Block Polyelectrolyte Networks from Poly(acrylic acid) and Poly(ethylene oxide): Sorption and Release of Cytochrome C

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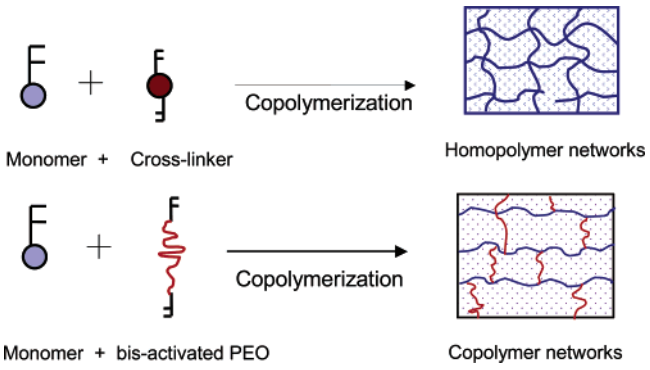
A new family of block polyelectrolyte networks containing cross-linked poly(acrylic acid) (PAA) and poly(ethylene oxide) (PEO) was synthesized by copolymerization of acrylic acid and bisacrylated PEO (10 kDa). Two materials with different PEO/PAA ratios were compared with a weakly cross-linked PAA homopolymer network. The networks bound a cationic protein, cytochrome C, due to the polyion coupling, leading to the network contraction. After binding the protein the block polyelectrolyte networks were more porous compared to a homopolymer network, facilitating protein absorption within the gel. The protein was released by adding Ca^{2+} ions or a polycation. Ca^{2+} ions migrated within the gels and reacted with PAA chains, thus displacing the protein. The polycation transfer into hydrogels, as a result of polyion substitution reactions, was inhibited by the excess of PEO chains in the block polyelectrolyte networks. Overall, these findings advance development of functional polyelectrolyte networks for immobilization and controlled release of proteins.

Introduction

Hydrogels formed by cross-linked polymer networks have gained wide attention in many areas including tissue engineering and drug delivery.[1−5] They are biocompatible, have high water content, exhibit rubbery behavior, respond to changes in environmental conditions, and can protect biologically active agents from hostile environments. The swelling behavior of hydrogels depends on the polymer composition and configuration, density and homogeneity of cross-links, quality of the solvent, and other factors. Polyelectrolyte networks (PNs) contain charges confined within the hydrogel. An excess of elementary counterions accumulated in such hydrogels produces an osmotic pressure and enhances the swelling. Similarly to linear polyelectrolytes, PNs form highly cooperative electrostatic complexes with oppositely charged molecules, such as surfactants, synthetic polyions, DNA, and polypeptides. These reactions are driven by a release of condensed counterions from the gel in the external media. Formation of such complexes induces a sharp conformational transition of the network and results in a collapse of the gel.[6]

The ability of the PN to absorb and release biomacromolecules of opposite charge is important for drug delivery. PNs were shown to bind very significant amounts (20−30 wt %) of biopolymers.[7−10] Polypeptides and other biopolymers immobilized in PN are protected from hostile environments, such as proteolytic enzymes and low pH (e.g., in the stomach).[11] For example, Lowman and Peppas immobilized insulin in microgels of cross-linked poly(methacrylic acid) (PMA) grafted with poly(ethylene oxide) (PEO).[12] Such immobilized insulin successfully enhanced the hypoglycemic effects upon oral administration. Our group developed nanogels from cross-linked polyethylenimine (PEI) and PEO.[10,13] Interactions of PEI chains with oppositely charged molecules led to the condensation of nanogels, which formed stable dispersions due to the effects of nonionic PEO chains. Nanogels were shown to deliver immobilized nucleotides across cellular barriers and protect them from degradation by the metabolic systems within the cells.[10,14,15]

Scheme 1. Synthesis of Cross-Linked Hydrogels by Free Radical Reactions Using (1) Short Cross-Linker, MBAAm, and (2) Bisactivated PEO[a]



[a] Each hydrogel represents homopolymer networks and copolymer networks, respectively.

This work explores a new family of cross-linked PN materials called block polyelectrolyte networks (BPNs). These materials contain cross-linked poly(acrylic acid) (PAA) and PEO chains (PEO-*cl*-PAA) and were synthesized by copolymerization of acrylic acid and a high molecular weight cross-linker, a telechelic bisacrylated PEO (Scheme 1). The long PEO chains serve as spacers separating the polyelectrolyte (PAA) chains in the gel. As a result of double functionality, BPNs exhibit combined properties of a PN and a hydrophilic nonionic network. The properties of these networks can be varied in a very broad range by changing the length of PEO, the PAA/PEO ratio, and the density of the cross-links. BPNs are compared with a

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conventional homopolymer PN, a weakly cross-linked PAA (*cl*-PAA) synthesized by radical copolymerization of acrylic acid and a low molecular weight cross-linker, *N*,*N*′-methylenebisacrylamide (Scheme 1). While both networks display similar swelling and deswelling behaviors in the presence of elementary salts and changes of pH, there is a considerable difference in the reactions of these networks with a cationic protein, cytochrome C, as well as release of this protein in the external media. Overall, by introducing nonionic polymer cross-links in the BPNs one can expand the range of biomaterials available for immobilization of biopolymers and the extent to which the properties of these materials can be fine-tuned for different biomedical applications.

