Polyelectrolyte Complexes between (Cross-linked) *N*-Carboxyethylchitosan and (Quaternized) Poly[2-(dimethylamino)ethyl methacrylate]: Preparation, Characterization, and Antibacterial Properties

Elena Yancheva,† Dilyana Paneva,† Vera Maximova,‡ Laetitia Mespouille,§
Philippe Dubois,§ Nevena Manolova,† and Iliya Rashkov*,†

Laboratory of Bioactive Polymers, Institute of Polymers, Bulgarian Academy of Sciences, Acad. G. Bonchev Street Bl. 103A, 1113 Sofia, Bulgaria, Department of Gene Regulations, Institute of Molecular Biology, Bulgarian Academy of Sciences, 1113 Sofia, Bulgaria, and Laboratory of Polymeric and Composite Materials, University of Mons-Hainaut, Place du Parc 20, B-7000 Mons, Belgium

Received October 28, 2006; Revised Manuscript Received December 19, 2006

Novel polyelectrolyte complexes (PECs) between *N*-carboxyethylchitosan (CECh) and well-defined (quaternized) poly[2-(dimethylamino)ethyl methacrylate] (PDMAEMA) have been obtained. The modification of chitosan into CECh allows the preparation of PECs in a pH range in which chitosan cannot form complexes. The CECh/PDMAEMA complex is formed in a narrow pH range around 7. The quaternization of the tertiary amino groups of PDMAEMA enables complex formation with CECh both in neutral and in alkaline medium. Cross-linked CECh is also capable of forming complexes with (quaternized) PDMAEMA. The antibacterial activity of (cross-linked) CECh, (quaternized) PDMAEMA, and their complexes against *Escherichia coli* has been evaluated. In contrast to (quaternized) PDMAEMA, (cross-linked) CECh exhibits no antibacterial activity. The complex formation between cross-linked CECh and (quaternized) PDMAEMA results in a loss of the inherent antibacterial activity of the latter in neutral medium. In acidic medium, the complexes exhibit strong antibacterial activity due to complex disintegration and release of (quaternized) PDMAEMA.

Introduction

Polyelectrolyte complexes (PECs) are formed by interaction of oppositely charged polyions. The stoichiometry of PECs depends strongly on the nature of polyelectrolytes. Nowadays, interpolymer complexes are considered as promising carriers in drug delivery[1,2] and gene therapy.[3−6] Other applications of PECs include preparation of membranes for dialysis and ultrafiltration,[7] as well as medical implants.[8] Because of their biodegradability and biocompatibility, polyelectrolyte complexes based on natural polysaccharides and their derivatives attract attention in the field of pharmacy and medicine.

Chitosan is a natural polysaccharide that is obtained by deacetylation of chitin and is an attractive material because of its nontoxicity, biodegradability, and physiological activity. It is known that it possesses antibacterial and hemostatic properties.[9] The complex formation between chitosan and synthetic or natural polyelectrolytes in aqueous medium allows the preparation of polymer materials with novel desired properties.[10−12] In certain cases, the limited solubility of chitosan in aqueous media (only at pH $\leq$ 6) may restrict its fields of application. That is the reason for the growing interest in chitosan derivatives having water solubility that does not depend on the pH value of the medium. An example of such a chitosan derivative is *N*-carboxyethylchitosan (CECh). It is worth noting that CECh, similarly to its precursor chitosan, is a biocompatible and biodegradable polymer. CECh is characterized by higher biodegradability as compared to chitosan, which is due to its higher solubility in water. In aqueous solutions, the presence of mono- and disubstituted *N*-carboxyethylglucosamine units in the structure of CECh determines the formation of betaine-type structures under certain conditions of the medium. That is why CECh combines the properties of a polyzwitterion and a polyampholyte and it can be considered as a polyampholyte-polyzwitterion. Recently, it has been demonstrated that depending on the pH value of the medium CECh can form PECs with both polyanions and polycations.[13] Poly(2-acrylamido-2-methylpropanesulfonic acid) (PAMPS), poly(acrylic acid) (PAA), and poly(ethylene imine) (PEI) have been used as partners for the preparation of CECh/synthetic polymer complexes. The stoichiometry of the complexes depends strongly on the pH value of the medium, and the complexes are stable in a narrow pH range.

The (quaternized) (co)polymers based on 2-(dimethylamino)ethyl methacrylate (DMAEMA) have their own biological activity. Recently, it has been demonstrated that quaternized derivatives of DMAEMA-based copolymers exhibit fungicidal and bactericidal activity.[14] Moreover, the ability of DMAEMA-based (co)polymers to form complexes with DNA makes them promising candidates for the preparation of non-viral gene delivery systems.[3−5] Recently, the complex formation between (quaternized) PDMAEMA and PAMPS has been studied.[15] It has been shown that the stoichiometry of the complexes depends on the degree of quaternization (QD) of the tertiary amino groups of PDMAEMA and on the pH of the medium. The DMAEMA-based polymers used in the studies have been obtained by atom transfer radical polymerization (ATRP), one of the most suitable modern synthetic tools for the preparation

---

* Corresponding author. E-mail: rashkov@polymer.bas.bg.
† Laboratory of Bioactive Polymers, Bulgarian Academy of Sciences.
‡ Department of Gene Regulations, Bulgarian Academy of Sciences.
§ Laboratory of Polymeric and Composite Materials, UMH.

10.1021/bm061029j CCC: $37.00

of well-defined polymers, which can find application in the biomedical field.[16−19] The complex formation between CECh and (quaternized) PDMAEMA is expected to lead to purposeful modification of the properties of the partners and to the preparation of novel polymer materials with desired behavior. To the best of our knowledge, there are no data concerning complex formation between CECh and PDMAEMA or its quaternized derivatives.

In the present work, the preparation and characterization of novel polyelectrolyte complexes between (cross-linked) CECh and well-defined (quaternized) PDMAEMA is described. The effect of the pH of the medium and the QD of PDMAEMA on the stoichiometry and yield of the complexes has been studied. In view of the potential biomedical applications of these novel polymer materials, the antibacterial activity of PECs based on cross-linked CECh and (quaternized) PDMAEMA has been evaluated.

Experimental Section

Materials. High molecular weight chitosan (MW 6×10^5, deacetylation degree 80% as determined by IR spectroscopy) and acrylic acid (AA) were purchased from Fluka. AA was distilled under reduced pressure prior to use. 2-Ethylbromoisobutyrate (EB^{i}B, 98%), 1,1,4,7,10,10-hexamethyltriethylenetetramine (HMTETA, 97%), and copper(I) bromide (CuBr, 98%) were supplied by Aldrich and used as received. Tetrahydrofuran (THF, 99+%, from Chem-Lab) and 2-(dimethylamino)ethyl methacrylate (DMAEMA, from Aldrich) were passed through a column of basic alumina to remove the stabilizing agents. The monomer was stored under nitrogen at −20 °C. Triethylamine (99%, from Fluka) was dried over barium oxide for 45 h at room temperature and distilled under reduced pressure. All other reagents including the salts for preparation of the buffer solutions were of analytical grade and used without further purification. The following buffer solutions were prepared: pH 4.8, 5.9 (CH_3COOH/NaOH), pH 7.0 (KH_2PO_4/Na_2HPO_4), and pH 9.0 ($NaHCO_3$/Na_2CO_3). The concentrations of the reagents were chosen in such a way as to achieve a value of the ionic strength equal to 0.1. For instance, the buffer solution of pH 4.8 and ionic strength 0.1 was obtained by mixing 388 mL of 1 M CH_3COOH and 100 mL of 1 M NaOH and subsequent dilution with distilled water to 1 L. A strain of *Escherichia coli* (LE 329) was obtained from the Collection of the Department of Gene Regulations, Institute of Molecular Biology, Bulgarian Academy of Sciences (Sofia, Bulgaria), and it was stored at 4 °C. Bacto trypton and Bacto yeast extract were purchased from Scharlau, Barcelona, Spain. Bacto agar was supplied by Difco, U.S.

