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Topical Review

Adsorption and protonation of peptides and proteins in pH responsive gels

Gabriel S Longo[1] **and Igal Szleifer**[2,3,4]

[1] Instituto de Investigaciones Fisicoquímicas Teóricas y Aplicadas (INIFTA), UNLP, CONICET, La Plata, Argentina
[2] Department of Biomedical Engineering, Northwestern University, Evanston, IL, USA
[3] Chemistry of Life Processes Institute, Northwestern University, Evanston, IL, USA
[4] Department of Chemistry, Northwestern University, Evanston, IL, USA

E-mail: longogs@inifta.unlp.edu.ar

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Abstract

To describe the non-trivial features of the equilibrium protonation and physical adsorption of peptides/proteins in pH-responsive hydrogels, we summarize our recent theoretical work on the subject. In these systems, molecular confinement in nanometer-sized environments modifies the balance between chemical state, physical interactions and molecular organization, which results in a behavior that is qualitatively different from what is expected from assuming the bulk solution protonation. To enhance adsorption, the pH-dependent deprotonation curves of all amino acids of adsorbed proteins are adequately shifted and deformed, which depends, in a complex fashion, on the specific amino acid. This possibility of modifying different acid–base equilibriums gives the adsorbed protein degrees of freedom to regulate charge and enhance electrostatic attractions under a wide range of experimental conditions. Protein adsorption modifies the microenvironment inside the hydrogel, particularly the gel pH. As a result, the state of protonation of the network is different before and after adsorption. The physicochemical considerations described in this review can be useful in the design of functional materials involving protein adsorption.

Keywords: responsive gel, adsorption, acid–base equilibrium

(Some figures may appear in colour only in the online journal)

1. Introduction

There is presently a growing interest in the pharmaceutical use of peptides and proteins (Vermonden *et al* 2012). Proteins are ubiquitous in biological processes and have highly specific functions and low toxicity, which are both difficult to achieve with the traditional small-molecule drugs. However, in spite of their increasing use in vaccination, disease diagnostics and treatment, and tissue engineering, efficient delivery of functional proteins is still an important challenge in biomedical research (Islam *et al* 2014).

Although most small-molecule drugs in the market are orally delivered, therapeutic peptides and proteins are generally administered through injections (Renukuntla *et al* 2013, Koetting and Peppas 2014). Oral uptake, being the least invasive path, is the desirable drug delivery route having several advantages that improve patient quality of life, including high compliance, ease of administration, avoidance of irritation and pain, and relatively low production cost (Renukuntla *et al* 2013, Koetting and Peppas 2014). However, due to the digestive system's natural mechanisms for breaking down ingested proteins into substituent amino acids, oral delivery of peptides and proteins without some means of protection results in extremely low bioavailability (Renukuntla *et al* 2013, Koetting and Peppas 2014). There is currently a considerable amount of research in attempting to overcome the

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physiological, enzymatic and chemical barriers that the gastrointestinal (GI) environment imposes to the administration of therapeutic peptides and proteins. To circumvent these barriers, some researchers have considered pH-responsive hydrogels for the delivery of therapeutic proteins to the upper small intestine. The rational behind this choice is the change in pH occurring along the GI tract from low in the stomach to high in the upper small intestine.

Hydrogels, formed by chemically or physically crosslinked polymer chains, are particularly suitable for the encapsulation/immobilization of proteins. Hydrogels are filled with water and display a polymer network structure with properties that resemble biological tissue (Myers *et al* 1984, Eisenberg and Grodzinsky 1987). When immobilized inside hydrogels, proteins are less susceptible to denaturation and aggregation (Asayama *et al* 2008, Sawada and Akiyoshi 2010). Several studies have shown that proteins released from hydrogels retain function and structure (Vermonden *et al* 2012).

The use of stimuli-responsive hydrogels is increasingly appealing in biomaterials research and as the smart component in different medical applications (Hoffman 2002, Miyata *et al* 2002, Peppas *et al* 2006, Hoffman 2013), including biomolecular sensors (Brahim *et al* 2002, Suri *et al* 2003, Zhang *et al* 2012, Islam *et al* 2014), biomimetic materials (Hilt and Byrne 2004, Wu *et al* 2010), scaffolds for tissue engineering (Lee and Mooney 2001, Matricardi *et al* 2013), tumor markers (Miyata *et al* 2006), and radio chemotherapy (Azhdarinia *et al* 2005) to mention a few examples. In drug delivery, stimuli-sensitive hydrogels can encapsulate and release low-molecular weight drugs, therapeutic peptides and proteins, enzymes, DNA and RNA molecules (Qiu and Park 2001), etc. These intelligent materials can be engineered to modify their physicochemical properties in response to external stimuli that can be physical, chemical or biological perturbations, including temperature changes (Tanaka 1978, Park and Hoffman 1992, Chen and Hoffman 1995, Yoshida *et al* 1995), applied electric (Tanaka *et al* 1982, Kwon *et al* 1991, Osada *et al* 1992) or magnetic field (Szabó *et al* 1998), exposure to light (Suzuki and Tanaka 1990), solution pH (Tanaka *et al* 1980, Park and Hoffman 1992, Philippova *et al* 1997, Kiser *et al* 1998, Eichenbaum *et al* 1999, Torres-Lugo and Peppas 1999, Zhao and Moore 2001) and/or salt concentration (Ohmine and Tanaka 1982, Jeon *et al* 1998, Zhao and Moore 2001), solvent composition (Tanaka *et al* 1980, Ilavsky 1982, Hirokawa and Tanaka 1984, Matsuo and Tanaka 1992), and concentration and activity of biomolecules such as glucose (Hassan *et al* 1997, Podual *et al* 2000), proteins (Miyata *et al* 1999a, 1999b) and enzymes (Ulijn 2006).

pH-responsive hydrogels are particularly appropriate for developments in oral drug delivery due the change in pH that occurs along the GI tract, from the acidic environment in the stomach (pH ~ 1.2–2) to neutral or alkaline media in the intestines (pH ~ 7–8). Polyacid networks are relatively collapsed at the low stomach pH, which prevents the encapsulated agent from escaping the hydrogel. At the alkaline conditions in the intestines, the hydrogel swells. Release occurs due to diffusion through the swollen network since the drug is only physically entrapped inside the hydrogel. For example, pH-responsive hydrogels have been considered for potential applications in the controlled oral delivery of several proteins including, among many others, insulin (Brøndsted and Kopeček 1991, Lowman *et al* 1999, Nakamura *et al* 2004, Yamagata *et al* 2006, Carr and Peppas 2010), calcitonin (Torres-Lugo and Peppas 1999, Kim *et al* 2003), lysozyme (van de Weert *et al* 2000a, Hoven *et al* 2007, Shi *et al* 2008, Zhang *et al* 2008), amylase (Liang-chang *et al* 1992), bovine serum albumin (BSA) (Zhang *et al* 2011, Gao *et al* 2012, Suhag *et al* 2015), human growth hormone (Carr *et al* 2010) and interferon-β (Kamei *et al* 2009). In addition, pH-sensitive hydrogels can be used for delivery of therapeutics to specific tissue, which has not only been considered in basic research but also in clinical trials (Cabral and Kataoka 2014). Cancerous, wounded or inflamed tissue exhibits lower than physiological pH values (Vaupel *et al* 1989, Rofstad *et al* 2006, Schmaljohann 2006). Some cellular compartments have acidic pH values (Grabe and Oster 2001, Watson *et al* 2005, Schmaljohann 2006, Casey *et al* 2010).

Applications requiring peptide/protein adsorption inside pH-responsive hydrogels face several challenges, and despite all the aforementioned research efforts the physical chemistry involved in protein adsorption and protonation is not completely understood. For example, calcitonin is frequently administered via injections in the treatment of osteoporosis, because the high isoelectric point of the protein hinders its oral delivery using pH-responsive hydrogels. Carr *et al* (2010) showed that at the pH of the small intestine, calcitonin remain highly positively charged and attracted to the negatively charged polyacid network, which prevented protein release. In designing biosensors, pH-responsive hydrogels are usually modified with enzymes whose activity can change the local environment and particularly the pH inside the hydrogel (Qiu and Park 2001). How does the interplay between environment, physical interactions, chemical equilibrium and molecular organization determine the resulting adsorption of proteins inside pH-responsive hydrogels? How does this adsorption modify protein and network protonation and the microenvironment inside the hydrogel? Molecular simulations can provide answers to these questions that might not be easy to address experimentally. Comprehending the physical chemistry involved in protein adsorption will lead to biomaterials with improved performance.

