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Poly(2-alkylacrylic acid) polymers deliver molecules to the cytosol by ph-sensitive disruption of endosomal vesicles

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Abstract

The permeability barrier posed by cell membranes represents a challenge for the delivery of hydrophilic molecules into cells. We previously proposed that poly(2- alkylacrylic acid)s are endocytosed by cells into acidified vesicles and are there triggered by low pH to disrupt membranes and release the contents of endosomes/lysosomes to the cytosol. If this hypothesis is correct, these polymers could be valuable in drug delivery applications. This report provides functional comparisons of a family of three poly(2-alkylacrylic acid)s. Poly(2-propylacrylic acid) (PPAA), poly(2-ethylacrylic acid) (PEAA), and poly(2-methylacrylic acid) (PMAA) were compared in red blood cell haemolysis assays and in a lipoplex gene transfection assay. We also directly examined the ability of these polymers to disrupt endosomes and lysosomes in cultured human cells. Our results show that (i) unlike membrane-disruptive peptides, the endosomal-disruptive ability of poly(2-alkylacrylic acid)s cannot necessarily be predicted from their hemolytic activity at low pH, (ii) PPAA (but not PEAA or PMAA) potently facilitates gene transfection by cationic lipoplexes, and (iii) endocytosed poly(2-alkylacrylic acid)s are triggered by lumenal acidification to selectively disrupt endosomes (not lysosomes) and release their contents to the cytosol. These results will facilitate the rational design of future endosomal-disrupting polymers for drug delivery.

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Poly(2-Alkylacrylic Acid) Polymers Deliver Molecules to the Cytosol by pH-Sensitive Disruption of Endosomal Vesicles

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Keywords: endocytosis; pH-sensitive polymers; haemolysis; gene delivery; endosomal disruption.

SYNOPSIS

The permeability barrier posed by cell membranes represents a challenge for the delivery of hydrophilic molecules into cells. We previously proposed that poly(2-alkylacrylic acid)s are endocytosed by cells into acidified vesicles and are there triggered by low pH to disrupt membranes and release the contents of endosomes/lysosomes to the cytosol. If this hypothesis is correct, these polymers could be valuable in drug delivery applications. This report provides functional comparisons of a family of three poly(2-alkylacrylic acid)s. Poly(2-propylacrylic acid) (PPAA), poly(2-ethylacrylic acid) (PEAA), and poly(2-methylacrylic acid) (PMAA) were compared in red blood cell haemolysis assays and in a lipoplex gene transfection assay. We also directly examined the ability of these polymers to disrupt endosomes and lysosomes in cultured human cells. Our results show that (i) unlike membrane-disruptive peptides, the endosomal-disruptive ability of poly(2-alkylacrylic acid)s cannot necessarily be predicted from their hemolytic activity at low pH, (ii) PPAA (but not PEAA or PMAA) potently facilitates gene transfection by cationic lipoplexes, and (iii) endocytosed poly(2-alkylacrylic acid)s are triggered by lumenal acidification to selectively disrupt endosomes (not lysosomes) and release their contents to the cytosol. These results will facilitate the rational design of future endosomal-disrupting polymers for drug delivery.

INTRODUCTION

Synthetic polymers have significant potential for the delivery of molecules to cells both in vitro and in vivo. In therapeutic applications, they offer many advantages. Conjugation of small molecules to polymers has been shown to (i) prolong their plasma half-life by increasing their size above that required for renal exclusion [1], (ii) passively enhance their accumulation at sites of solid tumours (due to the "enhanced permeability and retention" effect [2]) (iii) reduce their toxicity and immunogenicity [2]. These features have been exploited in the design of a number of polymer-drug conjugates intended for cancer therapy. Currently, there are at least seven polymer-drug conjugates that have entered phase I/II clinical trial as anticancer agents [3].

A variety of synthetic polymers and peptides have also been used to deliver genes to cells. Several studies have shown that the cationic methacrylate/methacrylamide and polyethylenimine (PEI) polymers can mediate gene delivery [4, 5]. The precise molecular mechanisms by which polymers facilitate gene delivery are still largely unknown, however, it has been suggested that they do this by disrupting endosomes after uptake of complexes with DNA by endocytosis [4]. An elegant study showed that complexes of PEI and DNA aggregate at the cell surface before being internalized within endosomal vesicles, and that subsequently, there was evidence of vesicle disruption and the appearance of complexes inside nuclei [6]. It has been suggested that in the acidic environment inside endosomes/lysosomes, PEI acts as a "proton sponge", and that this behaviour would result in osmotic lysis of vesicles [7]. Anionic polymers [8, 9] and peptides [10-12] have also been used to transfect cells. These delivery-enhancing agents are triggered by mildly acidic pH to undergo a change from a hydrophilic to a hydrophobic membrane-disruptive form, which is thought to facilitate the release of endocytosed molecules from acidic endosomes to the cytosol. Studies of intact cells have confirmed this mechanism of delivery for pH-sensitive peptides [13], however, similar studies are yet to be performed with the anionic polymers.

Tirrell and coworkers first described the pH-dependent membrane-destabilizing

properties of poly(2-ethylacrylic acid) (PEAA) [14]. The red blood cell (RBC) membrane-disruptive activity of (20-30 kDa) PEAA is essentially zero at pH 7.4, but increases rapidly as the pH decreases from 6.3 to 5.0 [15]. PPAA, with an additional methylene unit (Figure 1), was (on a molar basis) 15 times more efficient than PEAA at haemolysis, and reached maximum hemolytic activity at pH 6 (PEAA was most active at pH 5; [15]). These pH profiles and activities also have been shown to depend on molecular weight [16]. The mechanism by which PEAA and PPAA disrupt membranes is unknown but is probably related to their increased hydrophobicity when the carboxylate ions become protonated at low pHs. Under these conditions, the polymers may partition into the cell membrane and disrupt the packing of the lipid bilayer [17]. PPAA has been shown to facilitate the delivery of genes into cells when incorporated into lipoplexes formed with dioleyltrimethylammonium propane (DOTAP) [18]. The only previous study of PEAA as a transfection reagent demonstrated that a single concentration of PEAA tested did not significantly enhance the delivery of an anti-sense oligonucleotide into CHO cells [19]. The relative abilities of PPAA and PEAA to facilitate transfection have not been compared. Moreover, the hemolytic activity of PMAA and its ability to promote the delivery of genes into cells have not previously been tested.

In most cases, polymers are expected to be internalized by cells via endocytosis. Thus, if using a polymer to deliver a hydrophilic molecule into the cell cytosol, it is necessary to overcome the permeability barrier posed by the membranes of endosomes or lysosomes. Earlier work established a good correlation between the abilities of different endosomal-disruptive peptides to disrupt RBCs and to facilitate gene delivery, indicating that the convenient haemolysis assay could be used to identify peptides with high endosomal-disruptive activity [13]. We examined whether poly(2-alkylacrylic acid)s showed a similar correlation in activities by testing the relative ability of PEAA, PPAA and PMAA to lyse RBCs and to transfect cells with a reporter gene. Furthermore, we previously proposed that poly(2-alkylacrylic acid)s are endocytosed by cells into acidified vesicles and are there triggered by low pH to disrupt membranes and release the contents of endosomes/lysosomes to the cytosol. We recently showed that PPAA was able to mediate the delivery of an antibody-streptavidin complex into the cytosol of Jurkat cells [20]. However, the ability of poly(2-alkylacrylic acid)s to disrupt specific endosomal compartments, and the pH-

dependence of this action, have not previously been measured. Therefore, we also tested the ability of poly(2-alkylacrylic acid)s to disrupt endosomes and lysosomes in cultured human cells and determined whether this action was dependent upon vesicle acidification.

EXPERIMENTAL

Materials

DMEM:F-12 was obtained from Trace Biosciences (Sydney, Australia). DMEM was from Gibco (MD, USA). Fetal bovine serum (FBS) and sterile tissue culture plasticware was from either Trace Biosciences or Gibco. Dioleyltrimethylammonium propane (DOTAP) was purchased from Roche (IN, USA). Lipofectamine was from Invitrogen (Melbourne, Australia). Acridine orange, bafilomycin and calcein were from Sigma (MO, USA). Monoclonal antibodies reactive with "early endosomal antigen 1" (EEA1) and lysosomal-associated membrane protein-1 (Lamp-1) were obtained from Pharmingen-Transduction Laboratories (CA, USA). Alexa 488-conjugated goat-anti-mouse Ig was from Molecular Probes (OR, USA). O-methyl-serine dodecylamide hydrochloride (MSDH) was obtained as a kind gift from G. Dubowchik (Bristol-Myers Squibb, CT, USA). The pEGFP-N1 plasmid, encoding enhanced green fluorescent protein, and the pCMV□ plasmid, encoding □-galactosidase, were obtained from Clontech (CA, USA). The pCatB-GFP plasmid, encoding a fusion protein consisting of rat cathepsin B fused at its C-terminus to enhanced green fluorescent protein was obtained as a generous gift from G. Gores (Mayo Medical School, MN, USA). All other chemicals were of reagent quality and were obtained either from Sigma (MO, USA) or Ajax Chemical Co. (Sydney, Australia).

Monomer and Polymer Synthesis

All chemicals and solvents were purchased from Sigma (MI, USA) and were of analytical grade unless otherwise stated. Ethyl and propyl acrylic acid monomers were prepared as previously described [21]. Methylacrylic acid monomer was purchased from Lancaster (NH, USA). The structures of the monomers were verified by NMR. Poly(2-methylacrylic acid) (PMAA), poly(2-ethylacrylic acid) (PEAA), and poly(2-propylacrylic acid) (PPAA) were prepared as described previously [15]. Briefly, polymers were synthesized by free radical polymerization using 2,2'-azobisisobutyronitrile as the initiator at 60 ^{o}C for 48-72 hours. The polymers were dissolved in methanol and purified by ether precipitation. The molecular weights of PMAA (52 kD), PEAA (43 kD), and PPAA (44 kD) were determined by aqueous gel

permeation chromatography using polyethylene glycol standards and 100mM phosphate buffer, pH 8.0 as the eluent.

Haemolysis Assay

The pH-dependent activity of the polymers was tested utilizing a RBC haemolysis assay described previously [15, 22]. The release of hemoglobin from the RBCs was used as a measure of the membrane-disruptive activity of these polymers. Fresh human RBCs were isolated from whole blood by centrifugation and washed three times with 0.15 M NaCl. The RBCs were resuspended in either 100 mM phosphate or 75 mM citrate-phosphate buffer at the desired pH, then diluted in the appropriate buffer to obtain approximately 10^8 RBCs/ml. Polymer (PMAA, PEAA, or PPAA) was added to the buffered RBC suspension and the samples were incubated in a 37 ^oC water bath for 1 hour. In experiments testing the effects of concentration and pH on polymer-mediated haemolysis, the polymers were used at 5 – 50 □g/ml and 10 □g/ml, respectively. The samples were then centrifuged for 4 minutes to pellet any remaining intact RBCs. The absorbance of the supernatant from each sample was measured at 541 nm using a spectrophotometer. Each test was performed in triplicate. Haemolysis levels were normalized to both negative and positive controls. In controls, RBCs were incubated as above in either buffer alone or buffer containing 1 mg/ml dextran (negative control) or in distilled water or buffer containing 10 mg/ml Triton-X 100 (positive control).

