Available online at www.sciencedirect.com

ScienceDirect

Current Opinion in
Colloid & Interface Science

How protonation modulates the interaction between proteins and pH-responsive hydrogel films

Gabriel S. Longo[1], Néstor A. Pérez-Chávez[1] and Igal Szleifer[2]

Abstract

Hydrogels of pH-responsive polymers are promising candidates for the design of functional biomaterials. In this context, understanding the complexity of the interaction between these materials and proteins is essential. A recently developed molecular-level equilibrium theory for protein adsorption on hydrogels of cross-linked polyacid chains allows for modeling size, shape, charge distribution, protonation state and conformational degrees of freedom of all chemical species in the system; proteins are described using a coarse-grained model of their crystallographic structure. This review summarizes our recent studies, which have focused on understanding how the interaction between proteins and pH-responsive hydrogel films depends on the pH and salt concentration, both in single protein solutions and mixtures. In particular, we discuss the key role that protonation plays in mediating the polymer-protein electrostatic attractions that drive adsorption. Deprotonation of the polyacid network modifies the nano-environment inside the hydrogel; the local pH drops inside the film. In single protein solutions, protonation of amino acid residues in this lower-pH environment favors adsorption to the hydrogel. Upon adsorption, the net charge of the protein can be several units more positive than in the solution. The various amino acids protonate differently, in a non-trivial way, which gives flexibility to the protein to enhance its positive charge and favor adsorption under a wide range of conditions. In binary and ternary protein solutions, amino acid protonation is the decisive factor for selective adsorption under certain conditions. We show that the polymer network composition and the solution pH can be used to separate and localize proteins within nanometer-sized regions.

Addresses

[1] Instituto de Investigaciones Fisicoquímicas Teóricas y Aplicadas (INIFTA), UNLP-CONICET, La Plata, Argentina

[2] Department of Biomedical Engineering, Department of Chemistry and Chemistry of Life Processes Institute, Northwestern University, Evanston IL, USA

Corresponding author: Longo, Gabriel S. (longogs@inifta.unlp.edu.ar)

Current Opinion in Colloid & Interface Science 2019, **41**:27–39

This review comes from a themed issue on **Theory and Simulation**

Edited by **Nily Dan** and **Zbigniew damczyk**

For a complete overview see the Issue and the Editorial

https://doi.org/10.1016/j.cocis.2018.11.009

Keywords

Protein adsorption, pH-responsive hydrogels, Acid-base equilibrium, Protonation.

Introduction

Hydrogels consist of a highly hydrated, generally biocompatible, cross-linked polymer network. These materials can resemble biological tissue and be engineered to respond to environmental changes such as variations in temperature, pH, ionic strength and in the concentration of some biomolecules. As a result, polymer hydrogels are currently promising candidates for the development of a variety of biomaterials with applications to biosensing [1,2], tissue engineering [3,4], bone regeneration [5], biomimetic materials [6,7], drug delivery [8,9] and many other biomedical applications [10]. The aqueous environment inside hydrogels can protect proteins from denaturation and aggregation [11–13], while they remain active and structured when delivered from hydrogels [14]. In oral drug delivery, hydrogels with pH response have been largely investigated as functional vehicles that can encapsulate and deliver proteins, preventing their degradation in the gastrointestinal environment [15–17].

Controlling the function and behavior of a biomaterial requires understanding its interaction with proteins. For example, contact lenses based on pH-responsive poly(-methacrylic acid) (PMAA) are exposed to tear fluid, which contains hundreds of proteins. Adsorption of some of these proteins must be prevented as it affects wear comfort and it can lead to inflammation [18,19]; however, selective adsorption of proteins having antibacterial and anti-inflammatory properties, such as lysozyme, might be beneficial [19]. A complex interplay between different degrees of freedom, however, governs the interaction between proteins and polymer surfaces [20**]; the ability of both the adsorbate and adsorbent material to protonate/deprotonate, regulate their electric charge, and modify the near-by environment, adds to this complexity. For example, using isothermal calorimetry, Welsch et al. [21*] have emphasized the importance of amino acid protonation in the adsorption of lysozyme to core–shell microgels based on poly(acrylic acid) (PAA).

The first studies to draw special attention to the role of protonation considered protein adsorption to pH-responsive polymer brushes. Wittemann et al. [22**]

reported the strong interaction of bovine serum albumin (BSA) with nanoparticles whose surface was modified with a PAA brush. Their findings have revealed that protein adsorption to the weak polyelectrolyte brush decreases with increasing salt concentration. Moreover, there is significant adsorption at pH values above the protein's isoelectric point (pI), where both BSA and the brush are negatively charged.

Since then, a few theoretical models have been developed to explain these results. Biesheuvel and Wittemann [23*] and de Vos et al. [24**] constructed mean-field theories to study BSA adsorption to weak polyelectrolyte brushes having different geometries. These works show that the pH drops inside the brush, which increases the protein's degree of protonation inside the negatively charged polymer surface. This displacement of chemical equilibrium can even lead to charge reversal when the solution pH is close to the isoelectric point of the protein. Other studies have suggested that an inhomogeneous charge distribution on the protein surface and the interaction of polyelectrolyte chains with positively charged regions of the protein are responsible for the adsorption above pI [25,26]. Using a self-consistent field approach de Vos et al. [27] showed that both effects, an inhomogeneous charge distribution and charge regulation, contribute additively to adsorption above pI. However, they concluded that the main effect results from protein protonation at the lower pH inside the brush. Here, we are not particularly concerned with the adsorption of proteins above their isoelectric point, but these studies on polyelectrolyte brushes certainly highlight the importance of protonation and that new phenomena can emerge as a result. In this review, we describe how adsorption results from the interplay between a different environment inside the hydrogel and protein charge regulation. Both of these effects are the consequence of the ability of these molecules, polyacid network and proteins, to locally displace the chemical equilibrium of their different ionizable units.

Several experimental studies have considered protein adsorption to polymer hydrogels using a variety of methods [21,28]. Theory and molecular simulations are a valuable tool to investigate the underlaying phenomena in protein adsorption to different materials, providing information that cannot otherwise be accessed experimentally. However, theoretical studies of protein adsorption to hydrogels are not abundant in the literature. Johansson et al. [29*] developed a mean-field cell model to describe lysozyme adsorption to poly(NIPAM-co-acrylic-acid) nano/microgels. This work indicates that the driving force for protein incorporation into the polymer network results from the interplay between electrostatic attractions and entropic effects associated with the translational freedom of salt counterions. Upon adsorption at neutral pH, the protein acts as a multivalent ion that allows for the release of network counterions to the bulk solution; initially, these counterions are confined to keep the interior of the microgel electroneutral. This entropic effect that drives adsorption weakens with increasing ionic strength. By use of titration calorimetry experiments, Yigit et al. [30*] developed Langmuir binding models to describe lysozyme adsorption to core—shell microgels, where the state of charge of these gel particles can be modified by the adsorption. These models were used to quantify and separate the contributions to protein adsorption from electrostatic, hydrophobic and van der Waals interactions. Adroher-Benítez et al. [31*] extended these models to incorporate the effect of the surface charge distribution of the proteins at the dipole level. This dipolar contribution favors interfacial adsorption rather than partition inside the gel particle. Angioletti-Uberti et al. [32*] developed a classical dynamic density functional theory approach to study the adsorption kinetics of lysozyme to charged polymer-coated nanoparticles. Oberle et al. [33*] developed a multicomponent cooperative binding model to investigate competitive adsorption of proteins to a soft polymeric layer.

The aforementioned theoretical research on polyelectrolyte brushes and gels have shed light on many new underlaying aspects of protein adsorption to pH-responsive materials. In these works, the protein is modeled as a cylindrical or spherical object without a detailed information of its three dimensional structure. Then, these studies cannot evaluate the effect that the distribution of titratable amino acids on the protein structure has on the adsorption and protonation behavior. Molecular dynamics (MD) has been widely applied to investigate the adsorption of proteins to different surfaces and nanoparticles [34—38]. By use of MD simulations, Sun et al. [39*] addressed the adsorption and complexation of the fragment antigen-binding of trastuzumab to a polyvinyl alcohol hydrogel at different pH values. In most molecular simulation studies, however, the protein is considered to have a fixed charge, and the protonation/deprotonation of its amino acids ignored. With the recent development of molecular simulation methods that can account for amino acid protonation, particularly Monte Carlo methods and constant-pH molecular dynamics, the interaction of charge-regulating proteins with charged surfaces and polyelectrolytes have been studied [40—43]. In the next few years these methods will surely be applied to investigate molecular-level details of protein adsorption to pH-responsive gels.

