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Biochimica et Biophysica Acta 1613 (2003) 28–38
BIOCHIMICA ET BIOPHYSICA ACTA
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Characterization of the membrane-destabilizing properties of different pH-sensitive methacrylic acid copolymers

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Received 5 November 2002; received in revised form 19 March 2003; accepted 30 April 2003

Abstract

The intracellular delivery of active biomacromolecules from endosomes into the cytoplasm generally requires a membrane-disrupting agent. Since endosomes have a slightly acidic pH, anionic carboxylated polymers could be potentially useful for this purpose since they can destabilize membrane bilayers by pH-triggered conformational change. In this study, five different pH-sensitive methacrylic acid (MAA) copolymers were characterized with respect to their physicochemical and membrane lytic properties as a function of pH. pH-dependent conformational changes were studied in aqueous solution by turbidimetry and spectrofluorimetry. The hydrophobic domains that formed upon a decrease in pH were found to be dependent on copolymer's composition. Hemolysis and cytotoxicity assays demonstrated that the presence of the hydrophobic ethyl acrylate monomer and/or sufficient protonation of the carboxylic acid groups were important parameters for efficient membrane destabilization. Excessive copolymer hydrophobicity was not associated with membrane destabilization, but resulted in high macrophage cytotoxicity. Overall, this study gave more insights into the structure–activity relationship of MAA copolymers with membrane bilayers. Gaining knowledge of modulation of the physicochemical properties of copolymers and the optimization of copolymer–lipid interactions may lead to the elaboration of much more efficient drug delivery systems.

Keywords: Methacrylic acid copolymer; pH-sensitivity; Membrane bilayer; Endosomal release

1. Introduction

The efficient delivery of therapeutic macromolecules (e.g. DNA, proteins) to the cytoplasm is often limited by their intracellular trafficking pathway. Although colloidal complexes can be internalized into targeted cells via receptor-mediated endocytosis, they are most of the time rapidly trafficked from endosomes to lysosomes where degradation may occur [1,2]. The escape of biomolecules from endosomes generally requires a membrane-destabilizing agent, which can release the internalized compounds into the cytoplasm before they reach the lysosomes. Since endosomes exhibit a slightly acidic pH (5.0–6.5), a possible strategy to achieve such a goal consists of using a carrier that destabilizes the endosomal bilayer under mildly acidic conditions and is nondisruptive at pH 7.4.

Carriers based on attenuated viruses have been studied extensively in gene delivery [3–5]. At low pH (4.8–6.5), viral fusion glycoproteins adopt an α-helix or a β-sheet conformation, which interacts with cell membranes and induces their destabilization [6]. Although they provide high transfection efficiencies, these agents can elicit host immunogenic reactions [7]. Safety issues have prompted the development of synthetic fusogenic peptides that mimic the process by which viruses destabilize endosomal membranes [8,9]. However, like the parent viral vector, these peptides are likely to be immunogenic in vivo. To obtain safer agents, much attention has been focused on the development of nonviral delivery vectors such as synthetic polymers. Among these, synthetic pH-sensitive polyelectrolytes are of particular interest as they can, like viruses, interact with membranes in a pH-dependent manner. The mechanism of membrane destabilization varies, depending

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doi:10.1016/S0005-2736(03)00137-8

on whether the polyelectrolyte is a weak base (polycation) or a weak acid (polyanion).

The first class of polyelectrolytes is composed of cationic aminated polymers, such as poly(L-lysine) (PLL), poly(ethylenimine) (PEI), and poly(amidoamine) (PAA). These polymers promote membrane destabilization at acidic pH via electrostatic interactions between protonated amines and the negatively charged membrane. PLL is a linear polymer, which efficiently binds DNA and protects it from degradation by nucleases [10–12]. However, its usefulness is limited as it is relatively cytotoxic and poorly endosomolytic. Thus, PLL does not by itself induce the endosomal release of DNA unless another endosomolytic agent is added [13,14], or unless the polymer is chemically modified to increase its lytic activity [15,16]. Highly branched cationic polymers such as PEI and PAA dendrimers are efficient transfection agents that are thought to act by a proton-sponge effect. The high buffering capacity of these polymers prevents acidification of the endosomal compartment, and induces ion influx in endosomes, causing osmotic swelling and vesicle destabilization [17,18]. Recent work on these polymers has demonstrated that chain flexibility would be a key feature for this effect, since polymer expansion appears to be an important parameter leading to endosomal swelling and subsequent disruption [19].

The second category of pH-sensitive synthetic polymers consists of anionic carboxylated polymers, such as acrylic and methacrylic acid (MAA) copolymers. These polymers bear pendant carboxylic acid groups and destabilize membrane bilayers by pH-triggered conformational change. They collapse from an expanded hydrophilic coil at physiological pH to a hydrophobic globule in an acidic environment [20–23]. So far, poly(ethylacrylic acid) (PEAA) is the anionic carboxylated polymer that has been studied most extensively for its membrane-destabilizing properties. PEAA is currently being investigated in liposomal delivery systems to induce pH-triggered release of liposome content [24–27]. It is also being evaluated as an endosomolytic agent [28,29]. At acidic pH and low concentrations, it can permeabilize cell membranes, whereas high polymer concentrations completely solubilize phospholipid bilayers [30]. PEAA interaction with membrane bilayers is well characterized in the literature. However, little information is available on the structure–activity relationship of methacrylic and acrylic acid copolymers. This manuscript is aimed at characterizing five different MAA copolymers with respect to their membrane lytic properties as a function of pH. To gain more insights into their structure-dependent interaction with membrane bilayers, the physicochemical properties of the copolymers are correlated with their ability to destabilize vesicles (red blood cells (RBC) or liposomes), and with cytotoxicity assays. Ultimately, these copolymers could be used as soluble macromolecular drug carriers. Drugs could be covalently attached to the polymer backbone via cleavable linkage. Alternatively, MAA copolymers could be complexed by means of electrostatic interactions to positively charged DNA delivery systems such as polyplexes and lipoplexes.

2. Materials and methods

2.1. Materials

MAA copolymers were prepared by free radical polymerization and provided by Röhm GmbH (Darmstadt, Germany). The lactate dehydrogenase (LDH) assay kit and cytochalasin B were purchased from Sigma (St. Louis, MO). Egg phosphatidylcholine (EPC), dioleoylphosphatidylethanolamine (DOPE), and dimiristoylphosphatidylglycerol (DMPG) were obtained from Northern Lipids Inc. (Vancouver, BC, Canada). Cholesterol (Chol) was obtained from Avanti Polar Lipids (Alabaster, AL). 8-Hydroxypyrene-1,3,6-trisulfonic acid (HPTS) and *p*-xylene-bis-pyridinium bromide (DPX) were procured from Molecular Probes (Eugene, OR). Unless otherwise stated, all other products, including pyrene, fluoresceinamine (isomer I) and 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT), were obtained from Aldrich (Milwaukee, WI).

