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Research paper

Evaluation of pH-dependent membrane-disruptive properties of poly(acrylic acid) derived polymers

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Abstract

Anionic pH-sensitive membrane-disruptive polymers have evolved as a new class of bioactive excipients for the cytosolic delivery of therapeutic macromolecules. A large variety of anionic copolymers and analogues of poly(acrylic acid) (PA) was investigated and compared to a cationic PA copolymer. The pH-responsive membrane-disruptive properties were characterized by employing three in vitro models, such as pH dependent shift of pyrene fluorescence, liposome leakage and lysis of red blood cells. The pH-dependent increase of polarity and membrane disruption in the different model systems was in good agreement for all tested PA polymers. The efficacy of polymer-induced membrane disruption was concentration-dependent and significantly affected by the composition of the membrane. The sensitivity of relatively complex membranes of mammalian cells can be ranked between plain diphosphatidylcholine (DPPC) liposomal membranes and the more rigid cholesterol-containing DPPC membranes. Among the various studied PA polymers, medium and low molecular poly(ethacrylic acid) (PEA) and poly(propacrylic acid) (PPA) were identified as displaying significant pH-dependent disruptive activity. Relative to the disruptive cationic PA polymer (PDMAEM) the ranking is PEA < PPA < PDMAEM. The fine tuning of the pH-responsive hydrophilic–hydrophobic balance is likely to be responsible for the superior effect of PEA and PPA compared to other anionic PA polymers. This thorough investigation of a large variety of different anionic PA polymers and the comparison with an efficient, although rather toxic cationic PA polymer provides a good assessment for further therapeutic applications.

Keywords: Membrane disruption; pH-dependence; Poly(acrylic acid) derivative; Hemolysis; Liposome; Pyrene fluorescence

1. Introduction

Efficient trafficking to the correct intracellular compartment is a critical step for the efficacy of many protein and DNA therapeutics which need to be delivered intact to the cytosol or even to the nucleus of their target cells. In order to gain access to the cytosol, exogenously delivered DNA first needs to escape endosomal degradation before it can enter into further steps towards transcription to the encoded protein. Antisense oligonucleotides must reach distinct compartments inside the cell such as the cytosol and/or nucleus and interact with the target mRNA in order to exhibit an antisense effect. Furthermore, the delivery of exogenous antigens to the cytosol of professional antigen-presenting cells is mandatory for vaccine therapeutics against intracellular pathogens and cancer [1,2].

A plethora of exogenous material can be internalized by mammalian cells, either non-specifically (adsorptive pinocytosis, unspecific phagocytosis) or specifically via a targeting ligand (receptor-mediated endocytosis/phagocytosis). In either case, this material ends up in endosomes or phagosomes, which ultimately fuse with lysosomes [3–5]. Release of therapeutic compounds from these compartments into the cytoplasm is believed to be the rate limiting step for many intracellular applications. As a result, there have been substantial efforts focusing on the delivery of therapeutic compounds to the cytosol of target cells.

Fusogenic peptides structurally derived from viruses have been designed in order to specifically disrupt intracellular endosomal or lysosomal compartments [6]. Due to

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their pH-dependent shift in conformation, these peptides display membrane-disruptive activity only at endosomal or lysosomal pH of about 5.0–6.5, but not at physiological pH. However, immunogenicity has been regarded as a major problem facing routine use of viral vectors [7] and it is likely that fusogenic peptides derived from viral vectors are also immunogenic in vivo.

Totally synthetic so-called pH-sensitive polymers have been proposed to circumvent these problems. A high density of protonable amino nitrogen atoms in cationic polymers makes them an effective 'proton sponge' which causes lysosomal swelling and rupture, and provides subsequent escape for molecules entrapped in endosomes or lysosomes [8–12]. However, many cationic compounds have been associated with major cytotoxicity, which is particularly pronounced when it comes to high intracellular accumulation which is typical for phagocytic cells [13–19].

A new class of anionic pH-sensitive polymers with potentially lower cytotoxicity is based on α-alkyl acrylic acids. Poly(ethacrylic acid) and poly(propacrylic acid) have been demonstrated to display pH-dependent membrane-disruptive properties in liposomes and red blood cells dependent on their molecular weight [20–22]. Moreover, a copolymer composed of ethyl acrylate and acrylic acid has been suggested to display a significant pH-dependent membrane-disruptive effect on red blood cells [20–22].

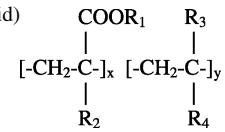
In the present study, we screened a large variety of structurally different anionic polymers and copolymers and analogues of poly(acrylic acid) (PA) by employing three in vitro methods, namely pH-dependent shift of pyrene fluorescence, liposome leakage and lysis of red blood cells. A cationic derivative of PA, which has already been proven to successfully mediate gene transfer in phagocytic and non-phagocytic cells [16,19], was used as a control in order to determine the pH-responsive membrane-disruptive efficiency of the various anionic PA polymers. All three in vitro assays revealed that the pH-dependent membrane-disruptive efficiency was highly affected by the hydrophobicity of the PA polymers. The disruptive activity was also influenced by the composition of the membrane. Membrane disruption by some anionic PA polymers was as efficient as that found for the cationic PA polymer control. Thus, this study provides a detailed investigation of a large variety of different anionic PA polymers with regards to their potential for further therapeutic applications.