Cell Culture and Transfection

U937 (human myelomonocytic) cells were obtained from the American Tissue Type Culture Collection (ATCC; MA, USA) and HEK-293 (human embryonal kidney epithelial) cells were a kind gift from P. Poronick (University of Sydney, Sydney, Australia). These cell types were cultured in DMEM:F-12 containing 10% (v/v) FBS. NIH3T3 (Swiss mouse embryonal) cells (also obtained from ATCC) were cultured in DMEM supplemented with 10% FBS. All cell cultures were maintained in a 37 ^oC incubator with 5% (v/v) CO_2. In preparation for experiments testing the ability of polymers to facilitate transfection, NIH3T3 cells (7.5 x 10^4 cells per well) were seeded into twelve-well plates and incubated at 37 ^oC for 24 hours in serum-containing medium. DOTAP lipoplexes were prepared following a specific mixing protocol. DOTAP was added to the plasmid DNA (pCMV□) and complex formation

allowed to proceed for 30 minutes. The polymer (PMAA, PEAA, or PPAA) was then added to the DOTAP/DNA mixture for an additional 30 minutes. For control mixtures, HEPES-Buffered Saline (HBS, pH 7.4) was added to DOTAP/DNA formulations instead of polymer. All formulations contained 1.6 μg/ml DNA. For formulations containing polymer, the DOTAP concentration was set to 10 μg/ml and polymer concentrations were adjusted to reach the specified theoretical particle charge ratio. The theoretical particle charge ratio was determined as the molar ratio of positively charged amines in DOTAP to the cumulative total of all negatively charged phosphate and carboxyl moieties in the DNA and polymer, respectively. For control formulations, DOTAP concentrations were varied to achieve the proper particle charge ratio. Cells were transfected in serum-free DMEM with these DOTAP formulations for 4 hours at 37 ^{o}C. All formulations were tested in triplicate wells. After 4 hours, the transfection particles were removed and replaced with DMEM supplemented with 10% FBS. The cells were then left to incubate at 37 ^{o}C for 48 hours to allow for protein expression.

β-Galactosidase gene expression was evaluated using the β-Galactosidase Enzyme Assay System (Promega, WI, USA). o-Nitrophenyl-β-D-galactopyranoside was added to cell lysates and the amount of β-galactosidase activity measured spectrophotometrically at 415 nm. The protein content in each of the lysates was determined using the BCA protein assay (Pierce, IL, USA) and β-galactosidase activities expressed relative to protein content. To transiently transfect cells with plasmids encoding EGFP or cathepsin B-GFP fusion protein, HEK-293 cells were seeded into 6-well plates (2 x 10^5 cells per well) and incubated for 18-24 hours at 37 ^{o}C. The cells were then transfected using Lipofectamine and 3 μg of plasmid DNA/well, following the manufacturer's instructions (Invitrogen, Melbourne, Australia). Cells were assayed 48 hours later for expression by confocal or epifluorescence microscopy.

Measuring Polymer-Mediated Release of Tracer Molecules from Intracellular Vesicles

Calcein, a membrane-impermeable fluorophore, was used as a tracer molecule to follow the uptake of the external medium by constitutive endocytosis, and the effects

of polymers on internalized vesicles. In these experiments, 2 x 10^6 U937 cells were centrifuged (for 5 minutes at 300 g) and resuspended in 0.5 ml of polymer buffer (PB; 145 mM NaCl, 5 mM KCl, 0.5 mM HEPES, pH 7.4) containing 2 mg/ml calcein with or without the addition of one of the polymers from stock solutions at up to 16 mg/ml in 0.1 M NaOH. Following the addition of the polymer, and prior to adding to cells, the pH of the PB/calcein/polymer solution was re-adjusted to pH 7.4. Cell suspensions were incubated for 30 minutes at 37 ^{o}C and then washed 3 times with DMEM:F-12 before being resuspended in 10% FBS/DMEM:F-12 and incubated for various lengths of time at 37 ^{o}C. Subsequently, the distribution of calcein within cells was examined by confocal microscopy (see below). In some experiments, 1 □M bafilomycin (a specific inhibitor of the endosomal/lysosomal H^+-ATPase [23]) was added to cell suspensions during calcein uptake and subsequent incubations at 37 ^{o}C. In related experiments, the concentration of HEPES buffer in PB was increased to 50 mM, which was expected to have the effect of alkalinizing the contents of endosomal vesicles [24]; in these experiments, control cells were incubated in 0.5 mM HEPES-buffered PB which had been adjusted to an equivalent osmotic pressure by the addition of 75 mM sucrose. In other experiments, transfected HEK-293 cells expressing a cathepsin B-GFP fusion protein were incubated for 30 minutes at 37 ^{o}C in PB with or without the addition of 50 □g/ml PPAA (or PEAA or PMAA). These cells were then washed with DMEM:F-12, incubated at 37 ^{o}C for up to 4 hours in 10% FBS/DMEM:F-12, and analyzed by confocal microscopy.

Confocal Microscopy and Image Analysis

Confocal microscopy was performed using a Leica DMIRBE inverted microscope coupled to a Leica TCS SP confocal system (Leica Microsystems, Sydney, Australia). Cells were excited at 488 nm and fluorescence collected using an emission window set at 500-540 nm. All signals collected were adjusted to remain within the linear range of the detectors. Images were collected using the TCS NT software (Leica Microsystems, Sydney, Australia). Images incorporated into Figures were exported as TIFF files and prepared for publication by Adobe Photoshop v5.0 software (Adobe Systems, Sydney, Australia).

In initial experiments measuring the release of calcein or cathepsin B-GFP from intracellular vesicles, TCS NT software was used to calculate the average pixel

intensity of calcein fluorescence within regions of interest (ROI) drawn onto collected images. In these cases, for each treatment, images of 15-25 individual cells were analysed as follows. For each cell, the mean fluorescence intensity was determined for the cytoplasm and the "background"; this was done by drawing three ROI inside the cell (to exclude any calcein-containing vesicles; these represented cytoplasm) and three ROI outside the cell (to represent background). The values for each set of three ROI were averaged, then the mean backgound value was subtracted from the mean value for cytoplasm and the result for each cell obtained in arbitrary fluorescence units. Differences between treatments were analysed using the Student's t-test or Oneway ANOVA (JMP software, v3.0.2, SAS Institute Inc., Cary, NC, USA). In experiments analyzing the effects of treatments on Lamp-1 immunofluorescence, the TCS NT software was used to measure, in a constant arbitrary plane, the diameter of "globular" areas of fluorescence within cells (see Figure 9B). In the small number of cases in which there was more than one globular area of fluorescence within a cell, only the largest was measured. The measurement taken was used to score each cell as being in one of four categories: (i) no globular regions of fluorescence (i.e. diameter (grf) = 0 μm), (ii) diameter (grf) = 0.1-1.5 μm, (iii) diameter (grf) = 1.6-3.0 μm, or (iv) diameter (grf) > 3.0 μm. For each treatment, this was done for 50 randomly chosen cells and the results plotted as histograms (see lower panels in Figure 9B).

Immunofluorescence Assays

To examine the morphology and distribution of endosomes and lysosomes, cells were fixed and permeabilized before staining them with monoclonal antibodies that recognize antigens specifically associated with endosomes or lysosomes. In these experiments, cells were treated as described for measuring the release of calcein from endosomal vesicles, except that calcein was deleted from the initial incubation mixture. Following uptake, the cells were incubated for 4 hours at 37 ^{o}C in 10% FBS/DMEM:F-12 before processing for immunofluorescence. In some experiments, polymers were omitted from the initial incubation buffer but 70 μM MSDH (an acidotropic detergent known to disrupt lysosomes [25]) was added to the medium used for the subsequent 4 hour incubation. The cells were then centrifuged (5 minutes at 300 g), resuspended in 0.4 ml of 4% (w/v) paraformaldehyde in phosphate buffered saline (137 mM NaCl, 2.7 mM KCl, 1.5 mM KH_2PO_4, 8 mM Na_2HPO_4, pH 7.4; PBS)

and incubated with shaking at room temperature for 30 minutes. The fixed cells were then washed with PBS and permeabilized by incubating them for 15 minutes on ice in 0.4 ml of 0.5% (v/v) Triton X-100 in PBS. Fixed, permeabilized cells were washed with 0.1% (w/v) bovine serum albumin (BSA) in PBS (0.1% BSA/PBS), then suspended in 50 μl of either anti-EEA1 or anti-Lamp-1 antibodies (diluted 1:50 in 0.1% BSA/PBS) and incubated on ice for 1 hour. EEA1 is an antigen localized in early endosomes [26], while Lamp-1 is a major lysosomal membrane protein [27]. The cells were then washed with BSA/PBS, suspended in 50 μl of goat-anti-mouse Ig-Alexa 488 (diluted 1:50 in BSA/PBS) and incubated on ice for 1 hour.

Lysosomal Staining

To further test the effects of polymers on lysosomes, we stained live, unfixed control cells and cells that had been exposed to polymers (as described above for the immunofluorescence assays) with acridine orange, a fluorescent acidotropic probe. Acridine orange accumulates to high concentrations in the lysosomal lumen where it forms red-fluorescing dimers; lysosomal disruption can be detected as a decrease in red fluorescence [28]. Cells were incubated in DMEM:F-12 containing 5 μg/ml acridine orange for 15 minutes at 37 ^{o}C, then washed with PBS before being analysed by flow cytometry (see below). In some experiments, cells were treated with MSDH (as described above) before staining with acridine orange.

Flow Cytometry

Flow cytometric analysis of acridine orange-stained cells was performed using a FACSort flow cytometer (Becton Dickinson, Sydney, Australia). The cells were excited with a 488 nm Argon laser and red fluorescence collected using a 650 nm long pass filter. In later experiments measuring the effects of bafilomycin and increased buffer strength on the polymer-mediated release of calcein from endosomes, we exploited the fact that release of calcein to the cytosol was associated with an increase in overall cell fluorescence. This presumably resulted from (i) a reduction in self-quenching of calcein fluorescence that otherwise occurred when calcein was present at high concentration inside endosomes, and (ii) the transition of calcein from a pH less than 6.0 (inside some endosomes) to greater than 7.0 in the cytosol, which would increase calcein fluorescence [29]. In these experiments, cells were excited at

488 nm and green fluorescence collected using a 530 +/- 30 nm band pass filter; cells with a fluorescence 3.5 times (or more) that of the peak channel number for control cells were scored as having “high calcein fluorescence” (these represent only that fraction of cells with extensive, high-level release of calcein to the cytosol). Preliminary experiments with bafilomycin indicated the same pattern of results using either this approach or the more laborious confocal microscopy method outlined above (data not shown). In all experiments, for each sample, 10,000 events were acquired. Data was analysed using CellQuest software (v3.1; Becton Dickinson, Sydney, Australia); where appropriate, the statistical functions of this software were used to quantify the proportion of cells with fluorescence different to that of controls.

RESULTS

Haemolysis Assay

The haemolysis assay was used to compare the membrane-disrupting activity of the different poly(alkylacrylic acid)s, which were of similar molecular weights (43-52 kDa). The ability of PMAA, PEAA, and PPAA to hemolyze RBCs at polymer concentrations ranging from 5 to 50 □g/ml was evaluated at both pH 6.2 and 7.4. At pH 6.2, which is representative of the lower pHs encountered in early endosomes, PPAA had the highest hemolytic activity of all the polymers tested (Figure 2). Complete haemolysis was practically attained with 5 □g/ml of PPAA, whereas only low to negligible levels of haemolysis were achieved with both PEAA and PMAA at all concentrations tested (a maximum of about 20% haemolysis for 50 □g/ml PEAA). PPAA also exhibited low activity at high concentrations at physiological pH (pH 7.4), with approximately 30% haemolysis measured at 50 □g/ml. A subsequent experiment evaluated the pH-sensitivity of haemolysis for each polymer. The extent of haemolysis for each polymer was tested at pHs ranging from 5.0 to 7.4, mimicking the pH range the polymer molecules are expected to encounter during their translocation through the endocytic pathway, and at a concentration of 10 □g/ml for each polymer. PMAA showed little hemolytic activity at all pHs tested, reaching a maximum of about 2% at pH 5.0 (Figure 3). In contrast, both PEAA and PPAA showed significant levels of haemolysis (Figure 3). As the pH decreased, the hemolytic activity of PEAA increased, peaking at pH 5.4. In contrast, the hemolytic activity of PPAA was reduced at low pH but increased with pH to a maximum at pH 5.8-6.2. An apparent slightly negative hemolytic value was obtained for PPAA at pH 5.0; this can be attributed to a higher level of haemolysis observed in the negative controls at low pH, which affected the calculation of the normalized level of haemolysis.