By use of molecular-level theory, we have studied the thermodynamics of hydrogels of cross-linked polyacid chains, including bulk gels [44], free-standing surface-deposited films [45], and surface-grafted films [46]. More recently, we have applied this theoretical framework to consider peptide and protein adsorption to hydrogel nanofilms of cross-linked polyacid chains [47—50]; currently, the most general version of this theory

can be found in Ref. [50**]. This method represents an extension of the molecular theories developed by Szleifer and co-workers to investigate protein adsorption on grafted polymer layers [51–53], and the behavior of grafted weak polyelectrolyte layers [54,55]. The predictions of this theory have been shown to be in excellent quantitative agreement with experimental observations for a variety of polymeric systems [56–60].

This theoretical approach allows for a description of size, shape, charge distribution, protonation state and conformation of all molecular species in the system, including the proteins and the polyacid network. This is achieved through the formulation of a general free energy that includes all the relevant contributions: the acid–base equilibrium, the entropic loss of molecular confinement, the conformational degrees of freedom of the network and the proteins, and the electrostatic, van der Waals and steric interactions. Our work has focused on understanding how the adsorption to these hydrogel films depends on pH and salt concentration, both in single protein solutions and mixtures. In this method, the protonation state of protein residues and that of network segments are not assumed a priori depending on the solution (bulk) pH, rather they are locally predicted as a result of the group position and its local environment. Our studies highlight the non-trivial role that amino acid protonation plays in protein adsorption. As aforementioned, there are currently a few molecular simulation methods that can describe the acid–base equilibrium of protein residues. However, to the best of our knowledge, our theory is the only molecular-detailed method that has been applied to consider the effect of protonation on the adsorption of proteins to a pH-sensitive material.

In this review, we discuss how protonation mediates peptide and protein adsorption to hydrogel films of cross-linked polyacid chains. To this goal, we include results corresponding to the adsorption from NaCl solutions of hexahistidine, cytochrome *c*, lysozyme, and myoglobin to poly(methacrylic acid) hydrogels. The solution composition (pH, NaCl and protein concentrations) is the relevant independent variable of our studies. In order to apply our theory, a molecular model must be defined to describe the chemical species that compose the system. Proteins have been modeled through applying a coarse-grained model on their crystallographic structure (protein data bank entries 3RGK, 193L, and 2B4Z for myoglobin, lysozyme and cytochrome *c*, respectively), where each amino acid residue is represented by a single particle (see Figure 1). These coarse-grained units can be either electroneutral or titratable; this latter group includes the acidic and basic residues. For the peptide, we use the same coarse-grained scheme, but all its conformations are generated using a rotational isomeric state model. Using this coarse-grained model, Figure 1 shows the net charge of the proteins in dilute solution as a function of pH. The calculated isoelectric points are 7.1, 10.8 and 9.8 for myoglobin, lysozyme and cytochrome *c*, respectively, which agree with the experimental values.

In the next section, we discuss how the chemical equilibrium of acidic units of the hydrogel network is displaced towards higher protonation (less electric charge). This behavior results in a different microenvironment inside the hydrogel, which significantly conditions protein adsorption. Adsorption from single peptide and protein solutions is discussed in Section Adsorption to pH-responsive hydrogels from single protein solutions, where we describe the non-trivial protonation of different amino acids. In Section Competitive protein adsorption, we consider protein adsorption from binary and ternary mixtures, where again protonation plays an important and unexpected role to induce selective adsorption.

Hydrogel films: pH-Response in salt solutions

Ideal behavior

Hydrogels of cross-linked polyacid chains are sensitive to changes in the solution pH or salt concentration. This response is due to the protonation/deprotonation of acidic units in the polymer network that comprises the backbone of these macromolecules. In this section, we will briefly remind the reader of some concepts on the protonation behavior of isolated acidic/basic molecules under ideal conditions. These concepts will later help us understand the displacement of acid–base equilibrium that occurs when the acidic units are confined to a polymer network instead of free in solution. In addition, these same notions apply to describe amino acid protonation when proteins adsorb to the hydrogel.

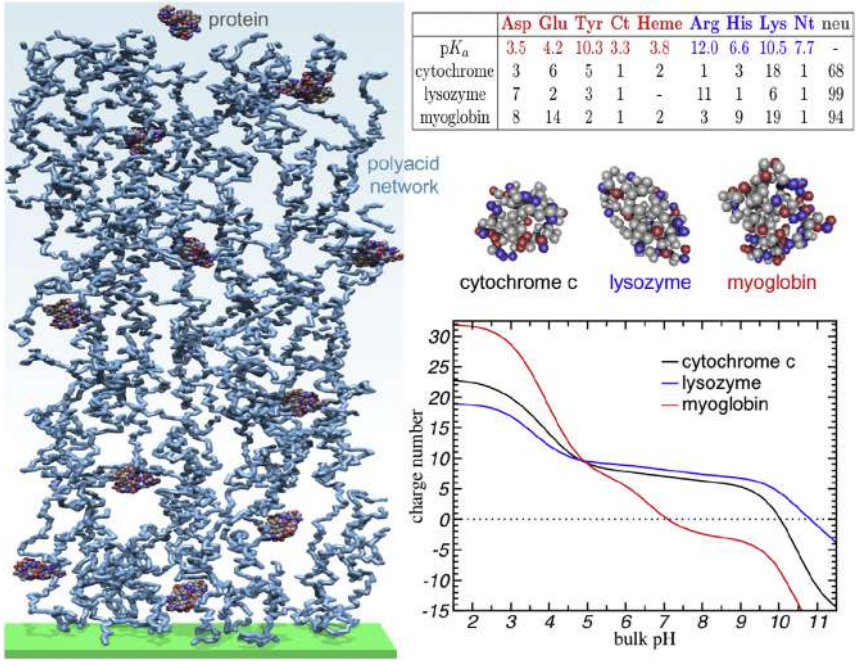
Consider a dilute aqueous solution of molecules bearing a titratable group. These molecules can exist in either one of two possible chemical states, protonated or deprotonated. The degree of proton dissociation of these titratable groups, f_d, gives the fraction of molecules that occupy the deprotonated state:

$$f_d = \frac{1}{1 + 10^{\mathrm{pKa}-\mathrm{pH}}} \quad (1)$$

For acidic groups, the protonated state is charge neutral while the deprotonated species is negatively charged; namely, f_d gives the fraction of charged molecules or degree of charge, f_c. For basic groups, on the other hand, the degree of charge is $f_c = 1 - f_d$, because the protonated species is positively charged while the deprotonated state is charge neutral.

In this dilute solution, f_d (and f_c) is completely determined by the solution pH and the intrinsic pKa of the

Figure 1



	Asp	Glu	Tyr	Ct	Heme	Arg	His	Lys	Nt	neu
pK_a	3.5	4.2	10.3	3.3	3.8	12.0	6.6	10.5	7.7	-
cytochrome	3	6	5	1	2	1	3	18	1	68
lysozyme	7	2	3	1	-	11	1	6	1	99
myoglobin	8	14	2	1	2	3	9	19	1	94

Left: Scheme showing a hydrogel film in contact with a protein solution. Right: The molecular model used to describe the proteins, where each amino acid residue is represented by a single coarse-grained particle that can be either electroneutral (grey spheres), acidic (red spheres) or basic (blue spheres). The top-right table presents the pKa values of some of the titratable coarse-grained units [50], which correspond to average values over different proteins obtained from several experimental results [61]. Using this pKa scheme, the bottom–right graph shows the net charge number of the proteins in dilute solution.

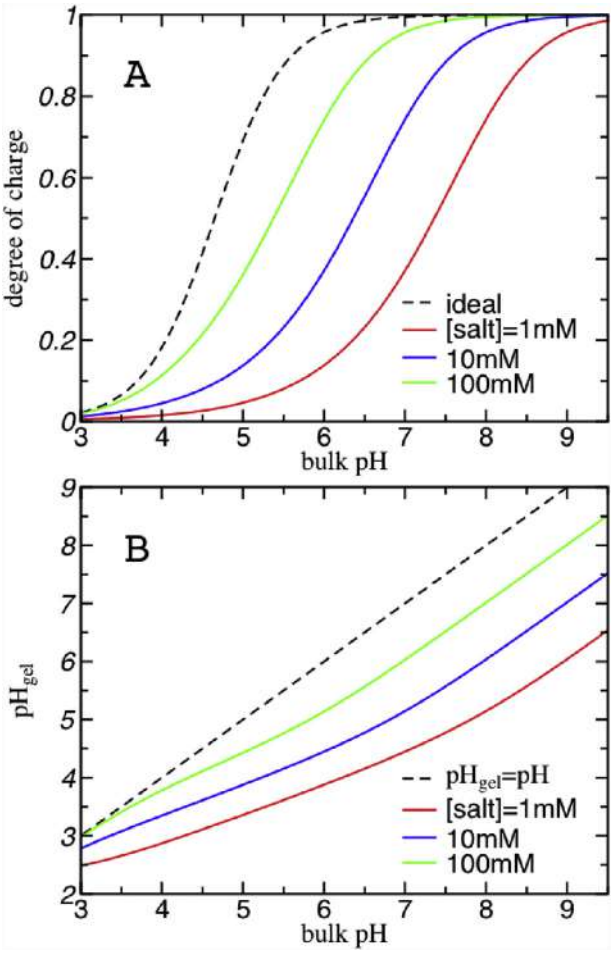
acid/base. When pH = pKa, half of the titratable groups are in the protonated state ($f_d = 0.5$). When pH = pKa − 1 less than 10% of molecules are deprotonated ($f_d < 0.1$), while when pH = pKa + 1 more than 90% of these molecules occupy this state ($f_d > 0.9$). Namely, when pH increases around pKa, the transition from 10 to 90% deprotonation occurs within two units of pH of the ideal solution. Oftentimes, such ideal solution considerations are used to estimate the degree of charge of acidic units inside the hydrogel polymer network. However, we will see next that confinement of these units to a polymer network significantly modifies their protonation behavior.

