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Protein Purification by Selective Phase Separation with Polyelectrolytes

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Protein Purification by Selective Phase Separation with Polyelectrolytes

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Abstract

Proteins phase separate in the presence of synthetic polyelectrolytes as a consequence of electrostatic interactions. This phenomenon may form the basis of protein separations and has therefore been of technological interest. In this review, we consider previous reports of such phase separation, and attempt to describe the effects of various relevant factors, such as polymer MW, ionic strength, and macromolecular concentration. Models for protein-polyelectrolyte complexation are discussed. Current challenges and problems are noted.

1. Introduction

Proteins of various degrees of purity are raw materials for the food, cosmetic and pharmaceutical industries. Rapid developments in biotechnology have greatly increased the variety and availability of high-value proteins, which must be provided at high purity. Protein separation and purification procedures are therefore becoming more and more important, and the cost and efficiencies of these processes profoundly influence the commercialization of bioengineered proteins and the productivity of the relevant industries.

Proteins are usually concentrated and purified in a series of many steps. Although chromatography plays a key role in the final purification steps, precipitation, ultrafiltration and dialysis are also important (1). A relatively little-known technique has

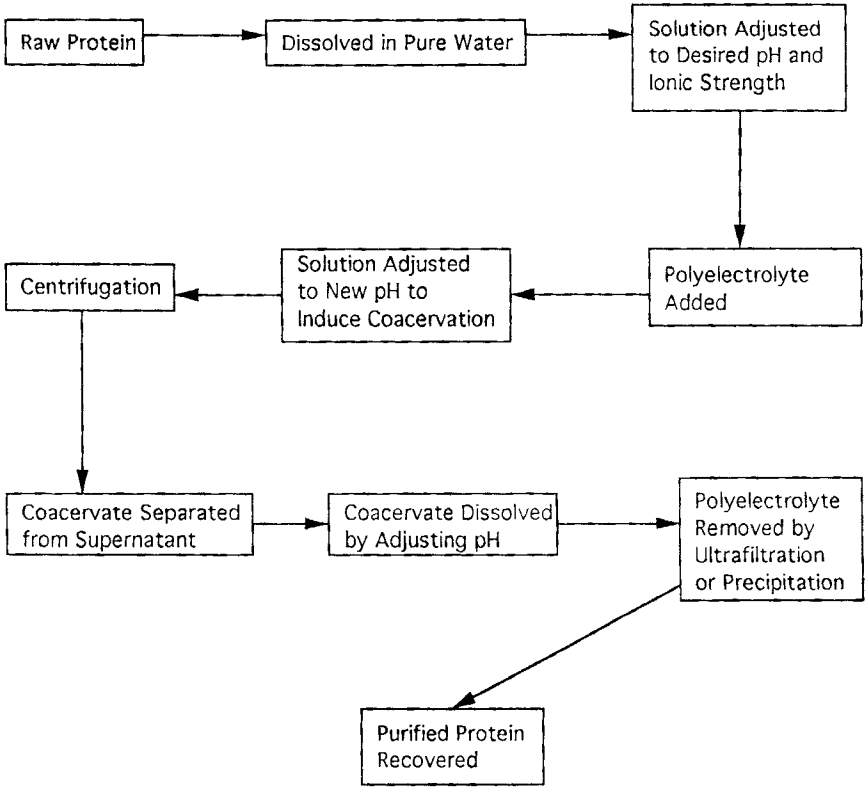
as its basis the phase separation that takes place when proteins are combined with polyelectrolytes under conditions of appropriate pH and ionic strength. This phase separation may either be precipitation or the formation of a second liquid phase, highly concentrated in both macroions. The second process closely resembles a phenomenon first observed with mixtures of oppositely charged polyelectrolytes, referred to as "complex coacervation" (2). This behavior is apparently characteristic of certain polyelectrolyte-colloid systems as well, namely, those in which the polymer and the colloidal particle are of opposite charge. Since complex coacervation can take place under near-equilibrium conditions, it is probably preferable to precipitation as a means of protein removal. In this review, we refer to "protein-polyelectrolyte coacervation" for brevity, with "complex" being understood. Selective coacervation of protein with polyelectrolytes seems to be a very promising method not yet incorporated into typical procedures. Its virtues, in principle, include high yield and low cost.

