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Polyelectrolyte–protein complexes

C.L. Cooper, P.L. Dubin *, A.B. Kayitmazer, S. Turksen

Department of Chemistry, IUPUI, 402 N. Blackford Street, Indianapolis IN 46202, United States

Abstract

The field of polyelectrolye–protein complexes is now benefiting from the convergence of three themes whose disciplinary distinctions formerly produced fragmentation. Theory and simulations, previously restricted to isotropic models of colloids and polyelectrolytes, are now taking into account the heterogeneity of charges in both macroions and providing far more realistic depictions of proteins. Applications of protein–polyelectrolyte systems have been strongly influenced by the opportunities presented by multilayer assembly, and the dichotomy between semi-synthetic systems and the protein–biopolyelectrolyte cognate pairs of biochemical interest has been giving way to less parochial recognition of universal effects. The junction of these disciplines, along with the development of many new methods of investigation, form the subject of this review focused on recent developments and their foundations.

1. Introduction

1.1. Scope

We delineate the scope of this review by limiting the terms "polyelectrolytes" and "proteins". The polyelectrolytes considered here are all hydrophilic, excluding "ion-containing polymers" that are water-insoluble, or polyamphiphiles that are hydrophobically modified. We treat only flexible chain polyelectrolytes, thus excluding the completely unique and distinct nucleic acids. We consider only globular proteins (whose well-defined tertiary structures reflect their roles in biosynthesis, regulation, transport, storage, and protection) and exclude contractile and structural proteins which typically assume filamentous or fibrillar states. These limitations are not indications of perceived importance but are intended to add more reliability to generalizations reached. Studies of DNA-binding proteins and of complexes between synthetic polyelectrolytes and gelatin may be found in abundance elsewhere, but are fundamentally different from the subject here. On the other hand, we propose that biopolyelectrolytes with no secondary or tertiary structure, namely ionic polysaccharides of plant or animal origin, including alginates, pectins, anionic "gums" and glycosaminoglycans, are not physicochemically distinct from synthetic polyelectrolytes.

1.2. Categories

The distinction between synthetic and natural polyelectrolytes having been raised, we can state that when secondary structure is absent from the latter, there is little physicochemical difference between the two, many of the principles of polyelectrolyte behavior having been founded on observations with macroions of natural origin. However, consideration of biopolyelectrolytes without reference to their biological role is like consideration of synthetic polyelectrolytes with no knowledge of their synthesis: something is lost. A second difference is conceptual. The literature on complexes of synthetic polyelectrolytes and proteins in vitro has been separate from the literature on the interaction of biopolyelectrolytes with proteins under physiological conditions. In an intermediate position but much closer to the first are systems in which both polyelectrolyte and protein are of natural origin but have no biological relationship. We can thus create three protein–

* Corresponding author.
E-mail address: dubin@chem.umass.edu (P.L. Dubin).

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polyelectrolyte categories: semi-synthetic systems, natural (but non-cognate) systems, and cognate (i.e. co-evolved) systems. In general, we find the first represented in areas of basic research and technology (an example would be protein immobilization for biosensors), the second in cosmetic and food applications (e.g. gum acacia and whey proteins), and the third in the biological literature (as an example, the interaction of pectins with their cognate galacturonases). The overlap among these three fields is only beginning to become evident, especially as the diversity of structure and function among plant and animal ionic polysaccharides becomes more evident. One goal of this review is to facilitate this process.

Early studies of semi-synthetic systems were motivated by the need to purify proteins [1,2]. Control or stabilization of enzyme activity by complexation was observed by Morawetz [3] and by Larionova et al. [4] But for the most part, few followed up on these early investigations, as interest in the biological relevance of polyelectrolytes focused almost exclusively on DNA, although activity persisted in food-related polyelectrolyte–protein systems. The field is currently being re-energized by enthusiasm for polyelectrolyte multilayers (including those bearing proteins), and opportunities in biosensor technology. The growing realization of the importance of glycosaminoglycans–protein interactions we see as an additional motivation for exploration.

1.3. Other reviews

No review on polyelectrolyte–protein complexes has appeared since that of Xia and Dubin [5] in 1994, but several quite comprehensive closely related reviews have been presented recently. Tribet [6••] described the association of amphiphilic polymers with proteins, with particular focus on membrane proteins. This last book chapter is nominally on amphiphilic polyelectrolytes but in fact covers many aspects about the nature of the protein–polyelectrolyte interaction, methods of investigation, and speculations about structure. In view of this coverage, we avoid detailed description of polyamphiphile–protein systems. Protein–polysaccharide interactions have been especially well-covered in three reviews [7–9]. Specific protein–polysaccharide systems of interest to the food industry comprise the subject of a review by Dickinson [10]. We de-emphasize complexes unique to food applications, such as mixtures of whey proteins or β-lactoglobulin with carrageenan, gum Arabic, xanthan and acacia gum, not because of any lack of interest, but because they are so well covered elsewhere.

1.4. Recent advances

The last decade has been characterized by rapid progress mainly reflecting the convergence of three factors: applications, instrumentation and simulations. The intersection of the fields of polyelectrolyte multilayers and protein–polyelectrolyte complexes has opened opportunities in protein–polyelectrolyte layer-by-layer fabrication, with particular focus on the preparation of biosensors with improved storage and operational stability for detection of inter alia glucose, cholesterol and antibodies. The investigation of these multilayers has been greatly aided by new techniques for surface characterization by optical waveguide laser spectroscopy, surface plasmon resonance and small angle reflectometry (see below). New techniques applicable to the characterization of protein–polyelectrolyte complexes in solution include frontal analysis continuous capillary electrophoresis and affinity-based separation methods. It is also in the area of solutions that simulations have made the greatest impact during the last decade, providing considerable insight into the structure and phase behavior of complexes. The recognition of polyelectrolyte–protein complex coacervate phases as unique and distinct from precipitates or suspensions has profited from the remarkable array of new microscopic techniques that probe dynamics and structure on the nm–μm length scale. Finally, since polyelectrolytes provide a gentle immobilizing environment for proteins in coacervates and microcapsules, it is not surprising to find that polyelectrolytes as gels or brushes can be used to entrap and deliver proteins for pharmaceutical applications. Work in these areas, highlighted in this review, is of course grounded on a body of exploratory literature before 1995. The convergence of biochemistry, material science, polymer and colloid chemistry and other disciplines at present is equally likely to form the basis for imaginative and relevant science in the next decade.

1.5. Forces (interactions)

In principle, a number of non-covalent forces can contribute to complex formation between polymers and proteins. It is reasonably certain that the interaction between proteins and poly(vinylpyrrolidone) or other hydrophilic nonionic polymers and proteins involves the former as hydrogen bond acceptors, the effect of pH here being on the availability of the protein's carboxylic acids groups as donors [11]. All solutes are hydrophobic relative to water, which makes the total absence of hydrophobic interactions in polyelectrolyte–protein complexes difficult to deny, and such interactions like ghosts, are hard to disprove. The strongest evidence for hydrophobic interactions arises from the effects of systematic structural variations, or from thermodynamic data that exhibit the temperature dependence of the entropy and enthalpy of association that is characteristic of hydrophobic interactions, but such measurements are not common. The predominance of electrostatic interactions is widely accepted but there are two points of discussion. The first is whether such electrostatic interactions can in some way promote non-electrostatic forces, rather like DNA-binding proteins exhibit "loose

electrostatic binding" which then is superimposed on more specific hydrogen bonds when the protein is properly situated; or whether the opposite of such synergism occurs, the two forces competing in some manner; and in either case whether the energies of short-range forces and electrostatic forces are additive. The second issue involves the definition of electrostatic forces.

There is a surprising range of interpretations of "electrostatically driven complexation" which turns out, upon inspection, to depend on underlying paradigms, often implicit, and which encounter varying degrees of success for different experimental conditions. For example, if complexation is carried out in the absence of salt and with strong polyelectrolytes, stoichiometric precipitation is observed: the precipitate shows a pH-dependent but otherwise constant protein:polyelectrolyte combining ratio, which is well correlated with the ratio of polyelectrolyte charge : net protein charge. This behavior (which implies loss of counterions) is reminiscent of "polyelectrolyte colloid titration", and provided a model for many investigations by Kokufuta [12]. While Kabanov and coworkers [13] also employed a model based on oppositely charged polyelectrolyte "interpolyelectrolyte complexation", the stoichiometric "salt bonds" formed between protein and polyelectrolyte (typically quaternized polyvinylpyridine) were reported to involve only some fraction of the protein's ionic (typically carboxylate) units. The consequent two-state model in which all charged units are either free or engaged in salt bonds, leads to an equilibrium treatment and was used to quantitatively explain inter alia pH shifts upon complex formation, and exchange reactions in which either the protein or polyelectrolyte is displaced by another macroion. Yet another view of electrostatically driven complexation is seen from Ballauff and coworkers [14], for whom "positive patches on the protein become multivalent counterions of the (polyanion) charge segments". This "ion-exchange model" promulgates a major role for the entropy gain upon release of the original polyelectrolyte and protein counterions.

If "electrostatic forces" were loosely defined as all effects involving oppositely charged groups, we should also include the "salt bridges" and "ion pairs" that form in the low-dielectric environment of the protein. These intimate interactions clearly complement other short-range forces. In the classical biochemical model, protein-ligand specificity arises from such directionally specific short-range forces leading to structurally precise ligand–host complexes. The existence of such atomistically defined complexes in our case is questionable because: (1) we are in general not dealing with biological cognates; (2) even in that case, precise fit between the protein and the polyelectrolyte would require severe contortions of the latter; and (3) since the polyelectrolyte is a "statistical coil", an exact local arrangement of the bound segments would entail a loss of configurational entropy much greater than for low MW ligands. A much more reasonable scenario is that a large array of local chain configurations of similar energy characterize the bound polyelectrolyte, and the binding energy arises primarily from the sum of the numerous pairwise Coulomb interactions (both repulsive and attractive) between protein and polyelectrolyte fixed charges. Thus, we define electrostatic forces here as those governed by Coulomb's law alone, hence solely dependent on effective point charges and their distance from each other, with no directional specificity. A number of consequences follow from such a characteristic length scale of ca. 4–40 Å: (1) hydration is not lost: (2) short-range forces play minimal roles, and the interaction between uncompensated charges is much more important than between local dipoles; and (3) the interactions are subject to salt-suppression due to Debye–Huckel screening (and not to "ion-exchange"). As noted above, exceptions to this scenario occur for complexation between proteins and strong polyelectrolytes in salt-free solutions, and for hydrophobically modified polyelectrolytes, and these will be mentioned where relevant; otherwise it will be assumed that non-electrostatic forces play minimal roles and that electrostatic forces rest on the Poisson–Boltzmann equation with its characteristic salt screening.

1.6. States

The physical states of polyelectrolyte–protein complexes correspond to varying degrees of solvation. *Soluble complexes* are strongly hydrated; depending on the microscopic stoichiometry (number of protein molecules bound per polymer chain, n), 5–15% of the hydrodynamic volume of the complex may be occupied by protein (a much smaller fraction due to polymer). The net charge of an intrapolymer complex is to a first approximation $Z_T = Z_P + nZ_{pr}$, where the subscripts correspond respectively to polyelectrolyte and protein. When Z_T approaches zero (for polyanions this can only occur at pH <pI) retention of counterions is reduced and intrapolymer complexes can combine to form less hydrated *coacervates* containing typically 20–30% protein and polymer. Under conditions of higher complementarity and stronger interactions — high charge density polymers, pH far from pI, and low salt — phase separation becomes liquid-solid i.e. *precipitation*. As noted above this type of phase separation is characteristic of systems explored by Kokufuta [12], who also reported that enzyme activity could be retained even in such environments. An additional solid state of growing interest is the polyelectrolyte multilayer, in which protein either replaces one polyelectrolyte, or is an additional component. Finally, there are two conditions under which proteins are constrained by containment in polyelectrolyte *gels*. In the first case, incorporation of the protein into a preformed gel, as demonstrated by Skobeleva and coworkers [13] provides a way to control delivery of protein drugs and also an interesting system for studying protein diffusion. In the second, the protein itself can participate in

the gelation process. Aside from gelation with polymeric amphiphiles, in which the hydrophobic protein domains act as cross-linking sites, mixed gels of BLG and carrageenan were reported to form below pI, electrostatic forces creating a continuous network [15].

2. Experimental techniques

Several techniques have frequently been used for the characterization of protein–polyelectrolyte complexes. Some of the methods included in this section have been summarized in previous review articles [5,6••]; therefore, we will concentrate on recent developments and applications, introducing the latest techniques. The instrumental techniques reviewed in this article offer information on morphological, structural, and optical properties, and on dynamic and binding behavior of protein–polyelectrolyte complexes. Table 1 summarizes these methods, with cross-references to the other sections. Because the purpose here is to present the applications of these instrumental methods in the protein–polyelectrolyte research field, the reader is directed to the references and the articles therein for a detailed explanation of these techniques.