2. Materials and methods

2.1. Materials

All investigated polymers and their characteristics are listed in Table 1. Poly(acrylic acid) (M_w 30 000) and poly(methacrylic acid) (M_w 9500) as 30 and 40% w/w solutions, respectively, poly(acrylic acid) (M_w 450 000) and poly(acrylic acid-co-acrylamide) (ca. 20% w/w acrylic acid), were all purchased from Aldrich Chemical (USA). Poly(methacrylic acid) (M_w 236 000) was obtained from Fluka Chemika (Buchs, Switzerland). Poly(acrylic acid-co-ethyl acrylate) (molar ratio 1:1) and poly(acrylic acid-co-butyl acrylate) (molar ratio 1:1) were purchased from Polysciences (USA). Poly(methacrylic acid-co-ethyl

Table 1
Investigated analogues and copolymers of poly(acrylic acid)



Polymer	R_1	R_2	R_3	R_4	Ratio *x*:*y*	M_w	Designation
Poly(acrylic acid)	H	H	–	–	1:0	30 000	PAA30
Poly(acrylic acid)	H	H	–	–	1:0	450 000	PAA500
Poly(methacrylic acid)	H	CH_3	–	–	1:0	9 500	PMA10
Poly(methacrylic acid)	H	CH_3	–	–	1:0	236 000	PMA200
Poly(ethacrylic acid)	H	C_2H_5	–	–	1:0	15 700	PEA10
Poly(ethacrylic acid)	H	C_2H_5	–	–	1:0	33 200	PEA30
Poly(ethacrylic acid)	H	C_2H_5	–	–	1:0	180 000	PEA200
Poly(propacrylic acid)	H	C_3H_7	–	–	1:0	7 800	PPA10
Poly(propacrylic acid)	H	C_3H_7	–	–	1:0	25 000	PPA30
Poly(acrylic acid-co-ethyl acrylate)	H	H	$COOC_2H_5$	H	1:1	1 300 000	PAA–EA
Poly(acrylic acid-co-butyl acrylate)	H	H	$COOC_4H_9$	H	1:1	600 000	PAA–BA
Poly(acrylic acid-co-acrylamide)	H	H	$CONH_2$	H	1:4	200 000	PAA–AC
Poly(methacrylic acid-co-ethyl acrylate)	H	CH_3	$COOC_2H_5$	H	1:1	135 000	PMA–EA
Poly(methacrylic acid-co-methyl methacrylate)	H	CH_3	$COOCH_3$	CH_3	1:1	135 000	PMA–MM 1:1
Poly(methacrylic acid-co-methyl methacrylate)	H	CH_3	$COOCH_3$	CH_3	1:2	135 000	PMA–MM 1:2
Poly(butyl methacrylate-co-[2-dimethyl aminoethyl]methacrylate-co-methyl methacrylate)	$C_2H_4N(CH_3)_2$	CH_3	CH_3, C_4H_9	CH_3	1:2:1	150 000	PDMAEM

acrylate) (molar ratio 1:1, Eudragit L 100 55), poly (methacrylic acid-co-methyl methacrylate) (molar ratio 1:1, Eudragit L 100 and molar ratio 1:2, Eudragit S 100) and poly(butyl methacrylate-co-[2-dimethyl aminoethyl]-methacrylate-co-methyl methacrylate) (molar ratio 1:2:1, Eudragit E 100) were all obtained from Roehm Pharma (Germany). Poly(ethacrylic acid) and poly(propacrylic acid), both at low and high molecular weight, were synthesized and characterized, according to the procedure outlined by Ferritto and Tirrell [23]. Briefly, 2-ethylacrylic acid was synthesized by reaction of diethyl ethylmalonate (Fluka) with formaldehyde followed by hydrolysis, dehydration and decarboxylation. Propylacrylic acid was prepared similarly, starting from diethyl propylmalonate (Acros Organics, Geel, Belgium). Polymerization was performed in bulk using 2,2′-azobis(isobutyronitrile) as initiator. The reaction mixture was magnetically stirred for 12–20 h at 60 °C (PEA200 for 72 h at 40 °C) under an argon atmosphere. The PEA polymers precipitated (white) during the reaction while PPA polymers stayed transparent but became solid. All polymers were dissolved in aqueous sodium hydroxide and dialysed against water. After freeze drying the polymers were obtained in a yield of 8–20%. The polymers were characterized by size exclusion chromatography (SEC) on a Shodex OH-pak SB 804 HQ column (Socochim, Lausanne, Switzerland) with eluent 0.7 M $NaNO_3$, 0.1 M Tris (pH 9), 0.3 ml/min. As reference PAA standards were used. All other chemicals used were of analytical grade unless otherwise specified and obtained from Fluka.

