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DNA Interpolyelectrolyte Complexes as a Tool for Efficient Cell Transformation

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SYNOPSIS

A tool was developed for enhancement of plasmid penetration into an intact cell, based on increasing DNA hydrophobicity via inclusion into a soluble interpolyelectrolyte complex (IPC) with polycations. The characteristics of formation of DNA IPC with synthetic polycations [poly(N-ethyl-4-vinylpyridinium)bromide (PVP) and PVP modified with 3% of N-cetyl-4-vinylpyridinium units (PVP-C)] were studied using ultracentrifugation and polyacrylamide gel electrophoresis methods. The conditions were established under which the mixing of DNA and polycation aqueous solutions results in the self-assembly of soluble IPC species. Incorporation of DNA into IPC results in the enhancement of DNA binding with isolated *Bacillus subtilis* membranes. A considerable increase in the efficiency of transformation of *B. subtilis* cells with pBC16 plasmid resulted from incorporation of the plasmid into the IPC with PVP and CVP.

INTRODUCTION

Recently it was suggested that the modification of biopolymers with hydrophobic residues might enhance binding to cell membranes and penetration into cells.[1] This approach was successfully applied for protein[2] and oligonucleotide[3] introduction into intact mammalian cells.

It is expected that binding of nucleic acids to a membrane is also an essential step for penetration into the cell.[4,5] Such binding of nucleic acids can be achieved by chemical modification with hydrophobic residues as well. However, in the case of nucleic acids, which represent polyanions with high charge density, another route for introduction of a hydrophobic moiety can be proposed.

When an aqueous solution of a nucleic acid is mixed with a linear polycation, there follows cooperative binding of oppositely charged polyions resulting in formation of an interpolyelectrolyte complex (IPC).[6] If the polycation chain has a hydrophobic backbone, its "sticking" to the nucleic acid chain, accompanied by compensation of the phosphate group charge, results in formation of the hydrophobic site.[7] The length and the number of such hydrophobic sites is determined by the length (degree of polymerization) of a polycation and by the IPC composition φ, i.e., the molar ratio of polycation repeating units and nucleic acid phosphate groups.

The applicability of this approach for efficient gene transfer was recently demonstrated by *Bacillus subtilis* transformation with plasmid DNA incorporated into soluble IPC with carbochain polycations,[8] including those additionally modified by fatty residues.[9] Behr et al.[10] independently used a similar approach for the efficient transformation of various mammalian cells by DNA complexes with lipopolyamines that, actually, represent a variety of IPC.

This paper presents new results and experimental considerations relevant to the above strategy.

MATERIALS AND METHODS

Cells and DNA

The *B. subtilis* (strain MC 5) and *Escherichia coli* (strain HB 101) cells (from Research Center of Molecular Diagnostics, USSR Ministry of Health) were grown in L-broth.[11] The solid media contained 1.6% agar.

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The plasmids were isolated from *B. subtilis* (pBC16) and *E. coli* (pTZ19) according to the Birnboim and Doly method.[12] The DNA from λ phage was obtained from Sigma. The ^{14}C-labelling of plasmids with ^{14}C-thymidine (Chemapol) was performed via a method described in Ref. 13.

Polycations and Polycomplexes

Poly(N-ethyl-4-vinylpyridinium)bromide (PVP) with various degrees of polymerization (P_w = 18, 200, and 400), and PVP modified with 3% of N-cetyl-4-vinylpyridinium units (PVP-C; P_w = 400), were obtained, purified, and characterized by a previously described method.[14] Poly(L-lysine), M_w = 1.5–3.0 × 10^4, was purchased from Sigma.

The polycomplexes were formed by mixing of the aqueous solutions of the DNA and polycations. The DNA, PVP, and CVP concentrations in the system obtained were determined by absorbance measurements at 260 nm.

Sedimentation Measurements

Ultracentrifugation of DNA and its polycomplexes was carried out at 20°C in the Beckman E. analytical ultracentrifuge at 20,000 rpm. The scanning was measured at 260 nm.

Gel Electrophoresis

The DNA and IPC were analyzed by electrophoresis in 0.8% agarose gel in standard Tris-borate buffer, pH 8.6.[11] The densitograms were obtained using the laser densitometer, Ultrascan (LKB).