2.2. Purification and molecular weight determination

Prior to use, the copolymers were dissolved in ethanol and dialyzed against water for at least 72 h. Absolute number (M_n)- and weight (M_w)-average molecular weights (MWs) as well as the polydispersity index (PI) were determined by size exclusion chromatography (SEC), using a Waters 1525 pump (Waters, Milford, MA) equipped with a high-sensitivity differential refractive index detector (Waters 2410) and PD 2000 light scattering detector (Precision Detectors, Franklin, MA). The eluent was either a 50 mM tris(hydroxymethyl)aminomethane (Tris) buffer, pH 8, or *N*,*N*-dimethylformamide (DMF) containing 10 mM lithium bromide (LiBr). Four Waters Ultrahydrogel (120, 250, 1000, and 2000) or three Waters Styragel columns (HT2, HT3, and HT4) were placed in series. Flow rate and temperature were set at 1 ml/min and 35 °C, respectively.

2.3. Evaluation of the precipitation pH of MAA copolymers

The copolymers were dissolved in phosphate-buffered saline (PBS, 34 mM, NaCl 75 mM), pH 7.4, to a final concentration of 75 mg/l. Polymer solutions were progressively acidified with HCl to pH 3.2 and aliquots were withdrawn at different pH values. The precipitation pH was determined by 90° turbidimetry at 480 nm under stirring at 37 °C, using a Series 2 Aminco Bowman spectrofluorimeter (Spectronic Instruments Inc., Rochester, NY) [31]. Light scattering intensities were plotted as a function of pH. Experiments were run in duplicate.

2.4. Study of the pH-dependent conformational transition of copolymers by pyrene fluorescence

The copolymers were dissolved in PBS, pH 7.4, to a final concentration of 200 mg/l. Polymer solutions were progressively acidified to pH 4.2 and aliquots were withdrawn at different pH values. Pyrene (2×10^{-7} M) was also dissolved in phosphate buffer and added to the copolymer solutions. The samples were stirred overnight in the dark at room temperature. Fluorescence emission spectra were recorded at 37 °C ($\lambda_{ex} = 334$ nm), and intensities of emission peaks I (372 nm) and III (383 nm) served to monitor the formation of hydrophobic domains within the copolymers. Experiments were also performed without pyrene to eliminate excess light scattering caused by precipitated copolymers. Each experiment was performed in quadruplicate.

2.5. Hemolysis assays

This procedure was described previously by Murthy et al. [28]. Human RBC were selected as the endosomal membrane model. The cells were collected from a healthy donor in vacutainer tubes (Fisher Scientific, Montreal, QC, Canada) containing 5.5 mg of liquid K_3 EDTA. The tubes were centrifuged at $200 \times g$ for 5 min at 4 °C, and the cells were washed three times (centrifugation followed by redispersion) with PBS, pH 7.4, or saline 0.9% (w/v). RBC were counted with a hemacytometer and diluted either in isotonic phosphate or 2-(*N*-morpholino)ethanesulfonic acid (MES, 200 mM, NaCl 110 mM) buffer of the appropriate pH. The copolymers were dissolved in PBS or in mildly alkaline saline 0.9% (w/v). The hemolysis assay was performed by adding RBC suspended in the appropriate medium to the copolymer solution (final cell concentration = 10^8 RBC/ml). The samples were incubated for 30 min under stirring at 37 °C, cooled on ice, and centrifuged at $5300 \times g$ for 5 min at 4 °C. To determine the extent of membrane disruption, hemoglobin absorbance in the supernatant was measured at 541 nm, using a PowerWave microplate reader (Biotek Instruments, Winooski, VT). To obtain 100% hemolysis, cells (10^8 RBC/ml) were lysed by dispersion in water. Controls were prepared by mixing the RBC with buffer or saline. RBCs were tested within 24 h after collection.

2.6. Assessment of copolymer–lipid interaction using liposomes as membrane models

Unilamellar liposomes (20 mM total lipid) composed either of EPC or EPC/Chol/DOPE/DMPG (61:17:16:6 molar ratio) were prepared by hydration of dried lipids, followed by repeated extrusion through a 0.2-μm pore size membrane at room temperature. Liposomes containing the highly water-soluble fluorophore HPTS and the collisional quencher DPX were prepared by including these probes in the lipid hydration buffer (*N*-(2-hydroxyethyl)-piperazine-*N*′-(2-ethane sulfonic acid) (HEPES) 20 mM, HPTS 35 mM, DPX 50 mM, pH 7.4). Untrapped dye was removed by gel filtration in Sephadex G-100 (Pharmacia, Baie d'Urfé, QC, Canada). Liposomes and the copolymers dissolved in mildly alkaline saline were added to either isotonic PBS or MES buffer of the appropriate pH. The release of liposome content was monitored for 20 min at 37 °C by fluorescence dequenching assay [32]. The extent of content release was calculated from HPTS fluorescence ($\lambda_{ex} = 413$ nm, $\lambda_{em} = 512$ nm) relative to measurement after liposome lysis in 0.5% (v/v) Triton X-100, which gave the complete release of encapsulated HPTS and DPX.

2.7. Measurement of liposome size by dynamic light scattering (DLS)

The hydrodynamic mean diameter and size distribution of liposomes were determined at an angle of 90° and a temperature of 37 °C by DLS, using differential size distribution processor intensity analysis (N4Plus, Coulter Electronics, Miami, FL). The copolymers were dissolved in PBS at concentrations ranging from 0.25 to 4 mg/ml. The solutions were filtered through a 0.45-μm pore size filter before liposome addition. Size measurements were conducted in triplicate.

2.8. Labeling of copolymer B with fluoresceinamine and study of adsorption

Fluoresceinamine (1.6×10^{-3} mmol) dissolved in distilled anhydrous tetrahydrofuran (THF) was added to a THF solution of copolymer B (250 mg, 2.2 ml). The mixture was degassed for 15–20 min under argon, and the carboxylic acid group activator *N*-ethoxycarbonyl-2-ethoxy-1,2-dihydroquinoline (1.9×10^{-3} mmol, 20% excess) was added. The reaction was carried out for 72 h under inert atmosphere in the dark at room temperature. THF was then evaporated under vacuum, and the crude product was dissolved in phosphate buffer, pH 9. It was dialyzed (MW cutoff 3500) for 3 days against a water/methanol mixture (50:50 v/v). The methanol was subsequently replaced by pure water, and dialysis was continued for another 2 days. The purified product was finally freeze-dried, and the absence of free fluoresceinamine was confirmed by thin layer chromatography, using an ethyl acetate/hexane/acetic acid (78:10:2 v/v) mobile phase. The fluorescein content of the copolymers was assayed by spectrofluorimetry in PBS and found to be 0.04 mol% (yield: 66%). The precipitation pH of labeled copolymer was measured as described above (Section 2.3).

For the adsorption study, 10^8 RBC suspended in the appropriate isotonic buffer (phosphate or MES) at pH varying from 6.0 to 7.4 were incubated for 30 min under stirring at 37 °C, with the labeled copolymer dissolved in mildly alkaline saline (final concentration = 75 or 150 μg/ml). Controls were prepared by adding only saline to the RBC. The samples were then centrifuged gently at $50 \times g$, and the fluorescence of the supernatant ($\lambda_{ex} = 485$ nm,

λ_{em} = 530 nm) was determined in a FL600 microplate reader (Biotek Instruments). To obtain 100% fluorescence, the copolymer was mixed with the appropriate buffer devoid of RBC. Each experiment was performed in quadruplicate.

2.9. Evaluation of the cellular toxicity of copolymers by MTT assay

Inhibition of cell proliferation was measured by tetrazolium salt MTT assay [33]. J774 mouse macrophage-like cells (a gift from Professor Michel Desjardins, Université de Montréal, Montreal, QC, Canada) were suspended in Dulbecco's modified Eagle cell culture medium (DMEM) containing 10% (v/v) heat-inactivated (56 °C, 30 min) fetal bovine serum (FBS), 100 U/ml penicillin G, and 100 μg/ml streptomycin (Invitrogen, Burlington, ON, Canada). The cells were plated in 96-well tissue culture plates (100 μl DMEM–FBS containing 5×10^3 viable cells) and allowed to adhere for 24 h at 37 °C in a humid atmosphere containing 5% CO_2. Twenty microliters of copolymer solutions in PBS and sterilized by filtration through a 0.2-μm pore size filter were then added to the wells in triplicate (final concentration ranging from 3 to 500 μg/ml). Controls (100% cell survival) were prepared by adding PBS to the cells. Incubations were conducted for 48 h, and MTT dissolved in PBS (10 μl of a 5 mg/ml solution) was added to each well. After a further 3-h incubation, sodium dodecyl sulfate (100 μl of a 10% w/v solution containing 0.01 N hydrochloric acid) was added to each well to dissolve reduced MTT. Absorbance was measured 24 h later at 570 nm. Each experiment was undertaken in quadruplicate. Experiments using the phagocytosis inhibitor cytochalasin B were performed in 24-well tissue culture plates (500 μl DMEM–FBS containing 5×10^4 viable cells). After 24 h of incubation, 50 μl of a solution of cytochalasin B (6 μM final concentration) were added to each well in triplicate. The plates were incubated for 20 min, and 108 μl of copolymer E solution prepared in PBS were added to the wells (final concentration ranging from 125 to 1000 μg/ml). Inhibitor cell toxicity was assessed by adding PBS to the cells instead of the copolymer solution. The cells were incubated for another 4.5 h, rinsed twice with serum-free culture medium, and fresh DMEM–FBS medium was added. The MTT assay was performed after 48 h as described above. Experiments were conducted in triplicate.

2.10. Evaluation of the cellular toxicity of copolymers by LDH assay

Cellular death due to necrosis is associated with release of the cytoplasmic enzyme LDH [34–36]. To determine the in vitro toxicity of the copolymers, J774 cells were plated in 96-well tissue culture plates as described above for the MTT assay. After 24 h of incubation, copolymer solutions were added to each well in triplicate (final concentration ranging from 3 to 500 μg/ml). Controls were prepared by adding PBS to the cells. The cells were incubated for another 48 h, and LDH activity was assessed in 4 μl aliquots of the supernatant of each well with a modified commercial LDH assay kit [36]. Total LDH content was measured by incubating the cells with Triton X-100 (1% v/v final concentration) to induce cell lysis.

3. Results

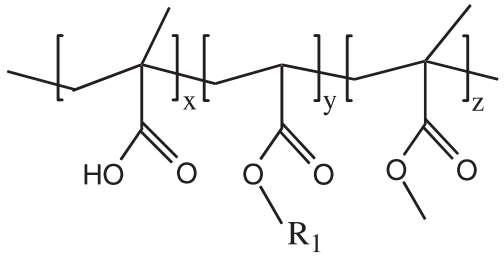
3.1. MW determination

The chemical compositions and respective MWs of the MAA copolymers are shown in Fig. 1 and Table 1. Although most copolymers were analyzed in an aqueous mobile phase (e.g. Tris buffer), it was necessary to use DMF with 10 mM LiBr for the more hydrophobic copolymer E. This copolymer, which contains only 10 mol% MAA, was found to interact with the Ultrahydrogel columns. The MWs obtained ranged from 20,000 to 30,000. For all copolymers, the PI lay between 1.2 and 1.4, which is acceptable for polymers synthesized by free radical polymerization.

3.2. Evaluation of the pH-dependent conformational transition of MAA copolymers

Fig. 2 shows the precipitation pH for the five MAA copolymers, while Table 1 presents the pH transition ranges. Since turbidimetry is a method sensitive to the formation of particles with size neighboring the light wavelength, the reported values do not correspond to the true onset of phase transition but rather to the stage where the polymer starts to aggregate substantially. Most of the copolymers exhibited relatively sharp transitions that encompassed 0.4–0.7 pH units, and started to precipitate at least 1.8 pH units below 7.4. Copolymer E presented a much broader transition that covered about 2 pH units and started to phase separate at pH 7.0. The fact that this copolymer contains only 10 mol% carboxylic acid groups may explain the more gradual precipitation profile.

To gain more information on the hydrophobicity of the aggregates that are formed at acidic pH and to determine whether hydrophobic microdomains can be detected prior to a turbidity increase, pH-dependent conformational transition



R_1=CH_3, CH_2CH_3

Fig. 1. Chemical structure of the MAA copolymers tested.

Table 1
Molecular weight and precipitation pH of the different copolymers tested

Copolymer	Chemical composition (molar ratio)	Molecular weight			pH transition range
		M_n	M_w	PI	
Type A[a]	MMA/MAA (50:50)	20640	28390	1.38	3.8–4.5
Type B[a]	EA/MAA (50:50)	21630	26240	1.21	4.7–5.1
Type C[a]	MA/EA/MAA (35:35:30)	24270	31510	1.30	5.0–5.6
Type D[a]	MMA/MAA (70:30)	19130	23030	1.20	4.8–5.3
Type E[b]	MA/MMA/MAA (45:45:10)	26940	32680	1.21	Broad (4.5–7.0)

MMA = methyl methacrylate; MA = methyl acrylate; EA = ethyl acrylate; MAA = methyl acrylic acid.
[a] MW evaluated in Tris buffer, pH 8.
[b] MW evaluated in DMF containing 10 mM LiBr.

of the different copolymers was monitored by spectrofluorimetry, using pyrene as a probe. Pyrene is commonly used to assess the degree of hydrophobicity of its surrounding environment because its emission intensity and vibronic band structure are sensitive to solvation [37]. For pH-sensitive polymers, the formation of hydrophobic microdomains under mildly acidic conditions can be monitored by an increase in total fluorescence intensity and a decrease in the ratio of the first (372 nm) and third (383 nm) peaks of the emission spectrum (I_1/I_3 ratio). Fig. 3A shows the emission spectrum of pyrene in solution with copolymer B at two different pH, whereas Fig. 3B depicts the I_1/I_3 ratio plotted as a function of pH for the different copolymers. Both an increase in the total fluorescence intensity of pyrene and a decrease in the I_1/I_3 ratio were observed as the pH was decreased from 7.4 to 4.8. These results reflect the progressive conformational change of the copolymer, and are typical of the presence of hydrophobic domains in which pyrene is preferentially solubilized. Copolymers A and B underwent the sharpest transitions, followed by copolymers C and D. Interestingly, copolymer E presented no transition

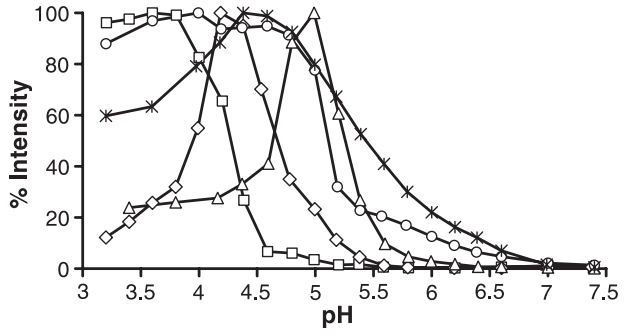


Fig. 2. Precipitation pH of MAA copolymers A (squares), B (diamonds), C (triangles), D (circles), and E (stars) at 37 °C. The copolymers were dissolved in PBS, pH 7.4. Polymer solutions were progressively acidified to pH 3.2 and aliquots were withdrawn at different pH values. Polymers' precipitation pH was measured as an increase in light scattering intensity at 480 nm.

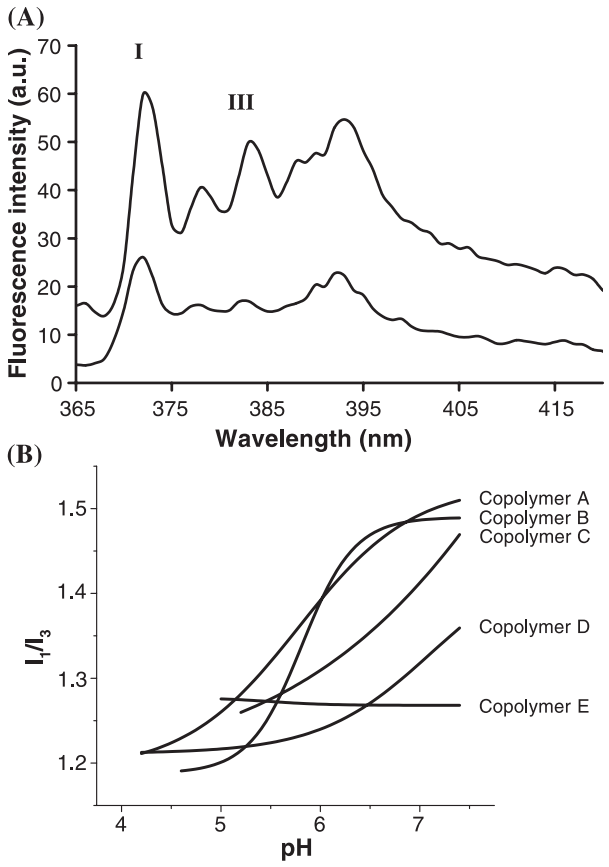


Fig. 3. (A) Pyrene emission spectra in the presence of copolymer B at 37 °C and pH 7.4 (lower curve) and 4.8 (upper curve). (B) Variation, with pH, of the I_1/I_3 ratio of pyrene codissolved at 37 °C with the different MAA copolymers tested (data points have been removed for clarity).

at all. It is noteworthy that the sharpness of the transition is related to the amount of MAA present in the copolymers, namely 50 mol% for copolymers A and B, 30 mol% for copolymers C and D, and only 10 mol% for copolymer E (Table 1). Also, as observed by turbidimetry, the transitions of copolymers A and B occurred at the lowest pH values, followed by copolymers C and D. However, these experiments suggest that hydrophobic microdomains already exist before polymer self-association is detected by turbidimetry. By comparing Figs. 2 and 3B, one can observe that at the onset of turbidity increase, pyrene has already partitioned into a highly hydrophobic environment (i.e. I_1/I_3 ratios < 1.3).

3.3. Evaluation of the membrane-destabilizing properties of copolymers at neutral and acidic pH

Destabilization of membrane bilayers by the copolymers was first studied at neutral pH, using RBC as the endosomal membrane model. Fig. 4 shows the copolymers' hemolytic activity at physiological pH. Copolymers B, C, and D were the most hemolytic at pH 7.4, whereas copolymer E presented limited hemolytic activity, reaching a maximal

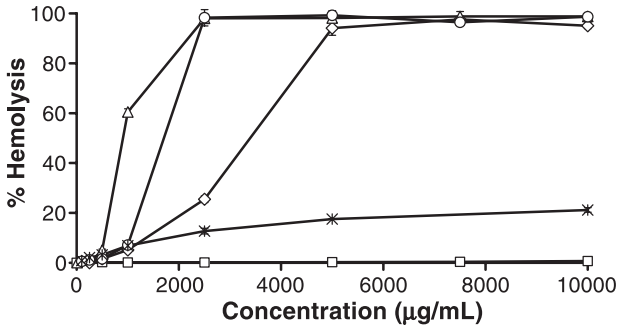


Fig. 4. RBC hemolysis at physiological pH induced by MAA copolymers A (squares), B (diamonds), C (triangles), D (circles), and E (stars) at 37 °C and physiological pH, as determined after 30-min incubation by the absorbance of released hemoglobin at 541 nm. RBC suspended in PBS, pH 7.4, were added to copolymers dissolved in the same buffer at varying concentrations (10–10 000 μg/ml). Mean ± S.D. (n = 3).

value of 20% hemolysis at 10 mg/ml. Copolymers B, C, and D became significantly hemolytic (5%) at a concentration of approximately 0.5 mg/ml. Between 0.5 and 5.0 mg/ml, copolymer B was less hemolytic than copolymers C and D, which demonstrated similar activities. These results are in agreement with the transitions detected by turbidimetry and pyrene fluorescence spectroscopy. The closer the transition was to pH 7.4, the more hemolytic the copolymer was at that pH.