Several molecular simulation studies have considered protein adsorption on surfaces (Latour 2008, Zhang and Sun 2010). Lysozyme has been generally chosen as a model protein for these simulations (Ravichandran and Talbot 2000, Carlsson *et al* 2004, Pellenc *et al* 2008, Kubiak-Ossowska and Mulheran 2010, Wei *et al* 2011, Kubiak-Ossowska and Mulheran 2012, Wei *et al* 2012), because it is a well-characterized globular protein that maintains its native conformation under most conditions (Gekko and Hasegawa 1986, Norde and Anusiem 1992, Vaney *et al* 1996, van de Weert *et al* 2000a, 2000b, Sauter *et al* 2001). Compared to other proteins, lysozyme undergoes minor conformational changes when it adsorbs on negatively charged surfaces (Blomberg *et al* 1994, Haynes and Norde 1995). Different levels of modeling have been used to describe lysozyme, from full all-atom

simulations (Kubiak-Ossowska and Mulheran 2010, Wei *et al* 2011, Kubiak-Ossowska and Mulheran 2012, Wei *et al* 2012) to coarse-grained models (Ravichandran and Talbot 2000, Carlsson *et al* 2004, Yu *et al* 2014).

In contrast, there are few examples of molecular simulations considering protein adsorption on responsive hydrogels. Recently, Sun *et al* (2014) considered the adsorption of the fragment antigen binding of trastuzumab (a monoclonal antibody) inside a polyvinyl alcohol hydrogel modified with charged moieties. Using molecular dynamics simulations, these authors investigated the effect of pH on the stability of the adsorbed protein that showed no indication of denaturation under different conditions. Masoud and Alexeev (2012) used dissipative particle dynamics, a particle-based mesoscale simulation method (Yeh and Alexeev 2015), to study the controlled release of nanoparticles and macromolecules from stimuli-responsive microgels.

The total charge of proteins is sensitive to local environment variations (Kirkwood and Shumaker 1952). Lund *et al* (2005) suggested that the charge capacitance of the protein contains all the information needed to describe the charge regulation mechanism. Protein adsorption on ionic polymeric materials is driven by polymer-protein electrostatic interactions (Kato *et al* 1995). Several studies have suggested that protein charge regulation strongly affects the adsorption behavior on surfaces (Ståhlberg and Jönsson 1996, Menon and Zydney 2000, Biesheuvel *et al* 2005, Biesheuvel and Wittemann 2005, Lund *et al* 2005, Lund and Jönsson 2005, Hartvig *et al* 2011). When considering protein adsorption on weak polyelectrolyte networks, the problem becomes significantly more complex because the adsorbent material itself can regulate charge in response to environment modifications (Longo *et al* 2011).

Therefore, when addressing the adsorption of peptides and proteins inside pH-responsive hydrogels, appropriate treatment of the acid–base equilibrium of each titratable species (protein/peptide amino acid residues, network ionizable segments, etc) is essential. These different chemical equilibriums can be locally displaced into the charged or the neutral species following spatial pH variations (given by changes in the local proton concentration). Accounting for this complex coupling between chemical equilibriums, physical interactions and molecular organization is a formidable task for most molecular simulation methods, though the protonation of protein residues can be treated using Monte Carlo simulations in a semi-grand canonical ensemble (Lund and Jönsson 2005, Evers *et al* 2012). In this review, we describe the physical chemistry of peptide/protein protonation after adsorbing in pH-sensitive hydrogels. Such a description is based on results using a theoretical approach where the states of protonation of different acid and basic groups are not assumed *a priori* but instead predicted depending on the local environment.

2. Thermodynamics and molecular theory

To study the thermodynamics of peptide/protein protonation upon adsorption in pH-sensitive hydrogels, we have recently developed a theory that explicitly accounts for size, shape, charge distribution and conformational degrees of freedom of all molecular components, including the adsorbate (peptide or protein) and the crosslinked polymer network that makes the hydrogel backbone. Using this molecular theory, the states of protonation of different acid and basic groups are not assumed but predicted depending on the local nano-environment. Namely, chemical states are coupled to the physical interactions, which include van der Waals attractions, steric repulsions, and electrostatic interactions as well as conformational degrees of freedom and entropy loss of molecular confinement. This coupling results from writing a general thermodynamic potential that can capture the local interplay between chemical state, physical interactions and molecular organization.

This molecular theory is an extension of a more general approach that has been previously used to study responsive polymer layers and hydrogels (Gong *et al* 2007a, Longo *et al* 2011) and protein adsorption on polymer-grafted surfaces (Szleifer 1997). This method can quantitatively predict the properties of poly(acrylic acid) (PAAc) layers as a function of pH and salt concentration (Gong *et al* 2007b). Protein adsorption on different polymer-modified surfaces has also been studied using this approach (McPherson *et al* 1998, Satulovsky *et al* 2000, Ren *et al* 2009, Lau *et al* 2012). The amount of adsorbed protein predicted with this molecular theory is in good agreement with experimental results. Modeled proteins include lysozyme, fibrinogen and streptavidin. We have recently used this theory to study protonation of peptides and proteins upon adsorption in polyacid hydrogel thin films (Longo *et al* 2014a, Narambuena *et al* 2015). In this review, we summarize our knowledge on the subject using results calculated using this molecular theory. Next, we briefly highlight the most important features of the method while a detailed description can be found in Longo *et al* (2014a) and Narambuena *et al* (2015).

Let us consider the general problem of adsorption of a molecule bearing acid and/or basic groups in a pH-sensitive hydrogel formed by crosslinked polymer chains. This hydrogel is in contact with an aqueous solution of controlled composition, which contains dissociated monovalent salt (say NaCl) and the adsorbate. The first step in our theoretical approach consists in writing the Helmholtz free energy of the system,

$$F = -TS_{\mathrm{tr}} - TS_{\mathrm{nw}} - TS_{\mathrm{ads}} + F_{\mathrm{chm,nw}} + F_{\mathrm{chm,ads}} + U_{\mathrm{el}} + U_{\mathrm{vdw}} + U_{\mathrm{st}}. \quad (1)$$

In this expression, T represents the temperature of the bath (bulk) solution, S_{tr} is the translational entropy of small free species in the solution (water molecules, hydroxyl ions, protons, chlorine and sodium ions), S_{nw} describes the conformational entropy of the polymer network, and S_{ads} accounts for the translational, rotational and conformational freedom of the adsorbate. The chemical free energy of the network is $F_{\mathrm{chm,nw}}$ and $F_{\mathrm{chm,ads}}$ is that of the adsorbate; these terms represent the contributions from the acid–base equilibrium of titratable groups of the network and adsorbate, respectively. The energetic contributions to the free energy are U_{el} that describes electrostatic interactions, U_{vdw} that accounts for van der Waals attractions, and U_{st} that incorporates steric (excluded volume) repulsions.

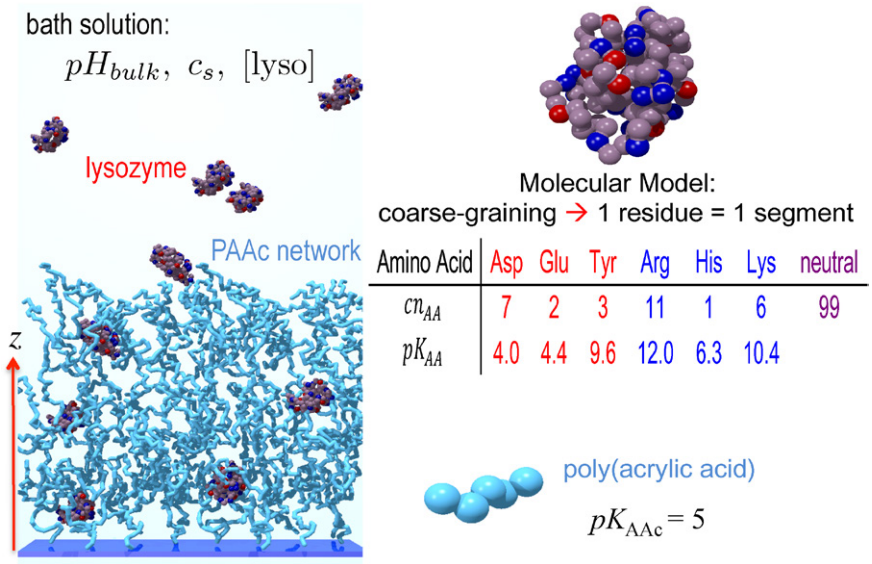

Figure 1. Scheme illustrating the coarse-grain molecular model used in Narambuena *et al* (2015) to study lysozyme adsorption in pH-responsive hydrogel thin-films. A network composed of crosslinked poly(acrylic acid) chains is in contact with a protein solution whose chemical composition is experimentally controlled. The table gives the frequency (composition number) and pKa of each amino acid in lysozyme.