DNA Restriction

Two microliters of the solution, containing two units of the nuclease (Alu I or Pvu II), were added to 1.7–2.0 μg of plasmid DNA in the free form or incorporated into the IPC plasmid DNA, dissolved in 30 μL of buffer. (The nucleases and the corresponding buffers for the DNA digestion were obtained from Boehriger.) The systems obtained were incubated at 37°C. The reaction was stopped by addition of 1 μL of 60 m*M* EDTA, pH 8.0.

The DNA-containing polycomplexes were decomposed by the sodium polymethacrylate added to the system as a threefold molar excess related to the polycation. The digestion products were analyzed by gel electrophoresis as described above.

DNA Binding With Membranes

The *B. subtilis* membranes were isolated via a previously described method.[15] The protein content in the preparations of the membranes was determined by the Lowry method.[16]

DNA binding with *B. subtilis* membranes was studied by the method described in Ref. 17. An aqueous solution (30 μL) of ^{14}C-labeled pTZ19 plasmid (21 cpm/ng) or of its complex with PVP were added to 1 mL of suspension of *B. subtilis* membranes (protein concentration 1 mg/mL) in 10 m*M* phosphate buffer, pH 7.0, containing 0.15 *M* NaCl. The system was incubated at 37°C and then filtered through a 0.2-μ*M* Synpore filter. The filters were washed with 5 mL of 10 m*M* phosphate buffer, pH 7.0, containing 0.15 *M* NaCl. Their radioactivity was measured in GS-106 scintillation liquid using a Rackbeta (LKB) counter.

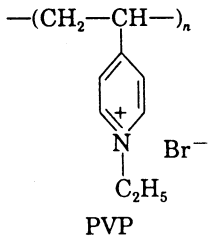
Transformation Experiments

The competent *B. subtilis* MC 5 cells were transformed with pBC16 plasmid by Spizizen's method.[18] An aqueous solution (50 μL) of pBC16 plasmid or of its complex with a polycation were added to 1 mL of 10^7 *B. subtilis* cells suspended in Spizizen II solution. Cells were incubated with DNA at 37°C for 1 h. They were seeded at the agar media[18] containing 10 μg/mL tetracycline. The number of tetracycline-resistant clones was determined. The yields of transformation presented in this paper are the average values obtained for three petri dishes.

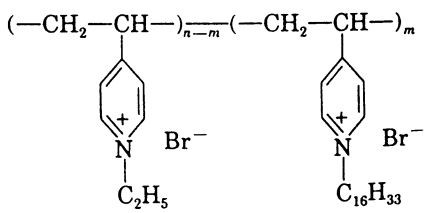
RESULTS

IPC Formation

The characteristics of formation of IPC of λ-DNA and quaternized poly(4-vinylpyridines):



PVP



PVP-C ($n : m$ = 97 : 3)

were studied by the method of ultracentrifugation.

The contour length of PVP and PVP-C polycations (P_w = 400 corresponds to $M_w \approx 8 \cdot 10^4$) was considerably lower than the contour length of the DNA ($M_w \approx 3 \cdot 10^7$).

Only one step is observed from the sedimentation curves of the samples obtained by mixing of the DNA and PVP solutions in the range of basic mole ratios [PVP]/[DNA] = 0–0.5. The sedimentation coefficient corresponding to this step continuously increases from 18×10^{-13} s for the free DNA to 23×10^{-13} s (Figure 1). These data give evidence of formation of the soluble nonstoichiometric IPC, the composition of which (φ) is equal to the initial ratio of the polymer components ($0 < \varphi < 0.5$). In other words, the PVP chains are completely complexed and uniformly distributed among the DNA molecules. Further addition of PVP causes a typical disproportionation phenomenon.[7] Parallel with the soluble IPC ($\varphi = 0.5$, $S = 23 \times 10^{-13}$ s) the insoluble complex with higher PVP content is formed, which precipitates in the ultracentrifuge at low rotation rates.

The regularities of PVP-C interaction with DNA are analogous to those observed for PVP. However, in this case the region of formation of the soluble IPC corresponds to a rather narrow interval of mole ratios [PVP-C]/[DNA] = 0–0.25 (Figure 1). Moreover, the uniform distribution of the PVP-C chains among the DNA molecules is observed only at relatively low ionic strength. At higher ionic strength (for example, in 10 mM phosphate buffer, pH 7.0, containing 0.15M NaCl), a series of IPCs with various compositions as well as free DNA are revealed in the sedimentation curve. (The difference in the sedimentation coefficients of the free DNA observed in Figure 1 evidently results from the difference in the ionic strengths.)

