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# Self-assembly and molecular 'recognition' phenomena in solutions of (bio)polyelectrolyte complexes

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**Abstract. Results of recent studies on polyelectrolyte complexes formed by both oppositely charged synthetic polyions and natural polyelectrolytes (proteins, enzymes, nucleic acids) are summarised. The properties of these complexes and the phase behaviour of their solutions are shown to obey general trends found in the studies of co-operative interpolyelectrolyte interactions. The principles of functioning of polyelectrolyte complexes that underlie the self-assembly of complexes in solution and determine the ability of the complexes to retain stability over a specified broad range of changes in external conditions and then to quickly and reversibly respond with high sensitivity to changes in the environment by changing their molecular characteristics and phase state are considered. The successful use of the results of fundamental research of (bio)polyelectrolyte complexes for solving topical problems of biotechnology, materials science and medicine are demonstrated. The bibliography includes 111 references.**

## I. Introduction

The last twenty-five years in the 20th century were marked by rapid progress of physical chemistry of polyelectrolyte complexes (PECs) that are formed as a result of co-operative electrostatic interaction of polycations with polyanions when mixing aqueous solutions of oppositely charged polyelectrolytes. The interest in PECs is to a great extent due to great prospects for the use of these systems for solving important practical tasks in industry, environment and medicine.[1] Investigations of PECs based on synthetic polyelectrolytes were carried out in line with intensive research on protein – polyelectrolyte complexes (PPCs) in which a positively or negatively charged globular protein acts as a partner of an oppositely charged polyelectrolyte. Numerous data on the formation and properties of PECs and PPCs were summarised in reviews.[2–9] There is an intimate relation between the mechanisms of formation and degradation of both types of complexes, structural organisation of water-soluble PECs and PPCs and changes in the phase state of their solutions. Common features of the behaviour of the complexes formed by pairs of oppositely charged synthetic polyions and those involving natural polyelectrolytes made it possible to formulate criteria for the existence of such systems and to reveal universal principles of their functioning.

The unified approach to the description of PECs of various origin served as a basis for further development of this avenue of research, extension of the range of systems under study and discovery of new fields of practical applications of the systems in question. Synthetic polyelectrolytes of different structure, which can act as PEC components, include charged block copolymers with neutral hydrophobic[10,11] or hydrophilic[8] blocks; various generations of hydrophilic[12] or hydrophobic[13] dendrimers; polybetaines;[14,15] star-like polyelectrolytes;[16] polyelectrolyte brushes[17] and loose polyelectrolyte hydrogels.[1] This list is far from being completed. It should be emphasised that complication of the structure of the components causes no fundamental changes in the properties of systems, which makes it possible to predict the behaviour of newly synthesised PECs and to use complexation as an efficient tool of targeted modification of the properties of polymers in order to impart the properties necessary for practical applications.

This also to the full extent concerns 'biopolyelectrolyte' complexes in which one or both components are charged biopolymers. An example is provided by the studies on the interaction of polycations with DNA (natural polyanion of complex structure). Systematic investigations of products of this interaction (the so-called 'polyplexes') began fifteen years ago (see a review[5]). At present, this avenue of research has been progressing rapidly. Polyplexes are used for, *e.g.*, the design of polyelectrolyte non-viral carriers for gene therapy or for efficient isolation of plasmid DNA from cell lysates. The functioning of such systems, which are of undoubtful practical interest, is based on the same principles of co-operative electrostatic interactions that are responsible for the self-assembly of complexes and their behaviour as 'smart' polyelectrolytes.

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The aim of this review is to draw the attention of researchers working in allied areas of polymer chemistry, biochemistry, enzymology, biothechnology and materials science to unique properties of PECs and PPCs. We shall emphasise the universal behaviour, simplicity of synthesis, high stability and ready involvement in competing interpolyelectrolyte reactions, which can be highly selective.

Considerable attention is paid to analysis of factors affecting the selectivity of interpolyelectrolyte interaction, because the molecular 'recognition' effects underlie the ability of complexes to self-assembly in aqueous solutions and determine the high sensitivity of the complexes to changes in the environment. It was established that PECs can retain stability over a wide range of changes in external conditions (pH value, ionic strength, temperature) and then rapidly and reversibly respond to minor variations of these parameters by radical changes in the molecular characteristics and phase state. Of particular importance is that structural rearrangements of the complexes can occur under conditions that are favourable for the functioning of natural polyions (proteins including enzymes, nucleic acids). This makes it possible to use polyelectrolyte systems for solving applied problems in biology and medicine. In this review we will consider and analyse in detail the results of recent applied-oriented researches on biopolyelectrolyte complexes, which indicate continuous progress and great potential of the approach in question.

## II. Complexation in solutions of oppositely charged synthetic polyelectrolytes

Highly charged linear synthetic polycations and polyanions are relatively simple, available and convenient model objects for elucidation of principles of interpolyelectrolyte interaction. Polyelectrolyte complexes formed as a result of co-operative coupling reaction between two oppositely charged polyions represent a specific family of polymeric compounds. The degree of conversion ($\Theta$) of polyions in this reaction is defined as the ratio of the equilibrium number of ion pairs formed under given conditions to the maximum possible number of ion pairs. If at least one component of the mixture is a weak polyelectrolyte, the degree of conversion $\Theta$ can be controlled by varying the pH value of the medium. The parameter $\Theta$ is calculated using the potentiometric titration curves of weak polyelectrolytes in the presence of oppositely charged polyions, as described in a review.[1] From comparison of the plots of experimental '$\Theta$-*vs*.-pH value' dependences with those of the 'ionisation degree-*vs*.-pH value' dependences for weak polyelectrolytes it follows that the weak polyelectrolytes become stronger in the presence of oppositely charged polyions, *i.e*., the highly charged polymer causes an additional ionisation of weak acidic or basic groups of the partner in the complex (see a review [3]). This significantly extends the existence range of stable PECs, thus increasing the number of polyanions and polycations suitable for preparation of stable PECs.

Charging of a weak polyelectrolyte on a highly charged polymer matrix has a number of interesting practical applications. For instance, it was used for (i) significant enhancement of the efficiency of target delivery of genetic material to cell nuclei,[18] (ii) stabilisation of PECs and PPCs in solution and at the interface in a specified range of pH values [19–21] and (iii) imparting the ability to release the low-molecular-mass filler (dye), in response to adsorption of the polyion from solution, to the ultrathin multilayer polyelectrolyte films and nanocapsules.[22]

Polyelectrolyte complexes possess two properties that at first glance seem to be mutually exclusive, namely, high stability and lability. Being rather stable, PECs readily undergo competing interpolyelectrolyte reactions, which are accompanied by transfer of polyions from some complexes to other. Combining these properties and selectivity of co-operative electrostatic binding provides self-assembly of PECs in aqueous solutions and creates prerequisites for the design of polyelectrolyte systems that are highly sensitive to changes in the environment under particular conditions.

### 1. Stability of polyelectrolyte complexes

Co-operative character of multipoint electrostatic bonding makes PECs very stable to dissociation into initial macromolecular components. The effective PEC dissociation constants abruptly decrease as the degree of polymerisation ($P$) of the short-chain component (oligomer) decreases. This follows from the results of theoretical analysis of the co-operative interaction in polymer–oligomer systems, which was first reported in Refs 23 and 24. More recently, the results obtained in these studies were analysed in detail in reviews.[25,26] For instance, expression (1) relating the equilibrium constant of formation of an interpolymer complex of an oligomer with a polymer matrix ($K_n$) to the equilibrium constant of binding of one oligomer unit to the matrix ($K_1$) has the form:

$$K_n = K_1^n = \exp\left(-\frac{n\Delta G_1^\circ}{RT}\right), \qquad (1)$$

where $n$ is the number of repeating units in the oligomer chain and $\Delta G_1^\circ$ is the change in the free energy caused by the interaction of an oligomer unit with the matrix. Equation (1) and derivative expressions relating the selectivity of the interpolymer interactions to the polymer chain length and structure were derived as a result of statistical thermodynamic analysis of the general model for polymer–polymer interaction and are valid for all types of interpolymer complexes, as was emphasised in the review.[26]

The applicability of this theoretical approach to the description of interpolymer interactions involving polyelectrolytes is confirmed by experimental data. The selectivity of interpolyelectrolyte binding was first proved by fractionation of a copolymer-based polyelectrolyte with respect to the length and structure of polymer chains, which was attained by complexation with an oppositely charged partner.[24]

An important role of the polyelectrolyte chain length in co-operative electrostatic interaction was considered in a review [27] and confirmed experimentally. The dissociation constant for a PEC comprised of a high-molecular-mass polymethacrylate (PMA) anion (polyanion) and oligomeric quaternised polyethyleneimine (polycation) abruptly decreased with an increase in the oligomer chain length, approaching zero for a relatively short oligocation. The 'critical' chain length, $n$, necessary for the formation of a stable PEC in aqueous solution varies from 4 up to 6 and depends on the nature of polyions.[3,27] In other words, if the number of consecutive charged units in the chain exceeds 4–6, the PECs formed in the absence of low-molecular-mass electrolytes almost do not dissociate into individual components.

