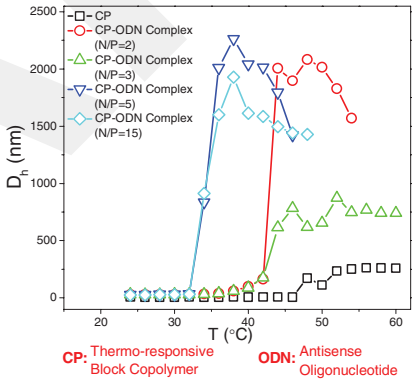
Thermo-Responsive Complexes of c-Myc Antisense Oligonucleotide with Block Copolymer of Poly(OEGMA) and Quaternized Poly(4-Vinylpyridine)

Murat Topuzogullari, Yeliz Basaran Elalmis, Sevil Dincer Isoglu*

Solution behavior of thermo-responsive polymers and their complexes with biological macromolecules may be affected by environmental conditions, such as the concentration of macromolecular components, pH, ion concentration, etc. Therefore, a thermo-responsive polymer and its complexes should be characterized in detail to observe their responses against possible environments under physiological conditions before biological applications. To briefly indicate this important issue, thermo-responsive block copolymer of quaternized poly(4-vinylpyridine) and poly(oligoethyleneglycol methyl ether methacrylate) as a potential nonviral vector has been synthesized. Polyelectrolyte complexes of this copolymer with the antisense oligonucleotide of c-Myc oncogene are also thermo-responsive but, have lower LCST (lower critical solution temperature) values compared to individual copolymer. LCST values of complexes decrease with molar ratio of macromolecular components and presence of salt. Dilution of solutions also affects solution behavior of complexes and causes a significant decrease in size and an increase in LCST, which indicates possible effects of severe dilutions in the blood stream.



1. Introduction

It is obvious that polyethyleneimine (PEI) opened a new age for synthetic polymers in gene therapy studies as a nonviral vector.[1] Since then, using synthetic polymers in gene therapy studies have gained a tremendous acceleration but, they are behind the viral vectors in transfection efficiency. Using stimuli-responsive, such as temperature- or pH-responsive, monomers and polymeric blocks is one of the methods for targeting to and accumulation in tumor tissues to improve vector's transfection efficiency.[2–4]

Polyelectrolyte complex (PEC) micelles are a significant class of genetic material carriers, in which negatively charged genetic material electrostatically associates with a block or graft copolymer having nonionic hydrophilic chain and positively charged chain.[5,6] PEC micelles may also become thermo-responsive by adding thermo-responsive monomer or block to copolymer structure.[7] However, the ratio of macromolecular components,[8] ion concentration,[9] pH,[10] and hydrophobic interactions[11]

Dr. M. Topuzogullari, Dr. Y. B. Elalmis
Department of Bioengineering
Yildiz Technical University
Istanbul 34210, Turkey
Prof. S. D. Isoglu
Bioengineering Department
Abdullah Gul University
Kayseri 38170, Turkey
E-mail: sevild77@gmail.com

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directly affect solution behavior of thermo-responsive polymers. The size of the particle is a result of solution behavior of the macromolecule and it is one of the most important factors that affect physiological function of PEC micelles. Studies about extravasation of macromolecules from vessels to tumors reveal clearly that size of the particle directly affects its diffusion to tumor tissue.[12,13] Even 40 nm difference may affect extravasation way of particle.[13] In some situations, for example in the absence of bulk flow, particles may diffuse back to vessels.[14] Therefore, it is a necessity to analyze thermo-responsive behavior, size, and structure of PEC micelle before in vivo and in vitro applications to better understand its response against environmental changes such as temperature and pH change through physiological conditions or very high dilutions in the blood stream.

N-isopropylacrylamide (NIPA) is the mostly used thermo-responsive monomer to date. Its copolymers with polycations, such as polylysine,[15] PEI,[16] and poly(2-(dimethylamino)ethyl methacrylate),[17] have been investigated in depth. In recent years, another thermo-responsive polymer, poly(oligoethyleneglycol methyl ether methacrylate) (POEGMA), a brush-shaped derivative of PEG, has been commonly used in PEC micelles[18–20] owing to its higher biocompatibility and easy synthesis using controlled polymerization techniques. LCST of POEGMA can be adjusted by altering PEG chain length or combining different OEGMA monomers having varying length PEG chains.[21] We synthesized[22] a series of block copolymers of OEGMA with 4-vinylpyridine (4VP) as amphiphilic block copolymers for delivery of hydrophobic drugs but, it was unsuitable for PEC micelle formation with anionic genetic material since the copolymer was nonionic in neutral pH. Polymers of vinylpyridines may form complexes with the genetic material after quaternization of tertiary amine groups in their pyridine rings or protonation at pH values lower than 5. Polyelectrolyte complexes of quaternized poly(4-vinylpyridine) (P4VP) or poly(2-vinylpyridine) with genetic material, such as plasmid DNA, were already investigated[23,24] and it was shown that this polymer may be used in gene therapy studies.