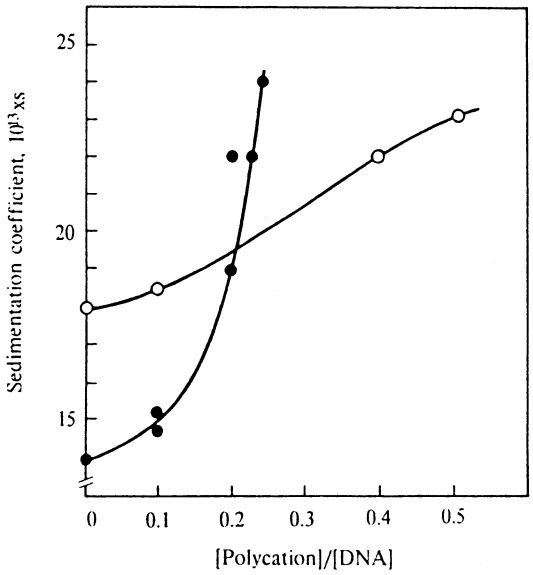


Figure 1. Dependence of the sedimentation coefficients of λ-DNA IPC with (○) PVP and (●) PVP-C on the basic mole ratio of the polycation and DNA concentrations. The data relate the experimental conditions corresponding to the uniform distribution of the polycation chains among DNA molecules: 10 mM phosphate buffer, pH 7.0, containing 0.15M NaCl (DNA–PVP complex) or 10 mM phosphate buffer, pH 7.0 (DNA–PVP-C complex).

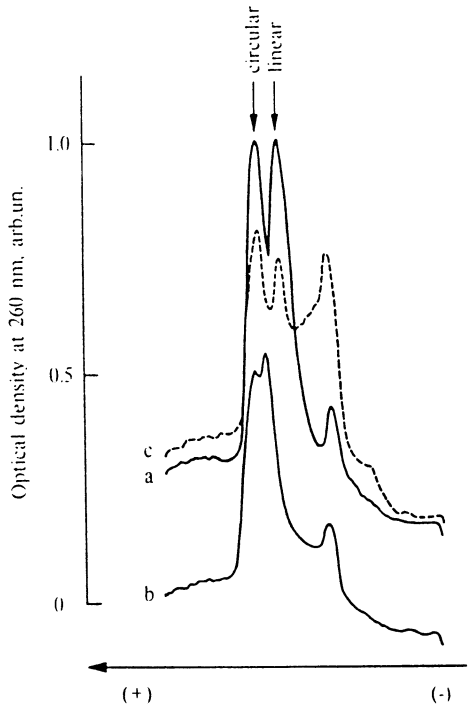


Figure 2. Gel electrophoresis densitograms of (a) free pBC16 plasmid or of its IPC with (b) PVP and (c) PVP-C. The PVP and PVP-C degree of polymerization P_u = 400. The composition of IPC φ = 0.2. The vertical arrows indicate the bands corresponding to the circular and linear forms of plasmid.

One can follow the formation of the IPC by gel electrophoresis data. As determined from the densitograms in Figure 2, the incorporation of the plasmid DNA in the soluble complex with PVP causes a shift of the bands, corresponding to the circular and the linear forms of plasmid. The disproportionation phenomenon is revealed for the plasmid–PVP-C complex. In this case, alongside the bands of free DNA (its circular and linear forms), a broad band corresponding to the IPC is observed.

The addition of the sodium polymethacrylate solution to a solution of the DNA–polycation complex results in the displacement of the DNA molecule by

polymethacrylate anions. This results in release of free DNA, which is detected by sedimentation as well as by electrophoresis data (not shown in figures).