Each of these terms of the free energy can be expressed as a functional of one or more of the following functions: (1) the probability distribution of polymer network conformations, (2) the local density of each of the free species including that of the adsorbate, (3) the local degree of protonation of titratable groups, and (4) the local electrostatic potential. We then build a semi-grand canonical potential as the Legendre transform of the Helmholtz free energy. This transformation accounts for the fact that the hydrogel is in contact with a bath solution, which means that the chemical potential of all free species is the same in all spatial regions of the system. Optimization of this semi-grand canonical potential leads to explicit formulas for all the densities, probability distributions, and local protonation states, as a function of two position-dependent interaction potentials, which are the electrostatic potential and the osmotic pressure. Such dependence of all physicochemical contributions to the free energy on these two interaction potentials makes clear the coupling existing between chemical state, molecular organization and physical interactions. These interaction potentials can be numerically calculated solving at each spatial position the two sets of local equations that result from the formulation of the theory, the incompressibility of the fluid system that describes excluded volume repulsions, and the Poisson equation. Once the interaction potentials are calculated the free energy of the system is known, from which any thermodynamic quantity of interest can be derived.

Evaluation of this theory for a particular system requires defining a molecular model for each of the constituent species, in particular the polymer network and the adsorbate (see Figure 1). In this review we discuss the adsorption of peptides and proteins in polyacid hydrogel films whose polymer network is chemically grafted to a supporting surface. Each network segment is a coarse-grained particle that bears an acidic group with logarithmic dissociation constant pKa = 5 to model a carboxylic acid such as acrylic acid. For peptides and proteins, we have used a coarse-grain model where all atoms of each amino acid residue are combined into a single particle placed at the position of the alpha carbon. The relative position of all atoms can be obtained from crystallographic data. We have performed case studies of hexahistidine and lysozyme adsorption into poly(acrylic acid) hydrogel thin-films; however, both the theoretical framework and molecular model are general and can be readily applied to investigate a variety of different systems. Full details of the theory and molecular models employed can be found in Longo *et al* (2014a) and Narambuena *et al* (2015).

3. pH-responsive hydrogels

We first address the behavior of pH-sensitive hydrogels when placed in contact with salt solutions that contain no peptides or proteins. This discussion provides the background to understand how the adsorption of proteins/peptides modifies the chemical equilibrium and physical properties of both the adsorbed species and the adsorbent material. This background will also help us to understand the non-trivial nature of protein adsorption. Adding proteins to the solution results in emergent behavior. In this section, we describe the behavior of a polymer network bearing acidic groups, while for a network having basic units the discussion is completely analogous.

We have concentrated our research on hydrogel nanofilms whose polymer network is either physically deposited on a surface (Longo *et al* 2012) or chemically grafted to it (Longo *et al* 2014b). The discussion presented here, however, is completely general and can also be applied to describe bulk hydrogels. As we will see, only a few nanometers from the solution–gel interface, these nanofilms already display the microenvironment of bulk hydrogels. The reason for considering thin films is their fast response time as compared to bulk hydrogels. Indeed, one of the most important aspects

to consider when developing applications based on stimuli-sensitive hydrogels is the response time. The perturbation-response time of 3D bulk hydrogels is generally prohibitively long for many applications. For example, upon changing the solution pH, weak polyelectrolyte gels swell/deswell within several minutes to a few hours, depending on the hydrogel size. Using hydrogels as the smart component in real-time biosensors and many other applications requires the response to occur almost immediately after the perturbation. Tanaka and Fillmore (1979) predicted that the stimulus-response delay must be approximately proportional to the square of the smallest dimension of the hydrogel, which points to the use of hydrogel thin-films in applications requiring fast response. In particular, a response within seconds can be achieved if the film thickness is less than 10 μm (Tokarev and Minko 2009). Indeed micro- or nano-sized hydrogel films are considered for a variety of applications requiring fast response, including photonic materials (Kang *et al* 2007), continuous glucose sensors (Suri *et al* 2003, Zhang *et al* 2012) and mechanical micro-actuators (Sidorenko *et al* 2007, Kim *et al* 2010).

3.1. Chemical equilibrium inside the polymer network

3.1.1. Ideal dissociation. Consider a dilute (ideal) aqueous solution of acid molecules. Each of these molecules can be found in one of two protonation states either having no electric charge (AH, protonated state) or bearing a negative unit charge (A^-, deprotonated or dissociated state). The acid dissociation constant Ka ($\mathrm{pKa} = -\log_{10} \mathrm{Ka}$) describes chemical equilibrium between the charged and protonated species through the following relation:

$$\mathrm{Ka} = \frac{[A^-]\,[H^+]}{[AH\,]} \tag{2}$$

In the case of acrylic acid, we use $\mathrm{pK_{AAc}} = 5$ in our molecular model. The degree of charge, for an acid, measures the fraction of molecules in the deprotonated state. In an ideal solution, this quantity is completely determined by pKa and the pH of the medium, such that

$$f_{\mathrm{ideal}} = \frac{[A^-]}{[AH\,] + [A^-]} = \frac{1}{1 + 10^{\mathrm{pKa}-\mathrm{pH}}} \tag{3}$$

where square brackets indicate molar concentrations. On average, less than 10% of the molecules are in the dissociated state if $\mathrm{pH} = \mathrm{pKa} - 1$ ($f_{\mathrm{ideal}} = 1/11 \approx 0.1$), while more than 90% of the molecules are dissociated if $\mathrm{pH} = \mathrm{pKa} + 1$ ($f_{\mathrm{ideal}} = 10/11 \approx 0.9$). Thus, the dissociation transition that goes from 10% to 90% charged molecules occurs in two units of pH around pKa. The middle point of this transition, where exactly half of the molecules in the solution are charged ($f_{\mathrm{ideal}} = 0.5$), occurs when $\mathrm{pH} = \mathrm{pKa}$.

3.1.2. Network dissociation. In contrast to a dilute solution, hydrogel acid groups are tied to a polymer network. This confinement modifies the balance between physical interactions and chemical states, which determines the conditions of thermodynamic equilibrium. Dissociation in the polymer network can lead to strong electrostatic repulsions between nearby charged segments. Ultimately, as solution pH increases and network dissociation proceeds, these intra-network electrostatic repulsions drive swelling of the polymeric structure. To reduce such repulsions the amount of charge that establishes in the polymer can be significantly less than that expected from dilute solution considerations. Namely, calculating the ideal degree of dissociation using equation (3) (with the intrinsic pKa obtained from chemical tables) gives little if any information on the actual state of charge of acid groups along the polymer network.

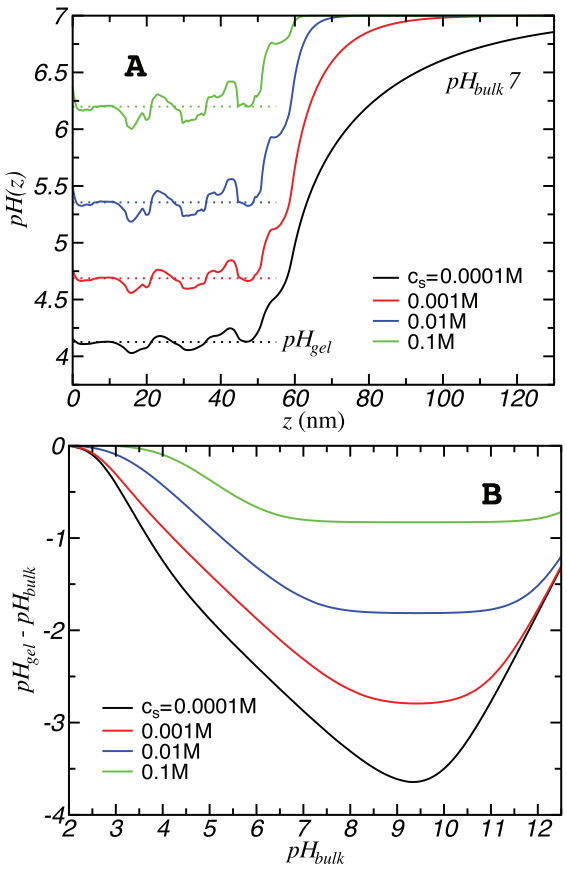

Figure 2. Poly(acrylic acid) hydrogel thin-film in contact with an aqueous salt solution. (A) Plot of local pH as a function of the distance from the surface that supports the hydrogel, z; curves for different concentrations of salt are shown (solid lines); solution pH is 7; dotted lines denote the average pH established inside the film $\mathrm{pH_{gel}}$. (B) Plot of drop in pH, $\mathrm{pH_{gel}}$–$\mathrm{pH_{bulk}}$ as a function of the solution pH for solutions having different concentrations of salt. The volume fraction of polymer is 0.03 when the network is in the protonated state (very low $\mathrm{pH_{bulk}}$).