2.2. Pyrene fluorescence assay

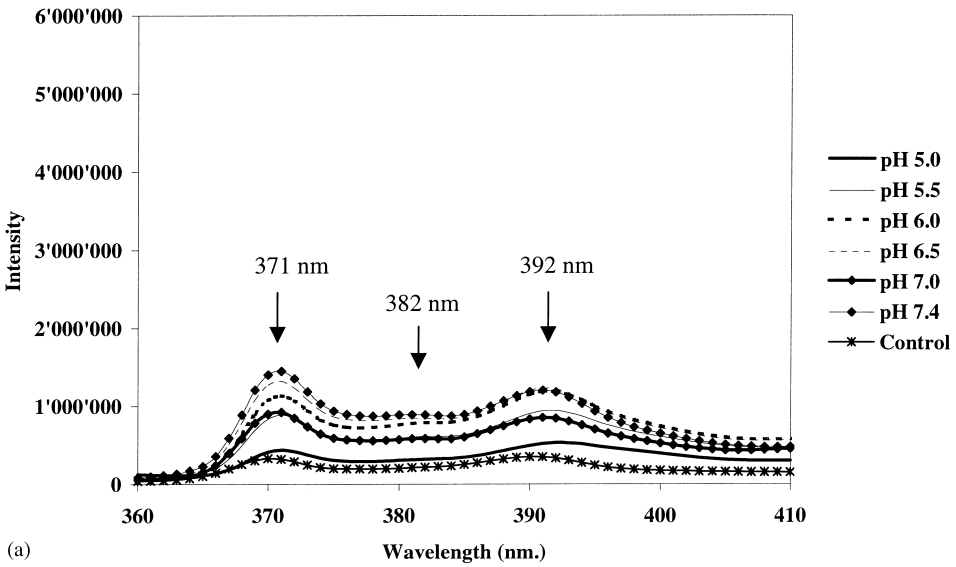
Stock solutions of all tested polymers were prepared in either 0.1 N NaOH or water, depending on their solubility, except PDMAEM which was dissolved in 1 N HCl, following the manufacturer's information. The polymer stock solutions were diluted with phosphate buffer solution of various pH from 5.0 to 7.4 to a final polymer concentration of 1 mg/ml. The pH of each sample was checked and re-adjusted if necessary. Pyrene (Fluka) was used as fluorescent probe and added to each sample at a final concentration of 5 x 10^{-6} M. Emission spectra of pyrene were recorded on a spectrofluorometer (excitation at 337 nm, FluoroMax, Spex Industries, NJ, USA) at room temperature and the intensity of emission peaks at 382 and 392 nm was determined.

2.3. Liposome leakage assay

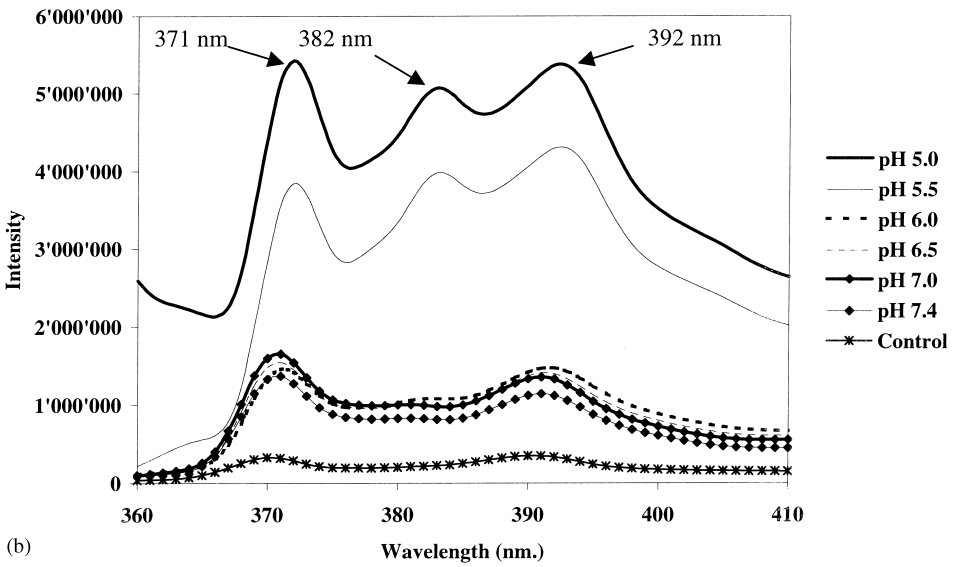
Liposomes consisting of dipalmitoylphosphatidylcholine (DPPC, Sygena, Liestal, Switzerland) or a combination of DPPC and cholesterol (Fluka), at a ratio of 4:1 by weight, were prepared by hydration of dried lipid films. Total lipid amount of 100 mg was first dissolved in 10 ml of anhydrous chloroform in a round bottom flask. Chloroform was then removed by a rotary evaporator under reduced pressure of 474 mbar at 40 °C with a rotational speed of 150 rpm. An aqueous phase containing 10 mM calcein (Sigma Chemical, MO, USA), dissolved by addition of 3.75 equivalents of NaOH, was added to hydrate the dried lipid film. The mixture was subsequently ultrasonicated at 50 °C for 10 min. The obtained liposomes were subjected to five freeze (dry ice/ethanol) and thaw (at 37 °C in water bath) cycles and then extruded ten times through 0.2-μm double polycarbonate filters by using the Extruder (Lipex Biomembranes, Canada). Non-encapsulated calcein was separated by ultracentrifugation (Optima L 60 ultracentrifuge, Beckman Instruments, CA, USA) at 35 000 rpm for 1 h. The liposomes were washed three times with water until the supernatant was clear. For the leakage assay in 96-well microtiter plates at various pH (5.0–7.4), the liposome solution was diluted in assay buffer and mixed with the polymer solutions resulting in 240 μg/ml of total lipid in each sample. Control experiments were performed in the absence of polymer and 100% leakage was assessed in the presence of 1% Triton X-100. Samples were incubated at 37 °C for 1 h under light protection and constant shaking. Calcein fluorescence intensity was assayed at an excitation wavelength of 485 nm and an emission wavelength of 530 nm by using an automated fluorescence plate reader (FluoroCount, Packard Instrument, CT, USA). Percentage of leakage was then calculated, based on control and 100% leakage. All experiments were done in triplicate. Standard deviations were below 15% and were not plotted for better clarity of the graphical presentation.

