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Effect of Hydrophobic Interactions on Properties and Stability of DNA−Polyelectrolyte Complexes

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Polyplexes are polyelectrolyte complexes of DNA and polycations, designed for potential gene delivery. We investigated the properties of new polyplexes formed from cholesterol-modified polycations and DNA. Three complexes were tested; their cholesterol contents were 1.4, 6.3, and 8.7 mol %. UV spectroscopy and fluorescence assay using ethidium bromide proved the formation of polyplexes. The kinetics of turbidity of polyplexes solutions in physiological solution showed that the colloid stability of polyplexes increases with increasing content of cholesterol in polycations. Dynamic, static, and electrophoretic light scattering, small-angle X-ray scattering, and atomic force microscopy were used for characterization of polyplexes. The observed hydrodynamic radii of polyplexes were in the range of 30−60 nm; they were related to the polycation/DNA ratio and hydrophobicity of the used polycations (the cholesterol content). The properties of polyplex particles depend, in addition to polycation structure, on the rate of polycation addition to DNA solutions.

Introduction

Gene therapy is a rapidly progressing method devised for the treatment of a variety of diseases.[1] Since native DNA is successfully used only for local applications, but not in transport to distant targets in vivo, more sophisticated delivery vectors have to be used.

One of the methods is the development of *polyplexes*: polycation/DNA complexes prepared by self-assembly of polyions in solution. The advantage of synthetic polycations for self-assembly with DNA consists of an easy, cheap, and reproducible synthesis of such material. The possibilities of structure optimization of the polycations by selecting the monomers and optimum composition of polycationic copolymers, of changes in molecular weights and the density of positive charge along the polymer chain, as well as of modification of the polycation/DNA complexes with biologically active substances are some of factors determining the choice of synthetic polymers for gene delivery vectors. Polycations and DNA form compact polyelectrolyte complexes (PECs) in solution held together by electrostatic forces between negative DNA phosphate groups and the charged groups of polycations. A variety of polycations have been already tested,[2−43] both in vitro and in vivo.

To optimize biological activity of the polyplexes, it is important to better understand their physicochemical properties. Several

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articles have been focused on characterization of the self-assembly, structure, and solution behavior of polyplexes.[2,10,19,28,42] It was found that their formation, stability, and size depend on several parameters. From the general point of view, the formation and properties of polyplexes are similar to those of the complexes of synthetic polyions. The crucial parameter that controls the PEC stability due to electrostatic binding is the (molar) mixing ratio X of polycations and polyanions. The composition and properties of polyplexes are reported in many papers.[2,41,42] The DNA-rich (negatively charged) and polycation-rich (positively charged) regions usually are selected, depending on composition. Between the two regions, a critical composition exists where the complex may be insoluble. A divergence of molecular weight and size of the polyplex is typically observed in the vicinity of charge compensation.[2,42] In some papers it was reported that polycation binding to DNA increases thermal stability of DNA.[19] The stability of PEC depends strongly on the nature of the added salt and characteristics of the polycations, e.g., their degree of polymerization and the charge density. The presence of bulky substituents such as primary, tertiary, or quaternary amino groups has proven to be the another factor.[10,15,21,23,30,41] Much less is known about the influence of polycation hydrophobicity on the stability and physicochemical properties of polyplexes.

Therefore, we have systematically examined the formation and properties of new polyplexes formed by self-assembly of DNA and synthetic linear polycations (see Figure 1) modified with cholesterol moieties. The content of cholesterol ranged from 1.4 to 8.7 mol %. The presence of hydrophobic cholesterol moieties in copolymers should enhance the stability of polyplexes compared with cholesterol-free polyplexes.

The aim of the paper is the investigation of physicochemical properties and the stability of new polyplexes. The approach includes systematic comparison of polyplexes with different contents of cholesterol moieties. Major attention is devoted to the size and stability of polyplexes and conditions of their formation. The key values of the particular parameters followed are identified.

Experimental Section

Materials. Calf thymus DNA Type II (Sigma-Aldrich) was used as received. Static light scattering (SLS) yields a molar mass of ca. 7×10^6 g/mol or 10400 bp.

Synthesis of Polycations Modified with Cholesterol. Poly[(2-{[(cholesteryloxy)carbonyl]amino}ethylmethylacrylate)-*co*-{benzyl-[2-(methacryloyloxy)ethyl]dimethylammonium chloride}] copolymers were synthesized (Figure 1):

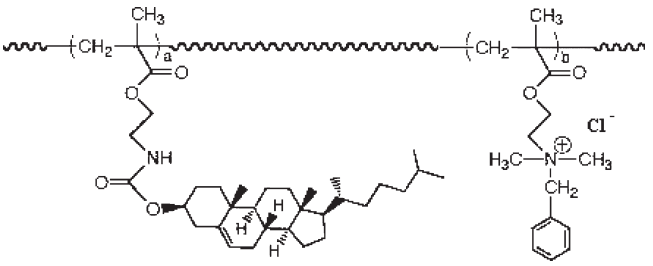

Figure 1. Chemical structure of polycations.

Polymer precursors of polycations were prepared by solution radical polymerization of cholesterol monomer and 2-(dimethylamino)ethyl methacrylate (DMAEMA) in dimethyl sulfoxide (DMSO). The polycations were obtained by quaternization of DMAEMA units in the polymer with benzyl chloride.

