Proton-Actuated Membrane-Destabilizing Polyion Complex Micelles

Marie-Andrée Yessine,[†] Marie-Hélène Dufresne,[†] Christian Meier,[‡] Hans-Ulrich Petereit,[‡] and Jean-Christophe Leroux*,[†]

Canada Research Chair in Drug Delivery, Faculty of Pharmacy, Université de Montréal, C.P. 6128 Succ. Centre-Ville, Montreal, Quebec, H3C 3J7, Canada, and Degussa, Pharma Polymers, Röhm GmbH and Co. KG, Kirschenallee, D-64293 Darmstadt, Germany. Received June 8, 2006; Revised Manuscript Received February 5, 2007

The efficiency of nucleic acid-based drugs is usually hampered by the fact that, following their uptake by the cell, these drugs end up in acidic organelles (i.e., endosomes/lysosomes) from which they barely escape. This work relates to the preparation and characterization of polyion complex micelles (PICM) formed by the self-assembly of three polyelectrolytes: a diblock cationic copolymer; a membranolytic, methacrylic acid copolymer; and an oligonucleotide. It is demonstrated that a synthetic membrane-active polyanion can be successfully integrated within the structure of PICM to yield well-defined, narrowly distributed micelles (30 nm) with a core/shell architecture. Besides their ability to protect the oligonucleotide against nuclease degradation, PICM partly dissociate under mildly acidic conditions, releasing chain clusters that destabilize bilayer membranes. This association/dissociation behavior illustrates the potential of these pH-sensitive PICM for the transport and efficient delivery of polyionic drugs.

INTRODUCTION

Recent advances in the field of nanotechnology have led to the design of highly sophisticated nanoscale constructs and supramolecular assemblies prepared from well-defined synthetic macromolecules (*1*). Of such assemblies, micelles formed by the self-association of block copolymers in aqueous medium have received much attention due to their potential applications in the biomedical and pharmaceutical fields (*2*, *3*). In particular, colloidal carrier systems prepared from the complexation of a polynucleotide and a diblock copolymer composed of polycation and poly(ethylene oxide) (PEO) segments are being developed for the delivery of nucleic acids (*4*−*7*). Neutralization of the charged segments through electrostatic interactions yields hydrophobic complexes that self-assemble into polyion complex micelles (PICM) presenting a core/shell architecture. These micelles present numerous advantages including their straightforward preparation, high colloidal stability, solubility in aqueous media, small size, and narrow size distribution. Furthermore, they represent a promising approach to improve the in vivo efficiency of nucleic acid-based drugs, since protection against nuclease degradation and prolonged circulation times in the blood compartment would allow for enhanced drug deposition at the target site (*8*).

However, despite these attractive properties, the intracellular bioavailability of polynucleotides remains limited due to their sequestration in the endosomal/lysosomal compartment following the uptake of PICM by the cell. The escape of biomolecules from endosomes generally requires a membrane-destabilizing agent, which will provoke the release of the internalized compounds into the cytoplasm before they reach the lysosomes. Synthetic titratable polyanions can achieve such a goal by destabilizing membrane bilayers at pH values typically found in endosomes. Indeed, polymers such as poly(ethylacrylic acid) (PEAA) and poly(propylacrylic acid) (PPAA) undergo a pH-triggered conformational change at slightly acidic pH values (*9*, *10*). Upon protonation and charge neutralization in the acidic environment of endosomes, PPAA was shown to disrupt selectively the endosomal membrane (*11*), thereby enhancing the cytoplasmic delivery of macromolecules such as plasmid DNA and polymer−antibody complex (*12*, *13*).

Recently, our group demonstrated that pH-sensitive methacrylic acid (MAA) copolymers can also be used to improve the cellular delivery of nucleic acid-based drugs (*14*). In that study, nanoparticles in suspension were obtained following the electrostatic complexation of an MAA copolymer, a model oligodeoxynucleotide (ODN), and a cationic lipid. The particles bore a positive surface charge which facilitated their internalization into cultured cells. However, given their cationic nature, the complexes were unstable in physiological conditions (Yessine et al., unpublished observations), thus compromising their applicability for systemic administration. The present work describes the incorporation of an MAA copolymer into a system that could be more suitable for in vivo applications. We demonstrate that the copolymer can be intimately integrated within the structure of neutral, water-soluble, and well-defined PICM. More importantly, we show that, upon a decrease in pH, the resulting PICM partially dissociate to release membrane-active fragments that efficiently destabilize lipid bilayers.