Polymer network dissociation

In this section we will describe the charging behavior of pH-responsive hydrogels of cross-linked polyacid chains. In contrast to dilute solutions, acidic units in a polymer network experience electrostatic repulsions if they are charged. To reduce the strength of intra-network repulsions, these groups dissociate significantly less than under ideal conditions. Figure 2A illustrates this behavior, and it shows the average degree of charge of the segments of a hydrogel film of poly(-methacrylic acid) (PMAA), which is in contact with solutions having different salt concentrations. At a given pH, an acidic unit of the network is significantly less likely to be charged than what is expected from ideal considerations. The salt concentration of the solution is the critical environmental variable that modulates this charge regulation behavior. At relatively high salinity, significant concentrations of both counter- and co-ions are found inside the hydrogel, resulting in the screening of electrostatic interactions, which effectively become short range. This screening of intra-network repulsions allows for the polymer to increase its degree of charge in order to lower the chemical free energy that describes the acid–base equilibrium. At sufficiently high salinity protonation approaches the ideal behavior. At low salt conditions, on the other hand, the entropic cost of confining ions inside the hydrogel increases. Only enough counter ions are present inside the network to neutralize the electric charge of the polymer. Under such conditions, the screening effect of salt ions weakens, and the electrostatic interactions effectively become longer range. As a result, the network charges less to prevent or reduce intra-network repulsions.

Another way to look at this behavior is considering the local pH, which we define at a spatial position **r** using the local concentration of protons:

Figure 2



Plot of the protonation behavior of a surface-grafted hydrogel of cross-linked PMAA in contact with aqueos solutions having different NaCl concentrations. A: Degree of charge of PMAA segments as a function of pH; the dashed line corresponds to the behavior of a dilute MAA solution (intrinsic pKa 4.65). B: Plot showing the average pH inside the hydrogel, pH_{gel}, as a function of the solution pH; the dashed line serves as a reference and describes the situation where the pH inside the film takes the bulk value. (Data partially published in Ref. [50].)

$$\mathrm{pH}(\mathbf{r}) = -\log_{10}\left(\left[\mathrm{H}^{+}\right](\mathbf{r})\right) \tag{2}$$

A lower dissociation (higher protonation) of polymer acidic units can be described in terms of a local-pH drop inside the material. Let us define pH_{gel} as the average of the local pH inside the hydrogel film. Our recent results have shown that this quantity is well defined [46]; here we will emphasize the importance of pH_{gel} and pH(**r**) in terms of the information they provide, the state of charge/protonation of all titratable units in the vicinity.

If a different pH establishes inside the hydrogel, the charge on the polymeric structure can be estimated using pH_{gel} instead of the bulk pH in Equation (1) [49**]. A similar procedure allows for calculating the local state of protonation of the different amino acid residues of adsorbed proteins. However, though this seems to simplify the problem of establishing the net charge of any species inside the material, including the polymer network and adsorbed proteins, determining changes in local pH has the same complexity as the original problem (*i.e.*, determining the charge of the network). The local pH that establishes inside the material, as well as its value in the interface between the polymer and the aqueous solution, is the result of the complex interplay between molecular organization, chemical equilibria, and physical interactions that determines thermodynamic equilibrium at the externally imposed conditions (pH, salt concentration). For example, Figure 2B shows the pH inside a PMAA hydrogel film as a function of bulk pH and salt concentration, calculated using our molecular theory.

Adsorption to pH-responsive hydrogels from single protein solutions

Peptide adsorption

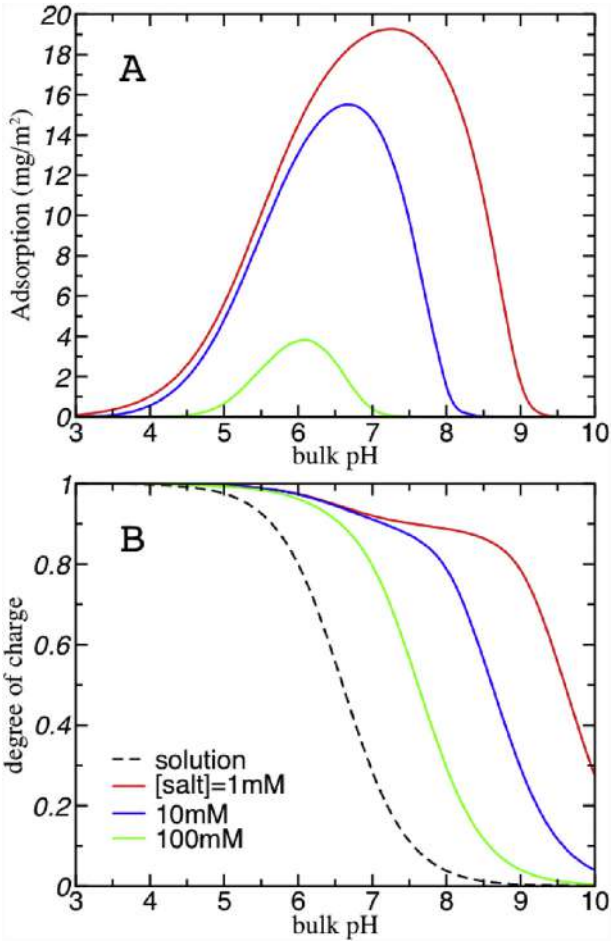
Before describing the adsorption of proteins, let us briefly consider a relatively easier problem, the adsorption behavior of a short homopeptide. We choose histidine peptides, known as his-tag, which are widely used as protein tags in chromatography [62]. Histidine is positively charged at low pH with pKa around 6 − 7, near that of the acidic units of the hydrogel. To further simplify our discussion, we will not consider the presence of the peptide chain's terminal nitrogen and carbon. To quantify the partition inside the hydrogel film, we define the adsorption as:

$$\Gamma = \int_{V} d\mathbf{r}(\rho(\mathbf{r}) - \rho_{\mathrm{bulk}}) \tag{3}$$

where $\rho(\mathbf{r})$ and ρ_{bulk} are the local and bulk densities of the adsorbate and V is the volume of the system. Hence, the adsorption gives the peptide mass in a particular volume in excess of the bulk contribution. In hydrogel films, Γ provides the excess amount of adsorbate inside the material, but it also recibes contributions from the polymer−solution interface.