2.4. Haemolysis assay

Human red blood cells (RBCs) were harvested by centrifuging whole blood from healthy donors for 10 min at 2000 × *g* from buffy coats (blood bank Zurich, Switzerland). The plasma and the white layer of leucocytes at the interface were discarded. The erythrocytes were washed three times with 150 mM NaCl or several times until the supernatant was clear, and subsequently resuspended in 150 mM NaCl to yield the initial volume. The haemolysis assay was carried out by adding the polymer solution to 10^8 RBCs suspended in the buffer solution. The final volume of the sample was adjusted to 1 ml with the buffer solution, if necessary. Samples were thoroughly mixed by vortexing and incubated at 37 °C for 1 h under constant shaking. After removal of the cells by centrifugation at 13 500 × *g* for 5 min, haemolysis was determined by measuring the absorbance of the supernatant at 541 nm (Uvikon 930 spectrophotometer, Kontron Instruments, Italy), reflecting the amount of haemoglobin released into the supernatant. As controls, 10^8 RBCs were suspended in distilled water and in buffer solution, in order to determine 100% lysis and theoretical 0% lysis, respectively. The percentage of haemolysis was calculated, based on theoretical 0% lysis and 100% lysis. All experiments were conducted in



(a)



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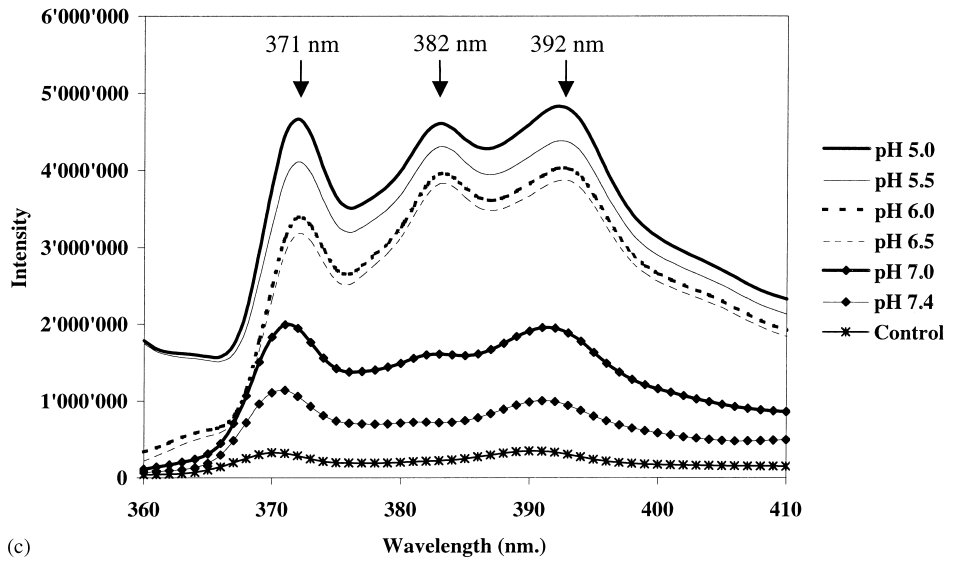

(c)

Fig. 1. Emission spectra of pyrene in the presence of (a) poly(acrylic acid) M_w 30 000 (PAA30), (b) poly(ethacrylic acid) M_w 33 200 (PEA30), and (c) poly(propacrylic acid) M_w 25 000 (PPA30) at different pH values. Control was pyrene without polymer.

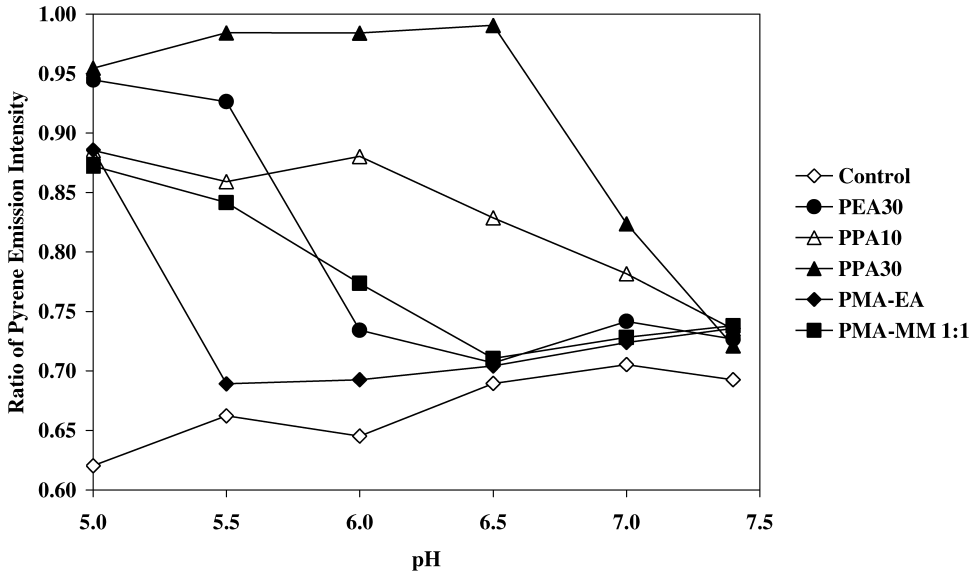

Fig. 2. The ratio of pyrene emission intensity at 382 nm to that at 392 nm in the presence of various polymers as a function of pH.

triplicate. Standard deviations were below 15% and were not plotted for better clarity of the graphical presentation.

3. Results

3.1. Detection of pH-dependent conformational shift of the PA polymers by pyrene fluorescence assay

Pyrene is a commonly used fluorescent probe for environmental polarity, because its emission intensity is dependent upon solvation [24]. The transition from an expanded, hydrophilic coiled polymer at high pH to a compact, globular structure in acidic solutions can be detected in the presence of pyrene [24,25]. Thus, we monitored the pH-dependent conformational transition of PA polymers by measuring the steady-state fluorescence of co-dissolved pyrene [25]. Enhanced intensity of the emission curves at low pH was only detected for PEA30 and PPA30, but not for PAA (Fig. 1). Interestingly, significantly different critical pH values of 5.5 for PEA30 and 6.5 for PPA30 were identified for the two polymers to induce a major shift of the emission curves (Fig. 1b,c). Besides the peaks at 371 and 392 nm, an additional peak at 382 nm appeared in the presence of PEA30 and PPA30 below their critical pH. This peak was neither detected with pyrene in the absence of polymer (control) nor with pyrene in the presence of PAA at all pH levels, or/and PPA above the critical pH (Fig. 1). Thus, the ratio of the emission intensity at 382 nm to that at 392 nm was calculated and was used to identify the critical pH for the conformational shift of the various PA polymers (Fig. 2). In addition to the homopolymers listed in Fig. 2, PEA10 induced significant enhancement of pyrene emission comparable to PEA30 (not shown). High molecular weight PEA200, on the other hand, also caused pyrene emission enhancement, but independent of pH (not shown). Interestingly, the enhancement caused by PPA30 was considerably more pronounced than that of the low molecular weight PPA10 (Fig. 2).

Among the copolymers, only two of them displayed a noticeable pH-dependent effect at the critical pH of around 6.0 for PMA–MM 1:1 and of 5.0 for PMA–EA (Fig. 2). PAA–BA, PMA–MM 1:2 and the cationic copolymer PDMAEM exhibited pyrene emission enhancement in a pH-independent manner (data not shown).

3.2. Detection of pH-dependent membrane-disruptive properties of the PA polymers in the liposomal leakage assay

All polymers were tested for their pH-dependent membrane-disruptive properties by calcein leakage from liposomes with two different types of liposomal membranes, DPPC liposomes and the more rigid cholesterol-containing DPPC liposomes (Fig. 3). In pure DPPC liposomes, low and medium molecular weight PEA and PPA (7–33 kDa) displayed the strongest pH-dependent membrane-disruptive effect (Fig. 3a). Membrane disruption was negligible at pH 7.0 and 7.4 and was induced by lowering the pH with complete disruption occurring at pH 6.0 (Fig. 3). No effect was observed with PAA and PMA. All other anionic copolymers displayed a pH-sensitive membrane-disruptive activity in the range of 30–80% at pH < 5.5. This is shown representatively for PMA–MM in Fig. 3a.

High molecular weight PEA (180 kDa) and the cationic PDMAEM non-selectively disrupted the DDPC liposomal membranes at all investigated pH (data not shown).

The membrane-disruptive effect of all polymers considerably decreased by employing the more rigid cholesterol-containing liposomes. Low and medium