Effects of Polymers on Transfection Efficiency

Transfection experiments were performed to determine the ability of cationic lipoplexes incorporating PMAA, PEAA or PPAA to transfect cells effectively. DOTAP lipoplexes were utilized as the cationic carrier to deliver the pCMV□ plasmid DNA (which encodes □-galactosidase) plus polymer into NIH3T3 murine fibroblasts.

Initial experiments compared transfection efficiencies attained with DOTAP formulations prepared with each polymer at varying particle charge ratios. Results from these formulations were compared in turn with DOTAP control formulations that lacked polymer. The highest level of gene expression was obtained with DOTAP particles containing PPAA prepared at a 1.0 (+/-) charge ratio (see Materials and Methods), which produced about a 25-fold enhancement of expression compared to that of the control (Figure 4). These particles contained the highest quantity of PPAA tested. In addition, relative to controls, DOTAP/DNA/PPAA formulations prepared at 1.3 (+/-) and 1.6 (+/-) charge ratios showed statistically significant increases in gene expression (Student's t-test, $p < 0.05$). Compared with controls, formulations containing PMAA and PEAA showed little or no increase in the transfection efficiency. DOTAP control formulations prepared at higher charge ratios did not enhance expression efficiencies (data not shown).

Endocytosed Polymers Release Calcein from Intracellular Vesicles

When U937 cells were incubated in buffer containing the membrane-impermeable fluorophore calcein, they subsequently showed fluorescent intracellular vesicles, consistent with constitutive endocytosis of the external medium (Figure 5A, panel a). When PEAA was included in the buffer together with calcein, there was a time-dependent release of calcein from the vesicles to the cytosol. After 1.5 h, many cells showed increased cytosolic calcein fluorescence, indicating partial release of calcein from endosomes (Figure 5A, most cells in panel b); in some cells, endosome fusion was apparent prior to substantial calcein release (same panel, cell indicated with white arrow, and data not shown). After 3.5 h, virtually all cells showed extensive release of calcein to the cytosol (Figure 5A, panel c). Similar results were obtained when the same experiments were repeated with Jurkat cells (data not shown). PPAA and PMAA were also able to mediate the release of calcein from intracellular vesicles (Figure 5B). The reason(s) for the variability in the timing of calcein release between individual cells are unknown but it might result from differences in the rates of endocytosis occurring in individual cells during their incubation with polymer.

Quantitative analysis of images acquired by confocal microscopy indicated that, under the conditions tested, all three polymers induced a progressive release of calcein from vesicles to the cytosol. For PPAA and PEAA, calcein release to the cytosol was first

detected immediately following calcein uptake (Figure 5B). PPAA and PEAA induced calcein release with similar kinetics, although the extent of calcein release was generally slightly greater for PPAA. The release of calcein mediated by PMAA was slower and less that that mediated by the other two polymers (Figure 5B). The ability of the polymers to mediate calcein release showed a biphasic dependence on concentration. For any one polymer, the level of calcein release was comparable for polymer concentrations of 5-25 □g/ml but increased similarly to a higher level at 50 and 200 □g/ml of polymer (Figure 6). For each polymer, comparing the levels of calcein release at 25 □g/ml with that at either 50 or 200 □g/ml, statistically significant differences were obtained for both 50 and 200 □g/ml PPAA (Oneway ANOVA, $F_{4,50}$ = 13.87, pairwise Tukey-Kramer comparisons, $p < 0.001$) but not for either of the higher concentrations of PEAA or PMAA.

Polymers Release a Cathepsin B-GFP Fusion Protein from Intracellular Vesicles

Our previous results demonstrated that the poly(2-alkylacrylic acid) polymers could mediate release of the 622 Da fluorophore calcein from intracellular vesicles. To investigate whether the polymers could also mediate the release of macromolecules from vesicles, we transiently transfected HEK-293 cells with pCatB-GFP plasmid encoding a ~ 64 kDa fusion protein consisting of cathepsin B (which is concentrated in lysosomes but is also found in early and late endosomes [30]) fused at its C-terminus to enhanced green fluorescent protein. Cells expressing enhanced green fluorescent protein alone showed a largely homogeneous distribution of green fluorescence within the cell (Figure 7, panel a). However, cells expressing cathepsin B-GFP showed a restricted distribution of intracellular fluorescence consistent with the fusion protein being localized within endosomes/lysosomes (Figure 7, panel b; also see [31]). When these latter cells were incubated in PB containing 50 □g/ml PPAA for 30 minutes, and subsequently examined by confocal microscopy, the intracellular fluorescence was more diffuse (Figure 7, panel c), indicating partial release of cathepsin B-GFP to the cytosol. Quantitative image analysis (performed as described in Materials and Methods) indicated that under the conditions tested, uptake of PPAA caused a statistically significant 2.4-fold increase in the cytosolic level of green fluorescence in pCatB-GFP transfectants (Student's t-test, $p < 0.001$; data not shown). Similar changes in the distribution of fluorescence were also found when

pCatB-GFP-transfected cells were treated as above with PEAA or PMAA, but not when they were treated the same in the absence of any of the polymers (data not shown). Collectively, the above results indicate that endocytosed poly(2-alkylacrylic acid)s are able to mediate the release of small and macro-molecules from intracellular vesicles.

Polymer-Mediated Disruption of Intracellular Vesicles is pH-Dependent

Results shown here (Figures 2 & 3) and elsewhere [32] indicate that poly(2-alkylacrylic acid)s show enhanced membrane-disruptive activity at reduced pH. The default pathway for endocytosed materials is to be carried through vesicle compartments towards lysosomes for eventual degradation. During this trafficking, the contents of the vesicles become progressively acidified, reaching a pH of less than 5.0 in lysosomes [33]. We reasoned that endocytosed poly(2-alkylacrylic acid)s would probably follow this pathway and when the vesicle contents became sufficiently acidic, the polymers would adopt a conformation competent to disrupt membranes. Acidification of the lumen of endosomes/lysosomes is effected by an H^+-ATPase found in the vesicle membrane, which can be specifically inhibited by bafilomycin [34]. We showed that bafilomycin inhibited the release of calcein from intracellular vesicles, mediated by either PEAA (Figure 8), PPAA or PMAA (data not shown). In each case, the effect of bafilomycin was statistically significant (Student's t-test, $p < 0.02$). These results suggest that acidification of the vesicle contents effected by the H^+-ATPase is required for the polymer-mediated disruption of vesicles. This interpretation was confirmed by showing that increasing the buffering of the solution from which cells endocytosed calcein and polymers, from 0.5 mM to 50 mM HEPES, significantly inhibited the subsequent release of calcein to the cytosol (Student's t-test, $p < 0.001$) (Figure 8). Cells incubated with bafilomycin or 50 mM HEPES showed the same total level of calcein uptake as control cells and under conditions when cytosolic calcein fluorescence was reduced relative to that of control cells, calcein remained within membrane-bound compartments (data not shown).

The Effects of Poly(2-Alkylacrylic Acid)s on Different Endosomal Compartments

Endocytosis of the poly(2-alkylacrylic acid)s resulted in the subsequent release of cathepsin B-GFP fusion protein to the cytosol (Figure 7). This indicates that the

polymers disrupt one or more of the compartments in which cathepsin B is normally located. Furthermore, acidification of compartment(s) by the vesicle H^+-ATPase is required for efficient polymer-mediated disruption of vesicles (Figure 8). The major intracellular sites of cathepsin B, and intravesicular acidification, are the early and late endosomes and lysosomes [30]. Thus, one or more of these compartments are likely to be disrupted by the uptake of poly(2-alkylacrylic acid)s. In an attempt to investigate this further, we used immunofluorescence to examine the effects of individual polymers on the morphology and distribution of different vesicle populations.

Early endosomes, stained with anti-EEA1 antibody, appeared as roughly circular, brightly fluorescent patches distributed primarily at the cell periphery (Figure 9A). There was no visible effect of any of the polymers tested on the morphology or distribution of early endosomes (Figure 9A, compare the two panels, and data not shown). The lysosomal disruptive agent MSDH also had no effect on early endosomes (data not shown). The pattern of immunofluorescence obtained with anti-Lamp-1 antibody was quite different. Control cells showed one or (in some cases) two "globular" areas of bright fluorescence (2-4 □m in diameter) positioned within the cell, together with what appeared to be numerous smaller fluorescent vesicles distributed throughout the cell (Figure 9B, panel a). Treatment of cells with MSDH caused a significant change in the pattern of Lamp-1 immunofluorescence, resulting in disappearance of the large globular areas of fluorescence in most cells (Figure 9B, upper panel b versus upper panel a). MSDH did not appear to affect the many, smaller fluorescent vesicles, which persisted. None of the polymers tested obviously affected these smaller vesicles either. However, like MSDH, the polymers did cause a reduction in the size and frequency of occurrence of "globular" areas of Lamp-1-specific fluorescence in cells (Figure 9B, compare panels c-e to panel a). This effect was similar to, but less extensive than that produced by MSDH, with PMAA showing the smallest effect. Image analysis confirmed this interpretation (see histograms in the lower panels of Figure 9B).

Lamp-1 is a major lysosomal membrane protein but is also found in the membranes of late endosomes and the trans Golgi [27]. The lumen of late endosomes is acidified to a pH of about 5.5, and that of lysosomes down to as low as pH 4.6-5.0 [35]. In

contrast, the Golgi is only very weakly acidified, with a lumenal pH of about 6.5 [36]. Thus, the changes in the pattern of Lamp-1 immunofluorescence induced by uptake of the polymers probably result from disruption of the strongly acidified late endosomes or lysosomes. To investigate this further we used acridine orange to test the effects of polymer uptake on lysosomes. Flow cytometric analysis of cells stained with acridine orange indicated that MSDH induced loss of lysosomal integrity but that uptake of any of the polymers did not (Figure 9C). In addition, in related experiments we found that uptake of any of the polymers had only limited effects on lysosomal morphology, visualized by confocal microscopy of cells stained with LysoTracker Yellow (Molecular Probes, OR, USA) (data not shown). Collectively, these results suggest that at 4 hours after uptake, poly(2-alkylacrylic acid)s had not physically disrupted lysosomes. Lastly, to confirm that poly(2-alkylacrylic acid)s do not disrupt the trans-Golgi, we demonstrated that their uptake had no significant effect on the pattern of immunofluorescence obtained using a monoclonal antibody reactive with golgin-97, a trans-Golgi-localized antigen [37] (data not shown).