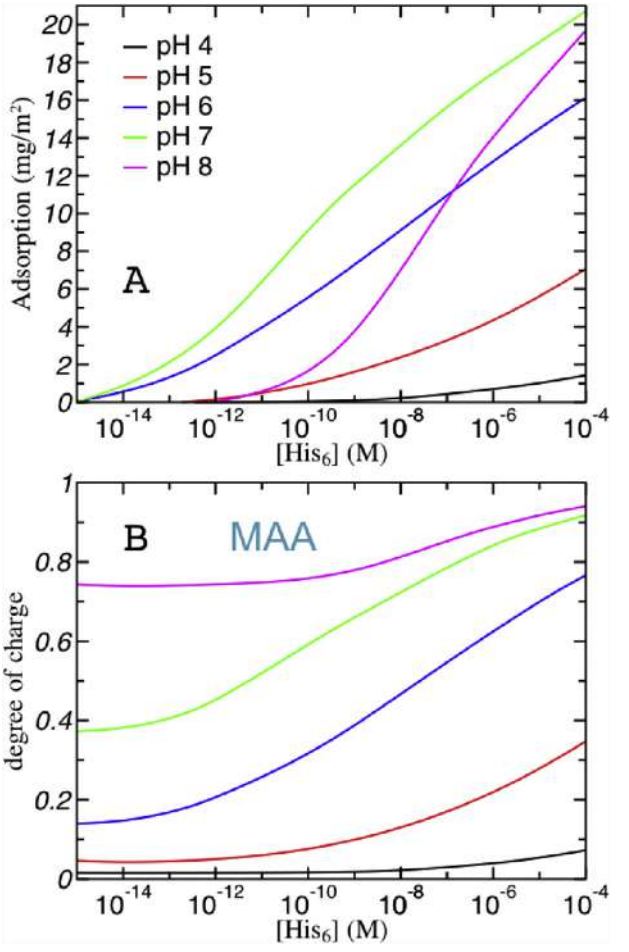
The adsorption of his-tag (hexahistidine) to a PMAA hydrogel film is a non-monotonic function of the solution pH, as shown in Figure 3A. This behavior is not completely unexpected; it can be predicted from ideal solution considerations given that adsorption is driven by electrostatic attractions. At low pH, solution histidine is mostly charged, but the polymer network is uncharged (see dashed line curves in Figures 2A and 3B, respectively). On the other side of the pH scale, the

Figure 3



A: Plot of the adsorption of hexahistidine to a PMAA hydrogel film as a function of pH, for solutions having different salt concentrations. Panel B shows the average degree of charge/protonation of His residues corresponding to adsorbed (solid lines) and solution peptides (dashed line).

Figure 4



A: Hexahistidine adsorption isotherms to a PMAA hydrogel film for different pH and 1mM NaCl. Panel B shows the average degree of charge of the network's MAA segments for the same conditions as panel A.

polymer network is sufficiently charged but solution histidine is uncharged. Under both conditions, there are no electrostatic attractions to drive adsorption. At intermediate pH values, on the contrary, both adsorbate and adsorbent material are significantly and oppositely charged for the adsorption to occur, which leads to the non-monotonic behavior observed in Figure 3A.

The predictive reach of ideal solution considerations, however, stops with the non-monotonic behavior. For example, the solution pH of maximal adsorption cannot be predicted in this way. As seen in Figure 3A, the magnitude of adsorption depends on the salinity of the solution. We have seen in Section Polymer network dissociation that the intrinsic pKa of the acidic segment and the solution pH do not provide the charge state of the polymer network. In addition to this, the charge of adsorbate molecules changes significantly due to the protonation of histidine units upon adsorption to the lower pH environment inside the hydrogel, as seen in Figure 3B. If we define the apparent pKa of histidine residues as the solution pH at which half of residues are protonated (charged), we see that for adsorbed molecules this quantity can be several units larger than the intrinsic histidine pKa. This displacement of chemical equilibrium occurs to increase the net positive charge of the peptide and enhance attractions with the polymer network. This behavior depends critically on the solution salt concentration. The lower the solution salinity, the more his-tag molecules protonate when they adsorb.

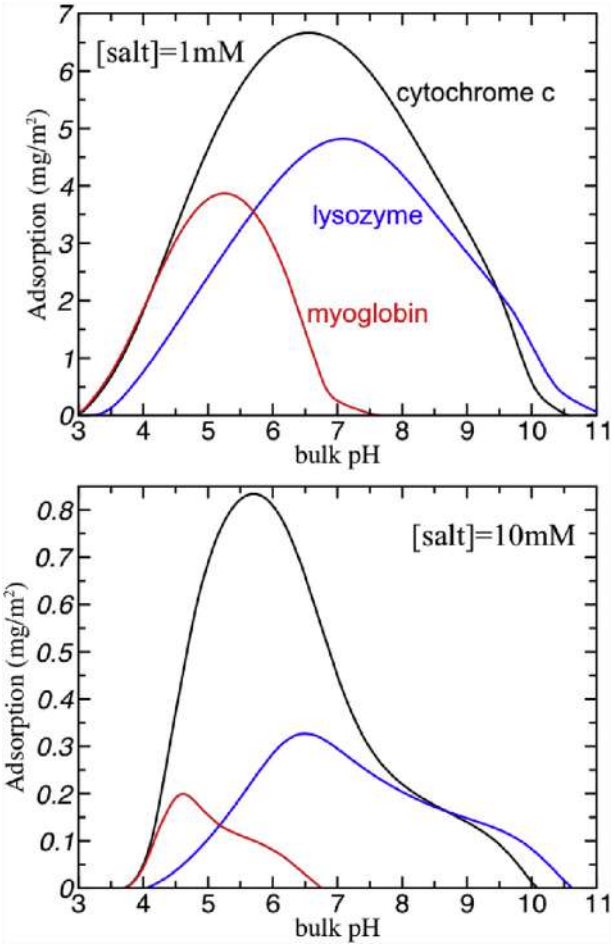
In addition, his-tag adsorption depends on the solution peptide concentration, as seen Figure 4A that presents adsorption isotherms at relatively low salt conditions. At sufficiently high but still relatively low high-tag concentrations, the charge of the network depends not only on the pH but also on the peptide concentration (see

Figure 4B). Namely, his-tag adsorption can modify the state of charge of the polyacid network [47*]. In other words, adsorption modifies the local pH inside the hydrogel. This behavior again occurs to enhance the His-MAA electrostatic attractions that drive adsorption. For all the conditions displayed in Figure 4, His residues of adsorbed peptides are highly charged ($f_c > 0.85$; results not shown).

Protein adsorption

We have recently investigated the thermodynamic adsorption of proteins to hydrogel films of cross-linked polyacid chains [48–50]. Lysozyme, cytochrome *c* and myoglobin have been studied. For these proteins, adsorption is a non-monotonic function of solution pH (see Figure 5), behavior that can be understood in similar terms the adsorption of histidine peptides. At low pH, these proteins are highly, positively charged but the polyacid network is only weakly ionized (see Figures 1 and 2A). At sufficiently high pH, on the other hand, the polymer is strongly negatively charged but the proteins are either weakly positively charged or even negatively charged. Under such (very) acidic or alkaline conditions, the electrostatic interactions are weakly attractive or repulsive. There is no driving force for adsorption. At intermediate pH values, on the contrary, where both protein and polyacid network are strongly and oppositely charged, significant adsorption occurs with a necessary maximum under such conditions.

Figure 5



Plot of protein adsorption to a hydrogel film of PMAA. These results correspond to 10 μM single protein solutions. The two panels correspond to different NaCl concentrations. (Data partially published in Ref. [50].)

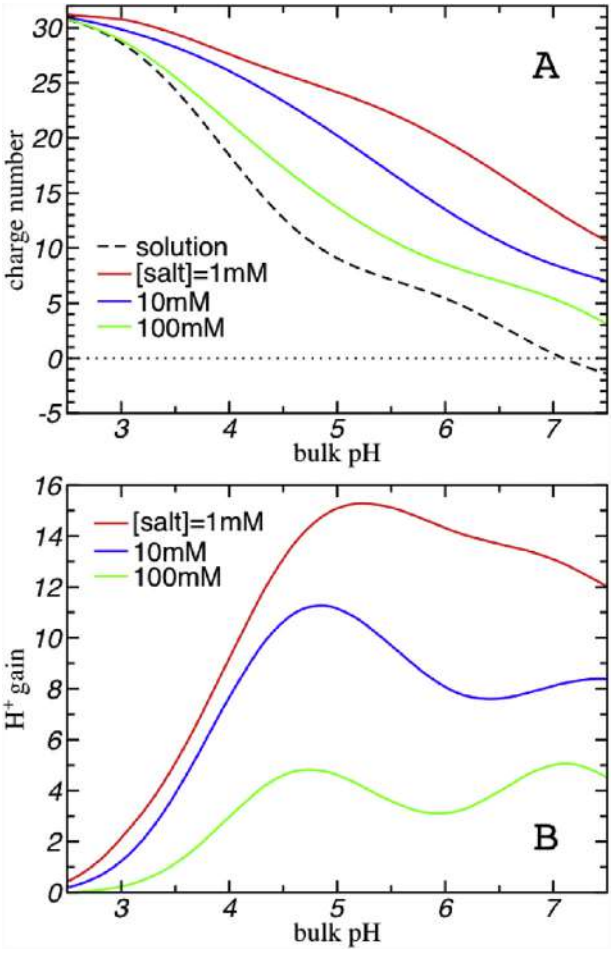
Protein adsorption depends critically on the solution salt concentration. This behavior is illustrated in Figure 5 that shows the adsorption of cytochrome *c*, lysozyme, and myoglobin to a PMAA hydrogel film. Decreasing salt concentrations enhances adsorption and widens the pH range of adsorption. For example, both panels of Figure 5 display roughly one order of magnitude decrease in adsorption when comparing 1mM and 10mM NaCl solutions. The pH of maximum adsorption also depends on the solution salinity. This behavior is even more interesting when considering that a lower salt concentration leads to a more weakly charged network, as we described in Section Polymer network dissociation. In other words, the more weakly charged polymer network, as the salt concentration decreases, adsorbs more protein. This last statement is true at the protein (10 μM) and salt concentrations of Figure 5, where adsorption only slightly modifies the degree of charge of the network.