EXPERIMENTAL PROCEDURES

Materials. Poly(ethylene oxide)-*b*-poly(aminoethyl methacrylate) (PEO-*b*-PAEMA, 75/25 mol %, M_n 4700) was synthesized by atom transfer radical polymerization as described previously (*15*). Poly(methacrylic acid-*co*-ethyl acrylate-*co*-butyl methacrylate) (P(MAA-*co*-EA-*co*-BMA), 50/40/10 mol %, M_n 19 800) was provided by Röhm GmbH (Darmstadt, Germany). It was prepared by free radical polymerization involving dodecyl mercaptane as chain transfer agent. The polymer was dissolved in ethanol and dialyzed against water for at least 5 days prior to use. P(MAA-*co*-EA-*co*-BMA) was labeled with Lissamine rhodamine B ethylenediamine (Molecular Probes, Eugene, OR) as described previously (*14*). Twenty-mer phosphorothioate ODN (5′-GTTCTCGCTGGTGAGTTTCA-3′) and its fluores-

* Corresponding author. Tel: (514) 343-6455; Fax: (514) 343-6871; E-mail: Jean-Christophe.Leroux@umontreal.ca.

[†] Université de Montréal.

[‡] Röhm GmbH and Co..

10.1021/bc060159m CCC: $37.00
Published on Web 04/03/2007

cein- labeled derivative were provided by Medicorp, Inc. (Montreal, QC, Canada).

Preparation of the Ternary Polyion Complex Micelles (PICM). PEO-*b*-PAEMA (500 μg) was incubated with P(MAA-*co*-EA-*co*-BMA) (216 μg) in tris(hydroxymethyl)aminoethane (Tris) buffer (10 mM, pH 7.4) for 5 min at room temperature under mild agitation. The ODN (20 μg) was then added, and the formulation was incubated further for 5 min. The charge ratio was calculated from the degree of ionization of the polymers, as determined from titration experiments (Supporting Information). Micelles containing PMAA were prepared using a similar procedure using the same amounts of each component. Salt-containing samples were prepared by adding 0.5 mL of micelles to 0.5 mL of Tris 0.1 M, pH 7.4, containing 300 mM sodium chloride (NaCl) to yield a final salt concentration of 150 mM (isotonic conditions).

Determination of the Critical Association Concentration. The critical association concentration (CAC) was measured by the steady-state pyrene fluorescence method (*16*) based on the I_{336}/I_{333} intensity ratio of the excitation spectra of pyrene following its translocation from water to the PICM hydrophobic core. Serial dilutions of a stock solution of ternary PICM in Tris buffer, pH 7.4, were prepared to contain 2×10^{-7} M of pyrene (Sigma, St-Louis, MO) and were stirred overnight in the dark. Fluorescence intensity was measured ($\lambda_{ex1} = 333$ nm, $\lambda_{ex2} = 336$, $\lambda_{em} = 390$ nm) at room temperature or at 37 °C using a Series 2 Aminco Bowman fluorimeter (Spectronic Instruments, Inc., Rochester, NY). Experiments were performed in triplicate.

Dynamic Light Scattering, Multiangle Static Light Scattering, and ζ-Potential Measurements. Size, M_w, and ζ potential of the ternary PICM were all determined in triplicate in Tris buffer of the appropriate pH following filtration of the micelles through a 0.45 μm GHP filter. The mean hydrodynamic diameter, PI, and scattering intensity of PICM were determined using a Malvern Autosizer 4800 or a Malvern ZetaSizer Nanoseries ZS (Malvern Instruments, Worcestershire, U.K.). Measurements were performed at a scattering angle of 90° and temperatures of 25 and 37 °C. The *CONTIN* program was used to extract size distributions from the autocorrelation functions. Alternatively, MASLS measurements were conducted using a Malvern Autosizer 4800 at 25 °C at seven angles ranging from 70° to 130° for four micelle concentrations. The M_w values of the complexes were derived from eq 1, which describes the intensity of light scattered from a dilute solution of macromolecules. In this equation, c stands for the solution concentration

$$\frac{\mathrm{K}c}{R_0} = \frac{1}{M_w P(\theta)} + 2A_2 c + 3A_3 c^2 \qquad (1)$$

in PICM, R_0 is the Rayleigh ratio, and A_2 and A_3 are the second and third virial coefficients. K is in turn defined by

$$\mathrm{K} = \frac{4\pi^2 n_0{}^2 \left(\frac{\mathrm{d}n}{\mathrm{d}c}\right)^2}{\lambda^4 N} \qquad (2)$$

where n_0 is the refractive index of the solvent, λ the laser wavelength in vacuo, and N Avogadro's number. The specific refractive index increment ($\mathrm{d}n/\mathrm{d}c = 0.174$) was determined using a differential Rudolph J157 automatic refractometer (Rudolph Research Analytical, Flanders, NJ), at a wavelength of 589.3 nm. ζ potential was evaluated with a Malvern ZetaSizer Nanoseries ZS.