Preparation of *N*-Carboxyethylchitosan (CECh). Chitosan (5 g, 0.03 mol) was dissolved in 250 mL of distilled water containing 4.3 g of acrylic acid (0.06 mol). The reaction mixture was heated for 24 h at 90 °C under constant stirring. After the mixture was cooled, an aqueous solution of NaOH (1.25 mol/L) was added to the reaction mixture until a pH of 10 was attained. The sodium salt of *N*-carboxyethylchitosan was purified by dialysis against distilled water for 2 d. The distilled water was changed repeatedly, and the dialysis was conducted until the pH of the reaction mixture reached a value of 7 (yield, 90%). ^{1}H NMR (500 MHz, D_2O, 70 °C, δ ppm): 2.06 (*Glc*−NH−CO−C*H*$_3$), 2.42 (*Glc*−NH−CH$_2$−C*H*$_2$−COOH), 2.68 (*Glc*−N(CH$_2$−C*H*$_2$−COOH)$_2$), 2.89 (H-2 of *Glc*−NH$_2$), 3.19 (H-2 of *N*-alkylated aminoglucoside units (*Glc*−NH−CH$_2$−CH$_2$−COOH)), 3.45−4.15 (H-3, H-4, H-5, and H-6 of *N*-aminoglucoside (*Gl*-NH$_2$) and *N*-acetylated aminoglucoside units (*Glc*−NH−CO−CH$_3$), H-2 of *Glc*−NH−CO−CH$_3$ and *Glc*−NH−C*H*$_2$−CH$_2$−COOH), and 4.52−5.09 (H-1).

Preparation of PDMAEMA by ATRP. PDMAEMA was synthesized by ATRP using EB^{i}B and CuBr ligated by HMTETA as an initiator and a catalyst, respectively ([DMAEMA]/[EB^{i}B]/[CuBr]/[HMTETA] = 170/1/1/2). Briefly, CuBr (0.105 g, 0.73 mmol) and HMTETA (0.336 g, 1.46 mmol) were introduced in a round-bottom flask, which was closed with a three-way stopcock capped by a rubber septum and purged by three freeze−thaw cycles. Next, THF (19.9 mL) and DMAEMA (19.64 g, 0.125 mol) previously filtered through a basic alumina column to remove their stabilizers were introduced in the round-bottom flask through a steel capillary under nitrogen flow. The mixture was purged with N_2, and the initiator EB^{i}B (0.144 g, 0.73 mmol) was added under nitrogen flow. The reaction was carried out at 60 °C for 21 h. PDMAEMA was selectively isolated by precipitation in 7-fold volume excess of cold heptane and was dried to constant weight (yield, 80%; conversion, 89%). The copper catalyst was removed by filtration of the PDMAEMA solution in THF through a basic alumina column. The residual copper content was determined using a Jobin Yvon 38-Plus inductively coupled plasma atomic emission spectrometer according to the following procedure: approximately 250 mg of polymer was accurately weighed, dissolved in distilled water containing 2 wt % nitric acid, and the obtained solution was diluted to 50 mL. The aqueous polymer solution containing an unknown amount of copper was introduced into the argon plasma, and the copper emission at 324.754 nm was measured and compared to that found for stock solutions containing 10, 5, 3, 1, 0.5, and 0.1 ppm Cu; a blank solution containing 2 wt % nitric acid in distilled water was used as the seventh calibration solution. The detection limit estimated by the manufacturer for the above-mentioned emission wavelength was around 0.001 ppm. The residual copper content of purified PDMAEMA was less than 1.0 ppm, as determined by ICP-AES analysis. ^{1}H NMR (300 MHz, CDCl$_3$, δ ppm): 0.6 (C(C*H*$_3$) PDMAEMA), 1.4 (C*H*$_3$−CH$_2$−O initiator), 1.8 (C*H*$_2$ PDMAEMA), 2.0 (C*H*$_3$ initiator), 2.3 (N(C*H*$_3$)$_2$ PDMAEMA), 2.65 (C*H*$_2$N(CH$_3$)$_2$ PDMAEMA), and 4.1 (O−C*H*$_2$ PDMAEMA and CH$_3$−C*H*$_2$−O initiator).

Preparation of PDMAEMAQ50 and PDMAEMAQ100. 5 g of PDMAEMA homopolymer (31.8 mmol tertiary amine) was introduced in a round-bottom flask and dissolved in THF (20 mL) under stirring at room temperature. Next, a defined amount of methyl iodide (CH_3I) (17.5 mmol for PDMAEMAQ50 and 38.2 mmol for PDMAEMAQ100) dissolved in 20 mL of THF was added. After 18 h, the quaternized homopolymer was recovered in the form of white powder by evaporating the solvent and residual CH_3I and drying under vacuum until a constant weight was achieved (yield, 100%). ^{1}H NMR (300 MHz, D_2O, δ ppm): 0.9 (C(C*H*$_3$) PDMAEMA), 1.2 (C*H*$_3$−CH$_2$−O initiator), 1.8 (C*H*$_2$ PDMAEMA), 2.0 (C*H*$_3$ initiator), 2.3 (N(C*H*$_3$)$_2$ PDMAEMA), 2.55 (O−CH$_2$−C*H*$_2$−N(CH$_3$)$_2$ PDMAEMA), 3.2 (N(C*H*$_3$)$_3$$^+I^-$), 3.8 (C*H*$_2$N(CH$_3$)$_3$$^+I^-$), 4.1 (O−C*H*$_2$−CH$_2$−N(CH$_3$)$_2$ PDMAEMA and CH$_3$–C*H*$_2$−O initiator), and 4.5 ppm (O−C*H*$_2$−CH$_2$−N(CH$_3$)$_3$$^+I^-$). The degree of quaternization (QD) was calculated from the relative intensity of the β-methylene amino protons at 4.1 ppm (O−C*H*$_2$−CH$_2$−N(CH$_3$)$_2$) and 4.4 ppm (O−C*H*$_2$−CH$_2$−N(CH$_3$)$_3$$^+I^-$). The same QD values were determined from the relative intensity of the α-methylene amino protons at 2.55 ppm (O−CH$_2$−C*H*$_2$−N(CH$_3$)$_2$) and 3.8 ppm (O−CH$_2$−C*H*$_2$−N(CH$_3$)$_3$$^+I^-$).

Characterization of the Polymers. Size exclusion chromatography (SEC) of PDMAEMA was performed in THF + 2 wt % triethylamine (NEt$_3$) at 35 °C using a Polymer Laboratories liquid chromatograph equipped with a PL-DG802 degasser, an isocratic HPLC pump LC 1120 (flow rate = 1 mL/min), a Marathon autosampler (loop volume = 200 μL, solution concentration = 1 mg/mL), a PL-DRI refractive index detector, and three columns: a PL gel 10 μm guard column and two PL gel Mixed-B columns. The molar mass and molar mass distribution were calculated with reference to poly(methyl methacrylate) standards.

The potentiometric titration of CECh for determination of the pK_a values of the carboxyl and amino groups in its structure was carried out according to a procedure previously described by Didukh et al.:[20] 170 mg of CECh was dissolved in 100 mL of 0.083 N NaOH or 0.1 N HCl and titrated at 25 °C with 0.1 N HCl or 0.083 N NaOH, respectively. The pH values were recorded using a PHM201 pH meter with a combined electrode.

