Buffer Capacity of Biologics—From Buffer Salts to Buffering by Antibodies

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Controlling pH is essential for a variety of biopharmaceutical process steps. The chemical stability of biologics such as monoclonal antibodies is pH-dependent and slightly acidic conditions are favorable for stability in a number of cases. Since control of pH is widely provided by added buffer salts, the current study summarizes the buffer characteristics of acetate, citrate, histidine, succinate, and phosphate buffers. Experimentally derived values largely coincide with values calculated from a model that had been proposed in 1922 by van Slyke. As high concentrated protein formulations become more and more prevalent for biologics, the self-buffering potential of proteins becomes of relevance. The current study provides information on buffer characteristics for pH ranges down to 4.0 and up to 8.0 and shows that a monoclonal antibody at 50 mg/mL exhibits similar buffer capacity as 6 mM citrate or 14 mM histidine (pH 5.0–6.0). Buffer capacity of antibody solutions scales linearly with protein concentration up to more than 200 mg/mL. At a protein concentration of 220 mg/mL, the buffer capacity resembles the buffer capacity of 30 mM citrate or 50 mM histidine (pH 5.0–6.0). The buffer capacity of monoclonal antibodies is practically identical at the process relevant temperatures 5, 25, and 40°C. Changes in ionic strength of $\Delta I=0.15$, in contrast, can alter the buffer capacity up to 35%. In conclusion, due to efficient self-buffering by antibodies in the pH range of favored chemical stability, conventional buffer excipients could be dispensable for pH stabilization of high concentrated protein solutions.
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Introduction

Over the past two decades monoclonal antibodies (mAbs) took over a leading role in the biopharmaceutics sector. Numerous mAbs are in the pipelines of biotech and pharmaceutical companies nowadays and over 20 antibody drugs are already on the market.[1] The increasing interest in the development of monoclonal antibodies as therapeutics is motivated by the following major advantages. First of all, antibodies inherently possess the potential to act in a very specific manner. Second, they can be used as shuttles for conjugated drug-molecules by which the drug is more efficiently delivered to a specific target. Therefore, high potency and efficacy are attributed to antibody-based therapeutics and they are generally expected to cause fewer side effects than small molecular drugs.[2]

However, monoclonal antibodies are complex molecules that are susceptible to a variety of degradation routes. Chemical degradation damages the amino acid building blocks and the backbone of the protein molecules affecting the protein primary and secondary structure. In addition, physico-chemical processes can impair the tertiary structure whose integrity is crucial for the biologically active state. Biopharmaceutical development therefore needs to address the degradation issues and seek for optimal stability of the antibody therapeutic. A further challenge lies in finding the correct application form of the therapeutic. Because of their proteinogenic origin, mAbs suffer from poor bioavailability when administered via the oral route. Therefore, antibody drug products for parental administration are developed. In many cases a high dosage (around 100 mg) is required to obtain significant therapeutic efficacy of these drugs. Furthermore, the volume of injected product is often limited by medical compliance issues (for example injection volume shall not exceed 1.5 mL for subcutaneous applications[3]). Therefore, to reach the clinical therapeutic dose, antibody formulations containing highly concentrated protein (up to more than 100 mg/mL) are strongly needed and become more and more popular. Aside from the medical requirements high concentration formulations are also advantageous as they bring about decreased storage and material cost. Also, costs for residence in hospitals and hospital personnel costs can be reduced if self-administration of a high concentration formulation via the subcutaneous route can be considered.

Chemical degradation of proteinogenic drugs typically includes deamidation, oxidation, proteolysis, β-elimination, and cleavage of disulfide bonds. Being strongly pH dependent, these degradation pathways can be controlled by choosing a formulation buffering at a specific pH. Studies on model peptides[4–7] and antibodies (examples collected by

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review authors[2]) revealed that low degradation rates are generally observed in the range of pH 4.0–7.5. Also, physico-chemical properties, that is, colloidal and conformational stability depend on the pH value of the antibody solution. The optimal pH value for ensuring desirably high physico-chemical stability varies over the whole pH range and is very specific for a certain mAb molecule. In general, however, control of pH is a crucial demand during mAb processing and storage. Conventionally, this is achieved by adding buffering agents such as phosphate, citrate or acetate to the antibody solutions.

The antibodies inherently possess a considerable amount of readily ionizable and thus potential buffering groups, that is, the amino acid side chains of histidine, aspartic acid, glutamic acid, arginine, tyrosine, cysteine, and lysine. The side chains of aspartic acid, glutamic acid, and histidine exhibit pK_a values of 3.7, 4.3, and 6.5 respectively,[8] and are therefore capable of providing buffer capacity in the range of pH 3.0–7.5 which covers the pH regime of high physiological compatibility and of slowest chemical degradation mentioned before. With the upcoming need for highly concentrated mAb solutions used for human application (introduced above), the concentration of buffering amino acid side chains in the mAb solutions increases and their buffer capacity becomes relevant.[9] Actually, the role of proteins providing buffer capacity is prevalent in any cellular and intercellular environment.[10] Besides the bicarbonate buffering system, proteins buffer in blood[11]. Moreover, buffering in saliva between pH 3.4 and 5 is even primarily accomplished by proteins.[12]

The goal of the present work is to characterize buffer agents widely used in biopharmaceutic manufacturing and generally protein solutions in terms of buffering power at various pH ranges between down to pH 4 and up to pH 8. Furthermore, we investigate the buffer capacities of antibodies in high-concentration solutions to evaluate their potential to provide self-buffering function. This function could on one hand be utilized for stabilizing pH but also might impair pH adjustment of a formulation if it dominates over buffer excipients.

For both conventional buffer agents and antibodies, we determine buffer capacities experimentally in titrations and compare them to ab initio derived values. To allow for calculating buffer capacities of the buffering agent at a certain concentration for practical use, we also determine the concentration dependence of buffer capacity. As the biopharmaceutical development process also comprises steps where buffered solutions are kept at different temperatures, we check for the influence of temperature on buffer capacities. Also, the dependence on ionic strength is covered in our study.

Material and Methods

Four monoclonal antibodies (mAbs1–4) used in this study were produced by mammalian cell culture technology.[13] Base and acid standard solutions (1 or 0.1 N HCl and accordingly 1 or 0.1 N NaOH; volumetric) used for the titration studies were purchased from Sigma-Aldrich (St. Louis, MO). All solutions were prepared using water for injection (WFI).

The buffer systems being investigated in terms of buffer capacity were acetate, citrate, succinate, phosphate, and histidine buffers. Chemicals used in the preparation thereof were of analytical grade. The buffers were prepared at concentrations of 10, 25, and 50 mM via weighing of the acid and corresponding base in a beforehand determined, defined ratio for obtaining pH 5.2 (acetate), pH 6 (citrate, succinate and histidine), or pH 7 (phosphate). To simulate conditions close to the situation in the development of subcutaneous formulations, all solutions also contained 130 mM NaCl (acetate buffer), 125 mM NaCl (citrate and succinate buffer), 135 mM NaCl (phosphate), 140 mM NaCl (histidine buffer). These saline contents each plus the contribution of 25 mM buffer salt adjust the solutions to isotonicity. The osmolality of the 10 or 50 mM buffer solutions therefore was slightly lower or higher respectively.

The antibody starting materials were received in different formulations specific to each antibody. Generally, the formulations were at pH 5.4–6.2 containing 25 or 50 mM buffer excipient plus maximally 170 mM NaCl. Based on these solutions the specific antibody solutions used in the current study were prepared as follows. For antibodies mAbs1–3, the bulk was first ultrafiltered[14] over a tangential flow filtration system (Sartorius, Goettingen, Germany) employing a polyethersulfone membrane (Sartocon Slice 200, cutoff 30 kDa, Sartorius, Goettingen, Germany) to a final concentration of approximately 100 mg/mL. In a second step, the concentrated solution was diafiltered against a solution containing 160 mM NaCl (or 10 mM as indicated in the text). Here, the minimum of diavolume exchanges was at least eight exclusion volumes. This procedure aims at a practically complete replacement of the antibody storage buffer since, based on calculations, after eight diafiltration volumes at a constant protein concentration, the solution is up to 99.96% diafiltered in the new solution if the membrane is 100% permeable to small molecules.[14] Protein dilutions were prepared in 160 mM NaCl (or 10 mM as indicated).

mAb4 was ultrafiltered over the tangential flow filtration system (polyethersulfone membrane Sartocon Slice 200, cutoff 30 kDa, Sartorius, Goettingen, Germany) against at least eight exclusion volumes WFI and concentrated up to a protein concentration $>$200 mg/mL. The solution was then adjusted to isotonicity ($\approx$ 300 mosmol/kg) by spiking with NaCl or trehalose (as indicated). Dilutions of mAb4 were prepared with aqueous NaCl or trehalose retaining around 300 mosmol/kg of the solution being titrated.

Final protein concentrations of the mAb solutions were determined via their absorption at λ=280 nm employing the mAb specific extinction coefficients.