The successful application of polyelectrolyte-protein coacervation must entail the redispersion of the coacervate, which is accomplished readily by pH adjustment, and the removal of the polyelectrolyte from the target protein, which may be carried out by ultrafiltration, or by co-precipitation with a second polyelectrolyte of opposite charge. The placement of these steps is as shown in the flow-chart below. However, in this chapter we devote our discussion to studies of the coacervation process alone. We present first an overview of the general phenomenon, followed by a discussion of the effects of various parameters (chemical structure and solution conditions), and then a review of studies concerning the molecular mechanism of the process. Finally, we review some practical applications and discuss the problems that remain to be resolved.

2. Basic Considerations

Protein-polyelectrolyte phase separation arises from intermolecular association, which is a consequence of cohesive forces such as electrostatic interactions, hydrogen bonds, and hydrophobic forces, i.e. the same forces that are responsible for protein aggregation in the absence of polymers. The observable consequences of this association may be the formation of soluble complexes (3), amorphous precipitates (4-6), or coacervates (7-10). Coacervation is the formation of a second liquid phase, rich in solute, in equilibrium with dilute phase, and it is characteristic of non-crystalline macromolecules. Coacervation that occurs as a result of the interaction of two different polymers has been called "complex coacervation" but recently the term "associative phase separation" has been suggested for similar phenomena (11).

Flow chart for Protein Purification with Polyelectrolyte



One of the earliest observations of complex coacervation involved mixtures of gelatin and gum arabic, in which coacervation could be induced by addition of strong acid (12). This system serves as a paradigm for coacervation between oppositely charged macroions, displaying the following characteristic features: (a) at least one component is a flexible chain polyion; (b) the components may be completely synthetic, completely natural, or mixed; (c) the charge distributions on the two polyions are not complementary, that is the charge spacings are asymmetric; and (d) solution variables, such as pH, typically play an essential role. While coulombic forces dominate, they cannot be too strong or too efficient if coacervation is to occur. Thus, oppositely charged symmetric strong polyelectrolytes, such as sodium poly(styrenesulfonate) and

poly(vinylbenzyltrimethylammoniumchloride) display irreversible precipitation (13), with efficient ion-pairing, and the expulsion of small ions. When complexes form between asymmetric or weak polyions, counterions are retained in the complex, along with substantial hydration. Thus, the osmotic swelling of the complex may be considered as one reason for liquid-liquid as opposed to liquid-solid phase separation. These considerations have been discussed in a number of reviews and books (14-16) and in various works on inter-polyelectrolyte phase separation (17-21)

Coacervation is not restricted to systems of oppositely charged polyelectrolytes, but is also characteristic of weakly- or asymmetrically-charged polyion-colloid systems of opposite charge. The two primary examples are polyelectrolyte-micelle systems (22) and polyelectrolyte-protein systems. In both cases, the combination of high particle surface charge density, ß, high polymer linear charge density, ç, and low ionic strength, I, promotes precipitation; but decrease in ß and ç and increase in I favors coacervation. Turbidimetry alone may not discriminate clearly between coacervaton and precipitation, but the two can be easily distinguished by visual observation after low-speed centrifugation, or by microscopy. In at least one system (23) protein-polyelectrolyte coacervate appears in the form of 1-10 µm droplets which, in time, fuse and settle.

Polyelectrolyte coacervation (PC) offers several advantages over other means of separation. Compared to chromatography, ultrafiltration or liquid-liquid extraction (as in polyethyleneglycol-dextran partitioning (24) PC is a concentrating, not a diluting step, with a reduction of 100X-1000X in the aqueous content. On the other hand, the coacervate is an aqueous phase, and the target protein is not likely to be denatured. The amount of polymer required is relatively small: Berdick and Morawetz (25) reported the precipitation of 0.24% catalase with 20X more dilute polyacrylic acid, with 97% yield of protein at pH 5. Clark and Glatz (4) obtained 96% yield of lysozyme with carboxymethylcellulose (CMC) at CMC/protein mass ratio of 0.04. In contrast to chromatography, the expense of scale-up in PC is likely to be small, relative to the equipment and stationary phase costs of large-scale column separations.

On the other hand, there are evident challenges to be faced in developing PC for protein mixtures. There is considerable question as to how much selectivity for the target protein can be achieved in the presence of other proteins. Nor should selectivity be attained at the expense of yield. The polyelectrolyte must be removed from the target protein and this process should not diminish yield or lower protein activity. In attaining these goals, a rather large set of variables needs to be optimized. These include polyelectrolyte

chemical structure and molecular weight, concentrations of protein and polyelectrolyte (both relative and absolute), and ionic strength and pH. Given these variables, along with the virtually infinite plausible protein mixtures that could be examined, a purely Edisonian approach to separation optimization is a daunting task, and the reader is likely to be confronted with a collection of fragmentary and anecdotal studies from which generalizations are difficult. Nevertheless, even rather specific studies can provide some sort of broad understanding of the general phenomenon.

3. Methodology

Polyelectrolyte coacervation (PC) of proteins is a complicated process, which will, in the long run, require numerous techniques. There are essentially three types of studies that may be carried out. First, it appears very likely that coacervation is preceded by the formation of soluble polyelectrolyte-protein complexes (26), so an understanding of these complexes is important to understanding all aspects of PC. Soluble complexes can be investigated by virtually all of the techniques used to characterize polymer solutions, and protein solutions, including static and dynamic light scattering, fluorescence, electrophoresis, circular dichroism and ultracentrifugation. Many of these are described in a recent review article (27). Second, the solution can be brought beyond the phase separation point and analysis of protein content carried out. In this way, the yield or selectivity can be determined as a function of variables such as polymer MW or pH. The third type of study is based on the observation that both complex formation and coacervation appear to be phase transitions, occurring abruptly at well-defined conditions. Thus, for example, gradual increase in the pH for a protein-polycation system displays a well-defined phase separation point (pH_f); and it is possible to discern, at lower pH, an early transition corresponding to incipient complex formation (pH_c). The dependence of either pH_f or pH_c on e.g. ionic strength, thus constitutes a phase boundary, which contains important practical information, as well as fundamental insight. These critical points may be located by changes in scattering intensity, often by simple turbidimetry. In this laboratory, the experiment which leads to the pH dependence of turbidity at constant ionic strength, and protein and polymer concentration, is called a "type 1" titration. One may also follow the evolution of the turbidity upon addition of either protein to polymer ("type 2") or polymer to protein("type 3") and these titrations can provide information about the stoichiometry of complex formation (23,28,29).

Most of the systems described in the literature are comprised of combinations of one of a half-dozen proteins, with one out of a larger number of polyelectrolytes. Specifically, the

proteins studied most intensively are bovine serum albumin (BSA), human serum albumin, ribonuclease (RNase), ovalbumin, lysozyme and hemoglobin, and nearly all the references cited pertain to one or more of these six. Polyanions studied include poly(methacrylic acid) (PMA)(6,30), poly(acrylic acid) (PAA)(6,25,31,32), poly(styrenesulfonate) (PSS)(33), polyvinylsulfonate (PVS)(34,42), poly(acrylamidomethylpropane sulfonate) (PAMPS)(34) and its copolymer with N-vinylpyrrolidone (NVP-AMPS)(34), carboxymethylcellulose (CMC)(4), hydrolyzed maleic anhydride-styrene copolymer (MAS)(25) and hydrolyzed maleic anhydride-vinyl methyl ether copolymer (MAVE)(25). Among the polycations examined are poly(dimethyldiallyl ammonium chloride) (PDMDAAC) (9,23,26,29,33,35,36), methylacrylamidopropyltrimethylammonium chloride homopolymers (PMAPTAC) and copolymers with acrylamide (35), and poly(ethyleneimine) (PEI) (36) . Recently, phase separation with polyampholytes has been described (37). These polymers self-precipitate at their own IEP in the absence of proteins, and this same property could prove useful in the polymer-removal step of the process.

4. Variables Affecting the Coacervation Process

4.1. Polymer MW

The energetics of the polyelectrolyte-protein interaction appears to be independent of polymer MW. Morawetz and Hughes (38) reported that the equilibrium of the BSA-PMA system, as evaluated from measurement of the amount of BSA in the supernatant, was independent of polymer MW. Similarly, Brittain and Dubin (39) recently found that neither pH_c nor pH_f is affected by polymer MW for BSA-PDMDAAC. On the other hand, Sakamoto et al. (40) concluded, on the basis of the inhibitory effect of polymer on enzymatic p-nitrophenolate hydrolysis, that the binding constant for PAA-BSA increases with MW. Other experiments show, more definitively, that polymer MW can affect the yield and efficiency of coacervation. Shieh et al. (6) reported that higher MW PAA was more efficient in the removal of egg white protein, which was similar to the result obtained by Sternberg and Hershberger (31) that the MW of PAA had little effect on the yield of lysozyme. Wang and Dubin (41) observed that the yield of BSA coacervated with PDMDAAC increased with polymer MW, but leveled off at MW ¡= 10^5. For the same system, higher pre-coacervation turbidities were observed with higher-MW polyions (41); these are likely to correspond to larger soluble complexes.

4.2. Polyelectrolyte Charge Density

Park et al. (34) used turbidimetry to study the interaction of two basic proteins (RNAse and lysozyme) and one acidic protein (BSA) with polycations and polyanions of different charge densities: acrylamide/cationic acrylamide copolymers and PDMDAAC; and NVP-AMPS copolymer, PSS, PVS and PAMPS, respectively. From these results, it appeared that the geometric surface charge density, calculated with consideration of polymer diameter, correlated better with the strength of the interaction than the linear charge density. On the other hand, in some cases for polyanions the critical pH increased with polyion linear charge density ξ. These polyions with higher negative charge density were able to bind proteins at higher negative net charge. Park et al. (34) proposed that polyions with larger ξ could bind more strongly to a local positive region ("patch") on the protein and thus overcome the longer range repulsion between the polyion and the globally negative protein charge.

4.3. Polymer and Protein Concentrations

Berdick and Morawetz (25) found that the extent of precipitation of catalase with polyacids in 0.1 M acetate or phosphate buffer decreased at low polymer concentration, and that the range of pH over which complete precipitation was achieved increased with polymer concentration. In pure water, Kokufuta et al. (29,42) found that the amount of PDMDAAC needed to precipitate HSA is linearly proportional to the amount of HSA in solution. Results obtained by Berdick et al. for catalase and polyacids (PAA, MAVE and MAS) in buffer gave a similar finding: for each system, at any given pH, there is a critical ratio of polymer:protein corresponding to maximum precipitation. Higher or lower concentrations of polyelectrolyte tend to produce soluble complexes. Also, for the phase separation of hemoglobin with PAA (21), lysozyme with PMA (30), BSA with MAS ((25)), lysozyme with CMC (4) and BSA with PDMDAAC (23), when the concentration ratio for protein:polymer deviates from some optimal value, some of the insoluble phase redissolves.

In the case of both PC precipitation and coacervation some evidence appears for a type of stoichiometric interaction, but the nature and the explanation of this stoichiometry is different in each case. For example, Kokufuta et al. (29,42) have found that precipitates formed from proteins and polyelectrolytes in pure water have well-defined stoichiometries corresponding to net neutrality of protein carboxylates, amine-containing residues, and polyion repeat units. Thus, upon addition of polymer to protein, the amount

of precipitate formed increases linearly with added polymer to reach a maximum value at the "end-point". Implicit in this model is a very large protein-polyion binding constant such that one has free protein below the end-point, free polymer above it, but never both at once. The situation may be different at moderate ionic strength. Ahmed et al. (23) observed that the ratio of BSA:PDMDAAC corresponding to maximum turbidity did not depend on protein concentration at low ionic strength, but did vary at high I where coacervate formed. This may suggest that the composition of the coacervate was not constant, but became ionic strength dependent. Recently Li et al. (43) carried out QELS, turbidmetry and ELS of BSA-PDMDAAC in 0.02 M NaCl and found that the turbidity maximum corresponded to zero electrophoretic mobility. As the ionic strength increases, resolubilization of the coacervate is readily observed upon increase in protein:polymer. In summary, maximum phase separation is observed at certain stoichiometries which may be loosely correlated with charge neutralization, but there are two very different interpretations. One is that complexes are inherently insoluble and of fixed composition, but the yield is governed by the limiting reagent (much like a "colloid titration"). The other is that complexes of various structures and compositions may form, but will phase separate only when their net charges approach zero. At present it appears likely that the first of these explanations is most suitable in pure water, the second at moderate ionic strength.