In practice this means that for preparation of PECs by mixing dilute aqueous solutions of polymeric components one can use non-fractionated samples of highly charged polyelectrolytes in which a large proportion of polyelectrolytes has relatively short chains. Clearly, an excess of the non-fractio-

nated polymer permits fractionation with respect to molecular mass, which is due to preferred complexation of longer chains.

Analogously, a copolymer consisting of charged and neutral units can form a stable PEC with the highly charged partner, if at least one segment of the copolymer includes the necessary number of consecutive charged units. Clearly, the formation of such a segment in a random copolymer requires a rather large number of charged groups. But with allowance for the fact that the probability of formation of a segment with the desired length increases with an increase in the degree of polymerisation of the copolymer at a specified charge density, one can assume that conventional long-chain copolymers can form stable PECs even if the relative content of charged units in the copolymer is low. In addition, high flexibility of vinyl polyelectrolytes, which are most widely used in the synthesis of PECs, provides the possibility of mutual alignment of fragments of the reacting chains, thus favouring co-operative electrostatic bonding. As a result, the stability of PECs changes only slightly as the charge density on the high-molecular-mass copolymer changes. This is confirmed by the results of studies on the stability of PECs formed by mixing aqueous solutions of PMA (polyanionic component) and random poly(*N*-ethyl-4-vinylpyridinium-*co*-4-vinylpyridine) copolymers (PEVP-β) with different number of charged pyridinium units $\beta$ (polycationic component).[28,29] As $\beta$ decreased from 92% to 25%, the stability of the complexes involving the copolymers with $P = 600$ (Ref. 22) and $P = 900$ (Ref. 29) remained almost unchanged. The same was observed for the DNA complexes with PEVP-β cations[30] or ionene polycations with various charge-to-charge separations in the chain.[31] In both systems, the decrease in the charge density on the cationic partner of DNA had little effect on the stability of the polyplex in salt media.

The possibility of varying the charge density on the polyion over a rather wide range without significant destabilisation of PECs considerably increases the range of polyelectrolytes suitable for complexation and simplifies the preparation of such systems. For instance, conventional procedures for preparation of high-molecular-mass polyelectrolyte sorbents for separation of mixtures of (bio)polymers involve total chemical modification of neutral units of the starting polymer (*e.g.*, polysaccharide), which is usually carried out to prepare highly charged samples. Now there is no longer need for this step. The same goal can be attained by charging the polymer chain with a relatively small number of charges without modification of the remaining functionally important groups of the sorbent or by introducing fragments consisting of relatively small number of charged units necessary to form a stable PEC into the polymer chain (by, *e.g.*, graft copolymerisation or attachment of fragments to terminal groups).

Finally, the small extension of the fragment of electrostatic binding allows the protein globule containing a small number of neighbouring ionogenic groups on the surface to form stable PPCs with the oppositely charged polyions. Electrostatic co-operative interaction of the protein globule with the polyion can occur in a broad range of pH values including the region near the isoelectric point of the protein. Moreover, the charge of a weak polyelectrolyte (here, protein) on the highly charged polymer (see above) permits electrostatic interaction between the components of the PPC at such pH values at which the protein and polyelectrolyte have charges of the same sign; of course, this is possible if the pH values do not differ too much from the isoelectric point of the protein.[9,32–35]

The addition of a low-molecular-mass electrolyte, which shields electrostatic interaction between the oppositely charged components of the PEC and breaks down ion pairs, leads to an increase in the 'critical' chain length necessary to form stable PECs. The stability of complexes formed in the interaction of PMA anion with poly(*N*-ethyl-4-vinylpyridinium) cations in mixtures of water–salt solutions abruptly increases as the polycation chain length increases up to $P \approx 100$; at higher $P$ values, the polycation chain length has no effect on the PEC stability.[36] Other pairs of polyelectrolytes behave analogously.

Thus, stabilisation of PECs in water-salt media requires the use of relatively high-molecular-mass polyelectrolytes. For instance, the 'critical' polycation chain length should be at least 20 units to ensure the formation of complexes between PEVP cations and PMA anion[36] or DNA[30] that are stable at physiological values of the ionic strength of solutions. If practical considerations (*e.g.*, the preparation of soluble complexes from sulfur-containing polyanions possessing an abnormally high polyamine affinity, see below) require reduction of the PEC stability, this can be done using charged short-chain oligomers.

The stability of a PEC also depends on the nature of salt added. The order of anions and cations of 1,1-electrolytes with respect to the ability to break down PMA/PEVP ion pairs is as follows: $Br^- > Cl^- > F^-$ and $Li^+ > Na^+ > K^+ \gg Me_4N^+$ (Ref. 37). The specific affinities of (i) halide anions to polyamines and (ii) alkali metal cations to polycarboxylic acids obey the same pattern. Since bulky tetramethylammonium cation, $Me_4N^+$, weakly binds polyanions due to steric hindrances and the $F^-$ anion has the lowest polyamine affinity, the presence of these species in solution has almost no effect on the stability of PECs. Thus, the efficiency of the influence of various anions and cations on the stability of complexes can be best studied using tetramethylammonium salts and metal fluorides, respectively. In addition, these salts allow the phase behaviour of water–salt PEC solutions to be changed over a wide range.[37]

It is noteworthy that replacement of one anion by another has a stronger effect on the PEC stability than replacement of the cation (Table 1). Thus, by introducing a certain halide anion into solution one can efficiently influence the PEC existence region.

**Table 1**. Critical salt concentration corresponding to complete dissociation of polyelectrolyte complex.

| Complex | Critical salt concentration /mol litre$^{-1}$ | | | | | | | | | Ref. |
|---|---|---|---|---|---|---|---|---|---|---|
| | LiCl | NaCl | KCl | KBr | KI | KF | $Me_4NBr$ | $Me_4NCl$ | $Me_4NF$ | |
| PMA/PEVP[a] | 0.41 | 0.47 | 0.56 | – | – | – | 0.72 | 1.6 | > 6 | 37 |
| DNA/PEVM[b] | 0.22 | 0.23 | 0.25 | 0.14 | 0.07 | 1.0 | 0.18 | 0.43 | > 2 | 30 |

[a] $P_{PMA} = 2150$, $P_{PEVP} = 390$, 20 °C. [b] $P_{PEVP} = 100$, 20 °C.

## 2. Water-soluble non-stoichiometric polyelectrolyte complexes and their self-assembly in solutions

In spite of high stability towards dissociation into individual components, PECs can undergo interpolyelectrolyte reactions accompanied by transfer of polyions from some complexes to other ones. This conclusion was first drawn in studies of the properties of an insoluble stoichiometric PEC (SPEC) formed by mixing equimolar amounts (with respect to charges) of ionene polycation and polystyrenesulfonate (PSS) anion.[38] As PSS was added to a suspension of insoluble SPEC, the system gradually became more transparent. In the presence of a threefold excess of the polyanion, the suspension completely dissolved owing to transfer of the polycation from the SPEC to the PSS chains added and the formation of a soluble non-stoichometric PEC (NPEC).

The discovery of soluble polyelectrolyte complexes was a breakthrough in studies of interpolyelectrolyte reactions. Indeed, in this case polyions were transferred under homogeneous conditions, which allowed the potential of the whole bunch of methods developed to study polymer solutions to be used for investigations of these reactions. In addition, solubility of NPECs in aqueous media significantly enhances prospects for practical applications of these systems.

However, the solubility of NPEC in aqueous media may also lead to undesired results. For instance, the formation of soluble NPECs reduces the efficiency of separation of biological mixtures and precludes layer-by-layer adsorption of differently charged polyions, which recently has been widely used for fabrication of ultrathin multilayer polyelectrolyte films and nanocapsules. Therefore, the conditions for formation of soluble NPECs, as well as their structure and properties deserve particular attention.

