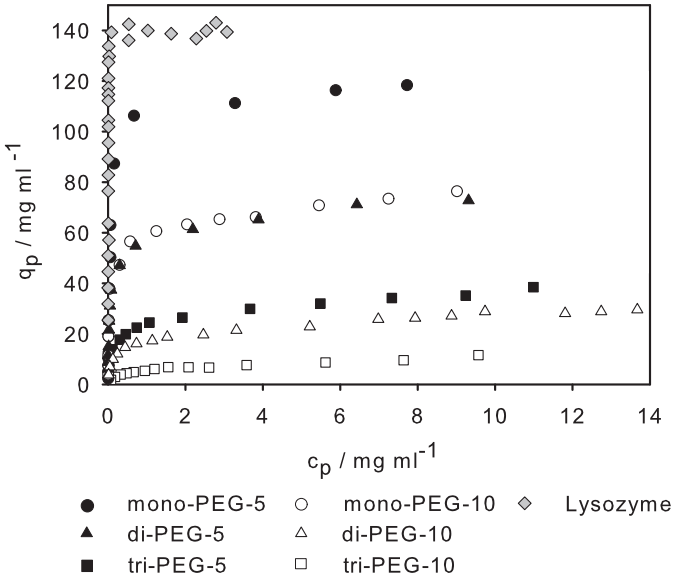

Fig. 3. Equilibrium adsorption isotherms for native lysozyme and all PEGylated forms in 25 mM sodium phosphate buffer, pH 6.0, 25 °C, without NaCl on Toyopearl Gigacap S-650M. The adsorbed phase concentration q_p is plotted versus the liquid phase concentration c_p.

as this is usually chosen as the reference state. For other concentrations the activity coefficients in both phases would have to be considered. In this work, we present a method to overcome this drawback. The reference state is no longer predefined as refering to infinite dilution. Rather, we allow for choosing it freely as refering to any concentration of interest on the equilibrium isotherm. More precisely, if the reference state, which is labeled here with an asterisk *, is chosen to refer to a certain concentration c_p^{ref*} for the liquid phase, the reference state for the adsorbed phase is chosen to refer to the loading q_p^{ref*} which is in equilibrium with c_p^{ref*}. These new reference states lead to differently defined activity coefficients which, however, are unity in the equilibrium state of interest. Applying this idea allows carrying out the same analysis as described above for every point on the adsorption equilibrium isotherm and not only for infinite dilution.

$$\Delta g_p^{ads\,ref*} = \Delta h_p^{ads\,ref*} - T \cdot \Delta s_p^{ads\,ref*} \tag{6}$$

Again, the specific enthalpy of adsorption in the reference state $\Delta h_p^{ads\,ref*}$ is equal to the measured value in the ITC experiment Δh_p^{ads} at this concentration. The equilibrium constant K_p^{ads*} can be determined from the slope of the equilibrium isotherm at the concentration of interest.

$$K_p^{ads*} = \left.\frac{dq_p}{dc_p}\right|_{c_p = c_p^{ref*}} \tag{7}$$

Hence, this new approach paths the way for analyzing the enthalpy and entropy contributions to the adsorption of a protein over the whole concentration range on the isotherm. Note that the different reference states are no longer independent, but this is no drawback for the type of analysis they are applied for. In the conventional approach the state of infinite dilution has a distinguished role. This distinction is, however, an arbitrary choice which we do not keep. Rather, we treat all states of the equilibrium isotherm in the same way. The theoretical background and the derivation of the new methodology is presented in full detail in Appendix in supplementary material.

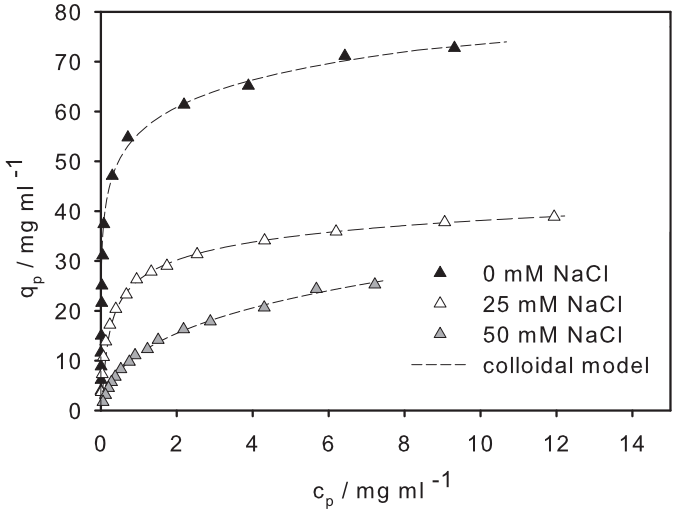

Fig. 4. Equilibrium adsorption isotherms of di-PEG-lysozyme-5 kDa on Toyopearl Gigacap S-650M in 25 mM sodium phosphate buffer, pH 6.0, 25 °C. Three sodium chloride concentrations are shown together with the corresponding colloidal model fits.

