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

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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.

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(2-methacrylic acid) (PMMA) 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, polyanion 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 underlying 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 underlying 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 complexion 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 polyanion 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 polyanion 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 ployacid 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 polycacid 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 crystalllographic 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 isometric 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 micro-environment 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 polycacid 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:

$$f_d = \frac{1}{1 + 10^{pK_a - \text{pH}}}
$$

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
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 intranetwork 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 \(\mathbf{r}\) using the local concentration of protons.
pHðrÞ ¼ \frac{C0}{\log10\left(\frac{C0}{C2}\right) H^+ + C3}{C1} \quad \text{(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\text{gel} as the average of the local pH inside the hydrogel film. Our recent results have shown that this quantity is well defined\cite{46}; here we will emphasize the importance of pH\text{gel} and pHðrÞ 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\text{gel} instead of the bulk pH in Equation (1)\cite{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\cite{62}. Histidine is positively charged at low pH with p\text{Ka} 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:

\[ \Gamma = \int \rho(r) - \rho_{\text{bulk}} \, dV \]  \quad \text{(3)}

where \rho(r) and \rho_{\text{bulk}} are the local and bulk densities of the adsorbate and \emph{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, \Gamma provides the excess amount of adsorbate inside the material, but it also receives 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
The 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.

**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 \(\mu\)M) 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...
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 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].)

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...
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 explored 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 equilibria, 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.

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