This displacement of acid–base equilibrium towards the protonated species depends critically on the solution salt concentration, c_s. When a hydrogel is immersed in a low salt solution, confining ions inside the polymer network is entropically costly. Only enough counterions (positively charged) adsorb to neutralize the net charge of the hydrogel. Effectively, electrostatic repulsions between charged segments extend for several nanometers. To reduce these intra-network repulsions, dissociation is significantly weaker than what the acid–base equilibrium favors (ideal dissociation). Entropy loss of confining ions inside the hydrogel decreases as solution salt

concentration increases. Both coions (negatively charged) and counterions (positively charged) adsorb, even if the network is weakly charged. These ions screen intra-network electrostatic repulsions, which become relatively short range and effectively extend for a few nanometers. When solution pH increases, the network ionizes with little displacement from ideal dissociation.

3.1.3. The gel pH. Another consequence of confining acid groups to a polymer network is that the pH inside the hydrogel can be significantly different from the solution pH, pH_{bulk}, which is the experimentally controlled variable. Indeed, we can define a local pH at position $\mathbf{r}$ as $pH(\mathbf{r}) = -\log_{10}[H^+(\mathbf{r})]$, where $[H^+(\mathbf{r})]$ is the local concentration of protons. Lower local pH inside the hydrogel and weaker network dissociation are really two sides of the same coin. However, as we will see in next chapter, the pH established inside the hydrogel controls the electric charge of adsorbed peptides/proteins, which highlights the importance of this local quantity in understanding adsorption.

Figure 2(A) shows the local pH for a surface-grafted PAAc hydrogel nanofilm in contact with a salt solution of controlled composition. This particular polymer structure, being covalently attached to a surface, can only adjust its distribution of polymer in the direction perpendicular to the surface in response to changes in the solution pH or salt concentration. Film thickness, h_{gel}, can be calculated as twice the first moment of this polymer distribution,

$$h_{gel} = \frac{\int_0^\infty 2z\left\langle \rho_{pol}(z)\right\rangle \mathrm{d}z}{\int_0^\infty \left\langle \rho_{pol}(z)\right\rangle \mathrm{d}z} \tag{4}$$

where z measures the distance from the grafting surface ($z = 0$), and $\left\langle \rho_{pol}(z)\right\rangle$ is the local number density of polymer segments. Angle brackets indicate an ensemble average over the different conformations of the polymer network.

Three different spatial regions can be clearly identified when describing the local pH of polyacid hydrogel nanofilms (figure 2(A)). Far from the surface, $pH(z)$ approaches the bulk solution pH, $pH(z \to \infty) = pH_{bulk}$. Inside the hydrogel film ($0 < z \leqslant h_{gel}$), $pH(z)$ significantly drops with respect to pH_{bulk}, depending on the solution salt concentration. In this region, local pH can be characterized calculating its average over the film thickness,

$$pH_{gel} = \frac{1}{h_{gel}} \int_0^{h_{gel}} pH(z)\mathrm{d}z. \tag{5}$$

Figure 2(B) shows how the pH inside the hydrogel can be several units lower than the experimentally controlled solution pH (up to four units for the cases shown). This drop in pH, pH_{gel}–pH_{bulk}, depends critically on the solution salt concentration as well as the bulk pH. The last spatial region to describe is the interface between bulk solution and hydrogel, where local pH varies smoothly from pH_{bulk} to pH_{gel}. The width of this interface depends critically on the solution salt concentration (Longo *et al* 2012, 2014b).

The drop in pH inside the hydrogel depends on the polymer network density. A higher volume fraction of polymer results in a shorter average distance between network segments. Thus, increasing the density of polymer disfavors dissociation because this leads to stronger intra-network repulsions between closer charged units. Such displacement of the chemical equilibrium of network acid groups towards higher protonation implies that the gel pH drops even more.

We have recently studied the pH-responsive behavior of chemically grafted polyacid hydrogel thin-films in electric fields. An applied voltage between the supporting surface and the bulk solution only modifies the local environment in the few nanometers closest to the surface. Varying the applied voltage allows the pH at the surface to be controlled without altering the gel pH, which results from the chemical composition of the bulk solution (pH_{bulk} and c_s) and the density of the polymer network.

4. Physical adsorption and protonation of peptides/proteins in pH-responsive gels

In this section, we discuss the adsorption and protonation of pH-sensitive molecules in polyacid hydrogels. We show that significant adsorption can occur under conditions where the polymer network and adsorbate are expected to have like-sign charge, or when the adsorbate is uncharged in solution. This behavior occurs because the protonation of both components displaces from ideal behavior. This protonation behavior is not trivial, particularly when different titratable groups are present in the adsorbate. We describe in detail the individual protonation/deprotonation upon biomolecule adsorption of some of its amino acid residues. We concentrate on two case studies: the adsorption of his-tag and lysozyme in PAAc hydrogel thin-films.

4.1. Isolated adsorption

Let us first consider the adsorption inside a hydrogel of a small molecule bearing a single basic unit with logarithmic dissociation constant pK_{ads}. An expression analogous to equation (3) can be written to relate local degree of charge and pH,

$$f_{ads}(\mathbf{r}) = \frac{[AdsH^+](\mathbf{r})}{[AdsH^+](\mathbf{r}) + [Ads](\mathbf{r})} \approx \frac{1}{1 + 10^{pH(\mathbf{r}) - pK_{ads}}} \tag{6}$$

where $[AdsH^+](\mathbf{r})$ and $[Ads](\mathbf{r})$ are the local concentration of protonated (charged) and deprotonated adsorbate, respectively. In the bulk solution, far from the hydrogel $pH(\mathbf{r}) = pH_{bulk}$, and the degree of charge corresponds to ideal protonation of the adsorbate. If the adsorbate concentration in the bulk solution is dilute, then we can safely assume that adsorption does not modify the environment inside the hydrogel. Thus, on average, inside the polyacid hydrogel we can approximate

$$\left\langle f_{ads}\right\rangle \approx \frac{1}{1 + 10^{pH_{gel} - pK_{ads}}} \tag{7}$$

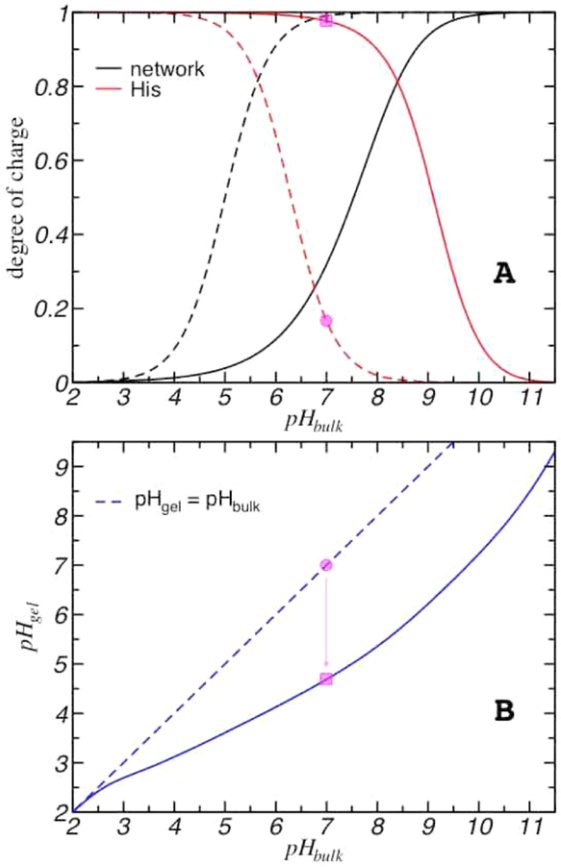

Figure 3. Graph illustrating adsorption in a poly(acrylic acid) hydrogel from a dilute adsorbate solution. (A) Plot of network and adsorbed histidine degree of charge as a function of solution pH (solid lines); the corresponding dashed-line curves represent ideal network dissociation and the degree of charge of histidine in the bulk solution. (B) Plot of gel pH as a function of solution pH. In both panels the bulk concentration of histidine is 1 nM, $c_s = 1$ mM, and polymer volume fraction of the protonated network is 0.03. At this histidine concentration, all hydrogel properties (gel pH, film thickness, network degree of dissociation, etc) are the same as those when the solution contains no histidine.

where pH_{gel} is the gel pH established in the absence of adsorbate, as described in section 3.1.3. Namely, gel pH controls the adsorbate charge inside the material.