3.3. Evaluation of ITC experiments

The evaluation of the ITC data and the calculation of the specific enthalpy of adsorption were carried out as previously described in [9] and are not discussed here in detail. Preliminary experiments showed that the contribution of the adsorption of buffer ions and the heat of dilution of buffer ions to the measured signal can be neglected here. Hence, the enthalpy of adsorption of the proteins was determined from the adsorption experiments by subtracting only the enthalpy of mixing, which was determined in a blank experiment. The specific enthalpy of adsorption was then determined from

$$\Delta h_p^{ads} = \frac{\Delta H_p^{ads}}{\Delta m_p^{ads}} \tag{8}$$

where Δm_p^{ads} is the amount of protein adsorbed upon each injection. Δm_p^{ads} was determined from the adsorption equilibrium isotherm. Experimental error of caloric data is hard to asess as it

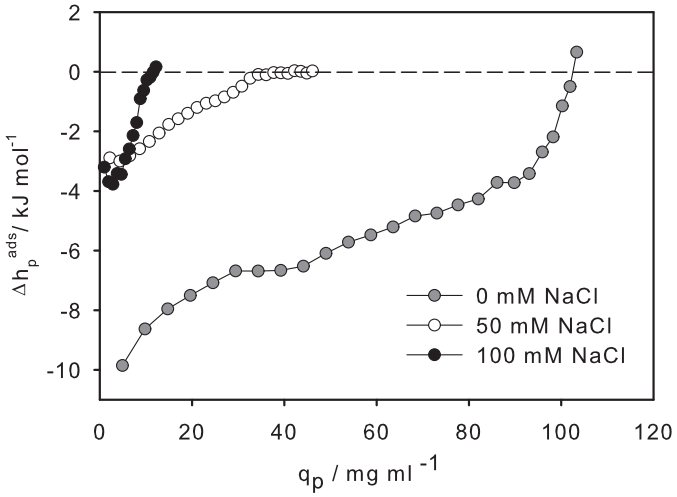

Fig. 5. Specific enthalpy of adsorption of mono-PEG-lysozyme-5 kDa on Toyopearl Gigacap S-650M in 25 mM sodium phosphate buffer, pH 6.0, 25 °C. Three sodium chloride concentrations are shown. The specific enthalpy of adsorption Δh_p^{ads} is plotted versus the adsorber loading q_p.

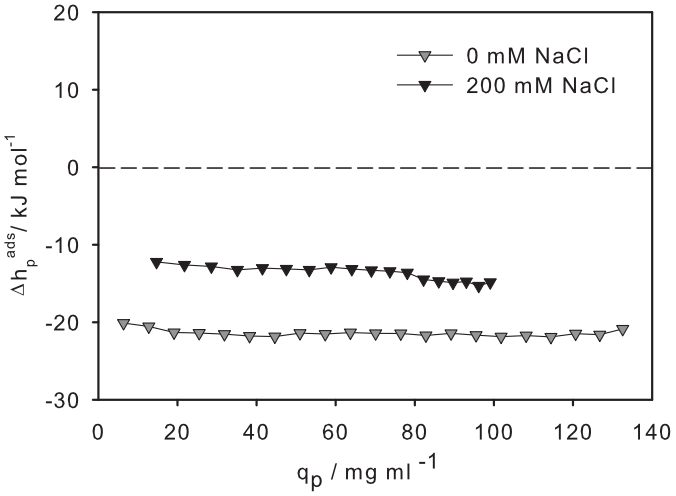

Fig. 6. Specific enthalpy of adsorption of native lysozyme on Toyopearl Gigacap S-650M in 25 mM sodium phosphate buffer, pH 6.0, 25 °C. Two sodium chloride concentrations are shown. The specific enthalpy of adsorption Δh_p^{ads} is plotted versus the adsorber loading q_p.