This dependence of the adsorption on the salt concentration has three main reasons: First, there is the screening of protein-network electrostatic attractions by salt ions. The lower the salt concentration, the weaker the screening of protein-network interactions, which enhances adsorption. Second, as the salt concentration decreases the pH inside the hydrogel drops (at a given bulk pH). This implies that adsorbed proteins are more positively charged upon adsorption (as [NaCl] decreases). Third, the entropic gain of counterion release from the polymer network is higher as the salt concentration decreases, which also favors protein adsorption.

Non-trivial amino acid protonation

Our recent studies have shown that amino acid protonation plays a key role in protein adsorption to pH-responsive hydrogels. The local pH drops inside the polyacid hydrogel, which modifies the net electric charge of adsorbed proteins. For example, Figure 6A shows that the net charge of myoglobin is more positive upon adsorption to a PMAA hydrogel film. Depending on the conditions, the protein can gain several protons upon adsorbing to the hydrogel, as seen in Figure 6B. This behavior increases the net positive charge of the protein and favors attractions with the polyacid network. Above the isoelectric point of the protein, where the solution charge is negative, this behavior can induce

Figure 6

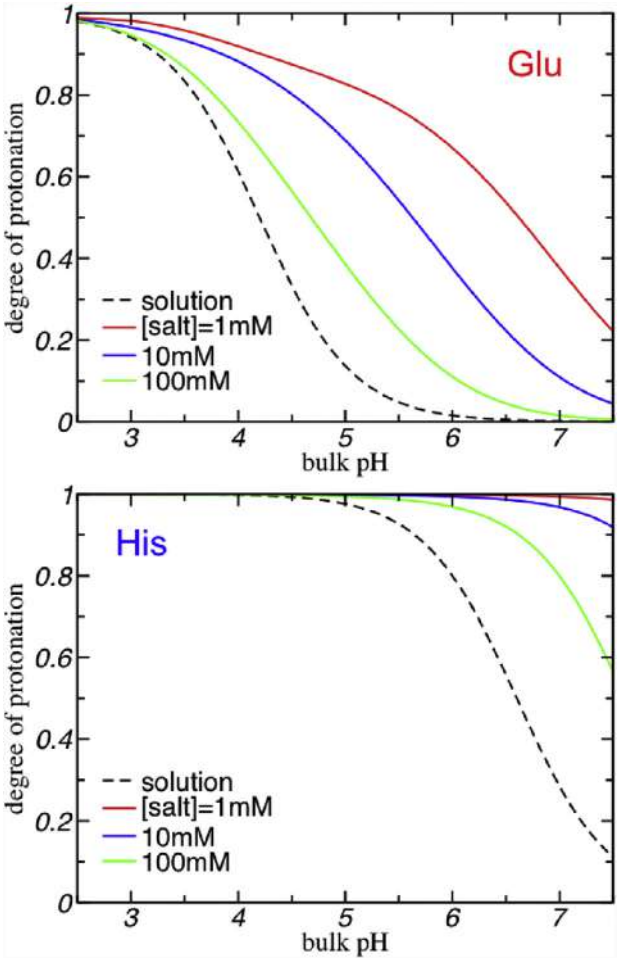


Plot of myoglobin charging behavior upon adsorption to a PMAA hydrogel film. Panel A shows the average net charge number of adsorbed (solid lines) and solution (dashed line) proteins as a function of pH, for different salt concentrations. Panel B shows the number of protons that myoglobin gains upon adsorption. These results correspond to 10 μM myoglobin solutions. (Data partially published in Ref. [50].)

charge reversal; that is, adsorbed proteins can be positively charged even when the solution pH is above their isoelectric point. This protonation behavior, including charge reversal, occurs in a few nanometers from the film top surface, in the hydrogel–solution interface [48*].

Protein charge regulation implies that amino acid residues displace their chemical equilibria into the direction of protonation. The molecular theory that we have developed allows for individually considering the contribution to charge regulation from each particular type of amino acid. Upon adsorption, the chemical equilibrium of all titratable amino acids is displaced in the direction of higher protonation. This means that acidic residues are less likely to be negatively charged inside the hydrogel, while basic residues are more likely to be positively charged. We illustrate this behavior in Figure 7 that shows the degree of protonation of glutamic acid (acidic) and histidine (basic) residues upon myoglobin adsorption to the hydrogel in comparison to those of solution proteins. This protonation behavior depends non-trivially on the experimental conditions. Displacement from ideal behavior is different for each particular amino acid, including the pH-width of the deprotonation transition (described in Section Hydrogel films: pH-response in salt solutions) and the relative change in apparent pKa. Therefore, having residues with different intrinsic pKa's gives the protein great flexibility to modify its net charge, under different conditions, to adjust the interactions with the polymer network that favor adsorption [49].

Figure 7



Plot of the average degree of protonation of myoglobin's glutamic acid (top panel) and histidine (bottom panel) residues as a function of pH. Solid line curves correspond to proteins adsorbed to the PMAA hydrogel at different salt concentrations, while dashed lines represent solution proteins. These results correspond to the same conditions as those of Figure 6. (Data partially published in Ref. [50].)

Calorimetry experiments of lysozyme adsorption to a PAA-based core–shell microgel indicate that the protein gains approximately one positive charge upon entering the gel under certain conditions [21], which agrees with our theoretical predictions [48]. Furthermore, the analysis of the protonation curves of lysozyme residues (similar to those displayed in Figure 7 for myoglobin), at the same conditions as the experiment, allows to conclude that this behavior results from the protonation of the single histidine residue when lysozyme adsorbs.

Competitive protein adsorption

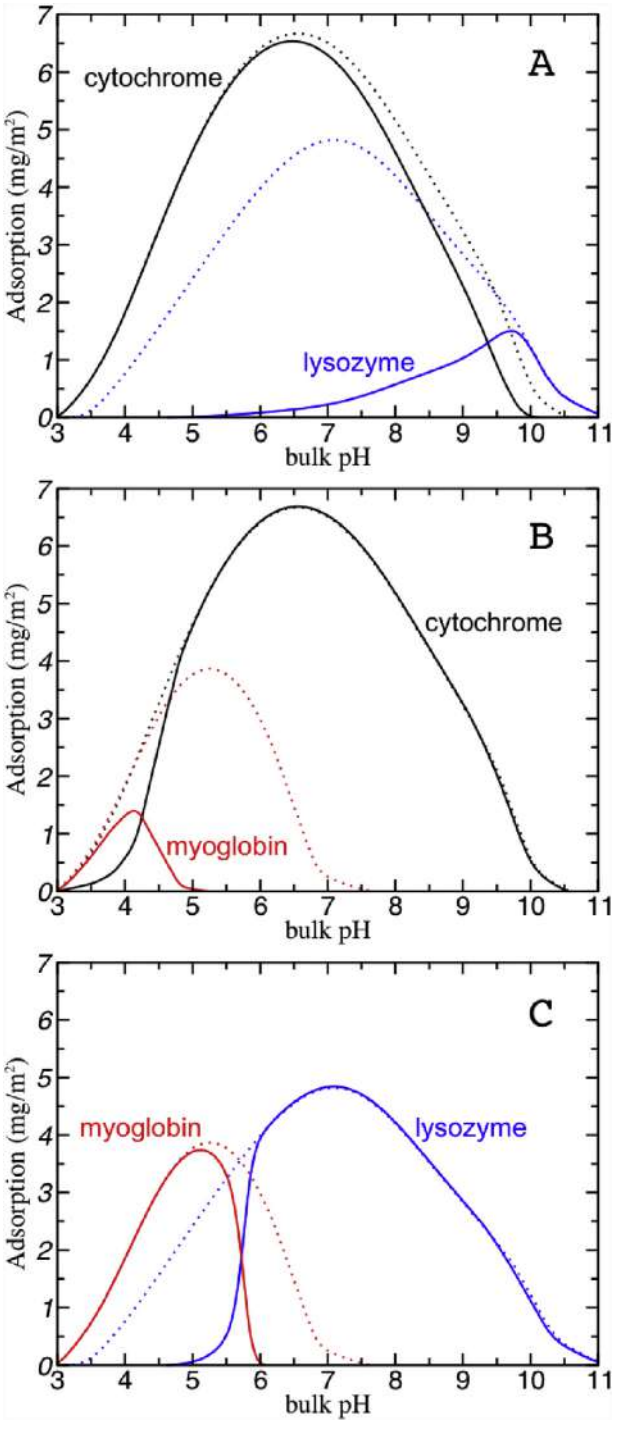
The decisive role of protonation in selective adsorption from binary/ternary mixtures

When applied as biomaterials in biological environments, pH-responsive hydrogels will be exposed to multicomponent protein mixtures. Experimentalists have long known that the adsorption from single protein solutions cannot predict the behavior of mixtures [63]. This concept is true for binary solutions, which display a rich pH-dependent behavior, let alone multicomponent biological protein soups. The simplest example of this emergent behavior is the following: the presence of a different protein can completely prevent the adsorption of another, which would otherwise strongly adsorb from a single protein solution at the same conditions. These emergent phenomena imply that understanding the physical chemistry that governs competitive protein adsorption from mixtures is essential in the rational design of biomaterials that make use of pH-sensitive hydrogels as the functional component.

