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Formulation and characterization of poly(propylacrylic acid)/poly(lactic-co-glycolic acid) blend microparticles for pH-dependent membrane disruption and cytosolic delivery

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Abstract

Poly(lactic-co-glycolic acid) (PLGA) is widely used as a vehicle for delivery of pharmaceutically relevant payloads. PLGA is readily fabricated as a nano- or microparticle (MP) matrix to load both hydrophobic and hydrophilic small molecular drugs as well as biomacromolecules such as nucleic acids and proteins. However, targeting such payloads to the cell cytosol is often limited by MP entrapment and degradation within acidic endolysosomes. Poly(propylacrylic acid) (PPAA) is a polyelectrolyte polymer with membrane disruptive capability triggered at low pH. PPAA has been previously formulated in various carrier configurations to enable cytosolic payload delivery, but requires sophisticated carrier design. Taking advantage of PPAA functionality, we have incorporated PPAA into PLGA MPs as a simple polymer mixture to enhance cytosolic delivery of PLGA-encapsulated payloads. Rhodamine loaded PLGA and PPAA/PLGA blend MPs were prepared by a modified nanoprecipitation method. Incorporation of PPAA into PLGA MPs had little to no effect on the size, shape, or loading efficiency, and evidenced no toxicity in Chinese hamster ovary epithelial cells. Notably, incorporation of PPAA into PLGA MPs enabled pH-dependent membrane disruption in a hemolysis assay, and a three-fold increased endosomal escape and cytosolic delivery in dendritic cells after 2 h of MP uptake. These results demonstrate that a simple PLGA/PPAA polymer blend is readily fabricated into composite MPs, enabling cytosolic delivery of an encapsulated payload.

Keywords

pH-responsive polymer; endolysosomal escape; drug delivery; controlled release; polymer-blend microparticles; PLGA

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Introduction

Poly (lactic-*co*-glycolic acid) (PLGA) copolymers of lactic acid and glycolic acid are one of the most widely studied biocompatible, biodegradable biomaterials. One of the numerous applications for PLGA is as a vehicle for the intracellular delivery of bioactive agents. Nano- to micron-sized PLGA particles (MPs) are an attractive system to deliver pharmaceutically relevant compounds, therapeutic peptides and/or proteins, and nucleic acids for gene therapy [1]. Depending on the physical and chemical nature of the payload molecule, MPs can typically be prepared by formation of an oil in water (O/W) single emulsion for loading hydrophobic drugs or water oil water (W/O/W) double emulsion for loading hydrophilic drugs. Solvent evaporation is then performed to obtain loaded MPs [2,3]. The size and shape of the MPs can be tuned by varying the matrix composition, concentration of agents in the emulsion, and homogenization time and speed [2]. Alternatively, nanoprecipitation methods incorporating a water miscible organic solvent can also successfully be used for PLGA MP fabrication and payload encapsulation [4]. Payload release kinetics from PLGA MPs have been well characterized, and are dependent on inherent factors such as the polymer molecular weight, composition, and the physiochemical properties of the loaded material. The pH, temperature, and hydrophilicity of the MP external environment also significantly affect release kinetics [3,5]. Additional functionality is afforded by available active groups on the MP surface, where receptor specific ligands and biomacromolecules can be conjugated for targeted delivery to cells or tissues [6,7]. Several compounds have been incorporated into PLGA matrices to derive blended formulations with improved stability, loading, and delivery capacity. These blended matrices consist of PLGA mixtures with different lactic/glycolic acid ratios [8], as well as other polymers such as poly(ε-caprolactone) (PCL) [9], poly ethylene glycol (PEG), triphenylphosphonium [10], poly(beta-amino ester) (PBAE) [11], chitin [12], and chitosan [13].

Polymers demonstrating pH-sensitive conformation with membrane destabilizing properties at acidic pH have been used as carriers for enhanced cytosolic delivery via endolysosomal escape [14,15]. For example, a number of polyelectrolyte polymers containing either weak acidic (polyanion) or basic (polycation) moieties belong to this category [15,16], where pH dictates the degree of ionization of these moieties. This ionized conformation promotes interactions with the hydrophobic backbone (and pendant hydrophobe, if present), which destabilize cellular membranes [17,18]. This membrane disruptive activity is analogous to pathogenic mechanisms employed by proteins such as influenza hemagglutinin and diphtheria toxin [19,20]. Such polyelectrolyte polymers have been employed for the intracellular delivery of biologically active molecules either by direct conjugation, encapsulation within amphiphilic diblock copolymer micelles [21–24] or electrostatic polyplex formation [25,26].