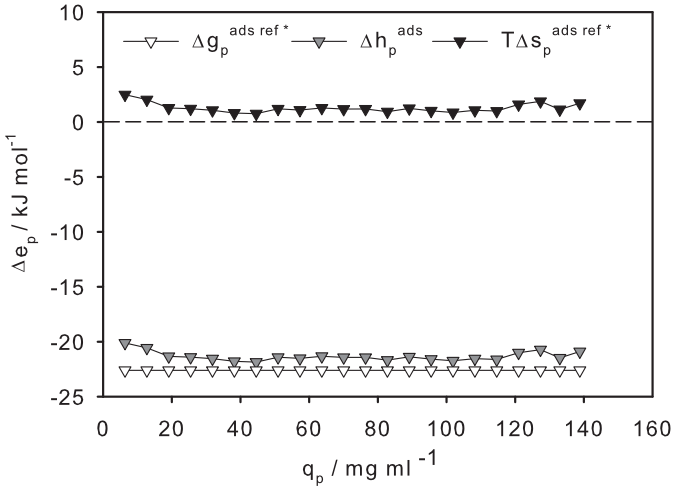

Fig. 8. Thermodynamic analysis of the adsorption of native lysozyme on Toyopearl Gigacap S-650M in 25 mM sodium phosphate buffer, pH 6.0, 25 °C. The reference gibbs energy $\Delta g_p^{ads\,ref*}$ and the corresponding contributions of the reference entropy $\Delta s_p^{ads\,ref*}$ and the specific enthalpy of adsorption Δh_p^{ads} to $\Delta g_p^{ads\,ref*}$ are plotted versus the adsorber loading q_p.

depends on a variety of multiply coupled parameters which can vary from point to point and with experimental set-up. A general statement on the error would be inappropriate.

4. Results and discussion

In this work, microcalorimetric experiments and corresponding equilibrium experiments were carried out for lysozyme and all PEGylated forms (mono-, di-, tri-) for two PEG chain lenghts (5 kDa, 10 kDa) at three NaCl concentrations for each protein. The temperature for all experiments was 25 ° C and the pH value was held constantly at pH 6.0 using a 25 mM sodium phosphate buffer. The NaCl concentrations were chosen according to the elution conditions in the production process (Figs. 1 and 2). This leads to 21 equilibrium isotherm experiments and 21 corresponding ITC experiments. As this is a large body of experiments, we will only discuss the major findings and results using representative examples as a basis for the discussion. The full set of results will be reported in [20].

4.1. Equilibrium adsorption isotherms

Fig. 3 shows the equilibrium adsorption isotherms for native lysozyme and all PEGylated forms obtained in experiments with solutions without NaCl. Native lysozyme has an almost ideally rectangular equilibrium isotherm which indicates a very strong interaction between the protein and the adsorber. The adsorber capacity and the equilibrium constant decrease with increasing PEGylation degree (total molecular weight of added PEG). All isotherms were fitted with the colloidal model (see Section 3.1), and the corresponding parameters for all measured isotherms are given in Table 2. An interesting finding is that there is no difference between the adsorption equilibrium of mono-PEG-lysozyme-10 kDa and di-PEG-lysozyme-5 kDa which have the same molecular weight. Even more, there is also no difference in their adsorption energetics (see Section 4.2). This indicates, that the influence of the size of the overall attached PEG chains on charge

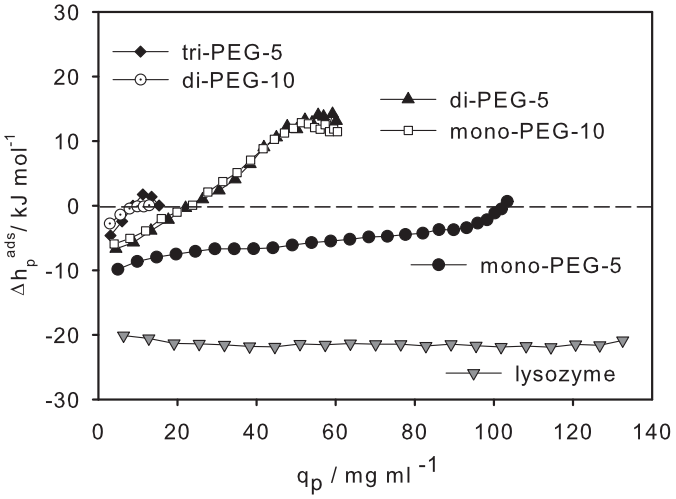

Fig. 7. Specific enthalpy of adsorption of native lysozyme and PEGylated forms with different PEGylation degree on Toyopearl Gigacap S-650M in 25 mM sodium phosphate buffer, pH 6.0, 25 °C. The specific enthalpy of adsorption Δh_p^{ads} is plotted versus the adsorber loading q_p.

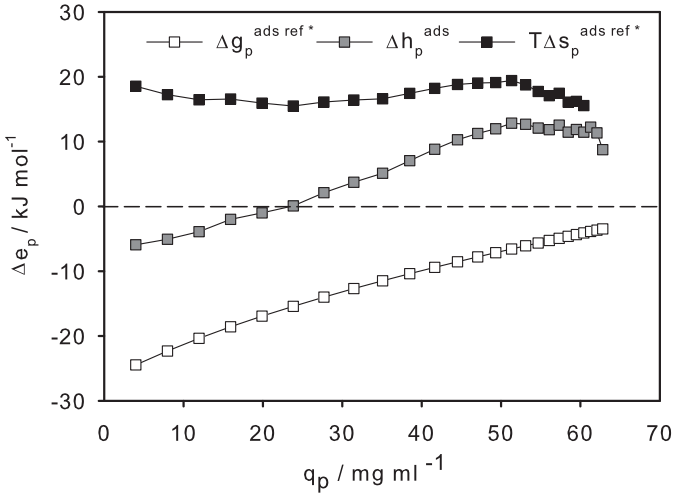

Fig. 9. Thermodynamic analysis of the adsorption of mono-PEG-lysozyme-10 kDa on Toyopearl Gigacap S-650M in 25 mM sodium phosphate buffer, pH 6.0, 25 °C. The reference gibbs energy $\Delta g_p^{ads\,ref*}$ and the corresponding contributions of the reference entropy $\Delta s_p^{ads\,ref*}$ and the specific enthalpy of adsorption Δh_p^{ads} to $\Delta g_p^{ads\,ref*}$ are plotted versus the adsorber loading q_p.

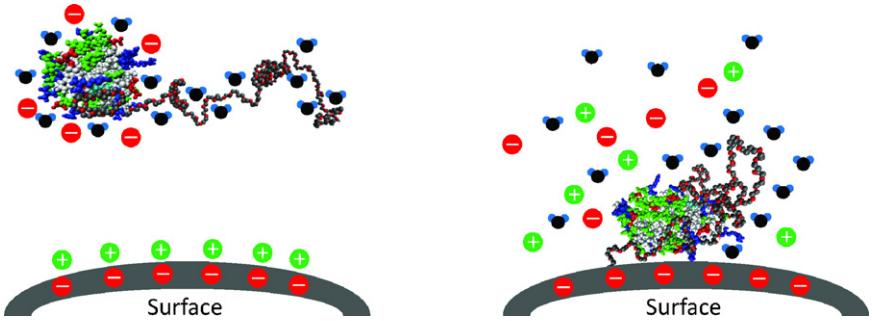

Fig. 10. Molecular picture of the adsorption of mono-PEG-lysozyme-5 kDa on a cation exchange resin. Left: The molecule is surrounded by counterions and the PEG chain is strongly hydrated. The adsorber surface is also covered with counterions. Right side: Adsorption of the molecule leads to rearrangement of charged groups, folding of the flexible PEG chain and a change in the state of hydration. Note, that this is only a representation of the effects contributing to entropy changes. No simulation of adsorption was carried out.

effects and adsorption is much stronger than the loss of a single charge due to an additional PEG chain.

The parameters of the colloidal fits are shown in Table 2. It can be stated that the colloidal model gives a good fit for all PEGylated lysozyme forms. Fig. 4 shows the salt concentration dependancy of the adsorption equilibrium of di-PEG-lysozyme-5 kDa. Only in the case of native lysozyme without sodium chloride, the colloidal model cannot achieve an acceptable fit due to the rectangular shape of the isotherm. Here, a simple linear relationship was used to describe the steep, initial part of the isotherm ($K_p^{ads} = 9153$). Data at low concentrations were checked internally by expanded plots which do not yield substantially new information.

4.2. Specific enthalpy of adsorption

The specific enthalpy of adsorption of mono-PEG-lysozyme-5 kDa is shown in Fig. 5 for different NaCl concentrations. The adsorption process is exothermal ($\Delta h_p^{ads} < 0$) but with increasing adsorber loading the absolute number of Δh_p^{ads} decreases and approaches zero. A similar behaviour was found by Dieterle et al. [9] for the adsorption of monoclonal antibodies on two strong cation exchange resins and explained by a preferred adsorption in energetically favoured configurations at low adsorber loadings. At higher loadings less attractive sites get filled and protein–protein interactions on the adsorber surface become stronger, which influences Δh_p^{ads}. See [9] for a more detailed discussion.