Soluble NPECs can be prepared from various pairs of polyelectrolytes of different structures. The conditions for preparation of NPECs are considered in reviews.[3, 39] A simple and universal method of synthesis of NPECs involves mixing of nonequimolar amounts (with respect to charges) of aqueous solutions of oppositely charged polymeric components at such pH values at which they coexist in the charged state. Mixtures are prepared in the presence of a relatively small amount of a low-molecular-mass electrolyte. Apparently, mixing of polyion solutions unavoidably leads to local supersaturations and subsequent formation of complexes of various chemical composition including insoluble SPECs. Nevertheless, the final product, that is, the soluble NPEC, has a homogeneous composition. A light scattering study of solutions [40] confirmed that the molecular mass and size of NPECs are independent of the order in which solutions of the components are mixed, being comparable with the molecular characteristics of the starting polymers. In other words, NPECs should be treated as new macromolecular compounds consisting of a finite number of electrostatically bound chains. In particular, NPECs can be comprised of a relatively long polyanion chain and a number of short polycation chains, which is governed by the ratio of the charges in the mixture.[40] These data provide a direct proof of self-assembly of NPEC particles in water-salt solutions, which occurs by transfer of polyions from one complex on another until an equilibrium state corresponding to uniform distribution of short polycations over all long polyanion chains.

Spontaneous formation of a soluble negatively charged NPEC by mixing aqueous solutions of PSS anion and ionene polycation [38] points to a rather fast transfer of polyions in this system, which does not require the addition of a low-molecular-mass electrolyte to favour the exchange reaction due to weakening of the binding between the components of the complex. Most probably, interpolyelectrolyte interaction in this system is initially weakened by the violated structural complementarity between the reacting chains due to a long charge-to-charge separation in the ionene polycations.

The results of a study of mixtures of high-molecular-mass PMA anion with PEVP-β cations of various length and charge density clearly indicate that good correspondence between the charges on the interacting chains plays a negative role because it hampers self-assembly of NPECs.[41] If mixing of aqueous solutions of electrostatically complementary highly charged PMA and PEVP ($\beta = 92\%$, $P = 100$) led to formation of an insoluble complex, the use of PEVP-β having the same chain length and a halved number of charges accelerated the exchange reaction to such an extent that attainment of the equilibrium state of the system, which corresponded to the formation of soluble NPECs, took a few minutes. It is noteworthy that a sixfold elongation of the polycation chain had no effect on the behaviour of the system. In this case the use of the highly charged PEVP (degree of alkylation of 91% and $P = 600$) also imposed a kinetic prohibition on exchange, whereas the PEVP-β-cation with a degree of alkylation of 50% readily entered the reaction, as indicated by rapid formation of a soluble NPEC. These data clearly indicate that the exchange kinetics is governed by the density of the charge on chains rather than the number of ion pairs formed.

Exchange between polyions can be accelerated by simply adding small amounts of a low-molecular-mass electrolyte, which will also cause local distortions in the system of ion pairs formed by complementary chains. For instance, the introduction of sodium chloride into a kinetically 'frozen' mixture of aqueous solutions of highly charged PMA and PEVP has such a strong effect that the formation of a soluble NPEC in a 0.05 M solution of the salt is completed during the mixing of the starting polyelectrolytes. Note that the NPEC remains quite stable at this ionic strength of the solution and complete dissociation of the NPEC requires that the salt concentration be ten times higher.[41]

According to terminology used in Ref. 3, a soluble NPEC consists of a lyophilising polyelectrolyte (LPE, host) and an oppositely charged blocking polyelectrolyte (BPE, guest). The molar ratio ($\varphi$) of the charged groups of BPE and LPE should not exceed a certain critical value, $\varphi = [\mathrm{BPE}]:[\mathrm{LPE}] < \varphi_{cr}$, close to 0.5. Ion-pair stabilised fragments of the LPE and BPE chains form hydrophobic domains that are prone to aggregation in aqueous media with the formation of a hydrophobic core. A hydrophilic shell of the hydrophobic core consists of charged LPE segments responsible for solubility of the NPEC. These structural elements are exposed to solution in the form of loops or tails (Fig. 1). Negatively charged NPECs with relatively long lyophilising polyanions (type **A** NPECs), which meet the condition $\psi = P_{\mathrm{LPE}}:P_{\mathrm{BPE}} > 1$, have been best studied.[3] Type **B** complexes containing short lyophilising polyanions ($\psi < 1$) were obtained twenty years ago,[42, 43] but information on these systems was scarce until recently.

The formation of type **B** NPECs is accompanied by thermodynamically unfavourable decrease in entropy of the system owing to a decrease in the total number of polymeric particles. The shorter the LPE chains (i) the larger the number of these chains required to form a complex with a molar ratio of units no greater than $\varphi_{cr}$, which provides solubility of the NPEC, and, therefore, (ii) the smaller the number of polymeric particles in the solution. A consequence of this undesired factor is a low stability of the type **B** NPECs in salt media.[44, 45] The critical salt concentration corresponding to the onset of phase separation in solutions of type **B** NPECs

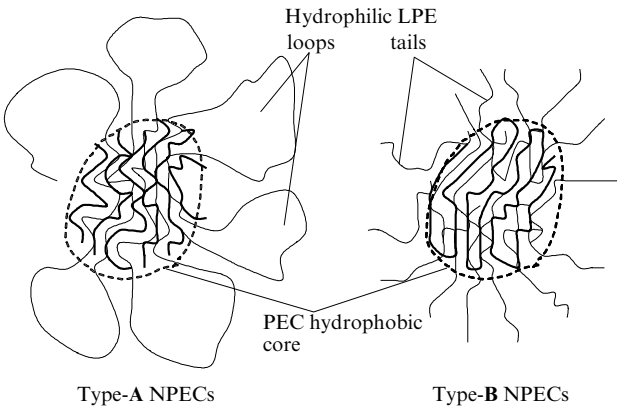



**Figure 1.** Schematic representation of the structure of soluble NPECs formed by lyophilising (LPE) and blocking (BPE) polyelectrolytes with different ratios of the degrees of polymerisation $\psi = P_{LPE} : P_{BPE}$.
**A**: $\psi > 1$, **B**: $\psi < 1$.

($\psi < 1$) abruptly decreases as $\psi$ decreases, being almost independent of the parameter $\psi$ in solutions of the type **A** NPECs ($\psi > 1$) (Fig. 2).

Thus, for the type **B** NPECs the interval of the ionic strength values in which the concentration of salt introduced is sufficiently high for intense exchange of the components to proceed but not too high for phase separation to occur abruptly narrows as $\psi$ decreases. This complicates the preparation of the type **B** NPECs by mixing water–salt polymer solutions.[46,47] It is also believed that the formation of soluble NPECs from pairs of polyelectrolytes characterised by $P_{LPE} : P_{BPE} < 1$ is impossible.

Preparation of positively charged soluble NPECs can be precluded by high affinity of conventionally used halide anions towards positively charged alkylated polyamines (*e.g.*, PEVP), which strongly reduces their lyophilising ability in the sequence $F^- \ll Cl^- < Br^- < I^-$. Iodide anions so strongly bind the pyridinium groups that the high-molecular-mass PEVP cation

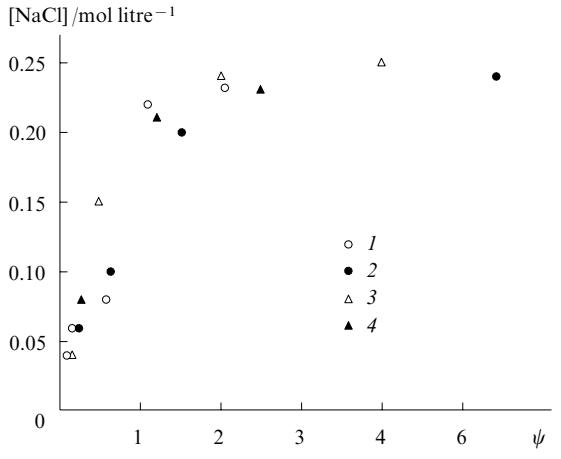



**Figure 2.** Critical salt concentration corresponding to the onset of phase separation plotted *vs.* ratio of the degrees of polymerisation of components ($\psi = P_{LPE} : P_{BPE}$) in solutions of negatively charged NPECs formed by the PMA anion (*1*, *2*)[45] or hydrolysed DIVEMA (copolymer of divinyl ether with maleic anhydride) polyanion (*3* and *4*)[44] and PEVP cations of different degree of polymerisation.
$P_{BPE}$: 900 (*1*), 560 (*3*) and 340 (*2*, *4*).

salts out and becomes insoluble even in a 0.1 M KI solution. To prepare a soluble NPEC with the lyophilising PEVP cation containing $Br^-$ anions as counterions the anions were replaced by $F^-$ counterions that almost do not participate in binding.[48]

Recently,[45] both types of positively charged soluble NPECs formed by the lyophilising PEVP bromide and blocking PMA anion (Fig. 3) were prepared and studied. As in the case of the negatively charged NPECs (see Fig. 2), the soluble type **A** complexes ($\psi > 1$) remained stable over a rather broad interval of ionic strength values, whereas the existence region of the soluble type **B** complexes ($\psi < 1$) became narrower as $\psi$ decreased; this is due to a decrease in the lyophilising ability of the PEVP cation. Oligomeric short-chain cations did not form soluble NPECs at all. All other things being equal, the positively charged NPECs were soluble in a narrower range of ionic strength values compared to the negatively charged analogues. This is consistent with the strong binding of $Br^-$ anions to pyridinium groups of the lyophilising PEVP.

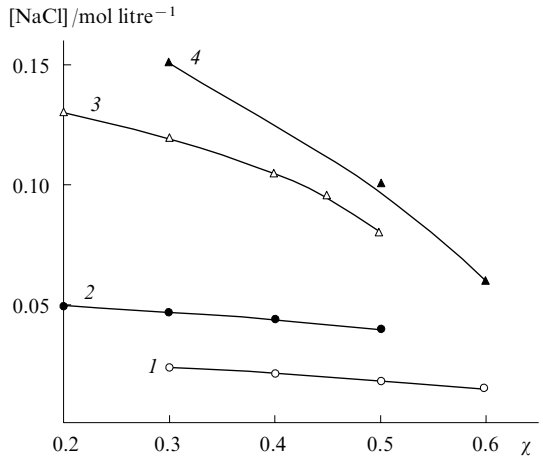



**Figure 3.** Critical salt concentration corresponding to the onset of phase separation plotted *vs.* ratio of charged groups ($\chi = [-]:[+]$) in solutions of positively charged NPECs formed by PEVP cation ($P = 1640$) and PMA anion (curves *2* and *3*) or DNA (curves *1* and *4*) at different $P_{LPE} : P_{BPE}$ ratios.
$\psi = 0.1$ (*1*), 0.4 (*2*), 2 (*4*) and 7 (*3*).
The curves were plotted using the data obtained in Ref. 45.

The data listed above can be of interest for determining conditions for quantitative transfer of the complex from solution to precipitate. In principle, undesired formation of a soluble NPEC can be avoided by taking equivalent amounts of positively and negatively charged groups in aqueous or aqueous salt solutions of the polyelectrolytes being mixed. However, in practice it is difficult and in some cases impossible to meet this condition.

For instance, layer-by-layer adsorption of differently charged polyelectrolytes (see above), which is widely used for preparation of multilayer films and capsules,[49–52] involves alternating treatment of the material with solutions of the polyanion and polycation; in each step the top layer of the adsorbed polyelectrolyte is exposed to a large excess of the oppositely charged partner. To prevent solubilisation of the complex and degradation of the material,[53–55] one can use relatively short-chain polycations containing $Br^-$ anions as counterions, because their overdosage will not result in the formation of a soluble, positively charged NPEC in water–salt media. Based on the same considerations, treatment of a film

with an excess amount of short-chain polyanions will help to avoid the formation of a negatively charged soluble NPEC, which leads to erosion of polyelectrolyte multilayers.

Interrelations between the phase behaviour of a soluble NPECs and the efficiency of formation of multilayer films and capsules from the polymer components were established.[56–58] This permits strict control of the transition from the stable layer deposition regime to the layer erosion regime upon variation of conditions (pH value, ionic strength of solution, ratio of the degrees of polymerisation of polyions, *etc*.). From the data collected and analysed in a review [59] it follows that the available information on the stability of NPECs, the phase state of their water–salt solutions and the kinetics of interpolyelectrolyte exchange can serve a basis for constructing a 'map' of the regions of stable growth or degradation of layers. This is necessary for successful formation of a multilayer polyelectrolyte material with specified properties.

Note that reversible transition between the stable layer deposition and the layer degradation regimes can be due to minor changes in external conditions. A remarkable example is provided by pH-dependent stabilisation of products of the interaction of polycarboxylic acid salts with polyamines.[19, 21] The stabilisation of products occurs under mild conditions in narrow ranges of pH values, being controlled by the acidity of the polymeric carboxylic acids and/or by the charge density on the polyamine chains.† Transfer of such systems to neutral or weakly acid media is accompanied by a stepwise increase in the ionic strength of the solution, which is necessary for full breakdown of the complexes owing to formation of a network of hydrogen bonds between protonated carboxylic groups of the polymeric acid in the complex.

## 3. Molecular 'recognition' in solutions of non-stoichiometric polyelectrolyte complexes

The use of soluble NPECs made it possible to carry out detailed studies of interpolyelectrolyte reactions in which two likely charged lyophilising polyions compete for binding to an oppositely charged blocking polyelectrolyte:

$$\mathrm{LPE}/n\,\mathrm{BPE} + \mathrm{LPE}^* \rightleftharpoons \mathrm{LPE}/(n-x)\mathrm{BPE} + \mathrm{LPE}^*/x\,\mathrm{BPE}. \qquad (2)$$

If the LPE* chains added have the same chemical nature as the LPE chains in the initial complex, transfer of the BPE chains is treated as an exchange reaction. Otherwise, the case in point is an interpolyelectrolyte substitution reaction.[3]

From the results of the kinetic and mechanistic studies of interpolyelectrolyte reactions (for the most comprehensive treatment, see Ref. 60) it follows that exchange of components in salt-free aqueous solutions is, as a rule, kinetically forbidden. To switch it on, it is sufficient to add a rather small amount of a low-molecular-mass electrolyte, which favours rapid attainment of an equilibrium state in the system corresponding to continuous transfer of the components from one soluble NPECs to another. Exchange is neither more nor less than a form of thermal motion of PECs that are products of reversible ionic interpolyelectrolyte reactions.

The position of equilibrium in reaction (2) can be shifted over a wide range from the equilibrium distribution of BPE chains between the LPE and LPE* of the same length ($x = n/2$) to highly selective binding of BPE to LPE* chains ($x \approx n$). The latter case is of particular value for the design of systems for targeted delivery of pharmaceuticals and for the development of self-regulatory polyelectrolyte systems; therefore, it deserves a detailed consideration.

The phenomenon of molecular 'recognition' in NPEC solutions and the factors responsible for selective binding of polyions were analysed in reviews.[9, 61] A polycation 'recognises' a polyanion that forms the most stable NPEC with the polycation even if the free energy difference per pair of oppositely charged units is rather small.

For instance, in mixtures of solutions of long-chain and short-chain PMA anions the PEVP cation preferably binds the long-chain PMA anions. If the degrees of polymerisation of polyanions differ appreciably, a competing reaction results in quantitative displacement of short-chain PMA anions ($P = 50$) by the long-chain ones ($P = 4100$) from the NPECs.[42] A substitution reaction involving polyphosphate (PPH) anions occurs in a similar fashion. In 0.3 M sodium chloride solutions, PPH anions with relatively high molecular mass ($P = 230$) replace almost all chains of the PMA anion in the PMA/PEVP complex, whereas the short-chain PPH anions ($P = 20$) cannot compete with the PMA anion for binding to the PEVP cation under the same conditions.[62]

Covalent attachment of hydrophobic pyrenyl residues, which additionally stabilise the PMA/PEVP complex, to the PMA chain can lead to selective binding of the polyanion to the polycation. Even a single pyrenyl group per 400 units in the PMA macromolecule is sufficient for unambiguous 'recognition' of the polyanion by the PEVP cation among unlabelled PMA anions,[63] despite the fact that the complex-stabilising groups are 'pygmies' compared with the competing polyanions. The high selectivity of the interaction offers great prospects for the design of polyelectrolyte systems for targeted delivery of pharmaceuticals and genetic material to cells, improvement of the efficiency of separation of biopolymer mixtures and for elaboration of highly sensitive analytical systems based on polyion conjugates with antibodies, antigens or antigenic determinants.

The role of stabilising groups ('anchors') can successfully be played by sulfate or sulfonate groups present in polyanions (sulfated/sulfonated polyanions). These groups make polyanions particularly 'attractive' to polycations. In mixtures of negatively charged polysaccharides the PEVP cation correctly recognises sulfated polysaccharides and binds them. If a mixture contains polyanions that bear no sulfate groups but are much highly charged, the binding remains selective [64] even if such polyanions are in large excess with respect to the sulfated/sulfonated polyanions. The pronounced preferableness of polyelectrolyte interaction of polyamines and sulfated/sulfonated polyanions makes it possible to use polyamines as a sensitive probe for determining polysaccharide chain fragments bearing sulfate groups and blocking them. The same principle can underlie investigations of structure–function relationships in the case of heparin-like polyanions.[65]

Polyanions with high density of sulfonate or sulfate groups in the chain [*e.g*., poly(styrenesulfonate), poly(vinyl sulfate) and poly(anetholesulfonate) anions, heparin] form abnormally stable complexes with polyamines. Very high specific affinity of the sulfated/sulfonated polyanions to polycations imposes severe kinetic restrictions on poly-ion exchange. This makes the synthesis of soluble NPECs much more difficult, because mixing of the strongly interacting components inevitably leads to formation of insoluble products of variable composition.

† The role of polyamines in such pairs can be played by base proteins, in particular, lysozyme or ribonuclease.[20] This makes it possible to prepare pH-sensible multilayer protein–polyelectrolyte films and capsules appropriate for the use under physiological conditions.

For this reason, polymers with high density of sulfate or sulfonate groups are most widely used to design polyelectrolyte multilayer films and capsules.

Some investigators believe that sulfated/sulfonated polyanions are inappropriate for the formation of soluble NPECs (see, *e.g.*, Refs 66 and 67). Nevertheless, by choosing the concentration of salt added (usually, for sulfated/sulfonated polyanions it should be ten times higher than for polycarboxylate or polyphosphate anions), one can minimise kinetic hindrances to exchange and to prepare a soluble NPEC. In the case of high-molecular-mass PEVP and PSS, this can be done by gradual mixing of water – salt solutions of the components containing 0.14 mol litre$^{-1}$ KCl.[68] Recently,[45] it was established that the existence region of soluble NPECs based on sulfated/sulfonated polyanions is much broader than those of other NPECs, because phase separation in their solutions occurs at much higher concentration of the salt added. As for other polyelectrolyte systems, the extension of this region is controlled by the ratio, $\psi$, of the degrees of polymerisation of the components.

Sulfated/sulfonated polyanions effectively compete with polycarboxylate or polyphosphate anions for binding to polyamines.[68, 69] This property underlies the use of soluble heparin-based NPECs as a 'depot' of polyamines that act as antiheparin agents.[3] Abnormally high affinity of sulfated/sulfonated polyanions to polycations is of great practical interest and may be used in, *e.g.*, the final stages of separation of (bio)polymer mixtures, where quantitative isolation of anionic components from insoluble PECs or PPCs is required. Irreversibility of the substitution reactions involving sulfated/sulfonated polyanions ensures complete regeneration of oligonucleotides, plasmids, proteins (enzymes) and protein conjugates.

If the polycation affinities of the competing polyanions do not differ much, the direction of interpolyelectrolyte substitution reaction can to a great extent be governed by the properties of the environment. For instance, competing binding of the PMA and PPH anions to the PEVP cation

$$\text{NPEC(PMA/PEVP)} + \text{PPH} \rightleftharpoons \text{NPEC(PPH/PEVP)} + \text{PMA} \quad (3)$$

is efficiently controlled by the nature of salt added.[62] The order of the alkali metal cations with respect to the ability to shift the equilibrium (3) to the right is as follows: $Li^+ > Na^+ > K^+$. This is in good agreement with the differences between the affinities of these cations to the PMA and PPH anions. Although these differences are rather small, replacement of one salt by another leads to fundamental changes in the behaviour of the ternary system. This fact deserves particular attention. For instance, the equilibrium in reaction (3) is completely shifted to the right in 0.3 M LiCl and to the left in 0.3 M KCl; the case of 0.3 M NaCl corresponds to an intermediate situation.

The equilibrium in reaction (3) is sensitive to temperature variation in the range 5 – 60 °C.[70] The PEVP cation preferably binds the PPH anion at low temperatures and the PMA anion on heating. Such a change in the direction of reaction (3) is consistent with the change in the stability of the complexes NPEC(PMA/PEVP) and NPEC(PPH/PEVP) in this temperature ranger. However, if the stabilities of the starting and resultant complexes differ significantly, the direction of substitution becomes temperature independent. For instance, in contrast to PEVP the poly-(*N*-methyl-4-vinylpyridinium) cation forms a much more stable NPEC with the PPH anion than with the PMA anion; therefore, the equilibrium of competing binding in this mixture is shifted towards the formation of the polyphosphate complex in the whole temperature range (from 5 to 60 °C).[70] Of course, in ternary systems 'polyanion – polycation – sulfated/sulfonated polyanion' in which the sulfated/sulfonated polyanions have an undisputable advantage in binding to polycations over another polyanions, the temperature factor is also insignificant.

Self-assembly of complexes and their transformations due to changes in external conditions occur as 'trials and errors' as a result of exchange or substitution reactions that can proceed selectively, reversibly and at a high rate. This concept of PECs and their properties is fundamental. The principles of competitive binding and chain transfer discovered in the investigations of solutions of synthetic polyions can be extended to reactions involving natural polymers. Soluble NPECs (products of co-operative interaction of polyions) can be treated as complexes of charged biopolymers. Both types of complexes are spontaneously formed in aqueous media, thus obeying general laws of polyelectrolyte interactions that are controlled by the same specific factors. This follows from the results of investigations of PECs formed by (i) synthetic polyelectrolytes with oppositely charged biopolymers, namely, nucleic acids, globular proteins (enzymes, antibodies) and (ii) protein conjugates with polyions.

## III. Interpolyelectrolyte reactions in polyplex solutions

High charge density of native DNA is due to negatively charged phosphate groups of the double helix. DNA–polyamine PECs attract increasing attention as potential non-viral agents for delivery of genetic material to cell nuclei. The use of such systems improves the efficiency of transfection of plasmid cells and protects DNA delivered from splitting by cell nucleases (see reviews 5, 7, 8, and 71 – 73). The promise of polycation-assisted DNA delivery to target cells in a packed form gave an impetus to intensive research of polyplexes and search for factors that allow fine tuning of their stabilities.

### 1. Stability of DNA-containing polyelectrolyte complexes

The formation and dissociation of DNA complexes can conveniently be monitored by the fluorescence quenching method‡ using intercalating dyes, in particular, ethidium bromide. The technique is based on the ability of ethidium cation to be spontaneously incorporated (intercalated) between bases of the DNA double helix, which causes a considerable increase in the dye fluorescence intensity. Competing displacement of the intercalating agent by the polycation added, which forms a stable complex with DNA, is accompanied by fluorescence quenching. Subsequent degradation of the PEC using a low-molecular-mass polyelectrolyte causes enhancement of fluorescence due to incorporation of ethidium cation into the releasing fragments of DNA. The applicability of this approach to investigations of polyplexes in solutions and competing reactions involving them was first experimentally substantiated in 1995.[75] Since then, numerous new data obtained using this technique were reported. The most interesting of them will be analysed here.

The efficiency of the destructive action of cations and anions introduced with respect the PEC (DNA/PEVP) decreases in the order $Ca^{2+} > Mg^{2+} \gg Li^+ > Na^+ >$

‡ The essentials and potential of the fluorescence quenching method as well as prospects offered by the technique have been comprehensively treated in a review.[74]

$K^+ \gg Me_4N^+$ (Refs 74–77) and $I^- > Br^- > Cl^- \gg F^-$ (Ref. 77), respectively. The affinity of the counterions to DNA and PEVP decreases in the same order. Similar interrelation between the ability of cations and anions to break down PECs and the affinities of these species treated as counterions to polymeric components were established in studies of the systems PPH/PEVP (Ref. 63) and PMA/PEVP (Ref. 37). A study of PECs of DNA with various quaternised polyamines revealed a similar order of cations, namely, $Ca^{2+} \geqslant Sr^{2+} \gg Li^+ > Na^+ \geqslant K^+ > Cs^+$ (Ref. 78).

The dependences of the stabilities of the complexes DNA/PEVP (Ref. 30) and PMA/PEVP (Ref. 36) on the PEVP cation chain length obey an identical pattern. The critical salt concentration necessary for complete dissociation of both complexes was independent of the chain length of the high-molecular-mass DNA and PMA anions, but abruptly increased with an increase in the PEVP cation chain length up to $P_{PEVP} \approx 100$ and then increased again, but to a lesser extent.

The nature of polycation amino groups strongly influences the stability of complexes.[79] Dissociation of the polyplexes formed by polycations with quaternised amino groups in the chain is independent of pH value, being most sensitive to the destructive action of the salt added. Polycations bearing primary amino groups form the most stable complexes with DNA; they are stable over a broad range of changes in pH value. Polyplexes based on polycations with tertiary amino groups are intermediate in stability, which strongly depends on the pH value of the medium.

The same trend was also observed in studies of lipoplexes, that is, DNA complexes with cationic micellae formed by $C_{12}$ surfactants containing amino groups of various structure in hydrophilic 'heads'.[80] The lowest stability was found for the lipoplexes formed by $n\text{-}C_{12}H_{25}N^+Me_3Br^-$ molecules in which the quaternised nitrogen atom was surrounded by bulky methyl groups. The decrease in the number of Me groups in the surfactant 'head' led to significant stabilisation of the complex. The critical salt concentration corresponding to complete dissociation of lipoplexes increased in the order $n\text{-}C_{12}H_{25}N^+Me_3Br^- < n\text{-}C_{12}H_{25}NMe_2 < n\text{-}C_{12}H_{25}NHMe < n\text{-}C_{12}H_{25}NH_2$. As in polyplexes, the lipoplex in which the DNA partner contained primary amino groups was most stable.

The data listed above indicate an important role of charge-to-charge separation in ion pairs; as this distance increases, interpolyelectrolyte interaction weakens appreciably. This is also confirmed by the results of a study of polyplexes with poly(*N*-alkyl-4-vinylpyridinium) cations containing *N*-alkyl substituents of various length.[18] An increase in the number of methylene groups in the substituent from 1 to 6 was accompanied by successive decrease in the salt concentration necessary for dissociation of the complex. This effect was particularly pronounced for polyamines with the longest methylene fragments that created steric hindrances to tight contact between charged pyridinium groups of the polycation and DNA phosphate groups.

It should be noted that enhancement of hydrophobicity of the side groups with an increase in their length did not preclude destabilisation of the complexes, although it is known that hydrophobic interactions between non-polar fragments of polycations and hydrophobic DNA bases can make polyplexes more stable towards the action of salt, as is the case of, *e.g.*, binding of DNA to soluble poly(pyridylphenylene) dendrimers.[13] Probably, in the case of linear polycations hydrophobic interactions contribute insignificantly to stabilisation of complexes and cannot compete with the pronounced weakening of electrostatic interaction between components. Nevertheless, hydrophobic interactions to some extent affected transfection; this manifested itself as an increase in transfection efficiency with chain elongation in the *N*-alkyl substituents in the polyamine [18] due to well-known effect of strengthening of the binding of the polyplex to cell membrane upon introduction of hydrophobic fragments into the cationic component.[81, 82]

Primary polyamines can form very stable PECs and are therefore widely used for preparation of polyelectrolyte multilayer films and capsules. Among polyelectrolyte systems employed to this end, polyallylamine/PSS pairs are most widely used, because abnormally high mutual affinities of the primary polyamine and sulfated/sulfonated polyanions impose strict kinetic prohibitions on interpolyelectrolyte exchange, thus excluding undesired formation of soluble NPECs in the course of film preparation.

Thus, the stability of DNA-containing complexes can be efficiently controlled by varying the chain length, charge density and structure of the polycation, as well as using various low-molecular-mass electrolytes. To make the effect more pronounced, one can combine these factors.[30, 79]

Owing to high charge density of phosphate groups and regular arrangement of these fragments on the rigid DNA double helix, the DNA offers an advantage over other anionic cell components in electrostatic binding with polycations. For instance, RNA molecules, which mainly consist of single-stranded fragments prone to formation of inner loops and hairpins, have a complex and disordered structure, which leads to weakening of electrostatic interaction with polycations. In addition, RNAs are characterised by a broad molecular mass distribution with a large proportion of oligomeric chains, which is due to the variety of RNA types in cell and the presence of their degraded forms. As a result, RNA release from complexes with polyamines upon addition of low-molecular-mass electrolytes completes at such a concentration of the salt added when the complexes of the same polyamines with DNA are still quite stable.[83, 84]

These differences in stabilities of DNA- and RNA-containing complexes may serve as a basis for separation of DNA/RNA mixtures. The efficiency of the procedure is determined by correct choice of the ionic strength of the solution, at which the polyamine – RNA complex completely dissociates and DNA bound to polyamine precipitates. In the case of quaternised polyamines, this occurs in the range 0.3 M – 0.5 M NaCl. Quantitative precipitation of DNA from DNA – RNA mixtures makes it possible to elaborate a simple, cheap, efficient and large-scale method for isolation of DNA (plasmids) from cell lysates.[85, 86]

## 2. Selectivity of interpolyelectrolyte interaction in polyplex solutions

Competitive reactions involving PPH polyanions and DNA (in both cases the charge is due to the presence of phosphate groups) have much in common. A substitution reaction in DNA solutions

$$\text{NPEC(PMA/PEVP)} + \text{DNA} \rightleftharpoons \rightleftharpoons \text{NPEC(DNA/PEVP)} + \text{PMA} \qquad (4)$$

is as sensitive to the concentration and nature of the low-molecular-mass electrolyte added as reaction (3) involving PPH. In terms of the ability to influence the direction of reaction (4), alkali metal cations can be arranged in the

sequence: $Na^+ > K^+ > Li^+$. At a specified concentration of salt added (0.25 mol litre$^{-1}$), the equilibrium (4) is essentially shifted to the right in the NaCl solution, being completely shifted to the left in the LiCl solution. An intermediate situation occurs in the case of KCl solution.[77] The order of cations presented above differs from the order $Li^+ > Na^+ > K^+$ established for reaction (3), which can be explained by abnormally high affinity of $Li^+$ cation to native DNA. Denatured DNA does not possess this property and the effect of the nature of cation added to denatured DNA solutions on the equilibrium (4) is similar to that obtained for reaction (3), namely, $Li^+ \approx Na^+ > K^+$.

In this case, the ratio of the degrees of polymerisation of components $\psi = P_{PMA} : P_{PEVP}$ also governs the direction of substitution.[76] If a negatively charged type **A** NPEC (PMA/PEVP) (see Fig. 1, $\psi > 1$) is used, PMA wins in the competition with DNA for binding to PEVP. If a type **B** complex ($\psi < 1$) is used, a decrease in $\psi$ makes the formation of a polyplex increasingly preferable due to entropy gain owing to an increase in the total amount of particles in solution.

The ability of aliphatic *n*,*n*-ionene cations to recognise and bind DNA or PMA in equimolar mixtures of these polyanions

$$\text{NPEC(PMA/ionene)} + \text{DNA} \rightleftharpoons \text{NPEC(DNA/ionene)} + \text{PMA} \quad (5)$$

is governed by the ionene chain length and the number ($n$) of methylene groups between charges in the ionene chain.[87] Oligomeric ionenes are not prone to recognition of competing high-molecular-mass polyanions; however, an increase in ionene chain length makes the interaction selective and the selectivity increases as $n$ increases. Highly charged ionenes preferably bind PMA anions, whereas low charged ionenes bind DNA.

The equilibrium (5) is completely shifted to the left for 3,3-ionene polycations with a sufficiently high degree of polymerisation ($P \approx 100$) and to the right for 10,10-ionene bromide.

Self-assembly of NPECs in aqueous DNA solutions and selectivity of competing reactions involving NPECs can be of great importance for the design of 'smart' polyelectrolyte carriers capable of releasing DNA delivered upon variation of external conditions.

### 3. Methods of improvement of transfection efficiency using polyplexes

Strong binding to negatively charged cell membrane requires that a polyplex be oppositely charged. A very small fraction of soluble, positively charged products of DNA–polycation interaction is involved in delivery of genetic material to cell.[88] In this connection, information on studies of soluble, positively charged polyplexes is of particular interest. Recently,[89] an intimate interrelation has been established between the efficiency of cell transfection using DNA molecules packed in a positively charged complex with a polyamine and the stability of this complex to the low-molecular-mass electrolyte added. The development of a strategy of preparation of soluble, positively charged polyplexes that remain stable at physiological values of pH and ionic strength is a challenge faced by researchers working in the field of physical chemistry of biopolymers.

Solution to this problem is precluded by low lyophilising ability of polyamines and high molecular mass of DNA. As mentioned above, positively charged soluble NPECs have relatively low stabilities in water–salt media owing to high affinities of halide counterions to amino groups of the lyophilising polycation. In addition, traditionally used polyamines are much shorter than plasmids and therefore form with them soluble type **B** NPECs (see Fig. 1), which are particularly sensitive to salt added. Phase separation in such systems occurs very rapidly upon salt addition,[45,83] being almost independent of the charge-to-charge ratio $\chi = [-]:[+]$ in the mixture (see Fig. 3, curves *1* and *2*).

Positively charged type **A** NPECs ($\psi \geqslant 1$) are more stable, specifically at low $\chi$ values (see Fig. 3, curves *3* and *4*). It is noteworthy that the left portion of curve *4* for the type **A** NPECs ($\psi \approx 2$) with relatively short DNA molecules (300–500 base pairs) is in the region of ionic strength values similar to physiological ones. Thus, preparation of a positively charged soluble polyplex from a rather long nucleic acid molecule requires the use of a long-chain polyamine that meets the condition $\psi > 1$. Lyophilising polyamines for oligonucleotide delivery to cell nuclei can be chosen much easier, because in this case the condition $\psi > 1$ can be met with ease for a broad range of conventional high-molecular-mass polycations.

Another approach to enhancement of cell transfection is to use the effect of charge of a weak polyelectrolyte on highly charged matrix[3] (see above). Transfection is a complex multistage process that begins with hydrophobic and electrostatic binding of a polyplex to the membrane and its penetration into a cell by the endocytosis mechanism. The key moment responsible for successful transfection is a fast release of the polyplex from endosomes and lysosomes to cytoplasm. The presence, in the polyamine molecule, of chain groups capable of ionising in weakly acidic media favours the process, which proceeds by the so-called 'proton sponge' mechanism.[90] In weakly acidic medium within endosomes and lysosomes, polyamine ionogenic groups act like sponges and uptake protons. Protonation causes their transformation to ionic groups and subsequent appearance of a charge gradient, which leads to supply of $Cl^-$ counterions, an increase in the osmotic pressure within endosomes and lysosomes, their swelling and subsequent break with DNA release to cytosol.

The hypothesis of 'proton sponge' was proposed for cationic polymers with $pK_a \approx 5.5$ that are capable of being protonated in a weak acid medium within endosomes and lysosomes. Partially alkylated PEVP-β cations, which are characterised by low $pK_a$ value ($pK_a = 3.25$), are protonated in the strongly acidic region;[91] however, the presence of DNA in the complex reduces the acidity by 2 to 3 units and favours the protonation of pyridine groups of the polymer, thus making the PEVP-β cations transfectionally active.[18] These data demonstrate the advantages of the use of partially alkylated tertiary polyamines as non-viral vectors for delivery of genetic material to cell nuclei.

## IV. Complexes of globular proteins with polyions

The kinetic and thermodynamic factors that control the formation of protein–polyelectrolyte complexes and their rearrangement in aqueous solutions were summarised and analysed in reviews.[9,92] Since soluble complexes of bovine serum albumin (BSA) with the PEVP cations were studied in detail, we will use this model system to illustrate the key features of the behaviour of PPCs.

Binding of the PEVP cations to negatively charged BSA globules may lead to formation of both soluble and insoluble complexes. Positively charged water-soluble type **C** PPCs (Fig. 4) are formed in the presence of a large (at least threefold)

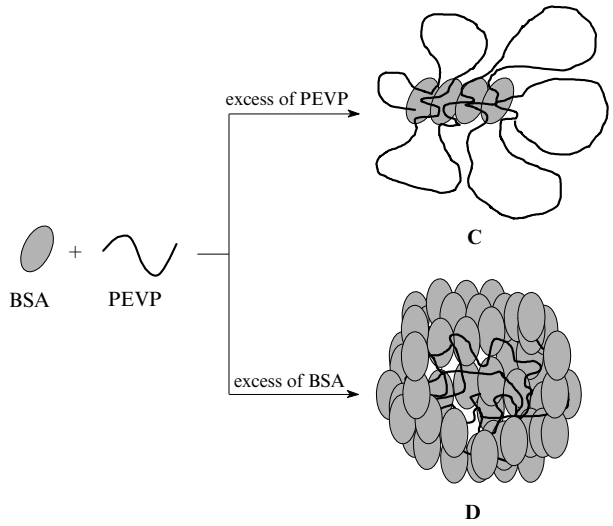



**Figure 4.** Structure of water-soluble PPC: a schematic drawing. **C** is the positively charged complex (excess of PEVP) and **D** is the negatively charged complex (excess of BSA).

excess of PEVP charged groups with respect to negatively charged groups of BSA that are arranged on the surface of protein globule and are accessible to interaction; PEVP chain fragments bearing non-compensated charges solubilise the complex.[93, 94]

The formation of soluble type **D** PPCs (see Fig. 4), in which the protein acts as the lyophilising component, requires a 2.5-fold excess of negatively charged groups of BSA.[95] Type **D** complexes contain one polycation chain and a number of protein globules that is necessary to make the PPC soluble. At intermediate concentrations of the positively and negatively charged groups, phase separation in the PEVP – BSA system occurs.

Spontaneous formation of water-soluble PPCs on mixing the components indicates that protein globules are transferred from one complex to another. Direct experimental proofs of transfer of globules in solutions of type **C** PPCs were reported.[96] The transfer kinetics was studied in solutions of a negatively charged type **D** PPC using a protein, a soybean trypsin inhibitor bearing a fluorescent label.[97] Protein transfer from one polycation to another occurred rapidly even in salt-free media and accelerated upon addition of a low-molecular-mass electrolyte. Competitive binding of protein to two different polycations was reversible and efficiently controlled by varying the content of a particular polycation in the mixture.

Molecular recognition of various plasma proteins by the PEVP cation was observed.[98] Soluble type **D** PPCs comprising of one high-molecular-mass polycation chain and one type of protein globules were detected in equilibrated mixture.

High-molecular-mass PEVP cation ($P = 650$) wins in the competition with oligomeric PEVP ($P = 30$) for binding to BSA.

$$\text{oligomer/BSA} + \text{PEVP} \rightleftharpoons \text{PEVP/BSA} + \text{oligomer}. \qquad (6)$$

The equilibrium in reaction (6) in type **D** PPC solutions is shifted to the right due to entropy gain owing to an increase in the total number of polymeric species in the solution.[99] The entropy factor also underlies the decrease in stability of the type **C** PPCs in salt media upon shortening of the PEVP cation chain. As for PECs, the destabilising effect becomes particularly pronounced for short-chain PEVP cations ($P < 100$).[99] The degree of polymerisation of charged chains also strongly affects their ability to solubilise type **C** PPCs at different (including elevated) temperatures. The importance of investigations of this effect is due to a great practical value of the problem of stabilisation of globular protein (enzyme) solutions.

The possibility of controlling protein solubility becomes of particular value due to the key role played by protein aggrega-

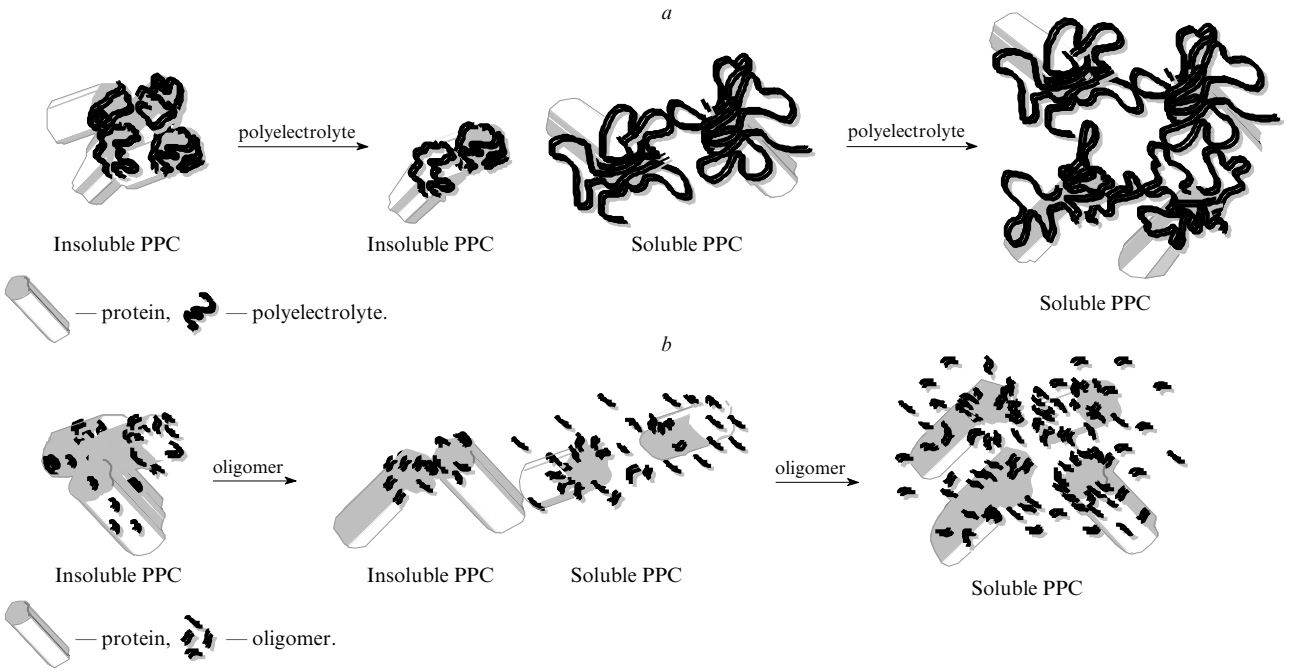



**Figure 5.** Conversion of insoluble PPC to a soluble complex upon adding high-molecular-mass polyion (*a*) or charged oligomer (*b*): a schematic drawing.