4.4. Added Salt

As recognized long ago (12), the primary effect of added electrolyte is to increase the ionic strength (I) and so screen coulombic forces between polyelectrolyte and protein. It has been shown (26,35) that complexation can be completely suppressed by increase in I. Indeed the ionic strength appears to govern complexation by way of its effect on the Debye length. Thus, if the critical pH at which complex formation is first detected (pH_c) is converted via the protein titration curve to the critical net charge Z_c, it is found, for the system BSA-PDMDAAC, that Z_c varies linearly with $I^{-1/2}$, i.e. with the Debye length (43). However, specific salt effects cannot be ruled out. Morawetz and Hughes (38) suggested for example, that anion-binding could change the net protein charge. Berdick and Morawetz (25) reported using 0.05 M KSCN to widen the region between the isoelectric points of catalase (normally 5.4) and BSA (normally 4.9) to improve the selective precipitation of catalase by PAA.

5. Selectivity

In many practical situations, the target protein must be recovered from a mixture containing other proteins. Given the nearly infinite combinations of target proteins,

extraneous proteins and polyelectrolytes, it may be difficult to identify paradigm systems. Indeed, there are few systematic studies on the separating efficiency of the process. Strege et al. (35) defined selectivity as

$$S = \{[A]_c/[B]_c\}/\{[A]_s/[B]_s\} \quad (1)$$

where A and B are two proteins, and the subscripts refer to the coacervate and supernatant phases. S = 1 corresponds to no separation, and S = ∞ (or 0) to complete separation. However, it should be noted that S = ∞ is of no practical value if the total yield of coacervate is small. For a given protein pair, S depends on many factors, including the pH, ionic strength, macroion concentrations, and the structure and MW of the polyelectrolyte. Despite this complexity, S is a measurable experimental quantity that, in principle, should be related to molecular parameters. Dubin and his coworkers (35) found for RNAse and BSA that S was nearly constant at 40 in the pH range 6-9. Large values of S are expected in this case where IEP's are so different, but more recently (41), S>8 was also found for proteins with much closer IEP, namely BSA/β-lactoglobulin (4.9/5.2) and BSA/©-globulin (4.9/5.3) (separated at pH 9, I = 0.1M, with high MW PDMDAAC). At present, an important question is to what extent the coacervation yield for a given protein is affected by the presence of another protein.

6. Models of Protein-Polyion Coacervation

The coacervation of protein-polyelectrolyte complexes may be considered from several points of view. One is as an example of polyelectrolyte complex coacervation, a phenomenon well-known in mixtures of oppositely charged polyelectrolytes (12). From a second perspective, polyelectrolyte-protein coacervation (PPC) may be thought of as a type of polymer-colloid flocculation, albeit with rather small colloids. Third, we may consider that the formation of soluble complexes, a sort of super-polyelectrolyte formed from protein and polymer, is the precursor to PPC and therefore must be considered as an integral part of the process. Yet another position is taken by Picullel and Lindman (11) who has considered the analogous case of the coacervation of polyelectrolyte-micelle complexes.

Polyelectrolyte complex coacervation was considered by Overbeek and Voorn (44), who concluded that spontaneous coacervation occurs as a result of the competition between electrical attractive forces that tend to accumulate oppositely charged polyions, and entropy forces which tend to disperse them. This theory applies to random coil polyions, with distributive electrostatic forces, and negligible solute-solvent interactions. These restrictions are thought to confine the relevance of the theory to high charge density

systems close to conditions of maximum coacervation yield (45). In contrast, Veis and Aranyi (46) considered coacervation as a two-step process. First, spontaneous coulombically-driven aggregation of oppositely charged polyions produces aggregates of low configurational entropy. These aggregates, or "coacervate sols", then rearrange to form the coacervate phase, which is driven by a gain in configurational entropy. The Veis-Aranyi theory seems to be more relevant to low charge density mixtures. This theory was modified by Tainaka (47,48) who proposed that aggregate pairs form in the dilute phase, as described by Veis, but without specific charge pairing. The aggregates condense to form the coacervate phase, in which they overlap. The driving force for coacervation is the electrostatic energy gain arising from an increase in ion density, and decrease in potential, in the overlapped domains.