Antisense oligonucleotide (ASODN) is a very selective therapeutic strategy for diseases to correct protein expression by gene therapy. Delivery of ASODNs specific to c-Myc as a potential treatment method for the breast cancer is one of the most popular approaches in cancer targeted antisense therapy. It was reported in numerous studies that c-Myc is overexpressed in various cancer cell types at both mRNA and protein levels.[16] A reduction in tumor growth and an increased survival have been observed once c-Myc specific ASODN is used in in vivo studies. Using c-Myc ASODN in the treatment of abnormal cancer cell growth has been applied with several nonviral vectors for more than two decades.[16,25–27] Thus, c-Myc ASODN is very well-known and an enormous data is available in the literature, which led us to use this genetic material as a model cargo molecule in our thermo-responsive polymeric vector.

In the study, we investigated the alterations in size and LCST value of thermo-responsive PEC micelles under different temperatures, dilution ratios, and salt concentrations. PEC micelles investigated in this study are composed of a block copolymer of POEGMA with quaternized P4VP[22] and ASODN of c-Myc oncogene, and may be used as a nonviral vector in gene therapy studies. Effects of ratio of macromolecular components, presence of salt, and dilution on LCST value and size of PEC micelles were investigated in detail by using dynamic light scattering spectroscopy. The study briefly indicates the importance of detailed investigation of size and LCST of thermo-responsive macromolecular architectures before in vitro and in vivo applications.

2. Experimental Section

2.1. Materials

Block copolymer of poly(diethyleneglycol methyl ether methacrylate-*co*-oligoethyleneglycol methyl ether methacrylate)-*b*-poly(4-vinylpyridine) (P(DEGMA-*co*-OEGMA)-*b*-P4VP) (M_n = 32.9 kDa, PDI = 1.07) was synthesized in the previous study[22] and will be henceforward abbreviated as POEGMA-*b*-P4VP. 1-Bromohexane and ethidium bromide were purchased from Sigma-Aldrich. Diethyl ether and methanol were obtained from Merck. The antisense oligonucleotide of c-Myc oncogene (AACGTTGAGGGGCAT) was obtained from Iontek. $NaH_2PO_4.5H_2O$, $Na_2HPO_4.12H_2O$, and NaCl was from Riedel-de Haen. All aqueous solutions were prepared using ultra-pure water obtained from Millipore MilliQ Gradient system.

2.2. Equipment

^{1}H NMR spectra were obtained from Bruker Avance III 500 MHz NMR spectrometry. Samples were dissolved in methanol (CD_3OD). Zetasizer Nano ZS (Malvern) instrument was used for dynamic and electrophoretic light scattering measurements to obtain hydrodynamic diameters (D_h) and zeta potentials (ζ) of PEC micelles. Size and zeta potential measurements were acquired at 25 °C. Temperature based analyses were acquired by increasing temperature 2 °C at each step and samples were equilibrated minimum 5 min at each step. Zeta potentials of complexes were acquired after dilution in the ratio of 1/16. Fluorescence emission spectra in ethidium bromide displacement assay were acquired from QM-4/2003 Quanta Master Steady State Spectrofluorometer (Photon Technology International, Canada). Samples were excited at 510 nm and the emission wavelength was 600 nm. Atomic force microscope (AFM) images of polyelectrolyte complexes were obtained using Shimadzu SPM 9600 scanning probe microscope. Dynamic mode was used for all samples. 5 μL of each sample was dropped on freshly cleaved mica surface, incubated for 5 min, then rinsed with ultra-pure water and dried.

2.3. Quaternization of Block Copolymer

4VP units of POEGMA-*b*-P4VP were quaternized using 1-bromohexane. The quaternized copolymer will be abbreviated as POEGMA-*b*-Q/P4VP. 10% (w/v) solution of the block copolymer was prepared in methanol, 1-bromohexane was added to the solution in the molar ratio of $N_{1\text{-bromohexane}}/N_{4VP}$ = 5/1 and the reaction vessel was sealed. The reaction was conducted in oil bath at 70 °C for 6 d. POEGMA-*b*-Q/P4VP was precipitated from cold diethyl ether. The precipitate was dried and dissolved in methanol. Then, the copolymer was re-precipitated from cold ether. This procedure was repeated two times. The final product was dried overnight in vacuum oven at 50 °C. Orange-colored POEGMA-*b*-Q/P4VP was kept in vacuum desiccator to prevent hydration. Quaternization degree of the final product was more than 90%, which was calculated from ^{1}H NMR spectrum.