Titration curves were generated by adding HCl or NaOH standard solutions to 5 mL of each buffer system in a clean 10-mL glass vial (Fiolax Clear Glass Vials, Schott Forma Vitrum, Müllheim, Germany), mixing and measuring the pH value (WTW pH 340, Wissenschaftlich-Technische Werkstätten, Weilheim, Germany). The pH meter had been calibrated via a two point calibration using standard buffer solutions pH 4.0 and 7.0 (Mettler-Toledo, Schwerzenbach, Switzerland). Titrations were performed starting from pH 6.0 to 4.0 or to pH 8.0, respectively. The concentration of the added standard solution (0.1 or 1 N) to change the pH in reasonable increments had been determined in preliminary tests. The titration time was kept as low as possible to minimize the CO_2-input and the interaction of the solution with the glass vial. All pH measurements were performed at 5, 25, or 40°C as indicated and the pH value was recorded after the value had stabilized for at least 10 s.

For evaluation, the pH was plotted vs. the microequivalents (μEq) of acid or base added for each titration normalized for the solution volume. For monoprotic and monobasic

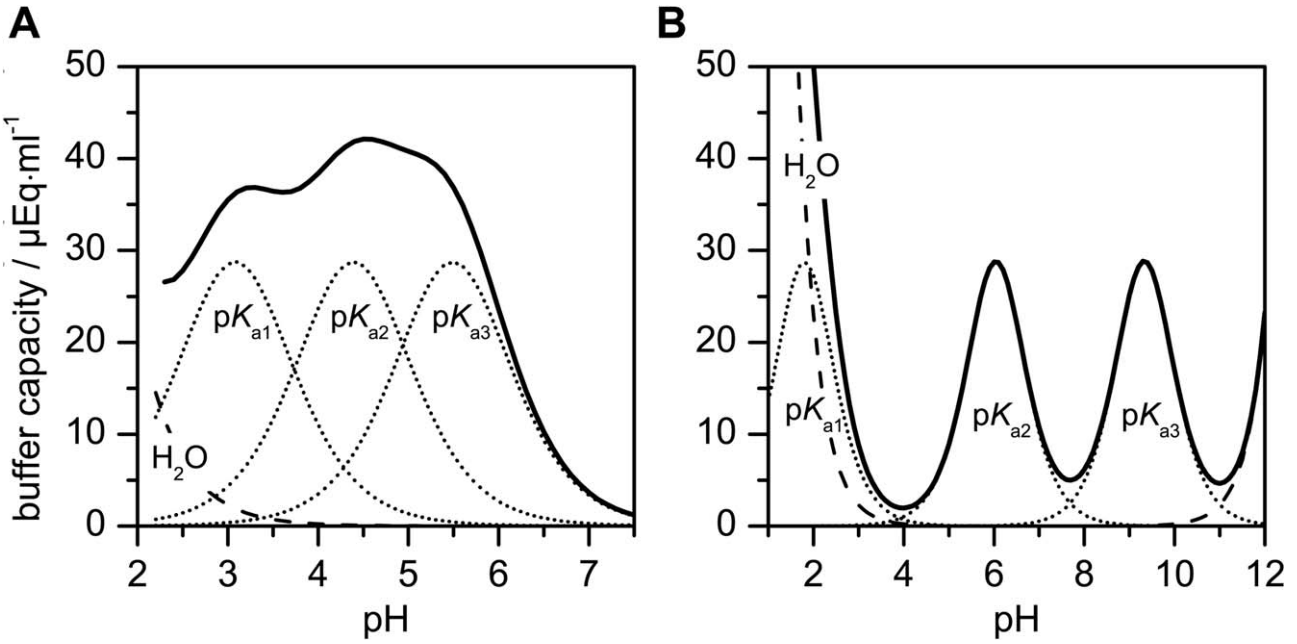

Figure 1. Buffer capacity of the ionizable groups of (A) citrate and (B) histidine (dotted lines, simulated for 50 mM buffering agent) in aqueous solution vs. pH and the resulting total buffer capacity (solid line).

The total buffer capacity also includes the contribution of the water ionization equilibrium that becomes significant at pH<3 (dashed line). Because of three carboxyl groups with pK_a values that differ around 1 unit, citrate exhibits considerable buffer capacity over a broad pH range (pH 2.5-6.0). The buffering by the amino, the carboxyl and the imidazole group in histidine hardly overlaps and practically relevant buffer capacity is only found in a narrow range around pH 6.0.

compounds, such as HCl and NaOH, the µEq is equal to the micromoles of titrant added. Titration curves were dissected in segments and the data points of each segment were fitted to a linear equation. The inverse value of the slope yields the average buffer capacity β for the chosen pH range. The buffer capacity describes how many mircoequivalents can be added to 1 mL of the buffer or antibody system before changing the pH one unit.

Finally, the buffer capacity of a given buffer system was plotted vs. buffer concentration and the linear fit thereof yields the "slope of capacity." Employing the "slope of capacity," buffer capacities of different buffer systems are compared and the buffer capacities at further concentrations can be extrapolated.

For theoretical determination of the buffer capacity the equation of van Slyke was used. According to van Slyke the buffer capacity (β) of an aqueous solution is described by Eqs. 1 and 2.[15]

$$\beta = 2.3\left(\frac{K_a C[H^+]}{(K_a+[H^+])^2} + [H^+] + [OH^-]\right) \quad (1)$$

$$\beta = 2.3C[H^+]\left(\frac{K_{a1}}{(K_{a1}+[H^+])^2} + \frac{K_{a2}}{(K_{a2}+[H^+])^2} + \ldots\right) + 2.3\left([H^+] + [OH^-]\right) \quad (2)$$

Here, C is the total buffer concentration and K_a is the acid dissociation constant of the buffer. Equation 1 is valid for buffer systems that are characterized by a single pK_a value only. More complex systems are described by Eq. 2 considering the contributions of further ionizable groups (with different pK_a values) to the total buffer capacity. As an example, the effect of three ionizable groups in citrate and histidine on the total buffer capacity is depicted in Figure 1A and 1B respectively. In citrate the pK_a values of three carboxyl groups are around 1 unit apart leading to a constantly large buffer capacity over a broad range (pH 2.5–6.0) (Figure 1A). In contrast, the pK_a values of the amino group, the carboxyl group and the imidazole moiety in histidine differ up to 4 units. As a consequence, the buffer effects of these groups hardly overlap and significant buffer capacity is found in narrow pH ranges only (Figure 1B).

Equation 2 yields the buffer capacity for discrete pH values. Therefore, the calculated average value within $\Delta pH = 1$ or 0.5 (as indicated) was compared with the experimentally determined value for the same range.

For calculation of the buffering capacity of an antibody Eq. 3 has been suggested by Gokarn et al.[9]

$$\beta_{mAb} = \sum_{i=Asp}^{His} N_i * C_{mAb} * \left\{ \left[\frac{[H^+]}{(K_a)_i+[H^+]}\right]_{pH} - \left[\frac{[H^+]}{(K_a)_i+[H^+]}\right]_{pH+1} \right\} + \left([H^+]_{pH} - [H^+]_{pH+1}\right) \quad (3)$$

In Eq. 3, the subscript i refers to the amino acids aspartic acid, glutamic acid and histidine (relevant for the buffering within the pH range of interest), N is the number of amino acids of the mAb, C_{mAb} is the molar concentration of mAb and K_a is the acid dissociation constant of the corresponding amino acid side-chain. The indices "pH" and "pH+1" denote the start and end value of the specific pH range $\Delta pH = 1$ being addressed.

Table 1 summarizes the pK_a values used for the calculations and Table 2 the number of the amino acids in mAbs1–4 contributing to the buffer capacity in the pH range of relevance.

Table 1. pK_a Values Used for Calculating Theoretical Buffer Capacities[8,16,17] (at Zero Ionic Strength, 25°C),[16] (0.1 M KCl at 25°C)[17]

	p*K*a1	p*K*a2	p*K*a3
Buffer			
Acetate	4.76		
Citrate	3.08	4.39	5.49
Histidine	1.8	6.04	9.33
Succinate	4.21	5.64	
Phosphate	2.15	7.20	12.35
Side chain of			
His	6.5		
Glu	4.3		
Asp	3.7		

Table 2. Number of Histidine, Aspartate, and Glutamate Residues in the Investigated Monoclonal Antibodies and Their Theoretical Isoelectric Point

	Number of Amino Acids Per mAb Molecule			
Antibody	His	Asp	Glu	Isoelectric Point
mAb1	24	48	68	8.3
mAb2	24	60	58	8.3
mAb3	26	50	72	7.8
mAb4	28	58	64	8.4

Results

Buffer capacity of standard buffers

To determine the buffer capacity β of acetate, citrate, succinate, phosphate, and histidine, the corresponding buffer solutions were titrated with acid or base and the resulting pH was measured. Figure 2A summarizes the titration of citrate buffer as a representative result. In the range of pH 4.0–6.0 a fairly linear dependence of pH and μEq is observed. For pH values greater 6.5, the buffer capacity of citrate is comparably low (cf., Figure 1A) and further addition of base leads to drastic pH shifts.