DISCUSSION

To minimize any contribution of the effects of molecular weight on polymer activity [16] to the comparative studies performed, the poly(2-alkylacrylic acid)s used in this study were all of similar molecular weight (43-52 kDa). At pH 6.2, the hemolytic activity of the poly(2-alkylacrylic acid) polymers increased from PMAA to PEAA and then PPAA, coinciding with their increasing hydrophobicity (Figure 2). This supports the interpretation that the hydrophobicity of the polymers is an important determinant of their membrane-disruptive activity. Both PEAA and PPAA were effective at haemolysis at the low pH values tested, however, PPAA was more efficient in the range pH 5.8-6.6 while PEAA was more efficient in the range pH 5.0-5.8 (Figure 3). At pH < 5.0, PPAA has a tendency to precipitate from aqueous solution (data not shown). Thus, the greater hydrophobicity of PPAA may explain why it had reduced hemolytic efficiency at the lower pH values tested; in these experiments, at pH 5.0-5.4, PPAA may have formed micro-aggregates with a reduced ability to disrupt membranes. Previous work suggested that the poly(2-alkylacrylic acid)s are likely to deliver molecules into cells by endocytic uptake followed by low pH-induced disruption of endosomes/lysosomes to release their contents to the cytosol [15]. The haemolysis results suggest that PPAA might achieve maximum membrane-disruptive activity during the earlier stages of endosomal acidification, whereas PEAA might only achieve this in the final most acidic endosomal compartments (but see below).

The ability of both PPAA and PEAA to hemolyze RBCs within the pH range found inside acidified endosomes led to the expectation that both polymers might enhance the cytosolic delivery of DOTAP cationic lipoplex vectors for gene transfection. PPAA was previously shown to enhance the transfection efficiency of DOTAP lipoplexes but the influence of polymer hydrophobicity on transfection efficiency was not investigated [18]. The current study shows that of the three poly(2-alkylacrylic acid)s tested, only PPAA significantly enhanced the transfection efficiencies obtained with DOTAP lipoplexes (Figure 4). This result was somewhat surprising, considering that effective haemolysis was also achieved with PEAA at acidic pHs. Given the structural similarity between the three polymers tested, it is likely that the increased

hydrophobicity of PPAA, compared with the other polymers, accounts for its increased transfection efficiency. This may allow PPAA (but not PMAA or PEAA) to maintain its ability to disrupt membranes at the concentrations available when the lipoplexes are taken up into endosomes.

In preliminary work using dual-color confocal microscopy, we showed that following a 30 minute incubation in medium containing biotinylated PEAA, U937 cells contained the polymer in punctate structures located primarily at the periphery of the cell, about 30% of which stained with anti-EEA1 antibody (data not shown). This suggests that the polymer, which is hydrophilic at pH 7.4, is taken up by endocytosis and transits through early endosomes before moving onto other vesicle compartments. Similarly, when U937 cells were incubated in buffer containing the membrane-impermeable fluorophore calcein, they subsequently showed fluorescent intracellular vesicles, consistent with constitutive endocytosis of the external medium (Figure 5A, panel a). Uptake of poly(2-alkylacrylic acid)s induced fusion of calcein-containing endosomes followed by release of calcein to the cytosol (Figure 5A). This effect appears similar to the PEAA-induced fusion of phosphatidylcholine vesicles observed in vitro at mildly acidic pH [38].

Time course studies indicated that PPAA and PEAA disrupted vesicles with similar kinetics, with PPAA releasing slightly more calcein from vesicles to the cytosol than PEAA. PMAA produced the least disruption of vesicles and its effects were the slowest of the three polymers tested (Figure 5B). Endosomal-disruptive peptides were shown to induce significant release of fluorescent dextran from intracellular vesicles 15 minutes after endocytic uptake, although no measurements of the time dependence of the extent of release were reported [13]. Both PPAA and PEAA mediated measurable release of calcein from vesicles immediately after uptake, however, the extent of release increased progressively with time reaching a maximum at about 4 hours after uptake (Figure 5B). Thus, although no direct comparisons have been made, it appears that once endocytosed, poly(2-alkylacrylic acid)s may act more slowly than pH-sensitive peptides to disrupt endosomes. One factor that may contribute to this apparent difference is that endosomal-disruptive peptides only require protonation of a small number of side-chain groups (e.g. 3) to bring about a transition to a membrane-disruptive conformation [13]. In contrast, poly(2-

alkylacrylic acid)s are polyanions at physiological pH; when internalised within endosomes they will act as polyvalent buffers and will require the transport of hundreds of H^+ ions for each polymer molecule before becoming membrane-disruptive. Another reason that poly(2-alkylacrylic acid)s may be slower acting than peptides is that the former appear unable to disrupt lysosomes (see below). If undegraded endocytosed materials are continuously recycled between late endosomes and lysosomes, as has been suggested [33], then the polymers may only mediate the release of molecules when they are intermittently and transiently resident in late endosomes.

The faster kinetics and greater extent of vesicle disruption mediated by PPAA and PEAA versus PMAA probably result from the greater hydrophobicity of PPAA and PEAA. Two observations suggest that, unlike endosomal-disruptive peptides [13], the endosomal-disruptive ability of poly(2-alkylacrylic acid)s cannot necessarily be predicted from their hemolytic activity at low pH: (i) despite poor hemolytic activity, PMAA is able to mediate release of calcein from endosomes, and (ii) despite different patterns of dependence of hemolytic activity on pH, both PPAA and PEAA release calcein from endosomes with similar kinetics. These findings may result from differences in the structure and physical properties of RBC and endosomal membranes that affect the action of poly(2-alkylacrylic acid)s but not that of membrane-disruptive peptides. Even when endocytosed from concentrations as low as 5 □g/ml (i.e. about 100 nM), all three polymers tested were able to mediate release of calcein from endosomes to the cytosol (Figure 6). The reason why the endosomal disruptive effect of the polymers shows a biphasic pattern of dependence on polymer concentration (see Figure 6) is unknown. However, it is possible that when present at higher concentrations in an acidic environment, individual polymer molecules may co-operate in some way to more efficiently disrupt membranes.

We demonstrated for each of the poly(2-alkylacrylic acid)s that their ability to disrupt vesicle membranes was substantially decreased when the contents of the vesicles were alkalinized by inhibiting the endosomal/lysosomal H^+-ATPase or by strongly buffering the medium from which the polymers were endocytosed (Figure 8). These results indicate that the progressive acidification of endosomes triggers internalized poly(2-alkylacrylic acid)s to become membrane-disruptive. We showed that poly(2-

alkylacrylic acid)s mediated the release of both a small molecule (622 Da calcein; Figure 5) and a macromolecule (a 64 kDa cathepsin B-GFP fusion protein) (Figure 7) to the cytosol. In addition, we recently showed that PPAA similarly mediated release of a large (~ 264 kDa) polymer-antibody-streptavidin complex to the cytosol [20]. Collectively, the results suggest that poly(2-alkylacrylic acid)s can sufficiently disrupt acidified endocytic vesicles to effect the indiscriminant release of their contents to the cytosol. The major types of acidified intracellular vesicles are the early and late endosomes and lysosomes. These are also the major compartments through which most endocytosed materials pass and the presumptive location of expressed cathepsin B-GFP [31]. Thus, we reasoned that one or more of these three compartments would be the site(s) disrupted by endocytosed poly(2-alkylacrylic acid)s.

There were no measurable effects of polymer uptake on the pattern of EEA1 immunofluorescence (Figure 9A), suggesting that early endosomes were unaffected. The early endosome is the first compartment that endocytosed materials are delivered to and its lumen is mildly acidic at pH 6.0-6.2 [39]. Despite that fact that PPAA has potent hemolytic activity at this pH (Figure 2), it does not appear to disrupt early endosomes (Figure 9A). As discussed above, this may result from differences in the structure of RBC and endosomal membranes. In contrast to the results with anti-EEA1 antibody, uptake of all three poly(2-alkylacrylic acid)s produced changes in the pattern of Lamp-1 immunofluorescence (Figure 9B). These results suggested that endocytosed polymers were disrupting late endosomes and/or lysosomes, which are highly acidified and together comprise the major cellular sites of Lamp-1 expression. Three lines of evidence indicate that the polymers tested did not disrupt lysosomes. Firstly, flow cytometric analysis of cells stained with acridine orange (which accumulates inside lysosomes [28]) 4 hours after uptake of polymers, when extensive release of calcein from vesicles could be measured, indicated that lysosomes remained structurally intact (Figure 9C). Secondly, we were unable to detect disruption of lysosomes by confocal microscopical examination of cells stained with LysoTracker Yellow (data not shown). Thirdly, extensive lysosomal bursting is known to induce cell death [40]; we found that even after continuous exposure of cells to 200 □g/ml of PPAA for 24 hours, about 90% of cells remained viable (data not shown). Lastly, we eliminated the possibility that poly(2-alkylacrylic acid)s might be transported from endosomes to the trans-Golgi (which expresses some Lamp-1

antigen), and there disrupt the Golgi, by demonstrating that the distribution of a trans-Golgi-localized antigen, golgin-97, was unaffected by polymer uptake (data not shown).

It is unclear why endocytosed poly(2-alkylacrylic acid)s do not physically disrupt lysosomes, which are highly acidified. However, two possible explanations (which are not mutually exclusive) are: (i) The lysosomal membrane may be resistant to the disruptive effects of the polymers. It is known that the lumenal face of the lysosomal membrane is heavily coated with a family of glycoproteins that are thought to provide the membrane with protection from the many lytic enzymes in the lumen [41]. This coating may also sterically shield the membrane from the actions of the protonated, hydrophobic polymers; (ii) At a pH of less than 5.0 which found in lysosomes, if present at a sufficient concentration, the heavily protonated, hydrophobic polymers may precipitate and become ineffective as membrane disruptants. Supporting the latter possibility, as stated above, PPAA precipitates from aqueous solution at pH < pH 5 (data not shown).

Collectively, the results indicate that (i) cells internalize poly(2-alkylacrylic acid)s via endocytosis, (ii) H^+-ATPase–mediated acidification of the lumen of endosomes triggers their disruption by the polymers and the release of vesicle contents to the cytosol, and (iii) early endosomes do not appear to be affected by the polymers and lysosomes are resistant to their effects, thus the major endosomal compartment disrupted is probably the late endosomes. Although all the polymers tested were able to mediate measurable endosomal disruption, only PPAA mediated efficient intracellular delivery of DNA. This suggests that the degree of polymer hydrophobicity may be an important parameter for the delivery of intact macromolecular therapeutics. It also indicates that the ability of the free polymers to disrupt endosomal membranes is not necessarily equivalent to their ability to deliver macromolecules in a delivery system, because their activity is expected to be altered when they are conjugated or complexed to more hydrophilic components. These important insights into the mechanism by which poly(2-alkylacrylic acid)s facilitate delivery of membrane-impermeable molecules into the cell cytosol will help in the rational design of future endosomal-disrupting polymers.

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ABBREVIATIONS LIST

BSA	bovine serum albumin
DOTAP	dioleyltrimethylammonium propane
FBS	fetal bovine serum
GFP	green fluorescent protein
PBS	phosphate-buffered saline
MSDH	O-methylserine dodecylamide hydrochloride
PEAA	poly(2-ethylacrylic acid)
PMAA	poly(2-methylacrylic acid)
PPAA	poly(2-propylacrylic acid)
RBC	red blood cell
ROI	regions of interest

FIGURE LEGENDS

Figure 1 Structure of the poly(2-alkylacrylic acid)s, (A) polymethylacrylic acid (PMAA), (B) polyethylacrylic acid (PEAA) and (C) polypropylacrylic acid (PPAA).

Figure 2 Concentration dependence of poly(2-alkylacrylic acid)-mediated haemolysis at (A) pH 6.2 and (B) pH 7.4 (see key). Data shown represent the means of triplicate measurements. In (B), the symbols for PMAA are essentially overlaid by those for PEAA and are thus difficult to see. The error bars shown are s.e.m. and in many cases are too small to be visible. The results shown are representative of several independent experiments.