Restriction of IPC-Incorporated DNA

Biological action of DNA necessarily requires specific recognition by competent enzymes. In this regard, restriction of DNA, incorporated into IPC, by specific restrictases was studied. Figure 3 presents the results of the restriction of free and IPC-incorporated pTZ19 plasmid with Alu I, representing an enzyme with broad specificity, i.e., recognizing a multitude of restriction sites in the DNA. The products of restriction formed in case of free DNA and its nonstoichiometric complexes with polycations

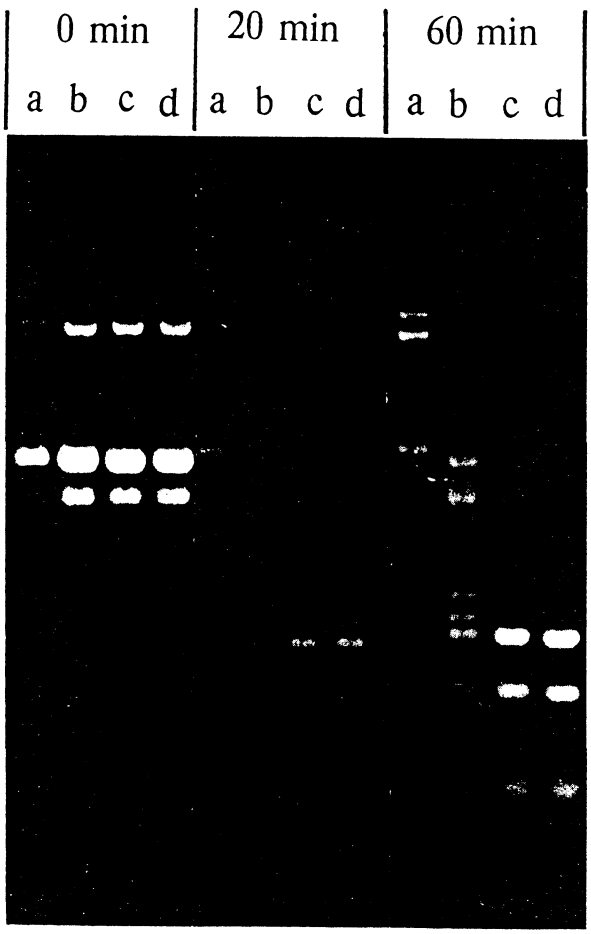


Figure 3. Pattern of the restriction products formed from (a–c) the IPC of pTZ19 plasmid with PVP and (d) the free plasmid after their incubation for various times in the presence of Alu I. The degree of polymerization of PVP P_w = 18. The composition of IPC: (a) φ = 1.0, (b) φ = 0.5, and (c) φ = 0.2.

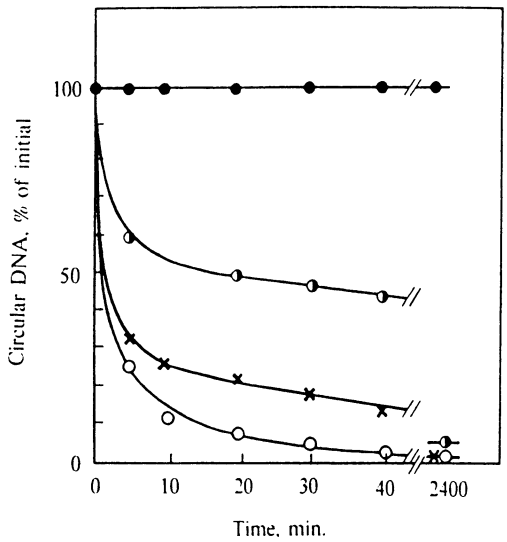


Figure 4. Dependence of the percentage of uncleaved circular form of pTZ19 plasmid on the time of incubation of (○) the free plasmid and (x, ◑, ●) its IPC with PVP in the presence of Pvu II. The degree of polymerization of PVP P_w = 18. The composition of IPC: (x) φ = 0.2, (◑) φ = 0.5, and (●) φ = 1.0.

are the same. This means that the polycation in the IPC structure does not prevent the recognition of DNA by the enzyme. This fact is in agreement with well-known dynamic properties of polyions incorporated into soluble IPC.[7] The polycation chains are not strictly fixed on the DNA polyanions. They can transfer from one site to another,[6] opening the DNA sequences required for restriction.

Figure 4 presents the data on the kinetics of the restriction of the pTZ19 plasmid with Pvu II. This enzyme recognizes only one site in the plasmid molecule. The treatment with Pvu II results in the opening of the circular form of the plasmid and in the formation of its linear form. As can be seen from Figure 4, the rapid cleavage of the native DNA is observed under the experimental conditions.