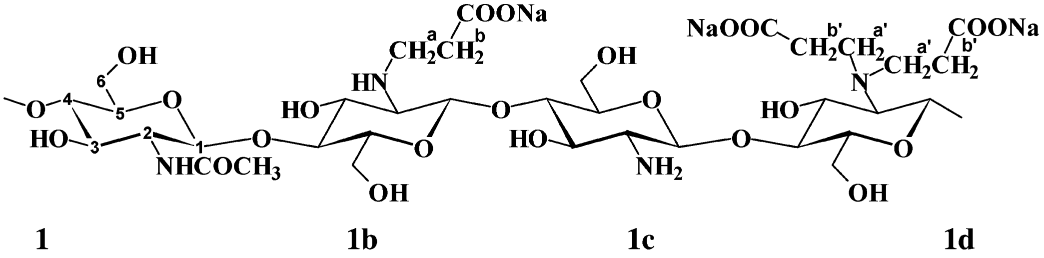

Figure 1. Fragment of the macrochain of the sodium salt of *N*-carboxyethylchitosan.

The reduced viscosity of CECh solutions of different pH and ionic strength was determined using a capillary Ubbelohde viscometer (inner diameter of the capillary $\phi = 0.45$ mm) at 25 ± 0.1 °C.

Preparation of Cross-linked *N*-Carboxyethylchitosan. An aqueous solution of glutaraldehyde (8 mL) with a concentration of 1.13×10^{-2} mol/L was added to a 1 wt % aqueous solution of CECh (50 mL) under constant stirring at room temperature ($[NH_2]/[CHO] = 1/1$). The mixture was stirred for 30 min, and subsequently it was poured into a Petri dish and dried at 40 °C until a film of constant weight was obtained. The obtained film was purified from unreacted glutaraldehyde and CECh by successive washing with distilled water.

Preparation and Characterization of PECs between (Cross-linked) CECh and (Quaternized) PDMAEMA. PECs were obtained at 25 °C by mixing (quaternized) PDMAEMA and CECh buffered aqueous solutions using a magnetic stirrer. Buffer solutions of pH 4.8, 5.9 ($CH_3COOH/NaOH$), 7.0 (KH_2PO_4/Na_2HPO_4), and 9.0 ($NaHCO_3/Na_2CO_3$) and constant ionic strength ($I = 0.1$) were used. The concentration of the polyelectrolyte solutions was 0.2 wt %, and they were mixed at different volume ratios: 1/9, 2/8, 3/7, 4/6, 5/5, 6/4, 7/3, 8/2, and 9/1. In a typical run, the polyelectrolyte solution with a larger volume was added to the one with a smaller volume. The mixtures were left for 48 h, and subsequently the white PEC precipitates were isolated by centrifugation for 40 min at 4000 rpm and dried. The yield was determined gravimetrically. The viscosity of the supernatants was measured using an Ubbelohde viscometer (inner diameter of the capillary $\phi = 0.45$ mm) at 25 ± 0.1 °C.

For the turbidimetric measurements, 0.01 wt % solutions of CECh and (quaternized) PDMAEMA in buffer solutions of pH 7.0 and 9.0 and constant ionic strength ($I = 0.1$) were mixed at the same volume ratios as in the case of the mixtures prepared for viscometric and gravimetric studies. The absorbance of the mixtures was recorded on a UV−vis SPECORD 71 spectrophotometer at 420 nm. The measurements were conducted on the fifth minute after mixing the polymer solutions.

Cross-linked CECh/(quaternized) PDMAEMA complexes were obtained according to the following procedure: pre-swollen films of cross-linked CECh (weight of the dry sample, 50 mg), which had reached an equilibrium swelling degree in buffer solutions (pH 4.8, 7.0, or 9.0 and ionic strength $I = 0.1$), were immersed in 3 wt % buffered solutions (pH 4.8, 7.0, or 9.0, ionic strength $I = 0.1$) of (quaternized) PDMAEMA for 48 h. The [CECh units]/[DMAEMA units] molar ratio was 1/1. The obtained complexes were dried at 40 °C to constant weight.

The surface morphology of the cross-linked CECh/(quaternized) PDMAEMA complexes was studied using a scanning electron microscope Philips SEM 515. For this purpose, the specimens were fixed onto sample holders by means of a double-sided adhesive tape, vacuum-coated with gold, and observed.

Determination of the Minimal Inhibitory Concentration (MIC) of (Quaternized) PDMAEMA and CECh against *Escherichia coli* (*E. coli*). The antibacterial activity of (quaternized) PDMAEMA and CECh against *E. coli* was evaluated using aqueous solutions of the polymers. A colony of *E. coli* LE 329 grown on agar (nutrient medium of Luria containing 1.5 wt % agar) was seeded in 5 mL of liquid nutrient medium of Luria (an aqueous solution of 1 wt % Bacto tryptone, 0.5 wt % NaCl, and 0.5 wt % Bacto yeast extract, pH 7.0). The bacterial culture was incubated overnight on a shaker at 37 °C. From the stationary night culture, a new seeding was made in liquid nutrient medium with a 1:100 dilution. The bacterial culture was incubated again on a shaker at 37 °C for 2 h. Samples with different concentrations of the investigated polymers (PDMAEMA, PDMAEMAQ50, PDMAEMAQ100, and CECh) were prepared in liquid nutrient medium, and bacterial culture in an exponential growth phase was added to the nutrient medium. The samples were incubated for 30 min. Each sample was titrated on solid nutrient medium. The seedings were incubated in a thermostat at 37 °C, and the number of colonies was counted on the following day. The *E. coli* control sample had a titer of 2×10^7 cells/mL.

Determination of the Bactericidal Activity of (Quaternized) PDMAEMA against *E. coli*. 100 μL of a stationary night culture of *E. coli* was added to 900 μL of distilled water containing PDMAEMA, PDMAEMAQ50, or PDMAEMAQ100 in concentrations 10 times higher than the respective MIC values determined by the above-described test. At different time intervals (5, 20, or 60 min), sample aliquots were taken and diluted to 10^{-6} and subsequently seeded on LB agar (solid nutrient medium). The seedings were incubated in a thermostat at 37 °C, and the number of colonies was counted the next day. The *E. coli* control sample had a titer of 2×10^7 cells/mL.

Determination of the Antibacterial Activity of Cross-linked CECh and Cross-linked CECh/(Quaternized) PDMAEMA Complexes. The tests were carried out according to the procedure employed in the determination of the MICs of the polymers: films of cross-linked CECh or cross-linked CECh/(quaternized) PDMAEMA complexes were immersed in nutrient medium, and bacterial culture was added to the medium. The samples were incubated for 30 min at 37 °C. Subsequent dilutions and seedings on agar were made for titration of the samples. The *E. coli* control sample had a titer of 2×10^7 cells/mL.

Results and Discussion

Properties of the Polymer Partners. CECh was synthesized according to a previously reported procedure[21] by a reaction of selective carboxyethylation of the amino groups of high molecular weight chitosan. A fragment of the structure of the sodium salt of CECh is shown in Figure 1. The degree of mono- and disubstitution (subunits 1b and 1d, Figure 1) was determined by ^1H NMR spectroscopy in D_2O (70 °C, 500 MHz) from the relative intensity of the methylene protons from mono- and disubstituted CECh units, $Glc{-}NH{-}CH_2{-}CH_2{-}COOH$ and $Glc{-}N(CH_2{-}CH_2{-}COOH)_2$, at 2.4−2.7 ppm and the intensity of the signal for the proton from the methine group in position 1 (H-1) in the aminoglucoside CECh subunit at 4.4−4.8 ppm.[13,20] The total degree of substitution was 76%. The content of mono- and disubstituted units, 51% and 25%, respectively, was calculated from the relative intensity of the protons from $Glc{-}NH{-}CH_2{-}CH_2{-}COOH$ and $Glc{-}N(CH_2{-}CH_2{-}COOH)_2$ at 2.42 and 2.68 ppm, respectively. The degree of acetylation calculated from the ^1H NMR spectrum was 17%. The content of non-substituted amino groups was 7%. As determined from the ^1H NMR spectrum, CECh consists of the following monomer units: *N*-acetylglucosamine (1a), glucosamine (1c),

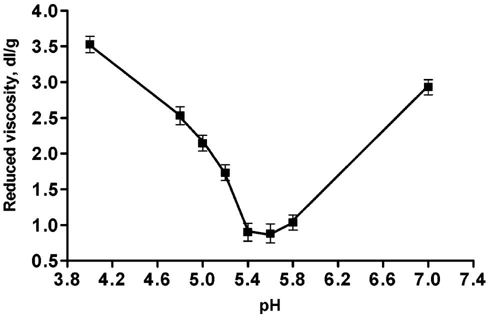

Figure 2. Dependence of the reduced viscosity of CECh solution on pH ($I = 0.1$, 25 °C, 0.1 wt %).

and mono- and disubstituted *N*-carboxyethylglucosamine units (1b, 1d) (Figure 1).