2-{[(Cholesteryloxy)carbonyl]amino}ethyl methacrylate was prepared by the reaction of 7.77 g (2 mmol) cholesterol with 3.10 g (2 mol) of 2-(methacryloyloxy)ethyl isocyanate in $CHCl_3$ in the presence of a catalytic amount (0.03 g) of dibutyltin dilaurate (95% Aldrich) and a small amount of inhibitor *tert*-octylpyrocatechol. The product was crystallized from ethyl acetate/$CHCl_3$.

Polycations were prepared by copolymerization of DMAEMA (dissolved in DMSO), and the cholesterol monomer (dissolved in toluene), in the presence of azobisisobutyronitrile (AIBN; 0.6 wt %) at 50 °C for 24 h. The monomer mixture (15 wt %) contained 2, 6, or 10 mol % of cholesterol monomer. After polymerization, the reaction mixture was evaporated in vacuum, and the DMSO solution was diluted with acetone. A 1.5 molar excess of benzyl chloride was added dropwise at room temperature, and the reaction mixture was stirred. The solutions became very viscous within 30 min of the reaction. The polycation obtained was purified by three precipitations of a DMSO solution of the copolymers into excess acetone. The yield of polycations was 70−80%. The content of cation in the polymers was determined by titration of chlorides with $AgNO_3$ using the modified Mohr method. The solutions of polycations were dissolved in a minimum amount of H_2O/MeOH (1:1) and titrated with 0.05 M $AgNO_3$ using K_2CrO_4 as the indicator.

Molecular weights of the polymers were determined by SLS in methanol; the results are presented in Table 1.

Preparation of Polyelectrolyte DNA Complexes. Polyplexes were prepared in water at pH lower than 7 by slow (a) or fast (b) addition of a polycation solution to the DNA solution at the molar ratio X.

(a) For light scattering experiments, the complexes were prepared directly in a measuring cell (a glass vial 15 mm in diameter). The DNA solution (1.6 mL, 2×10^{-5} g/mL) was introduced into the cells. A polycation solution of 2-fold molar concentration was slowly added (2 mL/h) under stirring (500 rpm) up to the desired mixing ratio X (molar ratio of polycation/amino groups). Polycation solutions were made dust-free by filtration through a polyvinylidene fluoride (PVDF) membrane of 0.2 or 0.45 μm pore size (Millipore). DNA was used as received and diluted with filtered Q-water (PVDF, 0.1 μm, Millipore).

(b) Polycation−DNA complexes (PECs) of the desired X were prepared by fast addition (< 1 s) of an appropriate volume of 2-fold molar concentration of polycation to a stirred solution of DNA (1.6 mL, 2×10^{-5} g/mL) in cylindrical glass vials 15 mm in diameter at a stirring speed of 1000 rpm with a magnetic stirrer 10 mm × 3 mm.

Possible denaturation of DNA, which might occur in salt-free solutions, should not affect the properties of polyplexes. Previously,[18] we tested the influence of NaCl salt on the polyplexes formed from block and graft copolymers *N*-(2-hydroxypropyl)methacrylamide (HPMA) with 2-(trimethylammonio)ethyl methacrylate and calf thymus DNA. We have observed that the hydrodynamic radius of the complexes is a function of NaCl

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Table 1. Characteristics of Polymers

polymer	M_w g/mol	R_h nm	dn/dc	mass/charge g/mol	cation mol %	cholesterol mol %	mass/charge g/mol
P17	1.0×10^4	6	0.235	290[a]	98.6	1.4	292[b]
P18	2.0×10^4	10	0.196	304[a]	93.7	6.3	321[b]
P19	2.0×10^4	10	0.184	338[c]	91.3	8.7	336[b]
DNA	6.7×10^{6}[d]		0.185[e]	325[e]			

[a]Calculated using titration results by ethidium bromide. [b]Calculated using titration of chlorides with $AgNO_3$ [c]NMR analysis. [d]Reference 13. [e]Reference 48.

concentration (ionic strength) but in the low salt concentrations region from salt-free up to 20 mM, the changes in R_h value are insignificant and about 10−20%. Because we did not find drastic changes in the properties of the complexes similar to the ones we have studied in our paper, we believe that the denaturation of DNA in salt-free solution is not a crucial factor in our case.

NMR. ^{1}H and ^{13}C NMR spectra of copolymer solutions in deuterium oxide were measured at 600.13 MHz with a Bruker Avance III spectrometer in 10 mm tubes. The integrated intensities were determined with accuracy of ± 1% using spectrometer integration software. The temperature was kept constant (25.0 ± 0.2 K) with a BVT 3000 temperature unit. Sodium 2,2-dimethyl-2 silapentane-5-sulfonate (DSS) was used as internal standard. The contents of monomer units in the copolymer were expressed by the ratio of integrated intensities of the corresponding signals for both copolymer blocks. For the 2-[(cholesteryloxycarbonyl)-amino]ethyl-2-methylacrylate units, the signal used was that of the two end methyl groups. For benzyl[2-(methacryloyloxy)-ethyl]dimethylammonium chloride, it was the signal of the two methyls of the dimethylammonium group. In the case of high content of cholesterol, the intensity of that peak is high enough to neglect the influence of the other peaks. Unfortunately, for samples with low concentration of cholesterol, the effect of overlap is too strong to be neglected, which made the analysis of results practically impossible.

Static Light Scattering. SLS measurements were carried out with an ALV goniometer. The instrument was equipped with an intensity-stabilized 22 mW He−Ne laser as a light source and a PC for data recording. The accuracy of the measurements was about 1%. The scattering curves were measured after each dose of a polycation. The samples were measured at a temperature of 25.0 ± 0.1 °C.