We have recently studied protein adsorption from binary and ternary mixtures of lysozyme, cytochrome *c* and myoglobin to polyacid hydrogel films [50]. In Figure 8 we illustrate the adsorption from binary solutions of these proteins at relatively low salt conditions. In myoglobin-lysozyme solutions, selective adsorption of one or the other protein can be achieved through changing the solution pH. Only myoglobin is present inside the hydrogel film at low pH, preventing the adsorption of lysozyme, which is significant for single protein solutions at the same conditions. At intermediate and high pH, lysozyme is the only species that adsorbs, preventing myoglobin adsorption. The transition from pure myoglobin to pure lysozyme adsorption as pH increases depends on the solution salt concentration. In this transition range of pH values, a mixture of both proteins occurs inside the film. Moreover, there are conditions where we observe adsorption of myoglobin inside the film while lysozyme strongly adsorbs at the film–solution interface [50].

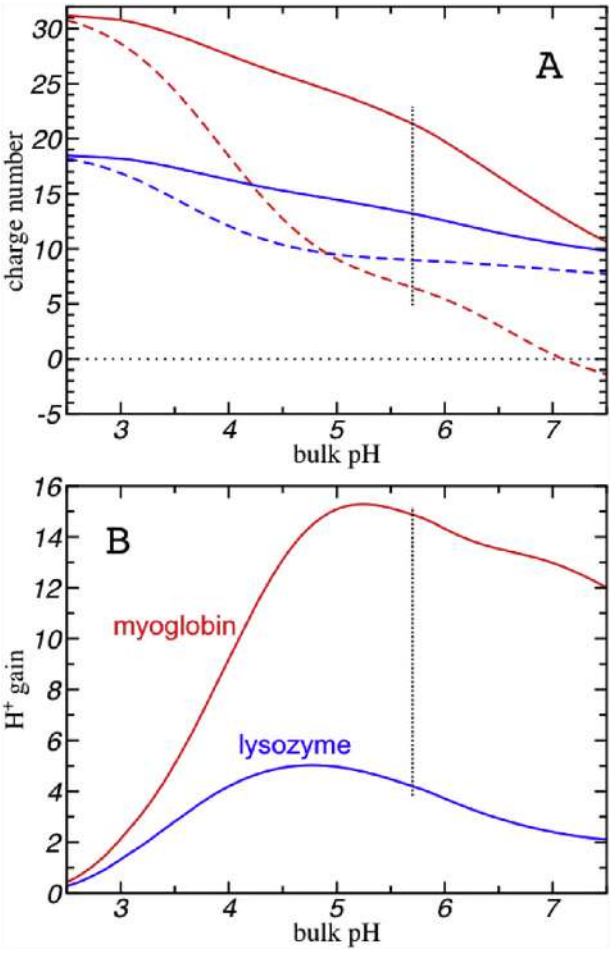
In this rich competitive adsorption behavior, both the size of the protein and its net charge at the lower-pH environment inside the hydrogel film play important roles. The chemical free energy cost of protonation is also a decisive factor in selective protein adsorption from protein mixtures. In binary solutions, there are conditions where only lysozyme adsorbs, even though myoglobin would be significantly more positively

Figure 8



Plot of the adsorption from binary protein solutions to a hydrogel film of PMAA as a function of pH (solid line curves). These results correspond to 1 mM NaCl solutions where the concentration of each protein is 10 μM. The dotted line curves give the adsorption from single protein solutions at otherwise the same conditions. (Adapted from Ref. [50].)

Figure 9



A: Plot of the net charge of myoglobin and lysozyme as a function of pH for adsorbed (solid lines) and solution (dashed lines) proteins. B: Plot of the number of protons gained by each protein upon adsorption to the PMAA hydrogel film. These results correspond to binary solutions with 10 μM concentration of each protein and 1 mM NaCl. The dotted line in both panels marks the pH where the myoglobin and lysozyme adsorption curves of Figure 8C intersect each other, which characterizes the transition from pure myoglobin to pure lysozyme adsorption as the bulk pH increases. (Adapted from Ref. [50].)

charged inside the film. For example, Figure 8C shows that only lysozyme adsorbs above pH 6, while Figure 9A indicates that the net charge of myoglobin would be significantly more positive than that of lysozyme should the former adsorb at these conditions. This counterintuitive adsorption behavior is not the result of the slightly larger size of myoglobin, although the relative size of proteins determines some quantitative details of this behavior [50]. The reason behind this phenomenon is that the adsorption of myoglobin under such conditions would require the gain of many more protons than the adsorption of lysozyme [50]. This behavior is illustrated in Figure 9B that shows the number of protons gained by each protein upon adsorption, but it can also be inferred from the wider gap between the adsorbed and solution charge of myoglobin seen in Figure 9A. Under such conditions, the adsorption of the more weakly charged protein is more favorable because it requires less chemical work to protonate it.

Selective adsorption upon changing the solution pH can also be observed in mixtures containing cytochrome *c* (as shown Figure 8A and B). However, cytochrome *c* significantly reduces the pH range and the amount of myoglobin and lysozyme adsorption, in both binary and ternary solutions [50]. Cytochrome is the smallest of the three proteins and its adsorption requires a similar degree of protonation as lysozyme.

Using pH gradients to control protein localization

Selective adsorption of proteins with specific properties can be beneficial for the function and durability of a biomaterial. Localization of specific proteins in different regions of the biomaterial (or the exclusion from those regions) can improve functionality of the material. We have recently explore these concepts to suggest the use of hydrogel films for protein separation and localization [50]. Changing the chemical composition of the polymer network, adding for example another acidic or a neutral comonomer, and playing with the solution pH allows for the localization of a specific protein to controlled spatial regions of the film with nanometer resolution. The different pH gradients these hydrogel films induce can lead to selective protein adsorption, where the solution pH provides a sensible dial to externally control protein separation. This phenomenon is associated with the complex interplay between the local pH that establishes in different regions of the hydrogel, the net charge that a protein acquires in these lower-pH environments, and the work required to protonate the protein's different amino acid residues, as discussed in this review.

Perspectives

Hydrogels of pH-sensitive polymers are promising candidates for smart, responsive biomaterials, which imposes the need for understanding their complex physicochemical interaction with proteins. Molecular simulations can provide insightful information to understand the mechanisms behind protein adsorption to pH-responsive gels, which can be challenging or impossible to obtain from experiments. Our work in recent years has focused on describing how the state of protonation of the polymer network of hydrogel films and that of the different amino acid residues of proteins affects or modulates their interaction. We have shown that a rich behavior emerges from the protein's ability to regulate its electric charge in the lower-pH environment that occurs inside the material. This behavior can be used for protein separation or localization within nanometer-sized spatial regions inside the material. We envision, for example, the development of multifunctional hydrogel-based materials where different

proteins are active in different regions of the polymer network. We will theoretically explore these concepts further in the near future.

There are now a few molecular simulations methods that can describe protonation equilibrium, which have been applied to investigate protein charge regulation under different conditions. We expect that in the next few years these methods will be applied to investigate the interactions between proteins and pH-responsive materials. Surely, these studies will reveal new and complex behavior arising from the ability of proteins and the adsorbent material to displace the protonation equilibria of its titratable molecular groups.

Conflict of interest statement

Nothing declared.

Acknowledgments

This work was supported by CONICET and ANPCyT (PICT-2014-3377, PICT-2017-3513), Argentina. N.A.P.-C. acknowledges a ANPCyT fellowship (PICT-2015-3425). I.S. acknowledges support from NSF, Div. of Chem., Bioeng., Env., & Transp. Sys. 1833214.

References

Papers of particular interest, published within the period of review, have been highlighted as:

* of special interest
** of outstanding interest

1. Zhang X, Guan Y, Zhang Y: **Ultrathin hydrogel films for rapid optical biosensing**. *Biomacromolecules* 2012, **13**:92–97, https://doi.org/10.1021/bm2012696.