By definition, the buffer capacity can only be determined for a discrete value by differentiating pH value with respect to acid/base equivalents added (Eq. 4, exemplarily B for base equivalents).

$$\beta = \frac{dB}{d\mathrm{pH}} \tag{4}$$

However, the average buffer capacity over a limited pH range (Eq. 5) serves as an acceptable approximation[15] and is moreover usually of practical interest.

$$\beta \approx \frac{\Delta B}{\Delta \mathrm{pH}} \tag{5}$$

Therefore, an averaged buffer capacity is derived from the linear fit of the data points within roughly linear segments of the titration curve of $\Delta\mathrm{pH}=1$ (rarely $\Delta\mathrm{pH}=0.5$ was chosen in case the titration curve was insufficiently described by a linear curve within $\Delta\mathrm{pH}=1$). This yields average buffer capacities for the assayed buffer solutions in defined pH ranges (cf., Table 3).

All titrations were performed at different buffer concentrations in the pharmaceutically relevant range and a linear dependence of buffer capacity and buffer concentration was observed (exemplarily shown in Figure 2B for citrate buffer in the range of pH 6.0–5.0). The linear fit yields the slope *a*, (called "slope of capacity" here). We point out that due to the employed evaluation procedure, the slope is specific for a certain pH-range. For example, a slope of capacity of 0.6 μEq/μmol for citrate buffer in the range of pH 6.0–5.0 was derived (Figure 2B). For acetate in the range of pH 4.0–5.0, we determined a slope of capacity of 0.47 μEq/μmol experimentally and 0.48 μEq/μmol when employing the van Slyke equation. For this system, Gokarn et al.[9] found values of 0.54 and 0.5 μEq/μmol experimentally and employing an alternative prediction, respectively.

Table 3 summarizes the experimentally derived parameters for the buffer excipients acetate, citrate, succinate, phosphate, and histidine which are widely used in protein science. The parameters allow for the computation of the average buffer capacity for these buffers in a certain pH range at any desired concentration by multiplying parameter *a* with the buffer concentration of interest. This procedure is successfully applied and allows gaining an estimate of how much acid/base needs to be added to a buffered solution of known concentration to achieve a defined pH shift.

In addition, Table 3 lists the corresponding parameters for values simulated employing the van Slyke equation. Except for phosphate buffer, we find a good correlation between experimentally derived values and predicted values. Larger deviations can be seen for pH ranges in which the corresponding buffer exhibits comparably low buffer capacity (pH far from p*K*a). Here, a larger susceptibility of the parameters to experimental errors is anticipated.

Buffer capacity of monoclonal antibodies

To determine the buffer capacity β of monoclonal antibodies, antibody solutions in 160 mM NaCl were titrated with acid or base and pH values were recorded. Titration curves of mAb3 are exemplarily shown in Figure 3 (filled symbols). The results obtained within the ranges of pH 6.0–5.0, 5.0–4.0, and 6.0–7.0 are representative for all monoclonal antibodies used in this study since we found no markedly significant differences between the studied antibodies. Within pH ranges of $\Delta\mathrm{pH}=1$, we find a practically linear correlation of the amount of base/acid added and the resulting pH just as described for the conventional buffers (previous section). Data points were fitted to linear equations (Figure 3, solid lines) and the derived buffer capacities β scale linearly with the protein concentration equally as shown for the buffer systems in the previous section (Figure 4).

We point out that a significant contribution by residual buffering components from the bulk formulation of the mAbs to the buffer capacity can be excluded since an eightfold (by volume) exchange to 160 mM NaCl had been performed in preparation of the solutions. Although the Donnan effect impedes the complete removal of residual buffer excipient,[18] the contribution to the total buffer capacity by the remaining molecules is neglectable. Exemplarily, Gokarn et al.[9] after diafiltration vs. a sorbitol solution detected 1.8 mM residual acetate in a 110 mg/mL mAb solution resulting in a ~6% contribution of acetate to the total buffer capacity. In another study, after diafiltration (nine diavolumes) of a 40 mg/mL protein solution vs. 10 mM histidine an amount of remaining 0.5 mM citrate was detected.[19] These observations are consistent with our experience. Generally, the amount of buffer ions subsequent to the described ultrafiltration/

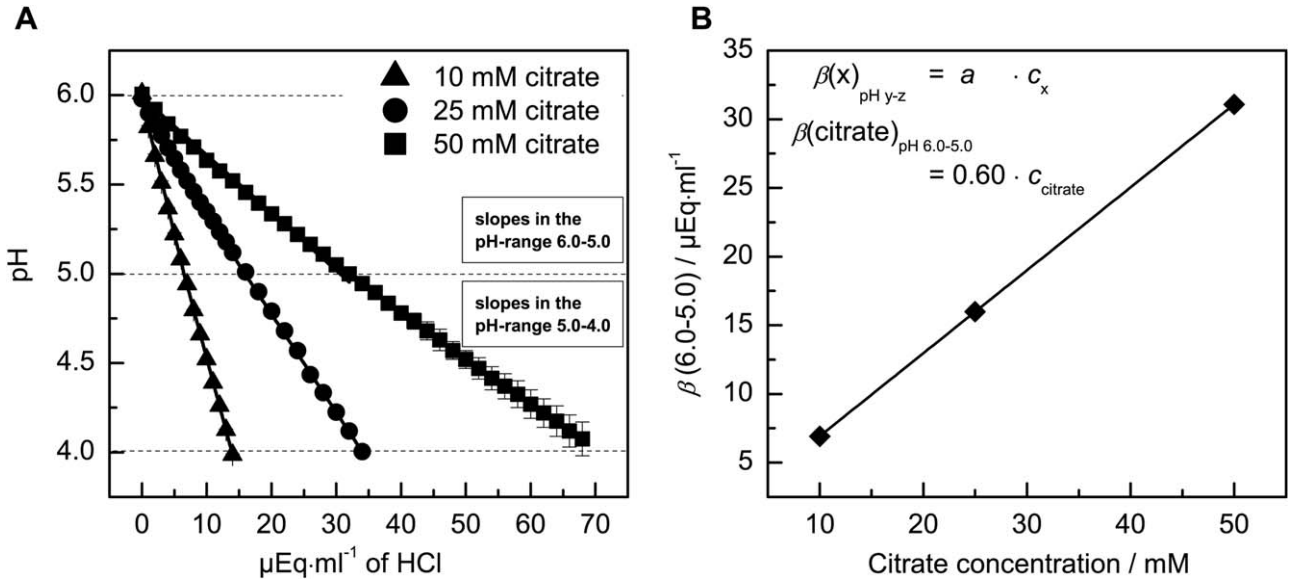

Figure 2. (A) Titration curves of citrate buffer with HCl at 25°C and at different citrate concentrations and (B) a corresponding buffer capacity vs. concentration plot.

The data values were determined by titrating 5 mL of citrate solution with 0.1 or 1 N acid. The addition of titrant was normalized to microequivalents of base or acid added per milliliter of solution. Error bars indicate the range for replicates. The lines represent linear fits to the data points. The inverse of the slopes yield the buffer capacity β plotted in (B) vs. the corresponding buffer concentration. From the fit to a linear equation the slope of capacity (parameter ***a***) can be derived and used to compare different buffer systems. In addition, buffer capacities at a certain pH-range can be predicted for various buffer concentrations. Results for histidine, acetate, citrate, succinate and phosphate are summarized in Table 3.

Table 3. Experimentally and Via the van Slyke Equation[15] Theoretically Derived Slopes of Capacity for Pharmaceutically Relevant Buffer Salts at Various pH Ranges

Buffer System	pH-Range	Experimental Slope of Capacity a_{exp} (µEq/µmol)	Slope of Capacity van Slyke Model $a_{vanSlyke}$ (µEq/µmol)	Ratio of Slopes $a_{exp}/a_{vanSlyke}$
Citrate	4.0–5.0	0.78	0.82	0.95
	5.0–6.0	0.60	0.70	0.87
	6.0–6.5	0.31	0.33	0.93
	7.0–8.0	0.04	0.03	1.16
Histidine	5.5–6.0	0.47	0.50	0.94
	6.0–7.0	0.49	0.43	1.15
	7.0–8.0	0.16	0.16	1.01
Acetate	4.0–5.0	0.47	0.48	0.97
	5.0–6.0	0.27	0.31	0.87
Succinate	4.0–5.0	0.79	0.64	1.23
	5.0–6.0	0.64	0.63	1.02
	6.0–6.5	0.28	0.39	0.72
Phosphate	6.0–7.0	0.47	0.32	1.45
	7.0–8.0	0.39	0.55	0.71

diafiltration procedure (methods section) at >100 mg/mL mAb ranges at sub-millimolar to low single-digit millimolar concentrations depending on the specific protein (Bahrenburg et al., unpublished results).

When titrating with NaOH (pH 6.0–7.0) a steeper slope corresponding to a lower buffer capacity than in the titration with HCl (pH 4.0–6.0) is observed. This effect reflects the low amount of amino acids buffering in the range of pH 6.0–7.0. As listed in Table 2, the side chains of 50 aspartic acids (pK_a=3.7) and 72 glutamic acids (pK_a=4.3), in theory, are capable of buffering up to about pH 5.5 whereas only 26 histidine side chains (pK_a=6.5) contribute to the buffer capacity in the range of pH 5.5–7.5. Similar proportions can be found for all mAbs studied here.