In aqueous solutions, the presence of mono- and disubstituted *N*-carboxyethylglucosamine units (1b, 1d) determines the possibility of formation of polyzwitterionic (betaine-type) structures at certain medium pH values. The pK_a values of carboxylate anions, imino groups, carboxyl groups, and protonated imino groups in the structure of CECh were determined by potentiometric titration of CECh with HCl or NaOH. The pK_a values of carboxylate anions and imino groups determined by titration of CECh with HCl were 4.38 and 8.31, respectively, and the pK_a values of carboxyl groups and protonated imino groups determined by titration of CECh with NaOH were 8.87 and 10.68, respectively. The obtained results are in good agreement with the data from the potentiometric titration of CECh with a similar degree of substitution,[13] as well as with the results obtained by Didukh et al.,[20] who have found that the pK_a values of the carboxyl groups of polyzwitterionic polymers are higher as compared to those of polycarboxylic acids. Because CECh in aqueous solutions behaves as a polyampholyte and a polyzwitterion, an important characteristic is its isoelectric point (IEP).

It is known that the dependence of the reduced viscosity of aqueous solutions of polyampholytes and polyzwitterions on pH at a given ionic strength is characterized by a minimum at the pH value equal to the IEP value.[13,22,23] The dependence of the reduced viscosity of 0.1 wt % aqueous solutions of CECh on the pH value of the medium is shown in Figure 2. As seen from the plot, the curve passes through a minimum in the pH range from 5.4 to 5.8. Further increase of the medium pH value leads to an increase in the values of the viscosity of CECh solutions. Therefore, CECh used in the present study has an IEP in the pH range from 5.4 to 5.8. In this pH range, CECh contains zwitterionic fragments in its structure, which form dipoles with significant dipole moments. The minimal values of the viscosity of polyzwitterionic polymers (corresponding to a considerable decrease in the hydrodynamic radius of the macromolecules) at pH values close to the IEP can be explained by the formation of self-assemblies of oppositely oriented dipoles.[24] These self-assemblies can be regarded as specific nodes of a dynamically changing physical network. The structure of this network depends strongly on the presence and concentration of low molecular weight salts. It is known that polyzwitterionic polymers exhibit the so-called “antipolyelectrolyte effect” at the IEP; the addition of a low molecular weight salt leads to an increase in the reduced viscosity.[23−25] The dependence of the reduced viscosity of CECh solution on the concentration of NaCl is shown in Figure 3.

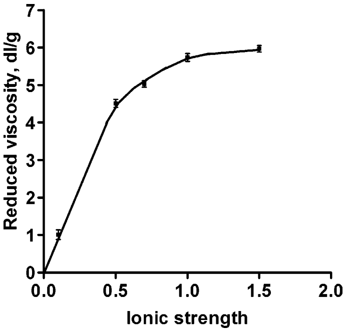

Figure 3. Dependence of the reduced viscosity of CECh solution on the ionic strength (pH 5.6, 25 °C, 0.1 wt %).

As seen, the increase in the ionic strength from 0.1 to 1.5 leads to an increase in the reduced viscosity of the solution of CECh. At low salt content, the intramolecular electrostatic interactions between the positive and negative charges in the macromolecule of CECh are dominant as a result of which the polymer chains collapse into a coiled structure.[25] Because CECh has a polyzwitterionic nature, the globulization can be a result of an interaction between dipoles. At high salt concentration, the electrostatic interactions are screened and CECh has a swollen or Gaussian conformation. The increase in the viscosity of the solution of CECh with the increase of NaCl concentration can be explained by disruption of the dipole−dipole intermolecular interactions and release of single CECh macromolecules.

PDMAEMA (Figure 4a) was synthesized according to a previously described procedure[18] by ATRP of DMAEMA in THF. In a further set of experiments, part of PDMAEMA (Figure 4a) was quaternized. The degree of quaternization of PDMAEMA was finely tuned by the initial tertiary amine/methyl iodide molar ratio.[16,19,26] Two quaternized PDMAEMA samples with QDs of 50% and 100% (Figure 4b and c), which will be further designated as PDMAEMAQ50 and PDMAEMAQ100, were synthesized and isolated.

The molecular characteristics of (quaternized) PDMAEMA are presented in Table 1. The QD value was determined by ^{1}H NMR spectroscopy as previously reported.[16,26] The average number molecular weight ($\bar{M}_n$) of (quaternized) PDMAEMA was calculated from the degree of monomer conversion and from the initial monomer-to-initiator molar ratio. The apparent $\bar{M}_n$ was determined by size exclusion chromatography (SEC) only in the case of PDMAEMA. Determination of the apparent $\bar{M}_n$ values of PDMAEMAQ50 and PDMAEMAQ100 has not been carried out because the quaternization reaction does not affect the chain integrity.

PDMAEMA is a weak polybase (pK_a 7.0).[17] Previous viscometric studies of (quaternized) PDMAEMA have revealed that the properties of PDMAEMA solutions are strongly dependent both on pH and on ionic strength in contrast to quaternized PDMAEMA.[15] This determines the difference in their behavior in complex formation with polyacids.

Complex Formation between (Quaternized) PDMAEMA and CECh. As it has already been mentioned, CECh due to its polyampholytic nature can form PECs with polyanions or polycations depending on the ratio of the positively and negatively charged groups in its structure. The protonation of amino and imino groups, as well as the ionization of carboxyl groups of CECh, depend on the pH value of the medium. A schematic presentation of the dependence of the type of ionized groups in the structure of CECh on the medium pH value is shown in Figure 5. The polymer partners (polyanions or polycations), which are able to form polyelectrolyte complexes with CECh depending on medium pH, are also presented in the figure. At pH values lower than 4.8, the positive charges

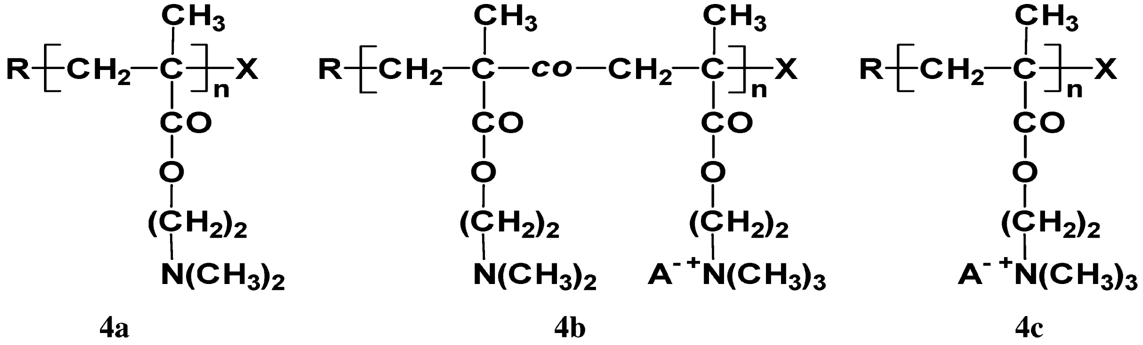

Figure 4. Fragments of the macrochains of PDMAEMA (a), PDMAEMAQ50 (b), and PDMAEMAQ100 (c); R = $C_2H_5OOC(CH_3)_2$; X = Br; A = I.