Consider, for example, the adsorption from a dilute solution of a histidine monomer ($pK_{His} = 6.3$) inside a PAAc hydrogel. Panel A of figure 3 shows the degree of charge of the adsorbed molecule as well as that of molecules in the bulk solution, as a function of pH_{bulk}. Panel B shows the pH inside the hydrogel. When $pH_{bulk} = 7$, for example, no significant adsorption is to be expected *a priori*, because histidine is mostly uncharged in the bulk solution. However, inside the hydrogel pH drops almost two units, such that adsorbed histidine molecules are strongly positively charged. The network is negatively charged under these conditions (roughly 30% of segments are ionized; see panel A). Adsorbate-network electrostatic attractions reduce the system free energy and drive histidine adsorption. Indeed, the adsorption of histidine displaces its ionization curve (with respect to bulk deprotonation) towards the charged species in the whole range of pH_{bulk}. This decrease in electrostatic energy exceeds the increase in the chemical free energy of the adsorbate, which favor ideal (bulk) protonation.

The results of figure 3 have been calculated using the molecular theory described in section 2; the bulk concentration of histidine is 1 nM to represent dilute conditions. Calculating network and histidine degree of charge using equations (3) and (7) respectively, yields results that are indistinguishable in the graph scale used in figure 3(A). Tough good approximations, the use of these two equations requires the input of pH_{gel}, whose calculation requires a more detailed description of the system accounting for all the different physicochemical contributions to the free energy (for example, the molecular theory as shown in panel B).

4.2. Adsorption depends on pH and salt concentration

As we have discussed, the solution pH, salt concentration and density of network acid units control local environment inside the polyacid hydrogel, because they define gel pH, the amount of charge established in the network as well as the effective extent of electrostatic interactions. Gel environment, in turn, controls the charge of pH-sensitive molecules adsorbed from dilute solutions. Thus, what is the effect of these independent variables on the adsorption at finite concentrations and protonation of peptide and proteins? We first describe the adsorption of hexahistidine in PAAc hydrogel thin-films. Then, we address the more complex protonation behavior that occurs upon lysozyme adsorption in these films.

To quantify the adsorption it is convenient to define the quantity

$$\Gamma = \iint_{V_{tot}} \left(<\rho_{ads}(\mathbf{r})> - \rho^{b}_{ads} \right) d\mathbf{r} \qquad (8)$$

which gives the excess amount of adsorbed molecules per unit area. In this definition, ρ^{b}_{ads} and $<\rho_{ads}(\mathbf{r})>$ are respectively the bulk and local number density of adsorbate, and V_{tot} is the total volume of the system, including the hydrogel and the bulk solution. Angle brackets in the local density represent a position-dependent ensemble average over adsorbate conformations. The adsorption, Γ, quantifies not only the excess amount of molecules partitioned in the interior of the hydrogel but it also accounts for contributions from the interfacial network-solvent region.

4.2.1. His-tag adsorption and protonation. Protein tags are short peptides attached to proteins for different purposes such as facilitating purification from a raw biological source, for example. In immobilized metal affinity chromatography (IMAC) (Porath *et al* 1975), a histidine oligomer (his-tag) is frequently attached to the sequence of recombinant proteins to enable separation from cultures of overexpressing cells (Biswas *et al* 1995). The most frequently used his-tag is that formed by six consecutive histidine residues (His_6). There are many examples of the use of hexahistidine for the immobilization of proteins and enzymes using polymer hydrogels. Recombinant his-tagged rhamnosidase was entrapped and purified using calcium alginate hydrogel beads (Puri *et al* 2010). Using IMAC, his-tagged green fluorescent protein

and glutamyl aminopeptidase was immobilized and purified within nickel functionalized poly(2-acetamidoacrylic acid) hydrogels and hydrogel beads (Ha *et al* 2008, 2012, 2013). Poly(ethylene glycol)-based hydrogels modified with metal-affinity ligands were employed for binding and release of his-tagged GFP (Lin and Metters 2007, 2008).

Both the adsorbent material and protein tag are generally pH sensitive. However, the effect of solution pH on the adsorption of his-tagged proteins has not been thoroughly studied. To achieve the immobilization of his-tagged proteins, the polymer is modified with metal-ion-chelating ligands. Thus, the specific binding interaction between histidine residues and metal ions is the driving force for protein entrapment. In this section, we describe the adsorption of hexahistidine due to electrostatic attractions between ionized units of a pH-sensitive hydrogel and charged histidine residues. We argue that hydrogel response to changes in pH and salt concentration can be used to trigger or control the adsorption/release of his-tagged proteins. We will show that peptide protonation and hydrogel response are complex, which requires the fundamental understanding of physical adsorption in the absence of metal ions before addressing the competition between peptide protonation and metal-histidine binding.

We have recently considered the physical adsorption of hexahistidine inside PAAc hydrogel thin-films (Longo *et al* 2014a). This adsorption presents a non-monotonic dependence on the solution pH (see figure 3(A)). At low pH_{bulk}, the network is only weakly ionized, which results in no electrostatic attractions with the peptide, and therefore no driving force for significant adsorption. At high pH_{bulk}, in contrast, the network is strongly charged, but histidine residues are mostly uncharged, which also results in negligible adsorption. At intermediate values of pH_{bulk}, both the network and the amino acids are significantly charged, leading to an adsorption that displays a maximum under these conditions.

Hexahistidine adsorption depends on the solution salt concentration (figure 3(A)). Decreasing c_s enhances adsorption, which is a clear sign that the driving force for adsorption results from network-adsorbate electrostatic attractions. High concentration of salt ions inside the hydrogel results in the screening of these electrostatic interactions, which effectively extend for only few nanometers. In contrast, if the concentration of adsorbed salt ions is low, attractions extend longer driving higher concentrations of oppositely peptide inside the hydrogel. In section 3.1.2, we have discussed that as salt concentration decreases, the network charges less. Interestingly, the weaker charged network adsorbs more peptide. This shows the critical importance of the effective extent of electrostatic interactions. However, the presence of peptide in the bulk solution has the additional effect of displacing network dissociation towards more charge, with respect to salt solutions containing no peptide. In particular, for moderately high peptide concentrations, network dissociation becomes ideal (Longo *et al* 2014a).

Upon adsorption, the equilibrium deprotonation of peptide residues displaces towards the charged species. At any pH_{bulk}, adsorbed residues are significantly more likely to be charged than residues in the bulk solution. In particular, at low

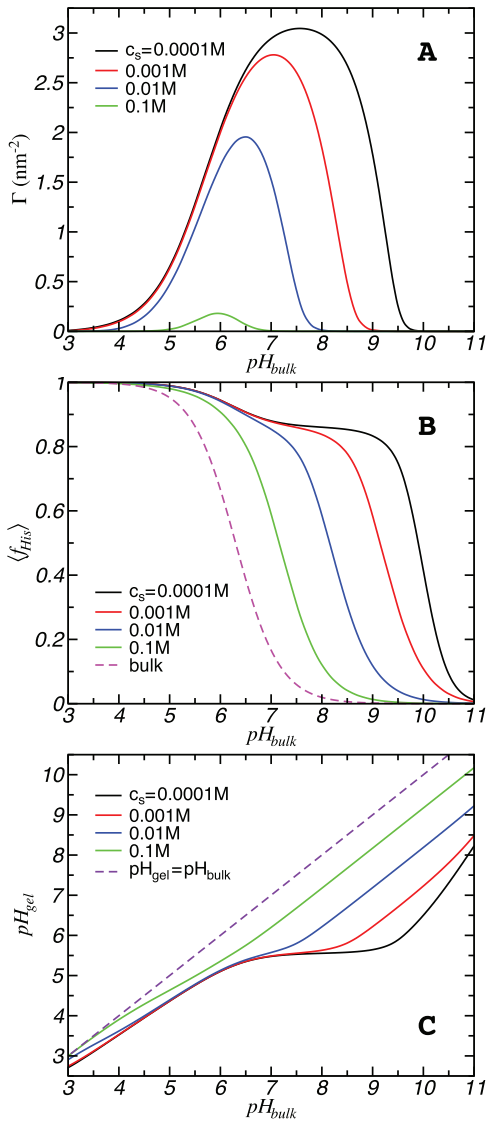

Figure 4. (A) Plot of the adsorption of hexahistidine inside a PAAc hydrogel thin-film as a function of the solution pH, for different concentrations of salt. (B) Average degree of charge of adsorbed (solid lines) as well as bulk (dashed line) histidine residues as a function of solution pH for the same conditions as those of panel A. (C) Gel pH versus solution pH for the same conditions as panel A. Polymer volume fraction is 0.03, when the network is protonated, and the bulk concentration of peptide is 10 μM.

c_s the adsorbed peptide residue remains charged several units of pH_{bulk} above pK_{His} (up to four units for the cases shown in figure 4(B)). This displacement of acid–base equilibrium towards the charged species occurs to enhance electrostatic attractions with the oppositely charged network. The price to pay for this decrease in electrostatic energy is increasing the chemical free energy, which accounts for the acid–base equilibrium of histidine residues. This chemical free energy favors ideal dissociation. At pH_{bulk} of maximum adsorption, this free energy contribution favors the deprotonated (neutral) residue, except at high concentration of salt.