Poly(propylacrylic acid) (PPAA) is a pH-sensitive polymer with membrane disruptive capacity that is well-suited for cytosolic delivery of biomolecules. The carboxylic acid moieties of PPAA possess an acid dissociation constant (pK_a) of ~6.7. At pH>pKa, the polymer deionized and water soluble. However, at pH<pKa, PPAA becomes protonated, more hydrophobic, and membrane interactive. This hydrophobic transition triggers disruption of endo-lysosomal compartments and cytosolic release following cellular uptake

of PPAA-based formulations. PPAA has been primarily leveraged in conjugates and as a component of copolymer micelles, both of which require specialized and difficult synthesis methods. This shortcoming has limited broad application and potentially delayed clinical translation of this attractive delivery approach [27].

In this work, we explore incorporating PPAA in PLGA MPs to develop a cytosolic delivery vehicle. These PPAA/PLGA blended MPs harness the pH-dependent membrane disruption capability of PPAA while maintaining the ease of fabrication and broad payload capacity of PLGA MPs (Figure 1). Thus, various PPAA/PLGA MP blend compositions were fabricated, characterized, and tested for loading efficiency, payload release, pH-dependent membrane destabilization capacity, cytotoxicity, and endosomal disruption/cytosolic payload delivery.

Materials and Methods

Reagents

Poly (lactic-*co*-glycolic) acid (PLGA, a copolymer of D,L-lactide and glycolide at a 50:50 molar ratio; M_n ~ 44,000 g/mol) was purchased from Purac Biomaterials (Netherlands). Poly(vinyl alcohol) (PVA, M_n ~ 15,000 g/mol) and the solvents methylene chloride (dichloromethane-DCM) and N, N-dimethylformamide (DMF) were purchased from Fisher Scientific (NJ, USA). All other chemicals were purchased from Sigma-Aldrich (St.Louis, MO, USA) at the highest purity available unless otherwise noted. Nanopure water (purified through Millipore Lab water systems) was used as the aqueous phase to form the emulsions while DCM or DMF was used as the organic solvent to dissolve the PLGA and PPAA polymers.

Synthesis of PPAA

2-propylacrylic acid (2-PAA) was synthesized according to the procedure outlined by Ferrito et al [28], utilizing diethyl propylmalonate (Alfa Aesar, Ward Hill, MA, USA) as a precursor. The 4-cyano-4-(ethylsulfanylthiocarbonyl) sulfanylpentanoic acid chain transfer agent was synthesized as previously described [24]. RAFT polymerization of 2-PAA to yield a PPAA homopolymer was carried out in bulk under a nitrogen atmosphere at 70°C for 48 hours using 2,2′-azo-bis-isobutyrylnitrile as the free radical initiator. The reaction mixture was put through three freeze-vacuum-thaw cycles and purged with nitrogen for thirty minutes prior to polymerization. The molar ratio of chain transfer agent:initiator was 1:1, and the monomer to chain transfer agent ratio was set so that a degree of polymerization of 190 would be achieved at 100% conversion. Following polymerization, the resultant polymer was dissolved in DMF and precipitated into ether 5 times before drying overnight *in vacuo*. Gel permeation chromatography (GPC, Agilent) was used to determine number average (M_n) and weight average (M_w) molecular weight of the PPAA homopolymer using HPLC-grade DMF containing 0.1% Lithium Bromide at 60°C as the mobile phase. Molecular weight calculations were performed with ASTRA V software (Wyatt Technology) and were based on experimentally-determined $d\eta/dC$ (concentration dependent change in the refractive index, η) values calculated through serial injections of increasing concentrations of polymer in conjunction with offline monitoring of the change in refractive index with a refractive index detector (miniDAWN TREOS, Wyatt Technology).

Polydispersity was calculated as the ratio of weight average to number average molecular weights (M_w/M_n).

Preparation of fluorescent dye loaded MPs

Microparticles loaded with rhodamine were prepared by nanoprecipitation. In a typical MP preparation, PLGA was combined with 0%, 1%, 3%, or 10% PPAA (w/w) to achieve a total weight of 100 mg and dissolved in 2.0 mL of DMF to which 0.5 mg of fluorescent dye rhodamine-6G was added. The resulting solution was homogenized in the presence of 4.0 mL of 5% PVA in water (pH 6.0) using a tissue-miser homogenizer (Dremel Power tools, Wisconsin, USA.) for 120 s to form a viscous emulsion. This emulsion was subsequently diluted into 100 mL of 1% PVA solution in nano-pure water and stirred for 24 hours in a continuous flow hood. The resulting MPs were collected from the solution by centrifuging at 10,000 × g for 10 min. The MPs were washed three times with Nanopure water by repeated centrifugation and re-suspension in Nanopure water to remove residual organic solvent. The final MP pellet was then flash-frozen in liquid nitrogen and lyophilized *in vacuo* overnight. Lyophilized MPs were stored at −20°C until use.

Characterization of MP composition and size

PLGA and PPAA/PLGA blend MPs were prepared for NMR analysis by dissolving 5-10 mg of lyophilized polymer in deuterated dimethyl sulfoxide (D_6MSO). Samples were analyzed in a 400 MHz spectrometer equipped with a 9.4 Tesla Oxford magnet and 5 mm Z-gradient broadband inverse (BBFO) probe with automatic tuning and matching capability controlled by a Bruker AV-400 console. The resulting spectra were analyzed with Bruker TopSpin 3.0 software. The mole % composition of propylacrylic acid (PAA) in the PPAA/PLGA blend MPs was determined by dividing the area per proton (H) of the peak associated with the methyl group of the PAA repeat (CH2-CH2-**CH3**, δ=0.8 ppm) by the area per proton of the peaks associated with the methyl group of the lactic acid unit of PLGA (C=O-CH(**CH3**)-O, δ=1.42 ppm), the alkyl group of the glycolic acid unit of PLGA (C=O-**CH2**-O, peak a), and the methyl group of PAA * 100%. The weight fraction of each component of the blended MPs (i.e., glycolic acid, lactic acid, and propylacrylic acid) was then determined by multiplying the mole % of each component by the corresponding molecular weight of that repeat unit (propylacrylic acid repeat = 114 g/mol, glycolic acid repeat = 58 g/mol, lactic acid repeat = 72 g/mol). The weight % of PPAA in each MP formulation was then determined by dividing the PAA weight fraction by the total of all weight fractions. The contribution of the chain transfer agent utilized in the polymerization of PPAA was not considered in composition calculations as it has a negligible contribution to the total weight of the blend MPs (i.e., the chain transfer agent is only 1.2% of the total weight of the PPAA polymer).

Dynamic light scattering measurements were performed using a Microtrac Nanotrac Dynamic Light Scattering Particle Analyzer (Microtrac, Montgomery, PA) at 25 °C using Nanopure water as the dispersion medium. Samples were briefly sonicated in a bath sonicator to disrupt any aggregates and provide uniform sized dispersion as well as to remove any dissolved air prior to analysis. Each sample was analyzed four times and the mean volume averaged size was determined employing the manufacturer supplied software.

A FEI XL-40 Field Emission Scanning Electron Microscope equipped with a Gatan EDAX system and TEAM Software for EDS analysis was used for imaging and visualization of MPs. SEM imaging was performed using an accelerating voltage of 8.00 kV. Microparticles were fixed to metal sample blocks with double sided tape and sputter-coated with gold prior to imaging. For each MP preparation, 75–100 individual particles in 3 separate SEM scans were analyzed for MP diameter using Image J software.

Loading and release for PLGA and PPAA/PLGA MP blends

MP preparations were dissolved in DMF, and the amount of rhodamine released from the MP solutions was quantified through fluorescence spectroscopy by fitting released amounts to a standard curve of known rhodamine concentrations in DMF. Data are presented as the ratio (weight %) of rhodamine retained to the amount of rhodamine loaded per mg of MPs.

Known weighed amounts of MPs from each blend were suspended in phosphate buffers of pH 5.0, and 7.0, containing 0.1% of Tween-20. Solutions were then placed in a shaker incubator at 37°C. After 4 hours, the tubes were centrifuged and the supernatants were collected. The pellets were resuspended in the same buffers and returned to the shaker incubator. This procedure was subsequently repeated at the time points indicated in Figure 5. The cumulative release of rhodamine was determined as a function of rhodamine fluorescence intensity per mg MP from each aliquot of supernatant collected.

CHO cell culture

Chinese hamster ovary (CHO-K1) cells were grown in Ham's F12 medium (Mediatech) containing 10% fetal bovine serum (FBS, Hyclone), penicillin (100 units/mL), and streptomycin (100 μg/mL) at 37°C in a humidified incubator with 5 % CO_2 [29]. Cell viability was determined via Trypan blue exclusion and was ≥ 97% with each passage.

Cytotoxicity assay

Toxicity of MPs was determined employing CytoTox96 Non-radioactive Cytotoxity Assay (Promega). CHO cells were plated at a density of 10,000 cells per well in a 96 well tissue culture plate in 200 μL of cell culture medium. Cells were maintained at 37°C in a humidified incubator in a 5% CO_2 atmosphere. MPs were added at a ratio of 5, 10, 20, 30, 40, and 50 particles/cell and incubated at 37°C for 24 h. The culture medium was transferred into wells of a clear bottom 96 well plate. Cells treated with 0.1% Triton X-100 were used as a positive control for 100% toxicity and cell culture medium alone was used for background subtraction for baseline lactate dehydrogenase (LDH) activity (background control). The cell supernatants were incubated with the developing reagents for 30 min per the manufacturer's instructions. The optical density of the developed color was determined by measuring absorbance at 490 nm. The background was subtracted from each experimental value (treatment) and normalized to the Triton X-100 value (positive control) in calculating the % viability as follows: 100% × (treatment – background control)/(positive control – background control).

Ex-vivo pH dependent human red blood cell hemolysis (RBC) assay

The pH-dependent endosomal disruptive potential of PLGA-PPAA blend microparticles was measured using a human red blood cell hemolysis assay. Following approval by Vanderbilt University Medical Center's Institutional Review Board, whole human blood was drawn from an anonymous donor, and the plasma was removed through repeated centrifugation and saline washes. Briefly, erythrocytes were washed three times with 150 mM NaCl and resuspended into phosphate buffers corresponding to physiologic (pH 7.4), early endosome (pH 6.8), late endosome (pH 6.2) and lysosomal (pH 5.6) environments. 20 μl of PPAA/PLGA blend microparticles or PPAA polymer in DMSO or 1.0% Triton X-100 in DMSO (positive control) and DMSO alone (negative control) were added into 180 μL of erythrocyte cell suspension to achieve final MP concentrations of 10, 50, and 100 μg/mL and incubated at 37° C for 1 hour. Intact erythrocytes were pelleted via centrifugation at 500 × g for 5 minutes and the supernatant was transferred to a new 96-well plate. The haemoglobin content was then measured via absorbance at 405 nm on a Tecan Pro plate reader. Percent hemolysis was calculated considering 100% hemolysis with 1.0% Triton X-100 (positive control) and red blood cell suspensions treated with DMSO alone as the negative control.

Isolation and culture of mouse dendritic cells (DCs)

DCs were isolated from mouse bone marrow tissues [7,30] according to the guidelines set forth by the University of Florida Institutional Animal Care and Use Committee. Dendritic cells (DCs) were obtained from 8 – 12 weeks old female C57BL6/j mice. Briefly, mice were euthanized by CO_2 asphyxiation followed by cervical dislocation. The marrow cells were obtained by flushing the shaft of the detached tibias and femurs bones with a 25 g needle using RPMI medium (MP Biomedicals, OH, USA) containing 1% fetal bovine serum (Lonza, Walkersville, MD) and 1% penicillin-streptomycin (Hyclone) and mixed by pipetting to make a homogenous suspension. The suspension was passed through 70 μm cell strainers (Becton Dickinson, NJ, USA) to remove debris. The cells were collected by centrifugation at 500 × g for 5 minutes. The cell pellet was then suspended in 3 mLs of ACK lysis buffer (Lonza, Walkersville, MD) and left for 5 minutes at room temperature to remove red blood cells by lysis, followed by centrifugation at 500 × g for 5 minutes to recover the leukocyte fraction. Leukocytes were then re-suspended in DC media (DMEM/F-12 with L-glutamine -Cellgro, Herndon, VA, 10% fetal bovine serum, 1% sodium pyruvate -Lonza, Walkersville, MD, 1% non-essential amino acids -Lonza, Walkersville, MD, 1% penicillin-streptomycin and 20 ng/ml GM-CSF -R&D systems, MN, USA) and plated in tissue culture flasks in order to remove adherent cells. After 2 days of culture, the floating cells were transferred to low attachment plates and cultured in fresh DC media for expansion of DC precursor cells. At day 7, cells were transferred to tissue culture plates to allow for DC adhesion and proliferation. At 10 days, DCs were harvested and used for various MP uptake studies.

Calcein release from endosomal vesicles

DCs were plated in 35 mm glass bottom culture dishes at a density of 5×10^4 cells in DC culture medium. DCs were incubated for 2 h at 37°C with cell membrane impermeable calcein (0.25 mg/ml) alone, calcein with PLGA MPs, or calcein with 10% PPAA blend MPs.

As a positive control, a portion of DCs were treated with the membrane permeable Acetoxymethyl ester of calcein (5 μM). Following treatment, cells were washed four times with phosphate buffered saline. New culture medium was added and the cells were imaged with a fluorescent microscope (Axiovert 200M microscope (Carl Zeiss, Oberkochen, Germany) using excitation/emission wavelengths of 495/515 nm at 200-fold magnification.

Image analysis

Images were analyzed by measuring the area of fluorescence per channel (green vs bright field). The values are reported as % relative fluorescence intensity per cell (RFI/cell).

Statistical analysis

The results reported in the study represent three independent experiments performed in triplicate unless otherwise noted in the figure legends. Statistical analysis was performed with a one-way analysis of variance (ANOVA) followed by Tukey's Post Hoc Test where p-values ≤ 0.05 were considered significant.

Results and Discussion

Synthesis and characterization of PPAA

While poly(propylacrylic acid) (PPAA) is commercially available, the polydispersity is relatively high due to the bulky α-alkyl substitution of the propyl moiety. To utilize a well-defined polymer chain length with minimal polydispersity, reversible addition fragmentation chain transfer (RAFT) polymer synthesis was performed as previously reported [26]. PPAA was synthesized from 2-propylacrylic acid utilizing RAFT polymerization from an ethyl cyanovaleric trithiocarbonate chain transfer agent. Gel permeation chromatography analysis of the synthesized PPAA showed the degree of polymerization to be 193. The number average molecular weight calculated using a calculated $d\eta/dC = 0.087$ (mL/g) was 22,010 g/mol. The polydispersity index of PPAA was 1.47, indicating a relatively monodisperse product.

Synthesis and characterization of the PPAA/PLGA blend MPs

A range of Blended PPAA/PLGA MP compositions were formulated by varying the starting weight percentage of PPAA to be incorporated within the base PLGA matrix. Blended PPAA/PLGA MPs were prepared using a nanoprecipitation method. Dimethylformamide (DMF), a polar, hydrophilic, aprotic solvent that is highly miscible with water was selected as the solvent. DMF was selected because PPAA is not readily soluble in most of the halogenated hydrocarbons that are commonly used for PLGA preparations. The aqueous phase was maintained at pH 6.0 to prevent deprotonation of PPAA and its loss into the aqueous phase during homogenization of the PPAA/PLGA mixture.

Compositions of the blended MPs were determined by H^1 NMR analysis, and a representative spectrum is shown (Figure 2). By comparing the relative areas of the peak corresponding to the methyl group of the α-alkyl substituted propyl moiety of PPAA (CH2-CH2-**CH3**, - peak g) to the peak areas corresponding to the methyl group of the lactic acid unit of PLGA (C=O-CH(**CH3**)-O, peak c) and the alkyl group of the glycolic acid unit of

PLGA (C=O-**CH2**-O, peak a), the weight % PPAA content was calculated for each blended MP formulation. We found that MPs formulated with starting PPAA amounts of 1.0%; 3.0%; and 10.0% (w/w) had final compositions of 1.2%, 2.3%, and 5.8% (weight % PPAA), respectively. This result indicates the amount of PPAA incorporated into the MP matrix correlates with, but may not be linearly proportional to the feed ratio. Reduced PPAA incorporation at higher starting amounts of PPAA is likely due to loss of PPAA into the aqueous phase during homogenization and is dependent on the ionization state of the carboxylate moiety present in the propylacrylic acid repeat unit (i.e., the deprotonated carboxylate anion is hydrophilic, whereas the protonated carboxylic acid is more hydrophobic).

The next set of experiments were designed to evaluate the effects of PPAA incorporation on the size and shape of the blended MPs, as polymer matrix composition has been shown to affect these parameters [31–33]. For example, targeting antigen delivery to dendritic cells using phagocytosable 2 μm MPs, versus soluble release of immunomodulatory proteins targeted to cell surface receptors via 30 μm MPs that are not internalized by cells is readily achievable [34]. The size and the shape of the MP preparations were assessed by scanning electron microscopy (SEM) and dynamic light scattering (DLS) techniques. Analysis by DLS of the PPAA/PLGA MPs yielded the volume average mean diameters for 0% (PLGA only), 1.0%, 3.0% and 10% PPAA/PLGA MPs to be 540 ± 60 nm; 440 ± 50 nm; 520 ± 90 nm and 470 ± 140 nm respectively (Figure 3, upper panels).

Scanning electron microscopic imaging was carried out to characterize MP morphology and verify the DLS sizing results. Images demonstrated MPs characterized as generally spherical and with a smooth surface. The mean diameters for 0%, 1.0%, 3.0% and 10% PPAA/PLGA MPs determined from analyzing SEM images were 360 ± 110 nm; 340 ± 100 nm; 390 ± 140 nm and 360 ± 120 nm, respectively (Figure 3,- associated lower panels). Thus, the size of the MPs obtained by SEM was 15%–20% smaller than that obtained from DLS measurements. This difference is anticipated and consistent with prior observations, as SEM is performed in a dry state whereas the aqueous environment in which DLS is performed results in hydration and increase in the MP hydrodynamic radius [35]. Both DLS and SEM analysis revealed that the incorporation of PPAA did not significantly alter the diameter of the blended PPAA/PLGA MPs.

Loading and release properties of PPAA/PLGA blend MPs

Incorporated rhodamine was solubilized into the solvent phase by dissolving the MPs in DMF. The fluorescence intensity of the resulting solution was compared to a standard curve to determine encapsulation efficiency (Figure 4). While there was small shift in average encapsulation efficiency with higher percentages of PPAA, at 95% confidence level the values were statistically equivalent. This finding indicates that the addition of PPAA does not appreciably influence the capacity to load rhodamine within the PLGA matrix. The efficiency of incorporation is influenced by multiple factors including the interactions of the MP matrix with the incorporated payload molecules (e.g., hydrophobic, hydrophilic, and Van der Waal interactions) and MP size. While a substantial portion of the rhodamine intended for encapsulation was lost during fabrication, others have shown comparable small

molecule drug encapsulation efficiencies (~60-80%) when similar methods were employed for MP preparation [36].

Successful application of MPs as an intracellular delivery vehicle depends on the ability to efficiently release the drug payload into the targeted subcellular environment (e.g., escaping endolysosomal entrapment for cytosolic delivery of intracellularly acting drugs). The pH of biological microenvironments varies from an extracellular pH of 7.0-7.4 to more acidic pH values of 5.0-6.5 encountered in intracellular endosomal/lysosomal compartments. These differing pH values can significantly influence the rate of release of encapsulated materials from polymeric MPs. While the rate of PLGA hydrolysis is known to increase in both acidic and alkaline conditions [37], PPAA is more hydrophobic at acidic than neutral/alkaline conditions. Therefore, it was unknown how composition of PPAA/PLGA blend MPs would affect release. In order to examine release kinetics of loaded materials, rhodamine release kinetics of blended MPs was analyzed at acidic (pH 5.0) and neutral (pH 7.0) conditions (Figure 5). