Coating of DNA/Poly(L-lysine) Complexes by Covalent Attachment of Poly[*N*-(2-hydroxypropyl)methacrylamide]

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N-(2-Hydroxypropyl)methacrylamide (HPMA) copolymers (pHPMA) containing 4-nitrophenyl ester (ONp) or thiazolidine-2-thione (TT) reactive groups in side chains and telechelic/semitelechelic pHPMA with TT groups were designed as highly hydrophilic biocompatible polymers suitable for chemical coating of polyelectrolyte-based DNA-containing nanoparticles bearing amino groups on the surface. The course of the coating reaction carried out in aqueous solution was evaluated on model self-assembling polyelectrolyte DNA/poly(L-lysine) (DNA/PLL) complexes either by monitoring the amount of residual polymer reactive groups by UV spectroscopy or by monitoring changes in the weight-average molecular weight and hydrodynamic size of the complexes using light scattering methods. Physicochemical stability of the coated complexes in buffered saline solution was also investigated. Contrary to uncoated particles, the coated complexes showed remarkable stability to aggregate in 0.15 M NaCl. Coating with pHPMA had practically no effect on the size distribution of the most stable complexes prepared by complexation of DNA with high-molecular-weight PLL (M_w = 134 000) as shown by dynamic light scattering. The coating reaction was faster and more efficient with multivalent HPMA copolymers containing TT reactive groups than that with HPMA copolymers containing ONp groups.

Introduction

Nanoparticles, e.g., polyelectrolyte DNA/polycation complexes (PECs), liposomes, and micelles, attract much attention as promising synthetic vectors for gene and drug delivery. However, these delivery systems are often quickly eliminated from the bloodstream following intravenous injection (e.g., plasma half-life is typically less than 5 min for PECs).[1] The nanoparticles are usually cleared quickly into the liver or spleen. In vivo experiments indicate significant phagocytosis of PECs by cells of the reticuloendothelial system (RES) (tissue macrophages such as Kupffer cells) and uptake in liver.[1] For the design of targeted gene delivery systems, a more prolonged plasma circulation of the DNA vector is essential. It is therefore a general aim to eliminate interactions of the vector with plasma proteins and cells of immune system, to decrease its uptake by liver, and to develop nanoparticles with long-term circulation.

The interaction of similar particulate drug delivery systems with the cells of RES is determined to a great extent by physicochemical properties of their surface and their size (larger particles being taken up faster).[2,3] Initial stages of phagocytosis involve physical attachment of the particle to the surface of the macrophage. An increase in particle hydrophobicity is known to increase uptake by forming hydrophobic interactions between the particle and the cell membrane.[3] To avoid capture of particles by cells of the RES, it is therefore important to reduce the hydrophobicity of the particle surface. Nanoparticles with polar and charged surfaces have an increased circulation time and reduced uptake by RES.[4] However, nanoparticles with a strong positive surface charge bind nonspecifically to any biological membrane, whereas those with a strong negative charge can be objects for phagocytosis via the macrophage polyanion receptor.[5] Hence, the best way to prolong particle blood circulation is to eliminate the presence of any charged groups from the particle surface.

In the process of uptake of the particles by the RES, an important role is played by the adsorption of blood components, mainly proteins, on particle surface. Blood contains a large number of high-molecular-weight proteins and glycoproteins (opsonins)[2] that can adsorb rapidly onto positively charged or hydrophobic particle surfaces. The adsorbed layer thus often determines the fate of particulate carriers. In most cases, due to its high concentration in blood, the protein adsorbed fastest is albumin. Other substances present in the blood at lower concentrations, exhibiting higher affinities to the particle surface (e.g., opsonins), then gradually diffuse to the particle surface and can displace the rapidly formed albumin layer.[5]

Therefore, several approaches to surface modification of nanoparticles have been evaluated to eliminate or reduce opsonization and interaction with macrophages and cells of the RES. One of the most successful strategies for obtaining long-circulating nanoparticles and liposomes has been attachment of a highly hydrophilic polymer (usually poly(ethylene glycol), PEG) onto the particle surface, to create a hydrophilic brush reducing interactions with proteins and cells (steric stabilization). This approach often named also STEALTH has been successfully applied in modification of liposomes and nano- and microparticulate drug delivery systems.[3,6,7] The *N*-(2-hydroxypropyl)methacrylamide copolymers (pHPMA) with 4-nitrophenyl ester (ONp) reactive groups and multivalent reactive polymer based on alternating segments of poly(ethylene glycol) and tripeptides bearing ONp reactive ester groups were successfully used for surface modification of PECs.[8−11] The polymer-coated complexes and adenoviruses exhibited significantly prolonged blood circulation, resistance to interaction with blood proteins, and uptake by macrophages.[8−12]

Here we evaluate the potential advantages of coating surface amino-bearing polyelectrolyte vectors, prepared by self-assembly

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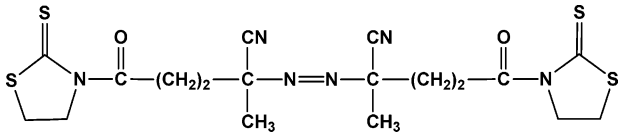
Scheme 1. Structure of the Initiator ABIK-TT



of DNA with poly(L-lysine) (PLL), with reactive polymers containing thiazolidine-2-thione (TT) groups. In this way, we aim to achieve stabilization, surface hydrophilization, and protection from undesirable molecular interactions. The reactive TT-containing polymers include new *N*-(2-hydroxypropyl)-methacrylamide statistical copolymers and also semitelechelic polymers. DNA/PLL complexes rank among the best studied synthetic gene delivery systems worldwide, and numerous data on their behavior are available. In this study, they are used as a simple model, enabling optimization of reaction conditions for coating of the nanoparticle surface and for study of the physicochemical behavior of the polymer-coated systems in aqueous solutions.

Materials and Methods

Materials. Calf thymus DNA (CT-DNA) (sodium salt) and poly(L-lysine hydrobromide) (PLL) (M_w = 23 400 and 134 000) were from Sigma Chemical Co. Methacryloyl chloride, 1-aminopropan-2-ol, glycylglycine, 6-aminohexanoic acid, thiazolidine-2-thione, 4-nitrophenol, *N*,*N*′-dicyclohexylcarbodiimide (DCC), 4-(dimethylamino)-pyridine (DMAP), 2,2′-azobisisobutyronitrile (AIBN), 4,4′-azobis(4-cyanopentanoic acid) (ABIK), dimethyl sulfoxide (DMSO), and 4-(2-hydroxyethyl)piperazine-1-ethanesulfonic acid (HEPES) were from Fluka (Sigma-Aldrich sro). All other chemicals and solvents were of analytical grade. The solvents were dried and purified by conventional procedures and distilled before used.

Synthesis of Monomers. *N*-(2-Hydroxypropyl)methacrylamide (HPMA), *N*-methacryloylglycylglycine (Ma-GlyGly-OH), and *N*-methacryloylglycylglycine 4-nitrophenyl ester (Ma-GlyGly-ONp) were prepared as described elsewhere.[13,14] *N*-Methacryloylated 6-aminohexanoic acid (Ma-ϵAhx-OH) was prepared accordingly.[15] Monomers, 3-(6-methacrylamidohexanoyl)thiazolidine-2-thione (Ma-ϵAhx-TT) and 3-(*N*-methacryloylglycylglycyl)thiazolidine-2-thione (Ma-GlyGly-TT), were prepared by the DMAP-catalyzed reaction of Ma-ϵAhx-OH and Ma-GlyGly-OH with thiazolidine-2-thione in the presence of DCC.[16]

Synthesis of 3,3′-[4,4′-Azobis(4-cyano-4-methyl-1-oxo-butane-4,1-diyl)]bis(thiazolidine-2-thione) (ABIK-TT). 4,4′-Azobis(4-cyanopentanoic acid) (2.0 g, 7.135 mmol), thiazolidine-2-thione (1.87 g, 15.7 mmol), and 4-(dimethylamino)pyridine were dissolved in tetrahydrofuran (20 mL); DCC (3.88 g, 18.8 mmol) was dissolved in THF (5 mL). Both solutions were cooled to −10 °C, mixed, and kept at −10 °C for 1 h and then at 5 °C for 24 h. Acetic acid (0.1 mL) was added and the mixture was stirred for an additional 1 h at room temperature. Precipitated dicyclohexylurea was filtered off, and THF was evaporated in a vacuum. The oily residue was dissolved in dichloromethane and crystallized from a dichloromethane−diethyl ether mixture. The structure of ABIK-TT initiator is shown in Scheme 1.

Yield 3.0 g (87%), mp 126−130 °C, Elemental analysis: calc/found: C 44.79/45.16, H 4.59/4.74, N 17.41/16.95, S 26.57/26.47. Molar absorption coefficient ϵ_{305} = 20 500 L·mol^{-1}·cm^{-1} (methanol). ^{1}H NMR: σ = 1.56 (s, 6H), 2.75 (m, 4H), 2.95 (m, 4H), 3.19 (m, 4H), 4.04 (m, 4H).

Synthesis and Characterization of Reactive Polymers. The HPMA copolymers were prepared by three polymerization procedures.

(a) The reactive multivalent HPMA copolymer (P-GlyGly-ONp) bearing 4-nitrophenyl ester reactive groups in the side chains, with weight-average molecular weight up to 30 000 was prepared by radical precipitation copolymerization of HPMA with Ma-GlyGly-ONp in acetone using AIBN as initiator at 50 °C.[17]

(b) Reactive multivalent HPMA copolymers bearing thiazolidine-2-thione groups in side chains were prepared by radical solution copolymerization of HPMA with Ma-ϵAhx-TT or Ma-GlyGly-TT in DMSO. The required weight-average molecular weight was achieved by changing initiator concentration and polymerization temperature from 50 to 60 °C.[16]

(c) Reactive telechelic pHPMA polymer with TT reactive group was prepared by radical solution polymerization of HPMA initiated with ABIK-TT in DMSO. HPMA (2.0 g, 0.014 mol) and ABIK-TT (0.235 g, 0.5 mmol) were dissolved in DMSO (13 mL). The solution was introduced into a polymerization ampule, bubbled with nitrogen, and sealed. The polymerization was carried out at 50 °C for 6 h. The polymer was isolated by precipitation into an acetone:diethyl ether mixture (1:1) and purified by reprecipitation from methanolic solution into an acetone:diethyl ether mixture (3:1). The polymer was filtered off, washed with diethyl ether, and dried in a vacuum. The yield was 1.6 g (73.8%).

The content of the ONp or TT groups was determined spectrophotometrically on a HEλIOS α (Thermochrom) spectrophotometer. The following molar absorption coefficients were used for calculation (ONp, ϵ_{274} = 9 700 L·mol^{-1}·cm^{-1}, DMSO; TT, ϵ_{305} = 10 700 L·mol^{-1}·cm^{-1}, methanol). Weight- and number-average molecular weights were determined by size exclusion chromatography on an Äkta Explorer HPLC (Amersham Biosciences, Sweden) equipped with a multiangle light scattering detector DAWN DSP−F (Wyatt Technology Corp., Santa Barbara, USA), UV and RI (refractive index) detector using a Superose 6 or Superose 12 column. 0.3 M sodium acetate buffer (pH 6.5) was used as a mobile phase. The flow rate was 0.5 mL/min. The ONp or TT reactive groups were aminolyzed with 1-aminopropan-2-ol before analysis.

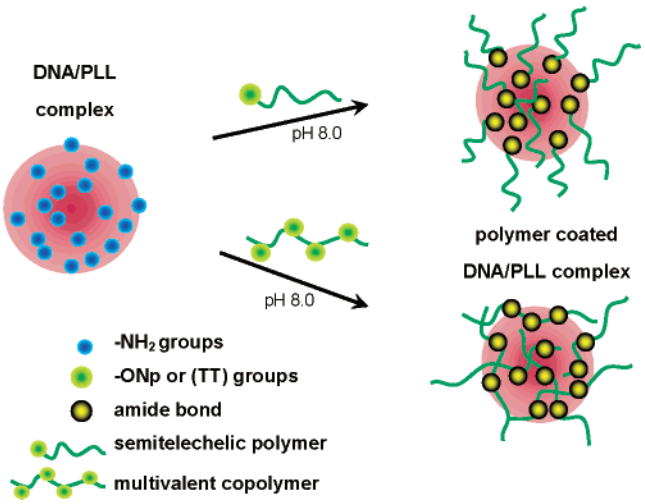
Formation of DNA/PLL Complexes and Their Coating with Reactive HPMA Copolymers. All complexes in this study were prepared in 0.01 M HEPES buffer (pH 7.4) at DNA concentration 20 μg/mL (concentration of phosphate groups was 61.5 nmol/mL). PLLs with the weight-average molecular weights of 23 400 and 134 000 were used for the complex formation. A PLL stock solution (2.25 mg/mL) was rapidly added to a stirred DNA solution in a single portion, so that the ratio of positive to negative charges (φ) was 1.2 and 2 (37 μL and 62 μL of PLL stock solution, PLL amino groups concentration was 73.8 and 123 nmol/mL, respectively). The complexes prepared at a mixing ratio of 2, in higher excess of PLL, provide more amino groups for coating than those prepared at the ratio 1.2. The high-molecular-weight PLL forms complexes with a higher amount of amino groups than the low-molecular-weight PLL because of conformation restrictions.[18,19] Thus, four types of complexes were available for coating experiments differing in the amount of amino groups exposed on the particle surface.

A freshly prepared solution of HPMA copolymer containing ONp or TT reactive groups in water (57 mg/mL) was then added to the complex solution followed by addition of 1 M HEPES buffer (pH 8.7; 0.2 mL) to reach a final pH of 8.0. The reaction was carried out at room temperature. The concentrations of HPMA copolymers used in the coating reaction ranged from 0.02 to 2 mg/mL. The coating process is schematically shown in Scheme 2.

Aminolysis and Hydrolysis of HPMA Copolymers with ONp and TT Reactive Groups. Rate of aminolysis and hydrolysis was followed spectrophotometrically as a decrease in absorbance of ONp and TT reactive groups of the HPMA copolymers. Due to high molar absorption coefficients of ONp and TT reactive groups, the measurements were carried out only at HPMA copolymer concentration 0.2 mg/mL.

In the study, the complexes were prepared as described above: the coating copolymer was added in final concentration 0.2 mg/mL, and the pH was adjusted to pH 8.0 or 8.2. The rate of aminolysis and hydrolysis of polymer-bound reactive groups was measured spectrophotometrically using the decrease in absorbance of a solution (concentration of polymer bound ONp reactive groups was measured at 272 nm and TT reactive groups at 305 nm for 6 h). The samples

Scheme 2. Schematic Feature of Coating of the DNA/PLL Complex by Covalent Attachment of Multivalent and Semitelechelic Hydrophilic Polymers



used for the measurement of the rate of hydrolysis were prepared in the same way, without addition of PLL to reaction mixture.

Static Light Scattering (SLS). Static light scattering measurements were carried out with a Sofica 42000 instrument (Wippler and Scheibling, Strasbourg, France). The Sofica instrument was equipped with a 10 mW He−Ne-laser (Spectra Physics) as light source. The accuracy of the measurements was about 2%.

The refractive index increments, ν, of the individual components of the complexes were taken from the literature. The ν values used were 0.185 and 0.188 for DNA and PLL, respectively.[20,21] Since the molar content of reactive groups attached to pHPMA backbones is low (about 7 mol %), $\nu = 0.167$ for neat pHPMA was used in calculations for all the coating copolymers. The estimation of the concentrations and refractive index increments of the complexes as a function of the molar mixing ratio was carried out on the basis of the model of complex formation.[22]

The static light scattering data were analyzed using the Zimm plot

$$\frac{Kc_{pc}}{R(q)} = \frac{1}{M_w^{pc}} + \frac{R_g^2 q^2}{3M_w^{pc}} \qquad (1)$$

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 the scattered beam, K is the contrast factor containing the optical parameters, c_{pc} is the complex concentration, M_w^{pc} is the weight average of the molar mass of the complex particles, and R_g is their radius of gyration. The data analysis based on a fitting procedure using theoretical model curves in a scaled representation[23,24] was used to obtain zero-angle limits of $R(\theta,c_{pc})/Kc_{pc} = M_w^{pc}$. The concentration dependence was neglected, which seems to be justified because of low concentrations of the PEC solutions ($\sim 10^{-5}$ g/mL). Since DNA solutions cannot be filtered (a danger of DNA degradation), data obtained at $\theta < 45°$ were not used for an analysis because of a nonnegligible dust contribution. The apparent molecular weight of coated complexes M_{wa}^{cc} was calculated assuming that changes of c_{pc} due to coating of copolymers are low. The scattered intensity of coating copolymers was subtracted from the total scattering intensity of the solution.

Kinetics of coating processes was monitored by changes of the apparent molecular weight of complexes $M_{wa}^{pc/cc} = R(90°)/Kc_{pc}$ measured at the scattering angle $\theta = 90°$. The time-resolved measurements of scattered intensity were performed on an ALV goniometer equipped with ALV 6000 correlator. The apparatus was selected because of its ability to correct the light scattering intensity by incident beam intensity securing in such a way a long-term stability of the measurement.

Dynamic Light Scattering (DLS). Polarized DLS measurements were made in the angular range 45−135° using a light scattering apparatus equipped with an He−Ne (632.8 nm) and an ALV 5000, multibit, multi-tau autocorrelator covering approximately 10 decades in delay time τ.

The inverse Laplace transform using the REPES[25,26] method of constrained regularization (a part of the GENDIST program), which is similar in many respects to the inversion routine CONTIN,[27] was used for 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 20 of components per decade) and determines their amplitudes. As a result, a scattered light intensity distribution function $A(\tau)$ of decay times is obtained which can be easily transformed into a distribution function of hydrodynamic sizes.

The average hydrodynamic radius R_h was calculated from the diffusion coefficient D using the Stokes−Einstein equation. At least 5 measurements were made of each sample to check repeatability. The experimental error of the R_h determination was typically ca. 3% for the complexes (unimodal fit) and less than 5% for coated complexes (bimodal fit).

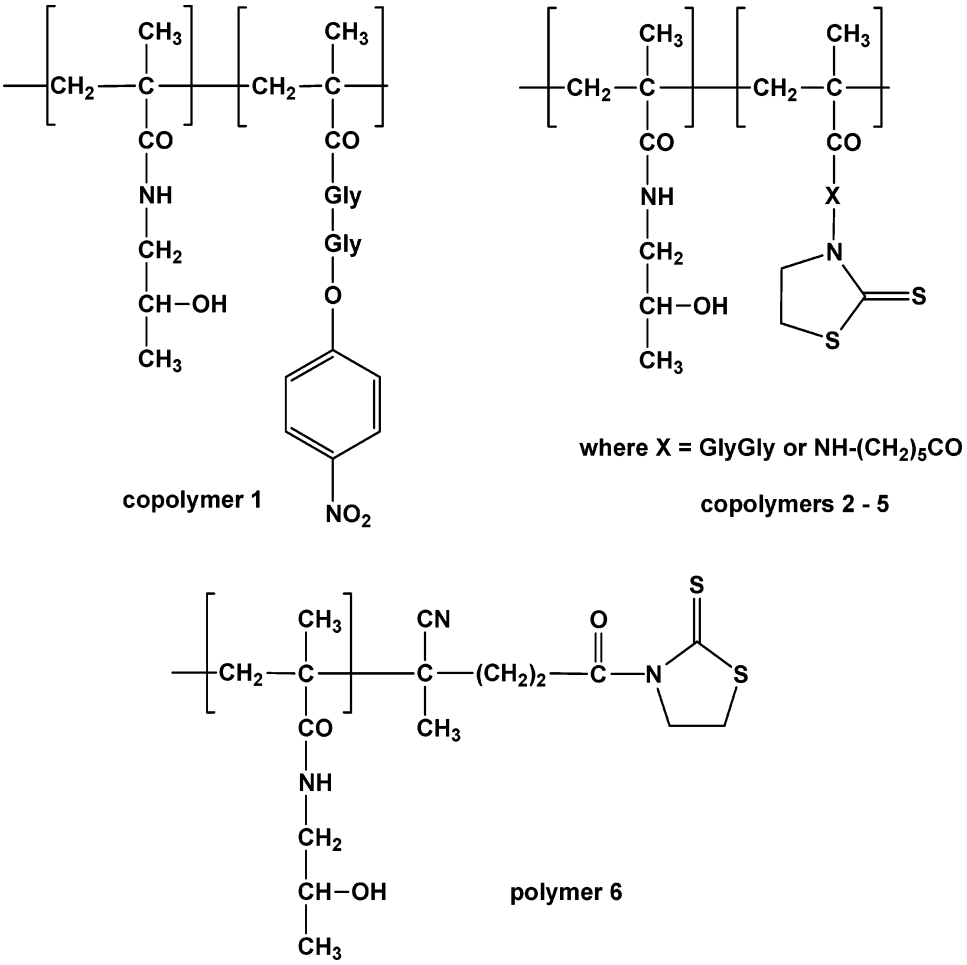
The size polydispersity of coated and uncoated complexes was evaluated by a force fitting of the time autocorrelation functions to the Gaussian distribution of characteristic relaxation times (a part of the GENDIST program).

Electrophoretic Light Scattering. Measurements of the zeta (ζ) potential were made using a Zetasizer ZS3600 (Malvern Instruments, U.K.). At least 10 measurements were made of each sample to check for reproducibility. Breaks of 5 min between the measurements were set to prevent heating of samples by electric current. The measurements of electrophoretic mobilities 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 for correct instrument operation.

Results and Discussion

Surface modification of DNA delivery vectors including PEC with hydrophilic polymers has been generally accepted as a strategy enabling prolonged blood circulation and protection of gene delivery vectors from biodegradation, from interactions with cells of immune and reticuloendothelial systems and from uptake by liver during their transport to the target cells.[3] Two systems were used in preparation of polymer-coated complexes. Complexes prepared from block and graft copolymers consisting of hydrophilic (PEG, pHPMA) and polycationic blocks exhibited lower density of the particle core and thus larger size compared with the complexes prepared by complexation of polycations with DNA followed by surface coating with reactive hydrophilic polymer. Advantages of the use of reactive HPMA copolymers containing ONp groups for coating of complexes and viruses were demonstrated.[8−12] The pHPMA-coated complexes exhibited higher hydrophilicity, better control of molecular weight and multivalency enabling further modification of particle surface with other biologically active molecules (e.g., targeting moieties and fusogenic peptides)[9,11] compared with PEG-coated complexes. Unfortunately, the major problem of using pHPMA with ONp reactive groups was associated with rather high rate of hydrolysis of ONp groups during aminolytic coating reactions carried out in aqueous media resulting in lower yield of the reaction. This is why we have developed two types of pHPMA containing TT reactive groups, semitelechelic polymer with TT group at the end of polymer chain and multivalent copolymer with TT groups statistically distributed along the polymer chain.

Scheme 3. Structure of pHPMA Copolymers Containing ONp and TT Reactive Groups and Semitelechelic Polymer pHPMA-TT



A new type of azo initiator (ABIK-TT) enabling direct introduction of reactive TT end groups during polymerization was synthesized. The HPMA polymer containing the TT reactive end groups was prepared by radical solution polymerization of HPMA initiated by the ABIK-TT initiator in DMSO. This procedure has several advantages in comparison with the synthesis of semitelechelic HPMA polymers by radical polymerization performed in the presence of 3-sulfanylpropanoic acid as a transfer agent.[28,29] The reactive TT group was introduced into polymer end in one reaction step during polymerization. The use of reactive initiator provides full control of molecular weight of the resulting polymers with weight-average molecular weight ranging from 20 000 to 100 000 by changing the initiator or monomer concentration or polymerization temperature. Unfortunately, the method has also a drawback. Depending on the termination reaction (reaction conditions), the number of terminal reactive groups can significantly exceed unity. In our case, the average number of reactive TT groups per polymer chain was 1.2, i.e., close to one and the polymer was considered as semitelechelic. The chemical structure of the polymers used for coating of complexes is shown in Scheme 3, and molecular characteristics are given in Table 1.

Two analytical methods were used to monitor coating reaction: (i) measurement of the amount of residual groups (ONp or TT) of a coating copolymer in solution by UV spectroscopy and (ii) measurement of changes in molecular weight (M_w) and hydrodynamic radius (R_h) of the PECs using static and dynamic light scattering methods.

Aminolysis and Hydrolysis of Copolymers with TT and ONp Groups. The HPMA copolymers containing ONp or TT reactive groups react with amino groups of lysine residues of DNA/PLL complexes to form amide bonds chemically stable under physiological conditions. Undesirable hydrolysis of reactive groups in HPMA copolymers results in the formation of an unreactive carboxylic group, thus reducing the yield of coating reaction. The rate of aminolysis and hydrolysis of reactive groups were followed spectrophotometrically using the difference in wavelength of absorption maxima of bound ONp and TT groups or released 4-nitrophenol and thiazolidine-2-thione. The hydrolysis of reactive groups is dominating the time dependence of the amount of unreacted TT groups in aqueous solution. The reactions were carried out at polymer concentration $c \sim 0.2$ mg/mL, which allowed continuous monitoring of the reaction course. The effect of the coating reaction (aminolysis) on time dependence of the content of residual TT and ONp groups at pH 8.0 and 8.2 is shown in Figures 1 and 2,

Table 1. Characteristics of Coating Polymers

coating polymer	structure	M_w^{cp}, g/mol	M_w^{cp}/M_n^{cp}	R_h^{cp}, nm	reactive groups, mol %
1	P-GlyGly-ONp	21 000	1.49	5.3	8.17
2	P-GlyGly-TT	24 300	1.72	5.5	7.00
3	P-ϵAhx-TT	24 000	1.60	4.8	7.48
4	P-ϵAhx-TT	45 400	1.85	6.7	7.8
5	P-ϵAhx-TT	174 000	3.44	12.3	6.47
6[a]	pHPMA-TT	45 200	1.72	6.5	4.67×10^{-5} mol/g

[a] Semitelechelic polymer, functionality 1.2.

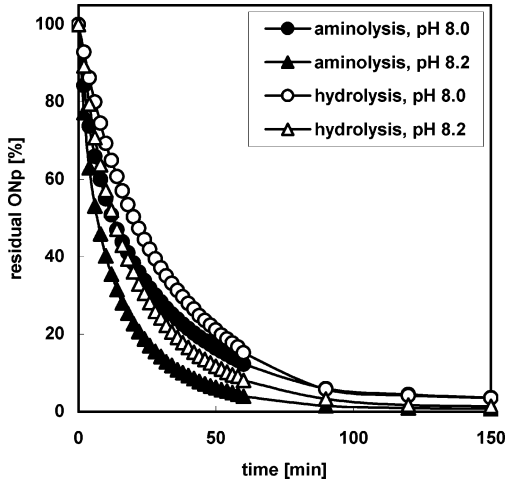


Figure 1. Decrease in the content of ONp reactive groups in copolymer **1** (P-GlyGly-ONp, M_w = 21 000, c = 0.2 mg/mL) in 10 mM HEPES solution due to coating of DNA/PLL (M_w = 134 000, φ = 2) complexes at pH 8.0 (●) and pH 8.2 (▲) and hydrolysis at pH 8.0 (○) and 8.2 (△). Standard deviation did not exceed 5%.

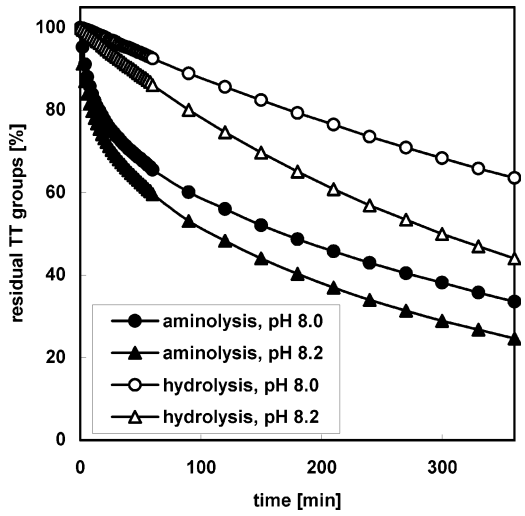


Figure 2. Decrease in the content of TT reactive groups in copolymer **4** (P-ϵAhx-TT, M_w = 45 400, c = 0.2 mg/mL) in 10 mM HEPES solution due to coating of DNA/PLL (M_w = 134 000, φ = 2) complexes at pH 8.0 (●) and pH 8.2 (▲) and hydrolysis at pH 8.0 (○) and 8.2 (△). Standard deviation did not exceed 5%.

respectively. The effect of hydrolysis of copolymers is shown for comparison. The difference between the course of the coating reaction curve and the hydrolysis curve is a measure of the coating reaction efficiency. Although the coating reaction carried out with the copolymers with ONp groups is fast and practically complete within 100 min after mixing of components, the coating reaction using the copolymers with TT groups is followed, after a very fast initial part (for several minutes), by a slow part continuing for more than 6 h. The difference between rates of coating (including aminolysis and hydrolysis) and hydrolytic reactions at pH 8.0−8.2 is much more pronounced for HPMA copolymers with TT groups than that found for copolymers with ONp groups. This means that, even if the absolute rate of coating reaction is slower with TT polymers, aminolysis significantly prevails in this case over hydrolysis and most of the reactive groups were involved in the reaction with amino groups exposed on the complex surface. Thus, multivalent HPMA copolymers with TT groups seem to be more suitable for coating of DNA complexes than the earlier developed copolymers with ONp groups.

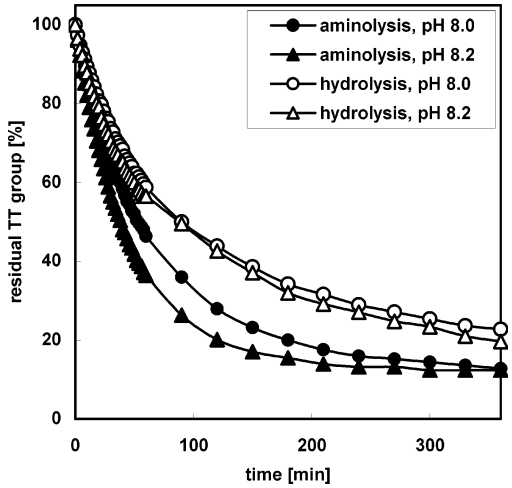


Figure 3. Decrease in the content of TT reactive groups in semitelechelic polymer **6** (pHPMA-TT, M_w = 45 200, c = 0.2 mg/mL) in 10 mM HEPES solution due to coating of DNA/PLL (M_w = 134 000, φ = 2) complexes at pH 8.0 (●) and pH 8.2 (▲) and hydrolysis at pH 8.0 (○) and 8.2 (△).Standard deviation did not exceed 5%.

The course of reaction of coating of DNA/PLL complexes with semitelechelic TT polymer is shown in Figure 3. Comparing Figures 2 and 3, it can be seen that the difference between aminolysis and hydrolysis curves, which is a measure of the coating efficiency of copolymers, is smaller for the semitelechelic polymer compared with the P-ϵAhx-TT one, especially at the beginning of the coating reaction. Thus, multivalent HPMA copolymers with TT groups are more efficient in coating of complexes than the semitelechelic copolymer.

Coating of DNA Complexes (Studied by SLS). Static and dynamic light scattering methods were used to monitor the efficiency of coating reaction of DNA/PLL complexes. Changes in the weight-average molecular weight (M_w) and hydrodynamic radius (R_h) of the PECs were examined. The sensitivity of the methods is demonstrated in Figure 4, panels a and b, where Zimm plots (a) and the R_h-distribution functions (b) for uncoated and coated DNA/PLL (134 000) complexes are shown. The complexes were prepared by fast addition of an excess of PLL (φ = 2) and coated with the copolymer **2**. The concentration of coating copolymer was 2 mg/mL. Results of light scattering measurements obtained for all four coating copolymers and complexes DNA/PLL (23 400) and DNA/PLL (134 000) prepared at mixing ratios φ = 1.2 and 2 are summarized in Table 2.

As can be seen in Figure 4b, no DNA was released from the complex during the coating reaction. The results given in Table 2 show that the coating reaction may result in aggregation of complexes especially in the case of complexes containing less amino groups (prepared at φ = 1.2)[8,10] and less stable complexes prepared by condensation of DNA with low-molecular-weight PLL (M_w = 23 400). Inadequate increase in R_h of nanoparticles after coating reaction is an evidence of such aggregation. An increase in the hydrodynamic radius of complexes, ΔR_h^{ct}, due to the formation of monolayer of a coating copolymer should be smaller than $2R_h^{cp}$ of the corresponding copolymer (see Table 1 and Scheme 2). Another evidence of particle aggregation is an increase in polydispersity of coated particles. The polydispersity of coated particles should be close to that of uncoated complexes (ratio of the halfwidth at the half-height of R_h distribution, ΔR_h to R_h, is typically 0.10 ± 0.01), whereas the aggregation manifests itself by broadening of R_h distribution. Using both these criterions we can state that only DNA/PLL (134 000) complexes prepared at mixing ratio φ = 2 are stable

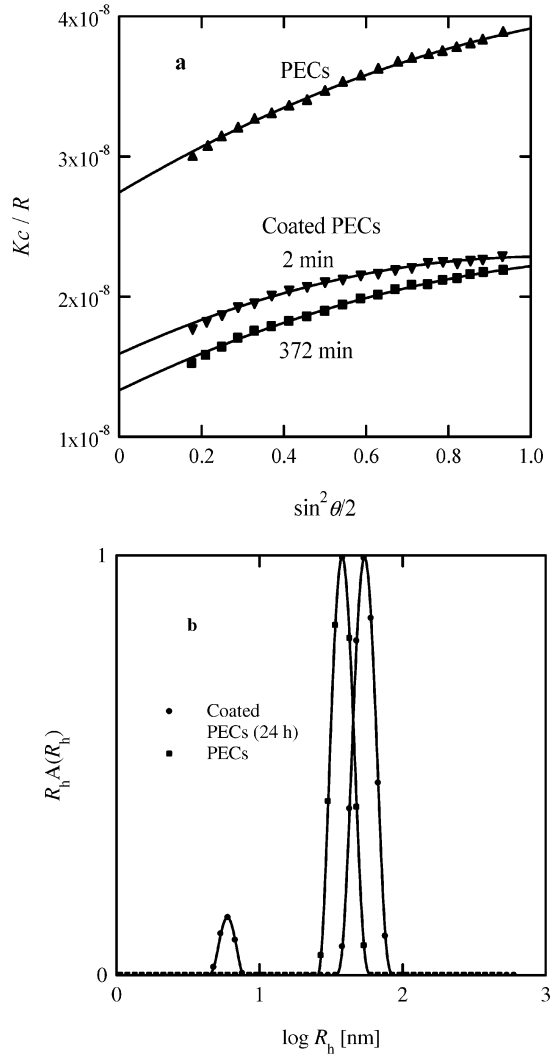


Figure 4. Zimm plots (a) and R_h distributions (b) for PECs (PLL 134 000, $\varphi = 2$) and coated PECs after incubation at the time indicated; coating copolymer **2**.

enough for coating with both the tested copolymers **2** and **4** bearing TT groups. The polydispersity $\Delta R_h/R_h = 0.12 \pm 0.03$ was found for coated nanoparticles with both the coating copolymers. In the case of the semitelechelic polymer **6** and copolymer **1** with ONp reactive groups, aggregation was observed only for complexes prepared with low-molecular-weight PLL (23 400) at $\varphi = 1.2$. The reason for aggregation of the complexes can be an increase in hydrophobicity of PECs due to consumption of positively charged amino groups of complexes in the coating reaction and a bridging of PECs with coating copolymers, particularly at slow reaction rates of coating. The latter process takes place only in coating reactions with statistical copolymers (see further in kinetics of coating reaction of DNA complexes).