2.4. Preparation of PEC Micelles

Stock solutions of ASODN and POEGMA-*b*-Q/P4VP were prepared separately using 0.02 M phosphate buffer at pH 7.0 and each solution was filtered through syringe filter with 0.2 μm pore size. Complexes were prepared in varying N/P ratios (1, 2, 3, 5, 10, 15 and 20). N/P is the molar ratio of positively charged amine groups of POEGMA-*b*-Q/P4VP to negatively charged phosphate groups of ASODN. Final concentration of POEGMA-*b*-Q/P4VP was 0.25 mg mL^{-1} in all samples and ASODN concentration was varied to obtain related ratios. All samples were also prepared in 0.02 M phosphate buffer containing 0.15 M NaCl to examine the salt effect.

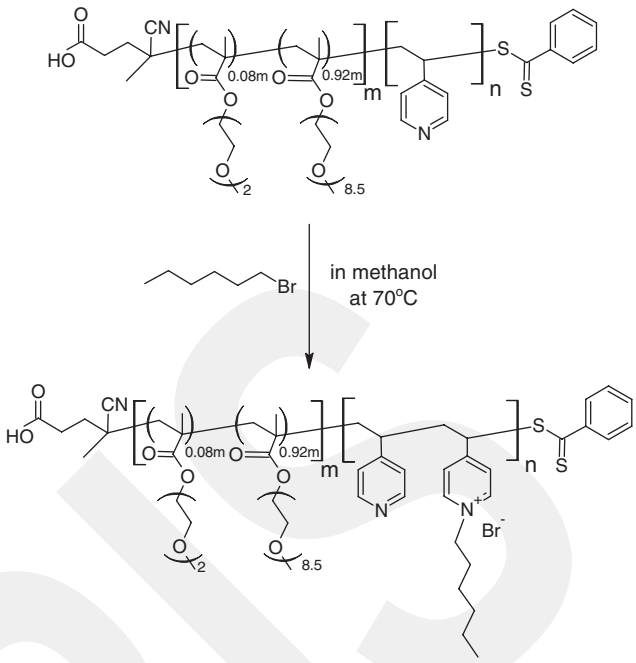
2.5. Ethidium Bromide Displacement Assay

Ethidium bromide (EtBr) was added to the stock solution of ASODN in the molar ratio of $N_{EtBr}/N_{\text{nucleotide base}}$ = 5/1. PEC micelles were prepared as mentioned before but using ASODN stock containing EtBr. For each N/P ratio; EtBr solution, ASODN solution with EtBr prepared with the identical concentration of PEC micelle were also analyzed with fluorescence spectrometry. Fluorescence intensity ratio of PEC micelles to individual ASODN was calculated according to Equation (1)[28]

$$\text{Fluorescence ratio } (F) = \frac{F_{\text{PEC Micelle}} - F_{\text{EtBr}}}{F_{\text{ASODN}} - F_{\text{EtBr}}} \times 100 \quad (1)$$

3. Results and Discussion

In our previous study, we synthesized a series of well-defined POEGMA-*b*-P4VP block copolymers, which have varying molecular weights and side PEG chains with varying lengths.[22] All of them were suitable for using as pH-responsive carriers for hydrophobic drugs and formed micelles in aqueous solutions. Block copolymers containing DEGMA and $OEGMA_{475}$ (OEGMA with M_n = 475 g mol^{-1}), which had LCST values between 25 and 30 °C, were suitable for thermo-responsive applications



Scheme 1. Quaternization reaction of POEGMA-*b*-P4VP.

under physiological conditions. In this study, we quaternized 4VP units of POEGMA-*b*-P4VP using 1-bromohexane to obtain a positively charged block copolymer suitable for genetic material delivery, which is shown in Scheme 1.

Pyridine groups of P4VP chain can be partially or almost fully quaternized using the reaction in Scheme 1 by adjusting reaction time. We conducted quaternization reaction for 6 d and analyzed quaternization degree of 4VP groups using ^{1}H NMR (Figure 1) spectrum. Protons of 4VP ring can be observed between 6.5 and 9 ppm in ^{1}H NMR spectra, because chemical shift values of protons of quaternized and nonquaternized pyridine rings change according to quaternization degree.[29] In Figure 1, peaks at 8.6 and 7.8 ppm belong to protons of quaternized pyridine rings, while peaks at 8.2 and 7.3 belong to nonquaternized pyridine rings. ^{1}H NMR spectrum of POEGMA-*b*-Q/P4VP gives a quternization degree higher than 90%.

After quaternization of block copolymer, PEC micelles were prepared by mixing POEGMA-*b*-Q/P4VP and c-Myc ASODN in varying molar ratios. These complexes were, first, analyzed using dynamic and electrophoretic light scattering spectroscopy at 25 °C (Figure 2). As seen in the figure, hydrodynamic diameter of POEGMA-*b*-Q/P4VP is about 7.5 nm and its zeta potential is +4.8 mV in aqueous solution without salt ions. Upon mixing with ASODN, the diameter of POEGMA-*b*-Q/P4VP increases to 22.1 nm (N/P = 1). At higher N/P ratios, sizes of complexes increase to a range between 25 and 30 nm. Complexes are negatively charged at lower N/P ratios (1, 2, 3, and 5) and zeta potential of complexes becomes neutral or slightly positive at higher ratios (10, 15, and 20), which confirms the

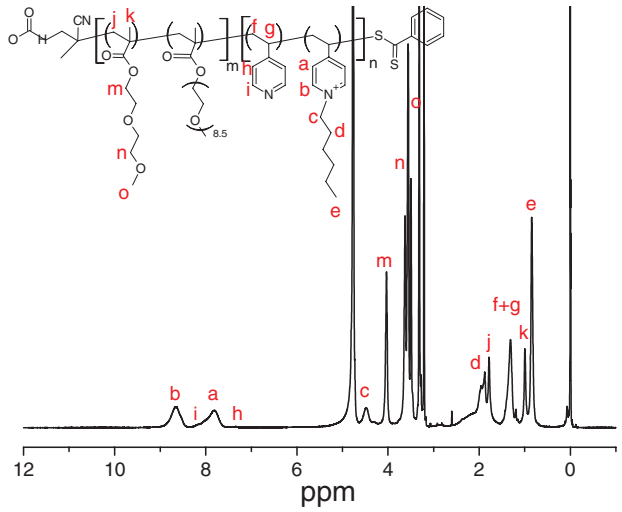


Figure 1. ^{1}H NMR spectrum of quaternized copolymer which was dissolved in methanol.

complex formation. Complexes prepared using phosphate buffer with 0.15 M NaCl had similar trends in both size and zeta potential analyzes, which exhibited an increase in zeta potential from negative values.

EtBr displacement assay was accomplished to prove electrostatic interaction between POEGMA-*b*-Q/P4VP and ASODN. In this method, EtBr molecules intercalate between negatively charged nucleotide bases of double stranded oligonucleotides and fluorescence intensity of EtBr increases. If a polymer forms complexes with oligonucleotide through electrostatic interactions with nucleotide bases, EtBr is excluded and its fluorescence intensity decreases.[30] Although EtBr displacement assay is mainly used for double-stranded oligonucleotides, it may also be used for single-stranded oligonucleotides, since EtBr may intercalate between bases found in the regional helices of single-stranded oligonucleotides.[31,32] Figure 3 shows fluorescence intensity ratios of complexes depending on N/P ratio. It is noticed that lower N/P ratios of complexes had fluorescence intensity ratios higher than 100%, which is unusual for this method compared to previous studies.[28,33] Here, it can be concluded that there is no complexation above 100% fluorescence intensity, but it is important not to forget that 100% fluorescence intensity is the maximum fluorescence intensity observed for free ASODN. Therefore, increase in fluorescence intensity above 100% should be a result of interactions with the copolymer. We assume this situation shows stronger interactions between EtBr and nucleotide bases or higher number of EtBr intercalation between bases which caused an increase in fluorescence ratio. Since this phenomenon was not observed in a previous study, we prepared the same N/P ratios of complexes using PEI (25 kDa) to understand if this phenomenon occurs due to the structure of ASODN but, in the experiments with PEI, fluorescence intensity of all complexes were lower than 100% (data not shown) as usual. Fluorescence ratio of complexes decreases with N/P ratio to the values lower than 100% which shows POEGMA-*b*-Q/P4VP and ASODN interaction. Complexes prepared in 0.15 M NaCl containing phosphate buffer had a similar trend but, at all N/P ratios, fluorescence intensities were closer to 100% than the complexes prepared in phosphate buffer without NaCl. Low molecular weight salt ions may have prevented or weakened electrostatic interactions between POEGMA-*b*-Q/P4VP and ASODN by screening effect.

We selected two different ratios to visually confirm the complexation with AFM. Therefore, a ratio above 100% fluorescence intensity (N/P = 2) and a ratio below 100% (N/P = 10) were analyzed. Figure 4 gives the AFM images of complexes of N/P = 2 and 10. In Figure 4a, image of N/P = 2 complex shows a uniform particle distribution with a diameter of 17.2 nm (±3 nm), which clearly shows the complex formation above 100% fluorescence intensity ratio in EtBr displacement assay. However, a

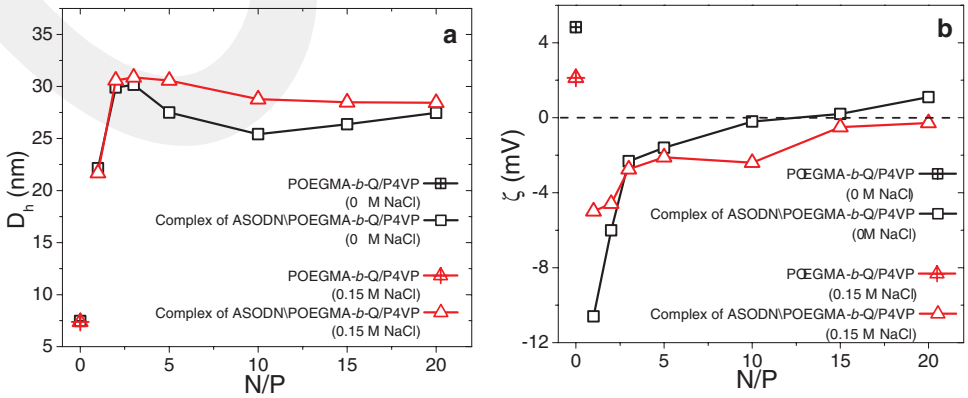


Figure 2. a) Hydrodynamic diameters (by volume) and b) zeta potential values of POEGMA-*b*-Q/P4VP and its complexes with ASODN depending on N/P ratio at 25 °C.

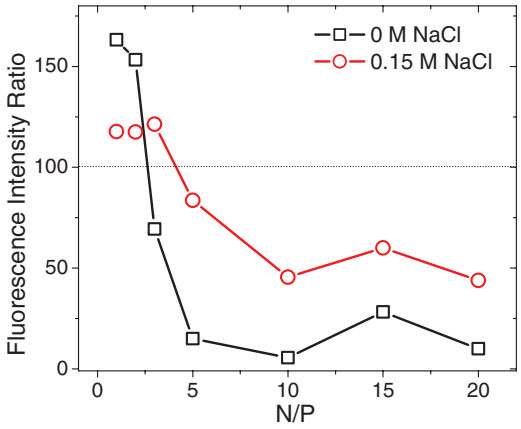


Figure 3. Fluorescence intensity ratio of PEC micelles of POEGMA-*b*-Q/P4VP and ASODN depending on N/P ratio.

small number of aggregates between complex particles can also be seen in Figure 4a. Complex particles of N/P = 10 (Figure 4b) also have a uniform distribution and their diameters are 15.1 nm (±2.5 nm). Diameters obtained from AFM are compatible with dynamic light scattering results, in which size of N/P = 2 is slightly higher than N/P = 10 in both techniques. However, diameters of complexes obtained from AFM are smaller than the values obtained from dynamic light scattering, which is explained by shrinkage of particles after drying on mica surface.[34] Diameter values obtained from AFM reveal the complex formation because they are even larger than the hydrodynamic diameter of individual POEGMA-*b*-Q/P4VP chains.

Altogether, POEGMA-*b*-Q/P4VP and ASODN have formed complexes at all N/P ratios in phosphate buffer solution with or without NaCl. Diameters of complexes are almost constant at all N/P ratios and are lower compared to plasmid DNA complexes.[8,35,36] Zeta potentials of nearly all complexes were negative or neutral, even at high N/P ratios. At low N/P ratios, it is most likely to obtain neutral or negatively charged complexes due to the less amount of polymer for the complex formation. However, the formation of positively charged complexes is expected at high N/P ratios. In this situation, negatively charged carboxylic acid chain ends of POEGMA block of the copolymer probably prevented the formation of positively charged complexes, due to POEGMA blocks, which extends from the shell of PEC micelle. The main question is about the lower N/P ratios, if they formed complexes or not. The zeta potential values of complexes obtained at lower N/P ratios (1, 2, and 3), their hydrodynamic diameters, and AFM image of N/P = 2 support fluorescence results and complex formation in lower N/P ratios. We assume that, in lower N/P ratios, POEGMA-*b*-Q/P4VP chains partly wrap around ASODN chains leaving free the rest of the chains, which may cause formation of more folding regions through ASODN chains and/or association of several ASODN chains together causing more intra- and/or interchain EtBr intercalations.

Dynamic light scattering spectroscopy is a much improved and accurate technique than turbidimetry to determine LCST of polymers, since it can characterize even small changes in size of the particle, not the precipitation. In Figure 5, change in hydrodynamic diameters of PEC micelles upon increase in temperature is given. All samples were prepared in phosphate buffers with and without 0.15 M NaCl. First, the response of individual POEGMA-*b*-Q/P4VP to temperature change was analyzed. In buffer without NaCl, LCST of POEGMA-*b*-Q/P4VP was 46 °C and its diameter increased to 260 nm above 46 °C. This value is quite higher when compared with the LCST value of nonquaternized form of the block copolymer at pH 7, which is about 25 °C.[22] The increase in LCST value depends on the increased hydrophilicity of copolymer due to quaternization pyridine rings and addition of positive charges to the structure. In the buffer without NaCl,

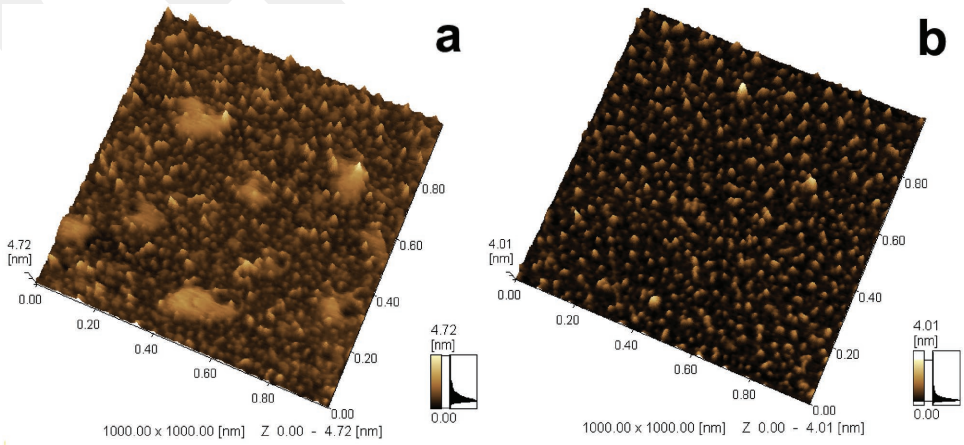


Figure 4. AFM images of PEC micelles of POEGMA-*b*-Q/P4VP and ASODN with the ratio of a) N/P = 2 and b) N/P = 10 at 25 °C in phosphate buffer without NaCl.

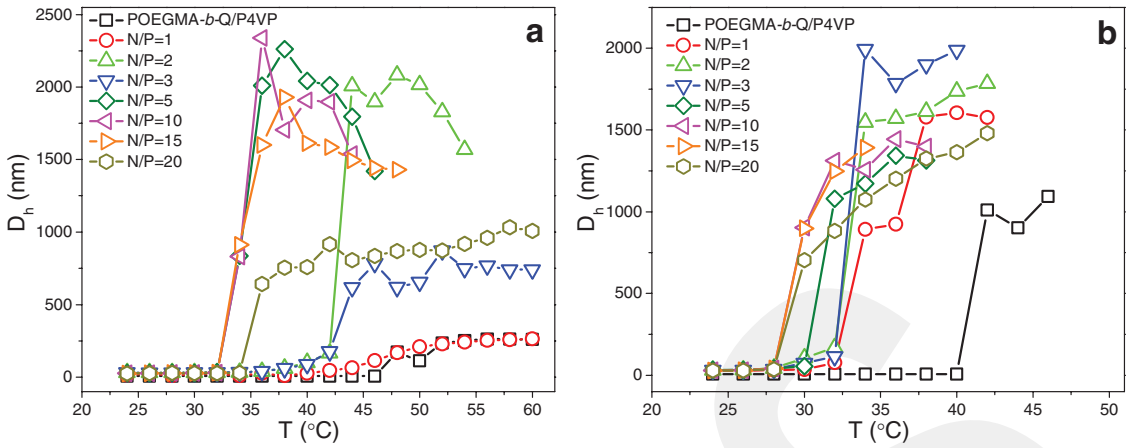


Figure 5. Effect of temperature on hydrodynamic diameters (by volume) of POEGMA-*b*-Q/P4VP and its complexes with ASODN prepared in phosphate buffer a) without and b) with 0.15 M NaCl.

turbidity was very low due to the formation of POEGMA-*b*-Q/P4VP micelles in which hydrophobic POEGMA blocks form the core while positively charged P4VP blocks form the shell of the micelle. In the buffer with 0.15 M NaCl, LCST of POEGMA-*b*-Q/P4VP decreased to 40 °C and higher turbidity was observed. It was shown that salt concentration affects LCST value of POEGMA polymers and an increase in NaCl concentration causes a decrease in LCST.[37] Salt ions increased hydrophobic interactions and caused collapse of POEGMA-*b*-Q/P4VP chains at a lower temperature to form larger aggregates and precipitates.

LCST of PEC micelles (Figure 5) are all lower than individual CP in both buffers. In addition, LCST values of complexes decrease with the increase in N/P ratio, which shows that increased POEGMA-*b*-Q/P4VP quantity in the structure of PEC micelle causes reduced LCST, both in the absence and presence of salt. By complexing with ASODN, positive charges on polymer chains are neutralized by negative charges of oligonucleotide chain and this increases the hydrophobicity of structure, which lowers LCST value in comparison with POEGMA-*b*-Q/P4VP. It is obvious that LCST values of all complexes are under normo-thermic temperatures in salt-containing buffer, which makes them nonsoluble and unsuitable for intravenous injection and extravasation from vessels. By simply altering monomer ratio, LCST values can be adjusted to a desired temperature range. However, one of our aims was especially to investigate and show the effect of complex formation of POEGMA-*b*-Q/P4VP (or a different thermo-responsive polymer) on LCST value, not to fine-tune LCST.