In Figure 4, the experimentally determined buffer capacities are plotted vs. the concentration of mAbs1–3. As the buffer capacity increases linearly with concentration, just as observed for conventional buffers, the data points were fitted to a linear equation. The slope, that is, the slope of capacity was determined and turns out to be nearly identical for mAbs1–3. This similarity was found for the overall pH-range of 4.0–8.0 (cf., Table 4).

The graphs in Figures 4A,B also include the experimentally determined buffer capacities of the conventional buffer citrate and histidine respectively at different concentrations in the pH-range pH 6.0–5.0 (secondary axis). We find that the monoclonal antibodies exhibit inherent buffer capacity that at 50 mg/mL resembles the buffer capacity of 6 mM citrate or 14 mM histidine buffer in the pH-range of pH 6.0–5.0. As 10 mM histidine is a commonly used buffer (e.g., end-formulation of Rituximab[2]), we conclude the self-buffering effect of a 50 mg/mL antibody solution is sufficient to replace a buffer excipient for pH stabilization.

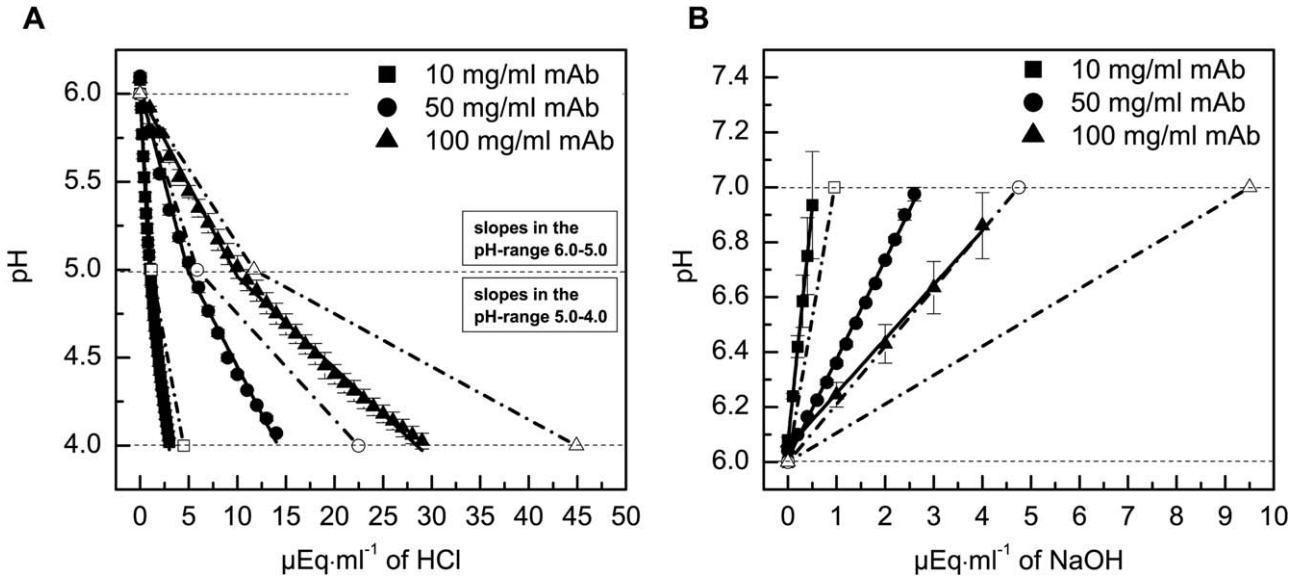

Figure 3. Titration curves of mAb3 in 160 mM NaCl at different mAb concentrations.

The data values were determined for 5 mL of a given solution using 0.1 or 1 N acid or base at 25°C. The addition of titrant was normalized to microequivalents of base or acid added per milliliter of solution. Error bars indicate the range for replicates. The solid lines represent linear fits to the data points. The slopes represent the buffer capacity plotted in Figure 4. From the theoretic slopes of capacity listed in Table 4 and thus the theoretic buffer capacities, the titration curves were also simulated for mAb3 at 10, 50, and 100 mg/mL (broken lines, open symbols) illustrating that in vitro the protein exhibits lower buffering than predicted from theory. For simplicity, the curves were simulated assuming a constant buffer capacity within $\Delta pH = 1$.

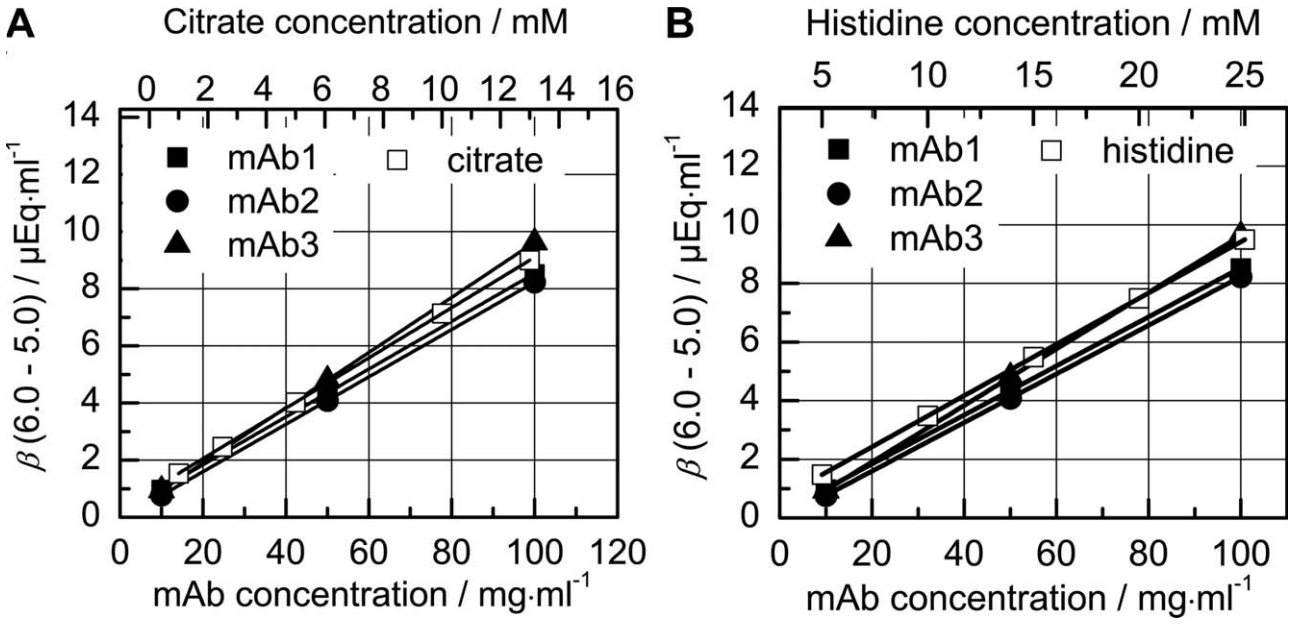

Figure 4. The experimentally determined buffer capacities β of mAbs1–3 and citrate (top *x*-axis, panel A) or histidine (top *x*-axis, panel B) plotted vs. the corresponding concentration. Data points were fitted to a linear equation.

The tested antibodies exhibit similar buffering characteristics and their buffer capacity at 50 mg/mL protein corresponds to 6 mM citrate or 14 mM histidine buffer.

Table 4 additionally lists the parameters for mAbs1–3 determined via the theoretical models (Eqs. 2 and 3). Here, the buffer capacities of all buffering amino acids (cf. Tables 1 and 2) are regarded as independently summing up to a total buffer capacity. However, deviations in the experimentally determined parameters and the theoretical values are observed. On average the slopes of capacity differ by a factor of 2. To illustrate the effect, we also simulated the corresponding titration curves for mAb3 in Figure 3 (broken lines, open symbols) employing the buffer capacities calculated from the predicted slopes of capacity in Table 4. The deviation of theory and experiment might be due to the fact that the pK_a values of the amino acid side chains can shift in the native protein environment compared to the theoretical

Table 4. Experimentally and Theoretically Derived Slopes of Capacity (a) for Monoclonal Antibodies mAb1, mAb2, and mAb3 at Various pH Ranges

pH-range	Experiment a_{exp}	Ab Initio prediction* a_{pred}	Ratio of Slopes a_{exp}/a_{pred}	Model of van Slyke† $a_{van\ Slyke}$	Ratio of Slopes $a_{exp}/a_{van\ Slyke}$
pH 4.0–5.0	μEq/mg	μEq/mg		μEq/mg	
mAb1	0.183	0.354	0.52	0.350	0.52
mAb2	0.160	0.322	0.50	0.320	0.50
mAb3	0.203	0.332	0.61	0.329	0.62
pH 5.0–6.0	μEq/mg	μEq/mg		μEq/mg	
mAb1	0.084	0.126	0.66	0.129	0.65
mAb2	0.080	0.111	0.71	0.113	0.70
mAb3	0.097	0.117	0.82	0.119	0.81
pH 6.0–7.0	μEq/mg	μEq/mg		μEq/mg	
mAb1	0.055	0.104	0.52	0.103	0.53
mAb2	0.042	0.095	0.44	0.093	0.45
mAb3	0.048	0.095	0.50	0.094	0.51
pH 7.0–8.0	μEq/mg	μEq/mg		μEq/mg	
mAb1	0.051	0.039	1.31	0.039	1.31
mAb2	0.031	0.036	0.85	0.036	0.85
mAb3	0.043	0.036	1.19	0.036	1.19

*The slope of capacity was derived from simulations employing a model suggested by Gokarn et al.,[9] Eq. 3.
†The slope of capacity was derived from simulations employing the van Slyke equation,[15] Eq. 2.