The refractive index increments, dn/dc, for the individual components of the complexes were measured with a Brice-Phoenix differential refractometer at a light wavelength of 633 nm or they were taken from literature. The used dn/dc values are collected in Table 1.

The calculation of the complex concentrations c_{PEC} and refractive index increments $(dn/dc)_{PEC}$ of the complexes as a function of the molar mixing ratio X (positive to negative charge ratio) were calculated on the basis of the model of complex formation given in our previous paper.[44]

The SLS data were analyzed using a Zimm plot:

$$\frac{Kc_{PEC}}{R(q)} = \frac{1}{M_w} + \frac{R_G{}^2 q^2}{3M_w} \quad (1)$$

where $R(q)$ is the Rayleigh ratio of the scattering intensity, $q = (4\pi/\lambda)\sin\theta/2$, λ is the wavelength in the medium, θ is the scattering angle between the incident and scattered beam, K is a contrast factor containing the optical parameters, and c_{PEC} is the complex concentration. SLS experiments in a polydisperse system give the z-average radius of gyration (R_G) and the weight-average molar mass (M_w).[45] The concentration dependence was neglected, which seems to be justified because of low concentrations of the PEC solutions (~10^{-5} g/mL). Extrapolation to zero scattering angle was carried out by linear or quadratic fits of the scattering curves. The experimental error of M_w determination for the complexes was typically about 2%.

Dynamic Light Scattering (DLS). DLS measurements were carried out on the same ALV instrument in the angular range 30−140°. An ALV 6000, multibit, multitau autocorrelator covering approximately 12 decades in the delay time τ was used for measurements of time autocorrelation functions. Most of the measurements were realized at the scattering angle $\theta = 90°$. The inverse Laplace transform using the REPES[46] method of constrained regularization, which is similar in many respects to the inversion routine CONTIN,[47] was performed in analysis of time autocorrelation functions. REPES directly minimizes the sum of the squared differences between the experimental and calculated intensity time correlation functions using nonlinear programming. This method uses an equidistant logarithmic grid with fixed components (here a grid of 20 components per decade) and determines their amplitudes. As a result a scattered light intensity distribution function $A(\tau)$ of delay times is obtained, which can be easily transformed into a distribution function of hydrodynamic sizes.

The hydrodynamic radius, R_h, was calculated from the diffusion coefficient, D, using the Stokes−Einstein equation.

$$R_h = kT/6\pi\eta D \quad (2)$$

where k is the Boltzmann constant, T is the absolute temperature and η (0.894 cP) is the viscosity of water at 25 °C. The experimental error of the R_h determination of the complexes was typically about 3%.

ζ-Potential. Measurements of the ζ-potential were made using a Nano-ZS Model ZEN3600 (Malvern Instruments, UK) at a temperature of 25.0 ± 0.1 °C. At least 10 measurements of every copolymer were carried out to check reproducibility. The measurements of the electrophoretic mobility were converted to ζ-potential (mV) using the Smoluchowski approximation. A reference measurement using the Malvern ζ-potential standard was run prior to each sample analysis to check correct instrument operation.

Fluorescence Assay. The degree of DNA condensation was determined as a function of the X ratio by a fluorescence method using ethidium bromide. It is well-known that the fluorescence intensity of ethidium bromide decreases with increasing X due to replacement of ethidium bromide molecules, intercalated in the DNA double helix, by interaction with polycations.[21] Fluorescence was measured using a light scattering apparatus equipped with an Ar-ion laser and a 560 nm cutoff filter for cutting off exciting laser radiation (λ = 514.5 nm). A DNA solution (2×10^{-5} g/mL) with ethidium bromide (2×10^{-6} g/mL) in water was used for the investigation. A polycation solution of 2-fold molar concentration was added stepwise (2 mL/h) to a DNA−ethidium bromide solution so that the X increased by 0.094 in each step. The main drop of I_f indicates the full charge

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compensation and DNA condensation ($X_c \cong 1$). All solutions were kept at room temperature.

Atomic Force Microscopy (AFM). The nanoparticles (2 mg/mL) were deposited on fresh mica substrate and characterized by AFM. The surface morphology (height image) and the sum of tip−sample interactions (phase image) were characterized by AFM. All measurements were performed under ambient conditions using a commercial atomic force microscope (NanoScope Dimension IIIa, MultiMode Digital Instruments, Santa Barbara, CA) equipped with a NCLR Pointprobe - Silicon SPM sensor (probe covered with reflex aluminum coating, NanoWorld AG, Switzerland; spring constant 45 N m^{1-}, resonance frequency 154 kHz). Tapping-mode AFM technique was used for collecting all images. This technique allows obtaining two- and/or three-dimensional information on both height and material heterogeneity contrast with high resolution when recording height and phase shifts simultaneously.

Small Angle X-ray Scattering (SAXS). A series of SAXS experiments were performed on the high brilliance beamline ID2 19 at the ESRF (Grenoble, France). The SAXS setup is based on a pinhole camera with a beam stop placed in front of a two-dimensional detector (image plate or X-ray image intensifier coupled to a charge-coupled device (CCD) camera). The X-ray scattering patterns were recorded on the detector that was most of the time located 6 m from the sample, using a monochromatic incident X-ray beam ($\lambda = 0.1$ nm). The available wave vector range was 0.10−0.68 nm^{-1}. The samples were placed in a thermostatted sample holder and measured at temperature 25.0 ± 0.5 °C.