tion in the course of formation of insoluble amyloid plagues leading to neurodegenerative diseases. It was found that the formation of soluble PPCs precludes protein thermal aggregation and that the polyion chain length can act as a factor of efficient control of the process.[100–102]

A number of studies were carried out with glyceraldehyde 3-phosphate dehydrogenase (GAPDH), an oligomeric enzyme comprising four subunits, as a model protein.[100–102] The enzyme was used to study protein folding mechanisms and protein behaviour under heat shock.[103] Not only are the GAPDH molecules prone to aggregation, but also participate in the formation of amyloid plagues responsible for the development of Alzheimer's and Huntington's diseases and bovine spongiform encephalopathy.[104] It was shown [101] that very short oligomeric PMA anions cannot have a strong effect on thermal aggregation of positively charged GAPDH.

Chain elongation in the PMA anion, as well as an increase in the proportion of the PMA anion in the mixture favour the formation of soluble PPCs (Fig. 5) and the use of a sufficient amount of high-molecular-mass PMA anions completely inhibits thermal aggregation of positively charged GAPDH. For the negatively charged GAPDH, the same result can be attained by introducing excess amounts of high-molecular-mass PEVP cations.[102] It is interesting that partially alkylated PEVP-β cations appeared to be better stabilisers than completely alkylated PEVP-β cations and the ability of the former to inhibit GAPDH thermal aggregation significantly increased with an increase in the content of neutral hydrophobic pyridine units in the copolymer.§

Thermodynamic restrictions imposed on the formation of soluble type **C** oligomer/protein complexes should also be taken into account and, if possible, used in solving practical tasks. For instance, inability of short charged chains to solubilise PPCs requires a more careful choice of polyions to prevent protein thermal aggregation. On the other hand, proteins can be safely precipitated from solution using charged oligomers, because their overdosage does not influence the efficiency of isolation and insoluble PPCs formed upon protein regeneration can be degraded using small amounts of the low-molecular-mass electrolyte. From the same considerations it follows that coating of protein outer layer of a protein–polyelectrolyte multilayer film with charged oligomers from solution should not lead to undesired formation of soluble PPCs, as does occur when using high-molecular-mass polyions.

## V. Conclusion

Analysis of the published data [9] showed that the properties of soluble NPECs and PPCs including self-assembly and molecular 'recognition' in these systems have much in common. The formation of both families of soluble complexes is governed by the hydrophilic-lipophilic ratio of their species, which contain minimum amounts of components necessary for dissolution. The similarity between NPECs and PPCs also manifests itself in the pattern of competitive reactions (direction and rate of transfer of components are controlled by the same factors), which indicates an identical character of the driving forces of exchange and substitution reactions. Phase separation in NPEC and PPC solutions also obeys the same pattern, namely, it occurs quantitatively, reversibly, in narrow ranges of changes

§ These data can serve as a basis for target synthesis of efficient prophylactics and pharmaceuticals.

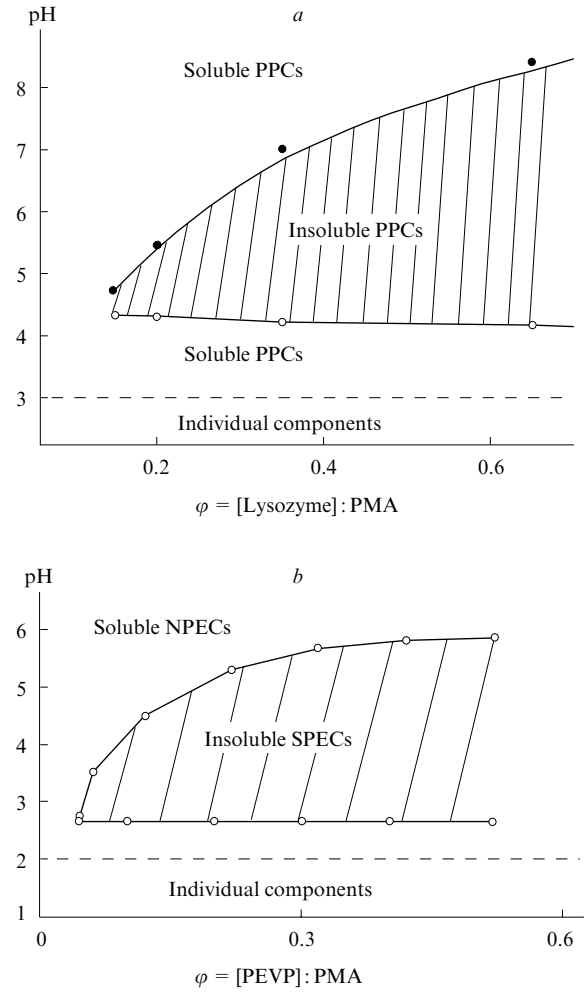



**Figure 6.** Phase diagrams of mixtures of PMA anion solutions with lysozyme (*a*) [105] and PEVP cation (*b*).[109]

in pH values or ionic strength of solutions and is efficiently controlled by the charge-to-charge ratio of the partners (Fig. 6).[105]

Specific features of the behaviour of PECs and PPCs are due to dissimilarity between the structure of components of the complexes and to the difference in the number and arrangement of ionic and ionogenic groups on the surface of globular proteins and on the chains of linear synthetic polyelectrolytes. For instance, BSA carboxylic groups accessible to interaction with the polycation are non-uniformly distributed over the surface of the protein globule (p$I$ = 4.7, 69 kDa) and occupy fixed sites. At pH 7.0, they form fifty-five ion pairs with the PEVP cation,[93] whereas the number of ion pairs on a chain segment of flexible PMA anion of the same molecular mass bound to the PEVP cation is an order of magnitude larger. Short extension and low ordering of the system of ion pairs in PPCs predetermine a relatively low stability of the BSA/PEVP complex in water–salt media [99] and provide the possibility of self-assembly of the soluble complexes on mixing aqueous solutions of the protein and polycation.

Unlike PPCs, spontaneous formation of soluble NPECs upon mixing solutions of strongly coupling, complementary polyions PEVP and PMA occurs only in the presence of a low-molecular-mass electrolyte. For the same reasons, competitive interaction between highly charged vinyl polyelectrolytes with

PPCs leads to quantitative displacement of protein globules from the complex to solution, whereas the protein specifically bound within PPC (through, *e.g.*, enzyme – inhibitor [106] or antigen – antibody [107] interactions) goes to the SPEC precipitate. This effect underlies the development of an improved method of immunochemical analysis, which involves determination of the target protein in solution using all advantages of homogeneous catalysis and subsequent precipitation of the components accompanied by simultaneous displacement of non-specifically bound proteins.[108]

Covalent binding of protein (enzyme) to polyelectrolyte imparts useful properties to the conjugate. The enzyme present in the chain has almost no effect on the interaction between the chain and the oppositely charged polyelectrolyte and does not preclude self-assembly of soluble NPECs in salt – water media. The soluble NPECs also show almost the same phase behaviour. The immobilised enzyme acquires the unique ability of NPECs to undergo reversible and quantitative phase separation under preset conditions upon minor changes in pH value, ionic strength or chemical composition of the mixture of polyelectrolytes.[109] The properties of the immobilised, reversibly soluble enzymes were studied in detail [110] and analysed in a review.[111] The use of such systems makes it possible to

— carry out an enzymatic reaction in solution using all advantages of homogeneous catalysis and remove the immobilised enzyme from the reaction sphere with ease upon reaction completion;

— significantly improve the thermal stability of the enzyme by transferring it to precipitate by varying the pH value of the solution;

— protect the enzyme from the action of high-molecular-mass inhibitors both in solution and precipitate;

— design self-regulatory enzymatic systems in which the process catalysed stops upon attainment of a preset degree of conversion of substrate and

— design reversibly soluble multi-enzyme systems.

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