Table 1. Molecular Characteristics of DMAEMA-Based (Quaternized) Polymers

sample	degree of quaternization, QD (%)[a]	$\bar{M}_n$ (calcd)[b]	$\bar{M}_n$ (SEC)[c]	$\bar{M}_w/\bar{M}_n$ (SEC)[c]
PDMAEMA	0	24 000	23 900	1.32
PDMAEMAQ50	52	35 000		
PDMAEMAQ100	100	45 000		

[a] As determined by ^{1}H NMR spectroscopy in D_2O from the relative intensity of amino methylene protons at 4.1 ppm [$O-CH_2-CH_2-N(CH_3)_2$] and 4.4 ppm [$O-CH_2-CH_2-N(CH_3)_3{}^+I^-$]. [b] As calculated using the following expression: $\bar{M}_n$ (calcd) = MW (EB^{i}B) + ([DMAEMA]$_0$/[EB^{i}B]$_0$) × conversion × [(QD/100) × (MW(DMAEMA) + MW (CH_3I)) + (1 − QD/100) × MW (DMAEMA)], where [DMAEMA]$_0$/[EB^{i}B]$_0$ is the initial monomer/initiator molar ratio, MW (CH_3I) is the molecular weight of CH_3I, and QD is the degree of quaternization. [c] As determined by SEC in THF + 2 wt % NEt_3 at 35 °C with reference to poly(methyl methacrylate) standards.

are dominant in the structure of CECh as a result of protonation of the amino and imino groups. The carboxyl groups are not ionized at these pH values. Thus, at pH < 4.8, CECh behaves as a polycation, and it can form PECs with polyanions. As was recently shown by some of us, CECh forms complexes in acidic medium with the polyacids PAMPS and PAA.[13] At pH values close to the isoelectric point of CECh (from 4.8 to 5.9), the ratio between the positively and negatively charged groups is close to 1. In this pH range, CECh exhibits the typical properties of a polyzwitterion due to compensation of the opposite charges in betaine-type structures: $-NH_2{}^+RCOO^-$ and $-NH^+RCOO^-$. Thus, the possibility for complex formation with polyanions or polycations is considerably smaller in the pH range from 4.8 to 5.9. It is worth noting that CECh forms a physical network in this pH range due to charge compensation and inter- and intramolecular ion/dipole interactions. At pH > 6.0, the carboxylate anions are predominant in the structure of CECh, which determines its ability to form complexes with polycations.

It is expected that mixing of aqueous solutions of DMAEMA-based polymers and CECh at pH values higher than the isoelectric point of the latter will lead to PEC formation as a result of electrostatic interactions between the ionized carboxyl groups of CECh and the protonated and/or quaternized amino groups of DMAEMA-based (co)polymers. The formation of PECs was studied viscometrically, gravimetrically, and turbidimetrically. As expected, at pH values lower than 4.8, as well as in alkaline medium (pH > 8), complex formation between CECh and PDMAEMA was not observed. The reason for the lack of complex formation in highly acidic medium (pH < 4.8) is the small percentage of carboxylate anions in the structure of CECh (Figure 5). The pH dependence of the protonation degree of PDMAEMA (pK_a = 7.0) determines the upper limit pH value, above which no complex formation occurs. It was found that a water-insoluble CECh/PDMAEMA complex is formed in a narrow pH range around 7. The dependence of the yield of the complex and of the specific viscosity of the supernatants of CECh/PDMAEMA solutions on the molar fraction of DMAEMA units (F_{DMAEMA}) in the mixture at pH 7.0 ($I = 0.1$) is shown in Figure 6. The molar fraction of DMAEMA units (F_{DMAEMA}) was calculated using the following equation: $F_{DMAEMA)} = n_{DMAEMA}/(n_{DMAEMA} + n_{CECh})$, where n_{DMAEMA} and n_{CECh} designate the number of moles of (quaternized) DMAEMA and CECh units, respectively, at a defined initial ratio of the polymer partners.

The complex formation was also studied by a turbidimetric method. The turbidity of CECh/PDMAEMA mixtures as a function of the molar fraction of DMAEMA units at pH 7.0 is shown in Figure 7. The maximum amount of complex corresponds to the maxima in the curves, which indicate the dependence of the yield and turbidity on F_{DMAEMA}, and to the minimum in the curve showing the dependence of the specific viscosity on F_{DMAEMA} (Figures 6 and 7). It was found that at pH 7.0 a maximum amount of complex was formed at a slight excess of DMAEMA units ($F_{DMAEMA} = 0.64$).

At pH values close to the pK_a value of PDMAEMA, the amount of protonated tertiary amino groups is reduced, and this determines the need of a slight excess of PDMAEMA for the preparation of a maximum amount of CECh/PDMAEMA complex. As demonstrated earlier, the partial quaternization of the tertiary amino groups of PDMAEMA determines the ability of DMAEMA-based polymers to form complexes in alkaline medium.[15] The present studies on the complex formation between CECh and PDMAEMAQ50 reveal that this dependence is valid for this pair as well. In contrast to the non-quaternized polymer, PDMAEMAQ50 forms a complex with CECh at pH 9.0. The turbidity of CECh/PDMAEMAQ50 mixtures as a function of the molar fraction of DMAEMA units at pH 7.0 and 9.0 ($I = 0.1$) is shown in Figure 8. As seen, at pH 7.0 a maximum amount of CECh/PDMAEMAQ50 complex is formed at an equimolar ratio between the partners ($F_{DMAEMA} = 0.53$). At this pH value, CECh contains a considerable amount of ionized carboxyl groups that are able to participate in complex formation, and the formation of betaine-type structures is insignificant. The quaternized amino groups and the protonated tertiary amino groups (ca. 50% of the non-quaternized amino groups) of PDMAEMAQ50 are positively charged and enable the formation of a complex with a stoichiometry close to the equimolar one. At pH 9.0, a slight excess of PDMAEMAQ50 is required ($F_{DMAEMA} = 0.63$) for the preparation of a maximum amount of complex. This result was expected because at this pH value a considerable amount (ca. 99%) of the non-quaternized tertiary amino groups of PDMAEMAQ50 are not protonated, and they do not participate in complex formation.