Experimental Section

Materials. The acrylic acid (AA) monomer and acryloyl chloride (AcCl) were purified by vacuum distillation. *N*,*N*′-Methylenebisacrylamide (MBAAm), ammonium persulfate, and sodium metabisulfite were used as received. Bisacrylate-terminated PEO (10 kDa) was synthesized from commercial polymers (Aldrich, St. Louis, MO) using acryloyl chloride as described previously.[16] The reaction was carried out in dichloromethane and triethylamine in the dark at room temperature overnight. The modified PEO was precipitated from toluene by addition of diethyl ether in an ice bath, recovered by filtration, washed with diethyl ether, and dried in vacuo. The structures of the products were confirmed by NMR spectroscopy at the University of Nebraska Medical Centerr NMR facility. Cytochrome C from horse heart (M_w 12 400 g/mol) was purchased from Aldrich. Poly(*N*-ethyl-4-vinylpyridinium bromide) (PEVP) was prepared as described previously.[17−20] The degree of polymerization of PEVP was 55. Other reagents were used as received.

Synthesis of PNs. *cl*-PAA and PEO-*cl*-PAA were synthesized by a free radical polymerization using the redox initiator system comprising sodium metabisulfite and ammonium persulfate. For the synthesis of *cl*-PAA, 10% (w/v) AA was polymerized in aqueous solutions (distilled water) in the presence of MBAAm at a molar ratio MBAAm/AA of 1:100. For the synthesis of PEO-*cl*-PAA, AA was mixed with bisacrylate-PEO at the molar ratios of 320:1 and 80:1, respectively. The concentrations of AA in the reaction mixtures were 30% (320:1) or 10% (80:1). Henceforth, PEO-*cl*-PAA gels with different cross-link densities are referred to as PEO-*cl*-PAA(320) and PEO-*cl*-PAA(80), respectively. In each case the amount of initiator added was 0.24 wt % with respect to the amount of the AA monomer used. The gels were synthesized in cylindrical glass tubes with an inner diameter of 11 mm at 40 °C for 24 h, then swollen in distilled water with 10^{-4} M NaOH for 48 h, and then washed repeatedly, at least every couple of days, with a large amount of distilled water for at least 8 weeks.

Swelling Studies. Wet gels, which were swollen in distilled water, were cut into small pieces and gently dabbed with Kim Wipes to remove the excess of water. The weights of the swollen gel and dry gels, obtained by lyophilization, were measured. The swelling ratio (Q) was determined as follows $Q = (W_s - W_d)/W_d$, where W_s and W_d are the weights of the swollen and dry gels, respectively. The swelling ratio in the distilled water in the absence of added salts is referred to as Q_0. The amounts of the gels used in subsequent studies were in the range of 0.5−1.2 g as determined for the swollen states.

Determination of the Concentrations of COOH Groups. The concentrations of the carboxylic groups in the PNs were determined by potentiometric titration. Direct measurement by a common titration of the PN was complicated by slow relaxation of the gels. Therefore, the indirect approach (inverse titration) was used. In brief, the carboxylic groups of PN were neutralized with a known excess of sodium hydroxide (V_0C_{NaOH}), and then the system was titrated with hydrochloric acid to neutralize the excess of sodium hydroxide. Therefore, the concentration of the carboxylic groups was obtained as the difference between the initial amount of sodium hydroxide and the amount of added hydrochloride ($V_{HCl}C_{HCl}$)

$$[\mathrm{COOH}] = V_0C_{\mathrm{NaOH}} - V_{\mathrm{HCl}}C_{\mathrm{HCl}}$$

Studies of the pH Effect. The gels swollen in distilled water were first neutralized with an excess of sodium hydroxide (ca. 1.5 equiv per the amount of the carboxylic acids of the gel) and then incubated in an aqueous solution of sodium hydroxide, pH 10 for 48 h. The pH was then changed by adding small amounts of HCl. At each point after the addition of HCl the gels were equilibrated for 48 h. The results were analyzed as the swelling ratio (Q) and the relative swelling (Q/Q_0) as a function of the pH, where Q_0 is the swelling ratio in distilled water and Q is the swelling ratio at a given pH.

Effects of Elementary Salts on the Gel Swelling. The known amounts of gels swollen in distilled water were immersed in the glass vial with 10 mL of distilled water. NaCl, $CaCl_2$, and $MgCl_2$ were introduced in these systems by adding small amounts of 1 M (NaCl) or 0.1 M ($CaCl_2$, $MgCl_2$) stock solutions of these salts. At each point after the addition of the salt the PNs were equilibrated for 48 h. The swelling ratio (Q) and relative swelling (Q/Q_0) as a function of the concentration of added salts were determined as described above.

Sorption and Release of Cytochrome C. The study used cytochrome C from horse heart muscle (Aldrich), M_w 12 400 g/mol (sphere d = 30 Å), pI = 10.3. It is known that a molecule of cytochrome C contains 13 carboxyl groups and 23 amino groups .[21] The PNs were immersed in 10 mL of distilled water, pH 5.8, or NaOH solutions, pH 9.5, and supplemented with cytochrome C. Two different molar ratios of total cytochrome C amino groups and PN carboxylic groups were used, $z = 0.25$ and $z = 1$. The initial concentrations of cytochrome C were varied from 0.15 to 0.45 mg/mL (from 1.2×10^{-5} to 3.6×10^{-5} M). The concentration of cytochrome C in the external solution was determined at different time points using a Lambda 25 UV/vis spectrophotometer (Perkin-Elmer) at 409 nm (extinction coefficient $\epsilon_{409} = 110\,000\ \mathrm{M^{-1}\ cm^{-1}}$). For the release studies the protein-loaded gels were placed in 10 mL of protein-free solutions and supplemented with NaCl, $CaCl_2$, or PEVP or in 10 mL of phosphate-buffered saline (PBS, pH 7.4). The conversion in sorption was determined as follows

$$F = (C_0 - C)/C_0$$

where the C_0 is the initial concentration of the added protein and C is the concentration of protein remaining (sorption study) or released (release study) in the external solution at each time point.