Figure 3 pH dependence of poly(2-alkylacrylic acid)-mediated haemolysis. In these experiments, the polymers (see key) were used at 10 □g/ml. Data shown represent the means of triplicate measurements. The error bars shown are s.e.m. and in many cases are too small to be visible. The results shown are representative of several independent experiments.

Figure 4 Effect of the complex charge ratio on polymer-mediated transfection efficiency. Histogram plot shows the relative transfection efficiencies of NIH3T3 cells (measured as expression of □-galactosidase activity) for different charge ratios (see Materials and Methods) of complexes formed with DOTAP, plasmid DNA and PPAA, PEAA or PMAA (see key). Data shown represent the means of triplicate measurements. The error bars shown are s.e.m.

Figure 5 Poly(2-alkylacrylic acid)s mediate release of calcein from endosomes to the cytosol. (A) Confocal microscopy images of intact U937 cells showing the distribution of calcein fluorescence in cells that had been incubated for 30 minutes in PB containing 2 mg/ml calcein (a; immediately after uptake) or the same concentration of calcein and 200 □g/ml of PEAA (at 1.5 hours (b) or 3.5 hours (c) after uptake). The white arrow in (b) indicates apparent vesicle fusion preceding calcein release. PPAA and PMAA induced similar changes (data not shown). Even many hours after uptake, cells that were not exposed to polymer showed a punctate

pattern of fluorescence similar to that in (a). (B) Plot showing the results of quantitative analysis of images like those in (A), showing for no added polymer, PPAA, PEAA and PMAA (see key) the dependence of cytosolic calcein fluorescence on the time after uptake. See the Materials and Methods for details of the analyses performed. Data points represent the means of values determined for 15-25 cells. The error bars shown are s.e.m. and in some cases are too small to be visible.

Figure 6 Dependence of endosomal calcein release on the concentration of poly(2-alkylacrylic acid)s. Plots show the level of cytosolic calcein fluorescence in U937 cells (determined as described in Materials and Methods), 4 hours after a 30 minute incubation in PB containing 2 mg/ml calcein and the indicated concentrations of PEAA, PPAA or PMAA (see key). Each data point represents the mean of values determined for 15 cells. The error bars shown are s.e.m.

Figure 7 Poly(2-alkylacrylic acid)s mediate release of cathepsin B-GFP fusion protein from endosomes to the cytosol. Confocal microscopy images of transiently transfected HEK293 cells showing (a) general cytoplasmic fluorescence in cells transfected with a plasmid encoding (cytosolic) GFP, (b) fluorescence restricted to intracellular vesicles in cells transfected with a plasmid encoding a cathepsin B-GFP fusion protein, and (c) the same type of transfectants shown in (b) 4 hours after incubation in PB containing 50 □g/ml PPAA, showing release of cathepsin B-GFP from vesicles to the cytosol. Release of cathepsin B-GFP to the cytosol was also visible at earlier time points following polymer uptake (data not shown). PEAA and PMAA were also able to mediate release of cathepsin B-GFP to the cytosol; this release did not occur in cells treated similarly but in the absence of poly(2-alkylacrylic acid)s (images not shown).

Figure 8 Alkalinization of endosomes inhibits poly(2-alkylacrylic acid)–mediated release of calcein to the cytosol. Results of flow cytometric analyses of U937 cells from two independent experiments measuring, 1.5 hours after a 30 minute incubation in PB containing 2 mg/ml calcein and (where indicated) other additions, only that proportion of cells with extensive, high-level release of calcein to the cytosol (see Materials and Methods). *Left histogram plots* show results for control (cells incubated

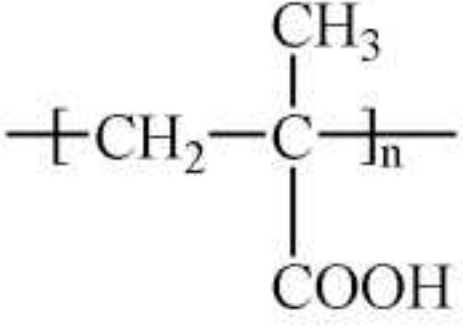
only with calcein) and PEAA (cells incubated with calcein and 200 □g/ml PEAA). Where indicated (PEAA/BAF), cells were also treated with 1 □M bafilomycin to inhibit H^+-ATPase-mediated acidification of endosomal vesicles. *Right histogram plots* show results from a second similar experiment in which, where indicated (HEPES), the buffering of the PB was increased from 0.5 mM to 50 mM HEPES. In all cases, the data shown represent the means of triplicate determinations; error bars are s.e.m. Pairwise comparisons indicated that bafilomycin and 50 mM HEPES significantly inhibited the release of calcein (Student's t-test, $p < 0.001$); the small difference between HEPES/control and control (right histogram plots) is not statistically significant. Both bafilomycin and 50 mM HEPES also caused significant inhibition of calcein release at 0 – 0.5 hours, and at 3.5 hours after uptake (data not shown).

Figure 9 Effects of the uptake of poly(2-alkylacrylic acid)s on different endosomal compartments. (A) U937 cells were fixed and permeabilized, 4 hours after after a 30 minute incubation in PB containing various additions, before being analyzed by confocal microscopy. Images show EEA1-positive vesicles (early endosomes) in cells incubated with no added polymer (left panel, control) or 200 □g/ml of PEAA (right panel). As for PEAA-treated cells, images of cells incubated with either PPAA or PMAA were similar to those of control cells (data not shown). (B) Confocal microscopy images of U937 cells prepared as in (A) except stained with anti-Lamp-1 antibody, showing: (upper panel) Lamp-1-positive vesicles in cells incubated with (a) no added polymer, (b) 70 □M MSDH (to disrupt lysosomes), or 200 □g/ml of (c) PEAA, (d) PPAA, or (e) PMAA. Each histogram plot (lower panel) summarises the results of quantitative image analysis of the diameter of "globular" areas of fluorescence in a constant arbitrary plane within 50 cells treated as in the image above (see Experimental for more details regarding this analysis). (C) Overlay flow cytometry histograms showing U937 cells stained with acridine orange 4 hours after a 30 minute incubation in PB containing no additions (thin solid line) or 200 □g/ml PEAA (dashed line), or after a 4 hour incubation in culture medium containing 70 □M MSDH (thick solid line). The result shown is representative of several independent experiments. In similar experiments, we showed that the uptake of PPAA and PMAA

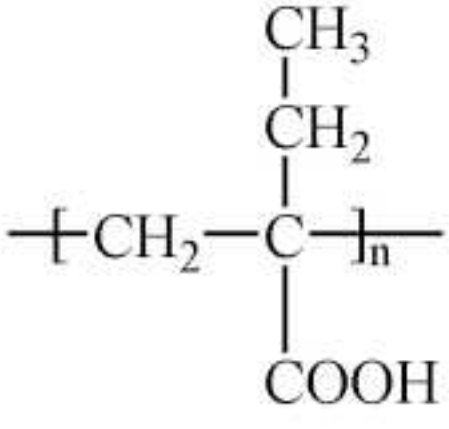
also had no effect on the level of red fluorescence associated with acridine orange-stained cells (data not shown).

Figure 1

A



B



C

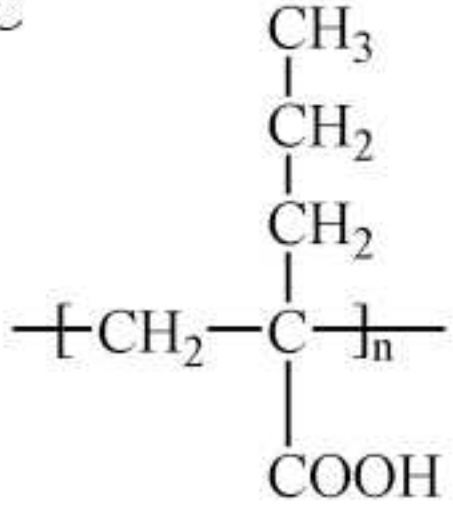


Figure 2

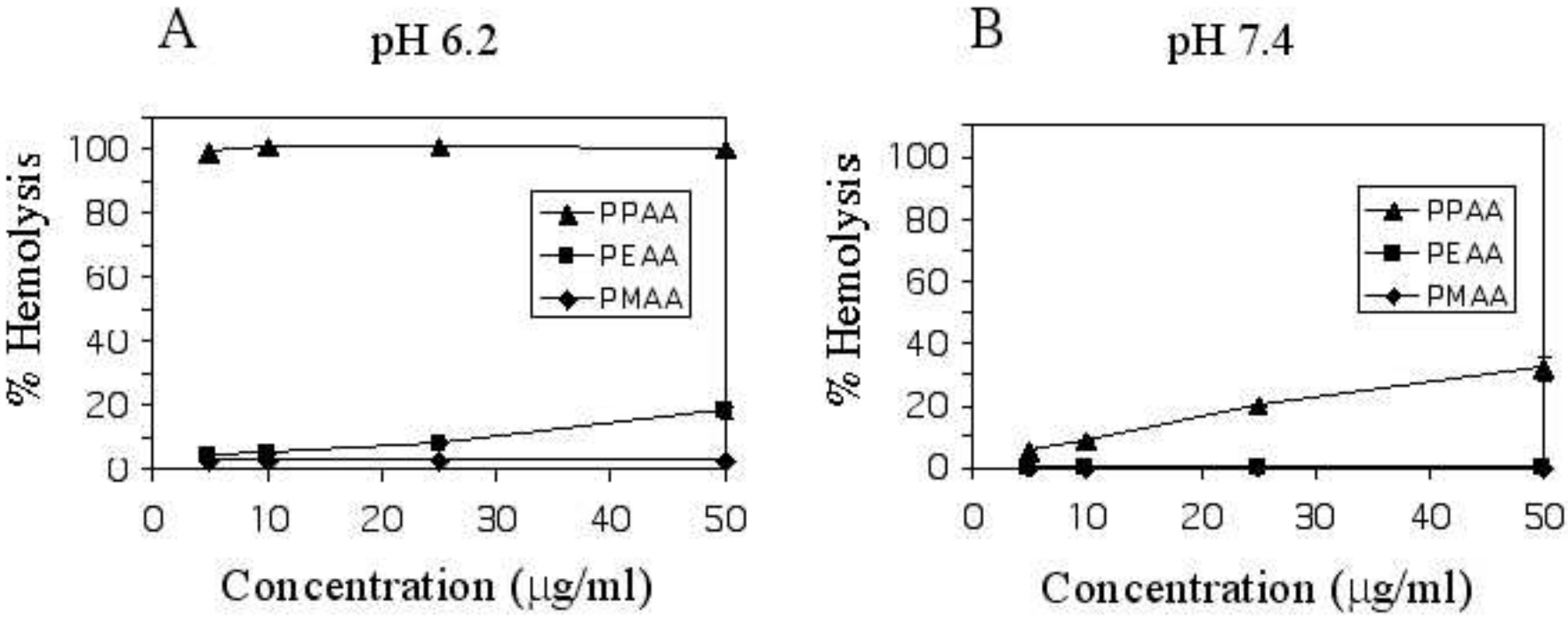

A
pH 6.2
% Hemolysis
100
80
60
40
20
0
0
10
20
30
40
50
Concentration (μg/ml)
PPAA
PEAA
PMAA
B
pH 7.4
% Hemolysis
100
80
60
40
20
0
0
10
20
30
40
50
Concentration (μg/ml)
PPAA
PEAA
PMAA