2. Islam MR, Gao Y, Li X, Serpe MJ: **Responsive polymers for biosensing and protein delivery**. *J Mater Chem B* 2014, **2**: 2444–2451, https://doi.org/10.1039/C3TB21657H. URL: http://xlink.rsc.org/?DOI=C3TB21657H.

3. van Vlierberghe S, Dubruel P, Schacht E: **Biopolymer-based hydrogels as scaffolds for tissue engineering applications: A review**. *Biomacromolecules* 2011, **12**:1387–1408, https://doi.org/10.1021/bm200083n.

4. Matricardi P, Di Meo C, Coviello T, Hennink WE, Alhaique F: **Interpenetrating polymer networks polysaccharide hydrogels for drug delivery and tissue engineering**. *Adv Drug Deliv Rev* 2013, **65**:1172–1187, https://doi.org/10.1016/j.addr.2013.04.002.

5. Bai X, Gao M, Syed S, Zhuang J, Xu X, Zhang XQ: **Bioactive hydrogels for bone regeneration**. *Bioact Mater* 2018, **3**: 401–417, https://doi.org/10.1016/j.bioactmat.2018.05.006.

6. Wu W, Mitra N, Yan ECY, Zhou S: **Multifunctional Hybrid Nanogel for and Self-Regulated Insulin Release at Physiological pH**. *ACS Nano* 2010, **4**:4831–4839, https://doi.org/10.1021/nn1008319.

7. Green JJ, Elisseeff JH: **Mimicking biological functionality with polymers for biomedical applications**. *Nature* 2016, **540**: 386–394, https://doi.org/10.1038/nature21005.

8. Annabi N, Tamayol A, Uquillas JA, Akbari M, Bertassoni LE, Cha C, *et al.*: **25th anniversary article: Rational design and applications of hydrogels in regenerative medicine**. *Adv Mater* 2014, **26**:85–124, https://doi.org/10.1002/adma.201303233. arXiv:NIHMS150003.

9. McClements DJ: **Designing biopolymer microgels to encapsulate, protect and deliver bioactive components: Physicochemical aspects**. *Adv Colloid Interface Sci* 2017, **240**:31–59, https://doi.org/10.1016/j.cis.2016.12.005. URL: http://www.sciencedirect.com/science/article/pii/S0001868616303281.

10. Rasool A, Ata S, Islam A: **Stimuli responsive biopolymer (chitosan) based blend hydrogels for wound healing application**. *Carbohydr Polym* 2019, **203**:423–429. URL: https://www.sciencedirect.com/science/article/pii/S0144861718311706?dgcid=rss{_}sd{_}all.

11. Asayama W, ichi Sawada S, Taguchi H, Akiyoshi K: **Comparison of refolding activities between nanogel artificial chaperone and GroEL systems**. *Int J Biol Macromol* 2008, **42**:241–246, https://doi.org/10.1016/j.ijbiomac.2007.11.003.

12. Sawada SI, Akiyoshi K: **Nano-encapsulation of lipase by self-assembled nanogels: Induction of high enzyme activity and thermal stabilization**. *Macromol Biosci* 2010, **10**:353–358, https://doi.org/10.1002/mabi.200900304. arXiv:arXiv:1507.02142v2.

13. Beierle JM, Yoshimatsu K, Chou B, Mathews MAA, Lesel BK, Shea KJ: **Polymer nanoparticle hydrogels with autonomous affinity switching for the protection of proteins from thermal stress**. *Angew Chem Int Ed* 2014, **53**:9275–9279, https://doi.org/10.1002/anie.201404881.

14. Vermonden T, Censi R, Hennink WE: **Hydrogels for protein delivery**. *Chem Rev* 2012, **112**:2853–2888, https://doi.org/10.1021/cr200157d.

15. Malmsten M, Bysell H, Hansson P: **Biomacromolecules in microgels - Opportunities and challenges for drug delivery**. *Curr Opin Colloid Interface Sci* 2010, **15**:435–444, https://doi.org/10.1016/j.cocis.2010.05.016.

16. Renukuntla J, Vadlapudi AD, Patel A, Boddu SH, Mitra AK: **Approaches for enhancing oral bioavailability of peptides and proteins**. *Int J Pharm* 2013, **447**:75–93, https://doi.org/10.1016/j.ijpharm.2013.02.030. arXiv:NIHMS150003.

17. Koetting MC, Peppas NA: **pH-responsive poly(itaconic acid-co-N-vinylpyrrolidone) hydrogels with reduced ionic strength loading solutions offer improved oral delivery potential for high isoelectric point-exhibiting therapeutic proteins**. *Int J Pharm* 2014, **471**:83–91, https://doi.org/10.1016/j.ijpharm.2014.05.023.

18. Luensmann D, Jones L: **Protein deposition on contact lenses: The past, the present, and the future**. *Contact Lens Anterior Eye* 2012, **35**:53–64, https://doi.org/10.1016/j.clae.2011.12.005.

19. Omali NB, Subbaraman LN, Coles-Brennan C, Fadli Z, Jones LW: **Biological and clinical implications of lysozyme deposition on soft contact lenses**. *Optom Vis Sci* 2015, **92**:750–757. URL: https://journals.lww.com/optvissci/Fulltext/2015/07000/Biological{_}and{_}Clinical{_}Implications{_}of{_}Lysozyme.5.aspx.

20. ** Wei Q, Becherer T, Angioletti-Uberti S, Dzubiella J, Wischke C, Neffe AT, *et al.*: **Protein interactions with polymer coatings and biomaterials**. *Angew Chem Int Ed* 2014, **53**:8004–8031, https://doi.org/10.1002/anie.201400546.

21. * Welsch N, Becker AL, Dzubiella J, Ballauff M: **Core-shell microgels as smart carriers for enzymes**. *Soft Matter* 2012, **8**: 1428–1436, https://doi.org/10.1039/C1SM06894F. URL: http://xlink.rsc.org/?DOI=C1SM06894F.

22. ** Wittemann A, Haupt B, Ballauff M: **Adsorption of proteins on spherical polyelectrolyte brushes in aqueous solution**. *Phys Chem Chem Phys* 2003, **5**:1671–1677, https://doi.org/10.1039/b300607g.

23. * Biesheuvel PM, Wittemann A: **A modified box model including charge regulation for protein adsorption in a spherical polyelectrolyte brush**. *J Phys Chem B* 2005, **109**:4209–4214, https://doi.org/10.1021/jp0452812.

24. ** de Vos WM, Biesheuvel PM, De Keizer A, Kleijn JM, Stuart MAC: **Adsorption of the protein bovine serum albumin in a planar poly(acrylic acid) brush layer as measured by optical reflectometry**. *Langmuir* 2008, **24**:6575–6584, https://doi.org/10.1021/la8006469.

25. Wittemann A, Ballauff M: **Interaction of proteins with linear polyelectrolytes and spherical polyelectrolyte brushes in aqueous solution**. *Phys Chem Chem Phys* 2006, **8**:5269–5275, https://doi.org/10.1039/b609879g.

26. Leermakers FAM, Ballauff M, Borisov OV: **On the mechanism of uptake of globular proteins by polyelectrolyte brushes: A**

two-gradient self-consistent field analysis. *Langmuir* 2007, **23**: 3937–3946, https://doi.org/10.1021/la0632777.

27. de Vos WM, Leermakers FA, De Keizer A, Stuart MA, Kleijn JM: **Field theoretical analysis of driving forces for the uptake of proteins by like-charged polyelectrolyte brushes: Effects of charge regulation and patchiness**. *Langmuir* 2010, **26**: 249–259, https://doi.org/10.1021/la902079u.

28. Zhang Z, Zhang R, Zou L, McClements DJ: **Protein encapsulation in alginate hydrogel beads: Effect of pH on microgel stability, protein retention and protein release**. *Food Hydrocoll* 2016, **58**:308–315, https://doi.org/10.1016/j.foodhyd.2016.03.015.

29. * Johansson C, Gernandt J, Bradley M, Vincent B, Hansson P: **Interaction between lysozyme and colloidal poly(NIPAM-co-acrylic acid) microgels**. *J Colloid Interface Sci* 2010, **347**: 241–251, https://doi.org/10.1016/j.jcis.2010.03.072. URL: http://www.sciencedirect.com/science/article/pii/S002197971000384X.

30. * Yigit C, Welsch N, Ballauff M, Dzubiella J: **Protein sorption to charged microgels: characterizing binding isotherms and driving forces**. *Langmuir* 2012, **28**:14373–14385, https://doi.org/10.1021/la303292z.

31. * Adroher-Benítez I, Moncho-Jordá A, Dzubiella J: **Sorption and spatial distribution of protein globules in charged hydrogel particles**. *Langmuir* 2017, **33**:4567–4577, https://doi.org/10.1021/acs.langmuir.7b00356.