In the study of Oupicky and his colleagues, LCST values of complexes of plasmid DNA with PEI-*g*-PNIPA slightly increased from 31–32 to 33 °C in comparison with individual PEI-*g*-PNIPA.[8] However, in our study, LCST value of POEGMA-*b*-Q/P4VP drastically changes after complexation with ASODN. It is obvious that neutralization of positive charges of P4VP increased hydrophobicity. In addition, we assume that six carbon alkyl chains of quaternizing agent found in the P4VP block might have increased the hydrophobic character of micelles and cause a further decrease in LCST value of PEC micelle. It was shown that increased hydrophobicity of the polymer causes a decrease in LCST of the thermo-responsive polymer.[11]

The ratio of N/P = 1 behaves differently from both POEGMA-*b*-Q/P4VP and other complexes in the absence of salt. Its size slowly increases with temperature, while all other complexes and POEGMA-*b*-Q/P4VP shows a sharp increase in size at LCST. Therefore, it is difficult to clearly determine its LCST but, it is obvious that size of N/P = 1 starts to increase at a temperature lower than POEGMA-*b*-Q/P4VP. This may be the result of high negative charge on N/P = 1 (Figure 6).

Zeta potentials of complexes acquired at 42 °C are given in Figure 6. As mentioned above, zeta potential of N/P = 1 is higher than all other ratios at 42 °C, which prevents the complex from formation of high aggregates and precipitates above LCST. Zeta potentials of PEC micelles at 42 °C are similar to values at 25 °C but slightly higher positive charges were obtained at N/P = 10, 15, and 20 in the buffer without NaCl.

Dilution is an important obstacle for drug delivery systems and nonviral vectors prepared by self-assembly, because dilution may cause disassembly of these particles. In addition, dilution may affect thermo-responsive behavior of temperature sensitive polymers.[38] Chilkoti and his colleagues clearly exhibited the increase in LCST of thermo-responsive polymeric drug delivery system upon dilution and selected a proper in vivo concentration.[3] It is known that total blood volume of a man is approximately between 4.75 and 6.23 L.[39] If 5 mL solution of a PEC micelle is administered intravenously to the body, the solution will be diluted almost up to 1000 times. Therefore,

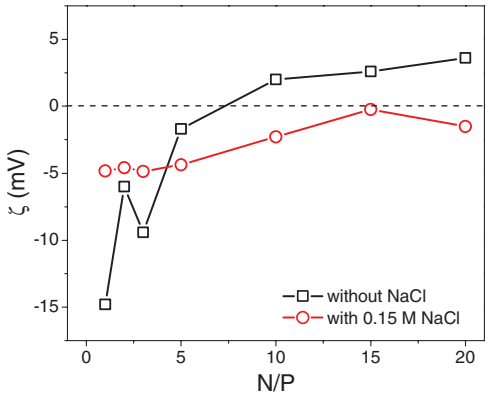


Figure 6. Zeta potential values of complexes of POEGMA-*b*-Q/P4VP with ASODN depending on N/P ratio at 42 °C. Samples were diluted in the ratio of 1/16.

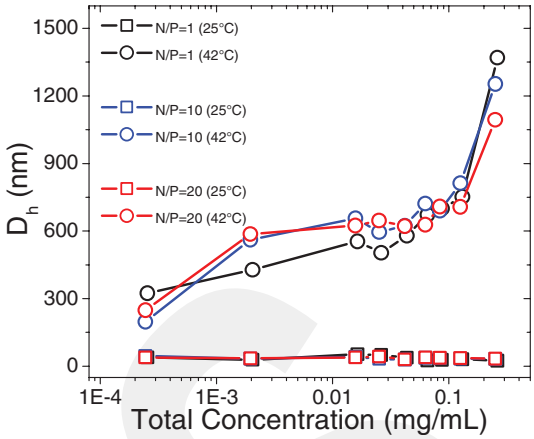


Figure 8. Hydrodynamic diameters of PEC micelles (N/P = 1, 10, 20) diluted to different total concentrations ($C_{POEGMA\text{-}b\text{-}Q/P4VP} + C_{ASODN}$) in phosphate buffer with 0.15 M NaCl at 25 and 42 °C.

the thermo-responsive properties of PEC micelles should be analyzed in a wide range of concentrations or dilution ratios. In order to investigate the effect of dilution, we prepared two N/P ratios of PEC micelles (N/P = 1 and 20) in the phosphate buffer containing 0.15 M NaCl and diluted them in varying dilution ratios (1/1, 1/16, 1/128, 1/1024) using the same buffer to simply simulate physiological conditions. Figure 7 shows the change in hydrodynamic radius of PEC micelles according to temperature. As seen in the Figure 7a, dilution of N/P = 1 complex obviously causes an increases in the LCST value and a sharp increase in size is not observed in all dilution ratios. The nondiluted complex has a lower LCST, and its size sharply increases at LCST. In addition, size of particles above LCST drastically decreases upon dilution and more dilution causes formation of smaller complex particles. At N/P = 20, a similar trend is observed. LCST value increases with dilution ratio and the size of particles above LCST value decreases drastically. It should be noted that difference in sizes of the particles in nondiluted and 1/1024 diluted solutions reaches up to 1 μm in both N/P = 1 and N/P = 20 complexes. The dilution of PEC micelle solutions increases the distance between particles and cause a decrease in intermolecular interactions between particles and prevents aggregate formation.[38] Therefore, smaller aggregates form above LCST value. Moreover, 6–8 °C increases are observed in LCST value of complex particles upon dilution. This alteration can be a problematic situation in local hyperthermia studies. If the effect of dilution on thermo-responsive behavior of complex particles is not analyzed before in vivo studies, accumulation may not be observed due to this crucial difference in LCST value.