Figure 3

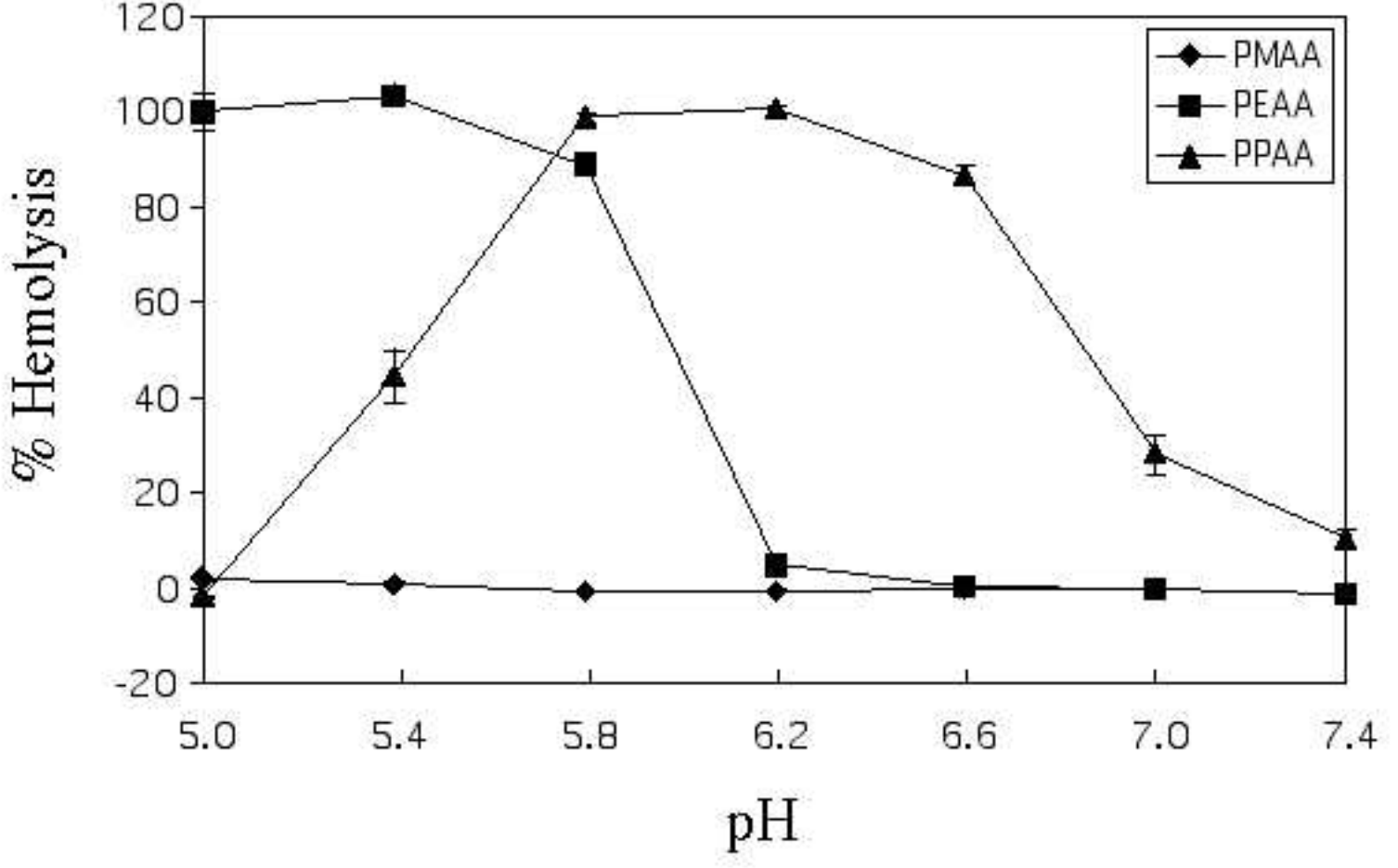

120
100
80
60
40
20
0
-20
% Hemolysis
5.0
5.4
5.8
6.2
6.6
7.0
7.4
pH
PMAA
PEAA
PPAA

Figure 4

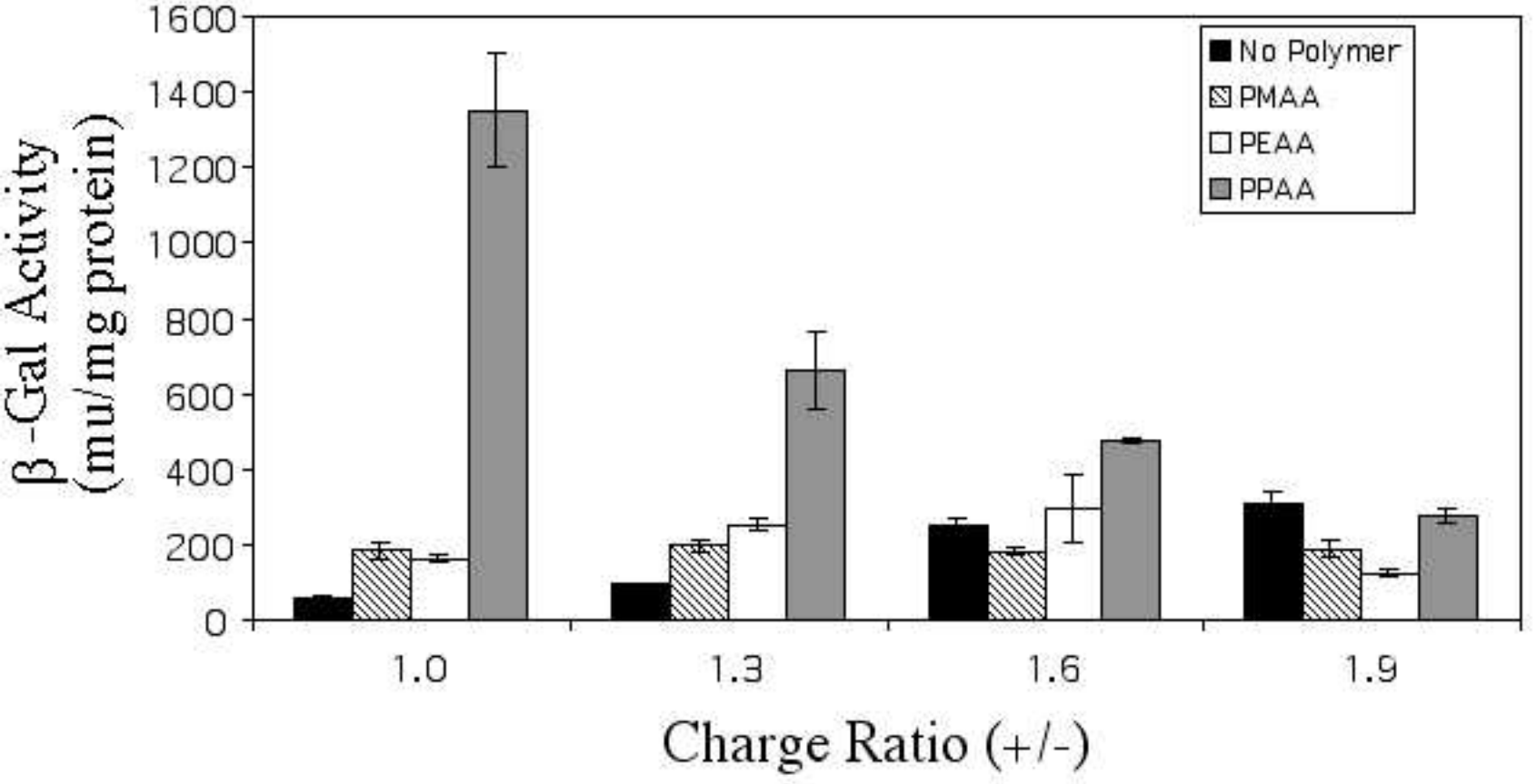

β -Gal Activity (mu/mg protein)
1600
1400
1200
1000
800
600
400
200
0
No Polymer
PMAA
PEAA
PPAA
1.0
1.3
1.6
1.9
Charge Ratio (+/-)