The picture observed for the IPC-incorporated DNA is completely different. In this case the kinetics of restriction is characterized by the presence of two different phases. The first phase evidently corresponds to the rapid cleavage of the DNA sites that are not covered by the polycation chains. We should mention that the amount of plasmid molecules that are cleaved during the first phase correlates with the composition of the IPC: at φ = 0.2 about 80% of the DNA sites are easily accessible to the enzyme; at φ = 0.5 this value is equal to 50%.

During the second phase, the slow restriction of the residual DNA proceeds. The complete cleavage

of the plasmid is observed only after 35 h of incubation with the enzyme. It is reasonable to conclude that the rate-determining stage during the second phase is the opening of restriction sites resulting from polycation transfer from one DNA chain to another.

As seen in Figures 2 and 3, the restriction does not occur in IPC at $\varphi = 1.0$ when the DNA is incorporated into unsoluble complex and therefore not accessible to the nuclease attack.

DNA IPC Binding with Membranes

The study of binding of free and IPC-incorporated ^{14}C-labeled pTZ19 plasmid with isolated *B. subtilis* membranes was carried out. The maximal binding of DNA (either free or IPC incorporated) was observed after 5 min of incubation with the membrane suspension.

As can be seen in Figure 5, the binding of DNA complexed with PVP is 1.5–1.8-fold higher than that of free DNA.

B. subtilis Transformation

To estimate the efficiency of the DNA transfer through a cell membrane, an experiment was carried out with transformation of the competent *B. subtilis* cells by pBC16 plasmid. The plasmid contains a determinant of stability with respect to tetracycline. It is known that the transmembrane transport of plasmid DNA is a rate-determining step of *B. subtilis* genetic transformation.[4]

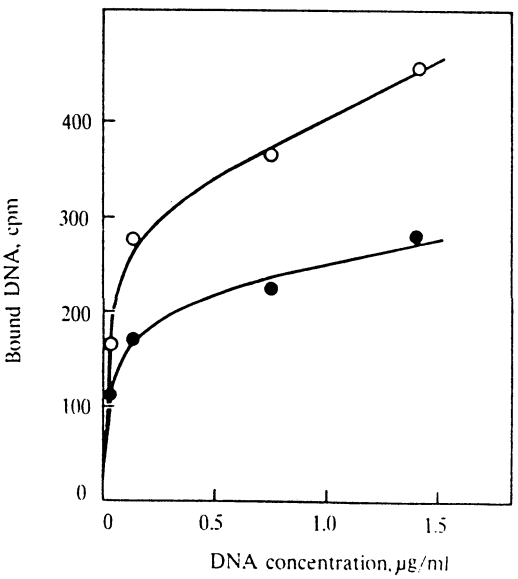


Figure 5. Dependence of the amount of pTZ19 plasmid bound with isolated *B. subtilis* membranes on the concentration of DNA during the membranes incubation with (●) the free plasmid or (○) its IPC with PVP. The degree of polymerization of PVP $P_w = 18$. The composition of IPC $\varphi = 0.2$.

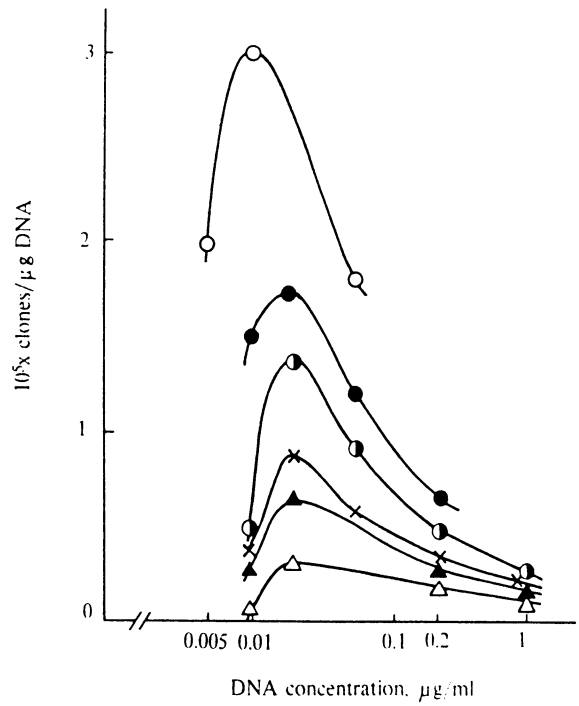


Figure 6. Dependence on the DNA concentration of the number of tetracycline-resistant clones produced as a result of *B. subtilis* transformation with (△) free pBC16 plasmid and its IPC with (▲) PVP-C or (x, ◑, ●, ○) PVP. The degree of polymerization of PVP-C and PVP: (△, x, ◑) $P_w = 400$, (●) $P_w = 200$, and (○) $P_w = 18$. The composition of IPC: (x) $\varphi = 0.1$ and (▲, ◑, ●, ○) $\varphi = 0.2$.