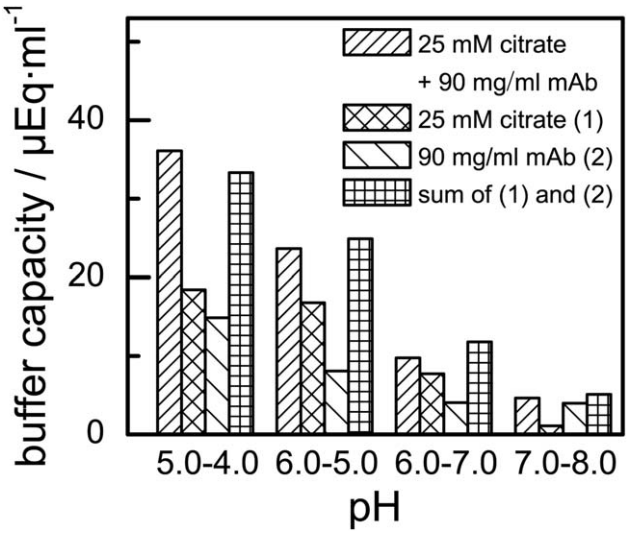

Figure 5. Experimentally derived buffer capacities β for mAb4 in citrate buffer and of mAb4 or citrate buffer only. The sum of the contributions by the single components matches the experimental buffer capacity of the mixture.

pK_a values for the ionizable side chain in isolation. As a result, they buffer in a pH range different from the predicted. We therefore suggest that the buffering characteristics should be derived experimentally for the antibody of interest.

In an additional experiment, we found that conventionally formulated mAb solutions, that is, mAb plus buffer salt in aqueous solution, exhibit a buffer capacity that is very similar to the sum of the component's buffer capacity (Figure 5). We do not observe nonlinear effects. Thus, for practical purposes the contribution in buffering by the protein at higher concentrations always needs to be considered for example when adjusting pH values of conventionally buffered samples.

Buffer capacity of a monoclonal antibody at the high-concentration regime

Recently, pharmaceutical development is heading towards protein solutions of very high concentrations. We therefore also characterized antibody solutions of more than 200 mg/mL protein in terms of buffer capacity. The number of aspartate, glutamate, and histidine residues of the corresponding antibody mAb4 is listed in Table 2. To approximate pharmaceutically relevant conditions, we titrated samples containing antibody in the presence of a nonbuffering excipient to adjust the osmolality of the solutions to around 300 mosmol/kg (i.e., close to isotonicity). Figures 6 and 7 summarize the titration curves for mAb4 plus NaCl and trehalose respectively at different protein concentrations including 220 mg/mL. We find adequate linearity of the titration curves for $\Delta pH=1$ in the range of pH 4.0–7.0. The buffer capacities for certain pH ranges were plotted vs. protein concentration (exemplarily shown in Figure 8) and the slope of capacity calculated. Table 5 summarizes the corresponding values from titrations of antibody solutions containing NaCl or trehalose. We find a linear increase of buffer capacity with increasing protein concentration to up to more than 200 mg/mL under conditions close to isotonicity. In the pH range of pH 4.0–5.0, the antibody solution of 220 mg/mL protein in trehalose or NaCl exhibits a buffer capacity resembling the buffer capacity of around 60 mM citrate buffer. In the range of pH 5.0–6.0 this highly concentrated antibody solution buffers like 50 mM histidine or 30 mM citrate buffer. These values underline that the buffer capacity of the protein can substitute for a buffering excipient in highly concentrated antibody solutions.

Buffer capacity of antibodies at varying temperature

During biopharmaceutical development antibody solutions can be exposed to different temperatures. For example, antibodies are commonly stored and shipped at 2–8°C and for accelerated high temperature stability studies temperatures up to 40°C are chosen. As any equilibrium constant (and therefore the pK_a) varies with temperature, the buffer capacity in a defined pH range is also subject to change. For proteins, a temperature change has further consequences (e.g., changing conformational equilibria) and an estimation of the total effect of a temperature shift on the self-buffering characteristics of proteins is not straight-forward. Therefore, to check whether the antibody inherent buffer capacity might

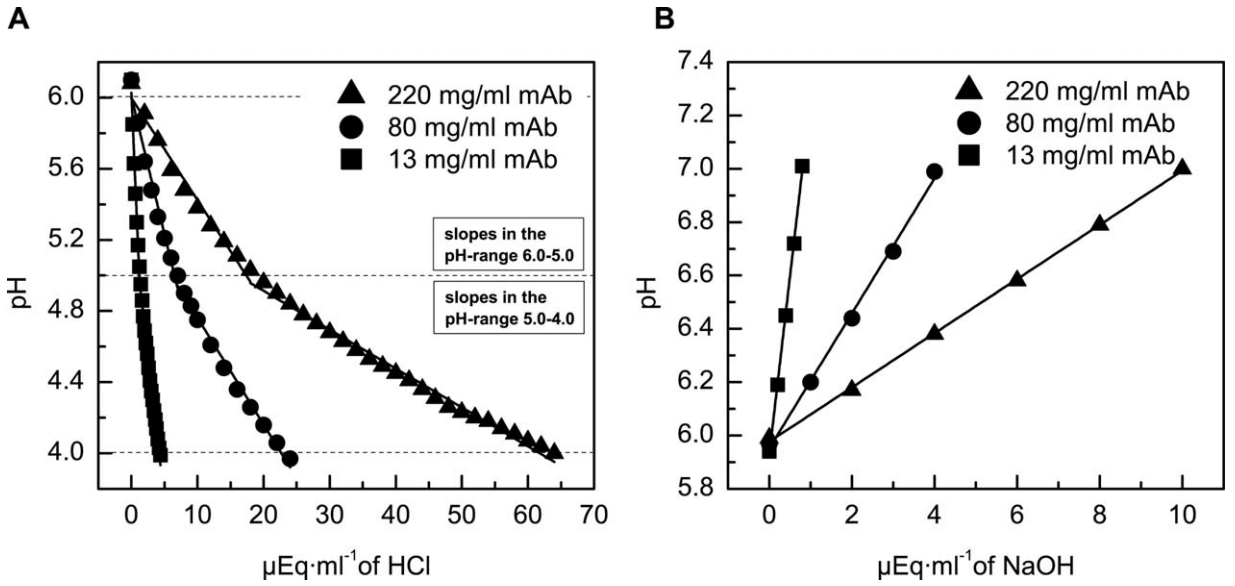

Figure 6. Titration curves of mAb4 at different mAb concentrations in the presence of NaCl such that a net osmolality of ≈300 mosmol/kg was obtained (i.e., isotonic conditions).

The data values were determined for 5 mL of a given solution using 0.1 or 1 N acid or base at 25°C. The addition of titrant was normalized to microequivalents of base or acid added per milliliter of solution. The lines represent linear fits to the data points. The slopes represent the buffer capacity plotted in Figure 8.

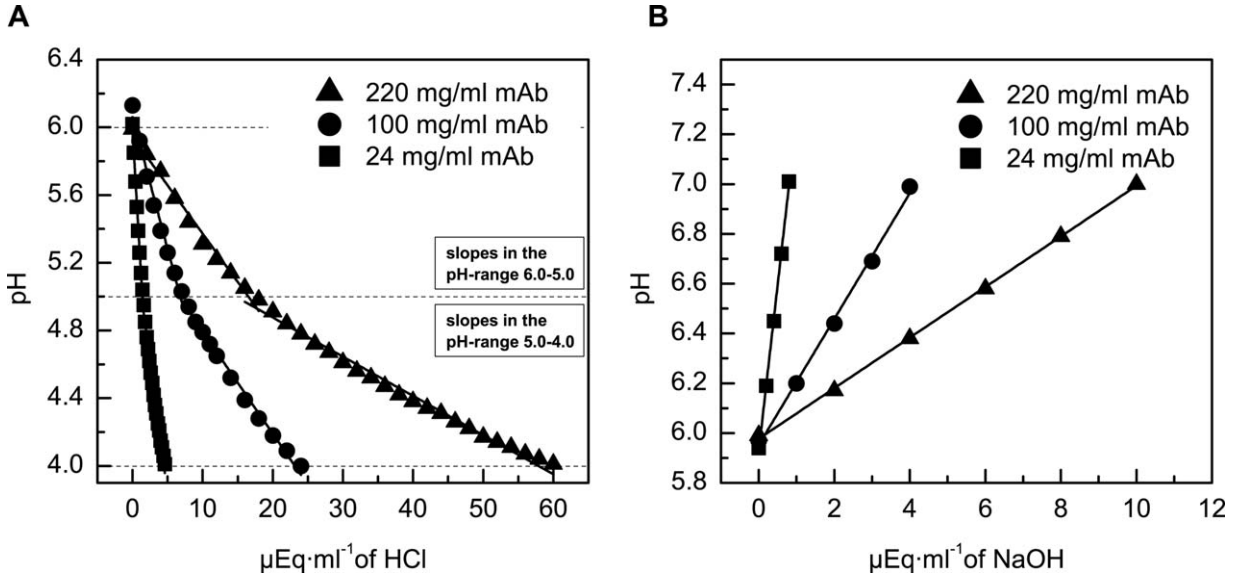

Figure 7. Titration curves of mAb4 at different mAb concentrations in the presence of trehalose such that a net osmolality of ≈300 mosmol/kg was obtained (i.e., isotonic conditions).