Figure 5A

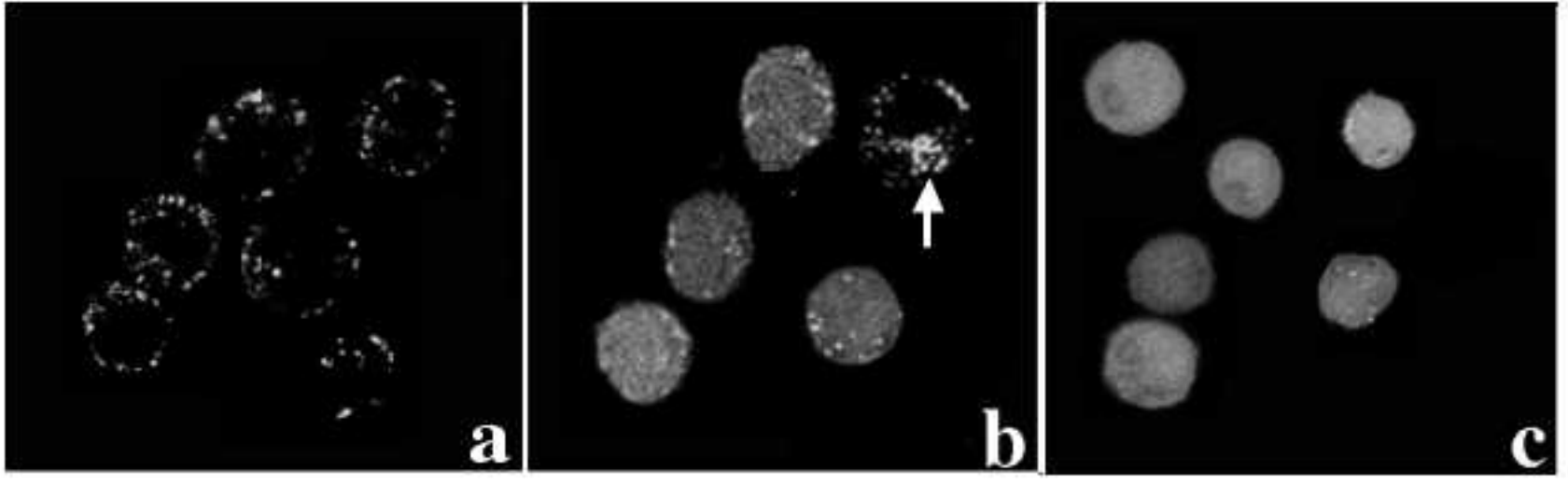


Figure 5B

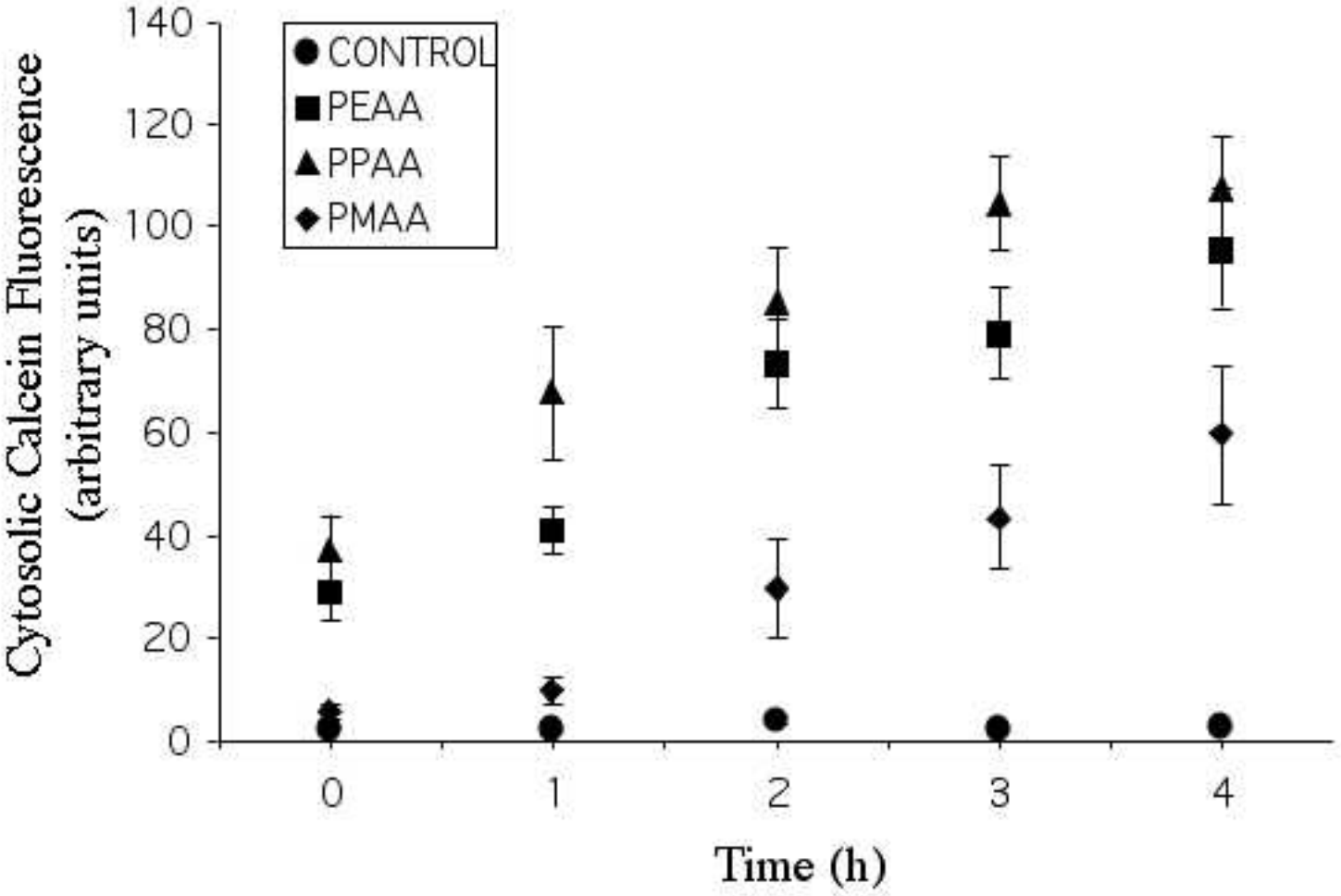

Cytosolic Calcein Fluorescence (arbitrary units)
140
120
100
80
60
40
20
0
CONTROL
PEAA
PPAA
PMAA
0
1
2
3
4
Time (h)

Figure 6

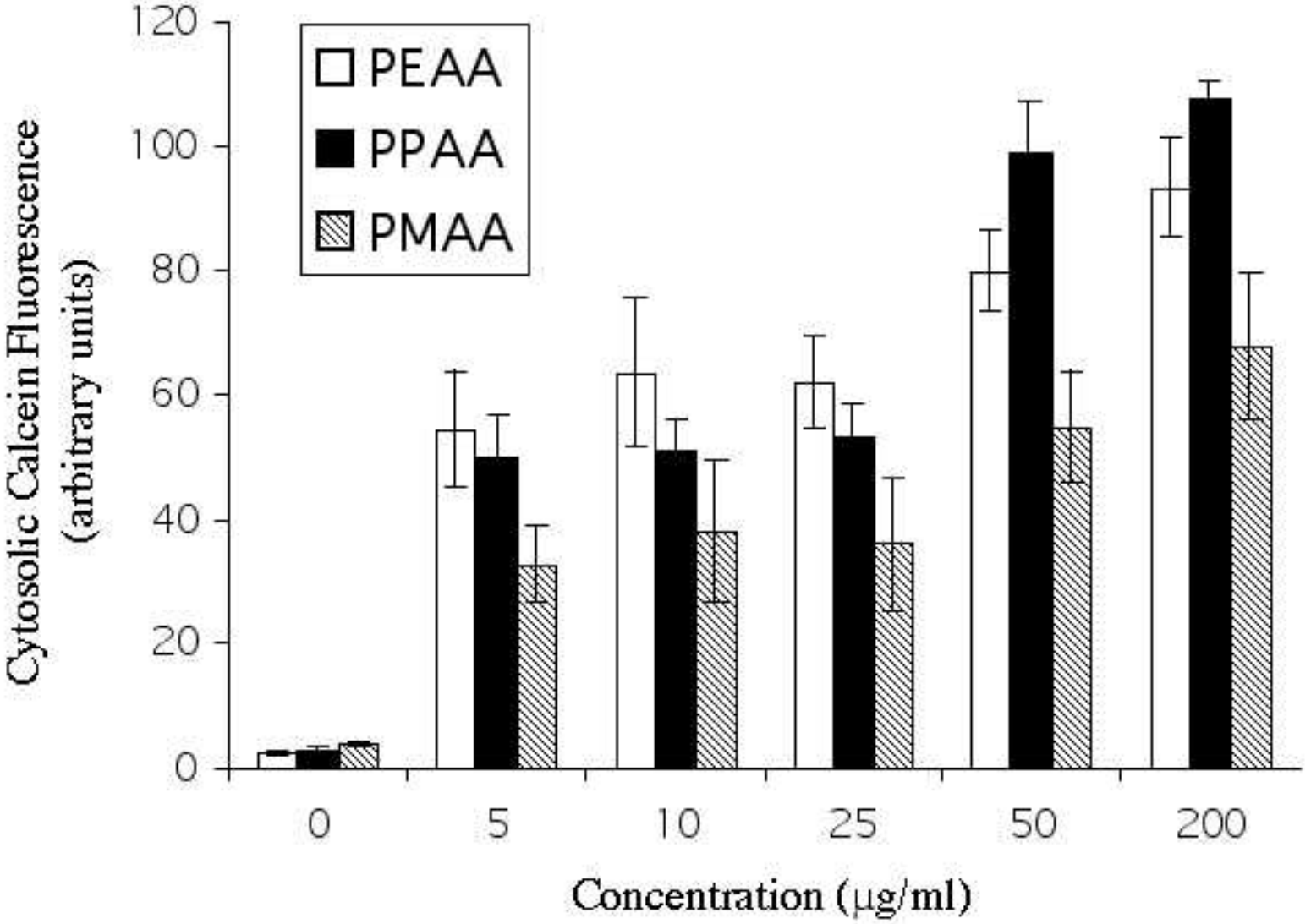

PEAA
PPAA
PMAA
Cytosolic Calcein Fluorescence (arbitrary units)
120
100
80
60
40
20
0
0
5
10
25
50
200
Concentration (μg/ml)

Figure 7

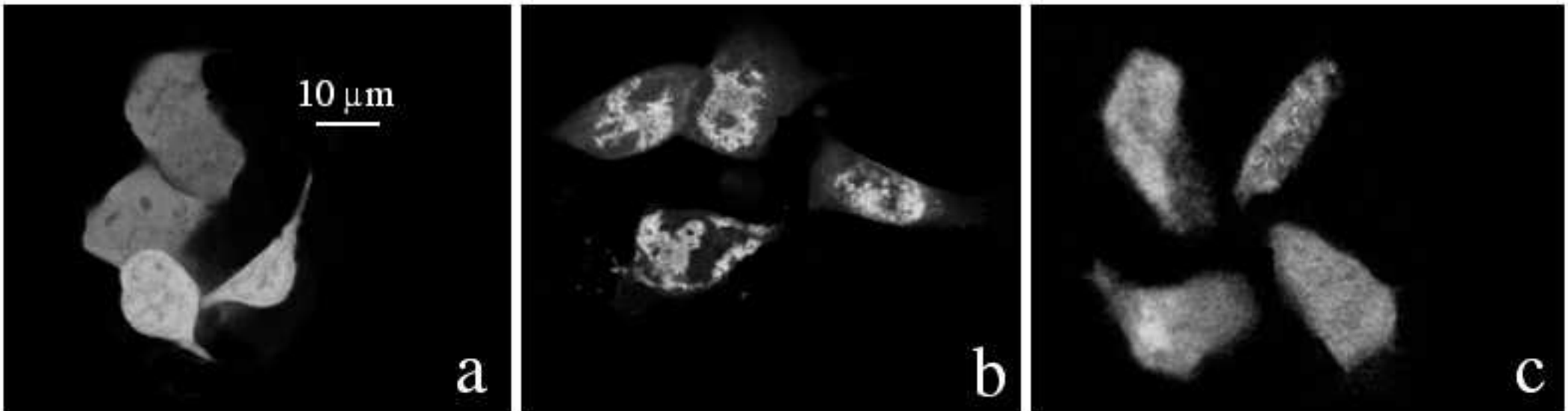


Figure 8

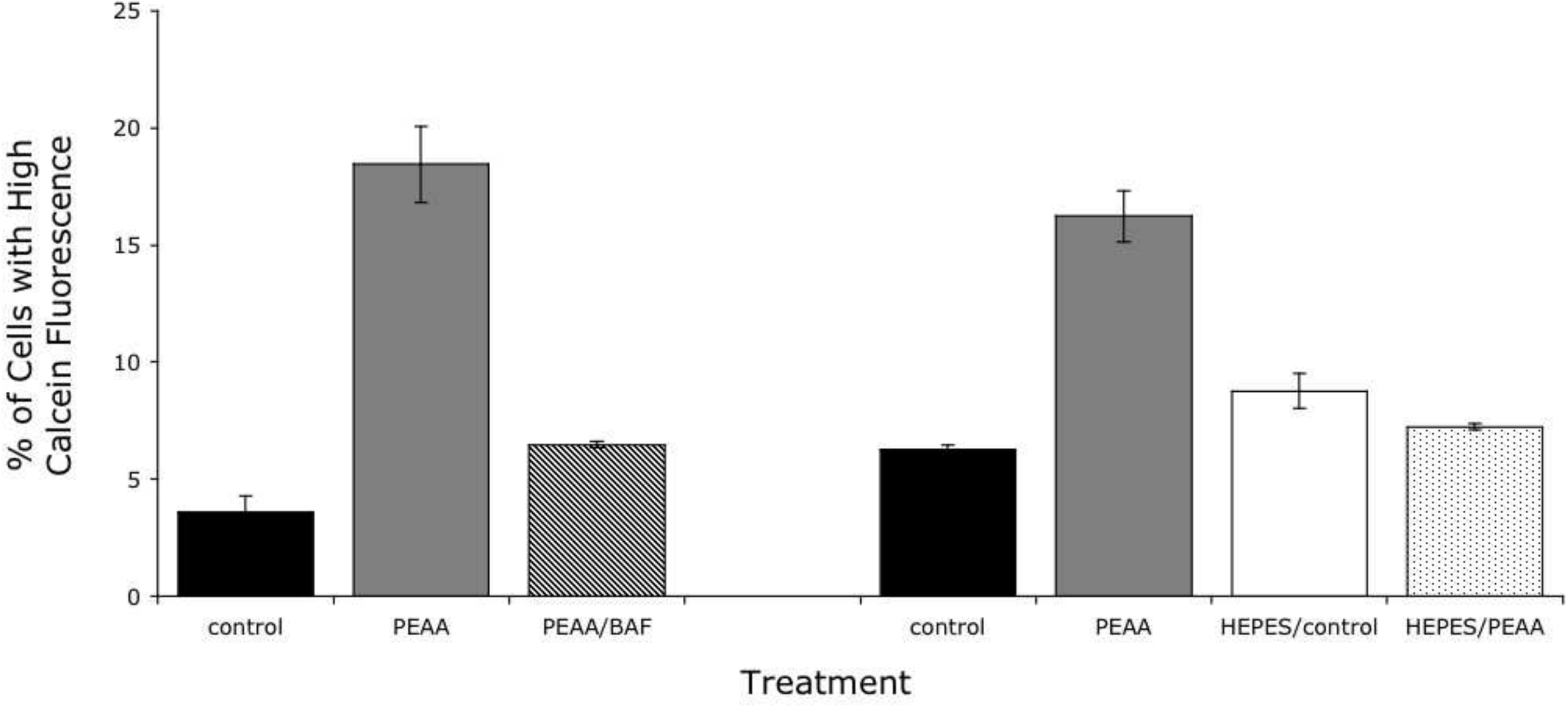

% of Cells with High Calcein Fluorescence
25
20
15
10
5
0
control
PEAA
PEAA/BAF
control
PEAA
HEPES/control
HEPES/PEAA
Treatment