The molecular-weight of pHPMA, ΔM_w^{ct}, fixed on the complex surface as a result of coating reaction, can be estimated from the M_w of coated and uncoated complexes. The procedure of ΔM_w^{ct} calculation is described in the Supporting Information. The results of ΔM_w^{ct} calculations are also given in Table 2. Taking into consideration only the results for DNA/PLL complexes formed with PLL of $M_w = 134\,000$ at mixing ratio $\varphi = 2$, the highest ΔM_w^{ct} was found for coating with copolymers having TT groups in side chains. A smaller value of ΔM_w^{ct} was found for copolymers bearing ONp groups and the lowest surface covering of PECs was found for semitelechelic polymers with TT groups. On the other hand, the danger of aggregation of complexes in the course of coating is lower for semitelechelic polymer than for multivalent copolymers. If ΔM_w^{ct} is known, the surface density of coating copolymer (per nm^2), d_{ct}, can be estimated; $d_{ct} = \Delta M_w^{ct}/4\pi(R_h^{pc})^2$. The results are shown for DNA/PLL complexes formed with PLL of $M_w = 134\,000$ at mixing ratio $\varphi = 2$ in Table 3.

The coating process also decreases the particle density of coated complexes $\rho_{cc} = 3M_w^{cc}/4\pi(R_h^{cc})^3$ as demonstrated in Table 3. $\rho_{pc} = 0.23 \pm 0.02$ found for PECs is higher than ρ_{cc} in all other cases. The lowest value of ρ_{cc} was found for complexes covered with the semitelechelic polymer **6**. The ρ_{cc} decrease is due to lower density of the coating shell compared with the PEC core of nanoparticles and/or to general swelling of the complexes covered with the hydrophilic coating copolymers. The number of pHPMA molecules fixed on the polyelectrolyte complex in coating reaction, n_{ct}, can be estimated from the ratio $\Delta M_w^{ct}/M_w^{cp}$, where M_w^{cp} is molecular weight of the coating copolymer (see Table 1). If we know the surface density of coating copolymer d_{ct}, a hypothetical surface covered by a coating molecule can be estimated as a ratio of M_w^{cp}/d_{ct}. The covered surface is 102−110 nm^2 for the semitelechelic polymer, which is a value close to the calculated value of coil cross-section, $\pi(R_h^{cp})^2 = 132$ nm. This means that the semitelechelic polymer molecules cover the PEC surface only with a low overlap with neighboring molecules. In the case of multivalent copolymers, the overlap and entanglement of coating molecules is more pronounced, and such polymers cover the particle surface more effectively (a better "stealth" effect can be expected).

Changes in the hydrodynamic radii due to coating after 22 h of incubation are also given in Table 2. The data demonstrate a higher sensitivity of the SLS method to the coating and aggregation compared with that of DLS.

Thus, the light scattering measurements also speak in favor of multivalent HPMA copolymers with TT reactive groups as a candidate for the optimal polymer for modification of DNA complex surfaces.

Effect of Molecular Weight of Coating Copolymers. For this investigation, the most stable DNA complexes prepared with the high-molecular-weight PLL ($M_w = 134\,000$) at $\varphi = 2$ were used. We have also restricted the study on coating with copolymers of the P-ϵAhx-TT type. Three coating copolymers with M_w^{cp} ranging from 24 000 to 174 000 were used for coating experiments. The light scattering results are shown in Table 4. Both the parameters $\Delta M_w^{ct}/M_w^{pc}$ and d_{ct} increase with increasing molecular weight of the coating copolymer M_w^{cp}. Thus, the high-molecular-weight coating copolymer is more efficient in coating a particle surface than the low-molecular-weight ones and the surface density of the coating copolymer is also significantly higher for the high-molecular-copolymer than for the low-molecular-weight ones.

Kinetics of Coating Reaction of DNA Complexes. The increase in weight-average molecular weight of complexes in the course of a coating reaction can be used for evaluation of the reaction kinetics. Since the most stable DNA complexes were those prepared with the high-molecular-weight PLL ($M_w = 134\,000$) at $\varphi = 2$, we have restricted further investigation to these PEC only. A time increase in M_{wa}^{cc}/M_{wa}^{pc} for the coating reaction with selected coating copolymers measured at the scattering angle $\theta = 90°$ is shown in Figure 5. The time dependence for the coating with multivalent copolymers **2** and **4** was practically identical and, therefore, the kinetics of the

Table 2. Coating of DNA/PLL Complexes with Reactive Copolymers (c_{cp} = 2 mg/mL) and Changes of the Weight-Average Molecular Weights and Hydrodynamic Radii

coating polymer	PLL kDa	φ	$M_w^{pc} \times 10^{-7}$, g/mol	$M_w^{cc} \times 10^{-7}$, g/mol	$\Delta M_w^{ct}/M_w^{pc}$	R_h^{pc}, nm	R_h^{cc}, nm	ΔR_h^{ct}	R_h^{cc} + NaCl, nm
1	134	2	2.62	3.59	0.37	35	46	11	50.0
1	134	1.2	3.47	4.45	0.28	39	50	11	53.4
1	23	2	2.39	3.39	0.42	32	51	19	63.4
1	23	1.2	2.61	4.41	0.69	33	54	21	69.3
2	134	2	3.42	5.34	0.56	38.2	52.8	14.6	58.9
2	134	1.2	3.90	5.48	0.41	38.5	55.1	16.6	63.5
2	23	2	2.75	5.54	1.02	36.8	66.8	30.0	80.8
2	23	1.2	3.26	7.76	1.38	38.0	81.7	43.7	102
4	134	2	2.89	4.50	0.56	37	49	12	55
4	134	1.2	3.80	6.77	0.78	40	69	29	74
4	23	2	2.56	4.86	0.90	38	61	23	70
4	23	1.2	3.10	6.38	1.06	37	68	31	97
6	134	2	2.78	3.57	0.28	37	48	11	52
6	134	1.2	4.80	5.63	0.18	44	59	15	66
6	23	2	3.28	3.84	0.17	40	53	13	55
6	23	1.2	3.29	4.65	0.41	41	57	16	62

Table 3. Characteristics of Coated PECs Prepared by the Standard Procedure with High-Molecular-Weight PLL (134 000) at $\varphi = 2$[a]

coating polymer	d_{ct}, g/nm²mol	n_{ct}	ρ_{cc}, g/mL	t_{ct}, min	ζ_{cc} (6 h), mV	ζ_{cc} (24 h), mV
1	650	460	0.15	22	-6.9 ± 0.2	-7.2 ± 0.2
2	1040	790	0.14	43	-3.3 ± 0.2	-4.3 ± 0.2
4	940	360	0.15	56	-2.6 ± 0.2	-4.4 ± 0.2
6	440	170	0.13	69	$+1.1 \pm 0.15$	$+0.3 \pm 0.15$
6[b]	410	220	0.11		-0.06 ± 0.15	-0.23 ± 0.15

[a] The concentration of coating polymer was 2 mg/mL. [b] The concentration of the coating semitelechelic polymer was 3 mg/mL.

coating with copolymer **4** is only shown in Figure 5. The coating reaction reveals two stages. The early stage is very fast, complete during the manipulation with the sample (typically 2 min).