The data values were determined with 5 mL of a given solution using 0.1 or 1 N acid or base at 25°C. The addition of titrant was normalized to microequivalents of base or acid added per milliliter of solution. The lines represent linear fits to the data points. The slopes represent the buffer capacity plotted in Figure 8.

be able to prevent pH shifts also at varying temperature, we determined the temperature effect on buffer capacity experimentally. Figure 9 depicts titration curves of mAb3 at 5, 25, and 40°C each at different protein concentration. Control experiments employing differential scanning calorimetry[20] showed the antibody in 160 mM NaCl is fully folded at 40°C. The first unfolding event takes place at temperatures above ~60°C (T_{m1}=67°C, data not shown).

In the titration curves at 5, 25, and 40°C, we find a linear dependence of pH value and added μEq of acid in the range of pH 4.0–5.0 and 5.0–6.0 at all temperatures. Moreover, the slope of the curve and hence the buffer capacity is nearly identical for all temperatures. This holds true for titrations at protein concentrations of 10 and 100 mg/mL. Also, in the range of pH 6.0–7.0 and 7.0–8.0, a nearly identical buffer capacity was determined from titrations at 5, 25, and 40°C (data not shown). The experiments indicate the inherent buffer capacity of mAbs is not significantly altered upon temperature changes within the range relevant for biopharmaceutic processes.

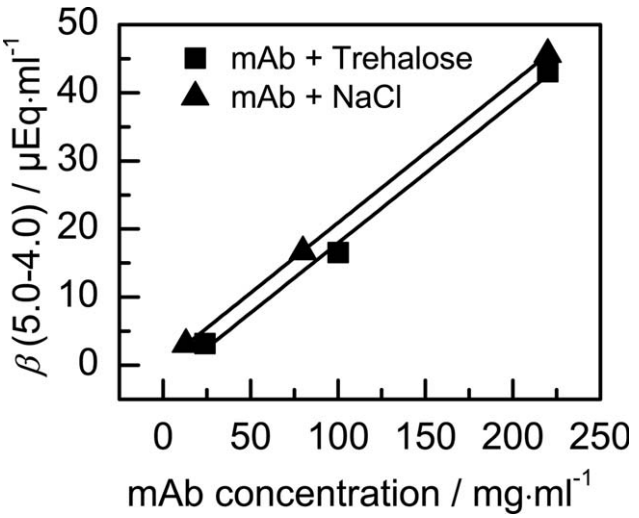

Figure 8. The experimentally determined buffer capacities β of mAb4 in the presence NaCl or trehalose (titration curves Figures 6 and 7) plotted vs. the corresponding mAb concentration.

Data points were fitted to a linear equation. The antibody exhibits a linearly increasing buffer capacity up to concentrations above of 200 mg/mL mAb.

Buffer capacity of antibodies and a conventional buffer at varying ionic strength

According to the Debye–Hückel theory[21] the activity coefficients of the species in an ionization equilibrium and hence the pK_a of acids depend on the ionic strength of a solution. A change in ionic strength can thus alter the average buffer capacity of a defined pH range. To check for the practical relevance of this effect, we determined average buffer capacities of buffers for different ionic strength conditions. The titration curves for mAb3 at different ionic strength can be found in Figure 10. We find that a change in ionic strength in the range of 10–160 mM NaCl (commonly employed in mAb formulations) induces a change in the buffer capacity of mAb3 in the range of pH 5.0–6.0 and 6.0–7.0 (change in β around 35 and 25% respectively). For the conventional buffer histidine the effect is rather negligible (change in β below 15 and 10% respectively).

Discussion

Buffering issues, here understood as buffering of pH, are omnipresent throughout the biopharmaceutical process. Changes in proton concentration can be caused by CO_2 intake, leaching or chemical reactions.[22] However, retaining a stable pH is essential for protecting the therapeutic agent (e.g., a monoclonal antibody) from degradation during manufacturing, storage, and application. Moreover, profound knowledge about buffering characteristics of a system in question is also necessary when controlled pH shifts take place (e.g., in virus inactivation steps, for ion exchange chromatography). This study summarizes buffer characteristics of buffer agents widely used in the biopharmaceutical setting, that is, acetate, citrate, histidine, succinate, and phosphate. Employing the slopes of capacity shown here, one can estimate the amount of protons (or hydroxide ions) a buffer system of a certain concentration can cope with before the pH is changed and vice versa how many acid/base needs to be added to achieve a requested pH shift. We find that parameters derived via the van Slyke equation (Eq. 2)[15] are widely consistent with experimental results. Thus, parameters can actually be calculated without any prior knowledge for these buffering agents employing the equation of van Slyke. At low (and high) pH the ionization equilibrium of water contributes to the total buffer capacity[15] (cf., Figure 1) but the contribution at the pharmaceutically relevant pH range of pH 4.0-7.0 is negligibly small at buffer concentrations >10 mM.

Table 5. Experimentally Determined Slopes of Capacity for Different pH Ranges and mAb4 in the Presence of Appropriate Amounts of Trehalose or NaCl Adjusting the Solution to Isotonicity

	Slope of Capacity a_{exp} (µEq/mg)	
pH-range	Trehalose	NaCl
5.0–4.0	0.205	0.206
6.0–5.0	0.083	0.079
6.0–7.0	0.048	0.044
7.0–8.0	0.046	0.044

The van Slyke equation was derived for the aqueous solution of the buffer agent only. Neglecting interactions between the molecules in the system, one can use the concentrations of the species in question just as we did here when employing the van Slyke equation and Eq. 3. However, one should always keep in mind that equilibrium constants (as featured in these equations) in the first place are defined via activities that are functions of the concentration and the activity coefficients. For a detailed background and implications, the reader is referred to textbooks in physical chemistry. In essence, one should not forget the following. On the one hand, the electrode in pH measurements responds to the proton activity that depends on the ionic strength I of the solution. Thus, the measurement gives only an approximate proton concentration. On the other hand, the ionic strength I contribution of the buffer itself and ionization equilibria of additional substances such as NaCl will also influence the equilibrium of the buffer (cf. simplified Debye–Hückel equation, Eq. 6, adapted from Scopes[23]). This effect will play a significant role for large changes in ionic strength. As an example the pK_a of the histidine imidazole moiety changes from 6.04 at $I=0$ to 6.21 at $I=0.5$ (calculated employing Eq. 6). For the range up to isotonicity the effect on buffer capacity close to the pK_a seems to be rather negligible. We found an about 10% change in buffer capacity for 25 mM histidine (pH range 5.0–7.0, that is, around pK_a=6) when changing the NaCl concentration from 10 to 140 mM. Equation 6 predicts a change in pK_a of 0.06 units under these conditions (neglecting the contribution of the ionic histidine species themselves). As a result, the slope of capacity changes by 7% (pH 5.0–6.0) or 5% (pH 6.0–7.0) consistent with the slight change in buffer capacity we have observed in the experiment. According to Eq. 6, the effect is more pronounced for multicharged buffers (e.g., for the third ionization of citrate $z=-2$).

$$pK_a^I = pK_a + \frac{0.5(2z-1)\sqrt{I}}{1+1.6\sqrt{I}} \quad (6)$$

pK_a^I–pK_a value at ionic strength I with z being the charge on the acid buffer form

As a matter of fact, uncharged excipients such as commonly used sugars,[24] for example 10% α-trehalose in Lucentis® (Genentech, San Francisco), do not influence the ionization of the buffer agent via this mechanism.