Kinetics of Coagulation of Complexes in 0.15 M NaCl Solutions. The kinetics of coagulation of the polycation/DNA complexes was examined by turbidity measurement in 0.15 M NaCl solutions. The complexes were prepared by fast addition of polycations under standard conditions ($c_{DNA} = 2 \times 10^{-5}$ g/mL and $X = 2$) in Q-water. Then the ionic strength of the solution was increased up to 0.15 M NaCl by fast addition of 4 M NaCl stock solution. The measurement of the solution turbidity was started immediately after the addition of NaCl at $\lambda = 500$ nm.

Results and Discussion

The results are presented in the following order. First, the important properties of polycations such as mass per charge and composition of copolymers obtained by dye exclusion titration and NMR spectra are evaluated. The effect of preparation conditions on properties of polycation/DNA complexes is given in the second part. Finally, the effect of hydrophobic interactions on colloid stability is measured and discussed.

Formation of Complexes Investigated by Dye Exclusion Assay. Complex formation was initially established by observing the changes in UV spectra of DNA in the presence of polycations. When a polycation was added to a solution of DNA, a red shift of UV absorption in the range 220−300 nm was found (see Supporting Information). Such UV shift is one of the typical signatures of complex formation.[2]

The ability of new polycations to promote condensation of DNA was also monitored by changes in fluorescence intensity of ethidium bromide. Ethidium bromide is a cationic dye that exhibits approximately 10-fold fluorescence enhancement upon binding to DNA. As the polycation interacts with DNA, it replaces the dye, and the fluorescence intensity decreases. The DNA condensation curve for the used polycations is a typical sigmoidal curve (see Figure 2) with the main drop of I_f close to the point of charge equivalence ($X_c = 1$). The low I_f independent of X beyond the main drop of I_f indicates practically total DNA condensation. The X value corresponding to the beginning of the I_f base should be close to full compensation of charges for low-molecular-weight polycations[21] ($M_w \sim 10^4$). This results from a high degree of compensation of negative charges of PEC by polycations with lower molecular weights due to their steric hindrance, which is lower than that for high-molecular-weight polycations.[21] Therefore, we have used the X-position of the I_f edge for calculation of the mass/charge ratio; the values are shown in Table 1. The obtained values are lower than those obtained by the titration of chlorides with $AgNO_3$ (Mohr method) in accord with our expectations.

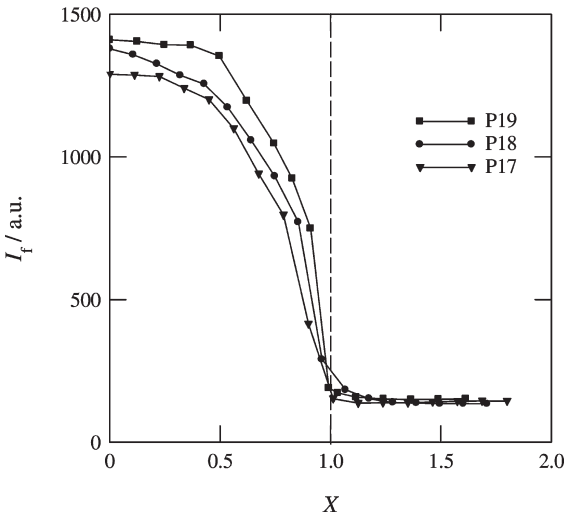

Figure 2. Dependence of fluorescence intensity of ethidium bromide (EB) I_f on X (slow addition of used polycations); $\lambda_{ex} = 514.5$ nm, $\lambda_{em} > 560$ nm, $c_{DNA} = 2 \times 10^{-5}$ g/mL, $c_{PEC} = 1.8 \times 10^{-5}$ g/mL, $c_{EB} = 2.0 \times 10^{-6}$ g/mL.

Since the I_f curve for PECs prepared using polycation P19 was influenced by aggregation and precipitation of the complexes in the vicinity of full charge compensation, we have used in this case for calculation of the mass/charge ratio and molar content of cholesterol moieties in copolymers the NMR results that were determined with high accuracy just for P19. Calculated from ^1H and ^{13}C NMR spectra, the content of cholesterol was 10 ± 1 mol %. I_f as a function of X is shown for all the investigated polycations in Figure 2. The corrected X values were calculated using the fluorescence and NMR mass/charge values from Table 1.

Effect of Preparation Conditions on Properties of Polycation/DNA Complexes. Since the properties of PECs prepared by slow and fast additions are usually different, particularly close to the point of charge equivalence ($X_c = 1$),[15] we have performed both of the procedures with the new hydrophobic polycations used for the investigation.

Slow polycation addition. The dependence of PEC parameters (M_w and R_h) as a function of the molar mixing ratio X is shown for complexes of polycation P17/DNA in Figure 3a and 3b. The quality of SLS measurements is demonstrated in Figure 3c, where the Debye plot of P17/DNA complexes is shown for a variety of mixing ratios X. The M_w values increase with increasing X to the maximum value at $X \approx 1.06$ (Figure 3a). No flocculation was observed at the 1:1 mixing ratio. While an increase in M_w is only small for the complexes prepared from P17, with the lowest content of cholesterol, a more pronounced increase in M_w due to enhanced aggregation followed by flocculation was observed for PECs prepared from polycations with higher contents of cholesterol (especially with polycation P19) in the vicinity of $X = 1$ (not shown). Thus, only negatively charged PECs could be prepared by slow addition of P18 and P19 polycations, which are not suitable for biological and medical applications.