Incorporation of the plasmid into IPC with PVP and PVP-C polycations leads to a considerable increase in the transformation efficiency (Figure 6). For IPC-treated cells the same yields of transformants are reached at much lower DNA concentrations than for cells treated with free plasmid. As it is seen in Figure 6, a considerable yield of transformants ($\approx 3 \cdot 10^{-4}$ of clones per 1 cell) is reached at the 0.01 μg/mL concentration of plasmid—conditions under which the cell transformation with free DNA is not observed.

The observed effect depends on the IPC composition φ and the polycation length (Figure 6). In particular, the change in the composition of the plasmid–PVP complex from $\varphi = 0.1$ to $\varphi = 0.2$, accompanied by an increase in the IPC hydrophobicity, results in a rise of the transformation efficiency. At constant φ the transformation efficiency can be reg-

ulated by variation of the polycation length: with a decrease in the degree of polymerization of PVP the observed effect increases.

The efficiency of the *B. subtilis* transformation by IPC-incorporated DNA evidently depends on the chemical structure of the polycation. Thus, the transforming activity of the plasmid in the complex with PVP-C is somewhat lower than its activity in the complex with PVP with an equal degree of polymerization (Figure 6). Therefore the transforming activity of DNA-containing IPC cannot be considered as a simple function of hydrophobicity of a polycation.

However, incorporation of the plasmid into the complexes with poly(L-lysine), the polycation with a polar backbone of the main chain, decreases transformation efficiency as compared with free DNA (not shown in the figure). This result apparently indicates that the hydrophobicity of the IPC is the necessary condition for enhancement of cell transformation.

DISCUSSION

The data presented demonstrates that DNA incorporation into soluble complexes with carbochain polycations results in the considerable increase of the efficiency of a DNA penetration into the cell and of its transforming activity. The mechanism of this effect is not clear. It is known that *B. subtilis* transformation represent a complex multistage process; an important stage involves adsorption of the DNA molecule by the membrane of a competent partial protoplast.[4,19] Inclusion of DNA into IPC evidently enhances the DNA binding with *B. subtilis* membranes. This presumably results from the interaction of the hydrophobic sites formed by a polycation at the DNA chain with nonpolar parts of lipid bilayers.

The next step of DNA penetration into bacillus cells is usually associated with DNA fragmentation and transfer into single-strand form.[4,19] The results of restriction experiments reveal that such specific enzymatic processing of DNA might also proceed in IPC bound to the membrane. However, we cannot exclude that the mechanism of transfer of IPC-incorporated DNA from the outer membrane into the cell might be entirely different from that for free DNA. The possible alternative mechanism of hydrophobic DNA penetration through bacillus[8] and mammalian cell membranes observed by Behr et al.[10] might be common, in spite of the considerable difference between both types of cells.

The approach previously described[8-10] can be undoubtedly used for efficient transformation of procaryotic and eucaryotic cells in vitro. However, its significance would be much higher if it can be employed for genetic correction in vivo. The arguments supporting this hope are following:

1. The polycation complexed with DNA represents a "building block" that can be easily conjugated with any target-recognizing molecule using a standard chemical technique, e.g., with an antibody against a certain receptor of a target cell. This might be a way to address DNA packed in IPC species to the target cell. Using this strategy, Wu et al.[20] have realized in vivo transformation of liver cells by a plasmid incorporated into IPC with poly(L-lysine) coupled with a liver-specific hormone.
2. Incorporation of DNA molecules into IPC with φ close to 1 can block cleavage by nucleases presented in living organisms, e.g., in the blood flow. This might be a method to protect DNA on its route to the target cells.

The important advantage of DNA IPCs arises from the fact that they are thermodynamically equilibrated self-assembling species that can be obtained by simple mixing of DNA and polycation solutions. The IPC species can be regarded as a mimic of "artificial virus" with the "core" formed by DNA (or RNA) and the "cover" containing receptor-recognizing molecules that can interact with the cell membrane and "infect" the cell.

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