Results and Discussion

Characteristics of PNs. As shown in Figure 1a the PNs of *cl*-PAA and PEO-*cl*-PAA(320) were transparent, while that of PEO-*cl*-PAA(80) was opaque. PEO-*cl*-PAA(80) had a higher content of PEO (PEO/PAA ratio) and a higher degree of cross-links in comparison to PEO-*cl*-PAA(320), and its opacity was indicative of some structural heterogeneity of the material. After the synthesis, PNs were washed and immersed in a large amount of distilled water for 8 weeks to achieve equilibrium swelling (Figure 1b). The swelling ratios determined by comparing the swollen and lyophilized PNs differed significantly for different materials. The swelling characterization was conducted at environmental pH 5.8, i.e., when the gels were partially neutralized. The swelling ratios, Q_0, were ca. 400 for PEO-*cl*-PAA(80), ca. 700 for PEO-*cl*-PAA(320), and ca. 1200 for *cl*-PAA (Table 1). The weight fraction of polyelectrolyte, i.e., PAA, in these materials increased in the same order. Furthermore, the density of the cross-links was lower in PEO-*cl*-PAA(320) than that in PEO-*cl*-PAA(80). Therefore, the observed differences in swelling were consistent with higher osmotic swelling

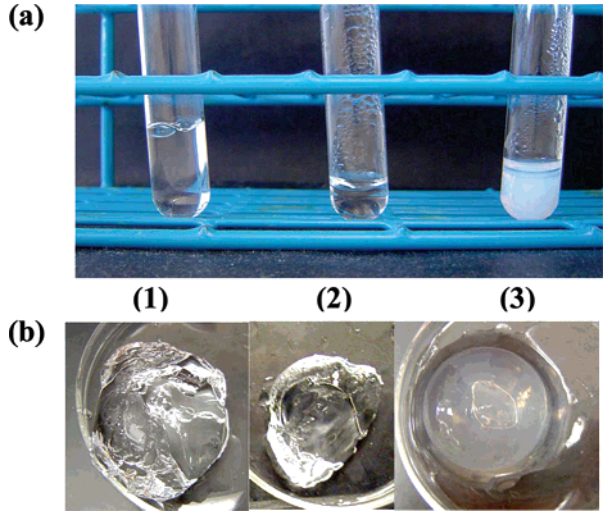

Figure 1. PNs (a) after polymerization in a test tube and (b) after swelling in distilled water, pH 5.8: (1) *cl*-PAA; (2) PEO-*cl*-PAA(320); (3) PEO-*cl*-PAA(80).

due to the effect of the counterions (for networks with higher PAA content) and lower steric hindrance to swelling (for networks with fewer cross-links). It is interesting to note that independent of the swelling ratios the concentrations of the carboxylic groups in equilibrium swollen hydrogels were similar for each of the three materials and varied from ca. 33 mM for PEO-*cl*-PAA(80) to ca. 41 mM PEO-*cl*-PAA(320) (Table 1). We also would like to point out that the PEO-*cl*-PAA(320) hydrogel was softer and easier to break in comparison to the two other hydrogels.

Effects of pH on the Swelling of PNs. Swelling the PN, Q as a function of pH at 25 °C is shown in Figure 2. It is apparent from the graph that all gels were completely collapsed below ca. pH 4, when the PAA was not charged. As pH increased the PNs progressively swelled due to the increasing amount of ionized carboxylic groups. At ca. pH 8.0 and above the swelling reached a plateau since the carboxylic groups of the PNs were completely deprotonated. Consistent with the previous result at any pH above 4.0 the swelling decreased in the following order: *cl*-PAA, PEO-*cl*-PAA(320), and PEO-*cl*-PAA(80) (Figure 2a). The relative swelling, Q/Q_0, normalized for each gel by the swelling ratio in the distilled water, pH 5.8, is also shown as a function of pH (Figure 2b). The fact that these curves were virtually identical for the homopolymer PNs and BPNs suggested that the ionization behavior of PAA chains was not affected by the presence of PEO chains in these materials. The only notable difference between these gels was that the PEO-*cl*-PAA(1:80) gel changed opacity as the pH was changed; it was opaque at pH 4−5 and transparent at higher pH. The PAA and PEO-*cl*-PAA(320) PNs did not reveal such behavior. It is possible that at lower pH the PEO chains of the PEO-*cl*-PAA(80) PN (having a high content of PEO) formed a complex with PAA resulting in a loss of transparency. The H-complexes stabilized by hydrogen bonds between the oxygen of the ethylene oxide units and the carboxylic groups of a polyacid

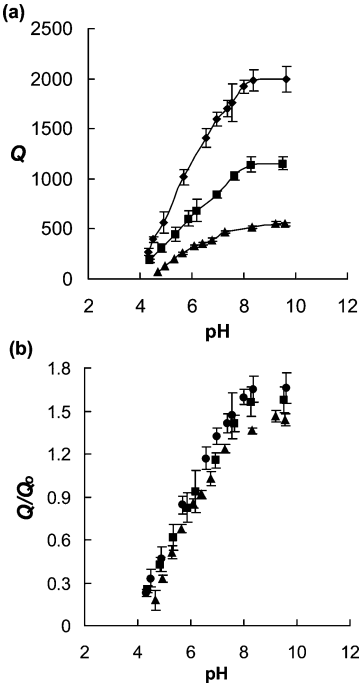

Figure 2. (a) Swelling ratio (Q) and (b) relative swelling (Q/Q_0) of PNs as a function of pH of the surrounding solution: (●) *cl*-PAA; (■) PEO-*cl*-PAA(320); (▲) PEO-*cl*-PAA(80). The data are mean ± standard error of the mean, $n = 3$.

are well-known for the homopolymer and block copolymer systems and also were discussed for PNs of PAA grafted by PEO.[22−25]