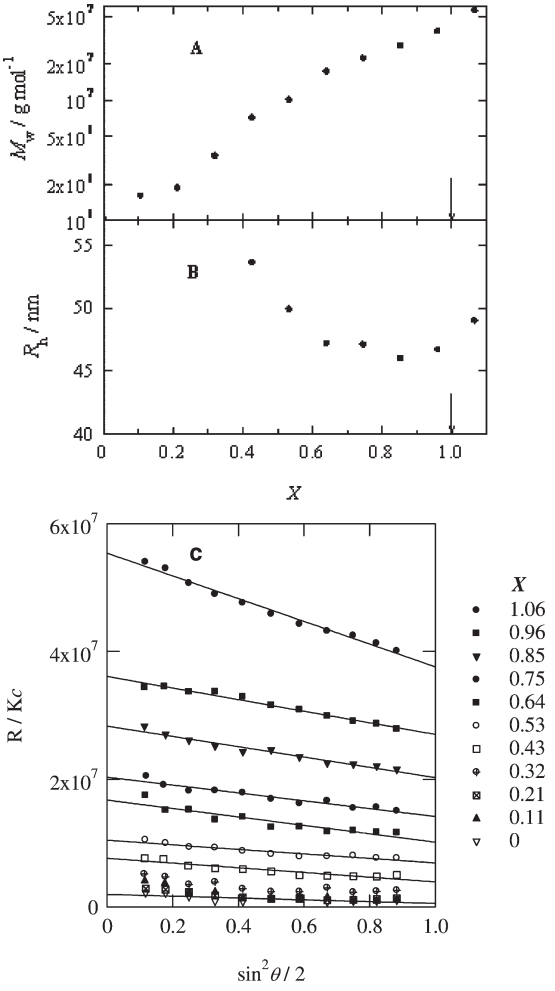

Figure 3. X-dependences of M_w (A) and R_h (B) of PECs prepared by a slow titration of P17 to DNA solution, $c_{DNA} = 2 \times 10^{-5}$ g/mL.

The hydrodynamic radius R_h of the complexes was estimated by DLS at a scattering angle θ of 90° for the P17/DNA systems. In the case of DLS, contributions of PECs to the scattering were separated from those of large impurities by a fitting procedure. The details of the fitting routine are described elsewere.[49] We believe that the observed impurities are larger particles that appeared due to flocculation. The results are given in Figure 3b for $X \geq 0.4$ only because the R_h data for low X (and low light scattering intensities of small PECs) were offended by the noise due to the presence of impurities. R_h decreases with increasing X up to $X = 0.95$ and then a small increase at $X = 1.06$ (probably due to small aggregation) is observed. R_h of PECs are smaller than 50 nm in all cases, therefore PECs fulfill the necessary condition for transport into cells by endocytosis.[50]

Fast Polycation Addition. Since positively charged PECs, which are more suitable for biological applications, cannot be prepared by slow addition of P18 and P19 polycations to DNA solution, we tried to prepare positively charged PECs at $X > 1$ by fast addition of polycations to DNA solutions. M_w and ζ-potentials of the PECs are shown as a function of X for all polycations in the range from $X = 0.8$ to 2 in Figure 4a,b, respectively. M_w decreases with increasing X for polycations P18 and P19 (higher contents of cholesterol). Such behavior was

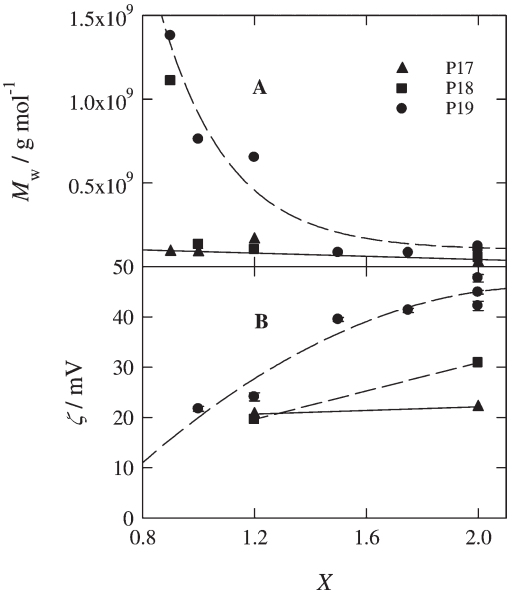

Figure 4. (a) M_w of PECs prepared by fast polycation addition plotted as a function of X; $c_{DNA} = 2 \times 10^{-5}$ g/mL. (b) ζ-Potential of PECs prepared by fast polycation addition plotted as a function of X; $c_{DNA} = 2 \times 10^{-5}$ g/mL.

frequently observed for hydrophobic polycations, as described, e.g., by Kabanov et al. and [2] Oupický et al.[18] M_w remains practically independent of X for P17 with the lowest cholesterol content. The effect of decreasing of M_w with increasing of X could be qualitatively explained by the enhanced stabilization of hydrophobic nuclei of DNA+polycations. Such hydrophobic nuclei aggregate with final macroscopic phase separation as a result of its significant hydrophobicity unless aggregation is terminated by any solubilizing agent—polycation molecules, in our case, which are in excess in a solution. If the adsorbed polycations reach a critical surface concentration, the nanoparticles are sufficiently solubilized and the growth of nanoparticles stops. Thus, the bigger X, the sooner aggregation will be terminated and nanoparticles with lower M_w will occur.