A useful endosomolytic agent should have membrane-destabilizing properties at the mildly acidic pH (5.0–6.5) found in endosomes. Assessment of copolymer pH-dependent lytic activity was first carried out with RBC (Fig. 5A). Only copolymers B and C caused hemolysis at 150 μg/ml in the pH range tested. Hemolytic activity was maximal at pH 5.0–5.5 for both copolymers. To determine the lowest hemolytic concentration at acidic pH, hemolysis caused by copolymers B and C (pH 5.0 and 5.5) was evaluated at concentrations ranging from 5 to 250 μg/ml (Fig. 5B). Copolymer B was more hemolytic at pH 5.5 than at 5.0, especially at 25 μg/ml (58% vs. 10% hemolysis, respectively). Copolymer C was less hemolytic at pH 5.5 than at 5.0 at all concentrations tested, but was globally less hemolytic than copolymer B. Thus, maximal hemolysis is not necessarily achieved at the most acidic pH. At low pH values, extensive phase separation (Fig. 2) could limit polymer interaction with cell membranes.

To assess the effect of membrane composition on their lytic properties, the copolymers were incubated for 20 min at 150 μg/ml and different pH, with artificial phospholipid vesicles prepared with EPC or EPC/Chol/DOPE/DMPG (61:17:16:6 molar ratio). The latter liposomes presented a phospholipid composition similar to lysosomal membranes [38]. The vesicles were loaded with fluorescent probe HPTS and a collisional quencher. Fig. 6A shows the results obtained with EPC liposomes. Compared to RBC, these vesicles were more readily destabilized. With the exception of copolymer E, the copolymers triggered complete probe release at pH 5.0. Copolymers B, C, and D also demonstrated high activity at pH 5.5 and 6.0. Copolymer A, which undergoes phase transition at lower pH values, required a more acidic pH for maximal activity. Incorporation of Chol and the negatively charged phospholipid DMPG into EPC liposomes decreased polymer-triggered content leakage (Fig. 6B). Release profiles from these liposomes almost mimicked those obtained with RBC (Fig. 5A), with copolymers A and E showing no HPTS release at any pH, and copolymers B and C being maximally lytic at pH 5.0 and 5.5.

3.4. Study of RBC adsorption

To determine whether the copolymers adsorbed to RBC, copolymer B was labeled with fluorescein. Labeling did not significantly modify the pH at which the polymer phase separates (data not shown). Although sharper, transition began at the same pH (5.5) as the unmodified copolymer and followed the same trend up to pH 5.0. The adsorption study was carried out at a maximal concentration of 150 μg/ml to avoid complete membrane solubilization at pH values ranging from 7.4 to 6.0. At all pH values, the proportion of total copolymer bound to RBC at 75 μg/ml was almost twice

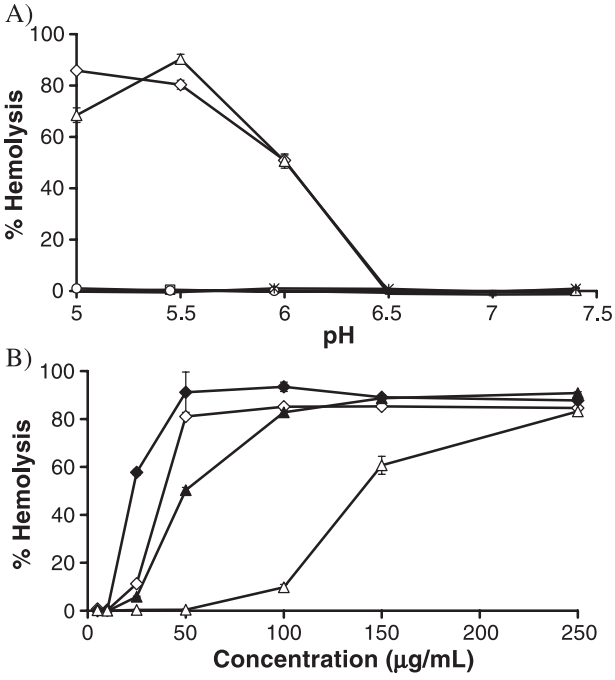


Fig. 5. pH-dependent RBC hemolysis induced by the copolymers tested at 37 °C after a 30-min incubation. (A) RBC suspended in isotonic phosphate or MES buffers of the appropriate pH were added to copolymers A (squares), B (diamonds), C (triangles), D (circles), or E (stars) dissolved in mildly alkaline saline at 150 μg/ml (final concentration). Mean ± S.D. (n = 3). (B) RBC hemolysis caused by copolymers B (diamonds) and C (triangles) at pH 5.0 (open) or 5.5 (closed) at various concentrations (5–250 μg/ml). Mean ± S.D. (n = 3).

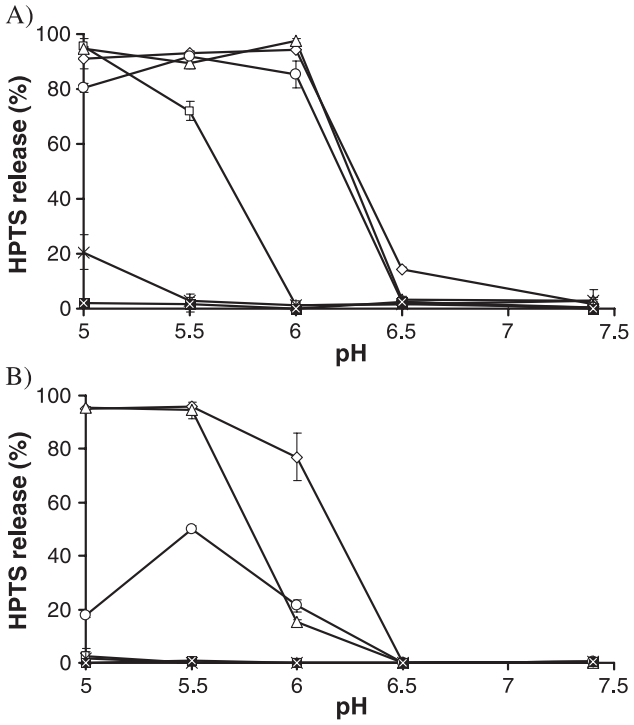


Fig. 6. Release of HPTS encapsulated in (A) EPC or (B) EPC/Chol/DOPE/DMPG (61:17:16:6 molar ratio) liposomes at 37 °C. The vesicles were incubated for 20 min without (cross) or with copolymers A (squares), B (diamonds), C (triangles), D (circles), or E (stars) at 150 μg/ml. HPTS release from liposomes was measured by an increase in HPTS fluorescence (λ_{ex} = 413 nm, λ_{em} = 512 nm). One hundred percent content release was obtained by adding Triton X-100 to the liposomes, with the data being expressed as a percentage of total HPTS release. Mean ± S.D. (n = 3).