Figure 9A

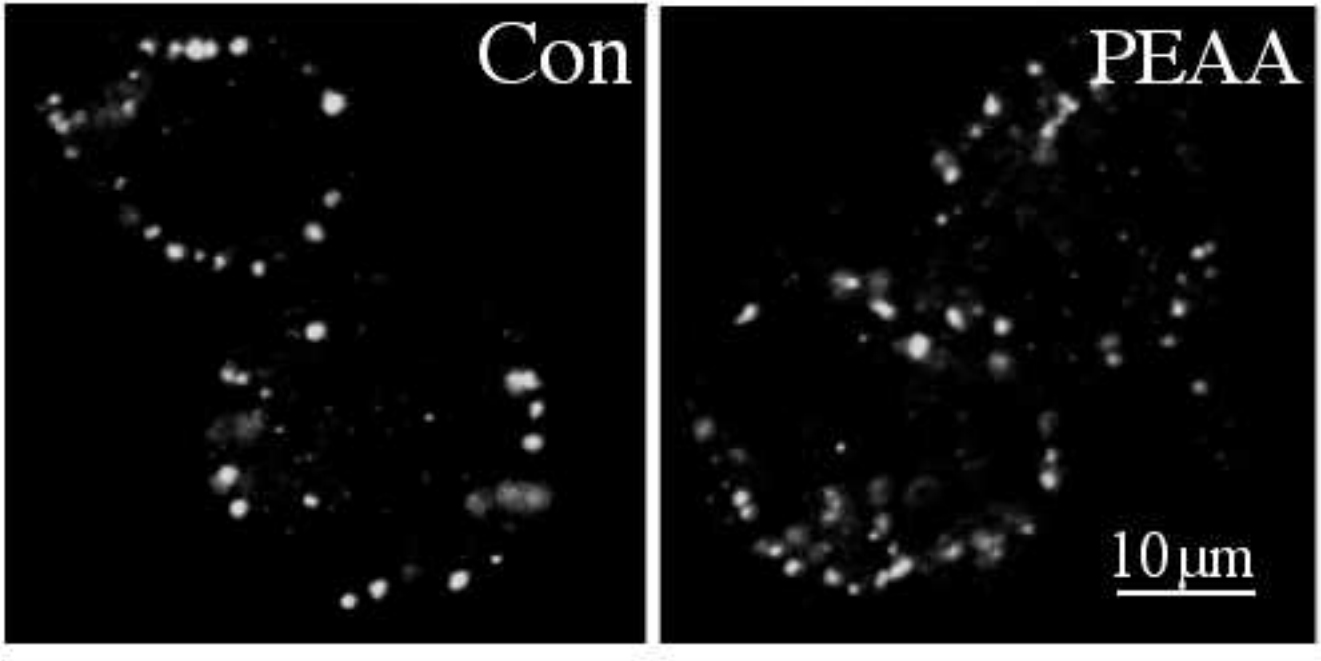


Figure 9B

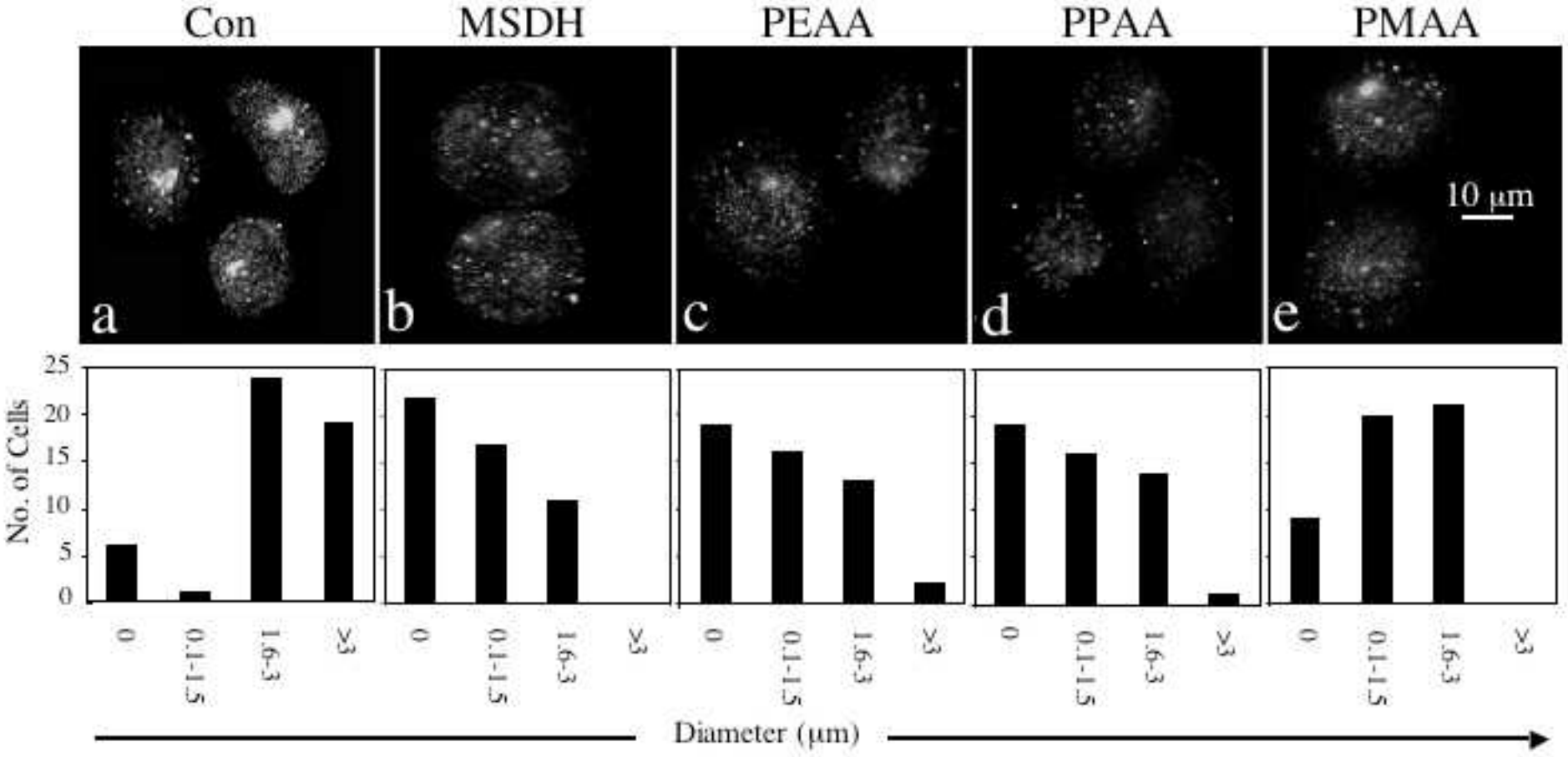
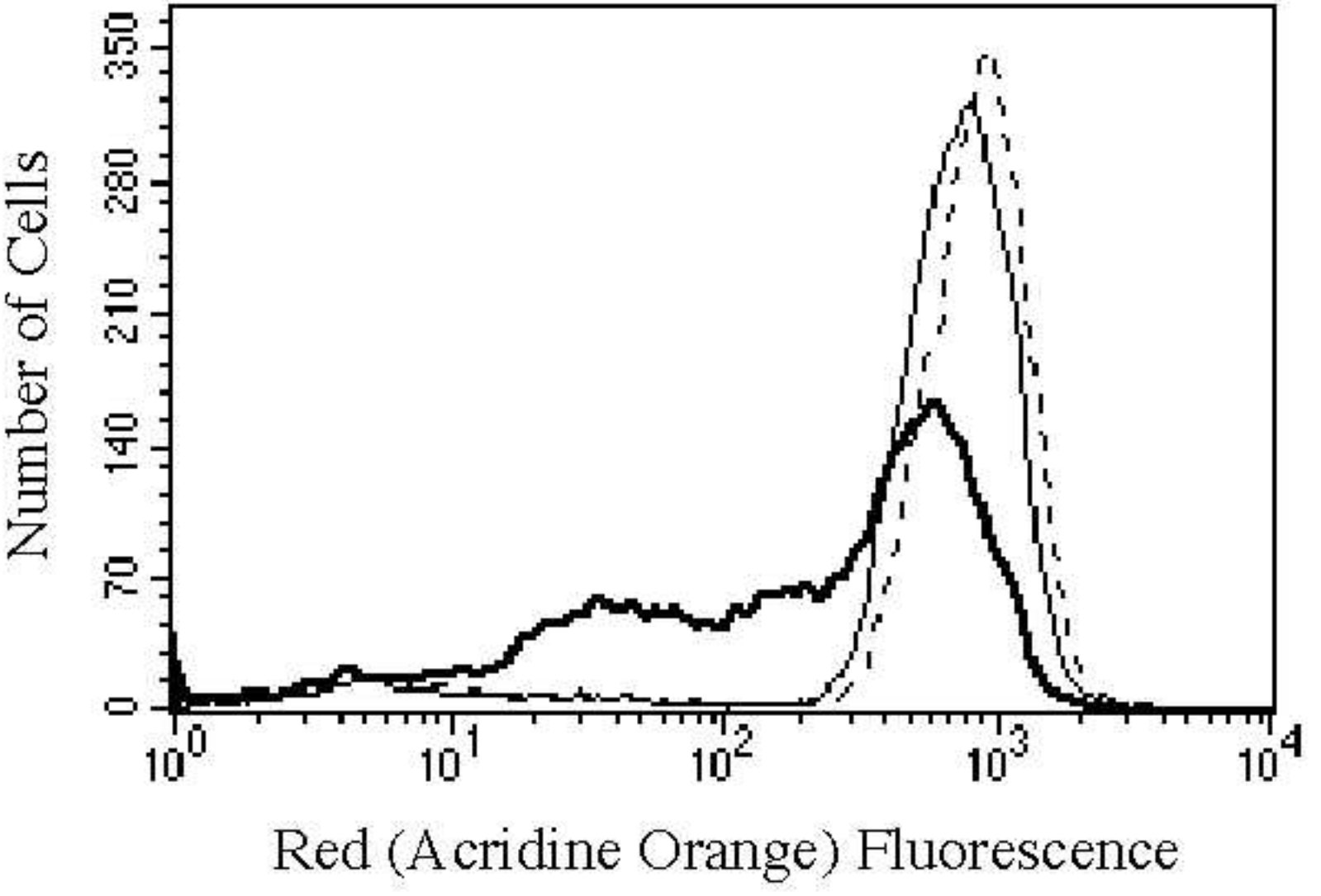

Con
MSDH
PEAA
PPAA
PMAA
10 μm
a
b
c
d
e
No. of Cells
25
20
15
10
5
0
0
0.1-1.5
1.6-3
≥3
Diameter (μm)

Figure 9C


Number of Cells
0
70
140
210
280
350
10^0
10^1
10^2
10^3
10^4
Red (Acridine Orange) Fluorescence