Transmission Electron Microscopy Imaging. Copper grids (200 mesh) were coated with pioloforme resin. Prior to sample deposition, a drop of an aqueous solution of poly(lysine) (17% w/v, Sigma) was placed onto the grid for 5 min. The excess solution was wicked off, the grid was allowed to dry, and a drop of ternary PICM (5 μL) in Tris buffer (pH 7.4 or 5.0) was placed onto the grid for 2 min. After removal of the excess solution, the sample was stained using uranyl acetate (3.5% w/v in water) for 5 min. The grid was then gently washed with water and allowed to dry. Prepared samples were imaged using a Philips CM100 transmission electron microscope (FEI Company, Japan) operating at 60 kV. Micrographs were taken at a 46 000 magnification.

Nuclear Magnetic Resonance Analysis. Polymer and micelle samples were prepared in deuterated Tris buffer (10 mM) adjusted to pH 7.4 using DCl and NaOD solutions (CDN Isotopes, Pointe-Claire, QC, Canada). Prior to analysis, the PICM were eluted on a Sephacryl 200 HR column (Sigma) to completely remove any free polymer in solution and concentrated at room temperature using a vacuum concentrator (Savant SpeedVac SC210A, Thermo Electron Corp., Waltham, MA). The NMR spectrum of the micelles was first acquired at pH 7.4 using a Bruker spectrometer operating at 400 MHz (Bruker, Milton, ON, Canada). The pH of the sample was then adjusted to 5.0 using DCl and the NMR acquisition repeated.

Enzymatic Degradation of ODN Incorporated into the Ternary PICM. Naked fluorescent ODN or fluorescent ternary PICM were incubated with DNAse I (50 U/μg ODN) (Sigma) in the presence of $MgCl_2$ (5 mM) at 37 °C. At predetermined time points, aliquots were withdrawn and degradation was stopped by adding EDTA (10 mM). Before being loaded onto a 20% polyacrylamide gel, heparin (280 μM) was added to the samples to induce ODN release from the PICM. Following migration, ODN was visualized directly on the gel after exposition to UV light using a ChemiImager 5500 imaging system (Alpha Innotech Corp., San Leandro, CA). The amount of intact ODN was quantified relatively to the fluorescent signal obtained with a sample containing 100% intact ODN (nonexposed to DNAse).

Assessment of Membrane-Destabilizing Properties of Micelles Using Liposomes as an Endosomal Membrane Model. Unilamellar liposomes (40 mM total lipid) mimicking the composition of the endosomal membrane (egg phosphatidylcholine/cholesterol/dioleoylphosphatidylethanolamine/dimiristoylphosphatidylglycerol, 61:17:16:6 molar ratio) (*17*) were prepared by hydration of a dried lipid film, followed by repeated extrusion through a 0.2 μm pore size membrane at room temperature. The highly water-soluble fluorophore trisodium 8-hydroxypyrene trisulfonate (HPTS) and the collisional quencher *p*-xylene-bis-pyrimidium (DPX) were included in the lipid hydration buffer (Tris 10 mM, NaCl 48 mM, HPTS 35 mM, DPX 50 mM, pH 7.4). Untrapped dye was removed by gel filtration on Sepharose 2B (Sigma). The membrane-destabilizing properties of the micelles were evaluated at 37 °C at different pH values by adding liposomes (10 μL) to the ternary PICM (previously purified on Sephacryl 200HR to remove free P(MAA-*co*-EA-*co*-BMA) or PMAA) in isotonic buffer of the appropriate pH. In all cases, the final MAA copolymer concentration was adjusted to 85 μg/mL. The release of liposome content was monitored for 20 min at 37 °C by a fluorescence dequenching assay (*18*). The extent of release was calculated from HPTS fluorescence ($\lambda_{ex} = 412$ nm, $\lambda_{em} = 513$ nm) relative to the fluorescence intensity measured following liposome lysis in 0.5% (v/v) Triton X-100, which accounted for the complete release of encapsulated HPTS and DPX.