that observed at 150 μg/ml (Fig. 7). Saturation of adsorption sites at 150 μg/ml may explain these results, since adsorption on membrane bilayers has been shown to be a saturable process for PEAA [39]. Between pH 6.5 and 7.4, there was about 20% adsorption to RBC at 75 μg/ml. At pH 6.0, adsorption increased to 60%. Since partial hemolysis was observed at that pH, this proportion was probably underestimated. After solubilization of RBC lipids, some copolymer may end up in the supernatant and may be included with the unbound fraction. The extent of polymer adsorption to RBC was estimated by dividing the number of adsorbed polymer molecules by the number of RBC. At 75 μg/ml, between pH 6.5 and 7.4, approximately 3×10^6 polymer molecules adsorbed to RBC, and this number increased to 1×10^7 at pH 6.0.

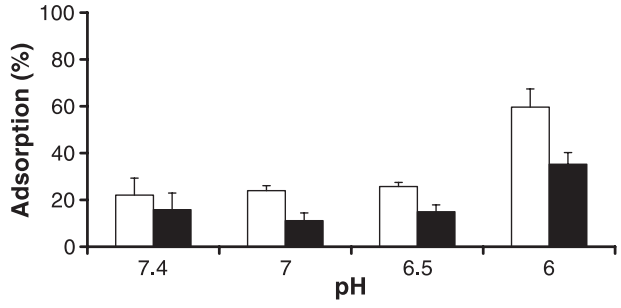


Fig. 7. Adsorption of labeled copolymer B to RBC. The labeled copolymer was incubated with RBC for 30 min at 37 °C at 75 μg/ml (open bars) and 150 μg/ml (closed bars). Samples were centrifuged at 50 × g, and fluorescence of the supernatant was measured (λ_{ex} = 485 nm, λ_{em} = 530 nm). Measurements are expressed relative to 0% adsorption obtained by incubating the labeled copolymer with the appropriate buffer devoid of RBC. Mean ± S.D. (n = 4).

3.5. Cytotoxicity assays

The cellular toxicity of the copolymers was evaluated on macrophage-like cells by MTT (cell proliferation, Fig. 8A) and LDH (cell necrosis, Fig. 8B) assays. The two assays gave comparable results. While copolymers A and D did not show any cytotoxicity, copolymer B was associated with less than 20% cell survival and LDH release of about 35% at concentrations exceeding 12.5 μg/ml. Copolymer C induced mild cytotoxicity (80% cell survival, 15% LDH release) above 250 μg/ml. It is noteworthy that at neutral pH copolymers B and C were hemolytic towards RBC at higher concentrations (≥ 0.5 mg/ml). This greater sensitivity of J774 cells vs. RBC may reflect differences in membrane intrinsic structural properties [40]. Surprisingly, copolymer E, which demonstrated weak hemolytic activity at pH 7.4, showed extensive cytoxicity towards J774 cells at the

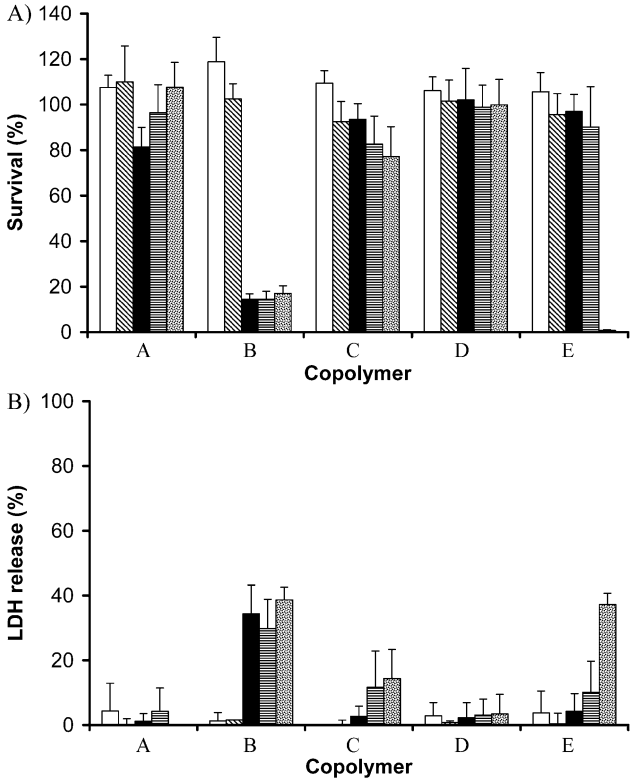


Fig. 8. Copolymer cytotoxicity on J774 cells as a function of concentration as determined by (A) MTT and (B) LDH colorimetric assays at (from left to right) 3.125 μg/ml (white), 12.5 μg/ml (striped), 62.5 μg/ml (black), 250 μg/ml (squared), and 500 μg/ml (dotted). Mean ± S.E. (n = 4).

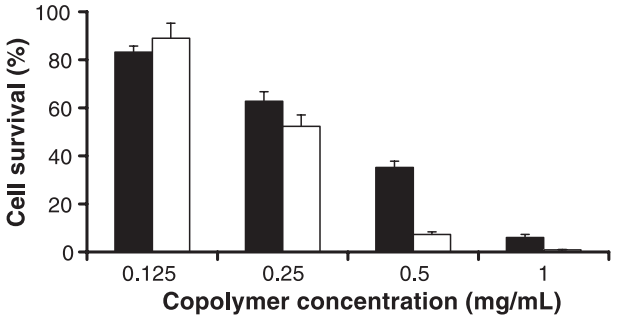


Fig. 9. J774 cell survival, as determined by MTT assay, as a function of copolymer E concentration. The cells were incubated with (closed bars) and without (open bars) 6 μM cytochalasin B. Mean ± S.E. (n = 4).

highest concentration tested (0.5 mg/ml). To determine whether phagocytosis of copolymer E was involved in the significant toxicity observed on macrophage-like cells, a cell proliferation assay was undertaken with this copolymer and cells were pretreated with cytochalasin B, a fungal metabolite frequently used to inhibit actin-dependent functions such as phagocytosis in living eukaryotic cells [41,42]. In the presence of cytochalasin B, there was a five-fold increase in cell survival at 0.5 and 1 mg/ml, the most toxic copolymer concentrations (Fig. 9). Reduction of cell death was less important at lower copolymer concentrations, probably because of cytochalasin B's intrinsic toxicity (about 20% cell death, data not shown).

4. Discussion

4.1. Molecular weight determination

Five different MAA copolymers were characterized with respect to their physicochemical properties, membrane lytic activity, and cytotoxicity. The synthetic copolymers used in this work are a priori not biodegradable. Thus, their elimination from the body would be strictly dependent on renal glomerular filtration. Seymour et al. [43] reported that a MW of 40,000 is the limiting threshold allowing renal elimination of nonbiodegradable *N*-(2-hydroxypropyl)methacrylamide (PHPMA) copolymers. It is known that uncharged polymers behaving as flexible coils (e.g. PHPMA, dextran, poly(*N*-vinylpyrrolidone)) can be filtered through glomerular pores even when their MW is higher than the threshold limiting glomerular filtration of globular, more rigid proteins [44]. The MAA copolymers examined in this study are polyanions in physiological solution. Accordingly, they have a relatively rigid, more extended conformation than corresponding uncharged polymers because of intramolecular electrostatic repulsion. This restricted deformability may decrease their filtration rate compared to flexible polymers with similar MW. Given that these polymers may eventually be administered parenterally, we decided to study copolymers with MWs well below the threshold value established for flexible polymers (Table 1). The MWs found by aqueous SEC at pH 8 may, however, be overestimated owing to aggregation. In water at pH 9, it was previously demonstrated that a random ethyl acrylate (EA)/MAA copolymer (50:50 molar ratio) forms aggregates of a few polymer chains. Aggregate formation would be driven by the EA segment, which is sufficiently blocky to induce hydrophobic associations in the backbone according to an open association mechanism [45,46]. Aggregation at high pH is also supported by a study showing that the addition of β-cyclodextrin to random EA/MAA copolymers reduces the MWs obtained by SEC [47]. Since β-cyclodextrin is known for its ability to shield hydrophobic associations, the resultant MWs probably correspond to those of single polymer chains. Aggregation at neutral pH has also been demonstrated for a random copolymer of acrylic acid containing 29% ethyl methacrylate [22]. Since the copolymers used in this work bear hydrophobic monomers (EA, methyl acrylate and methyl methacrylate) in proportions equal to or greater than 50 mol%, aggregation at neutral pH cannot be excluded.

4.2. Evaluation of copolymer–lipid interaction at neutral pH

The membrane-destabilizing activity of copolymers B and C on both RBC (Fig. 4) and J774 macrophages (Fig. 8) at neutral pH could be explained by the presence of hydrophobic EA units in the backbone. It has been suggested that ethyl chains facilitate interaction with hydrophobic components of the membrane [26,30]. Destabilization of membrane bilayers can still occur with a shorter alkyl chain (i.e. methyl) provided that the polymer is sufficiently hydrophobic (low I_1/I_3 ratio) and its concentration is high (e.g. copolymer D). However, the ability of such a copolymer to interact with the cell membrane at low concentrations is limited, as demonstrated by the weak toxicity of copolymer D towards J774 cells. Interestingly, copolymer E, which contains 10 mol% MAA and no EA, was poorly hemolytic towards RBC, even at 10 mg/ml. A control experiment also demonstrated that copolymer E was unable to trigger HPTS release from EPC liposomes at pH 7.4 (data not shown). Nevertheless, this copolymer was very cytotoxic towards J774 cells at only 0.5 mg/ml.

Why was copolymer E more toxic than copolymer D towards macrophages? The answer to this question probably lies in the conformation adopted by the copolymer in solution. The I_1/I_3 ratio of copolymer E at neutral pH is very low (Fig. 3B). Therefore, at pH 7.4, the copolymer is probably in a globule configuration and exhibits a relatively hydrophobic surface owing to the low concentration of MAA units. Such a conformation would make copolymer E more susceptible to phagocytosis by macrophages [48]. This hypothesis was verified by using a phagocytosis inhibitor. An increase in cell survival was observed at concentrations where the copolymer was the most cytotoxic. These results indicate that, following phagocytosis, the nonbiodegradable polymer

may persist within macrophages as an indigestible residue. Intracellular residue accumulation may ultimately lead to cell damage or death [48–50]. In contrast to J774 cells, RBC cannot internalize foreign particles. Thus, the limited RBC hemolysis observed at pH 7.4 may be explained by strong intramolecular interactions and poor insertion into the phospholipid membrane. The other copolymers were significantly less hydrophobic than copolymer E at pH 7.4 (higher I_1/I_3 ratio), and thus less prone to phagocytosis by J774 macrophages. A hydrophilic copolymer (high I_1/I_3 ratio) bearing no EA, such as copolymer A, is, in turn, inactive toward both RBC and J774 cells at neutral pH.

PEAA adsorption to lipid bilayers at neutral pH has been demonstrated in previous studies [20,39]. Accordingly, to follow polymer adsorption to RBC, copolymer B, which is highly hemolytic, was labeled with fluorescein. At neutral pH, the copolymer was sufficiently hydrophobic to physically bind to RBC (Fig. 7). With increasing concentrations, polymer adsorption can lead to complete membrane solubilization [30]. At copolymer B concentrations inducing 100% hemolysis (≥ 5 mg/ml), membrane solubilization was demonstrated by the absence of a pellet after RBC centrifugation. However, interaction with membrane bilayers with increasing polymer concentrations does not necessarily lead to complete membrane solubilization. We demonstrated by DLS that MAA copolymers (e.g. copolymers D and E) can destabilize EPC liposomes and trigger the release of their contents without solubilizing the vesicles (data not shown).

4.3. Evaluation of copolymer–lipid interaction upon decrease in pH

Polymers bearing pendant carboxylic acid groups undergo conformational transition after a decrease in pH [20–23]. As the acid/ester ratio is decreased, the transition from an extended chain to a compact globule occurs at higher values of ionization, since the charged groups are less numerous, and the charge effects are more rapidly overcome by hydrophobic forces [51]. This finding was made by both aggregation probed by turbidimetry and the creation of hydrophobic domains detected from pyrene fluorescence (Figs. 2 and 3). It is interesting that polymer aggregation and the formation of hydrophobic domains (large enough to accommodate pyrene molecules) do not occur in a concomitant manner. Systematically, hydrophobic domains start to form before the extent of the aggregation is sufficient to be detected by turbidimetry. Copolymer E, for example, appeared to lead to hydrophobic domains over the investigated pH range, while the onset of light intensity variation was observed at pH 7. Neither of these assays is, by itself, predictive of the copolymers' ability to destabilize membrane bilayers. Increase in the pyrene I_1/I_3 ratio allow the monitoring of subtle conformational changes (e.g. the formation of hydrophobic pockets within or between polymer chains) when the polymer is still in solution whereas turbidimetry detects the onset of extensive polymer aggregation. As explained below, the data obtained from both assays should be interpreted by taking into consideration other properties associated with the polymer structure.