Surprisingly, the ζ-potential increases with increasing X, especially for P19 where $\zeta = +45$ mV was found for $X = 2$ (see Figure 4b) The value is more than twice higher than those observed for PECs prepared at $X = 2$ with the other polycations.[48] A smaller ζ-potential was found for PECs prepared with P18, with an medium content of cholesterol at $X = 2$. PECs prepared with copolymer P17 show on X-independent ζ-potential of about +20 mV, like complexes with other polycations.[48] Thus, the ζ-potential of complexes prepared by fast addition of polycations to DNA solutions increases with hydrophobicity of polycations at $X = 2$. The effect could be explained by the following working hypothesis: The PECs are formed by electrostatic long-range attractive forces at the early stage after mixing polycation solutions with DNA. As a result, hydrophobic particles are formed in the vicinity of the charge compensation point ($X \sim 1$). DNA and polycation molecules are intermixed in the initially formed complex. Further, the particles start to attract hydrophobic polycations by hydrophobic interaction. The strongly hydrophobic cholesterol moieties in polycations are attached to the particle surface; remaining positively charged parts of polycations increase the surface ζ-potential of the particles. The growth of particle stops by repulsive interactions of positively charged PECs and polycations. The above suggested PEC structure is schematically shown in Figure 5. The same mechanism can also explain the growth of M_w of PECs at $X = 2$

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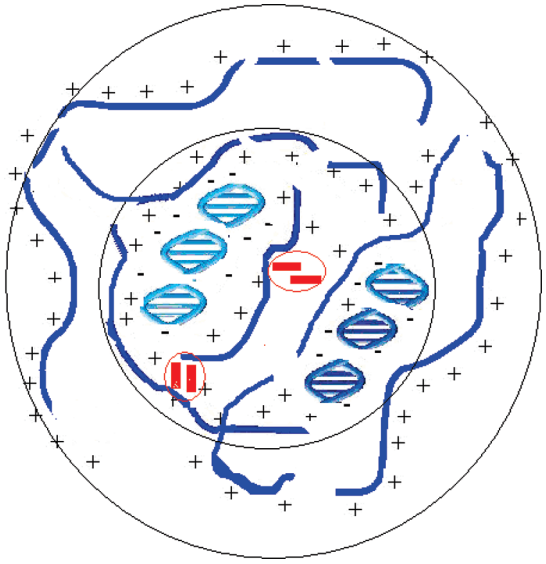

Figure 5. Scheme of polyplex structure by fast polycation addition to DNA solutions.

with increasing hydrophobicity of polycation (see Table 2). The polyplex has a core−shell structure as was predicted by Vasilevskaya et al.[51,52]

We also have to mention that P17 has a lower molecular weight, which will have similar influence as a decrease of hydrophobicity. This effect was demonstrated by Kabanov et al.[53]

The hydrodynamic radii R_h of the complexes were also estimated by DLS for PECs of all the polycations used at $\theta = 90°$. The results for $X = 1.2$ and 2 are given in Table 2. In contrast to the results for $X = 1.2$, R_h found at $X = 2$ increases with polycation hydrophobicity.

In order to characterize the density of polymers in the PEC particle, the structural density ρ_h was calculated from hydrodynamic volumes of particles V_H (in cm^3) and from their molecular weights M_w. The results are shown for $X = 1.2$ and 2 in Table 2. ρ_h increases with increasing hydrophobicity of polycations approaching $\rho_h = 0.205$ at $X = 2$. The aggregates of PECs observed at $X = 1.2$ for P19/DNA complexes are influenced by PEC aggregation in the vicinity of charge compensation point. Therefore, the found ρ_h is overestimated. The values $\rho_h \approx 0.1-0.2$ observed at $X = 2$ are comparable with those found for micelles in organic solvents.[54]

From M_w of the complexes, the DNA aggregation number, the ratio $N_{DNA} = M_w(PEC)/M_w^s$ can be estimated, where M_w^s is the molecular weight of complexes bearing only one DNA molecule.[44] Assuming full charge compensation of DNA molecules by polycations, N_{DNA} values were calculated using the data from Tables 1 and 2 for PECs formed at $X = 2$. The results are shown in Table 2. The lowest aggregation number 2 was observed for P17/DNA complexes. This indicates that single DNA complexes are predominating in solution. The aggregation number of PECs increases with increasing hydrophobicity of polycations (the cholesterol content).

The parameters of PECs prepared by slow and fast addition can be compared for P17/DNA complexes just slightly above $X = 1$. $M_w = 5.0 \times 10^7$ g/mol and $R_h = 49$ nm were found at $X = 1.06$ and $M_w = 8.8 \times 10^7$ and $R_h = 43$ nm at $X = 1$ for slow and fast procedures, respectively. Thus, slightly smaller and more compact particles are prepared by the fast procedure.

Two preparation modes gave different results because of the changes in structure of PECs. While the slowly prepared complexes, formed close to equilibrium conditions are homogeneous, the fast prepared PECs, formed under nonequilibrium conditions, should have more complex structure (see Figure 5) and, therefore, they are more compact in accord with experimental observations.