Another finding is that the absolute number of Δh_p^{ads} decreases with increasing sodium chloride concentration. This finding holds for all investigated proteins and can be explained by a decrease in electrostatic interaction energy through the shielding of protein charges by counter ions in solution.

However, for native lysozyme a distinctly different behaviour is observed (Fig. 6). The numbers for Δh_p^{ads} are independent of the adsorber loading. That implies that the observed influence of the adsorber loading of Δh_p^{ads} for the PEGylated lysozyme forms must be related to the PEG chains attached to the lysozyme. Regarding the influence of the PEGylation-degree the experiments yield two major findings (Fig. 7). The observed effect becomes less exothermal with increasing PEGylation degree. This effect can be explained by the increased molecular size of the higher PEGylated forms which leads to an increased distance between the protein's charges and the counterions on the adsorber surface. A detailed description and further experimental investigation of this phenomenon is given by Abe et al. [21].

The second finding for the results shown in Fig. 6 is that with increasing PEGylation degree the influence of the adsorber loading on Δh_p^{ads} gets stronger. Furthermore, for mono-PEG-lysozyme-10 kDa and di-PEG-lysozyme-5 kDa the process switches from exothermal at low loadings to endothermal at higher surface coverage.This implies that at higher loadings the adsorption process must be entropy-driven. This can be quantified using the approach described in Section 3.2. The results are exemplary shown for native lysozyme and mono-PEG-lysozyme-10 kDa in Figs. 8 and 9, respectively. In these figures the difference of the gibbs energy $\Delta g_p^{ads\,ref*}$ as determined from the equilibrium isotherm is shown together with the corresponding enthalpy of adsorption Δh_p^{ads}, determined from the ITC experiments, and the resulting difference of the entropy $T \cdot \Delta s_p^{ads\,ref*}$.

For native lysozyme a strongly exothermal process is observed. The specific enthalpy of adsorption is negative and constant along the adsorption isotherm. There is a small entropy gain over the whole concentration range, but overall this process is clearly enthalpy driven. For the adsorption of mono-PEG-lysozyme-10 kDa (see Fig. 9) a different behaviour is observed. At low loadings the process is exothermal. However, already at low loadings the entropic contribution to $\Delta g_p^{ads\,ref*}$ is dominant. At higher loadings the process switches to endothermal behaviour. Hence, adsorption does only occur due to the large entropy gain.

A picture of the adsorption process on the molecular level is given in Fig. 10. Preliminary molecular simulations of PEGylated lysozyme in water, based on the 53a6 Gromos 96 force field with SPC/E water, that were carried out with Gromacs 4.0 indicate that the PEG conjugate is folded or coiled around the lysozyme with long ends streching into the bulk water phase (Fig. 10, left). The PEG chain adds much flexibility to the rigid lysozyme molecule. In solution, the PEG chain is strongly hydrated and the protein's and adsorber's charged sites are shielded by counter ions. Adsorption of the molecule leads to a redistribution of the charged groups, structure rearrangements in the flexible PEG chain and therefore to a change in the state of hydration (i.e. a water release) which leads to an entropy gain upon adsorption. At high surface coverage increasing importance of protein–protein interactions modify this behaviour.

Compared to its PEGylated form, native lysozyme is a quite rigid molecule and structure rearrangements and water release are therefore much less important. This leads to the highly enthalpy driven adsorption of native lysozyme, mostly caused by simple electrostatic interaction effects. The PEGylation, on the other side, weakens this exothermal effect.

5. Conclusion

This work demonstrates that microcalorimetric studies can substantually contribute to a better understanding of the mechanisms of protein adsorption to chromatographic resins. A new

methodology was developed that allows evaluating the contributions of enthalpy and entropy effects to protein adsorption along the entire equilibrium isotherm, i.e. at different concentrations. The adsorption of PEGylated lysozyme forms on a strong cation exchange resin was found to be entropy driven although the adsorption of the native protein is enthalpy driven. This effect is mostly caused by the addition of flexibility to the protein molecule through the PEG chain. The flexibility of the PEG chain and its large hydration shell lead to structure rearrangements upon adsorption upon which water is released leading to an entropy gain. The detailed mechanisms of protein adsorption to chromatographic resins should be further investigated using the methody described in the present work combined with molecular simulation studies.

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Appendix A. Supplementary Data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.chroma.2011.05.063.

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