2.1. Microscopic techniques

2.1.1. Atomic force microscopy (AFM)

Atomic force microscopy measures the surface height through the vertical force between the probe and the specimen, and provides topographical information through 3D images. AFM is widely used for determining the film thickness and surface morphology of protein–polyelectrolyte multilayers and brushes. It is possible to determine surface roughness as low as 5–10 nm. Gergeley et al. [16] used AFM to investigate the film morphology of poly(L-lysine) (PLL) and poly(glutamic acid) (PGA) multilayers interacting with human serum albumin (see also Section 7.2); similarly, Ram and coworkers [17] used AFM to characterize for cholesterol oxidase embedded in poly (styrene sulfonate) (PSS) and poly(ethyleneimine) (PEI) multilayers. Czeslick et al. [18] utilized AFM to measure

Table 1
Experimental techniques applicable to protein–polyelectrolyte complexes and their formation

Methods	Complex state	Information provided	Section	References
AFM	ML, SPB	Surface topography	2, 7	[16–18]
Electron microscopy	all	Morphology, composition	2, 3, 6, 7, 8.4	[19••,21••,22••]
Optical microscopy	C, SC	Morphology, dynamic behavior	2, 6, 8.1, 8.4	[23]
Phase contrast microscopy	C, SC	Morphology	2, 6	[24,100••]
Confocal Scanning Microscopy (CLSM)	C, SC, SPB	Morphology	2, 6	[25•,26]
TIRF	ML, SPB, PPB	Morphology	2, 7	[27]
Dynamic light scattering (DLS)	C, SC, G, SPB	Hydrodynamic radius, diffusion constant, transitions	2, 6, 7, 8.4	[28–30,35,99]
Static light scattering (SLS)	C, SC, G, SPB	Radius of gyration, molecular weight	2, 7	[33]
SAXS	SPB	Crystallinity	2, 7	[28]
Electrophoretic light scattering	C, SC, SPB	Electrophoretic mobility	2, 6, 7, 8.4	[22••,34•,35,99]
Diffusing wave spectroscopy	C, SC, G	Diffusivity, size	2	[25•,37]
UV-VIS spectroscopy	C, SC, G, SPB	Absorption, concentration	2, 7	[21••,38,39]
FTIR	SPB, G, ML	Structure, composition	2, 7	[39–41]
ATR-FTIR and IR-MIR	ML, PPB	Structure, composition	2, 7	[25•,42,43••]
Fluorescence spectroscopy	SPB, C, SC, G	Adsorption, kinetics	2, 7	[22••,26,116]
Circular dichroism	C, SC, G, SPB	Protein conformation	2, 3, 7	[19••,24,38,41]
Potentiometric titrations	C, SC, G	pK, transitions	2, 8.1	[105,117]
Turbidimetric titrations	SC	transitions, stoichiometry	2, 6	[24,100••]
DSC	C, SC, G	ΔH, ΔS, stability	2	[40,48]
Isothermal titration calorimetry	C, SC, G	ΔH, ΔS	2, 3	[40,49•,87••]
HPLC	SC, C, G	Composition	2	[39]
SEC (GPC)	SC, C	Composition	2	[50,51]
Affinity chromatography	SC	Binding	2, 3	[52]
Scanning angle reflectometry	ML, PPB	Surface thickness, composition	2, 7	[57••]
X-ray and neutron reflectometry	ML, PPB	Surface thickness, composition	2, 7	[58•]
FPR	C, SC, G, SPB	diffusivity	2, 7	[37,59•]
FACCE	SC		2	[53••,54]
Optical waveguide lightmode spectroscopy	ML	Surface thickness, composition	2, 7	[16,59•]
Quartz crystal microbalance (QCM)	ML	Surface thickness, composition, viscosity	2, 7	[34•,60]
Surface plasmon resonance (SPR)	ML	Surface structure	2, 3,7	[19••,61•,62•]
Rheology	C, G	Viscoelasticity	2, 6	[38,63]
Affinity coelectrophoresis	SC	Binding constants	2, 3	[55,56•,87••,88•]

S: solution, SC: soluble complex, C: coacervates, coacervation, G: gel, P: precipitate, ML: multilayer, SPB: spherical polyelectrolyte brush, PPB: planar polyelectrolyte brush.

the sizes of spherical poly(acrylic acid) (PAA) brushes before the binding of human serum albumin (see also Section 7.3). However, in all of the above applications, AFM does not provide proof for direct adsorption of protein on the surface, due to the roughness of the polyelectrolyte multilayers.

2.1.2. Electron microscopy (EM)

Electron microscopy is widely used for imaging objects by illuminating the sample with an electron beam, thus providing microscopic resolution at dimensions >10 nm. EM is applicable to both stained and unstained samples and yields sample morphology, composition, and crystallinity. Pihlajamaa et al. [19••] studied the complexation of heparin with collagen IX by EM and used this information to approximate binding sites for heparin. Burgess [20••] determined the shape and stability of albumin/acacia and gelatin/acacia coacervate droplets in relationship to the coalescence of coacervate droplets measured by scanning electron microscopy. For multilayer systems, whether assembled on colloids or on flat surfaces, EM is a powerful tool for monitoring layer formation and film thickness. Lvov and et al. [21••] used EM to verify the calculated film thickness of multicomponent protein multilayers PEI and PSS. Caruso and Mohwald [22••] utilized EM to demonstrate uniform coating of polyelectrolyte-surface-modified polystyrene latex particles with FITC labeled bovine serum albumin (BSA) or immunoglobulin G multilayers (see also Sections 7.1 and 7.2).

2.1.3. Optical microscopy (OM)

In a conventional optical microscope, light from an illuminated sample is collected by lenses to form an image, providing morphological information with resolution of about 0.2 μ. With specific attachments and functionalities, optical microscopes can also provide information on dynamic behavior e.g. via particle tracking, using a fluorescent microscope with single molecule sensitivity. Ducel et al. [23] studied the morphology and size of plant protein coacervates upon change in gum acacia concentration and pH by optical microscopy. Various imaging modes can be used for image enhancement with different types of optical microscopes. Phase contrast imaging improves contrast in unstained biological samples by changing the phase of the scattered light and converting refractive index differences to light and dark image regions. Mekhloufi et al. [24] used it to monitor structural changes of BLG and acacia gum dispersions during acidification. Confocal microscopy offers several advantages over conventional optical microscopy, including the ability to control field depth, and elimination or reduction of background noise. A significant advance has been the introduction of laser excitation: Laser scanning confocal microscopy (LSCM) compared to optical microscopy produces better images for rough surfaces since it has a better lateral resolution. The major use of LSCM is to provide, in conjunction with fluorescent microscopy, 3D images of cells. Schmitt et al. [25•] characterized the formation of BLG/gum acacia coacervates both in the presence and absence of protein aggregates by using LSCM, which revealed fundamental differences in their structures. LSCM was used to demonstrate the reversible binding of fluorescent protein (mEosFP) to individual spherical PSS brushes [26].

Total internal reflection fluorescence microscopy (TIRF) restricts the excitation and detection of fluorophores to a thin region adjacent to the interface between two media having different refractive indices induced by an evanescent wave. It can be utilized to observe a single fluorescent molecule (i.e. protein) at surfaces and interfaces and has been employed to investigate the adsorption of proteins on polyelectrolyte modified surfaces [27].

2.2. Scattering techniques

2.2.1. Dynamic light scattering (DLS)

Dynamic light scattering (also known as Quasi Elastic Light Scattering or Photon Correlation Spectroscopy) is particularly utilized to determine the hydrodynamic radius from diffusivities. DLS was used to measure adsorption layer thickness for the binding of BSA onto spherical PAA or PSS brushes [28], and was used to characterize adsorbed layers of poly(diallyldimethylammonium chloride) (PDADMAC) on controlled pore glass, later used to bind BSA [29]. Seyrek et al. [30] used DLS to verify critical pH values obtained from turbidimetric titrations (see Section 2.4.b) for BSA–heparin complexation. DLS was also used to determine diffusion coefficients within BSA–PDADMAC coacervates [31••].

2.2.2. Static light scattering (SLS)

Static light scattering provides information on the molecular weight and radius of gyration, but is more difficult than dynamic light scattering, especially in the case of polyelectrolyte–protein complexes, because of the challenge of constructing Zimm plots when the concentration of complex is essentially unknown, requiring iterative techniques [32]. Tsuboi et al. [33] constructed Zimm plots from SLS data for complexes formed low-salt solution between potassium poly(vinyl alcohol) sulfate and several proteins such as papain, human serum albumin, lysozyme, ribonuclease, trypsin, and pepsin. The results used were utilized to estimate molecular weight and degree of aggregation.

2.2.3. Small angle X-ray scattering (SAXS)

X-rays are scattered by regions of varied electron density; the intensity is related to the number of such regions and their contrast. Thus, SAXS provides information on the electron density distribution of the sample and is used to analyze structure on a 1–200 nm scale. It is widely used in structural studies of non-crystalline materials at relatively low resolutions, has found many

applications in biological sciences. Recent SAXS studies of the adsorption of BSA and bovine pancreatic ribonuclease A on spherical PAA and PSS brushes represent one of the few applications in this field [28] (see also Section 7.3).

2.2.4. Electrophoretic light scattering (ELS)

Electrophoretic mobility can be obtained from the perturbations of Brownian diffusivity under a pulsating electrical field. ELS had been used for biocolloids, emulsions and charged polymers. Caruso and Mohwald [22••] measured the mobilities of bare and coated (PDADMAC/PSS/PDADMAC/FITC–BSA and $(PAH/PSS)_2$/IgG) PS latex particles. Richert and coworkers [34•] measured ζ-potentials of alternating multilayers of PLL and PGA before and after the multilayer was contacted with serum. Xia et al. [35] studied the electrophoretic mobility of ferrihemoglobin–PDADMAC complexes as a function of protein concentration, while Burgess and coworkers [36] measured ζ-potential of albumin–acacia coacervates.

2.2.5. Diffusing wave spectroscopy (DWS)

Diffusing wave spectroscopy extends classical dynamic light scattering to the multiple scattering regime by measuring the fluctuation of scattered light resulting from the variation of total path length with wavelength. DWS can probe particle motion on very short length scales, i.e. the motion of a 1 μm particle on a length scale <1 nm. Weinbreck et al. [37] used DWS to measured diffusion coefficients of all scattering particles in whey protein/gum Acacia coacervates as a function of pH. DWS was used to study the stability of BLG/acacia gum dispersions by measuring the backscattering intensity as a function of time [25•].

2.3. Spectroscopic techniques

2.3.1. UV-VIS

The use of UV-Vis spectrophotometery to measure protein and nucleic acid concentrations is well known. Bromberg [38] used it to measure residual concentrations of insulin after its interaction with poly(acrylic acid) and Jiang and Zhu [39] used it to determine myoglobin, cytochrome c, and pepsin entrapment within PMAA– or PAA–gelatin complex gels. UV-Vis spectroscopy was also utilized to monitor the assembly of multicomponent protein PSS/PEI multilayers [21••] (see also Section 7.1).

2.3.2. Infrared spectroscopy

Since the stability and activity of proteins is very important in several applications, determination of their secondary structure is crucial. IR has been used to this end for proteins in complexes [39], coacervates [40], and brushes [41]. Attenuated total reflectance Fourier transform IR (ATR-FTIR) can sample a surface by internal reflection of the light. In contrast to other techniques frequently used for multilayer films, ATR-FTIR can distinguish individual components on the surface. Müller et al. [42] used ATR-FTIR to study the adsorption of human serum albumin on several alternating polyelectrolyte multilayers; Schwinte et al. [43••] studied the secondary structure of fibrinogen embedded in PAH–PSS multilayers and found it to be protected (unperturbed) within the polyelectrolyte layers (see also Section 7.1). Multiple internal reflection FTIR (MIR-FTIR) enhances the ATR-FTIR technique, and was used to study the effect of PSS chain length on formation of protein–polyelectrolyte multilayer assemblies [25•] (see also Section 7.1).

2.3.3. Fluorescence

Fluorescence can be used to monitor protein structural changes, protein adsorption, and the dynamics of adsorbed proteins on both colloidal and planar surfaces. Caruso and Mohwald [22••] used it to follow the adsorption of FITC–BSA onto PDADMAC/PSS/PDADMAC-coated PS latex (see also Section 7.2). Anikin et al. [26] measured fluorescence emission of individual PSS brushes as a function of mEosFP protein to determine the efficiency of protein binding. Teramoto and coworkers [44] used fluorescence spectral change – especially the emission maximum of tryptophan – to investigate the conformational changes of BSA in heparin or hyaluronan complexes.

2.3.4. Circular dichroism (CD)

CD (related to wavelength dependence of optical rotation) provides information on protein secondary structure and has been used to determine protein conformational transitions in ferrihemoglobin–PDADMAC complexes [35] and BLG/acacia coacervates [24]. Gong et al. [45] used CD to investigate structural transformations of cytochrome c and apo cytochrome c induced by negatively charged sulfonated polystyrene with different degree of sulfonation and chain length. CD can also be related to the enzymatic activities of proteins adsorbed on polyelectrolyte brushes. Since spherical brushes are strong scatterers, direct analysis of adsorbed protein is not possible; however the proteins released can be analysed [41] (see also Section 7.3). CD was used to establish that (1) complexation with PAMPS in low salt nullified the effect of low pH <2 on the helical content of BSA (7% decrease in the absence of PAMPS); and (2) neither complexation (pH 6.56) nor coacervation (pH 9.5) with PDADMAC has any effect on the helical content of BSA [46].