Effects of Elementary Salts on Swelling of PNs. Figure 3a presents the relative swelling of the PNs as a function of a concentration of the added NaCl at pH 5.8. (The absolute data for swelling are presented in the Supporting Information.) In all cases considerable contraction of the hydrogels was observed as the concentration of the monovalent salt reached several millimolar, i.e., was comparable with the concentration of the charged groups within the gels. On the basis of the Donnan theory this behavior can be explained by a decrease of the elementary salt contribution to the osmotic swelling upon an increase of the salt concentration.[26−28] Notably, different PNs exhibited different deswelling behaviors, which may be due to the structural differences in the homopolymer PNs and BPNs. As is seen in the figure, the *cl*-PAA PN contracted less than the PEO-*cl*-PAA(320) PN. In the distilled water the average concentrations of the carboxylic groups in these hydrogels were close to each other (Table 1), which suggests that the contribution of the counterions to the swelling of these networks was similar. However, *cl*-PAA was likely to have a greater contribution from local interchain electrostatic repulsion to swelling, since the PAA chains connected by low molecular mass cross-links were located close to each other. This could explain the lower deswelling of *cl*-PAA compared to that of PEO-*cl*-PAA(320), in which the PAA chains were separated by relatively long PEO cross-links and, hence, the local interchain repulsion

Table 1. Characteristics of Synthesized PNs[a]

	cross-linker	cross-linker/monomer	COO^- per 1 g swollen gels (mM) ($n = 3$)	swelling ratio in distilled water (Q_0)
cl-PAA	MBAAm	1:100	38 ± 4	1200 ± 110
PEO-*cl*-PAA(320)	PEO (10 000)	1:320	41 ± 4	730 ± 80
PEO-*cl*-PAA(80)	PEO (10 000)	1:80	33 ± 2	380 ± 10

[a] Each value represents as mean ± standard deviation, $n = 3$.

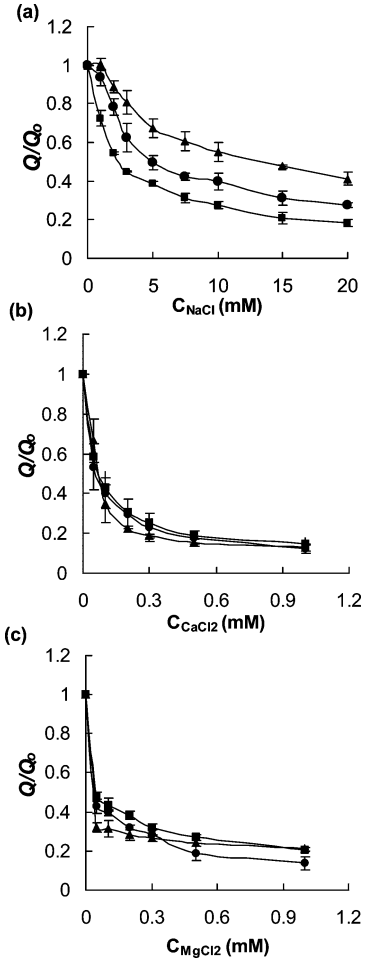

Figure 3. Effects of (a) NaCl, (b) $CaCl_2$, and $MgCl_2$ (c) on the relative swelling of PNs as a function of the concentration of added NaCl: (●) *cl*-PAA; (■) PEO-*cl*-PAA(320); (▲) PEO-*cl*-PAA(80). The data are mean ± standard error of the mean, $n = 3$. The equilibrium swelling ratios for these hydrogels in distilled water, Q_0 are presented in Table 1.

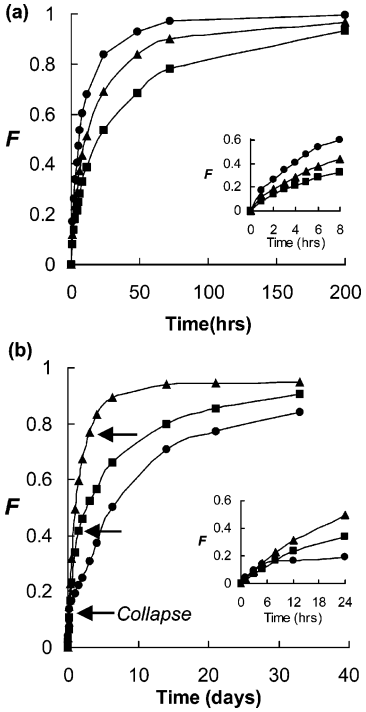

Figure 4. Kinetics of the sorption of cytochrome C in PNs at (a) $z = 0.25$ and (b) $z = 1$ and pH 5.8 by different hydrogels: (●) *cl*-PAA; (■) PEO-*cl*-PAA(320); (▲) PEO-*cl*-PAA(80). The insets present the data for shorter time scales. The horizontal arrows indicate the points at which hydrogels reached a collapsed state and subsequent protein sorption did not result in further gel contraction.

was less. As a result, the homopolymer PNs that swelled more in water also contracted less in the presence of the added salt. At the same time, the PEO-*cl*-PAA(80), which had the lowest swelling ratio of the three hydrogels, exhibited the lowest contraction in the presence of added NaCl. Notably, this network had a higher portion of PEO in comparison to PEO-*cl*-PAA-(320). Therefore, the mixing entropy of the nonionic PEO chains may have a greater contribution to the swelling of PEO-*cl*-PAA-(80) in comparison to that of PEO-*cl*-PAA(320) (and, of course, *cl*-PAA, which did not have PEO). As a result, the PEO-*cl*-PAA(80) hydrogel was less sensitive to the added monovalent salt in comparison to the two other materials due to the swelling contribution of the hydrophilic nonionic chains.

The effects of the salts of the divalent cations, $CaCl_2$ and $MgCl_2$, on the PN contraction were much more pronounced and observed at 10−100 times lower concentrations of the salt than the effects of NaCl (Figure 3). The divalent cations (Mg^{2+}, Ca^{2+}) are known to strongly ("specifically") interact with the carboxylic groups of the PAA, resulting in hydrogel collapse.[26] Consistent with the previous report, the cations of different sizes, Ca^{2+} and Mg^{2+}, produced virtually the same effects on the hydrogels.[26] Notably, the extent of the contraction was almost the same for all three hydrogel materials. It is likely that the binding of the divalent cations with the PAA chains induced strong interchain interactions resulting in a complete collapse of the hydrogels independently of the molecular architecture of the PN. As a result, the PEO chains in BPNs could not prevent the shrinking of the hydrogel induced by the divalent cations.

Sorption of a Protein. The kinetic profiles of sorption of cytochrome C by partially neutralized PNs (pH 5.8) are presented in Figure 4. The ratios of cytochrome C amino groups to the PN carboxylic groups were kept constant, $z = 0.25$ and $z = 1$. The data are presented as conversion in sorption, F, i.e., the amount of absorbed protein normalized by the added protein. Separately we demonstrated that for the duration of the experiment there was no loss of protein in the absence of the hydrogels. At a low content of added protein ($z = 0.25$) the sorption of cytochrome C in the hydrogels proceeded to completion within 6 days. The driving force for sorption was the neutralization of polyanion segments with the positively charged amino groups of the protein, resulting in the release of small counterions in the external media. As sorption proceeded, the red solution of cytochrome C became colorless, and simultaneously, the transparent PN turned red. In all cases the reaction proceeded from the surface to the interior of the gel (Figures 5a and 5b). This was evident by the appearance of the surface layer ("shell") of the colored collapsed gel, while the interior of the gel ("core") remained highly swollen and transparent. At $z = 0.25$ the rate of sorption decreased in the following order, *cl*-PAA, PEO-*cl*-PAA(80), PEO-*cl*-PAA(320) (Figure 4a), although these differences were relatively subtle and practically disappeared at pH 9.5 for a completely ionized gel (data presented in the Supporting Information). A strikingly different behavior was observed as the amount of the added protein was increased to $z = 1.0$. In this case the completion of the reaction was observed at different time points from approximately 7 days for PEO-*cl*-PAA(80) to approximately 30 days for *cl*-PAA. Furthermore, the block polyelectrolyte gel with the high content of PEO, PEO-*cl*-PAA(80), appeared to be

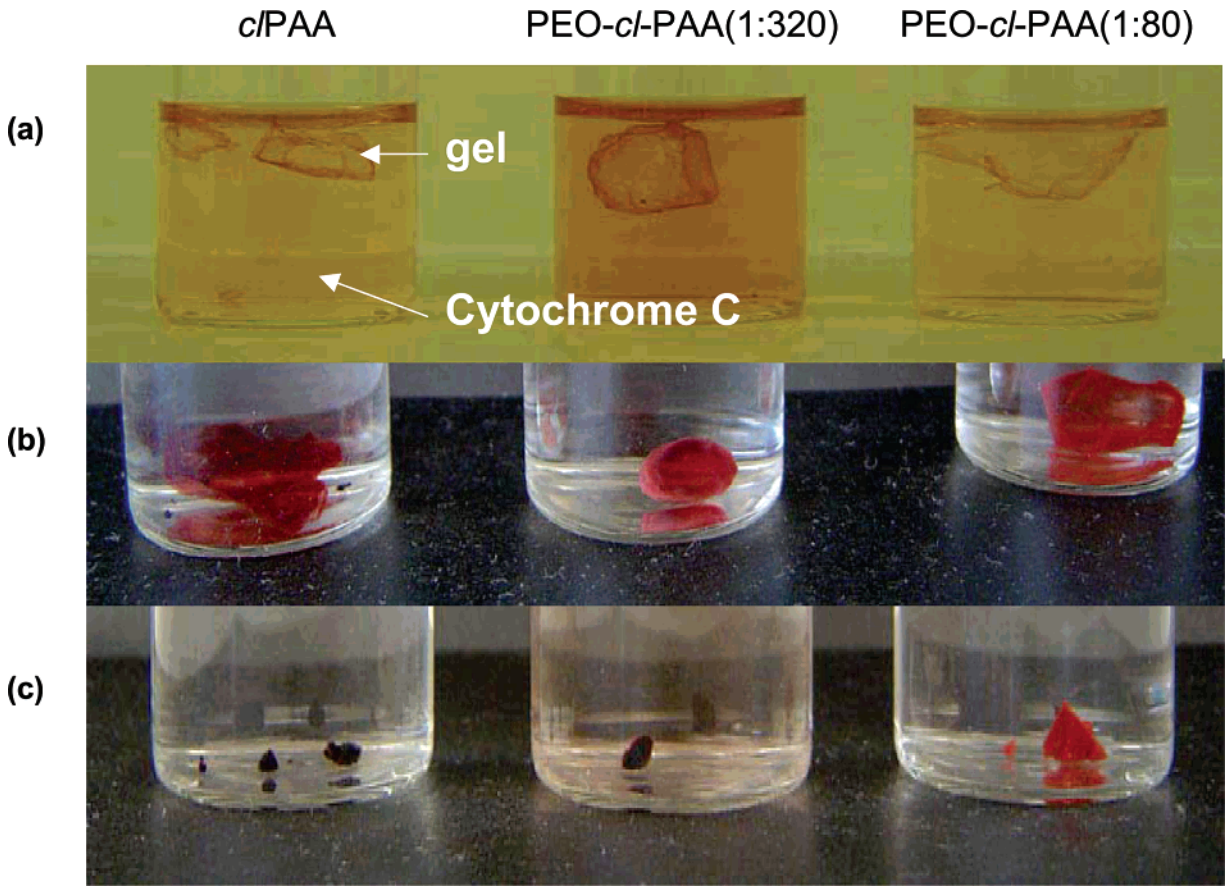

Figure 5. PNs immersed in cytochrome C solution (a) after 1 h, (b) after the completion of the reaction, 2 weeks, and (c) 2 days after addition of 1 mM $CaCl_2$ (1 mM) to the solution in part b. The conditions were as follows: $z = 0.25$; pH 5.8 (b and c) or pH 9.5 (a) (same behavior was observed at pH 5.8).

partially swollen (having a higher volume) at high conversions of sorption while the homopolymer gel and PEO-*cl*-PAA(320) were notably more shrunken even when sorption was far from completion (Figure 6). There was an obvious relationship between the ability of the gel to collapse and the rate of protein sorption. For example, as is seen in inserts in Figure 4b, the protein sorption in *cl*-PAA gel was strongly inhibited after several hours, when the collapse of this gel was observed. However, slow sorption continued and eventually all protein was immobilized in the gel. In contrast, the PEO-containing gels did not collapse, and the rates of sorption of the protein in them remained much higher. Eventually all of the gels reached a highly shrunken state although this happened at different conversions as shown by horizontal arrows in Figure 4b. Less shrinking of the PEO-*cl*-PAA(80) gel upon binding of the protein evidently was due to the lyophilizing effects of the PEO chains (and the higher degree of cross-linking compared to that of PEO-*cl*-PAA(320)) that counteracted the chain contraction. Furthermore, the shell formed in this case was likely to be more permeable and, perhaps, even porous, allowing for a more rapid transfer of the protein globules into the swollen core where the polyelectrolyte coupling reaction took place. It is possible that the PAA chains of the shell neutralized by cytochrome C segregated and formed dense clusters of the insoluble polyelectrolyte complex joined by swollen PEO chains. In contrast, materials with a lower content of PEO and/or less cross-linking density were likely to form a less porous shell, which was impermeable for the protein, resulting in a slower sorption.

Studies by V. A. Kabanov and Zezin have shown that the reactions of the slightly cross-linked PNs with the oppositely charged species begin at the surface of the gel and proceed as a propagating frontal polyion exchange reaction involving layer-by-layer displacement of the complex formed from the periphery to the center of the gel specimen.[7−9] In this case, as previously shown for the slightly cross-linked PN of PAA and oppositely charged proteins,[29,30] the polyion exchange reaction proceeds at the core−shell interface. This results in the formation of the vacancies (i.e., non-neutralized segments of PAA), which then migrate through the shell backward and react with the new supply of the protein in the external solution. As a result, the area of the collapsed gel spreads from the surface of the network into its center, following the front of the reaction. Using a previously published kinetic analysis,[29] this mechanism ("relay race") was confirmed in all cases reported here for the initial 4−8 h of the sorption process. In these cases the kinetic curves linearized according to equation $\ln[1 - F] = -3/2K_s t$, where K_s is the first-order rate constant and t is time (Supporting Information).[29] However, in all cases over longer periods of time there were striking deviations from linearity, consistent with the slowdown of the sorption following the collapse. Notably, the area of linearity corresponding to the relay-race mechanism was generally much more extended for the PEO-containing PNs in comparison to the homopolymer PNs. For all PN types at $z = 0.25$ the K_s values calculated in the areas of linearity were in the range from ca. 1×10^{-5} to 2.3×10^{-5} s^{-1}, which were close to the value previously reported for cytochrome C sorption in *cl*-PAA gels (5×10^{-5} s^{-1}).[29] However, at $z = 1$ these values were almost an order of magnitude less, ranging from ca. 4.2×10^{-6} to 5.9×10^{-6} s^{-1} (Supporting Information). These differences might be related to the differences in the extents of surface contraction since at the same degrees of conversion in sorption there is 4 times more

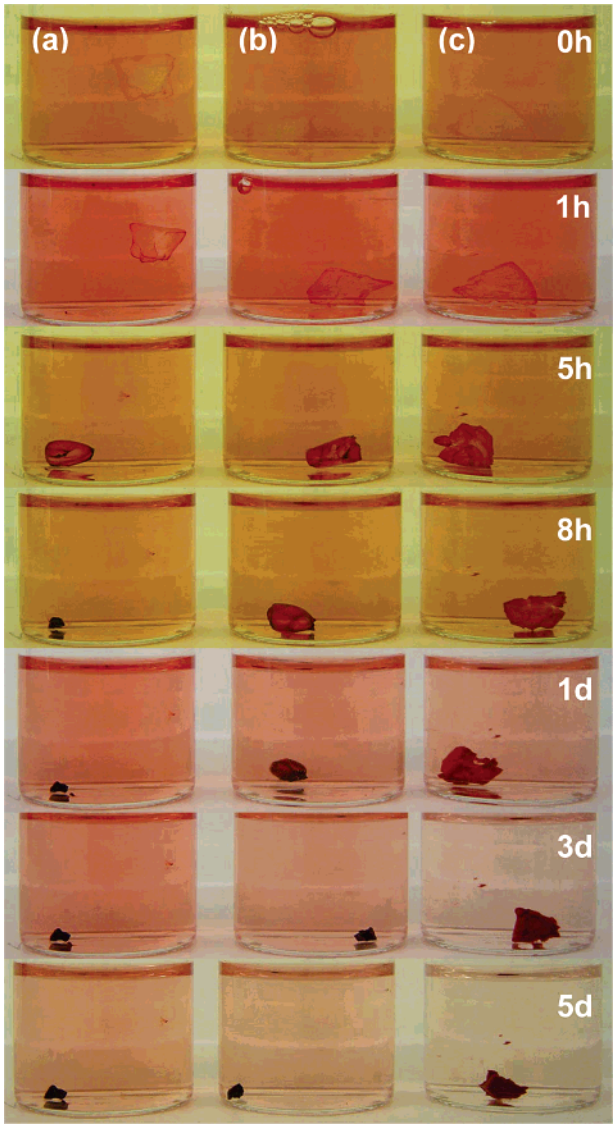

Figure 6. PNs immersed in the cytochrome C solution at $z = 1$, pH 5.8 at different time points: (a) *cl*-PAA; (b) PEO-*cl*-PAA(320); (c) PEO-*cl*-PAA(80).

protein bound to the gel at $z = 1$ in comparison to $z = 0.25$. At both compositions of the mixture, $z = 0.25$ and $z = 1$, the kinetic curves were not linearized in the coordinates of Fick's Law, F vs $t^{1/2}$, suggesting that diffusion was not the rate-limiting step.