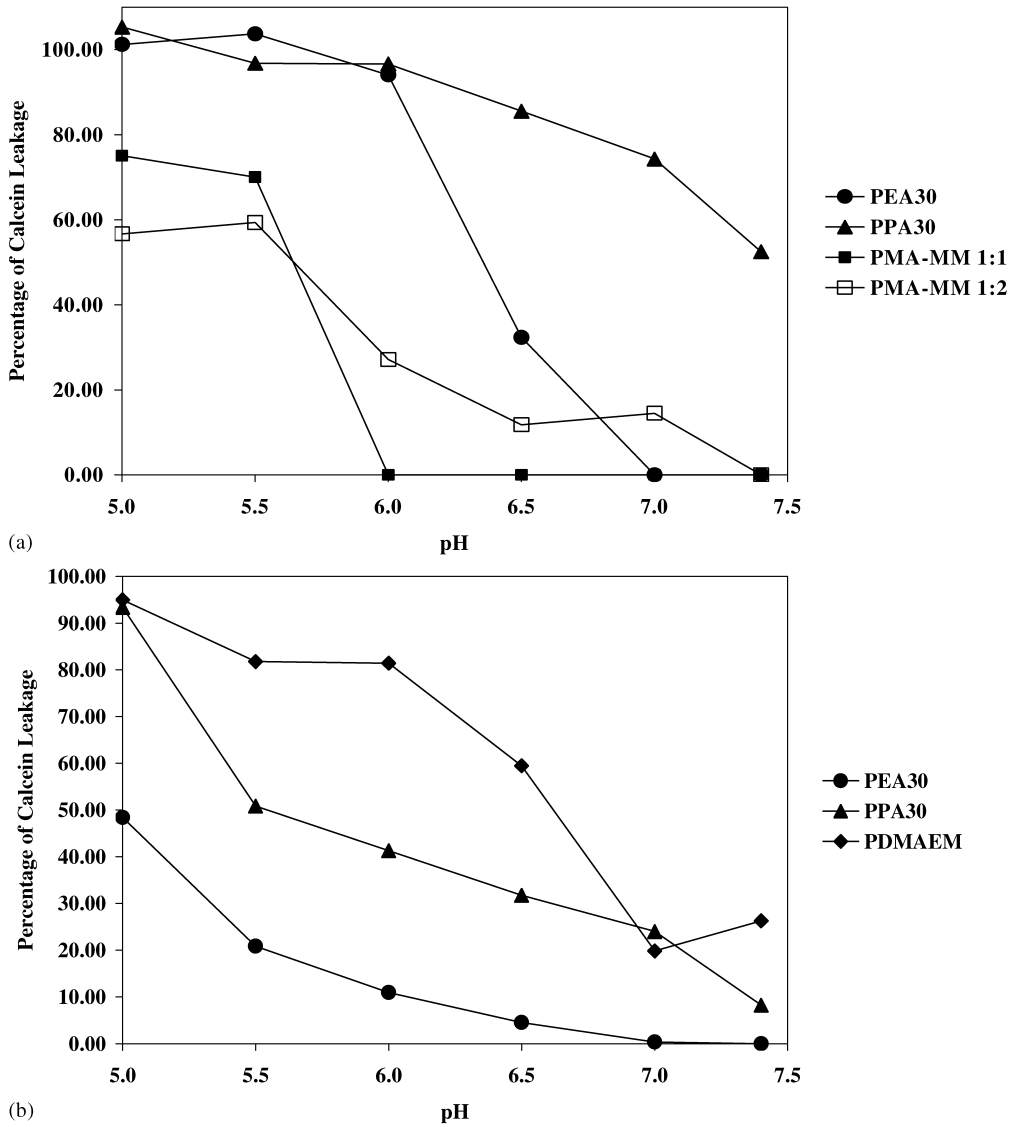

Fig. 3. Calcein leakage from liposomes as a function of pH in the presence of various polymers at a concentration of 240 μg/ml. (a) Dipalmitoylphosphatidylcholine (DPPC) liposomes, and (b) cholesterol-containing DPPC liposomes at a 4:1 ratio of DPPC to cholesterol. Each data point represents the mean of three determinations. The maximum standard deviations were 18% and 11% for a and b, respectively.

molecular weight PEA and PPA maintained their characteristic pH-dependent disruptive patterns with reduced efficiency (Fig. 3b), whereas high molecular weight PEA200 maintained its pH-independent disruptive effect to the reduced extent of 20–30% (data not shown). In addition to low and medium molecular weight PEA and PPA, only the cationic PDMAEM induced a major effect on the more rigid cholesterol-containing liposomes, which was now pH-dependent and led to 100% disruption at pH < 7.0 (Fig. 3b). All other copolymers displayed only a small pH-dependent leakage of less than 5–10% in these liposomes (data not shown).

Reducing the concentration of the polymers from 240 to 120 μg/ml resulted in similar effects as described above.

3.3. Detection of pH-dependent membrane-disruptive properties of the PA polymers in the haemolysis assay

In order to study the pH-dependent membrane-disrupting properties of the various polymers on the more complex cellular membranes, we measured haemolysis of human red blood cells upon incubation with polymer at different pH conditions. The effect of low and medium molecular weight PEA10, PEA30 and PPA10 was clearly pH-dependent at a concentration of 60 μg/ml showing no membrane disruption at pH 7.4 and high disruptive activity at pH 5.5 and 6.0, respectively (Fig. 4). The pH-dependent membrane-disruptive effects of PPA30 and PDMAEM were also dependent on the concentration of the polymers. Polymer concentrations below 15 μg/ml resulted in pH-sensitive

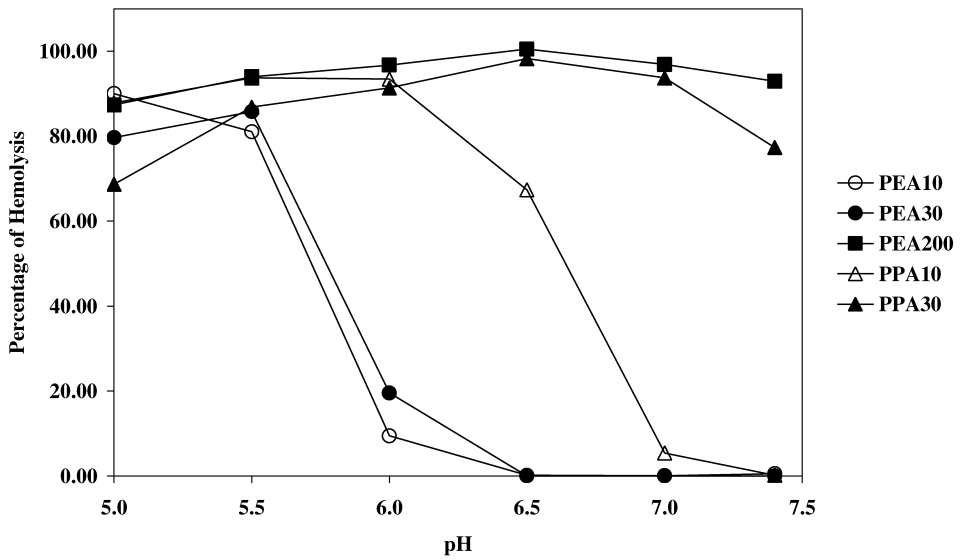

Fig. 4. Lysis of red blood cells as a function of pH in the presence of various polymers at a concentration of 60 μg/ml. Each data point represents the mean of three determinations. The maximum standard deviation was 4%.

membrane disruption, whereas high concentrations led to unselective membrane disruption at all pH (Fig. 5a,b). In contrast, high molecular weight PEA200 induced haemolysis with no clear dependency upon pH even at low concentrations (Fig. 5c). For all other polymers, only low activity below 10% haemolysis was observed at pH 5.0 and was not increased at concentrations up to 240 μg/ml (data not shown).

4. Discussion

The effect of pH on the transition of the polymer polarity differed greatly among the various polymers investigated in this study. The introduction of alkyl groups, such as ethylene groups in PEA and propylene groups in PPA, is required to induce a significant transition of the polymer polarity between pH 7 and 5. The critical pH of polymer transition shifted from pH 5.5 to 6.5 when the ethylene groups were substituted by propylene units. The more hydrophilic PMA and PAA have been reported to display pH-dependent transition below pH 5.0 which is outside the pH range investigated in this study [25]. The pH-dependent membrane-disruptive activity of the PA polymers is closely associated with the conformational transition of the polymers. The transition from an expanded conformation at high pH to a relatively hydrophobic, globular coil in acidic solution is triggered by the protonation of free carboxylic groups of the polymer [25]. Consequently, the collapsed polymer chain provides an increased number of hydrophobic site for enhanced polymer adsorption to phospholipid membranes [26].