Visualization of the Complexes by AFM. AFM was used as a direct method of obtaining information about the morphology of PEC particles consisting of size, shape, and polydispersity. The AFM amplitude images, obtained in the tapping mode, of some nanoparticles are presented in Figure 6a−d. As Figure 6a shows, at $X = 2$, when using polycation P18 in excess, complex particles are evident. The sizes of dried polyplexes obtained by analysis of AFM pictures are only slightly smaller (the radius ranging from 40 to 60 nm) than the R_h values presented in Table 2. The polyplexes exhibit a condensed, compact core surrounded by a thick and fluffy coating (Figure 6b−d). The fluffy shell can be attributed to the polycation, which condensed around the DNA core in agreement with above proposed polyplex structure. This is further evidence of the core−shell structure of polyplexes. The most uniform morphologies were observed for a P17/DNA $X = 2$ polyplex with the lowest content of cholesterol (Figure 6b). Increasing the content up to 10 mol %, the particles homogeneity strongly decreased (Figure 6d). One can see that it consists of several different subunits. The highly nonuniform structures found for this system are explained by a higher content of the hydrophobic cholesterol moieties, which self-assembles into micelle-like units on the polyplex surface (see Figure 5).

Colloid Stability of Complexes. The time evolution of turbidity, which is related to the kinetics of phase separation and coagulation of PECs in saline is shown in Figure 7. The kinetics of coagulation depends on the amount of bound cholesterol in the polycations used for preparation of PECs. The complexes prepared from polycations P17 with the lowest content of cholesterol showed the highest coagulation rate. The rate of coagulation was lower for PECs prepared by self-assembly of DNA with polycations bearing higher amounts of cholesterol. The fast increase in turbidity observed at the beginning of turbidity measurement for PECs with P19 is probably caused by aggregation of excess P19 in saline. The polycation P19 concentration is close to the solubility limit in water and hence it partly aggregates in 0.15 M NaCl solution. The small increase in turbidity observed at longer observation times corresponds to a very slow coagulation of polyplexes prepared from P19 polycations. Thus, we can state that the colloid stability of PECs increases with increasing content of side-chain cholesterol moieties. Our results correlate well with those obtained by Reschel et al.[21] The authors demonstrated a higher stability of poly[*N*-(2-aminoethyl)methacrylamide trifluoroacetate] (PAEMA)/DNA complexes, compared with poly(L-lysine) (PLL)/DNA complexes, due to the more hydrophobic nature of the polymer carbon chain. The stability decay time for P19, obtained by fitting the data with exponential growth function, is compatible with the most stable complexes, as shown also by Reschel et al.[21]

The effect of the cholesterol moieties on coagulation (turbidity) of the complexes can be explained as follows. The nonstoichiometric polycation/DNA complexes prepared in water with an

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Table 2. Characteristics of Polyplexes Prepared by a Fast Addition of Hydrophobic Polycations to DNA Solutions for $X = 1.2$ and 2

polycation	X	$M_w \times 10^{-7}$ g mol^{-1}	R_g nm SAXS	R_h nm	R_g/R_h	ρ_h g cm^{-3}	N_{DNA}	ζ mV
P17	1.2	16.4	80	80 ± 2	0.97	0.126		+21
	2	2.65	58	44 ± 1	1.33	0.127	2	+22
P18	1.2	10.1	88	80 ± 2	1.11	0.080		+20
	2	5.39	48	58 ± 2	0.83	0.112	4	+31
P19	1.2	26.2	89	66 ± 2	1.34	0.358		+24
	2	12.2	70	62 ± 2	1.13	0.205	9	+45

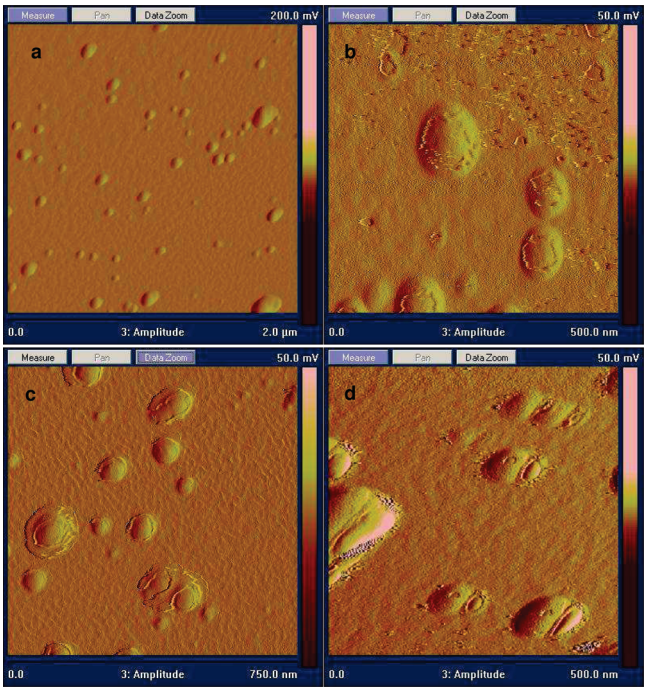

Figure 6. AFM amplitude images. (a) P18; $X = 2.0$ (resolution 2 μm); (b) P17; $X = 2.0$ (resolution 500 nm); (c) P18; $X = 2.0$ (resolution 750 nm); (d) P19; $X = 2.0$ (resolution 500 nm); $c_{DNA} = 2 \times 10^{-5}$ g/mL. Complexes are prepared by fast addition method.