Release of a Protein. Once the gels were loaded with cytochrome C they did not release the protein to the external media (distilled water) for at least 10 days. Furthermore, addition of relatively low concentrations of NaCl ($C_{Na^+}/C_{COOH/COO^-} \approx 1$) practically did not induce the protein release (Figure 7a). However, the protein release was induced by placing a gel in PBS ($C_{Na^+}/C_{COOH/COO^-} \gg 1$) (Figure 7b), adding 1 mM $CaCl_2$ ($C_{Ca^{2+}}/C_{COOH/COO^-} \approx 1$) (Figure 7c), or adding a polycation, 0.2 mM PEVP ($C_{PEVP}/C_{COOH/COO^-} = 0.2$) (Figure 7d). Obviously, addition of each of these components resulted in disintegration of the cytochrome C/PAA complex within the hydrogels. However, the mechanisms of disintegration were clearly different. The effect of PBS was due to the destabilization of the polyion complex in the presence of a relatively high concentration of salts (145 mM NaCl, 7 mM Na_2HPO_4, 3 mM NaH_2PO_4). Divalent Ca^{2+} ions migrated into the hydrogel and displaced the protein molecules bound to the PAA chains, resulting in the further hydrogel collapse. The PEVP chains also displaced cytochrome C in the complex with PAA, but the mechanism of

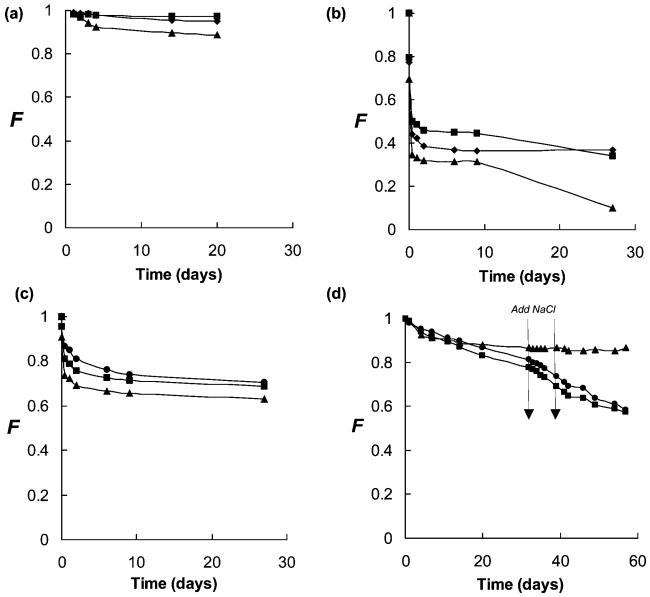

Figure 7. Release of cytochrome C from (●) *cl*-PAA, (■) PEO-*cl*-PAA(320), and (▲) PEO-*cl*-PAA(80) PNs loaded with protein at $z = 1$: (a) NaCl (2 mM); (b) PBS; (c) $CaCl_2$ (1 mM); (d) PEVP (0.2 mM, counting per repeating unit). In part d the vertical arrows indicate the points of addition of NaCl, and each data point between the arrows represents the concentrations of NaCl of 2, 4, 6, 10, and 15 mM, increasing from the left to the right arrows, respectively.

the reaction was different and involved a polyion substitution reaction that was initiated at the surface of the hydrogel and propagated along with the transfer of the PEVP chains inside the hydrogel. This difference was highlighted by a striking effect of the elementary salt on the rate of the protein release induced by PEVP (Figure 7d). In the absence of the added salt the release was very slow, but it was greatly accelerated by adding a small concentration of NaCl (which alone did not induce the cytochrome C release, data not shown). This effect was consistent with the increase in the rate of the polyion substitution reaction in the presence of NaCl.[31] Interestingly, the salt did not enhance the rate of the release of cytochrome C from PEO-*cl*-PAA(80) induced by PEVP (Figure 7d). This suggests that PEO chains in this BPN material impeded the polyion substitution reaction and the transfer of the PEVP chains inside the network. In this case only a small amount of protein was released, perhaps exchanged in the surface area of the network, and the exchange reaction did not proceed after that. Notably, there was no difference in the release of the protein in different networks induced by Ca^{2+} ions, suggesting that PEO chains did not impede the penetration of the small ions within the protein-loaded networks (Figure 7c).