The upcoming occurrence of high concentrated liquid formulations of monoclonal antibodies suggested the development of self-buffering antibody solutions.[9] In the literature,

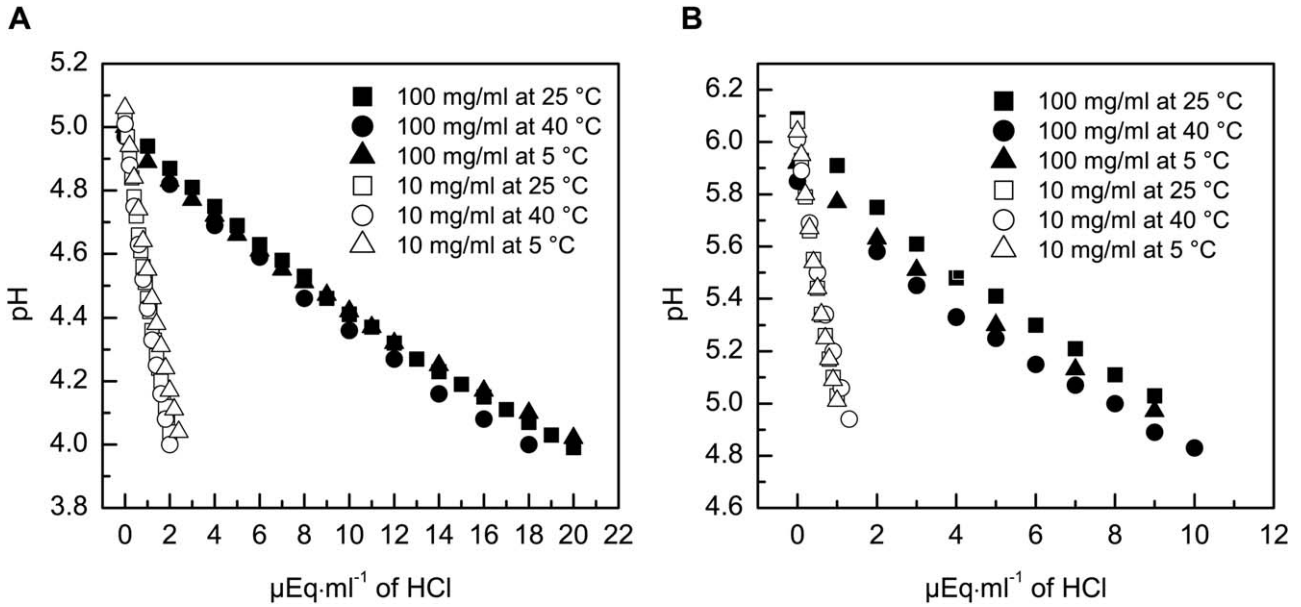

Figure 9. Titration curves for 10 and 100 mg/mL mAb3 each at 5, 25, and 40°C in the range of pH 4.0–5.0 (A) and pH 5.0–6.0 (B).

The mAb solution was titrated from the starting value of around pH 6–5 and subsequently to pH 4. The addition of titrant was normalized to microequivalents of acid added per milliliter of solution. The curves and the corresponding slopes (i.e., the inverse of β) are similar for all temperature conditions.

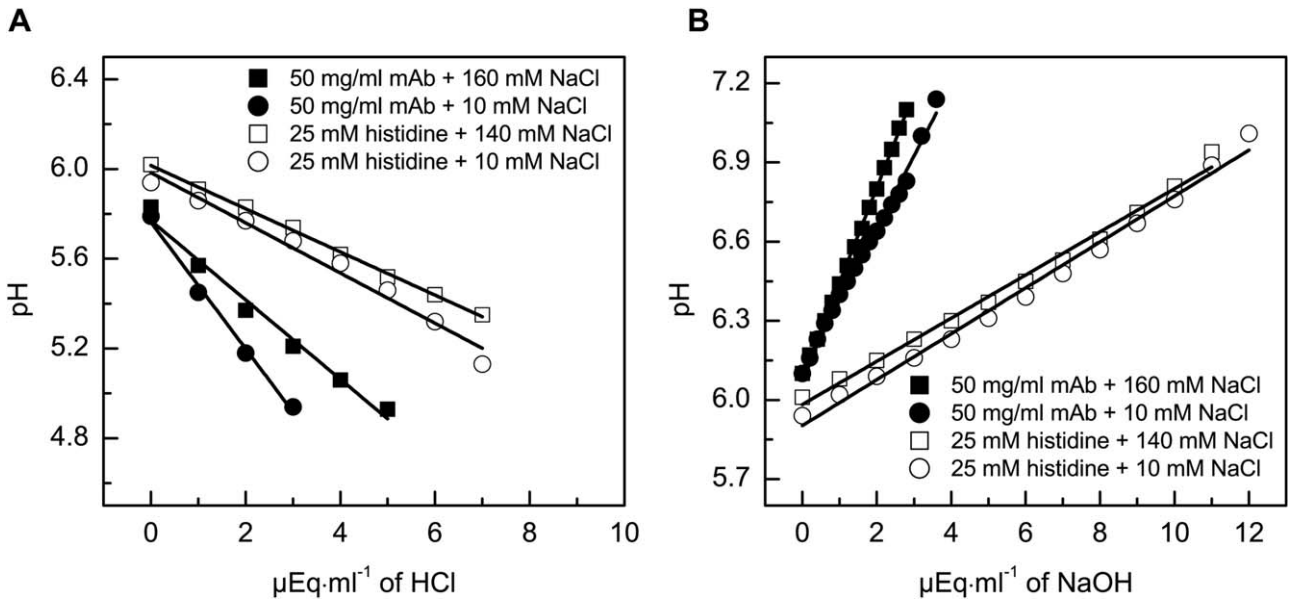

Figure 10. Titration curves for 50 mg/mL mAb3 and 25 mM histidine buffer at different ionic strength.

Titrations were performed at 25°C in the range of pH 5.0–6.0 (A) and 6.0–7.0 (B). The addition of titrant was normalized to microequivalents of acid added per milliliter of solution.

the characterization of proteins in vitro with respect to buffer attributes widely focuses on determination or prediction of amino acid side chain pK_a values and isoelectric points.[25–32] Findings in these studies touch on the subject of buffer capacity indirectly since any monovalent buffer exhibits a maximum molar buffer capacity of β_M=0.575 at the pK_a.[15] The buffering power is in principal equal for any (monovalent) buffer, just the pH range in which a certain average buffer capacity is observed, is specific. In addition, comprehensive information on buffer attributes can be found for proteins in physiological settings.[2,10,15,33] Findings on buffer capacity of antibodies in vitro for pH ranges down to pH 4.0 and up to 8.0 are summarized in the current study (Table 4). The prediction of buffering power by antibodies is not straightforward. Consistently with results of others,[9] models employed here that sum up the contributions of histidine, aspartate, and glutamate moieties in the protein overestimate the buffer capacity. Thus, the theoretically derived values might serve as setting an upper limit. In context of the protein tertiary structure pK_a values of the amino acid side chains can change drastically due to charged neighbors, hydrogen bonding or dehydration effects.[27,32,34,35] Changes up to three units have been observed for proteins (Asp76 in RNase T1 pK_a=0.5[36]). Similar phenomena are likely to occur in the studied antibodies and would totally diminish the buffering contribution of certain amino acids in the biopharmaceutically favored pH range of pH 4.0–

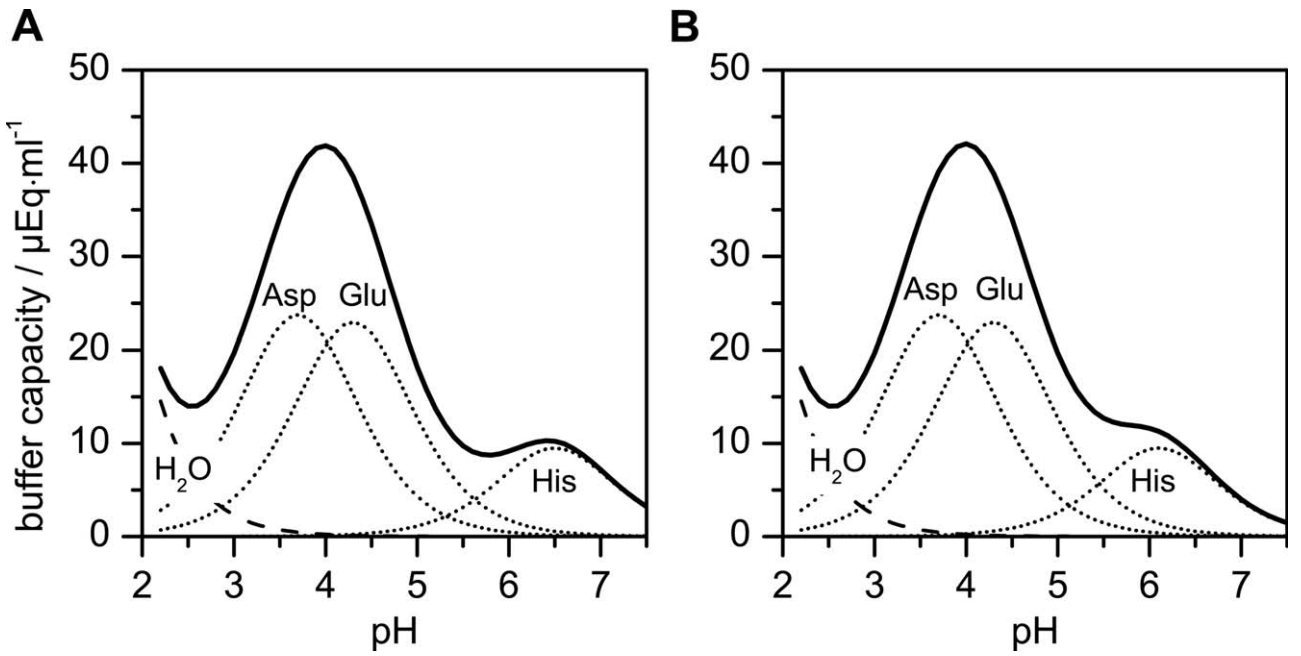

Figure 11. Buffer capacity β of a typical monoclonal antibody (simulated for 100 mg/mL mAb2) in aqueous solution vs. pH and the resulting total buffer capacity (solid line).