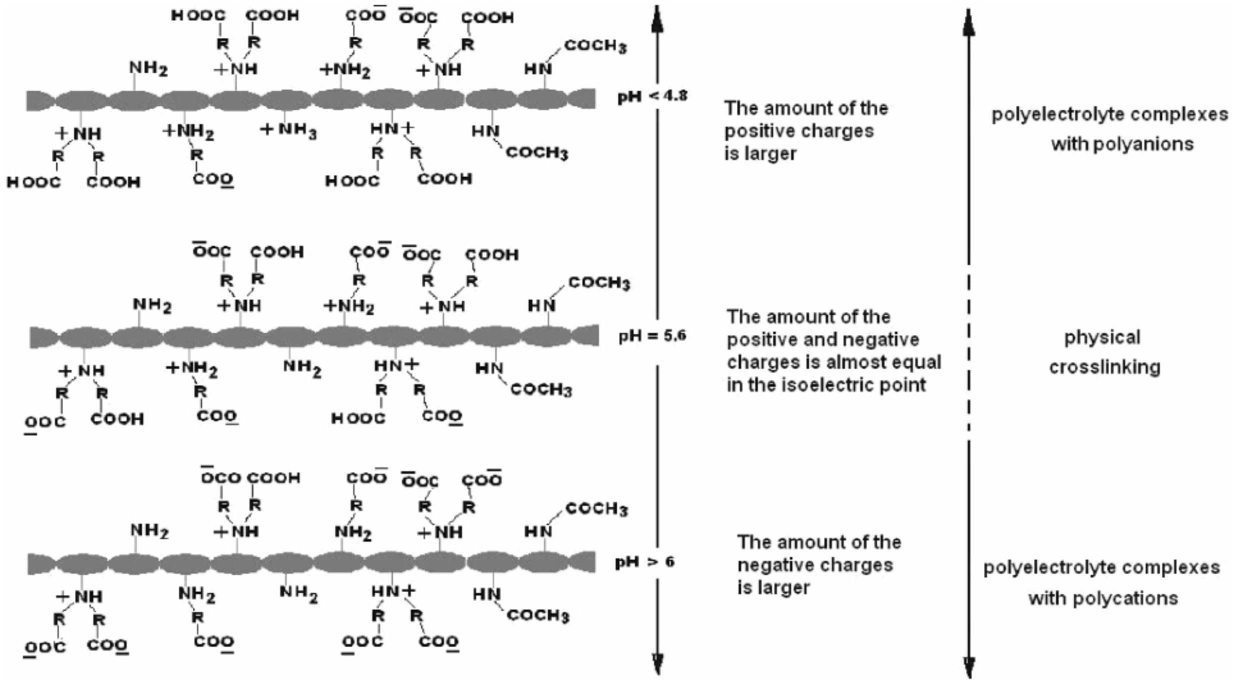

Figure 5. Schematic presentation of the structure of CECh at different medium pH values. The pH range in which CECh can interact electrostatically with polyanions or polycations is outlined as well.

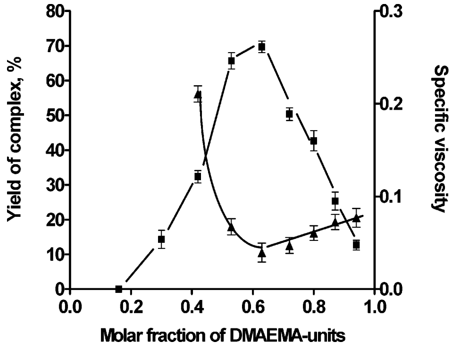

Figure 6. Dependence of the specific viscosity of the supernatants (▲) and of the yield (■) of CECh/PDMAEMA complex on the molar fraction of DMAEMA units at pH 7.0 (25 °C, *I* = 0.1, 0.2 wt %).

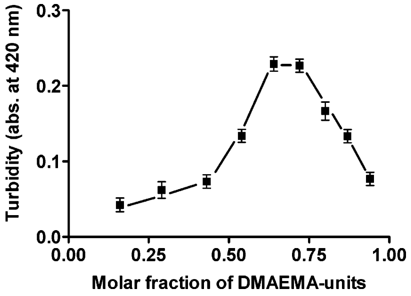

Figure 7. Dependence of the turbidity of CECh/PDMAEMA mixtures on the molar fraction of DMAEMA units at pH 7.0 (25 °C, *I* = 0.1, 0.01 wt %).

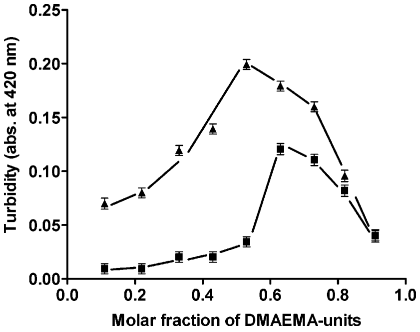

Figure 8. Dependence of the turbidity of CECh/PDMAEMAQ50 mixtures on the molar fraction of DMAEMA units at pH 7.0 (▲) and pH 9.0 (■) (25 °C, *I* = 0.1, 0.01 wt %).

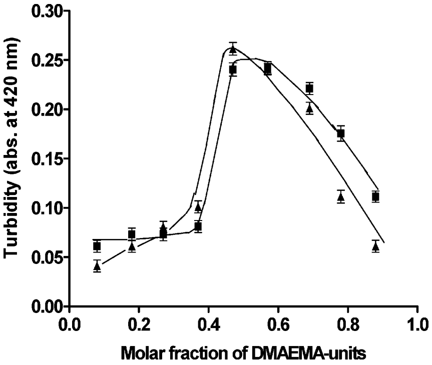

Figure 9. Dependence of the turbidity of CECh/PDMAEMAQ100 mixtures on the molar fraction of DMAEMA units at pH 7.0 (▲) and pH 9.0 (■) (25 °C, *I* = 0.1, 0.01 wt %).

The complete quaternization of the tertiary amino groups in the case of PDMAEMAQ100 allows the preparation of complexes with a stoichiometry close to the equimolar one both in neutral and in alkaline media. At pH 7.0, the molar fraction of DMAEMA units is 0.47, and at pH 9.0, it is 0.57. The dependence of the turbidity of CECh/PDMAEMAQ100 mixtures on the molar fraction of DMAEMA units is shown in Figure 9. The slight excess of CECh necessary for the preparation of a maximum amount of CECh/PDMAEMAQ100 complex at pH 7.0 may be explained by the presence of betaine-type structures in the macromolecules of CECh, while the slight excess of DMAEMA-based units at pH 9.0 may be attributed to steric hindrance determined by PDMAEMAQ100 similarly to the already reported PDMAEMAQ100/PAMPS pair.[15]

A summarized illustration of the pH range of complex formation between chitosan or its derivative CECh, and different polyanions and polycations, is presented in Figure 10. This summarized illustration reveals the possibility for the preparation

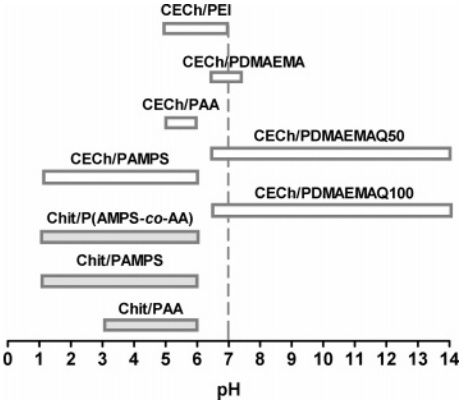

Figure 10. pH range of complex formation between chitosan (Chit) and (co)polymers of acrylic acid (AA)[27] and 2-acrylamido-2-methylpropanesulfonic acid (AMPS);[10,11] or between CECh and PAA or PAMPS,[13] PEI[13] or PDMAEMA, PDMAEMAQ50, and PDMAEMAQ100 (according to data from the present study).

of stimuli-responsive materials, the properties of which depend strongly on the PEC composition, that is, on the nature of the polymer partners. As seen, similarly to chitosan, CECh forms complexes with polyanions in acidic medium. The pH-independent water solubility and the polyampholytic nature of CECh extend its ability to form PECs in the whole pH range. It is worth noting that, depending on the desired material properties, CECh-based complexes with tailored pH stability and swellability can be prepared.

Complex Formation between Cross-linked CECh and (Quaternized) PDMAEMA. In previous studies, some of us have shown that CECh can be cross-linked by thermal treatment.[28] In this case, the carboxyl groups in the structure of CECh participate in the cross-linking reaction, thus limiting the ability of CECh to interact with polycations on a next stage. Therefore, the possibilites of using CECh for the preparation of new polymer materials based on PEC formation with polycations are restricted. Glutaraldehyde (GA), which is widely used as a chitosan-cross-linking agent, is capable of interacting with compounds containing amino, imino, and/or hydroxyl groups, and its reactivity decreases in the following order: primary amino groups > secondary amino groups > hydroxyl groups.[29] It was of interest to study the possibility to obtain pH-sensitive polymer networks based on CECh by interaction of its amino and imino groups with GA, while preserving its carboxyl groups free. No data were found in the literature concerning the preparation of CECh-based pH-sensitive networks by chemical cross-linking with glutaraldehyde.