Increasing the molecular weight was expected to enhance the effect of alkyl group substitution on the hydrophobicity of the polymers. The 2-fold higher molecular weight of PEA30 compared to PEA10 did not result in any detectable effect, whereas the 3-fold increase in molecular weight of PPA30 compared to PPA10 significantly affected the pH-dependent sensitivity and resulted in a more pronounced decrease in polarity upon acidification at lower pH. In contrast, the high molecular weight PEA200 displayed an impaired sensitivity to pH with already pronounced hydrophobicity at pH 7.4. In a previous study, it was found that only PEA with the lowest molecular weight (12 kDa) displayed a slightly different conformational transition compared to the other two (42 and 164 kDa) with the transition midpoint shifted to lower pH [24]. The authors conclude that the longer chains collapse in a higher cooperative fashion and are more effective in excluding water from the from the coiled domain at higher pH [24].

In addition to PA homopolymers, the design of PA copolymers offers manifold possibilities to tune the pH-responsiveness by polymer polarity, e.g. by introducing different ratios of acrylate groups esterified with alkyl chains of various lengths (Table 1). The introduction of methyl methacrylate or ethyl acrylate monomers eventually enabled the responsiveness of the otherwise too hydrophilic PMA. On the other hand, increasing the amount of methyl methacrylate in the PMA polymer even further led to a non-responsive polymer with impaired polarity such as PEA200. Similarly, substitution of PAA with butyl acrylate in combination with a high molecular weight resulted in the PAA–BA polymer with non-responsive, impaired polarity. The cationic PDMAEM polymer consists of a rather hydrophobic backbone due to the numerous substitutions with methyl and butyl side groups with all carboxylic groups esterified. Thus, in accordance with hydrophobic anionic PA polymers, the pronounced hydrophobicity of