2.4. Titrimetric techniques

2.4.1. Potentiometry (pH titration)

pH (potentiometric) titration yields the pK of titratable amino acids (lys, arg, glu, asp); since these pK's depend on the local electrostatic environment of the titratable groups, they are sensitive to protein conformational changes, and, in principle, to binding with polyelectrolytes.

Wen and Dubin [47] found that binding of PDAMDAC to BSA does, as expected, released H^+, but that the effect is subtle, only seen at low I, indicating loose binding of the polycation.

2.4.2. Turbidimetry

In one type of turbidimetric titration a polyelectrolyte is added incrementally to a highly dilute protein solution and the intensity of the light scattered by, or the turbidity τ due to complex/precipitate formation is measured as a function of polyelectrolyte added. Alternately, protein can be added to polyelectrolyte. In so-called colloid titration, the τ is recorded as a function of titrant volume (usually in salt free systems) and the end point as indicated by maximum turbidity is used to ascertain the stoichiometry of complex formation. Jiang and Zhu [39] used colloid titration to investigate PAA/gelatin complexation and Tsuboi et al. [33] used it to study complexation of papain with potassium poly(vinylsulfonate) (KPVS). In a different form of turbidimetric titration, pH is varied (at constant concentration of protein, polyelectrolyte and salt) and a departure from zero slope in the pH-dependence of τ is noted. This "Type 1 turbidimetric titration" was popularized Dubin and co-workers as a way to determine critical conditions for soluble complexation and phase changes.

2.5. Calorimetric techniques

2.5.1. Differential scanning calorimetry (DSC)

Differential scanning calorimetry measures heat changes that occur during controlled changes in temperature, and has been used to study thermodynamic parameters associated with these changes and to examine thermal denaturation of proteins in complexes. van de Weert et al. [40] applied DSC to complexation of heparin and lysozyme and observed a reduction in protein thermal stability. Ivinova et al. [48] used DSC to study the thermal denaturation of lysozyme and chymotrypsinogen without changing their enzymatic activity and reported that polyanions significantly reduce the initiation of this thermal denaturation.

2.5.2. Isothermal titration calorimetry (ITC)

Isothermal titration calorimetry probes the interaction of two species by titrating one binding partner with another while measuring heat released in a calorimeter cell. Girard et al. [49•] used ITC to study the interaction between BLG and low- or high-methoxyl pectin. A substantial amount of sample required for ITC; however, the abundance and low cost of food polyelectrolytes makes it still applicable to those systems.

2.6. Separation techniques

2.6.1. Size exclusion chromatography (SEC)

SEC is a liquid chromatography technique in which molecules are separated by their molecular size in solution. Andrianov et al. [50] investigated complexation of polyphosphazene polyelectrolytes and BSA by SEC to establish immunostimulating activity and to determine composition of the protein–polyelectrolyte complex. Wang et al. [51] utilized SEC to determine protein concentration in PDADMAC–BSA or PDADMAC–RNAse coacervates.

2.6.2. Affinity chromatography

Affinity chromatography is a chromatographic technique based on biological function: it can isolate proteins via their biospecific interaction with a ligand immobilized on a column and can be used to measure ligand–protein binding constants. In the field of protein–polyelectrolyte complexes, the bound ligand can be in fact the polyelectrolyte, e.g. heparin or gelatin [52].

2.6.3. Frontal analysis continuous capillary electrophoresis (FACCE)

In FACCE, a protein–polyelectrolyte mixture is eluted continuously. Free protein emerges as a frontal band which can be quantified, yielding its concentration without perturbing the binding equilibrium. The resultant binding isotherm, fitted to various forms, yields the binding constant, cooperativity, and number of protein binding sites on the polymer. Gao et al. [53••] used FACCE to study the binding of BLG to sodium PSS. Girard et al. [54] utilized FACCE to separate noncomplexed BLG from pectin and from β-lactoglobulin–pectin complexes; the results were converted to a binding isotherm from which the authors could calculate the stoichiometry of the complex.

2.6.4. Affinity coelectrophoresis (ACE)

Affinity coelectrophoresis provides another way to measure binding of biopolyelectrolytes (glycosaminoglycans, GAGs) and proteins under equilibrium conditions. In contrast to affinity electrophoresis and affinity chromatography where one ligand is immobilized, in ACE both protein and biopolyelectrolyte are mobile. San Antonio and Lander [55] reported a detailed ACE method to study heparin–protein complexation. Herndon and coworkers [56•] utilized ACE to study binding of nine GAG-binding proteins to GAGs and proteoglycans isolated from rat brain (see also Sections 4.1.b and 4.4).

2.7. Reflectometric techniques

2.7.1. Scanning angle reflectometry (SAR)

In scanning angle reflectometry the intensity of light reflected from thin films (100–150 nm) is measured as a function of angle of incidence. This provides information on refractive index, film thickness, and adsorbed mass per surface area. SAR facilitates determination of protein surface concentrations and of the thickness of protein–polyelectrolyte multilayers. Ladam and coworkers [57••] used SAR to study film thickness as a function of pH after

several proteins were terminally adsorbed onto PEI/PSS/PAH multilayers (see also Section 7.2).

2.7.2. X-ray and neutron reflectometry

The experimental setups and underlying theories of neutron and X-ray reflectometry are similar. In contrast to SAR, these techniques can provide interfacial density profiles normal to the surface interface. Both techniques are used to study the surface structure of thin-films or buried interfaces. They also provide information about the adsorption, adhesion and interdiffusion processes that may occur at surfaces. These reflectivity techniques are particularly well suited for multilayer characterization. However the absorption of X-rays by water limits its application to studies of protein adsorption on interfaces. Both techniques were used by Czeslik et al. [58•] to study the surface thickness of BSA bound to planar PAA brushes. Neutron reflectivity was used to find the protein density profile across the interface (see also Section 7.3).

2.8. Fluorescence photobleaching recovery (FPR)

FPR is used to measure the local mobility of fluorescently labeled particles in a matrix by measuring the fluorescent recovery of partially bleached samples. Even though the technique is almost 40 years old, it has not been extensively applied to protein–polyelectrolyte systems. FPR has been used to measure diffusivity of proteins adsorbed or embedded in polyelectrolyte multilayers [59•] (see also Section 7.1) as well as in their coacervate state [37]. Szyk et al. [59•] used FPR to measure the diffusion of HSA on both PSS and poly(allylamine hydrochloride) (PAH) terminating multilayers and determined surface concentration by using FPR equipment as a fluorometer. FPR was also utilized by Weinbreck and coworkers [37] to conclude that gum acacia and whey protein move independently in their coacervate phase.

2.9. Optical waveguide lightmode spectroscopy (OWLS)

In OWLS linearly polarized light is coupled by a diffraction grating into the waveguide layer, and provides information on film thickness and the refractive index of the deposited films/layers. OWLS is particularly well suited to the in situ study of protein adsorption kinetics on multilayer surfaces. Gergeley et al. [16] and Szyk et al. [59•] independently used this technique to study adsorption of human serum albumin onto polyelectrolyte multilayers (see also Sections 7.1 and 7.2).

2.10. Quartz crystal microbalance (QCM)

The QCM consists of a thin plate of quartz whose resonance frequency changes with the mass of adsorbed layer on its surface. This makes it possible to measure the mass of very thin surface bound layers and provide information about their viscoelastic properties. In contrast to other optical mass instruments, the mass from QCM includes hydrated layers. QCM has been used to determine the amount of encapsulated enzyme [60]. Richert et al. [34•] reported that the amount of chondrosarcoma cells adsorbed on a PLL terminating film remained almost constant as the number of layers increased whereas adsorption was not significant for PGA terminating multilayer films (see also Section 7.2).

2.11. Surface plasmon resonance (SPR)

The basic principle of SPR derives from quantum optical-electrical phenomenon arising from the interaction of light with a metal surface. The resonance wavelength can be determined by measuring the light reflected by a metal surface and consequently to observe refractive index changes at surfaces. SPR has been becoming more prominent in the past 10 years in studies on the formation and properties of thin films and self-assembled monolayers. Hernaiz et al. [61•] used SPR to investigate the binding of antithrombin III onto a heparan sulfate-modified biochip and found the existence of a low affinity interaction between them which was increased when heparin was modified with 3-O-sulfotransferase. Pihlajamaa et al. [19••] used SPR in a similar study and found that hydrophobic sensor chips yield more consistent results in SPR analysis. Caruso et al. [62•] used SPR to determine the layer thickness of sequential PAH/PSS multilayers on which immunoglobulin was immobilized (see also Section 7.1).

2.12. Rheology

Rheology, the study of deformation and flow of materials, is mainly used to investigate dynamic behavior of gels, and to characterize mechanical properties of coacervates. Borrega et al. [63] studied viscoelastic properties of hydrophobically modified PAA complexed with BSA, paparin, or lysozyme to form gels and found similarities between these reversible gels and chemically crosslinked macromolecules. Bohidar et al. [31••] measured frequency-dependent viscoelastic moduli of BSA/PDADMAC coacervates which showed a weak network that was solid-like at low strain but reformed after breaking by shear. Weinbreck and Wientjes [64] studied the viscoelastic properties of whey protein/gum acacia coacervates and verified that pH plays a major role in the microstructure of coacervates.

3. Structure–property relations

A complete understanding of protein–polyelectrolyte complexes would be tantamount to an ability to predict the full range of physicochemical properties of the complex from the chemical structures of its constituent macro-

molecules along with the solution conditions. Despite the clearly far-fetched nature of this goal (at present we can barely achieve it for the polymer and protein alone), it at least illuminates our ignorance and progress. What would constitute a thorough description of the complex? The list would certainly include:

1. Global (10–1000 nm scale) structural parameters, such as the number of proteins bound per polymer chain, the dimensions of such an interpolymer complex, its aggregation state (number of polymers in an aggregate), and the possible multimer state of the protein
2. Local (0.5–10 nm scale) structural features, such as the local arrangement of polymer segments in their "bound state", the domain of the protein active in the binding process (the consensus moving away from the notion that all of the protein is equally involved in binding)
3. Perturbation of the protein native state
4. The degree of retention of small ions and hydration
5. Energetics: the energy released per bound protein, and its resolution into enthalpic and entropic components, and the cooperativity of binding
6. Electrostatics (pK shifts).

From such a detailed picture, what might we predict, given key variables such as: polymer MW, charge density, chain stiffness (see Section 3.2), protein structure (i.e. pdb file), protein and polymer concentrations, and solution conditions (pH, ionic strength, and temperature)?

1. The phase state of the system
2. The concentration of free and bound polyelectrolyte and protein
3. The MW of the intrapolymer complex (number of proteins bound)
4. The degree of aggregation (number of primary complexes per aggregate)
5. The electrophoretic mobility
6. The nature of the protein-binding site on the polymer and the polymer-binding site on the protein.

The purpose of these lists is to demonstrate how far we are from a full understanding of these systems, by which we mean an ability to make verifiable predictions from information about the two macroions and solution conditions. Perhaps simulations offer the best possibility of such predictions, the key element being the feasibility of subjecting those predictions to experimental verification. But for the moment an understanding of the important parameters is central.

3.1. Hydrophobic effects

Polymers may be specifically designed to interact hydrophobically with proteins, and systems containing such polyamphiphiles are thoroughly described in the comprehensive review article by Tribet [6••]. Here we consider the more vexing problem of knowing when hydrophobic interactions do *not* make a significant contribution. On the one hand, since all proteins have hydrophobic regions with varying degrees of exposure, and since some would even argue that all vinyl polymers have "hydrophobic backbones", it is not easy to summarily dismiss such possibilities. On the other had, solvation (hydration) need not be sacrificed to establish strong electrostatic stabilization because of the long-range nature of those forces. For example, electrostatic considerations alone seems to supply a good explanation for protein–protein interactions as shown by Schreiber and co-workers [65•].

Hydrophobic interactions may resist the applications of Occam's razor and require stronger evidence either pro or con. Hydrophobic interactions have some characteristic signatures: they become stronger at high salt (but the salt concentration required for such effects is well above the 0.5 M upper limit usually explored); and they are entropy-driven (but the release of counterions upon complexing might show similar behavior). The clearest evidence for hydrophobic interactions comes from either (1) systematic variations of polymer structure, or (2) careful thermodynamic data for ΔS and ΔH.