The latter stage is slow; it is completed in several hours. The time dependence of the latter coating reaction may be approximated by an exponential function with a characteristic reaction time t_{ct}. Besides t_{ct}, the asymptotic value $M_{wa}^{cc}(\infty)/M_{wa}^{pc}$ and zero time value $M_{wa}^{cc}(0)/M_{wa}^{pc}$ of the ratio M_{wa}^{cc}/M_{wa}^{pc} are obtained from the force fitting procedure. The t_{ct} results are given in Table 3. In the case of HPMA copolymers with TT groups, the latter stage of coating reaction is slower than that of the copolymer with ONp groups, which reflects faster hydrolysis of ONp groups in the course of the reaction compared with TT groups. In other words, the ONp groups are faster deactivated by hydrolysis than TT. The reaction time is longer for the semitelechelic polymer **6** than for the copolymers, reflecting different behavior of the copolymers at the complex surface. The semitelechelic polymer **6** is in most cases bound only via a single end chain reactive group at the early stage of the reaction and the latter process is controlled by steric hindrance at the PEC surface. The binding of the multivalent

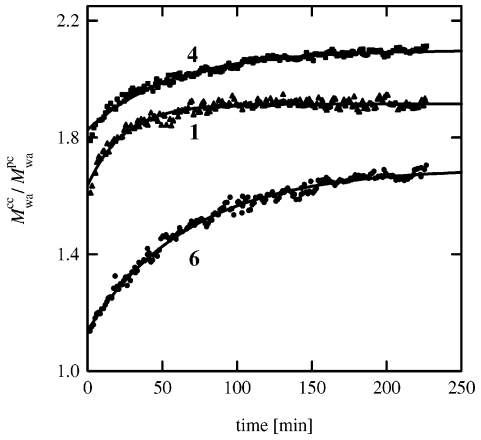


Figure 5. Increase in M_{wa}^{cc} of PECs (PLL 134 000, $\varphi = 2$) in the course of the coating reaction for copolymers **1** and **4** and polymer **6**; $\theta = 90°$.

copolymer is more complex. The coating process probably starts by attachment of the copolymer with a single bond at the early stage of the coating process followed by a sterically enhanced successive binding of the other reactive groups of the copolymer to the PEC surface.

The two stages of the coating reaction are also reflected in time variations of R_g^{cc} and R_h^{cc} of nanoparticles, which is demonstrated in Figure 6, where the time dependence of R_g^{cc} and R_h^{cc} for complexes DNA/PLL(134 000) prepared at $\varphi = 2$ and coated with copolymer **2** is demonstrated. Only R_h^{cc} is changed in the early stage of the reaction. This means that the coating copolymers form very fast a nondraining shell around the complex. R_g^{cc} is practically unchanged at this stage because the low density of the shell is not reflected in R_g^{cc}. In the latter stage, R_h^{cc} is practically unchanged and R_g^{cc} increases ap-

Table 4. Effect of the Molecular Weight of Coating Polymers (P-ϵAhx-TT) on Parameters of Coated PECs[a]

coating polymer	M_w^{cp}, g/mol	$M_w^{pc} \times 10^{-7}$, g/mol	$M_w^{cc} \times 10^{-7}$, g/mol	$\Delta M_w^{ct}/M_w^{pc}$	d_{ct}, g/nm²mol	R_h^{pc}, nm	R_h^{cc}, nm
3	24 000	2.19	3.33	0.52	739	35.0	44.7
4	45 400	2.89	4.50	0.56	935	37.0	49.2
5	174 000	2.05	3.92	0.91	1260	34.4	48.7

[a] PECs were prepared by the standard procedure with high-molecular-weight PLL (134 000) at $\varphi = 2$; the concentration of the coating polymer was 2 mg/mL.

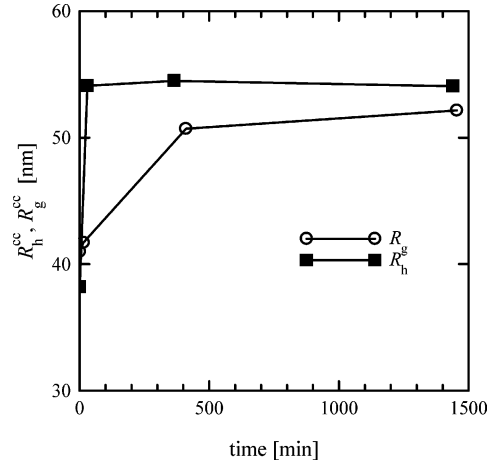


Figure 6. Time variations of R_h and R_g of PECs (PLL 134 000, $\varphi = 2$) in the course of the coating reaction; coating copolymer **2**.

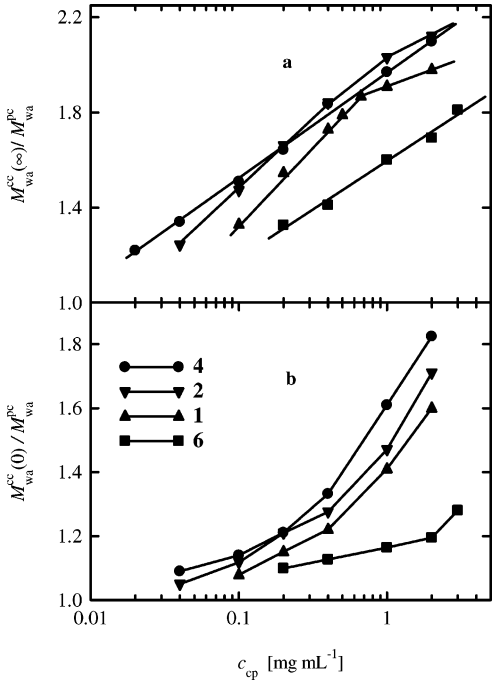


Figure 7. Effect of the concentration of coating copolymers **1**, **2**, and **4** and semitelechelic polymer **6**, c_{cp}, on the ratio $M_{wa}^{cc}(\infty)/M_{wa}^{pc}$(a) and $M_{wa}^{cc}(0)/M_{wa}^{pc}$ (b).

proaching the R_h^{cc} level. This means that the density of PEC shell increases in the latter stage of the reaction and nanoparticle become more and more homogeneous. We assume that a sterically hindered restructuring of the polymer chains on the particle surface due to the coating reaction facilitates additional reactions with remaining reactive groups of the coating copolymers, which is reflected in the slow kinetics observed in the latter stage of the reaction.

Effect of Copolymer Concentration on Efficiency of Coating Reaction. The time-resolved measurements were also used to evaluate the effect of the concentration of coating copolymer, c_{cp}, on the efficiency of PEC-coating. The results are shown in Figure 7a where $M_{wa}^{cc}(\infty)/M_{wa}^{pc}$ is plotted as a function of log c_{cp} for the most stable PECs (DNA/PLL (134 000), $\varphi = 2$). If the refractive changes due to coating of PECs are neglected then $M_{wa}^{cc}(\infty)/M_{wa}^{pc} \approx (1 + \Delta M_{wa}^{ct}/M_{wa}^{pc})^2$ (see the Supporting Information). The c_{pc}-dependences of M_{wa}^{cc}/M_{wa}^{pc} are linear functions of log c_{cp} in broad regions of log c_{cp}. The copolymer **1** with ONp groups and **2** with TT groups show a tendency to saturation at high log c_{cp}. The slopes of linear dependences are slightly higher for coating copolymers **1** and **2** (containing GlyGly spacer) for copolymer **4** (containing ϵAhx spacer) and semitelechelic polymer **6**, reflecting slightly different reactivity of reactive groups at the end of various spacers of multivalent copolymer and lower accessibility of TT group in semitelechelic polymer.

The zero time limits of $M_{wa}^{cc}(0)/M_{wa}^{pc}$, which are also obtained in the fitting procedure, provide information on the efficiency of the fast process. The results are shown in Figure 7b where $M_{wa}^{cc}(0)/M_{wa}^{pc}$ is plotted as a function of log c_{cp}. Although the $M_{wa}^{cc}(0)/M_{wa}^{pc}$ is practically independent of c_{cp} for the semitelechelic polymer, the $M_{wa}^{cc}(0)/M_{wa}^{pc}$ are an increasing function of c_{cp} for all the copolymers.

The characteristic reaction time of the slow process, t_{ct} is practically independent of c_{cp} for semitelechelic polymer ($t_{ct} = 74 \pm 7$ min). t_{ct} shows only small concentration variation for the copolymers. Thus, $t_{ct} = 43$ min for $c_{cp} = 2$ mg mL^{-1}, increasing with decreasing concentration to 66 min at $c_{cp} = 0.4$ mg mL^{-1} and finally decreases to 45 min at $c_{cp} = 0.04$ mg mL^{-1} for the copolymer **2**.

Stability of Complexes in 0.15 M NaCl Solution. One of the most important properties of PECs designed as a gene delivery system is their stability in physiological solutions. A combination of static and dynamic light scattering techniques was used to examine the stability of complexes in physiological saline (0.15 M NaCl) solution. Table 2 shows the results of stability measurements as obtained from dynamic light scattering measurements. It is known[18] that the uncoated DNA/PLL complexes precipitated very quickly forming large aggregates almost immediately after NaCl addition, whereas the coated complexes were stable at least for 24 h. A significant stabilizing effect of the polymer coating was also seen in our experiments. M_w^{cc} of the coated complexes have increased by several percent only showing practically negligible aggregation. R_h^{cc} of the complexes did not show any significant change either. The small increase in R_h^{cc} may be due to a small swelling of complexes resulting from screening of electrostatic interactions.

ζ-Potential of Coated PECs. ζ-potentials of coated PECs measured after 6 and 24 h of the coating reaction are shown in Table 3. Although the ζ-potential of uncoated PECs determined for $\varphi = 2$ is positive ($\zeta_{pc} = 24 \pm 3$ mV), coating of complexes with both HPMA copolymers containing ONp or TT reactive groups results in a ζ-potential drop to slightly negative values. The negativity of ζ-potential increases with increasing reaction time, and the effect is more pronounced for ONp containing copolymers. The negative ζ-potential of coated complexes results from negative charges of carboxylic groups formed in a side reaction (hydrolysis) of a part of reactive groups of the multivalent copolymers. A higher extent of hydrolysis of ONp groups-containing copolymers also explains higher negative ζ-potentials of complexes coated with ONp copolymers. The hydrolysis of such copolymers is faster and they form more carboxylic groups in the hydrophilic shell of the coated complexes than that of TT groups-containing copolymers (see Aminolysis and Hydrolysis of TT and ONp Groups).

ζ-potential of PECs coated with the semitelechelic polymer **6** at the concentration 2 mg/mL is slightly positive. This means that only a portion of the positively charged amino groups on the surface of PECs was modified by the polymer, which is in agreement with the calculated amount of TT reactive groups (93.4 nmol) in semitelechelic polymer and theoretical amount of amino groups originating from poly(L-lysine) (307 nmol). If

c_{pc} increases up to 3 mg/mL, the ζ-potential of the coated particles is slightly negative ($\zeta = -0.06 \pm 0.15$ mV after 6 h and -0.23 ± 0.15 mV after 24 h of the reaction); more charges of the amino groups accessible to the coating reaction are compensated and PEC is more effectively coated.

Conclusion

New highly hydrophilic *N*-(2-hydroxypropyl)methacrylamide copolymers and semitelechelic polymers with TT reactive groups (TT-polymers) were developed as polymers intended for efficient chemical coating of surfaces of nanoparticulate gene delivery systems bearing amino groups on the surface (polyelectrolyte complexes, viruses). Properties of the new TT-polymers and the efficiency of the coating procedure were compared with those of the previously described ONp group-containing copolymers[8−11] successfully used for surface modification of both viral and PECs systems. The study was carried out using a model of such system: self-assembling polyelectrolyte DNA/poly(L-lysine) complexes. The course of the reaction and efficiency of particle coating was followed either by monitoring an amount of the unreacted coating polymer in solution by UV spectroscopy or by monitoring the weight-average molecular weight, changes in hydrodynamic size and density of particles using the light scattering methods. The coating reaction was faster and more efficient for multivalent HPMA copolymers with TT reactive groups in side chains than for those with conventional ONp reactive group. pH ∼ 8.0 was found to be optimum for the coating reaction of DNA/PLL complexes with HPMA copolymers containing TT reactive groups. The coating with multivalent high-molecular weight HPMA copolymers with TT groups was more efficient than coating with low-molecular-weight polymer analogues, and it was superior to coating with corresponding semitelechelic polymers. By selecting optimal conditions, full surface coating of the nanoparticles can be achieved resulting in only a small increase in the particle size and in a significant improvement of particle stability in physiological saline solution (0.15 M NaCl).

The results show that due to higher accessibility of reactive groups in multivalent HPMA copolymers, the copolymers are more suitable for efficient coating of PECs than semitelechlic polymers. Moreover, multivalency of copolymers offers a possibility of further modification of coated complexes/particles facilitating, e.g., targeting of such gene delivery systems.

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Supporting Information Available. Calculation of the amount of bound coating copolymer. This material is available free of charge via the Internet at http://pubs.acs.org.

List of Abbreviations

M_w = weight-average-molecular weight (g/mol)
M_{wa} = apparent weight-average-molecular weight (g/mol)
R_h = hydrodynamic radius (nm)
R_g = radius of gyration
ζ = zeta potential of PECs (mV)
ρ = particle density of PECs (g/mL)
c = concentration (g/mL)
ν = refractive index increment
q = scattering vector (m^{-1})
φ = positive/negative charge ratio
d_{ct} = surface density of coating polymer (g/nm^2 mol)
n_{ct} = number of coating molecules fixed on PEC surface
t_{ct} = characteristic reaction time of the slow coating process (min)

Subscripts and Superscripts

cp = coating polymer
pc = uncoated polyelectrolyte complexes (PECs)
cc = coated PECs
ct = coating layer of PECs

Definitions

$\Delta M_w^{ct} = M_w^{cc} - M_w^{pc}$
$\Delta R_h^{ct} = R_h^{cc} - R_h^{pc}$

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