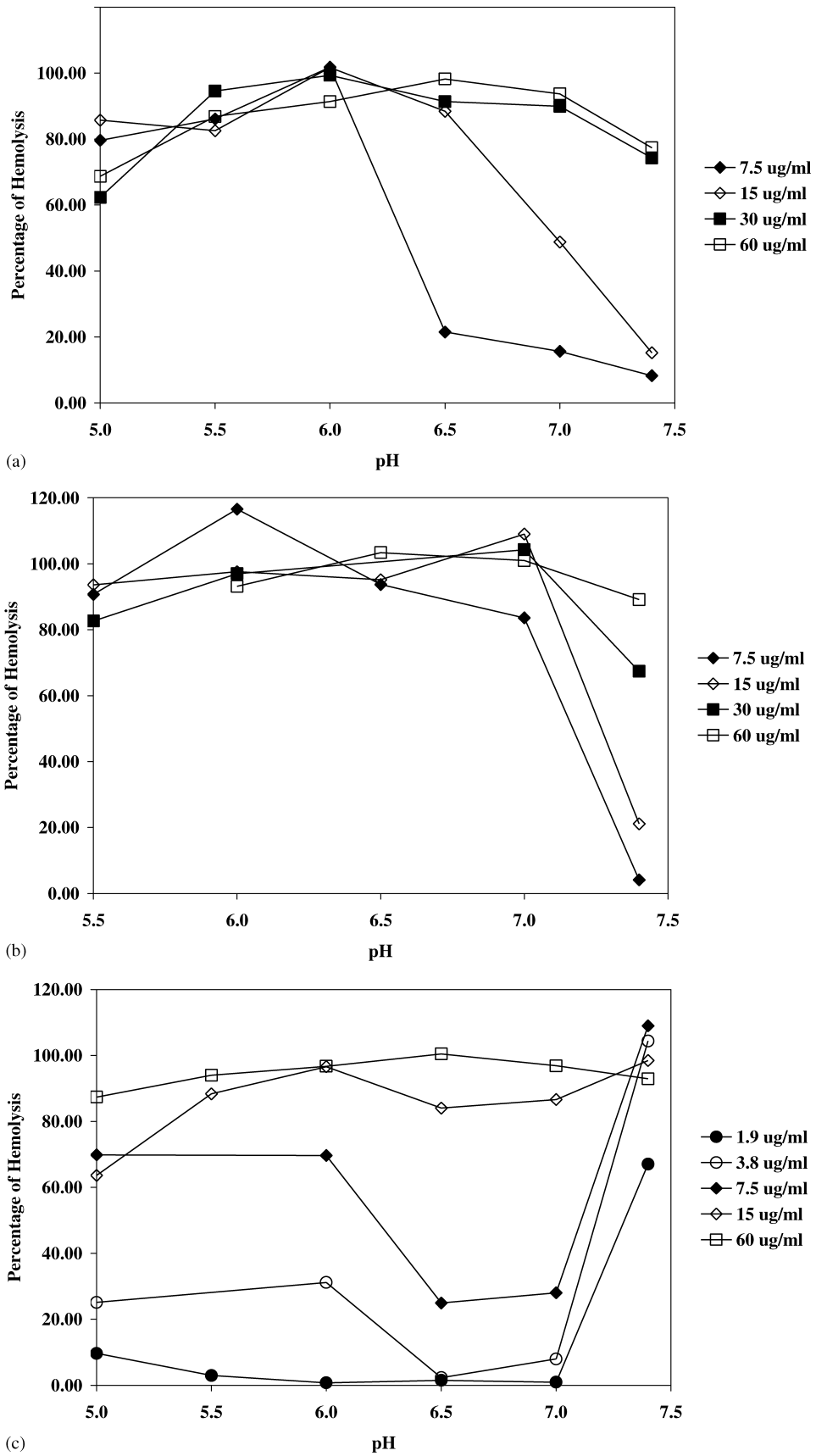

Fig. 5. Lysis of red blood cells as a function of pH at varying concentrations of (a) poly(propacrylic acid) M_w 25 000 (PPA30), (B) poly(butyl methacrylate-co-[2-dimethyl aminoethyl]methacrylate-co-methyl methacrylate) M_w 150 000 (PDMAEM), and (C) poly(ethacrylic acid) M_w 180 000 (PEA200). Each data point represents the mean of three determinations. The maximum standard deviations were 5%, 7%, and 18% for a, b and c, respectively.

the PDMAEM polymer did not lead to a detectable pH-responsive change in this study. From these results, we conclude that the fine tuning of polymer hydrophobicity in combination with its molecular weight results in appropriate pH-responsive polymers displaying transition of polarity in the pH range of 7–5.

Increasing the polymer surface energy by decreasing its polarity enhances association with phospholipid membranes and correlates well with the changes in the capacity to reorganize these membranes [22]. PEA has been reported to permeabilize liposomal membranes upon membrane association either by channel formation [27] or by membrane micellization [22,28]. In this study, we found a good agreement between the pH-dependent change of polymer polarity and disruption of DPPC membranes. While medium and low molecular weight PPA and PEA resulted in a pH-sensitive membrane disruption around pH 6.5, an impaired polarity as detected for PEA200 and PDMAEM resulted in a non-responsive pH-independent leakage of encapsulated dye from DPPC liposomes.

The reduced, but detectable membrane-disruptive effect of PPA at higher pH can be attributed to the fact that the critical pH for membrane reorganization is concentration-dependent. Thus, the critical pH is raised when the polymer concentration is increased [28]. A concentration-dependent effect has indeed been shown in this study by the haemolysis assay.

In addition to phospholipids, the lipid content of cellular membranes typically includes sterols, which increase the microviscosity of membranes with higher sterol/phospholipid ratios [29]. Intracellular membranes, such as lysosomal membranes, usually have molar sterol/phospholipid ratios lower than 0.3 while plasma membranes have ratios of 0.6–1.0. In this study, we demonstrated that the membrane-disruptive activity of the PA polymers was significantly reduced in liposomal membranes with a sterol/phospholipid ratio of 0.6 compared to plain DPPC membranes and was only detectable for PEA30 < PPA30 < PDMAEM. Thus, the highest activity was seen with the cationic PA polymer and was followed by the more hydrophobic PPA in comparison with PEA. The influence of membrane microviscosity was also observed with most fusogenic peptides, which were less active when applied to cholesterol-containing membranes [30]. While a reduced cholesterol content was reported for nascent endocytic membranes as compared to the plasma membrane [18], nascent phagosomal membranes contain a rather high sterol/phospholipid ratio of 1–1.5 [29]. This may have implications for the application of pH-sensitive compounds in phagocytic cells, such as macrophages or dendritic cells, when compared to endosome-forming cells. In order to avoid significant degradation of the therapeutic agent, the passage through degradative organelles should be minimized and the transfer to the cytosol should occur from early phagosomes or endosomes. Thus, the increased microviscosity and rigidity of the phagosomal membrane in phagocytes may require membrane-disruptive compounds with higher efficiency as compared to other cell types. The low efficacy of gene transfer in phagocytes may partly be accounted for by such phenomena [16,17,31].

The sensitivity of more complex membranes of mammalian cells, as observed here in red blood cells, was in the order of DPPC/cholesterol < RBCs < plain DPPC and was dependent on the polymer structure in a similar manner. The higher sensitivity of RBC membranes compared to DPPC/cholesterol liposomes is suggested to be partly due to the difference in acyl composition of the phospholipids, with about one half being unsaturated in cell membranes [29]. PEA-induced reorganization of membranes has been reported by others to be sensitive to the acyl composition [28], with less polymer required for phospholipid membranes containing unsaturated lipids.

In summary, this study provides a detailed investigation of a large variety of different anionic PA polymers with regard to their pH-dependent membrane-disruptive activity and their potential for further therapeutic applications. The pH-dependent increase of polarity and membrane disruption in the different model systems was in good agreement for all tested PA polymers. Among the various anionic PA polymers, only medium and low molecular weight PEA and PPA were identified to display significant pH-dependent disruptive activity which can be placed in relation to the cationic PDMAEM in the order of PEA < PPA < PDMAEM. The fine tuning of the pH-responsive hydrophilic-hydrophobic balance is likely to be responsible for the superior effects of PEA and PPA compared to other anionic PA polymers. The efficacy of polymer-induced membrane disruption was shown to be concentration-dependent and was significantly affected by the composition of the membrane. The sensitivity of the relatively complex membranes of mammalian cells can be placed between plain DPPC liposomal membranes and the more rigid cholesterol-containing DPPC membranes. Recent studies demonstrated that complexes of a cell-specific ligand and PPA were detected in the cytosol of a T-cell lymphoma cell line [21]. In addition, an enhancement of gene transfer was observed in vitro and in vivo with PA polymer in ternary formulations of PPA, DNA and cationic lipid [21,32]. Whether the incorporation of PPA or PEA into synthetic delivery systems could also enhance the cytosolic delivery of therapeutics in phagocytes by concurrently avoiding significant cytotoxicity needs to be evaluated.

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