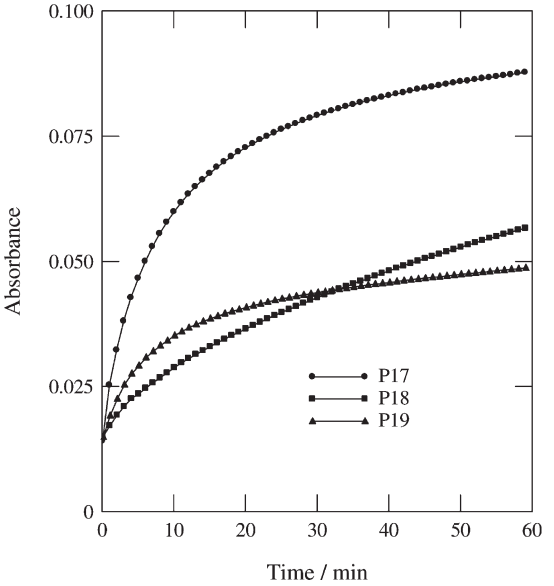

Figure 7. Time dependences of the turbidity of PECs solutions in 0.15 M NaCl; $X = 2$, $c_{DNA} = 2 \times 10^{-5}$ g/mL. Complexes are prepared by fast addition method.

excess of polycations ($X = 2$) possess a positive surface charge (see ζ-potential in Table 2 and Figure 4) protecting PECs from

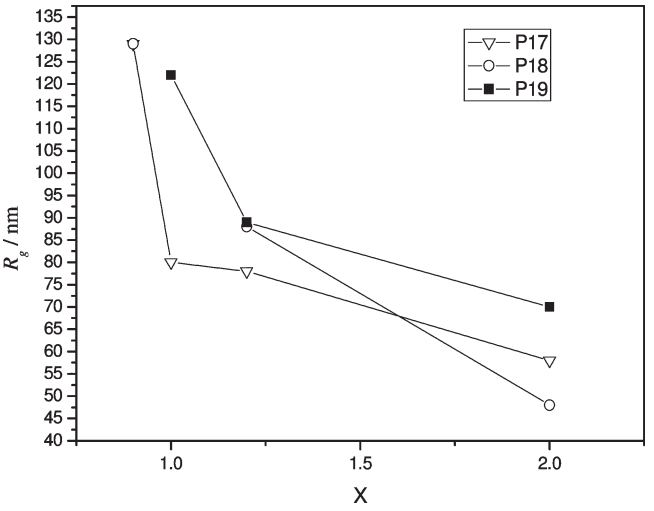

Figure 8. Dependence of R_g of PECs prepared by fast addition of polycations to DNA solutions as a function of composition ratio X for all the used polycations, $c_{DNA} = 2 \times 10^{-5}$ g/mL.

aggregation. In saline (0.15 M NaCl), positive surface charges are successively screened, and the complexes become more neutral and start to coagulate. The higher the surface charge (ζ-potential), the slower the PEC coagulation.

SAXS Results. Polyplex formation was also monitored by SAXS experiments for polyplexes with different composition ratios. The R_g values extracted by applying the Guinier approximation, $I(q) = I(0) \exp(-R_g^2 q^2/3)$, to the initial region of SAXS curves for PECs at $X = 2$ are shown in Figure 8. The advantage of this approach is that it can be used regardless of the shape of the object involved. We have neglected the concentration dependence $R_g(c)$ due to extremely low concentrations of components. The R_g values obtained from SAXS measurements are more accurate than those obtained from SLS measurements (lower effect of impurities); therefore they are used in Table 2. Both the R_g as well as R_h values reported above diminish with increasing polycation content in polyplexes. Another important ratio relating static and dynamic properties defined as the ratio of radius of gyration to hydrodynamic radius, is presented in Table 2. The values of R_g/R_h are mostly close to unit in accord with the results for PECs published, e.g., Šubr et al.[55] Such particles behave as "soft balls".[56] The P18/DNA complex at $X = 2$ behaves as hard spheres (R_g/R_h is close to 0.78).

Conclusion

In this study we investigated properties of new polyplexes formed from cholesterol-modified polycations and DNA. The effect of preparation conditions on the properties of polycation/DNA complexes was investigated. The positively charged PECs were prepared by fast addition of polycations to DNA solutions

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at $X > 1$. M_w and ζ-potential were determined in the X range 0.8−2. M_w decreased with increasing X for polycations P18 and P19 (with higher contents of cholesterol) and remained practically independent of X for P17 with the lowest cholesterol content. Surprisingly, ζ-potential increases with increasing X, especially for P19, where $\zeta = +45$ mV was found for $X = 2$. Thus, the ζ-potential of complexes prepared by a fast addition of cations to DNA solutions increased with hydrophobicity of polycations. The effect was explained by the following working hypothesis. The PECs are formed by electrostatic long-range attractive forces at an early stage, after mixing polycation solutions and DNA. As a result, hydrophobic particles are formed in the vicinity of the charge compensation point ($X \sim 1$). DNA and polycation molecules are intermixed in the initially formed complex. The particles start to attract hydrophobic polycations by hydrophobic interaction forming core−shell structure. The strongly hydrophobic cholesterol moieties of polycations are attached to the particle surface and, remaining positively charged parts of polycations, increase the ζ-potential of particle surface (see Figure 5). The particle growth is stopped by repulsive interactions of positively charged PECs and polycations.

The colloid stability of PECs increases with increasing content of side chains bearing cholesterol moieties, which is related to the level of surface charge (ζ-potential) and hydrophobicity of polycations (Figure 5).

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Supporting Information Available: Effect of DNA condensation on its DNA absorption UV spectrum near $\lambda = 260$ nm. This information is available free of charge via the Internet at http://pubs.acs.org/.