32. * Angioletti-Uberti S, Ballauff M, Dzubiella J: **Dynamic density functional theory of protein adsorption on polymer-coated nanoparticles**. *Soft Matter* 2014, **10**:7932–7945, https://doi.org/10.1039/C4SM01170H.

33. * Oberle M, Yigit C, Angioletti-Uberti S, Dzubiella J, Ballauff M: **Competitive protein adsorption to soft polymeric layers: binary mixtures and comparison to theory**. *J Phys Chem B* 2015, **119**:3250–3258, https://doi.org/10.1021/jp5119986.

34. Wei T, Carignano MA, Szleifer I: **Lysozyme adsorption on polyethylene surfaces: Why are long simulations needed?** *Langmuir* 2011, **27**:12074–12081, https://doi.org/10.1021/la202622s.

35. Ding F, Radic S, Chen R, Chen P, Geitner NK, Brown JM, *et al.*: **Direct observation of a single nanoparticle-ubiquitin corona formation**. *Nanoscale* 2013, **5**:9162–9169, https://doi.org/10.1039/C3NR02147E.

36. Ding Hm, Ma Yq: **Computer simulation of the role of protein corona in cellular delivery of nanoparticles**. *Biomaterials* 2014, **35**:8703–8710, https://doi.org/10.1016/j.biomaterials.2014.06.033. URL: http://www.sciencedirect.com/science/article/pii/S0142961214007248.

37. Tavanti F, Pedone A, Menziani MC: **Competitive binding of proteins to gold nanoparticles disclosed by molecular dynamics simulations**. *J Phys Chem C* 2015, **119**:22172–22180, https://doi.org/10.1021/acs.jpcc.5b05796.

38. Shao Q, Hall CK: **Protein adsorption on nanoparticles: Model development using computer simulation**. *J Phys Condens Matter* 2016, **28**:414019. URL: http://stacks.iop.org/0953-8984/28/i=41/a=414019.

39. * Sun TY, Liang LJ, Wang Q, Laaksonen A, Wu T: **A molecular dynamics study on pH response of protein adsorbed on peptide-modified polyvinyl alcohol hydrogel**. *Biomater Sci* 2014, **2**:419–426, https://doi.org/10.1039/C3BM60213C.

40. Lund M, Åkesson T, Jönsson B: **Enhanced protein adsorption due to charge regulation**. *Langmuir* 2005, **21**:8385–8388, https://doi.org/10.1021/la050607z.

41. Da Silva FLB, Jönsson B: **Polyelectrolyte-protein complexation driven by charge regulation**. *Soft Matter* 2009, **5**:2862–2868, https://doi.org/10.1039/b902039j.

42. Evers CH, Andersson T, Lund M, Skepö M: **Adsorption of unstructured protein β-casein to hydrophobic and charged surfaces**. *Langmuir* 2012, **28**:11843–11849, https://doi.org/10.1021/la300892p.

43. Torres P, Bojanich L, Sanchez-Varretti F, Ramirez-Pastor AJ, Quiroga E, Boeris V, *et al.*: **Protonation of β-lactoglobulin in the presence of strong polyelectrolyte chains: A study using Monte Carlo simulation**. *Colloids Surfaces B Biointerfaces* 2017, **160**:161–168, https://doi.org/10.1016/j.colsurfb.2017.09.018. URL: http://www.sciencedirect.com/science/article/pii/S0927776517305933.

44. Longo GS, Olvera de la Cruz M, Szleifer I: **Molecular theory of weak polyelectrolyte gels: The role of pH and salt concentration**. *Macromolecules* 2011, **44**:147–158, https://doi.org/10.1021/ma102312y.

45. Longo GS, de la Cruz MO, Szleifer I: **Molecular theory of weak polyelectrolyte thin films**. *Soft Matter* 2012, **8**:1344–1354, https://doi.org/10.1039/C1SM06708G.

46. Longo GS, Olvera de la Cruz M, Szleifer I: **Non-monotonic swelling of surface grafted hydrogels induced by pH and/or salt concentration**. *J Chem Phys* 2014b, **141**:124909, https://doi.org/10.1063/1.4896562.

47. * Longo GS, Olvera de la Cruz M, Szleifer I: **Equilibrium adsorption of hexahistidine on pH-responsive hydrogel nanofilms**. *Langmuir* 2014a, **30**:15335–15344, https://doi.org/10.1021/la5040382.

48. * Narambuena CF, Longo GS, Szleifer I: **Lysozyme adsorption in pH-responsive hydrogel thin-films: The non-trivial role of acid-base equilibrium**. *Soft Matter* 2015, **11**:6669–6679, https://doi.org/10.1039/C5SM00980D.

49. ** Longo GS, Szleifer I: **Adsorption and protonation of peptides and proteins in pH responsive gels**. *J Phys D Appl Phys* 2016, **49**:323001. URL: http://stacks.iop.org/0022-3727/49/i=32/a=323001.

50. ** Hagemann A, Giussi JM, Longo GS: **The use of pH gradients in responsive polymer hydrogels for the separation and localization of proteins from binary mixtures**. *Macromolecules* 2018, **51**:8205–8216, https://doi.org/10.1021/acs.macromol.8b01876.

51. Szleifer I: **Protein adsorption on surfaces with grafted polymers**. *Biophys J* 1997, **72**:595–612, https://doi.org/10.1016/S0006-3495(97)78698-3.

52. Fang F, Satulovsky J, Szleifer I: **Kinetics of protein adsorption and desorption on surfaces with grafted polymers**. *Biophys J* 2005, **89**:1516–1533, https://doi.org/10.1529/biophysj.104.055079.

53. Fang F, Szleifer I: **Controlled release of proteins from polymer-modified surfaces**. *Proc Natl Acad Sci* 2006, **103**:5769–5774, https://doi.org/10.1073/pnas.0509688103. URL: http://www.pnas.org/cgi/doi/10.1073/pnas.0509688103.

54. Nap R, Gong P, Szleifer I: **Weak polyelectrolytes tethered to surfaces: Effect of geometry, acid-base equilibrium and electrical permittivity**. *J Polym Sci Part B Polym Phys* 2006, **44**:2638–2662, https://doi.org/10.1002/polb.20896. URL: https://onlinelibrary.wiley.com/doi/abs/10.1002/polb.20896.

55. Gong P, Genzer J, Szleifer I: **Phase behavior and charge regulation of weak polyelectrolyte grafted layers**. *Phys Rev Lett* 2007, **98**:018302, https://doi.org/10.1103/PhysRevLett.98.018302.

56. Longo GS, Thompson DH, Szleifer I: **Ligand-receptor interactions between surfaces: The role of binary polymer spacers**. *Langmuir* 2008, **24**:10324–10333, https://doi.org/10.1021/la8009699.

57. Zhao Z, Matsui H: **Accurate immobilization of antibody-functionalized peptide nanotubes on protein-patterned arrays by optimizing their ligand-receptor interactions**. *Small* 2007, **3**:1390–1393, https://doi.org/10.1002/smll.200700006.

58. Wang D, Nap RJ, Lagzi I, Kowalczyk B, Han S, Grzybowski BA, *et al.*: **How and why nanoparticle's curvature regulates the apparent pKa of the coating ligands**. *J Am Chem Soc* 2011, **133**:2192–2197, https://doi.org/10.1021/ja108154a.

59. Tagliazucchi M, Azzaroni O, Szleifer I: **Responsive polymers end-tethered in solid-state nanochannels: When nanoconfinement really matters**. *J Am Chem Soc* 2010, **132**: 12404–12411, https://doi.org/10.1021/ja104152g.

60. Gong P, Wu T, Genzer J, Szleifer I: **Behavior of surface-anchored poly(acrylic acid) brushes with grafting density gradients on solid substrates: 2. Theory**. *Macromolecules* 2007, **40**:8765–8773, https://doi.org/10.1021/ma0710176.

61. Grimsley GR, Scholtz JM, Pace CN: **A summary of the measured pK values of the ionizable groups in folded proteins**. *Protein Sci* 2009, **18**:247–251, https://doi.org/10.1002/pro.19.

62. Wood DW: **New trends and affinity tag designs for recombinant protein purification**. *Curr Opin Struct Biol* 2014, **26**:54–61, https://doi.org/10.1016/j.sbi.2014.04.006.

63. Green RJ, Davies MC, Roberts CJ, Tendler SJB: **Competitive protein adsorption as observed by surface plasmon resonance**. *Biomaterials* 1999, **20**:385–391, https://doi.org/10.1016/S0142-9612(98)00201-4. URL: http://www.sciencedirect.com/science/article/pii/S0142961298002014.