The total buffer capacity also includes the contribution of the water ionization equilibrium that becomes significant at pH<3 (dashed line). For pH<8 the buffering is mainly contributed by the aspartate, glutamate, and histidine residues (dotted lines). Lysines, arginines, and tyrosines commonly do not buffer at pH<8 and their contribution is omitted in the figure. All cysteine residues are paired in disulfide bonds in this antibody and therefore do not contribute to the buffering capacity. In panel A the buffer capacity was simulated assuming a pK_a value of 6.5, in panel B a pK_a value of 6.1 for the histidine imidazole (e.g., this pK_a shift can take place upon a temperature change of 20 K). The comparison of A and B exemplifies how temperature dependent pK_a changes may affect the total buffer capacity in theory.

7.0. This might explain why the apparent buffer capacity of the proteins is lower than inferred theoretically employing p*K*a values that were determined from model compounds.

The studied antibodies exhibit strong buffering in the range of pH 4.0–6.0. This range is within the pH range of favored chemical stability of proteins (pH 4.0–7.0) and is advisable for a self-buffering formulation. The buffer capacities we determined experimentally indicate the antibodies can sufficiently buffer proton changes due to leaching occurring during storage and handling.[22,37,38] An antibody solution of 50 mg/mL at pH 6 buffers around 1 mM of protons before the pH drops to pH 5.75. This amount surpasses the amount of protons leached from borosilicate glass vials by a factor of 100 (Bahrenburg et al., unpublished observations).

The strong buffering by proteins can affect controlled pH shifts during bioprocessing. We observed that the buffer capacities of the single components in a buffer-excipient-containing mAb solution merely sum up. The contribution of the protein influences for instance the amount of base that needs to be added to neutralize the product pool subsequent to Protein A affinity chromatography acidic elution.[39] Adding a fixed amount of base that had beforehand been determined by neutralizing the buffer salt solution (e.g., acetate) will for various samples result in different pH values depending on the protein concentration of the sample. The contribution by the buffer at a certain concentration can be calculated from the data in Table 3 and added to the protein's buffer capacity to determine the amount of acid/base for the intended pH shift. As controlled pH shifts are omnipresent in bioprocessing (e.g., ion exchange chromatography), we suggest determining the buffer capacity of the protein of interest to enhance process knowledge and accelerate process development.

Biopharmaceuticals are handled at room temperature, stored at 5°C and kept at up to 40°C in stability studies. Thus, the effect of temperature on buffer capacity needs to be considered. The temperature dependence of an equilibrium reaction is factorized via the enthalpy of the ionization reaction. If the standard molar ionization enthalpy is close to zero (e.g., for carboxyl groups and the second ionization step of phosphoric acid as well as organic derivatives of them[40]), pK_a values are hardly affected by temperature changes. The buffer capacity in the tested antibodies is for the most part provided by the carboxyl side chains of aspartate and glutamate (Table 2, Figure 11). Consistently, we found that temperature changes in the range of 5–40°C do not significantly affect the buffer capacity of monoclonal antibodies in the range of pH 4.0–8.0. In the range of pH 6.0–8.0, a typical antibody is predominantly buffered by its histidine residues. As the buffering imidazole moiety features a temperature dependent pK_a value,[23] in theory, an alteration in buffer capacity would be expected especially for pH 7.0–8.0 (Figures 11A,B exemplarily depict how the total buffer capacity changes upon a temperature change of 20 K, that is, pK_a of histidine changes from 6.5 to around 6.1). As discussed before, however, each single aspartate, glutamate and histidine residue in a protein might exhibit a different pK_a scattered around the theoretical value. As a result, the net buffer capacity vs. pH curve will flatten and also temperature dependent changes might be different for certain histidine residues. Both factors will induce a lower change in total buffer capacity than inferred from summing up the contribution of standard amino acid side chains and shifting pK_a values in a concerted fashion upon temperature change.

In contrast, the influence of ionic strength on protein self-buffering can be considerable. For lysozyme considerable changes on the titration curve were observed in the range of pH 1.5–4.5 for ionic strengths of $I=1$ and $I=0.1$. Effects were only minimal between pH 4.5 and 9.[26] Moderate changes on the titration curve were also observed for ribonuclease at ionic strength of $I=0.01$ to $I=0.15$[25] and cytochrome c at 0, 0.1, and 0.5 M NaCl.[41] We found differing titration curves (pH 5.0–7.0) and therefore different buffer capacities in that pH range for a monoclonal antibody when changing NaCl concentration from 160 to 10 mM ($I=0.16$ and 0.01 respectively neglecting the contribution of charged antibody to the total ionic strength of the solution). Contrasting the change observed in the experiments with a predicted change (as done for histidine above) is not straightforward. For the theoretical treatment of the ionic strength effect on charged proteins one has to take into account that for the buffering amino acid side chains the ionic strength in proximity of the ionized, multivalent protein will be different from the bulk ionic strength. Even if a mAb does not contribute a lot to the ionic strength, for example, of a 50 mg/mL protein+160 mM NaCl solution in bulk, the local ionic strength caused by the protein will be significant. This might also explain why the ionic strength effect on buffer capacity is different for the protein in comparison with histidine (cf., above and Figure 10).

Ionic strength effects need to be considered for example during purification processes. During storage and handling practically no changes in ionic strength occur. The use of self-buffering high concentrated antibody solutions might thus be first of all recommended for the end formulation or as a preliminary preparation before end formulation.[42] Also the preparation of lyophilisates starting from a self-buffered protein solution seems an attractive option. Because of the fact that ionic substances lower the glass transition temperature, generally minimizing the amount of electrolytes is preferred for freeze-drying processes. But, a possible impact of the omission of buffer excipients on stability during lyophilization and lyophilization process itself will have to be considered in future studies.

In stability studies of Gokarn and colleagues the pH of a self-buffering antibody solution remained constant over a 12 month storage, formation of soluble aggregates at 50°C was least for the self-buffered in comparison to the conventionally buffered solutions and the antibody was not compromised by freeze-thaw cycles.[9] In this study, sorbitol was added to the antibody solution to ensure isotonicity but could also have influenced protein stability. In our study described here, isotonicity conditions were approximated by including corresponding amounts of NaCl or trehalose in the solution but the evaluation of the long-term stability of these solutions is awaited from future studies. To completely omit all additives from an antibody solution is at least not first-choice, as it has sometimes been observed that phase separation is induced upon transfer of protein into excipient free solutions (i.e., pure water).[43]

Omitting the buffer agents from high concentrated protein formulations appears to be advantageous for the following reasons. From a toxicological point of view, reducing the amount of components in a drug product (e.g., by leaving out the buffering excipient) is generally preferred. Also, in the case of unprecedented buffering excipients the toxicological evaluation can be omitted saving time and costs if a self-buffering formulation constitutes an equivalent alternative. Furthermore, the processing step of preparing buffer solution is skipped. Discoloration events by buffer components[44] and chemical instability reactions caused by impurities in buffer excipients have been reported and can be avoided employing the self-buffering option. Moreover, the physico-chemical stability of the antibody might be even improved as an increasing concentration of conventional buffer has proven to sometimes enhance aggregation.[9] However, destabilizing as well as stabilizing effects have been attributed to buffer components.[45] Hence, case to case assessments are still advisable and extended data on the stability of self-buffering formulations are awaited from future studies. Currently, the following advantages of self-buffering formulations can be summarized:

- Avoidance of buffer-excipient-caused protein destabilization and degradation.
- Reduction of excipient content and potentially occurring impurities in the excipient (both being generally beneficial from toxicological point of view).
- Avoidance of buffer-excipient-induced discoloration issues.
- Reduction of steps in bioprocessing.

Limitations of self-buffering formulations are:

- Restriction in the suitable pH range as it needs to be covered by sufficient buffer capacity of the protein of interest.
- Potential instability of the protein of interest in absence of buffer excipient.

Conclusion

The current study investigates buffering capacity characteristics of buffer excipients and proteins in aqueous solution. As shown in this study, buffer capacities of the common buffer excipients acetate, citrate, histidine, and succinate can be reliably calculated employing a model proposed by van Slyke[15] (Eqs. 1 and 2). The calculation of protein buffer capacities is not straightforward as the currently available approaches to determine buffer capacity based on the amino acid primary sequence are poorly predictive. Therefore, experimental data need to be generated for the protein of interest. For highly concentrated protein solutions the buffer capacity by the proteins becomes equipotent to conventional buffer excipients and can replace the latter in terms of pH stabilization. The usage of the corresponding self-buffering protein solutions has certain advantages and disadvantages and therefore has to be evaluated for the specific case. Nevertheless, for the development of a biopharmaceutic product a self-buffering formulation constitutes a noteworthy formulation option.

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