In Figure 8, PEC micelles (N/P = 1, 10, 20) diluted to varying ratios (1/1, 1/2, 1/3, 1/4, 1/6, 1/10, 1/16, 1/128, 1/1024) were analyzed at 25 and 42 °C. At 42 °C, sizes of complexes at all N/P ratios decrease significantly in the first dilution, 1/2. In further dilutions, sizes of complexes at all N/P ratios decrease slightly. A significant decrease in size is also observed in the dilution ratio of 1/1024. The differences between sizes of complexes in 1/1 and 1/1024 dilutions are 1046, 1056, and 845 nm for N/P = 1,

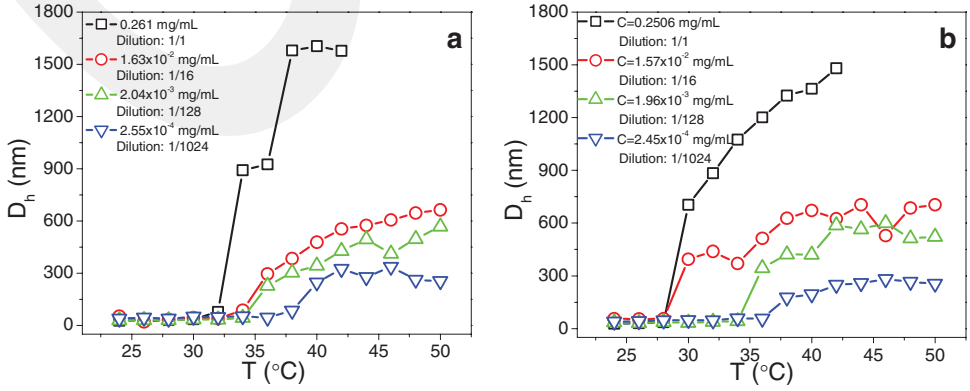


Figure 7. Effect of temperature on hydrodynamic diameters of PEC micelle (a) N/P = 1 and b) N/P = 20) diluted to different total concentrations ($C_{POEGMA\text{-}b\text{-}Q/P4VP} + C_{ASODN}$) and in phosphate buffer with 0.15 M NaCl.

10, and 20, respectively, at 42 °C. The reduction in concentration might cause fewer complexes to be involved in large aggregates. At 25 °C, sizes of complexes changed slightly in a range between 25 and 50 nm. In addition, it is necessary to note that size of N/P = 1 is different in Figures 7 and 8 at 42 °C in nondiluted solutions. These solutions were separately prepared for these two experiments. The difference in size between these complexes may be caused by nonstoichiometric aggregation of particles due to hydrophobic interactions.

Figure 8 obviously shows that complexes did not dissociate in all dilutions above and below LCST. Above LCST, the decrease in sizes of complexes is tremendous but, all the size values seem suitable for extravasation, and accumulating in tumor tissue. All in all, severe dilutions in blood stream may cause significant alterations in thermo-responsive behavior and physicochemical character of PEC micelles.

4. Conclusions

It was shown in our previous study that POEGMA-*b*-P4VP copolymer had thermo-responsive behavior even it is positively charged at acidic pH values, which made it a potential candidate for use in gene therapy studies using local hyperthermia techniques.[22] In the current study, we quaternized pyridine rings and made P4VP block permanently positive at all pH values, which is suitable for complexation with negatively charged ASODN.

POEGMA-*b*-Q/P4VP formed complexes with c-Myc ASODN at all N/P ratios and all complexes showed thermo-responsive behavior. N/P ratio and presence of salt ions directly affected size and LCST of complexes, but zeta potential values of complexes were not significantly different at temperatures above and below LCST. It is known that medicines given to human body are severely diluted in the blood stream. When diluted in phosphate buffer containing 0.15 M NaCl, LCST values of complexes significantly changed and sizes of them were tremendously reduced above LCST.

EtBr displacement assays showed the electrostatic interaction between POEGMA-*b*-Q/P4VP and ASODN but, unusual results were obtained in which fluorescence intensity ratios were higher than 100% at lower N/P ratios. Complex formation for the N/P ratios both above and below 100% fluorescence intensity was visually confirmed with AFM images. We examined the EtBr displacement assay with PEI and obtained similar results with literature, all values were below 100%. In further studies and with different polymers this phenomenon should be analyzed in detail.

This study shows that it is very challenging to make a direct relation between structure and function of thermo-responsive PEC micelles under physiological conditions due to local changes in ion concentration, dilution, and temperature because these parameters directly alter the structure of PEC micelle. Therefore, it is essential to characterize in detail the physicochemical properties of thermo-responsive complexes, which will be applied in vivo, in a broad perspective considering the physiological conditions.

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