A remarkable difference in the behavior of PNs of different architectures was observed in the case of the partially loaded gels at $z = 0.25$. After incubation of these gels with cytochrome C for 2 weeks (Figure 5b) the gels were treated with 1 mM $CaCl_2$. In all cases this induced further contraction of the gels (Figure 5c), suggesting that Ca^{2+} ions reacted with the carboxylic groups of the PAA chains. Surprisingly the release of the cytochrome C in the external solution was observed only in the case of the block polyelectrolyte gel with a low content of PEO, PEO-*cl*-PAA(320), while the two other gels, *cl*-PAA and PEO-*cl*-PAA(80), contracted without releasing the protein. As shown in Figure 8 subsequent addition of $CaCl_2$ resulted in further release of the protein in the case of PEO-*cl*-PAA(320), while with two other materials no release was observed for at least 30 days. This can only be explained by different distribu-

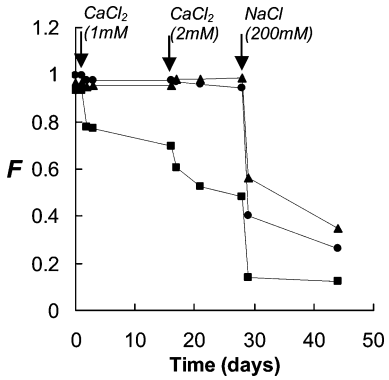

Figure 8. Release of cytochrome C from the PN/protein complex ($z = 0.25$) by addition of $CaCl_2$ and NaCl: (●) *cl*-PAA; (■) PEO-*cl*-PAA(320); (▲) PEO-*cl*-PAA(80). The arrows indicate the time points of addition of $CaCl_2$ (1 mM and 2 mM) and NaCl (200 mM), respectively.

tions of the binding of Ca^{2+} ions to PAA within the partially loaded PNs of different architectures. Evidently in *cl*-PAA and PEO-*cl*-PAA(80) Ca^{2+} ions reacted only with the free carboxylic groups of PNs and did not affect the carboxylic groups of PNs bound to the protein molecules. In this case Ca^{2+} did not displace cytochrome C, forming mixed (and possibly layered) complexes of the collapsed network that contained different areas of PAA neutralized by Ca^{2+} and the protein. In PEO-*cl*-PAA(320), Ca^{2+} displaced cytochrome C, resulting in the release of the latter in the external solution.

Such behavior may be explained in terms of the free energy at the core−shell interface of the partially loaded hydrogels. As was discussed above the PEO-*cl*-PAA(320) hydrogel was the most flexible of the three materials. As a result the interface between the contracted shell and the swollen core in this gel may have less excess of the free energy due to greater chain flexibility. In this case the displacement of the cytochrome C in the shell with Ca^{2+} ions is more favorable than contraction of the gel in the core. In the more "rigid" gels of *cl*-PAA and PEO-*cl*-PAA(80) the core−shell interface may have a higher excess of the free energy. Consequently, the reaction proceeds so that the interface area decreases and the core shrinks without the release of the protein bound in the shell. Obviously, this effect is more pronounced in the gels with a relatively low extent of loading with protein, which have a significant portion of non-neutralized PAA chains available for interaction with the Ca^{2+} ions.

The final aspect to be addressed in view of this study is why the protein release induced by Ca^{2+} ions appears to be incomplete. As is seen in Figure 7c for fully loaded hydrogels or in Figure 8 for partially loaded PEO-*cl*-PAA(320) hydrogels the kinetics of the release revealed a rapid phase followed by a "saturation" or a slow phase, after which practically no release was observed. Most likely, this behavior was due to the establishment of an equilibrium distribution of the Ca^{2+} ions bound to PAA and remaining in the external solution. As is shown in Figure 8 further addition of $CaCl_2$ to the hydrogel (PEO-*cl*-PAA(320)) resulted in additional rapid release of the protein followed by saturation (i.e., establishment of a new equilibrium). Notably, destruction of the PAA and cytochrome C complexes by adding 200 mM NaCl resulted in a rapid release of the protein in all three network materials.

Conclusion

This paper explores anionic BPNs of cross-linked PEO and PAA and characterizes the swelling behaviors of these networks and their reactions with low molecular mass counterions and cationic proteins. The divalent cations or cationic proteins bind to the polyanion segments of the network and induce its local contraction. The nonionic PEO chains of the BPNs hinder a complete collapse of the gel, which remains more swollen than the PAA homopolymer PNs. As a result, BPNs form partially swollen materials, in which the insoluble clusters of the polyion complex are joined by the hydrated PEO chains. These materials are relatively permeable, allowing for easier diffusion of the protein molecules within the gel and faster sorption of the protein from the external solution. The protein immobilized in such networks can be released by increasing ionic strength as well as displacement with Ca^{2+} ions or a polycation, PEVP, that binds to the PAA chains. The Ca^{2+} ions penetrate into the hydrogels by diffusion, resulting in protein displacement and release. The PEVP transfers within the hydrogels as a result of the polyion substitution reactions that initiate at the surface and proceed within the gels accompanied by the protein release. The PEO chains in BPNs with a high PEO/PAA ratio hinder the polyion substitution, resulting in abolition of the protein release induced by the polycation. The networks partially loaded with the protein represent layered structures with a collapsed shell containing a polyion complex and a swollen network core. Such materials formed from BPNs upon reacting with Ca^{2+} ions can either release protein from the shell area or absorb Ca^{2+} ions into the core depending of the ratio of the PEO and PAA chains. In both cases, the networks additionally collapse. This behavior was not observed for a slightly cross-linked homopolymer PAA. It was explained in terms of the differences in the excess free energy at the core−shell interface in the partially loaded BPNs. In more rigid gels the excess of the free energy at the interface is high. As a result Ca^{2+} ions bind with the chains in the core resulting in shrinking of the interface area. In more flexible gels the excess of the free energy at the interface is less, and Ca^{2+} ions displace the protein in the shell. These findings can open new possibilities for the control of the protein immobilization and release behavior, which can be used in different applications of polymer hydrogels, including controlled drug delivery.

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Supporting Information Available. Swelling profiles of PNs in solutions of NaCl, $CaCl_2$, and $MgCl_2$, kinetics of sorption of cytochrome C in PNs at $z = 0.25$ and pH 9.5, linearization of the kinetic data according to the equation $\ln[1 - F] = -3/2K_s t$ and in the coordinates of Fick's Law, and a table of rate constant values calculated from the kinetic data. This material is available free of charge via the Internet at http://pubs.acs.org.

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