In our theoretical studies, we impose global electroneutrality of the hydrogel-solution system. This means that the whole system must be electroneutral when considering hydrogel, bulk solution and gel–solution interface. However, in our

calculations we obtain local electroneutrality as a result. In particular, the interior of a few tens of nanometer-thick hydrogel film satisfies this condition. Then, at a pH_{bulk} such that the network is partially dissociated, the hydrogel must adsorb counterions to balance the charge in the polymer. This adsorption implies the confinement of such ions, which leads to a loss of entropy that depends on the concentration of ions in the bulk solution. Lowering the species bulk concentration increases the entropy loss of molecular confinement. The adsorption of a charged hexahistidine peptide offers more counterion charge to neutralize the hydrogel with the cost of confining just one molecule. As a result peptide adsorption increases as salt concentration decreases. Hexahistidine adsorption however requires displacing the acid–base equilibrium of the residue with the consequent increase of chemical free energy. In this complex context, the degree of charge of the peptide residues results from the balance between all these physicochemical contributions to the total free energy.

Adsorbed His residues are significantly more charged than those belonging to solution peptides, as seen in figure 4(B) for the adsorption in a polyacid hydrogel nanofilm. Where does this protonation occur? Figure 5(A) shows the local degree of protonation of His residues, $f_{His}(z)$, inside and above the polyacid hydrogel nanofilm. From this local quantity we can calculate the average value for adsorbed residues as

$$<f_{His}> = \frac{1}{h_{gel}} \int_0^{h_{gel}} f_{His}(z)dz. \quad (9)$$

We see that 20–30 nm from the top of the film the charge of the peptide is roughly that corresponding to the bulk solution. In this narrow interfacial region, the peptide becomes strongly protonated such that the protonation degree inside the film f_{His} is significantly displaced from that of the bulk solution, f^b_{his} (as seen in figure 4(B)). In particular, at $pH_{bulk} = 7 (c_s = 1\,mM)$, $f_{His}(z)$ changes from $f^b_{His} \approx 0.2$ to $<f_{His}> \approx 0.9$, which implies that his-tag gains four protons on average upon adsorption under these conditions (figure 5(A)).

Inside the hydrogel, pH drops. This can be seen in figure 4(C) that shows gel pH as a function of solution pH for a variety of conditions. In addition, figure 5(B) shows how local pH changes in different spatial regions of the hydrogel–solution system. For salt solutions without peptide, the drop in pH prevents the strong intra-network repulsions that would arise were the network state of protonation ideal (i.e. resulting from $pH_{gel} = pH_{bulk}$). For peptide solutions, this drop allows the adsorbed peptide to be more protonated than in the bulk solution (see the approximation given by equation (7)), which enhances electrostatic attractions with the oppositely charged network. Interestingly, the presence of the peptide increases gel pH with respect to that of bulk solutions without the peptide (see figure 5(B)). As a result, the network in contact with a peptide solution is more charged than when in contact with a salt solution, other conditions being the same (pH_{bulk}, c_s and volume fraction of polymer). In particular, if the peptide concentration is high, network dissociation becomes nearly ideal (Longo *et al* 2014a). This behavior not only enhances

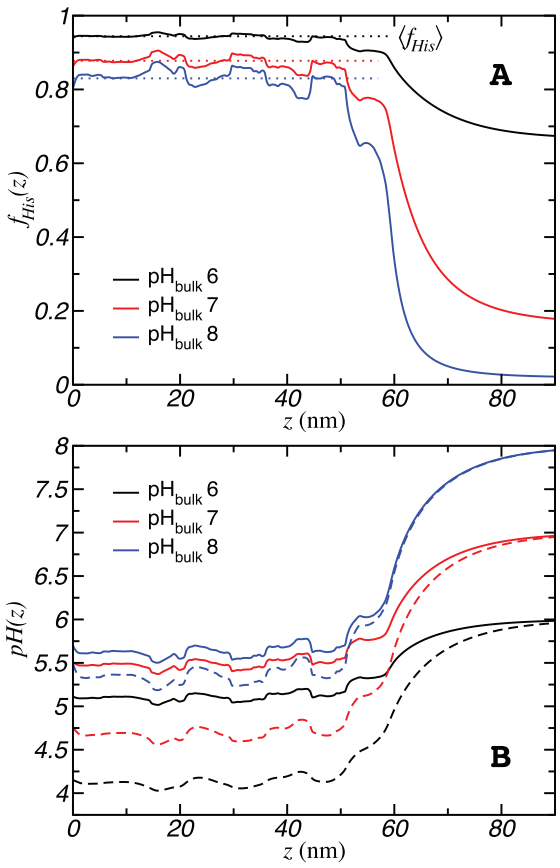

Figure 5. Poly(acrylic acid) hydrogel thin-film in contact with a aqueous solution containing 10 μM hexahistidine and 1 mM NaCl. Panel A shows the local degree of charge of His residues as a function of the distance from the surface that supports the hydrogel, z; curves for different solution pH are included (solid lines); dotted lines illustrate the average degree of charge of residues belonging to adsorbed molecules. Panel B shows local pH for the same conditions as panel A (solid lines); dashed lines represent the local pH at the same pH_{bulk} but for salt solutions containing no peptide. Polymer volume fraction is 0.03 (protonated network).

network-peptide attractions but also optimizes the chemical free energy of the network that favors ideal dissociation.

At low salt concentration, histidine protonation upon adsorption displays a very interesting feature that can be understood in terms of gel pH (see figures 3(B) and (C)). There is a range of solution pH where $<f_{His}>$ shows a plateau. Namely, varying pH_{bulk} within this range does not (roughly) modify the charge of adsorbed peptides. Constant $<f_{His}>$ results from a gel pH that displays the same feature in this pH_{bulk} range (figure 3(C)). Constant gel pH prevents peptide deprotonation and the consequent weakening of electrostatic attractions, which are strong since this behavior occurs around the pH_{bulk} of maximum adsorption (see figure 3(A)). The price to pay for keeping the strength of electrostatic attractions high is increasing the chemical free energy due to the dramatic displacement of the acid–base equilibrium of adsorbed residues towards protonation. This contribution to the free energy favors ideal peptide protonation.

Moreover, there is another consequence resulting from the buffering effect illustrated in figure 3(C) where pH_{gel} remains

constant for some units of pH_{bulk} depending on the salt concentration. Network dissociation also shows a plateau in the same pH_{bulk} range (results not shown). Namely, varying the solution pH does not alter the charge of the network under these conditions. This displacement of the acid–base equilibrium preventing network deprotonation bears the unfavorable effect of increasing the chemical free energy of the polymer. Finally, network dissociation in principle favors electrostatic attractions, the driving force for this buffering behavior. However, because the network is strongly dissociated at these conditions (results not shown), preventing peptide discharge is more favorable than a relative small increase in network charge.

4.2.2. Lysozyme adsorption. The same physicochemical concepts that explain peptide adsorption in pH-responsive hydrogels can be applied to describe protein adsorption. However, more complex behavior arises from the variety of amino acid residues in a single molecule, whose protonation/deprotonation occurs around different pKa's. Next, we will describe the adsorption and protonation of proteins using lysozyme as a case study. Although some of the behavior predicted is particular to this protein, most of the emerging concepts are broadly general and can be qualitatively applied to describe many other proteins.

We have recently studied the adsorption of lysozyme in polyacid hydrogel nanofilms (Narambuena *et al* 2015). Lysozyme has been modeled using a coarse-grain model where all the atoms of each amino acid are represented by single particle located at the position of the α-carbon. Atomic coordinates are obtained from crystallographic structure (PDB file 193L)(Vaney *et al* 1996). These coarse-grained particles are classified into two main groups, neutral and titratable. The latter category includes aspartate (Asp), glutamate (Glu), and tyrosine (Tyr), which are acidic amino acids as well as arginine (Arg), histidine (His) and lysine (Lys), which are basic amino acids. The intrinsic pKa's of these amino acids as well as the number of such residues in the protein (i.e. the composition number) are shown in figure 1. The rest of the amino acids are considered electroneutral. Lysozyme has 99 of these neutral residues.