Intracellular Distribution of the ODN. U937 cells were plated in 6-well tissue culture plates (1 mL medium containing 1×10^6 viable cells) containing coverslips and differentiated into macrophages using 80 nM phorbol myristate (Sigma). After 72 h, the phorbol was rinsed, and ternary micelles (2 mg PEO-

Table 1. Characteristics of the Ternary PICM

pH	diameter (nm) [PI]	scattering intensity (Kcps)	M_w[a] (Da)	ζ potential ± SD (mV)
7.4	29.5 [0.13]	41.2	829 400	0.0 ± 2.6
6.2	31.5 [0.19]	30.8	752 900	1.4 ± 0.9
5.5	28.1 [0.24]	19.7	658 700	2.1 ± 1.2
5.0	29.1 [0.21]	17.0	362 200	1.8 ± 3.6

[a] Determined by static light scattering. Below pH 7.4, the M_w is an average for whole micelles, chain clusters, and free polymeric chains.

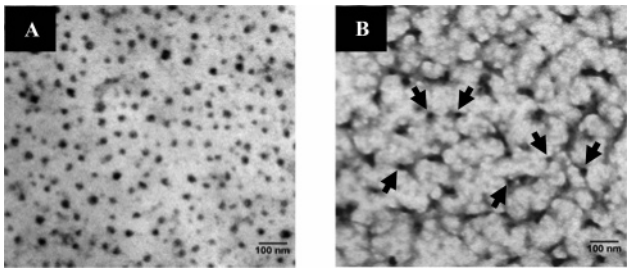

Figure 1. TEM analysis of ternary PICM at pH 7.4 (A) and 5.0 (B).

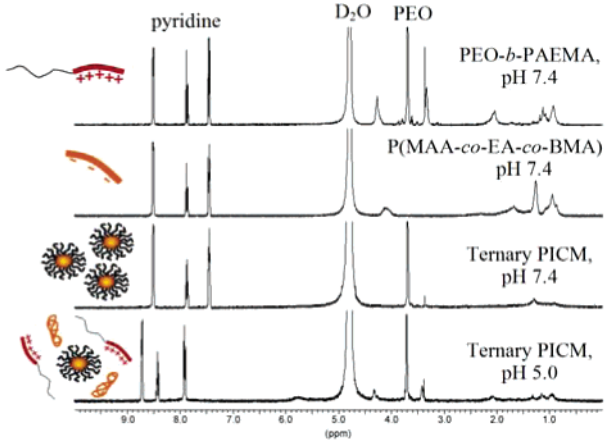

Figure 2. ^{1}H NMR spectra of the polymers alone and ternary PICM in deuterated Tris buffer. Pyridine was used as an internal standard.

b-PAEMA, 0.862 mg P(MAA-*co*-EA-*co*-BMA) or PMAA, 80 μg ODN) containing fluorescein-labeled ODN (50% of total ODN) were prepared as described above and incubated with cells for 4 h at 37 °C in a humid atmosphere containing 5% CO_2. Two hours before the end of the incubation period, LysoTracker red (Invitrogen, Burlington, ON, Canada) was added to each well at a final concentration of 80 nM. After 4 h, the cells were washed three times with cold PBS and mounted on slides which were kept on ice. Live cells were then examined by laser scanning confocal microscopy (LSCM) under a Leica DMIRBE inverted microscope coupled with a Leica TCS SP confocal system (Leica Microsystems, Heidelberg, Germany). The cells were excited at 488 or 568 nm, and fluorescence was collected by emission windows set at 505−555 and 585−635 nm, respectively. Images collected by *LCS Lite* software (Leica Microsystems) were exported as TIFF files and prepared for publication by *Adobe Photoshop* v 7.0 software (Adobe Systems, San Jose, CA).

RESULTS AND DISCUSSION

At pH 7.4 and room temperature, small-sized (30 nm), narrowly distributed (PI ≈ 0.1) and neutral PICM were obtained at the nearly stoichiometric ± ratio of 0.7/1 (PEO-*b*-PAEMA/P(MAA-*co*-EA-*co*-BMA)/ODN 68%/29%/3% w/w) (Table 1 and Figure 1A). The size and PI of the micelles barely changed at 37 °C (data not shown). Micelles were formed when the PEO-*b*-PAEMA concentration reached the critical values of 11.7 and

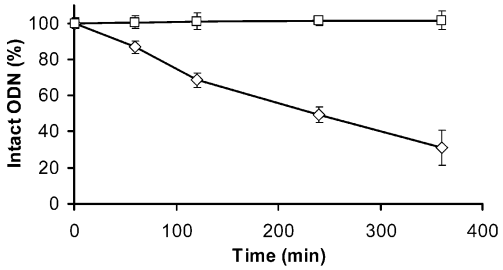

Figure 3. Stability toward nuclease degradation of naked ODN (◇) and ODN incorporated into ternary PICM (□) over a 6 h period.

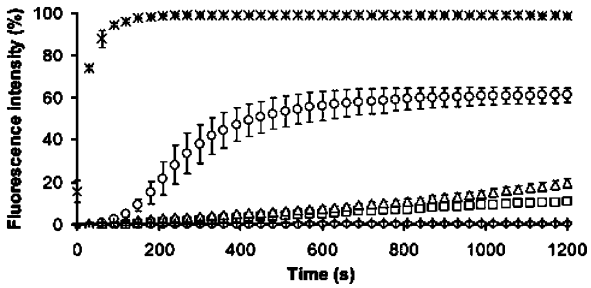

Figure 4. HPTS release induced by the ternary micelles at 37 °C and pH 7.4 (◇), 6.2 (□), 5.5 (△), and 5.0 (○). Star symbols represent the release induced by free P(MAA-*co*-EA-*co*-BMA) at pH 5.0 at equivalent concentration. Release induced by ternary micelles prepared with PMAA, pH 5.0, is represented by (+). Mean ± S.D. (n = 3).

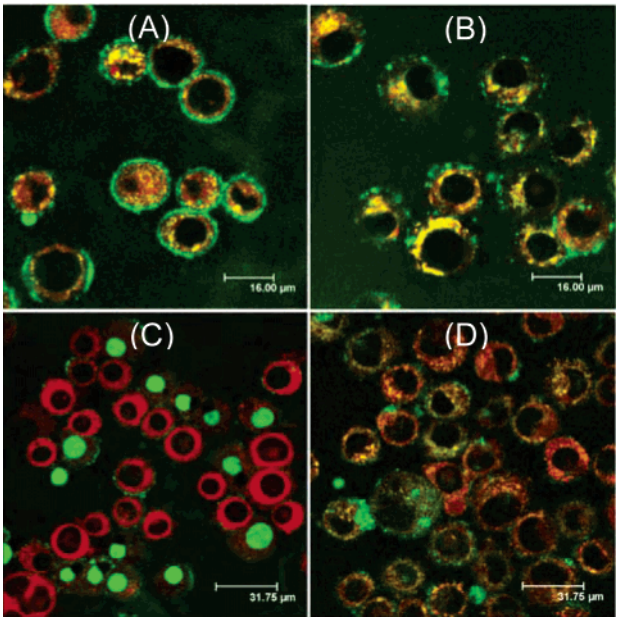

Figure 5. U937 macrophages on coverslips were incubated with ternary micelles prepared with P(MAA-*co*-EA-*co*-BMA) (A,C) or the non-endosomolytic polymer PMAA (B,D) for 4 h at 37 °C. Micelles were prepared using a fluorescein-labeled ODN, and acidic organelles were stained with LysoTracker red. After a 4 h incubation period at 37 °C, the cells were rinsed with cold PBS, mounted on slides, and kept on ice. Live cells were examined by LSCM 45 min (A,B) and 2 h (C,D) following the incubation period.

19.1 μg/mL at 25 and 37 °C, respectively, and remained stable in the presence of 150 mM NaCl (isotonic conditions). Incorporation levels of ODN and P(MAA-*co*-EA-*co*-BMA) reached 100% and 90%, respectively, as determined by gel electrophoresis and ultrafiltration of micelles containing a fluorescein-labeled ODN or a rhodamine-labeled copolymer (see Supporting Information). Table 1 reports the characteristics of the PICM at different pH values in Tris buffer. It is noteworthy that the individual copolymers did not self-associate in solution.

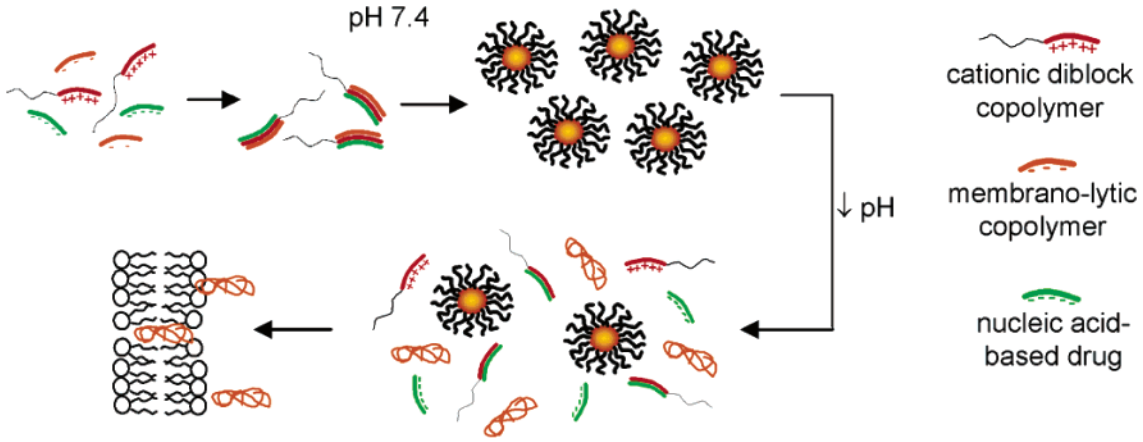

Figure 6. Schematic representation of the association/dissociation behavior of the ternary PICM as a function of pH.

At acidic pH, the micelle PI increased substantially. Moreover, upon lowering the pH from 7.4 to 5.0, a 2.3-fold decrease in the PICM M_w was observed, along with a decreased scattering intensity. This indicates partial dissociation and release of chain clusters and/or free polymeric chains in the external medium at acidic pH. The fact that 30 nm aggregates were still detected at pH 5.0 implies that micelle disruption was not complete. This was confirmed by TEM analysis (Figure 1B), which revealed the presence of small particles of appearance and size similar to those observed at neutral pH (see arrows), along with an excess of noncondensed material.

The association of the copolymers into a core/shell architecture was corroborated by ^{1}H NMR spectroscopy. Prior to analysis, the PICM were eluted on a Sephacryl column to completely remove any free polymer in solution. Figure 2 shows that PICM formation at neutral pH resulted in an important decrease in the intensity of the peaks corresponding to P(MAA-*co*-EA-*co*-BMA), while the signal of the PAEMA block completely disappeared. Interestingly, the peak attributed to the PEO block remained visible, confirming the micellization of the copolymers into core/shell assemblies (*19*). Indeed, the PEO chains forming the outer corona remain hydrated, while the (meth)acrylate monomers are segregated into a more viscous, less solvated internal core, causing a partial suppression of their signals. When the pH was reduced to 5.0, peaks corresponding to the cationic copolymer reappeared to some extent, which points toward enhanced solvation of the micellar core and/or polymer release due to partial micellar dissociation. No signal increase corresponding to P(MAA-*co*-EA-*co*-BMA) was observed at acidic pH because the relatively high concentrations required for the NMR analysis exceeded the copolymer solubility.

ODN stability toward nucleases was assessed by incubating free fluorescent ODN or ternary micelles containing the fluorescent ODN with DNAse I (50 U/μg ODN, 37 °C). Gel electrophoresis of aliquots withdrawn at predetermined time points showed that only 30% of the naked ODN remained intact after a 6 h incubation period. In contrast, no degraded ODN could be detected in the micellar samples at the end of the study (Figure 3). Thus, it is possible to protect the ODN upon incorporation in the ternary PICM structure.

The membrane-destabilizing properties of the micelles were evaluated at different pH values following removal of uncomplexed polymer. To do so, artificial phospholipid vesicles were used to mimic the endosomal membrane. The vesicles were loaded with the water-soluble fluorophore HPTS. They were then added to an isotonic micellar solution prepared at the appropriate pH value. Figure 4 illustrates the release profiles of the encapsulated probe. At pH 7.4, the ternary PICM did not destabilize the phospholipid bilayer as shown by the absence of released dye. As the pH was reduced, the extent of leakage progressively increased, reaching up to 60% at pH 5.0. When comparing the release kinetics obtained in the presence of free P(MAA-*co*-EA-*co*-BMA) to that associated with PICM at pH 5.0, one can note that the latter caused a lowered and delayed HPTS release. This suggests that the protonation of P(MAA-*co*-EA-*co*-BMA) led to its progressive removal from the micelles and to the subsequent destabilization of the vesicle membranes. Chung et al. (*20*) have reported that PEAA induces the formation of cation-selective channels through artificial membranes in a pH-dependent manner. Since P(MAA-*co*-EA-*co*-BMA) is structurally related to PEAA, a similar mechanism of vesicle destabilization may be hypothesized, although the exact nature of the membrane defects remains to be determined. Interestingly, control ternary PICM (35 nm) prepared using a well-known non-membranolytic polymer (PMAA) (*21*) instead of P(MAA-*co*-EA-*co*-BMA) were unable to trigger HPTS release at pH 5.0 (Figure 4), confirming that PEO-*b*-PAEMA does not destabilize the vesicles.

To verify whether the ternary micelles could facilitate the transfer of the ODN from the endosome to the cytoplasm, the intracellular distribution of the ODN in macrophages was examined by LSCM. In these in vitro experiments, macrophages were used, since the uptake of the PICM in non-phagocytic cells is inefficient due to the steric barrier created by the PEG corona (unpublished observations). Live cells were analyzed since it was previously demonstrated that cell fixation could affect membrane permeability and cause artifacts (*22*). Forty-five minutes after the incubation period, macrophages treated with ternary endosomolytic micelles showed multiple fluorescent intracellular vesicles, probably corresponding to phagocytic/endocytic compartments (Figure 5A). Colocalization of ODN and LysoTracker red was detected in most cells (yellow color), indicating that some micelles were localized in acidic organelles. Similar subcellular distribution was observed with the control micelles prepared with PMAA (Figure 5B). Two hours after the incubation period, bright green nuclear fluorescence could be seen in several cells incubated with endosomolytic micelles, indicating that the ODN was released from the endodomal/lysosomal compartments (Figure 5C) (*23*, *24*). These cells also exhibited a decreased red fluorescence indicating disruption of most of the acidic organelles. At that stage, the control, non-endosomolytic micelles were still mainly localized in intracellular globular compartments (Figure 5D), and staining of the nucleus of the cells was weak and, most of the time, undetectable. Collectively, these preliminary findings clearly demonstrate that cytoplasmic delivery of the ODN was facilitated by the incorporation of an endosomolytic polymer into PICM.

In conclusion, ternary PICM with a core/shell architecture, small size, and narrow size distribution were successfully

obtained at physiological pH following the sequential addition of a cationic diblock copolymer, an MAA copolymer, and an ODN. Besides their ability to protect the ODN against nuclease degradation, the ternary micelles were shown to release chain clusters capable of inducing membrane destabilization at slightly acidic pH and improving substantially the ODN subcellular distribution. This association/dissociation behavior is represented in Figure 6 and highlights the potential of these pH-sensitive PICM for the efficient transport and delivery of polyionic drugs. Future work will aim at evaluating the ability of the system to increase the efficacy of ODNs into human tumoral cells. To promote cellular internalization, the micelles will have to be decorated with a targeting ligand.

ACKNOWLEDGMENT

This work was financially supported by the NSERC and the Canada Research Chair Program. M.-A. Yessine acknowledges a scholarship from the FRSQ.

Supporting Information Available: Molecular weight determination, potentiometric titration, and ^{1}H NMR spectra of the copolymers in deuterated Tris. Experimental details on the determination of the critical association concentration of the ternary PICM and the quantification of complexed ODN and P(MAA-*co*-EA-*co*-BMA). This material is available free of charge via the Internet at http://pubs.acs.org.

LITERATURE CITED

(1) Hawker, C. J., and Wooley, K. L. (2005) The convergence of synthetic organic and polymer chemistries. *Science 309*, 1200−5.

(2) Kataoka, K., Harada, A., and Nagasaki, Y. (2001) Block copolymer micelles for drug delivery: design, characterization and biological significance. *Adv. Drug Delivery Rev. 47*, 113−31.

(3) Gillies, E. R., and Frechet, J. M. J. (2005) pH-responsive copolymer assemblies for controlled release of doxorubicin. *Bioconjugate Chem. 16*, 361−8.

(4) Rungsardthong, U., Ehtezazi, T., Bailey, L., Armes, S. P., Garnett, M. C., and Stolnik, S. (2003) Effect of polymer ionization on the interaction with DNA in nonviral gene delivery systems. *Biomacromolecules 4*, 683−90.

(5) Kataoka, K., Togawa, H., Harada, A., Yasugi, K., Matsumoto, T., and Katayose, S. (1996) Spontaneous formation of polyion complex micelles with narrow distribution from antisense oligonucleotide and cationic block copolymer in physiological saline. *Macromolecules 29*, 8556−7.

(6) Kabanov, A. V., Vinogradov, S. V., Suzdaltseva, Y. G., and Alakhov, V. Y. (1995) Water-soluble block polycations as carriers for oligonucleotide delivery. *Bioconjugate Chem. 6*, 639−43.

(7) Rungsardthong, U., Deshpande, M., Bailey, L., Vamvakaki, M., Armes, S. P., Garnett, M. C., and Stolnik, S. (2001) Copolymers of amine methacrylate with poly(ethylene glycol) as vectors for gene therapy. *J. Controlled Release 73*, 359−80.

(8) Kakizawa, Y., and Kataoka, K. (2002) Block copolymer micelles for delivery of gene and related compounds. *Adv. Drug Delivery Rev. 54*, 203−22.

(9) Murthy, N., Robichaud, J. R., Tirell, D. A., Stayton, P. S., and Hoffman, A. S. (1999) The design and synthesis of polymers for eukaryotic membrane disruption. *J. Controlled Release 61*, 137−43.

(10) Lackey, C. A., Murthy, N., Press, O. W., Tirell, D. A., Hoffman, A. S., and Stayton, P. S. (1999) Hemolytic activity of pH-responsive polymer−streptavidin bioconjugates. *Bioconjugate Chem. 10*, 401−5.

(11) Jones, R. A., Cheung, C. Y., Black, F. E., Zia, J. K., Stayton, P. S., Hoffman, A. S., and Wilson, M. R. (2003) Poly(2-alkylacrylic acid) polymers deliver molecules to the cytosol by pH-sensitive disruption of endosomal vesicles. *Biochem. J. 372*, 65−75.

(12) Cheung, C. Y., Murthy, N., Stayton, P. S., and Hoffman, A. S. (2001) A pH-sensitive polymer that enhances cationic lipid-mediated gene transfer. *Bioconjugate Chem. 12*, 906−10.

(13) Lackey, C. A., Press, O. W., Hoffman, A. S., and Stayton, P. S. (2002) A biomimetic pH-responsive polymer directs endosomal release and intracellular delivery of an endocytosed antibody complex. *Bioconjugate Chem. 13*, 996−1001.

(14) Yessine, M. A., Meier, C., Petereit, H.-U., and Leroux, J. C. (2006) On the role of methacrylic acid copolymers in the intracellular delivery of antisense oligonucleotides. *Eur. J. Pharm. Biopharm. 63*, 1−10.

(15) Dufresne, M. H., and Leroux, J. C. (2004) Study of the micellization behavior of different order amino block copolymers with heparin. *Pharm. Res. 21*, 160−9.

(16) Astafieva, I., Zhong, X. F., and Eisenberg, A. (1993) Critical micellization phenomena in block polyelectrolyte solutions. *Macromolecules 26*, 7339−52.

(17) Darnell, J., Lodish, H., and Baltimore, D. (1990) *Molecular Cell Biology*, 2nd ed., p 493, Scientific American Books, New York.

(18) Daleke, D. L., Hong, K., and Papahadjopoulos, D. (1990) Endocytosis of liposomes by macrophages: binding, acidification and leakage of liposomes monitored by a new fluorescence assay. *Biochim. Biophys. Acta 1024*, 352−66.

(19) Weaver, J. V. M., Armes, S. P., and Liu, S. Y. (2003) A "holy trinity" of micellar aggregates in aqueous solution at ambient temperature: unprecedented self-assembly behavior from a binary mixture of a neutral-cationic diblock copolymer and an anionic polyelectrolyte. *Macromolecules 36*, 9994−8.

(20) Chung, J. C., Gross, D. J., Thomas, J. L., Tirrell, D. A., and Opsahl-Ong, L. R. (1996) pH-sensitive, cation-selective channels formed by a simple synthetic polyelectrolyte in artificial bilayer membranes. *Macromolecules 29*, 4636−41.

(21) Thomas, J. L., You, H., and Tirrell, D. A. (1995) Tuning the response of a pH-sensitive membrane switch. *J. Am. Chem. Soc. 117*, 2949−50.

(22) Richard, J. P., Melikov, K., Vives, E., Ramos, C., Verbeure, B., Gait, M. J., Chernomordik, L. V., and Lebleu, B. (2003) Cell-penetrating peptides. A reevaluation of the mechanism of cellular uptake. *J. Biol. Chem. 278*, 585−90.

(23) Loke, S. L., Stein, C. A., Zhang, X. H., Mori, K., Nakanishi, M., Subasinghe, C., Cohen, J. S., and Neckers, L. M. (1989) Characterization of oligonucleotide transport into living cells. *Proc. Natl. Acad. Sci. U.S.A. 86*, 3474−8.

(24) Chin, D. J., Green, G. A., Zon, G., Szoka, F. C., Jr., and Straubinger, R. M. (1990) Rapid nuclear accumulation of injected oligodeoxyribonucleotides. *New Biol. 2*, 1091−100.

BC060159M