In the present work, a film of cross-linked CECh was obtained by reacting CECh with glutaraldehyde at a molar ratio $[NH_2]/[CHO] = 1/1$. The obtained film was insoluble in the whole pH range, evidence of the successful preparation of a polymer network. In a further set of experiments, the possibilities for preparation of cross-linked CECh/(quaternized) PDMAEMA complexes by immersion of pre-swollen films of cross-linked CECh in buffered (quaternized) PDMAEMA solutions were studied. In acidic medium (pH 4.8), no alterations were observed upon immersion of the film of cross-linked CECh into a solution of (quaternized) PDMAEMA. At this pH value, the amount of ionized groups in the structure of cross-linked CECh is not high enough for the occurrence of complex formation. At pH 7.0, the immersion of cross-linked CECh into the solution of the respective DMAEMA-based (co)polymer led to whitening and collapse of the film, thus indicating PEC formation. Similar behavior was observed after immersing cross-linked CECh in buffered solutions of PDMAEMAQ50 and PDMAEMAQ100 of pH 9.0. The obtained results show that the cross-linked CECh/PDMAEMA complex can be obtained only at pH values close to 7, whereas cross-linked CECh/quaternized PDMAEMA complexes can be obtained in neutral and alkaline medium.

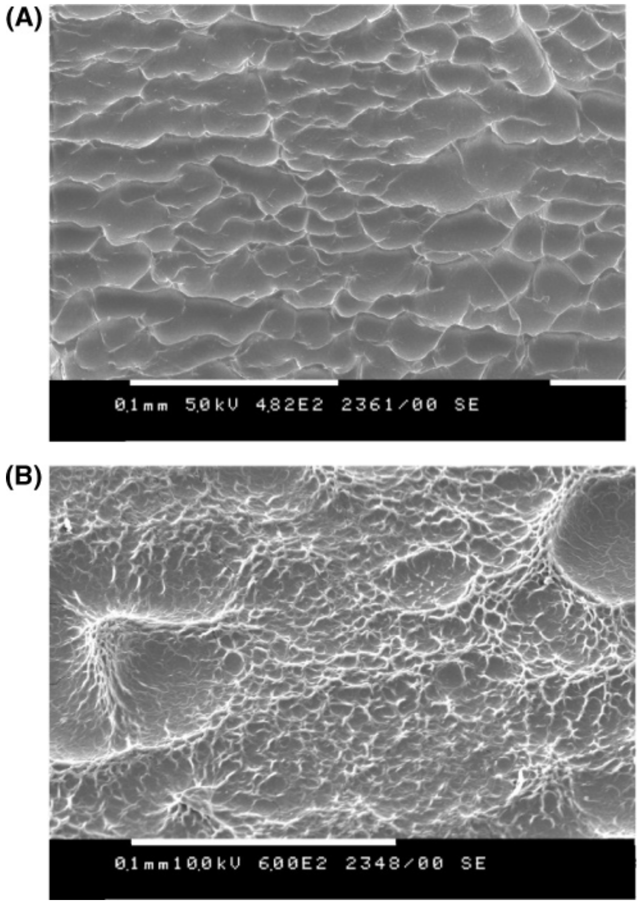

Figure 11. SEM micrographs of freeze-dried cross-linked CECh/PDMAEMAQ100 (A) and cross-linked CECh/PDMAEMAQ50 (B) complexes, respectively. Magnification: ×480 (A) and ×600 (B).

SEM micrographs of cross-linked CECh/quaternized PDMAEMA complexes (prepared using a buffer solution of pH 7.0, $I = 0.1$) are presented in Figure 11. The surface of the film of cross-linked CECh is smooth. The formation of CECh/(quaternized) PDMAEMA complexes on the surface of the film leads to wrinkling of the surface (Figure 11), which is characteristic of polymer material surfaces modified with PECs.[11,30]

Evaluation of the Antibacterial Activity of (Cross-linked) CECh, (Quaternized) PDMAEMA, and Their Complexes against *E. coli*. Chitosan is known as a natural disinfectant due to its ability to inhibit the growth of a wide spectrum of bacteria and fungi.[31] It should be noted that it is more active against Gram (−) bacteria (such as *E. coli*) than Gram (+) bacteria.[32] This is explained by an electrostatic interaction between the positively charged amino groups of chitosan and the negatively charged moieties of the bacterial cell walls. The *N*-carboxymethylation of chitosan leads to a decrease of its antibacterial activity, as shown in the case of *N*,*O*-carboxymethylchitosan.[31] No data were found in the literature concerning the antibacterial activity of (cross-linked) CECh. Similarly to chitosan, DMAEMA-based polymers as polycations exhibit antibacterial activity. It has been shown that block copolymers containing a quaternized PDMAEMA block exhibit biocidal activity against Gram (−) bacteria.[14] In the present work, the antibacterial activity of

Table 2. Determination of the Minimal Inhibitory Concentration (MIC) of PDMAEMA, PDMAEMAQ50, PDMAEMAQ100, CECh, and Chitosan against *E. coli*

polymer	MIC [mg/mL]
PDMAEMA	0.3
PDMAEMAQ50	0.3
PDMAEMAQ100	0.5
CECh	no antibacterial activity
chitosan	0.25

(cross-linked) CECh, (quaternized) PDMAEMA, and their complexes against *E. coli* has been evaluated.

In the first series of experiments, the minimal inhibitory concentration (MIC) of (quaternized) PDMAEMA, CECh, and chitosan was determined. MIC is a measure of the antibacterial activity of these polymers. It represents the lowest concentration of an antibacterial agent that inhibits the visible growth of a given microorganism. The initial concentration of the polymer stock solutions was 10 mg/mL in the case of (quaternized) PDMAEMA, and 20 mg/mL in the case of CECh and chitosan. The obtained data for the MICs are presented in Table 2. As seen from Table 2, CECh does not possess any antibacterial activity even at a concentration as high as 20 mg/mL. Contrarily, chitosan (the precursor of CECh) exhibits substantial antibacterial activity. Concentrations up to 0.25 mg/mL chitosan (solubilized by adding HCl, pH = 5) caused complete inhibition of the bacterial growth. The lack of antibacterial activity of CECh is due to the substitution of the amino groups, which are responsible for the antibacterial activity of chitosan.

As expected, (quaternized) PDMAEMA exhibited antibacterial activity (Table 2). All dilutions of PDMAEMA up to 0.3 mg/mL caused complete inhibition of the bacterial growth, and the sample containing 0.125 mg/mL of PDMAEMA caused a 3 log decrease of the *E. coli* titer. PDMAEMAQ50 exerted complete inhibitory effect in all dilutions up to 0.3 mg/mL. In the case of PDMAEMAQ100, all dilutions with concentrations up to 0.6 mg/mL caused complete inhibition of the growth of *E. coli*. In a concentration as high as 0.3 mg/mL, PDMAEMAQ100 exhibited significant antibacterial activity expressed by a 4 log decrease in the *E. coli* titer. The lower antibacterial activity of PDMAEMAQ100 as compared to PDMAEMA and PDMAEMAQ50 might be attributed to a steric hindrance of its pendent quaternized amino groups, which impedes the interaction of the polycation with the bacterial cell wall.

In the next set of experiments, (quaternized) PDMAEMA was studied in terms of its bactericidal activity, that is, its ability to cause bacterial death. It was found that all polymers exerted a fast bactericidal effect; a 3 log decrease of the titer of *E. coli* was observed in the case of PDMAEMA, and a 5 log decrease was found in the case of PDMAEMAQ50 and PDMAEMAQ100 within 5 min. After 20 min of incubation, the titer of *E. coli* in all cases was equal to zero, an indication of the absence of living cells.