Since membrane destabilization was expected to increase under acidic conditions, hemolysis assays as a function of pH were carried out at a low polymer concentration (150 μg/ml). Between pH 5 and 6.5, only the two copolymers bearing EA units (i.e. B and C) were found to be hemolytic (Fig. 5A). Interestingly, the hemolytic activity of both copolymers at pH 5.5 (Fig. 5) was similar to or more pronounced than that reported previously for PEAA and a random copolymer of acrylic acid and EA (1:1 molar ratio) [28]. For all copolymers, the hydrophilic–hydrophobic balance is comparable in terms of the ethyl to carboxyl group ratio (1:1). Murthy et al. [28] demonstrated that membrane lytic activity could be enhanced by increasing the alkyl chain length of monomer units. Poly(propylacrylic acid) exhibited higher hemolytic activity than PEAA, and achieved complete RBC lysis at 15 μg/ml below pH 6.1 [28,52].

Membrane composition was shown to influence the extent of pH-triggered content release. Suspectibility to destabilization by MAA copolymers was in increasing order RBC<EPC:Chol:DOPE:DMPG liposomes<EPC liposomes. RBC contain about 25% Chol [38] and are structurally rigid [40], making this model fairly resistant to destabilization. The addition of Chol to EPC liposomes promotes packing of the bilayer and increases its stability [53]. In the case of PEAA, higher Chol concentrations were shown to inhibit binding of the protonated polymer to the membrane [27]. In addition, the presence of a negatively charged lipid such, as DMPG can generate electrostatic repulsion between the bilayer and the copolymer, thus reducing its adsorption. The experiments carried out with liposomes were helpful in that they allowed discrimination between the membrane lytic activity of the polymers that seemed relatively inactive towards RBC (Fig. 5A). For example, at pH 5.5, copolymer D was more active than copolymer A towards liposomes (Fig. 6), whereas both copolymers were unable to destabilize RBC. This can be explained by the greater hydrophobicity of copolymer D at pH 5.5, as demonstrated by the pyrene fluorescence experiments (I_1/I_3=1.22 and 1.33 for copolymers D and A, respectively; Fig. 3B). However, despite high hydrophobicity at acidic pH, copolymer D was less efficient than B and C. Copolymer D is devoid of EA, which decreases its ability to insert into lipid bilayers.

Interestingly, GALA, an endosomolytic synthetic peptide, was found to be at least 12 times more potent than copolymers B and C in destabilizing EPC vesicles at pH 5.0 (data not shown) [54]. However, its hemolytic activity towards RBC was weak, with 50% hemolysis at 10 mg/ml [9]. At pH 5.0, GALA forms a stable amphiphatic α-helix secondary structure, which is thought to partition in the phospholipid membrane and form small pores or channels [54]. Changes in helical content as pH was decreased was correlated with GALA's lytic activity [8]. GALA's weak

hemolytic activity may result from the size of hemoglobin, which would be too large a molecule to escape the pores [9]. Additionally to helix formation, an increase in GALA's hydrophobicity upon a decrease in pH was associated with liposome lysis [8]. Adsorption of partially ionized (meth)-acrylic acid copolymers to phospholipid bilayers may involve several types of interaction, including hydrophobic interactions and hydrogen bonding. It has been suggested by Seki and Tirrell [20] that hydrogen bonding involving the lipid phosphodiester head group and protonated carboxyl groups of the polymer would be the major driving force for poly(carboxylic acids) binding on the lipid bilayer surface. Membrane destabilization at acidic pH probably comes from increased copolymer binding and pH-dependent change in polymer conformation. At this point, it appears difficult to determine the individual contribution of these two mechanisms since both phenomena are interdependent. Protonation of carboxylic acid groups under acidic conditions triggers coil-to-globule phase transition, but also increases polymer hydrophobicity, and may further contribute to augment binding to the membrane via hydrogen bonding. Interestingly, it was previously shown that acrylic acid copolymers permeabilize membranes by creating defects such as pores or channels. Chung et al. [55] have reported the formation of cation-selective channels through artificial membranes induced by PEAA in a pH-dependent manner. In their study, no ion channels were present at pH 7.6, but they formed rapidly when the pH was reduced to 6.0. Two hypothetical models were suggested to illustrate pore formation by PEAA. In the first model, one or multiple polymer molecules could align their segments across the bilayer, the inner surface of the pore being stabilized by the polymer. Another possibility would be the induction of a change in local curvature of the bilayer, with the PEAA chains localized on the outer surface of the pore. Since the copolymers studied in this work are structurally related to PEAA, similar mechanisms of membrane destabilization can be hypothesized, although the exact nature of the defects remains to be determined.

This study revealed that multiple parameters influence MAA copolymer–lipid interaction at neutral and at acidic pH. First, the presence of EA was found to greatly increase the polymer ability to destabilize membrane bilayers. Second, for efficient destabilization at acidic pH, the copolymers should exhibit a sharp phase transition as measured by turbidimetry or pyrene spectrofluorimetry. Third, the copolymer should not be too hydrophobic at neutral pH to avoid excessive uptake by macrophages. This can be readily assessed by pyrene fluorescence spectroscopy. Among the different copolymers studied in this work, copolymer C is of particular interest. The concentration at which it was highly hemolytic at pH 5.5 (approximately 50% hemolysis) was 10 times lower (50 μg/ml, Fig. 5B) than the concentration at which hemolysis appeared at neutral pH (500 μg/ml, Fig. 4). Moreover, copolymer C did not show any cytotoxicity on macrophage-like cells at concentrations where it was highly hemolytic at acidic pH. Thus, given its good lytic activity and safety profile, copolymer C would be a good candidate for complexation with a drug delivery system. However, to reduce the administered dose, promote cell uptake, and achieve sufficiently high copolymer concentration in the endosomal compartment, the complex should be further decorated with a targeting ligand.

Acknowledgements

This project was funded in part by the Fonds Québécois de la Recherche sur la Nature et les Technologies (scholarship to M.-A. Yessine) and the Canada Research Chair Program. Didier Hoarau is acknowledged for his technical help with the hemolysis assays.

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