Gao and Dubin [66] studied the binding of BSA to a series of copolymers of maleic acid and alkyl vinyl ethers, with side chains methyl, ethyl, butyl, hexyl, octyl and decyl, by frontal analysis continuous capillary electrophoresis (FACCE). The results were (1) methyl and ethyl bound identically, and (2) binding increased from C2 to C6, but then decreased from C8 to C10. The conclusions were that C1 and C2 side chains could not access BSA hydrophobic domains; and that intrapolymer micellization of hydrophobes competes with protein–polyelectrolyte interactions, gaining the upper hand for C8 and C10. Unfortunately, Gao could not quantitatively assess the energies of the competing processes. Seyrek et al. [30] were more successful with hydrophobically modified poly(acrylic acid) (PAA) and BSA, observing the effect of hydrophobes on K_{obs} by FACCE. The dependence of K_{obs} on I displayed a maximum at $I \approx 20$ mM, and this simply shifted up with addition of hydrophobic side-chains. Binding could then occur at high pH, but the maximum was retained, indicating that electrostatic and hydrophobic contributions can be simply additive. Gong et al. [45] found that PSS binding enhances the helical content of (normally random coil) apo cytochrome c, with a maximum effect at $I = 20$ mM (perhaps coincidental because apo cyt c is fully protonated at the pH 2.0 used). Using polystyrene of varying degrees of sulfonation they concluded that both electrostatic and hydrophobic polyelectrolyte–protein interactions are involved; however, the protein in fact interacted with polymer nanoparticles that formed when it was added to the protein from an organic/water solvent.

3.2. Chain stiffness

3.2.1. Simulations and theory

Beginning in 1996, Linse and coworkers [67,68] reported an extensive and comprehensive set of simulations of polyelectrolytes interacting with oppositely charge spheres many of which incorporated effects of chain stiffness. In 2001, Jonsson and Linse [69] extended an earlier model [70•] in which a 3 nm diameter sphere ("micelle") with +20 charges was allowed to interact with a 16 nm contour length polyelectrolyte chain comprised of forty 0.4 nm beads of charge −1. The chain stiffness was represented by the mean value of the angle $<\alpha>$ between consecutive beads, and its range of values from 90° to 175° was suggested to correspond to bare persistence lengths l_p° ranging from 0.5 to 10 nm. For the range of l_p° most relevant here (2–5 nm), typical snapshots from MC simulations show about half of the beads in contact with the sphere, the others disposed in small loops with amplitudes of 1–2 nm from the sphere surface. For $l_p^\circ > 6$ nm, the fraction of bead-sphere contacts falls to 15–25%. Despite the limitations of the model (short polyelectrolyte chain, absence of salt) the simulations still provide a uniquely useful depiction of the local arrangement of polyelectrolyte segments in the "bound" state. The 2001 paper employed the same model except for a two-fold reduction in sphere charge and a more realistic range of l_p° from 1–15 nm, but focused on the microstoichiometry of complexes and consequent charge state (from "undercharged" to "overcharged"). For stiff chains, only 4–5 segments could be in contact with the sphere, regardless of microstoichiometry, while a given sphere could contact from 7 to 12 polymer segments for flexible chains, with surprisingly little difference between $l_p^\circ = 1$ or 4 nm. Flexible chains allowed intrapolymer collapse when many spheres are bound, but stiff chains required large inter-sphere separations.

Stoll and Chodanowski [71] applied MC simulations to a model in which a 7 nm diameter sphere with surface charge density $\sigma = 0.1$ cm^{-2} was allowed to interact via a screened Verwey–Overbeek potential with polyelectrolytes of one hundred 0.7 nm segments, at ionic strengths ranging from $I = 0$ to 1 M. These simulations revealed a desorption/adsorption transition at I_{crit} ranging from ca. 200–350 mM, rather consistent with experimental results for micelle–polyelectrolyte systems. Increasing l_p° from 1.5 to 5 nm diminished binding, I_{crit} dropping by 20%. Further increase to 35 nm lowered I_{crit} an additional 35%. Flexible chains permit binding at higher I, with loops and tails present. These effects are shown in Fig. 1, where chain stiffness is represented by k_{ang}, the bead-chain angular spring constant.

Manning [72] put forward a simple theoretical model for the binding of a spherical micelle of radius r and surface charge density σ, to a polyelectrolyte of opposite charge, modeled as a flexible rod with stiffness $\sim l_p^\circ$. Free energy calculations for the case where $r \approx l_p^\circ$ reproduced several features of experiment [73]: (1) a bound state appeared at a critical value of σ, and (2) σ_{crit} increased with salt, and was larger (weaker binding) upon a slight increase in l_p°.

3.2.2. Experiment

The aforementioned effects of chain stiffness, although intuitively reasonable, are difficult to confirm by experiment, since chain stiffness in reality cannot be decoupled from other effects of structure. This is especially problematic for proteins: Hattori et al. [74] measured binding constants K_{obs} of BLG to PSS relative to poly(acrylamidomethylpropanesulfonate) (PAMPS), and noted that the stronger binding of PSS could arise from either its hydrophobicity or its greater flexibility; they therefore compared poly(vinylsulfonate) (PVS) ($l_p^\circ = 3$ nm) to PAMPS ($l_p^\circ = 5$ nm). The larger K_{obs} of the former relative to the latter was interpreted as a chain stiffness effect. Kayitmazer et al. [73] chose hyaluronic acid and acrylamide/AMPS copolymer (5 : 1) as a pair with equal charge spacing (1.1 nm), but bare persistence lengths of 2 and 4 nm, respectively, and found I_{crit} ranging from 10–220 mM for the stiff chain, and 10–400 mM for the flexible one. The ionic strength dependence of the persistence length effect lead to the definition of an empirical effective l_p^{eff}

$$l_p^{eff} \equiv l_p{}^\circ + \frac{1}{4} l_p^{el} \quad (1)$$

rationalized by suggesting that l_p should reflect polymer stiffness under the influence of the field of the oppositely charged colloid. In order to see persistence length effects more clearly, the same authors compared the binding to anionic micelles, proteins, and dendrimers of PDADMAC and chitosan (again equal charge density), with l_p° of 2 nm and ca. 10 nm, respectively [75]. Surprisingly, I_{crit} was identical, the expected differences appearing only for binding of the small (1.7 nm radius) dendrimer. Visualization of the two polymers by SPARTAN showed a "crumpled" structure for PDADMAC; this means that this chain may not propagate very far per unit of apparent contour length, but that it also cannot be easily configured to locate its charges near the colloid surface – in other words, measured persistence length and chain local stiffness need not be equivalent, although, – in the model world in which polyelectrolytes are beads linked by varying bond angles — these two may be the same.

Simulations often show polyelectrolytes "wrapping around" spheres, a result that would consistently underestimate the maximum number of proteins or micelles bound per unit contour length of polymer (for heparin/BSA, for example, the average contour length per bound protein is smaller than the protein mean diameter) [76]. It seems intuitively reasonable that steric constraints on real polyelectrolytes are likely to preclude contact so intimate as to cause desolvation of themselves and proteins, especially at moderate ionic strengths. Indirect evidence from studies of polyelectrolyte–micelle binding by the current author suggests that the mean locus of bound polyelectrolyte

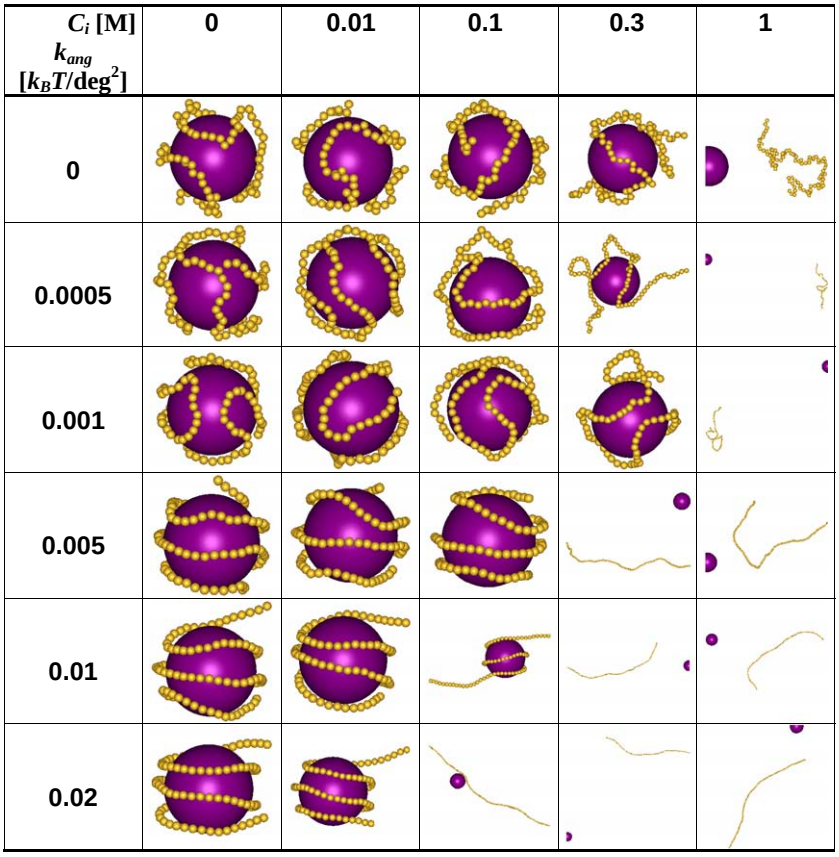

Fig. 1. MC Equilibrated conformations of semiflexible polyelectrolyte–particle complexes as a function of salt concentration and chain flexibility (from Ref. [70•]).

segments is about 5–7 Å from the colloid surface; this value is very close to an empirical result for protein–protein electrostatic interactions which appear to be controlled by potentials about 5 Å from the protein surface [65•].

The foregoing simulations all apply to the general case of a uniformly charged spherical colloid; application to proteins would be a daunting task. Simulations more nearly related to polyelectrolyte–protein systems will be discussed in Section 4.2. Nevertheless, simulations with simple spheres must be considered as a foundation for more complex particles.

3.3. Protein charge anisotropy

The role of protein charge anisotropy is immediately evident in (1) binding "on the wrong side of the pI" (particularly observed at I <100 mM), and (2) the absence of any noticeable change in phenomena at net charge of Z_{pr}=0. While there are few comparative studies of proteins with different levels of charge asymmetry, Seyrek et al. [30] showed that treating proteins as simple dipoles, using the respective centers of mass of negative and positive charges, accounted well for the positions of the maxima in K_{obs} vs. I (mentioned above), for BSA, BLG and insulin binding to a strong polyanion. These and other works [77] demonstrate the usefulness of Delphi [78] to visualize protein charge as an approaching polyelectrolyte does (see Fig. 2). Recent inspections of Delphi also indicate that, over a significant range of pH, protonation or deprotonation of BSA is occurring mainly in most positive domain; this explains the straightforward ionic strength dependence of Z_{pr} at the onset of binding [6••,46].

Kokufuta and coworkers [79] compared complexes formed between potassium poly(vinylalcoholsulfate) and lysozyme vs. ribonuclease. He attributed differences in the degree of aggregation (number of primary complexes per aggregate) to the difference in charge distribution between lysozyme and RNAse (the latter more heterogeneous). The results showed the importance of the complementarity (or lack thereof) of the respective charge spacings of polyanion and protein (here exclusively cationic at pH 2). The importance of intimate ion pairing was presumably amplified by the absence of salt [79].

Site-directed mutations or chemical modifications of ionic amino acids — an approach underexploited for non-cognate protein–polyelectrolyte systems — is often used to deduce a variety of biochemical mechanisms, and has been frequently applied to elucidate the contributions of charge interactions. For example, neutralization of lysines in the protein tropoelastin abolishes the ability of the intensely polyanionic glycosaminoglycan chondroitin sulfate (see Section 3.2) to promote the protein's conversion to elastin [80]. The results of such studies are interpreted from a point of view that is

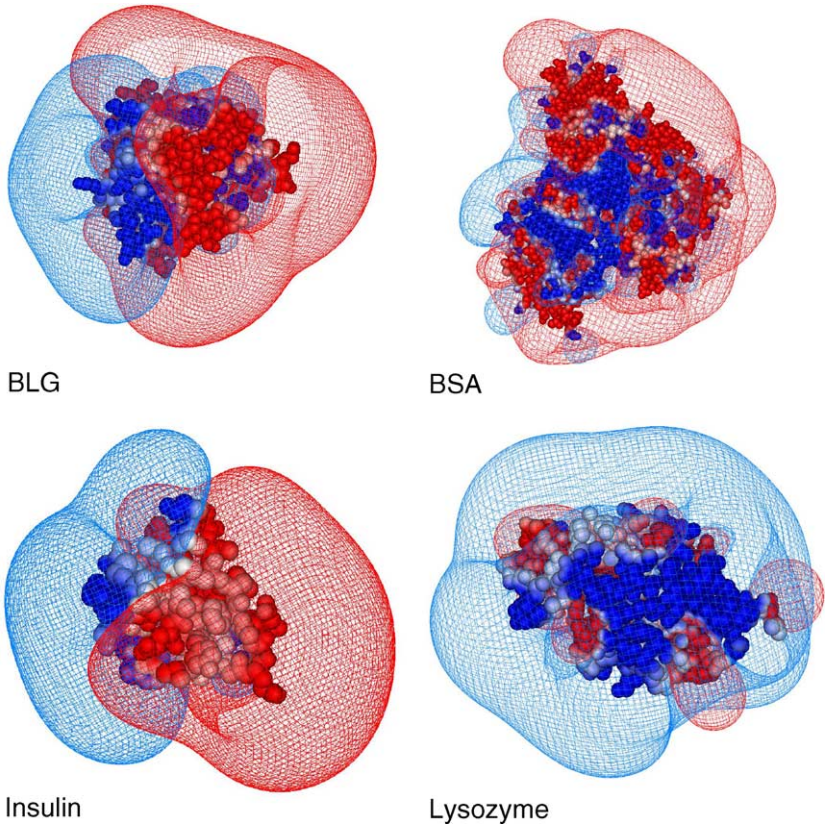

Fig. 2. Delphi representations of electrostatic potentials for different proteins at pH 7 [30]. −0.1 kT/e (red) and 0.1 kT/e (blue) potential surfaces around different proteins at pH=7, I=0.15 (from Ref. [30]). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

different from the potential domains visualized in Delphi, or the stoichiometric ion-pairing implicit in Kokufuta's [67] studies of salt-free complexation (which itself is subtly different salt bond/equilibrium approach of Kabanov [81]). When mutation of a given site strongly affects binding, a common inference is that site-specificity has been identified. We will return to this point in Section 4.