To evaluate the effect of complex formation on the antibacterial activity of DMAEMA-based polymers, microbiological studies on cross-linked CECh/(quaternized) PDMAEMA complexes were carried out. The concentrations of PDMAEMA and quaternized PDMAEMA in the cross-linked CECh/(quaternized) PDMAEMA complexes were 10 times higher than the MIC values of the polymers. First and as expected, the results obtained for cross-linked CECh attest for the absence of antibacterial activity. Interestingly enough, the cross-linked CECh/PDMAEMA complex caused complete inhibition of the growth of *E. coli*. The cross-linked CECh/PDMAEMAQ50 complex caused a slight inhibition of bacterial growth, leading to a 1 log decrease of the titer of *E. coli*. In the case of the cross-linked CECh/PDMAEMAQ100 complex, no inhibition of the bacterial growth was observed. The antibacterial activity of the cross-linked CECh/PDMAEMA complex reveals the presence of free protonated tertiary amino groups, which are able to interact electrostatically with the negatively charged moieties of the cell walls of *E. coli*. In general, the complex formation between CECh and quaternized PDMAEMA leads to a decrease of the antibacterial activity of the latter. This may be attributed to engagement of the positive charges of PDMAEMA in the complex formation with CECh. It should be noted that the performance of the antibacterial test in distilled water (pH 5) resulted in the exhibition of significant antibacterial activity in the case of the cross-linked CECh/(quaternized) PDMAEMA complex. This is explained by disintegration of the complexes at this pH value, followed by release of free DMAEMA-based polymers into the liquid medium. These materials are good examples for release of bioactive polymers depending on the medium pH value.

Conclusions

In conclusion, novel polyelectrolyte complexes based on CECh and well-defined (quaternized) PDMAEMA have been prepared. The stoichiometry and stability of the complexes depend strongly on the medium pH value and on the QD of PDMAEMA. The evaluation of the antibacterial activity of the polymer partners and their complexes revealed that PEC formation leads to loss of the inherent antibacterial activity of (quaternized) PDMAEMA. However, at pH values lower than 6, due to disintegration of the complexes these materials exhibit antibacterial activity. This is an indication of the possible use of these materials as pro-drugs exhibiting a pH-sensitive behavior.

Acknowledgment. This work was partly supported by the Bulgarian National Fund for Scientific Research (Grant CH-1414). N.M. is grateful to the Belgian F.N.R.S. for financing her stay as an invited professor at the University of Mons-Hainaut. L.M. and P.D. acknowledge support from the Belgian Office of Science Policy (SSTC-PAI 5/3). L.M. thanks the Belgian F.N.R.S. for financial support.

References and Notes

(1) de la Torre, M. P.; Enobakhare, Y.; Torrado, G.; Torrado, S. *Biomaterials* **2003**, *24*, 1499−1506.

(2) Said, A. E. H. A. *Biomaterials* **2005**, *26*, 2733−2739.

(3) Wetering, P.; Cherng, J. Y.; Talsma, H.; Crommelin, D. J.; Hennink, W. E. *J. Controlled Release* **1998**, *53*, 145−153.

(4) Wolfert, M. A.; Dash, P. R.; Nazarova, O.; Oupicky D.; Seymour, L. W.; Smart, S.; Strohalm, J.; Ulbrich, K. *Bioconjugate Chem.* **1999**, *10*, 993−1004.

(5) Pirotton, S.; Muller, C.; Pantoustier, N.; Botteman, F.; Collinet, S.; Grandfils, C.; Dandrifosse, G.; Degée, Ph.; Dubois, Ph.; Raes, M. *Pharm. Res.* **2004**, *21*, 1471−1479.

(6) Liu, W.; Sun, S.; Cao, Z.; Zhang, X.; Yao, K.; Lu, W. W.; Luk, K. D. K. *Biomaterials* **2005**, *26*, 2705−2711.

(7) Mark, H. F.; Bikales, N. M.; Overberger, C. E.; Mengwes, G. *Encyclopedia of Polymer Science and Engineering*; Wiley-Interscience: New York, 1988; Vol. 11, pp 720−739.

(8) Haisch, A.; Gröger, A.; Radke, C.; Ebmeyer, J.; Sudhoff, H.; Grasnick, G.; Jahnke, V.; Burmester, G. R.; Sittinger, M. *Biomaterials* **2000**, *21*, 1561−1566.

(9) Kumar, M. N. V. *React. Funct. Polym.* **2000**, *46*, 1−27.

(10) Stoilova, O.; Koseva, N.; Manolova, M.; Rashkov, I. *Polym. Bull.* **1999**, *43*, 67−73.

(11) Paneva, D.; Stoilova, O.; Manolova, N.; Rashkov, I. *e-Polym.* **2003**, no. 033.

(12) Lee, K.; Park, W.; Ha, W. *J. Appl. Polym. Sci.* **1997**, *63*, 425−432.
(13) Mincheva, R.; Manolova, N.; Paneva, D.; Rashkov, I. *Eur. Polym. J.* **2006**, *42*, 858−868.
(14) Lenoir, S.; Pagnouelle, C.; Detrembleur, C.; Galleni, M.; Jérôme, R. *e-Polym.* **2005**, no. 074.
(15) Paneva, D.; Mespouille, L.; Manolova, N.; Degée, Ph.; Rashkov, I.; Dubois, Ph. *J. Polym. Sci., Part A. Polym. Chem.* **2006**, *44*, 5468−5479.
(16) Bütün, V.; Armes, S. P.; Billingham, N. C. *Macromolecules* **2001**, *34*, 1148−1159.
(17) Bütün, V.; Armes, S. P.; Billingham, N. C. *Polymer* **2001**, *42*, 5993−6008.
(18) Ydens, I.; Moins, S.; Botteman, F.; Degée, Ph.; Dubois, Ph. *e-Polym.* **2004**, no. 039.
(19) Mespouille, L.; Degée, Ph.; Dubois, Ph. *Eur. Polym. J.* **2005**, *41*, 1187−1195.
(20) Didukh, A.; Koizhaiganova, R.; Khamitzhanova, G.; Bimendina, L.; Kudaibergenov, S. *Polym. Int.* **2003**, *52*, 883−891.
(21) Sashiwa, H.; Yamamori, N.; Ichinose, Y.; Sunamoto, J.; Aiba, S. *Macromol. Biosci.* **2003**, *3*, 231−233.
(22) Dautzenberg, H.; Jaeger, W.; Philipp, B.; Kötz, J.; Seidel, Ch.; Stsherbina, D. *Polyelectrolytes: Formation, Characterization and Application*; Carl Hanser Verlag: Munich, 1994.
(23) Dobrynin, A. V.; Colby, R. H.; Rubinstein, M. *J. Polym. Sci., Part B: Polym. Phys.* **2004**, *42*, 3531−3538.
(24) Georgiev, G. S.; Kamenska, E. B.; Vassileva, E. D.; Kamenova, I. P.; Georgieva, V. T.; Iliev, S. B.; Ivanov, I. A. *Biomacromolecules* **2006**, *7*, 1329−1334.
(25) Kudaibergenov, E. S. *Adv. Polym. Sci.* **1999**, *144*, 115−197.
(26) Ydens, I.; Sébastien, M.; Degée, Ph.; Dubois, Ph. *Eur. Polym. J.* **2005**, *41*, 1502−1509.
(27) Chavasit, V.; Kienzle-Sterzer, C.; Antonio Torres, J. *Polym. Bull.* **1998**, *19*, 223−230.
(28) Mincheva, R.; Manolova, N.; Paneva, D.; Rashkov, I. *J. Bioact. Compat. Polym.* **2005**, *20*, 419−435.
(29) Jayakrishnan, A.; Jameela, S. R. *Biomaterials* **1996**, *17*, 471−484.
(30) Koseva, N.; Stoilova, O.; Manolova, N.; Rashkov, I.; Madec, P. J. *J. Bioact. Compat. Polym.* **2001**, *16*, 3−19.
(31) Liu, X.; Guan, Y.; Yang, D.; Li, Z.; Yao, K. *J. Appl. Polym. Sci.* **2001**, *79*, 1324−1335.
(32) Chung, Y.; Su, Y.; Chen, C.; Jia, G.; Wang, H.; Wu, J.; Lin, J. *Acta Pharmacol. Sin.* **2004**, *25*, 932−936.

BM061029J