Similarly to his-tag, lysozyme adsorption in a polyacid hydrogel is a non-monotonic function of the bulk solution pH (see figure 6). This behavior can be explained by considering how the total charge of the protein depends on pH. In a dilute solution having low pH, lysozyme bears several positive unit charges. Most network segments, however, are uncharged at low pH, resulting in little adsorption due to the weak electrostatic attractions. At sufficiently high pH, in contrast, the polymer is strongly charged, but the net charge of the solution protein is negative. Network-protein electrostatic interactions are repulsive, which prevents adsorption. In an intermediate range of pH_{bulk} both the network is strongly charged negatively and the net charge of the protein is positive, which leads to significant adsorption.

Decreasing solution concentration of salt favors adsorption (see figure 6(A)). This is because of the weaker screening of network-protein attractions, but also due to the relatively lower entropy loss in confining the protein inside the hydrogel. The adsorption of this macro-ion offers the possibility of neutralizing several network charges with the cost of confining only one molecule. Increasing the polymer density also enhances adsorption (see figure 6(B)). A higher density of ionizable network units offers more net charge for proteins to neutralize upon adsorption. However, confining the protein can induce high steric repulsions inside the hydrogel. This effect is only appreciable at high pH_{bulk} where the protein is weakly charged. Under these conditions, a hydrogel with lower polymer density can adsorb more protein (see figure 6(B)). Recently, Koetting and Peppas (2014) have studied pH-responsive poly(itaconic acid-co-N-vinylpyrrolidone) hydrogels for delivery of high isoelectric point therapeutic proteins. Consistently with our results, they have found that lowering solution ionic strength during protein loading can significantly improve the delivery of calcitonin under physiological conditions.

The thickness of a hydrogel film in contact with a salt solution changes when placed in contact with a protein/peptide solution. Namely, protein/peptide adsorption modifies hydrogel thickness. We have shown this behavior for lysozyme and hexahistidine adsorption in PAAc hydrogel nanofilms (Longo *et al* 2014a, Narambuena *et al* 2015). This behavior can have technological relevance in the development of biomolecular sensors.

The isoelectric point, pI, gives the solution pH at which the net charge of the protein is exactly zero. In a dilute solution, the protein is positively charged if $pH_{bulk} < pI$, and negatively charged if $pH_{bulk} > pI$. Interestingly, lysozyme adsorption is predicted in figure 6 above the isoelectric point. Namely, a protein that is negatively charged in the bulk solution adsorbs inside a hydrogel that is strongly like-sign charged. This behavior results from the pH drop inside the hydrogel, which allows adsorbed proteins to regulate charge and remain positively charged even when $pH_{bulk} > pI$. Figure 7 demonstrates this behavior showing that the net average charge of adsorbed proteins, $<Z_{lyso}>$, is significantly displaced to higher positive values. The magnitude of this displacement depends on the composition of the bath solution (pH_{bulk}, c_s and protein concentration) as well as the polymer volume fraction of the hydrogel, as illustrated in panels A and B of figure 7.

There are several theoretical studies of protein adsorption on charged surfaces and polyelectrolyte layers (Biesheuvel *et al* 2005, Biesheuvel and Wittemann 2005, Fang and Szleifer 2006, de Vos *et al* 2008). Using a mean-field theory, Biesheuvel and Wittemann (2005) predicted that BSA can adsorb on spherical weak polyelectrolyte brushes, even if the solution pH is above the protein pI. These authors attributed this anomalous adsorption to the fact that the pH established inside the brush is indeed lower than BSA pI. Thus, upon adsorption the protein regulates charge, which leads to charge inversion allowing for the attractive electrostatic attractions with the polymer. Using a similar approach, de Vos *et al* (2008) investigated the adsorption of BSA on PAAc layers. This work also suggests that adsorption above pI results from charge regulation due to a lower pH inside the polymer, as opposed to previous studies suggesting that anomalous adsorption was caused by an inhomogeneous charge distribution on the protein (Wittemann

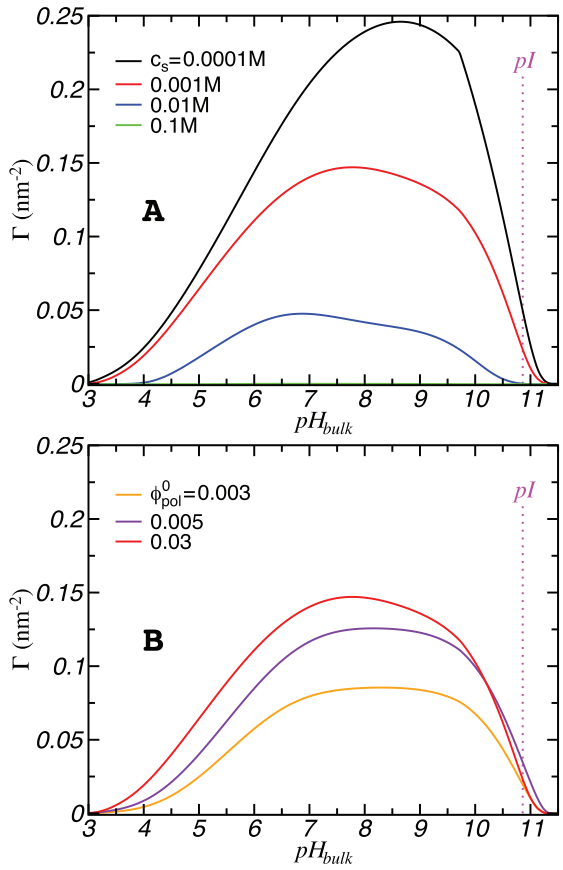

Figure 6. Adsorption of lysozyme from a 10 μM protein solution inside a poly(acrylic acid) hydrogel thin-film. (A) Plot of adsorption as a function solution pH for different concentrations of salt; the polymer volume fraction of the protonated network is $\phi^0_{pol} = 0.03$. (B) Adsorption versus solution pH for different polymer densities and 1 mM salt concentration. Vertical dotted lines mark the protein isoelectric point.

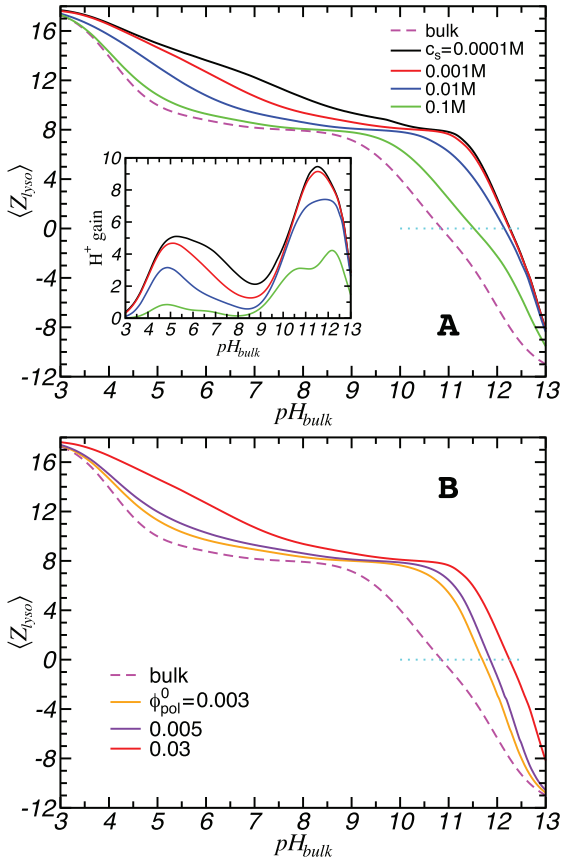

Figure 7. Net electric charge of lysozyme after adsorbing from a 10 μM protein solution inside a poly(acrylic acid) hydrogel thin-film. (A) Plot of net charge as a function solution pH for different concentrations of salt; the volume fraction of polymer is $\phi^0_{pol} = 0.03$ when the network is protonated; the inset shows the overall number of gained protons upon adsorption. (B) Net charge versus solution pH for different polymer densities and 1 mM salt concentration. In both panels dashed lines show the net charge of the protein in the bulk solution, and dotted horizontal lines mark the condition $<Z_{lyso}> = 0$.

and Ballauff 2006). The two effects, however, can contribute additively to adsorption above the protein isoelectric point (de Vos *et al* 2010). The theoretical description presented in section 2 incorporates both effects, an inhomogeneous charge distribution on the protein and charge regulation. Our results clearly point to charge regulation as the main effect in driving adsorption above the isoelectric point.