These thermodynamic treatments described oppositely charged polyelectrolytes, with both macroions as random coils, quite different from the present case. For PPC, per se, some empirical qualitative models have appeared. Kokufuta and co-workers (28,29) believe that specific salt linkages form between the polyion and complementary residues on the protein, and this ion-pair formation leads at once to precipitation, presumably via desolvation. This mechanism explains the stoichiometry they observe in "colloid titration" of protein with polymer or vice versa. Strege et al. (35) suggested that soluble complexes coacervate only when a value of zero is reached for the net charge, $Z_T = Z_P + -n\ Z_{pr}$, where Z_P is the polyion net charge (the degree of polymerization for a strong polyelectrolyte), and Z_{pr} the protein charge (a function of pH). –n, the mean number of bound proteins per polymer chain, is determined by the relative or absolute protein concentration in solution, and the protein-polyion binding constant (itself a function of pH and ionic strength, *inter alia*). In other words, the number of proteins bound is governed by a mass action equilibrium. This relation appears to be a simplification. Firstly, it has not been directly demonstrated, for example, that $\bar{n}$ varies inversely with Z_{pr}, as predicted. Second, phase separation can occur on "the wrong side of the isoelectric point", i.e. polycations may phase separate with lysozyme at pH<pI. Third, electrophoretic light scattering carried out in the pH regime between soluble aggregates and coacervates reveals that net charge persists, almost to the point of turbidity maxima (maximum coacervate yield) (43). It is certainly likely that soluble aggregates with high charge will not coacervate, but it is not at present clear just how much the charge must be reduced for phase separation to occur.

The fact that proteins may bind to polyelectrolytes even when the net charges of the two macroions are of the same sign, is analogous to the fact that proteins can be retained on

Table I. Examples of Protein Separation by Polyelectrolytes

Proteins	Polyelectrolytes	Conditions	Best Recovery
BSA (38, 1)	PMAA, MAVP, PVAm, MAS, DEMMA	pH 3.95-8.70 I: H_2O, NaCl solution	~93%
Catalase and BSA(25)	PAA	pH ~5.2	~100%
Catalase-Ferrihemoglobin (25)	PAA	pH~5.8	~92%
lysozyme from egg-white (31,50)	PAA	pH 5.45	~92.3
lysozyme from egg-white (13)	CMC	pH 4.5, I=0.07	~96%
RNA polymerase (51)	PEI	pH 7.0 I=0.2 NaCl	~90%
Whey Proteins (52)	CMC	pH 2.5-4.0	68%
Amyloglucosidase (53)	PAA	pH 3.2-4.0	95%
Lactase (53)	PAA	pH 3.2	100%
Microbial (53)	PAA	pH 3.2	85-96%
α amylase (53)	PAA	pH 4.0	93%
β amylase (53)	PAA	pH 4.0-4.5	77%
Lipoxygenase (53)	PAA	pH 4.8	76%
Calf Rennet (53)	PAA	pH 3.5	73%

positively charged ion-exchange ("anion-exchange") resins even at pH<pI, and vice-versa for cation-exchange resins; and the explanation in both cases involves protein surface charge heterogeneity or "charge patches". Furthermore, for some protein-polyion systems (25,34), because of the low degree of polymerization or unfavorable polyion chain conformation, the accessible polyion chain length may be comparable to or even less than the diameter of the globular protein. In these situations, one may imagine that the polyion chains see only the local charge patches on the protein, and it is the distribution of these

charge patches that matter. However, the "tightness" of binding for proteins in soluble complexes and in coacervates is at present quite unknown.

7. Applications, Problems and Future Prospects

Selective protein-polyelectrolyte coacervation has been successfully applied to a large number of specific purifications. Xia and Dubin (49) compiled a partial list of conditions for optimal recovery, shown in Table 1. Further examples of protein separations have also appeared in the patent literature. These results, however, are highly specific to certain systems, and generalizations are difficult to draw from somewhat andecdotal reports.

Rather little attention has been paid to the separation of the target protein from the polyelectrolyte after phase separation. Redissolution of a coacervate is readily accomplished by pH adjustment. In principle, high MW polymer could then be removed by ultrafiltration. If the polyelectrolyte is relatively inexpensive, it could also be removed by precipitation with a polyelectrolyte of opposite charge. Park found that PDMDAAC could be removed from re dissolved BSA-PDMDAAC complex by precipitation with NaPSS. BSA recovery of 85% was obtained with the pH appropriately adjusted, i.e. low enough to minimize complexation with PDMDAAC, and high enough to prevent precipitation with NaPSS.

Because understanding of complexation and coacervation at the molecular level is rather primitive, there are few well-defined principles or general rules in guiding the optimization of conditions, and so we see a case-by-case approach. Vigorous research together with the development of novel experimental methods and computational modeling may help provide more insight, and ultimately advance applications of selective coacervation to a higher level.

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