3.4. Polyelectrolyte charge sequence

Flexible chain polyelectrolytes of natural origin are typically carboxylated and/or sulfated polysaccharides. Since their biosyntheses, unlike nucleic acids or proteins, does not involved template transcription, carboxylation and sulfation arise from post-polymerization enzymatically controlled modifications. Therefore, bacterial "exopolysaccharides", seaweed polysaccharides, fruit pectins, and animal glycosaminoglycans inter alia commonly display charge patterns which may be said to be "random, but subject to genetic control". To the extent that their biological functions involve interactions with proteins, these charge patterns may be biologically significant. Often these interactions may involve proteins with pI <7, so that local attraction to a positive domain may be conjoint with global polyanion–protein repulsions [30]. The importance of synthetic polyanion sequence distribution in their binding to cationic micelles was presented by Feng et al. [82], but extension to protein binding has not appeared. A related observation is that partially neutralized PAA binds more strongly to BSA or to cationic micelles than does AMPS/Acrylamide copolymer of equal structural charge density. This is evidence of charge mobility in the former ("annealed" polymer) relative to the latter ("quenched") one, which makes it possible for it to develop a local charge sequence complementary to that of the colloid to which it binds [83]. The enhanced binding of a weak polyelectrolyte was also observed from simulations by Stoll and coworkers [84] In the domain of cognate polyelectrolyte–protein interactions, one can note that the biofunctionality of "blockiness" in pectins is a subject of current investigation [85] but the significance of polyelectrolyte charge distribution for other biochemically significant polyelectrolyte–protein interactions has been essentially ignored. The same theme will be touched on in Section 4.

4. Biopolyelectrolyte cognate systems

4.1. Definitions

The language of biochemistry is more metaphorical than that of chemistry and employs terms unique to the field.

Often these terms have nuances beyond their dictionary definitions that may elude the non-biochemist reader.

4.1.1. Cognate

The word "cognate", or "having a common origin", is used to describe any two molecules that interact in vivo at physiological conditions, thus co-evolved. While polyelectrolytes of animal or plant origin are able to interact with many different proteins, they interact with their cognate proteins to carry out specialized physiological functions. By contrast, non-cognate interactions may occur between any two molecules that would not normally interact with each other in a physiological milieu. Non-cognate pairs are often useful as model systems to elucidate the more complex interactions that occur among cognates.

4.1.2. Affinity

Binding affinity is a measure of the attraction between two molecules that form a well-defined complex. Typically, "binding" implies a guest molecule (ligand) and a larger host molecule (e.g. protein), but for polyelectrolyte–protein complexes the host–guest relationship may be unclear. This affects the measurement of binding isotherms and the definition of binding constants. As an example, in affinity chromatography, the most selective chromatographic method, which involves the specific interaction between a solution phase molecule and a second molecule immobilized on a solid phase, either the ligand or the host could be immobilized. The affinity of biopolyelectrolytes often refers to their ability to bind a given protein, with affinity constant k. However, the labeling of one or the other as the "ligand", or the choice of which one to immobilize, becomes somewhat arbitrary. Relative affinities of several polyelectrolytes for a protein could be qualitatively assessed from the salt concentration required to dissociate a given polyelectrolyte from a protein immobilized on a solid-phase resin. More quantitatively and without immobilization, k could be measured at any pH and I by capillary electrophoresis of protein–polyelectrolyte mixtures, or a variety of other techniques.

4.1.3. Specificity

The interaction of monoclonal antibodies (proteins) with antigens (any molecule capable of stimulating antibody production in the body) provides an example of specificity. Interaction occurs between the antigen and the epitope, or specific antigen-binding region, of an antibody. Thus, "specificity" implies that there are complementary structural features that are required for strong binding. A corollary is that several proteins with a common conserved domain bind with high affinity to the cognate molecule.

4.1.4. Selectivity

Selectivity, "to choose from among several", is usually believed to be a consequence of specificity. That is, if an interaction between two molecules is specific the interaction is also selective; however, the converse is not necessarily true. An example is affinity chromatography, in which an immobilized protein can preferentially bind one ligand in a mixture of several (selectivity), but the binding could involve different protein regions (non-specific). A protein–ligand interaction is not selective if the protein binds several ligands with similar affinity. In the present writing, selectivity could mean that a protein preferentially binds one biopolyelectrolyte or subpopulation thereof among several of similar structure; or that an immobilized biopolyelectrolyte preferentially binds one protein among several.

4.1.5. Biological activity

Biological activity, as discussed in this section, but not necessarily in others, is the action of a protein to carry out a function at physiological conditions. Because the biopolyelectrolytes of animals are often competitive inhibitors of the biological activity of many proteins, the concentration needed for fifty percent inhibition of biological activity is also an indicator of their biological activity.

In defining these terms, we have also noted that they carry, sometimes implicitly, assumptions about the relationship between structure and biological activity. A recent study of the binding of erythropoietin (Epo), a cytokine protein, to its dimeric receptor (EpoR) [86••] may challenge some of these assumptions. Mutations among the ten basic amino acids (cationic) located within the two Epo binding sites to alanine (uncharged) unsurprisingly reduced the rate of association k_{on} and the biological activity of EpoR. However, it was concluded that the selectivity of EpoR for Epo was based on global electrostatics rather than local structural modification, because (1) mutation to glutamic acid had a greater negative effect than mutation to alanine, and (2) the effects were similar regardless of whether mutations were made at site 1 or site 2 of EpoR. This demonstrates *selectivity* without *specificity*. The implication is that binding depends on the conjoint electrostatic forces arising from the combined influence of several charged amino acids and not from atomistically defined short-range bonds between particular pairs of protein and receptor amino acids. Since the interactions between proteins and biopolyelectrolytes clearly has a strong component of such concerted electrostatic forces, ascribing the binding to certain specific pair-wise interactions may be more questionable than for e.g. the binding of proteins to small molecules or to other macromolecules of fixed geometry.

4.2. Systems discussed

Our emphasis on glycosaminoglycan–(GAG–) protein systems within the greater conceptual framework of cognate biopolyelectrolyte–protein systems stems from three related motivations. First, this review focuses on protein interactions with linear, flexible biopolyelectrolytes. GAGs represent a special class of biopolyelectrolytes: they are linear, variably sulfated polysaccharides with no known secondary or tertiary

structure (see Fig. 3). Secondly, like many of the other polyelectrolytes discussed in this review, GAGs are structurally heterogeneous, owing to the series of ten sequential post-polymerization enzyme-catalyzed modifications in their biosynthesis. The selectivity and specificity of the heterogeneous structural subpopulations and the structures responsible for high affinity binding pose a unique challenge in biochemistry. Last is the current intense interest in GAG–protein interactions, related to the numerous physiological roles for GAG–protein interaction reported recently and briefly discussed here. Because of their structural variety GAGs are capable of interacting with proteins to affect a plethora of physiological functions, including among many others cell differentiation, angiogenesis, association with tropoelastin to promote elastogenesis, and the "recruitment" of chemokines in response to inflammation.

Here we present recent reports on (1) selectivity of different GAGs for a given protein, (2) structural modification of heparin to study selectivity (3) and a comparison of binding cognate and non-cognate GAGS to neural rat brain proteins. We include a study on protein–pectin interactions which may be involved in the inhibition of pectin degradation in plant cell walls caused by pathogen-secreted enzymes in response to physiological and environmental stressors. Exhibiting immeasurably less complex heterogeneity than GAGs, pectin offers a glimpse of how a plant exopolysaccharide can play an extracellular mechanical role, while also responding to the signals of changing systemic environment carried by proteins.

4.3. Goals/objectives

The goals of cognate studies, and the approaches used to address them, are distinct from those established for synthetic polyelectrolyte–protein systems. In contrast to the latter, a common goal of nearly all cognate biopolyelectrolyte–protein interaction studies is identification of the polyelectrolyte and protein structural elements required for binding. The corresponding strategies include measuring relative affinities of biopolyelectrolyte–protein interactions and determining the specificity and selectivity (assumed, as noted above, to go hand-in-hand) of these interactions. Site-directed mutagenesis of protein amino acids is utilized to identify biopolyelectrolyte binding sites on the protein. Frequently comparison is made between binding of cognates and non-cognates. A third goal is to elucidate the biological activities facilitated by biopolyelectrolyte–protein interactions.

4.4. Techniques/approaches

The following experimental techniques have been used to determine binding (or dissociation) constants: affinity chromatography, affinity co-electrophoresis (ACE), surface plasmon resonance (SPR), isothermal titration calorimetry (ITC), and ultrafiltration. In addition to measuring binding constants, ITC provides information about the enthalpy of binding and the number of protein molecules bound to each molecule of biopolyelectrolyte [87••]. Electron microscopy also provides information regarding the number, and also the location, of polyelectrolyte binding sites on a given protein [19••]. See Section 2 for more information about these techniques.

The paradigmatic GAG–protein interaction is heparin–antithrombin, hence, studies on this system have had a defining role for all GAG–protein investigations. But it is also understood that heparin is the name used for an extraordinarily polydisperse material, in which both intra- and inter-chain heterogeneity reflects the wide array of N- and O-sulfation, de-acetylation and carboxylation, and epimerization to which the various sugar rings are subject. The ubiquitous heparan sulfate (HS), differing from heparin largely in terms of its low sulfation (*sic*) is similarly polydisperse. While it is known that 3-O-sulfation is correlated with the high affinity binding of heparin to antithrombin (AT) and with its potent anticoagulant activity, the role of heparin sulfation in various physiological activities is not otherwise well understood, and a number of studies have been directed towards the effect of modifications of the GAG functional units. Interaction between HS and AT was investigated by SPR [61•]. Binding to solution AT was measured for HS lacking 3-O-sulfate groups, and for HS modified with 3-O-sulfotransferase, both immobilized on a biochip. The latter bound more strongly; but no control study was done to find out if the interaction was specific for the 3-O-sulfated HS or simply due to increasing the sulfate content. Kuschert et al. [87••] found that N- and O-desulfated

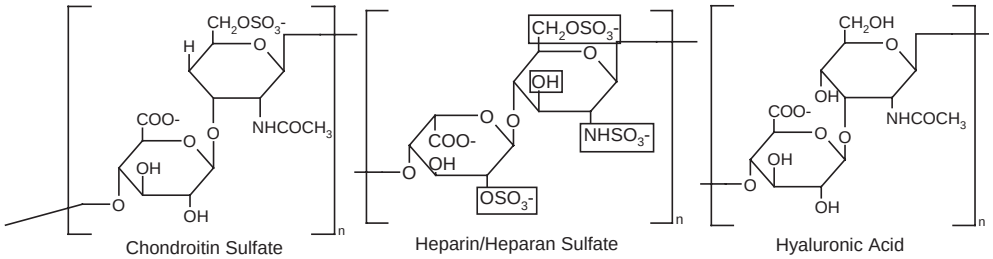

Fig. 3. Glycosaminoglycan (GAG) structures for chondroitin sulfate, heparin/heparan sulfate, and hyaluronic acid. The squares present in the heparin/heparan sulfate structure represent sites of possible sulfation during post-translational enzymatic modification.

heparins (especially the latter) were less effective than unmodified heparin in competing with chemokines (small immune proteins that interact with GAGs to promote cell response to inflammation). HS and other GAGs promote the cell internalization and transactivation function of HIV-1 Tat protein. While both HS and heparin bind to immobilized Tat [88•], Tat was bound more strongly by 6-O-desulfated heparins than 2-O-desulfated heparins, leading to the conclusion that Tat was "specific for certain structural units".

Protein sequences can be modified by site-directed mutagenesis or by other chemical modifications. The heparin-binding NC4 domain of collagen IX was mutated by site-directed mutagenesis from Lys-Arg-Arg to Asn-Gly-Leu [19••]. Affinity chromatography showed a reduction of affinity for the mutated amino acid sequence. In the extracellular matrix, lysine residues of tropoeleastin interact with chondroitin sulfate B (CSB) to form elastin, and heparin has a greater effect. When these residues were rendered neutral by acetylation the effects of CSB and heparin were negated, suggesting that the interaction is predominantly electrostatic [80].

Effects of salts have been used to determine whether interactions were specific or due to nonspecific electrostatic interaction between oppositely charged groups located on the protein and biopolyelectrolyte surfaces (a dichotomy that should be considered from the perspective offered in Section 4.1.e). Polygalacturonase-inhibiting proteins (PGIPs) protect pectin (a polygalacturonic acid) from pathogen-secreted endo-polygalacturonase (endo-PG). This interaction was evaluated in endo-PG activity assays performed in presence of PGIPs. In the absence of salt, endo-PG was inhibited for both alginates and polygalacturonic acid, but only the latter bound PGIPs in 0.1 M NaCl. Because both alginates and polygalacturonates have the same charge content, it was concluded that PGIPs were specific for polygalacturonic acid [89•].

Finally, the relative affinities of cognate versus non-cognate GAGs for proteins were evaluated by using affinity coelectrophoresis to measure dissociation constants. These results indicated that rat brain proteins, including fibronectin and thrombin, bound cognate rat brain GAG chondroitin sulfate (CS) more strongly than non-cognate CS from bovine trachea. These proteins bound to cognate (rat brain) heparan sulfate selectively with a range of affinities. One exception was that exogenous heparin bound more strongly than cognate GAGs, including heparan sulfate, to all rat brain proteins [56•].

5. Theory and simulations

5.1. Theory

The simplest relevant theory deals with the adsorption of polyelectrolytes on oppositely and uniformly charged planar surfaces. In one such recent treatment [90] polymer adsorption initially increases with addition of salt and then decreases with further increase of I. The second effect is obvious; more interesting is the explanation given for the increase of adsorption at low salt, which raises the possibility of intrapolymer repulsion in the absorbed state. Some experimental evidence for this may exist in the $I<20$ mM regime.

Adsorption of a polyelectrolyte to a spherical surface (1–10 nm) should be more relevant to polyelectrolyte–protein interactions than adsorption onto flat surfaces. It is now obvious that binding of a polyelectrolyte onto an oppositely charged spherical particle with uniform charge distribution should increase with the colloid surface charge density σ, the charge per polymer repeat unit (q) (or linear charge density ξ), and decrease with ionic strength. Experiment and theory both show that at any I and ξ, no interactions occur unless σ exceeds some critical value. In addition to these parameters, von Goeler and Muthukumar [91] also considered the effects of chain stiffness and molecular weight, and colloid surface radius as well as solution conditions such as temperature T and dielectric constant of the medium ε. At constant T, ε, I and ξ, adsorption is favored by larger colloid radius and diminished by chain stiffness (see also Section 3.2).

The effect of nonuniform surface charge distributions was considered for the adsorption of a polyanion onto a flat surface made up of heterogeneously distributed positive charges [92••]. The critical condition for adsorption is given by

$$\frac{12\pi l_B \sigma q}{\kappa^3 l_1}\left[1-(\kappa\Lambda+1)e^{-\kappa\Lambda}\right]\geq 1 \qquad (2)$$

where l_B is the Bjerrum length, κ is the inverse Debye length, Λ is the pattern size, and l_1 is a renormalized Kuhn length which depends on κ, q, and the molecular weight of the polymer. Monte Carlo simulations originating from this theory indicated a transient complex whose lifetime decreases as ionic strength increases. Also, numerous bound states not corresponding to perfect complementarity ("full registry") are attained. The presence of very long lifetimes could correspond to charge pattern distributions which preferentially bind the polyelectrolyte. This theory was subsequently advanced [93•] by considering a surface with a distribution of positive and negative charges (see Section 5.2).

Coming closer to the matter at hand, de Vries et al. [94] attempted to address the effect of protein charge anisotropy on protein–polyelectrolyte interactions. Considering proteins as randomly charged (annealed) spheres, they developed a rough analytical theory to estimate the critical pH of soluble complex formation with polyelectrolytes of low linear charge. The occurrence of binding on the wrong side of the isoelectric point, under conditions of low I and ξ was correlated with the existence of multiple small charge patches on the protein surface.

5.2. Simulations

Monte Carlo (MC) simulations have been used to investigate the effect of various parameters that have been pointed to by theory or experiments. A zeroth-order model considered the adsorption of a polyelectrolyte onto a heterogeneously charged planar surface composed of both positive and negative charges [93•]. The "spatial inhomogeneity" of the surface charges created regions of repulsive and attractive potential above the surface. When the net charge of the polyelectrolyte was the same as the surface, it would adopt conformations proximate to the regions of higher attractive potential, this reproducing the commonly observed binding "on the wrong side of pI".

Carlsson et al. [95••] carried out MC simulations between a flexible polyanion and lysozyme, modeling the latter as a hard sphere with atomic charges projected on or near the surface. For the case of net positive protein charge and in the absence of non-electrostatic interactions, a maximum in the number of adsorbed polymer segments appeared near $I = 10$ mM. This maximum was attributed to the stretching of the polyelectrolyte chain at low I due to intrapolymer electrostatic repulsion, leading to a reduction in polymer–protein contacts. (n.b. The requirement of protein charge opposite to that of polyelectrolyte does not appear in experimental systems which display similar maxima [30]). Only when short range interactions (i.e. hydrophobic) were included could these simulations agree with the observation that polyanions can bind to proteins with net negative charge (pH > pI).

The same model system [67] was used to examine effects of polyelectrolyte chain length, ionic strength and non-electrostatic protein–protein interactions on protein–polyelectrolyte cluster formation. Maximum electrostatic interactions were obtained at or near stoichiometric equivalence of net protein charge and polyelectrolyte charge. Further increase of polyelectrolyte concentration was found to trigger the redissolution of the complexes. It was also found that reduction in chain length and increase in I weakened cluster formation, while non-electrostatic protein–protein interactions upon addition of oppositely charged polyelectrolytes enhanced it.

de Vries [96] used MC simulations to study the interaction between a flexible low-charge polyanion (gum acacia) and α-lactalbumin or BLG (two whey proteins). They focused on "critical ionic strengths", below which soluble complexes formed "on the wrong side" of the pI. Although both refs 66 and 90 take the original charge group coordinates from the Protein Data Bank crystal structures, the positions of charged groups are preserved in (90) but moved below the surface of a hard sphere in (66). The net charge of the protein was maintained at zero by varying the dissociation constant of negative groups in (90), but allowed to vary in (66). The polyelectrolyte bending energy in (66) is replaced in ref (90) by a monomer–monomer interaction energy which also contains an electrostatic enhancement factor to take into account the protein's low dielectric constant. In agreement with experiments, α-lactalbumin was found to bind more strongly to the polyanion than BLG due to the single large positive domain on α-lactalbumin as opposed to multiple small patches on BLG.

6. Coacervates

In addition to a valuable early book chapter by Burgess [20••], which deals with theory, mechanism, and applications to microencapsulation, the subject of complex coacervation has also been covered in more recent reviews [9,97,98]. While various examples of coacervate systems were cited in these articles, generalizations were mostly drawn from systems involving either (a) non-globular proteins such as gelatin, (b) polypeptides such as polylysine, or (c) highly polydisperse systems with heterogeneous proteins, e.g. whey proteins or compositionally polydisperse polyelectrolytes, e.g. gum acacia. Thus, in this part of the review we emphasize simpler (model) systems. We also deal only with liquid–liquid phase separation, and exclude precipitating systems.

6.1. Definitions

Complex coacervation is the separation of a macromolecular solution, composed of at least two macromolecules (typically oppositely charged polyelectrolytes), into two immiscible liquid phases. This definition is also applicable to a mixture of interacting polyelectrolytes and oppositely charged colloid particles, e.g. proteins or micelles [99]. Protein–polyelectrolyte coacervation is a special case of polyelectrolyte–colloid coacervation in which the dense phase is rich in protein and polyelectrolyte while the dilute phase contains an equilibrium mixture of protein and the polyelectrolyte. Coacervation is in contrast to precipitation, which corresponds to solid–liquid phase separation. Visual observation after centrifugation is usually a satisfactory way to distinguish between precipitation and coacervation (see Fig. 4).

While the definition of "coacervation" is reasonably clear, that of "coacervate" is not, because it sometimes refers to a stabilized suspension of macroion-rich droplets in a dilute continuous phase, and at other times to the content of those droplets. In order to distinguish between these two definitions, one for a biphasic system, one for a monophasic system, we define the former as a "coacervate suspension". Thus, we have adopted the definition of Burgess [20••] who defined the coacervate as the droplet itself.

6.2. Process of coacervation

Kaibara et al. [100••] pointed out four consecutive steps in the formation of BSA–polycation coacervates upon increasing pH at constant ionic strength, and protein/polyelectrolyte ratio: (i) Formation of soluble "primary"

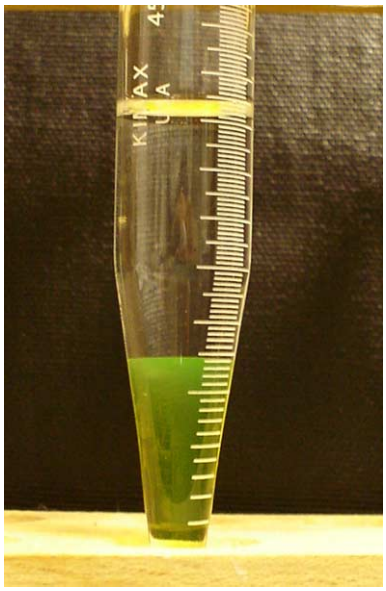

Fig. 4. Coacervate/dilute equilibrium phase separation of BSA (labeled with fluorescein isothiocynate (BSA-F))/PDADMAC coacervates prepared at pH 9.5 and I=0.1 M NaCl. The upper phase is the dilute equilibrium phase, and the coacervate is the lower which looks turbid due to fluorescence from BSA-F.

complexes between proteins and polyelectrolytes at a critical pH (which might be below the protein's isoelectric point). (ii) Completion of soluble complex formation. (iii) Initiation of the aggregation of primary complexes. (iv) Onset of formation of microcoacervate droplets at pH=pHϕ, (which must be above pI). Formation of coacervates was seen to be followed by additional processes in which coacervate droplets fuse and/or show morphological changes, presumably due to dehydration. In (i–ii), present are only soluble complexes and either excess free proteins or excess polymers, depending on the protein/polyelectrolyte ratio. Step (iii), on the other hand, includes the formation of quasi-neutralized primary complexes and involves the association of intrapolymer complexes into interpolymer aggregates. And finally in step (iv), coacervates form from charge neutralized the aggregates.

6.3. Theory

Experimental data on gelatin/acacia and gelatin–gelatin coacervation first motivated the development of several theoretical models for complex coacervation. These theories, described and compared by Burgess [20••] and de Kruif et al. [98], deal with complexation of oppositely charged flexible chain polymers, but nevertheless provide a starting point for the explanation of protein–polyelectrolyte coacervation. The Voorn–Overbeek theory describes the formation of a concentrated coacervate phase as a spontaneous process, driven by a gain in electrostatic free energy at the expense of a decrease in total entropy. This model assumes a distribution of random coil polyelectrolytes, negligible solvent–solute interactions, and the absence of site-specific interactions. On the other hand, in the dilute phase aggregate model developed by Veis and Aranyi, coacervation occurs in two steps: (1) aggregation of oppositely charged polyelectrolytes by electrostatic interaction to form ion-paired aggregates of low configurational entropy, followed by (2) re-arrangement of these aggregates to form coacervate, in equilibrium with ion-paired aggregates are present in the dilute phase. In addition to taking into account solvent–solute interactions, Veis and Aranyi considered the importance of the configurational entropy gain arising from the rearrangement of aggregates into a randomly distributed coacervation phase. While the boundary between soluble complex formation and coacervation may be too narrow to permit observation, examples of soluble complexes as precursors to coacervation are sufficient to strongly support the Veis model. Tainaka adapted the Veis model by considering the presence of possibility of neutral aggregates without specific ion pairing in both phases. According to Tainaka coacervation is driven by attractive forces between aggregates and is only possible within a certain range of polyelectrolyte linear charge density and molecular weight. The theory of Nakajima and Sato, which is a modification of Voorn–Overbeek theory (also agreeing that charges should be distributed uniformly in both phases), takes into account the presence of added salt in coacervates.

None of these theories are fully successful in explaining every aspect of complex coacervation. First, the Voorn–Overbeek and Veis–Aranyi theories are restricted to low charge density systems. Second, while all the theories account for high salt suppression of coacervation, they do not account for suppression of coacervation sometimes observed at low salt. In a qualitative way, Wang et al. [99] explained this effect with a model based on micelle–polyelectrolyte coacervation: excess salt suppresses all binding, and moves the system to a mixture of the two macroions; depletion of salt increases the binding so that the macroion in stoichiometric excess can produce complexes with net charges all of the same sign, which by mutual repulsion cannot coacervate.

6.4. Microstructure

Continuing to distinguish coacervates from coacervate suspensions, we separate work done on analysis of structure in the <1 μm length scale (the former), from studies at higher length scales which may refer to suspensions of coacervates.

6.4.1. Microstructure of coacervates

A recent study [31••] on coacervates of BSA and PDADMAC indicated the existence of heterogeneities on a length scale of ca. 0.1 μm. The origin of these heterogeneities was proposed to come from electrostatic protein–polyelectrolyte forces producing either (1) a dispersion of micro-

phases, large domains more dense in PDADMAC and BSA, or (2) a semi-dilute matrix of polymer–protein complexes. To our knowledge, this is the only experimental study that investigates the <1 μm microstructure of monophasic coacervates.

6.4.2. Microstructures of suspensions of coacervates

The BLG/acacia gum coacervates studied by Schmitt et al. [25•] formed either individual spherical vesicular particles or foam-like structures, with sizes from 1–30 μm. Although there was no subsequent physicochemical perturbation such as salt addition or dehydration, these structures showed time dependence. Thus, they attributed the phenomenon to partial coalescence of coacervates, possibly resulting in solvent entrapment inside the droplets.

Weinbreck and Wientjes [64] studied microstructures of coacervates suspensions (optically turbid phases) from whey protein and gum Acacia, a low charge density, highly complex and polydisperse anionic polysaccharide. From small-angle X-ray scattering (SAXS) experiments, the structures of coacervate were interpreted in terms of a network of compact gum acacia molecules whose degree of shrinkage depended on the strength of their electrostatic interaction with whey proteins [101]. Coacervates obtained at high salt and low pH were described as more heterogeneous and less structured. Rheology and SAXS results from the same group [64] led to a description of coacervate as a concentrated dispersion of gum acacia chains electrostatically crosslinked with whey proteins. The high viscosity at the pH and *I* of maximum coacervate yield was explained by the strong attractive electrostatic interactions between proteins and polyelectrolyte. Full recovery of viscosity at constant shear rates showed that the structural changes were reversible.

6.5. Limitations

Until recently, most of the studies of protein–polyelectrolyte coacervation have been application oriented, especially involving complex, heterogeneous and typically low charge density polysaccharides along with and polydisperse milk proteins. Studies that do not include food polymers and proteins are limited to human tropoelastin with heparin or chondroitin sulfate B [80], and coacervation of BSA with a synthetic polyelectrolyte, namely PDADMAC [31••,100••]. The majority of systems studied are thus those in which the protein, the polyelectrolyte, or both, are highly polydisperse, with respect to both MW and chemical composition. This impedes elucidation of the effects of variables such as polymer charge density and charge lability, polymer chain stiffness, protein charge anisotropy, and effects of ionic strength and pH that take place through these four variables. We thus suggest that some reduction of system polydispersity is necessary in order to bring experimental systems into better concordance with the worlds of theory and simulation.

7. Multilayers and brushes

Layer-by-layer polyelectrolyte multilayer formation, introduced by Decher [102••], involves consecutive adsorption of oppositely charged polyelectrolytes with rinsing between each layer. On the other hand, polyelectrolyte brushes are formed when only one terminus of the polyelectrolyte chain is bound to the surface. (Fig. 5a) Both of these methods have been adapted for protein immobilization. For brushes, noncovalent forces allow rather durable adsorption of proteins within the brush. For multilayers, the protein can be adsorbed on a previously formed multilayer (Fig. 5b) or more permanent immobilization can be attained if the protein is distributed within the polyelectrolyte multilayer, or allowed to form its own layer embedded among the polyelectrolyte multilayers (Fig. 5c).

In this review, proteins adsorbed on previously formed multilayers will be referred as “terminally adsorbed” and the multilayer assembly will be abbreviated as TAPPEM — terminally adsorbed protein/polyelectrolyte multilayers. Embedded protein–polyelectrolyte multilayers (EPPEM) can be divided into two groups. If the protein replaces one type of polyelectrolyte in the multilayer assembly, the multilayer assembly will be referred as EPSPEM-embedded protein/single polyelectrolyte multilayers. We refer to embedded proteins assembled along with multiple polyelectrolytes as EPMPEM-embedded protein/multiple polyelectrolyte multilayers.

7.1. Embedded protein–polyelectrolyte multilayers (EPPEM)

The role of the polyelectrolyte in all multilayers with embedded proteins is to maintain organizational stability by

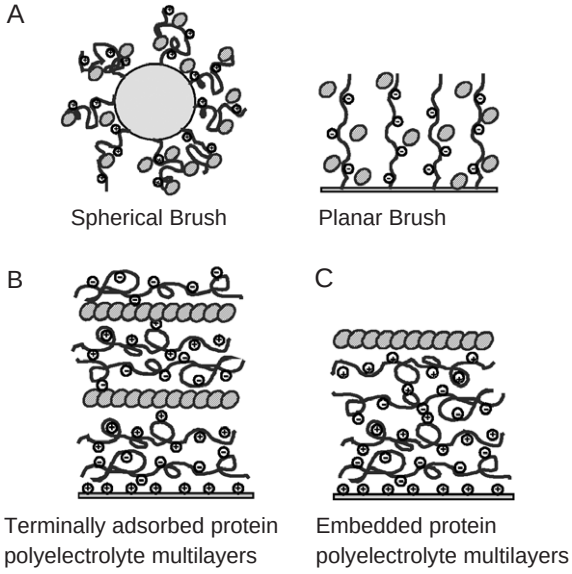

Fig. 5. Schematic of (A) protein polyelectrolyte brushes; (B) embedded protein polyelectrolyte multilayers (EPPEM); and (C) terminally adsorbed protein polyelectrolyte multilayers (TAPPEM).

bridging all components. This is because oppositely charged globular proteins will not allow assembly, although one protein and one polyelectrolyte can do so — see below. The bridging capacity of the polyelectrolyte is affected by its chain length. Houska et al. [25•] studied the effect of polyelectrolyte chain length on layer-by-layer assembly of albumin–PSS and albumin–heparin single polyelectrolyte multilayers, and found that for the primary protein layer the molecular weight of the polyelectrolyte did not have any effect on the multilayer assembly, yet the effect of chain length was clearly observed in the following layers. Lvov et al. [21••] used layer-by-layer adsorption of PSS and PEI to prepare EPMPEM systems containing more than one protein. They studied the formation and structure of 4 different assemblies in which several proteins are sandwiched between different multiple polyelectrolyte layers.

When a protein is incorporated in polyelectrolyte multilayers, the objective is to preserve biological recognition as demonstrated by retention of binding capacities or enzymatic activities. This requires preservation of protein native structure. In one EPSPEM system in which alternating multilayers of PSS and anti-Immunoglobulin G (anti-IgG) thin films were assembled up to nine layers with proteins, retention of biological activity was seen, whereas, when anti-IgG was embedded between multiple polyelectrolytes (PSS and PAH) only the outer protein layer was immunologically active [62•]. Schwinte et al. [43••] studied the secondary structure of fibrinogen (1) in a TAPPEM (PAH- or PSS-penultimate); and (2) in a EPMPEM system (PAH and PSS multilayers). In both cases it was found that fibrinogen retained its secondary structure. However thermotropic analysis of these multilayers showed that thermally induced structural transitions become strongly hindered for embedded fibrinogen, while the structural transition was clear for terminally adsorbed fibrinogen and fibrinogen in solution.

The retention of biological activity depends not only on the protein structure but also on protein mobility. There are conflicting expectations about the dependence of protein diffusion on the number and thickness of multilayers. Therefore, experimental studies of the diffusion of proteins adsorbed on or embedded in multilayer films are crucial. Szyk et al. [59•] studied the lateral mobility of human serum albumin in two systems where protein was embedded in multiple polyelectrolytes — (PEI(PSS/PAH)$_3$-HAS-(PAH/PSS)$_3$ or PEI(PSS/PAH)$_2$PSS-HAS-(PSS/PAH)$_3$) — by using fluorescence photobleaching recovery (see also Section 2.8) and found one protein population diffusing laterally along the multilayers whereas the other population is almost immobile.

7.2. Terminally adsorbed protein–polyelectrolyte multilayers (TAPPEM)

Instead of embedding proteins in multilayers, one can have them to adsorb on the surface of preformed multilayers. Once the modified surfaces come into contact with biological materials such as proteins, protein–polyelectrolyte interactions and protein coverage become the main concern.

Protein coverage can be varied because both positively and negatively charged proteins may adsorb on either negatively and positively charged multilayer surfaces. Ladam and coworkers [57••] studied adsorption of several proteins onto PEI(PSS/PAH)$_3$ and PEI(PSS/PAH)$_3$–PSS multilayers and found thicker protein layers if the terminating polyelectrolyte layer has a charge opposite to the protein, In that case the protein layer presents a net surface charge providing a surface for further protein adsorption. However, as subsequent protein layers formed, they became less structured and the process stopped. Gergeley et al. [16] studied HSA adsorption onto preformed PLL/PGA multilayers and showed that protein adsorption strongly depends on the terminating polyelectrolyte and pH conditions. At high pH, where both the protein and polyelectrolyte are negatively charged, PGA-terminating multilayers adsorbed HSA. Müller and coworkers [42] found that increasing I reduced HSA adsorption on PAA- or poly(vinylsulfonate) (PVS)-terminating multilayers with PEI, resulting in different protein coverages.

For controlled drug release, detachment of the protein is as important as the binding ability. Richert et al. [34•] studied the detachment forces of terminally adsorbed chondrosarcoma cells on alternating PLL/PGA multilayers by micromanipulation, which is a micropipette aspiration technique used to determine the forces needed to separate proteins from the surfaces to which they are attached. In the presence of serum, the detachment forces were significantly higher for PLL-terminated multilayers and decreased as the number of layers in the preformed multilayer increased; whereas PGA-terminated multilayers behaved as nonadherent films.

Investigations of multilayers on colloidal particles are few. Caruso and Mohwald [22••] used layer-by-layer technique for the fabrication of polyelectrolyte–protein multilayers on polystyrene latex particles. FITC-labeled BSA was terminally adsorbed onto PDADMAC/PSS/PDADMAC-coated PS latex, and IgG was deposited onto PAH/PSS/PAH/PSS coated particles. Provided the conditions were suitable for stable colloidal dispersion, it was found that the protein-multilayer assembly process is very similar to that on planar substrates.

7.3. Polyelectrolyte–protein brushes

A polyelectrolyte brush is formed when one end of a linear polyelectrolyte is affixed to a planar or curved surface and the average lateral distance between the attached polyelectrolyte chains is much smaller than their contour length. Brushes represent a new group of carrier particles for enzymes and proteins: spherical brushes are more popular than planar brushes due to the large surface area offered by colloidal particles.

Spherical polyelectrolyte brushes with PAA (weak polyelectrolyte) and PSS (strong polyelectrolyte) have been used to study the extent of protein binding [18], the protein stability [41], and the type of interactions involved [28]. Czeslik et al. [58•] studied BSA binding to PAA-modified planar brushes and found that at low ionic strength BSA was strongly bound to like-charged PAA and binding resistance was observed at higher ionic strengths. These results were similar to those obtained from spherical brushes indicating that surface curvature does not affect protein adsorption. Preservation of biological activity is indispensable for the retention of biological recognition by proteins in polyelectrolyte brushes. Wittemann and Ballauff [41] studied the adsorption of bovine pancreatic ribonuclease A, BSA and BLG onto spherical PAA and PSS brushes. They demonstrated that BSA interacted very strongly with PSS brushes (almost no free protein left in solution). BSA retained its secondary structure during adsorption onto PAA brushes, but showed a decrease in helical content with PSS brushes. However, for BLG on PSS brushes, there was almost no change in secondary structure, although adsorption diminished. The adsorption of RNase A with PSS brushes was found to be intermediate between BSA and BLG with minimal change in secondary structure (see also Section 2.3.b for secondary structure analysis). Recently Rosenfeldt et al. [28] showed that BSA and bovine pancreatic RNase A can enter into a PSS or PAA brushes, an important finding relevant to the biological activity of the bound proteins.

8. Applications

8.1. Ionic hydrogels for protein delivery and entrapment

8.1.1. Background

Hydrogels are three dimensional polymeric structures that can absorb large amounts of water [103•,104•]. In this section, we cover ionic hydrogels made from slightly cross-linked swollen polyelectrolyte networks, which can be used to control the release of proteins or protein-based drugs on demand, by triggering changes in the gel structure via environmental stimuli (i.e. temperature, pH, composition, etc.) [103•].

The swelling of ionic hydrogels can be manipulated by changes in the temperature, ionic strength, pH and polyelectrolyte charge density [104•]. Swelling, due to interchain electrostatic repulsions and increased hydrophilicity of the network, is promoted by pH increase for weak polyacids, and pH decrease for weak polybases. For most hydrophilic gels swelling increases with temperature, but different behavior is seen when hydrophobic groups are in the hydrogel structure, including overall suppression of swelling. The application of an external electric field is an example of external stimuli that also influence swelling.

Some have tried to explain the swelling of polyelectrolyte hydrogel as an outcome of various contributions to osmotic pressure of the gels. Ogawa and Kokufuta [105] investigated whether an osmotic pressure can arise from a difference in mobile ion concentration, by comparing the swelling behavior of homogeneously and heterogeneously distributed hydrogel charges. Thermally responsive cationic gels with immobilized urease were used to create a pH-gradient that would cause heterogeneous distribution of charges in the gel. Volume changes in the gel can be manipulated by pH changes inside the gel as a result of an enzymatic reaction, the products and substrates of which are free to diffuse inside the gel while the enzyme (urease) is immobilized. Their study has verified a direct correlation between charge inhomogeneity and overall gel swelling, an alternate explanation to osmotic swelling of hydrogels arising from mobile charges.

8.1.2. Control of protein release

The rate of protein release from ionic hydrogels is affected by various structural parameters of the protein and polyelectrolyte as well as by the environmental conditions inside the hydrogel. An increase in the concentration of ionizable charged groups in the polymer, i.e. the charge density of gel polyelectrolyte, might cause an increase or a decrease in protein release depending on the charge sign of the polymer. For example, the amount of myoglobin released from a cationic hydrogel was found to increase when a homopolymer of 2-hydroxyethyl methacrylate (HEMA) was replaced by a copolymer of cationic diethyl aminoethyl methacrylate and nonionic HEMA [106]. As expected, proteins with low molecular weights have faster release rates, and globular proteins are faster than ones with fibrous structure.

8.1.3. Systems, uses, and examples

The use of stimuli responsive gels for protein and peptide delivery has been reviewed by Bromberg and Ron [103•]. Protein-carrying ionic hydrogels have potential use in drug delivery systems; for the design of novel biocompatible materials, in chronobiology, i.e. the study of the temporal relationships of biological phenomena, and for the medical treatment of chronobiological diseases [104•,106]. Leonard et al. [107] used hydrophobically modified alginate hydrogels for protein-entrapment, and demonstrated controlled release of BSA and human hemoglobin. With an entrapped vaccine protein and encapsulated urease, the system could be applied to immunization. Gel-entrapped enzymes may be also biocatalysts for chemical conversion. Kokufuta and coworkers [108] developed amphoteric gels with immobilized glucose oxidase plus urease. These systems can convert biochemical energy into mechanical work through the swelling and shrinking of the gel due to inter-network electrostatic interactions.

8.2. Enzyme immobilization

The application of hydrogels for controlled drug release was reviewed in the previous section. On the other hand,

many applications in diagnostics and drug discovery require proteins immobilized on substrates. In this section we discuss the immobilization of proteins as active catalysts and biosensors.

8.2.1. Colloidal catalysts

Enzymes are proteins which can function as biocatalysts. Due to their large surface areas, spherical colloidal particles with either polyelectrolyte brushes in which enzymes are immobilized or with polyelectrolyte multilayers onto which proteins are adsorbed are both excellent candidates for biocatalysis [26,109].

β-glucosidase (β-GLS) was sandwiched between layers of PSS, creating several layers of β-GLS separated by PSS. The sandwich was assembled onto a precursor of four layers of PAH/PSS polyelectrolyte film deposited onto the PS latex particle to facilitate the adsorption of first β-GLS layer [109]. The yield of glucosidation reaction increased with the number of β-GLS layers, indicating that the enzymes within the lower layers were also taking part in the catalytic reaction. This also demonstrated that the substrate, dodecanol, can diffuse into the polyelectrolyte layers to interact with the active sites of immobilized enzyme. If the outermost layer is the polyelectrolyte, the catalytic activity of immobilized β-GLS decreased relative to particles with β-GLS as their outermost layer. This result could be attributed to the loss of enzyme from the surface into solution. If not protected with a polyelectrolyte layer some of the β-GLS can go into solution leaving fewer enzymes on the surface to take place in glucosidation reaction. However, for the embedded enzymes catalytic activity did not change since there was no significant enzyme loss into solution. Anikin and his coworkers [26] investigated the bioactivity of the immobilized fluorescent protein (mEosFP) onto polyelectrolyte PSS brushes. They reported the uptake and release of fluorescent protein molecules did not affect its structural integrity and bio-functionality was retained.

8.2.2. Biosensors

In general a biosensor can be considered as a device that detects, records, and transmits information regarding a physiological change or in response to the presence of various chemical or biological materials in the environment. The IUPAC definition stresses the fact that these devices must contain a biological recognition element in direct spatial contact with a transducer element, usually an electrode. This review focuses on biologically responsive materials, namely, polyelectrolyte-immobilized enzymes as biosensors.

Storage stability (shelf life) and operational stability are very important considerations in the process of biosensor preparation, because biosensors need to maintain their activities for extended period of time [110]. Thus the presence of polyelectrolytes used for immobilization on electrode surfaces generally improves biosensor stability. High MW polyelectrolytes in low concentration improved the operational stability of the biosensors much more than low MW ones [111]. Yu and Caruso [60] utilized PSS/PAH encapsulated catalase crystals to prepare an electrochemical biosensor. They formed PAH/PSS multilayers on the catalase crystals and deposited them on the electrode. The polyelectrolyte layers prevented enzyme leakage and controlled the permeability of the surface, hence increasing stability. Gibson et al. [110] increased biosensor stability by forming a glucose oxidase–polyelectrolyte complex on the surface of the electrode after enzyme immobilization. They also showed that the immobilization of glucose oxidase in the presence of polyelectrolytes increased thermal operational stability. Their results indicated the benefit of addition of polyelectrolytes to the carrier solutions used in flow analysis systems, where sensors are used for detection. Cholesterol biosensors were prepared through COX enzyme immobilization onto a polyelectrolyte-modified working electrode surface [17]. Polyelectrolytes were used to prevent direct contact between enzyme and electrode surface, thereby keeping the COX enzyme and its biospecific activities alive.

8.3. Protein separations

Polyelectrolytes can be used to recover proteins from their mixtures by selective phase separation (precipitation or coacervation), the considerations being selectivity, yield, and subsequent purification steps. These factors were considered in a review by Izumrudov et al. [112]. Dubin and co-workers early on noted that a simple commercial polycation (PDADMAC) showed remarkable selectivity for proteins of similar pI (BSA and BLG) , also retained when the polymer was adsorbed on glass. Dainak et al. [113] coupled to quaternized poly(4-vinylpyridine) an affinity ligand, (glyceraldehyde-3-phosphate dehydrogenase) to make an immobilized antigen. Antibody fragments could be purified separated active from inactive ones using this polycation–antigen complex.

8.4. Microencapsulation using protein–polyelectrolyte coacervates

Protein–polyelectrolyte coacervation is a common physicochemical method used in microencapsulation of oils and flavors for food and drinks; but, with a better understanding of the structure and stability of the process [20••,114•], it could also be used to encapsulate drugs, cosmetic additives, pesticides, live cells and vaccines.

Encapsulation is accomplished by dispersing the emulsion core-material, e.g. oil, in a stock solution of either polyelectrolytes or polyelectrolyte–protein mixtures, followed by a change in solution conditions (e.g. pH) to enhance coacervation around the core material. Complex coacervate microencapsulation is sensitive to the same parameters that affect coacervation, such as pH, I, total macromolecule concentration and protein/polyelectrolyte

ratio as seen in albumin/acacia, plant proteins/gum acacia, plant proteins/carboxymethylcellulose, and whey proteins/gum acacia [20••,23,114•]. The only additional consideration is the size of the core-material droplet; and it was found that small oil droplets (<50 μm) are more readily encapsulated than large ones [114•]. Optimal encapsulation of core material was accomplished at the pH of maximum coacervation yield where the viscosity is moderately high [20••,114•].

Coacervate stability is controlled by particle size, viscosity and interfacial rigidity, but stability can always be enhanced by cross-linking the coacervate layer chemically or enzymatically. However, cross-linking of coacervate droplets is not recommended for biocompatible and biodegradable microcapsule systems since cross-linking agents such as glutaraldehyde can be potentially toxic [20••].

8.5. Other applications

Poly[di(carboxylatophenoxy)phosphazene] (PCPP) is an example of immunostimulant polyphosphazene polyelectrolytes, with potential applications in the development of vaccines for enhancement of immune response to antigens. Andrianov et al. [50] used complexation between BSA and PCPP as a model system for the interaction of the polymer with antigens. In vivo studies showed that an increase in serum antibody levels when immunized with formulas prepared from BSA–PCPP complexes compared the formulas prepared from BSA. Enzyme–polyelectrolyte complexes combined with hydroxyl-containing compounds such as lacitol, lactose, maltilol and sucrose were found to have a stronger enzyme stabilization effect; i.e. sustained enzyme activity, compared to complexes without them [115]. An electrostatic "molecular cage" around the enzyme structure was proposed as the source of enhancement of enzyme stability. It was suggested that such effects could enhance the production of dehydrated enzymatically active vaccines.

9. Conclusions

The convergence of interests as diverse as food systems, bioseparations, GAG biochemistry, drug delivery and biosensors should encourage the investigation of protein–polyelectrolyte systems. The number of combinations of polyelectrolytes and proteins, and the array of applicable techniques is vast, an embarrassment of riches for experiment.

Relevant theories have been developed for polyelectrolyte adsorption on charge-patterned planes, and on spherical colloids. The obvious extension is to heterogeneously charged spheres interacting with polymers that may have their own charge patterns. Simulations have been pursued enthusiastically, and have surely rendered somewhat irrelevant the cartoons of the last century. Still, caution is appropriate, and the relationship between measured persistence lengths and bead-spring models should be looked into. Fidelity to atomistically defined protein structures, a more sophisticated view of polyelectrolyte structures, and explicit consideration of the role of water remain challenges for simulation.

The field of coacervates, previously associated with mid-century (brilliant) explorations, can be a fertile area for new techniques on multiple length and time scales, and for theories more relevant than those developed early on for flexible biopolyelectrolytes. Advances here may require a willingness of experimentalists to focus on systems less polydisperse than those of current applications.

Bridging the gulf separating biochemists and polymer/colloid chemists will open up enormous possibilities of collaboration. The latter will have to understand the language and conceptual frameworks of the former. Given the current hegemony of molecular biology relative to physical biochemistry, this may be a slow process. In the meantime, an effort should be made to work with biochemists amenable to the concept that selectivity and electrostatic interactions are not mutually exclusive, and that, when the latter predominate, the meaning of "specificity" should be reconsidered.

Opportunities abound in the developing area of protein–polyelectrolyte multilayers, partly due to unexpected ability of many proteins to function in such unnatural environments. With small particles, very high levels of protein loading are achievable, and the resultant materials could be of great technological value in biosensing and catalysis. Considered overall, the field of protein–polyelectrolyte complexes, located at the interface of multiple disciplines, is unusually rich for concerted progress in vivo, in vitro and in silico.

List of Abbreviations

AFM	Atomic force microscopy
AC	Affinity chromatography
ACE	Affinity coelectrophoresis
AMPS	2-acrylamido-2-methylpropane sulfonate
Anti-IgG	Anti-immunoglobulin G
AT	Antithrombin
ATR-FTIR	Attenuated total reflectance Fourier transform infra red spectroscopy
BLG	β-lactoglobulin
BSA	Bovine serum albumin
CD	Circular dichroism
CS	Chondroitin sulfate
CSB	Chondroitin sulfate B
DLS	Dynamic light scattering
DSC	Differential scanning calorimetry
DWS	Diffusing wave spectroscopy
ELS	Electrophoretic light scattering
EM	Electron microscopy
EPMPEM	Embedded protein multiple polyelectrolyte multilayers

Epo Erythropoietin
EpoR Dimeric receptor of erythropoietin
EPPEM Embedded protein polyelectrolyte multilayers
EPSPEM Embedded protein single polyelectrolyte multilayers
FACCE Frontal analysis continuous capillary electrophoresis
FITC Fluorescent isothiocyanate
FPR Fluorescence photobleaching recovery
GAG Glycosaminoglycan
GLS β-glucosidase
HSA Human serum albumin
HEMA 2-hydroxyethyl methacrylate
HS Heparan sulfate
IgG Immunoglobulin G
IR Infra-red
ITC Isothermal titration calorimetry
KPVS Potassium poly(vinylsulfonate)
LSCM Laser scanning confocal microscopy
MC Monte Carlo simulations
MIR-FTIR Multiple internal reflection Fourier transform infra red spectroscopy
MW Molecular weight
OM Optical microscopy
OWLS Optical waveguide lightmode spectroscopy
PAA Poly(acrylic acid)
PAH Poly(allylamine hydrochloride)
PAMPS Poly(2-acrylamido-2-methylpropane sulfonate)
PCPP Poly[di(carboxylatophenoxy)phosphazene]
PDADMAC Poly(diallyldimethylammonium chloride)
PEI Poly(ethyleneimine)
PG Polygalacturonase
PGA Poly(glutamic acid)
PGIPs Polygalacturonase-inhibiting proteins
PLL Poly(L-lysine)
PMMA Poly(methyl methacrylate)
PS Polystyrene
PSS Poly(styrene sulfonate)
PVS Poly(vinylsulfonate)
QCM Quartz crystal microbalance
RNase A Ribonuclease A
SAR Scanning angle reflectometry
SAXS Small angle X-ray scattering
SEC Size exclusion chromatography
SLS Static light scattering
SPR Surface plasmon resonance
TAPPEM Terminally adsorbed protein polyelectrolyte multilayers
TIRF Total internal reflection fluorescence microscopy

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