Depending on the environment, the protein can gain several protons upon adsorption in the hydrogel (see figure 7(A) inset). Where does this protonation occur, inside or outside the gel? Similarly to the behavior shown in figure 5 for his-tag, this protonation occurs within the few tens of nanometers closest to the polymer-solution interface. For example, for some of the conditions considered in figure 7, the protein can gain up to 9–10 protons within this narrow interface as it adsorbs.

4.2.3. Non-trivial protonation of amino acids. We have seen that the net charge of the lysozyme is more positive inside the hydrogel. How does this behavior result from the protonation of different amino acid residues? Figure 8 shows the average degree of charge of some amino acids corresponding to adsorbed proteins. All chemical equilibriums are displaced towards more protonation. Namely, at a given pH_{bulk} acidic residues are less likely to be negatively charged while basic residues are more likely to be positively charged than the same residues of proteins in the solution. When summed up considering the composition number, these individual displacements result in the more positive net charge of the adsorbed protein. The protonation of each amino acid depends on the salt concentration and the hydrogel density of polymer (see figure 8).

Deprotonation curves (as bulk pH increases) of adsorbed residues are not only displaced to higher pH_{bulk} values to favor positive charge, but these curves are also deformed with respect to bulk solution behavior. At the lowest salt and the highest polymer volume fraction shown in figure 8 (1 mM and 0.03, respectively), glutamic acid protonates very smoothly. The dissociation transition from $<f_{Glu}> = 0.1$ to 0.9 occurs in 4 pH_{bulk} units, twice as much as in the bulk solution (see ideal dissociation in section 3.1.1). This amino acid deprotonates in the same pH range as the polymer network (we use $pK_{Glu} = 4.4$ and $pK_{AAc} = 5$). Tyrosine, whose bulk deprotonation occurs near the protein pI where the network is fully

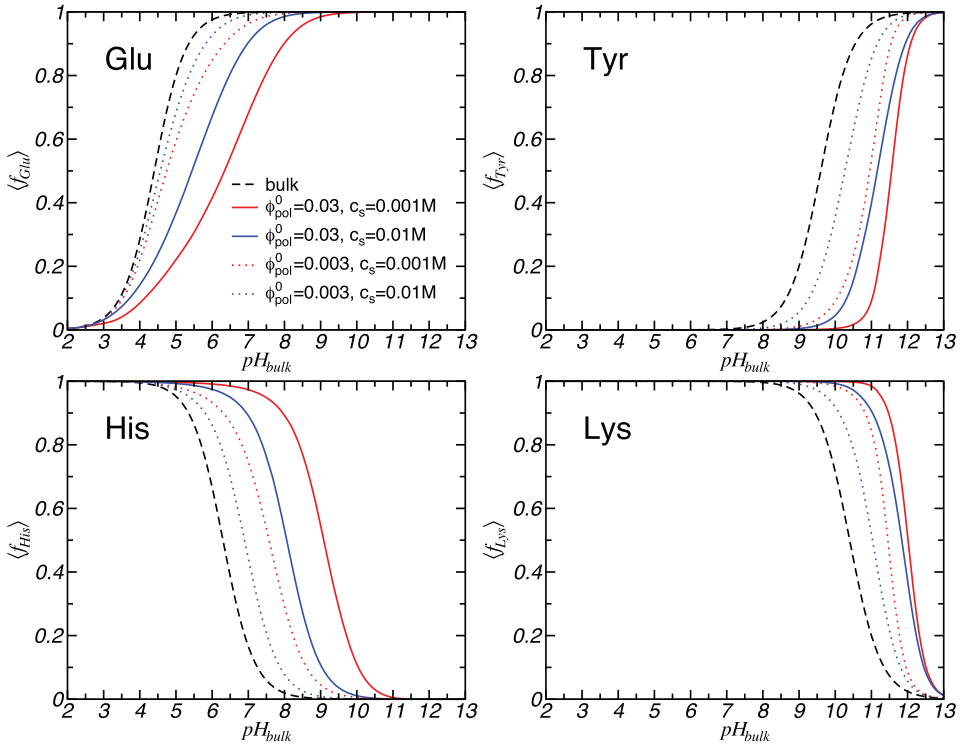

Figure 8. Deprotonation curves for some residues corresponding to lysozyme molecules adsorbed in a poly(acrylic acid) hydrogel thin-film. Each panel shows the average degree of charge of adsorbed residues as a function of the solution pH (solid and dotted-line curves); the degree of charge of bulk solution residues is represented with a dashed-line curve. The concentration of protein is 10 μM.

charged, dissociates sharply under those conditions. The dissociation transition of this amino acid occurs in about 1 pH_{bulk} unit, half as much as in the bulk solution. Analogous behavior is observed for the deprotonation of Lys. Histidine, on the other hand, suffers the most significant displacement towards more protonation. However, under the same conditions, the width of this discharging transition is similar to that in the bulk solution, roughly 2 pH_{bulk} units.

Therefore, the protein can displace and/or deform the chemical equilibrium of different amino acid to favor protonation and induce stronger electrostatic attractions with the hydrogel network. Displacement/deformation is different for the various amino acids and depends on the chemical composition of the solution and the density of polymer of the adsorbent material. Different residues give the protein more degrees of freedom to regulate charge under different environment conditions.

Using calorimetry, Welsch *et al* (2012) studied the protonation of lysozyme upon adsorption in core–shell microgels of poly(N-isopropylacrylamide-co-acrylic acid). They found that lysozyme gains one proton when entering the negatively charged gel at solution pH 7.2 and 10 mM salt concentration. Analysis of titration curves we have calculated at the conditions of the experiment allow us to suggest that this behavior corresponds to the protonation of the only protein histidine residue (Narambuena *et al* 2015).

4.3. Controlling surface pH

We have recently studied the swelling behavior of pH-sensitive hydrogels with chemically grafted networks in external electric fields. Our results show that the application of a voltage between the surface that supports the hydrogel and the bulk solution allows controlling the local pH in the region near the electrode (supporting surface), without changing the local pH in the rest of the material. Namely, gel pH is still controlled using pH_{bulk}, salt concentration, and density of polymer. However, next to the surface we can change local pH varying the applied electric potential. This prediction can have relevance for potential applications. We have shown that gel pH is critical for the adsorption of proteins inside the hydrogel, because it defines the network–protein electrostatic interactions. In the case of enzymes, for example, we can think of a device where the gel pH is set to drive adsorption inside the hydrogel, while activity near the surface is triggered with the applied voltage.

5. Perspectives and conclusion

When considering peptide/protein adsorption in pH-responsive hydrogels, drawing conclusions from chemical equilibrium in bulk solutions leads to qualitatively wrong interpretations. As discussed in this review, our recent studies have shown that in these systems the interplay between chemical equilibrium, physical interactions and molecular organization in nano-confined environments results in emergent behavior. We have discussed some of the non-trivial features of physical adsorption and protonation of proteins in polyacid hydrogels. The main driving force for adsorption results from the electrostatic attractions between the positively charged protein and the negatively ionized polymer network that makes the hydrogel.

Thus, adsorption depends on the solution pH and salt concentration as well as the network density of ionizable units. These are key variables to consider in the rational design of polymeric network/therapeutic protein delivery systems.

All amino acids of adsorbed proteins display deprotonation curves that are significantly different from those in solution. To enhance adsorption, these curves are adequately shifted and deformed, which depends in a complex fashion on the specific amino acid. This possibility of modifying different acid–base equilibriums gives adsorbed proteins considerable flexibility to regulate charge and enhance the electrostatic attractions with the network under a wide range of experimental conditions. In particular, when pH is above the isoelectric point, the protein is negatively charged in the solution. Charge regulation to favor adsorption under these conditions can lead to a dramatic sign reversal where the protein gains several protons within a narrow interfacial region. In addition, protein adsorption modifies the microenvironment inside the hydrogel, in particular the gel pH. As a result, the state of protonation of the network is different before and after adsorption.

In oral delivery of therapeutic proteins, pH-responsive hydrogels are investigated as potential carriers that overcome and profit from the barriers imposed by the gastrointestinal tract. However, a major breakthrough in effective oral delivery of peptides and proteins is still awaiting (Renukuntla *et al* 2013). Such a breakthrough is likely to be achieved through rational molecular design of pH-responsive hydrogels, which requires a deep understanding of the physical chemistry involved in protein adsorption inside these materials. His-tagged proteins and pH-sensitive hydrogels are frequently used in protein chromatography. Protein, tag, and the adsorbent material are all pH-responsive. Solution pH, however, is rarely considered as a tool to optimize or control system behavior. The results of our recent work provide molecular insights that can be used as guidelines in the design or optimization of functional biomaterials. For example, adsorption of lysozyme and his-tag is a non-monotonic function of pH. Decreasing the salt concentration can significantly enhance adsorption, even if the pH is above the isoelectric point of the protein.

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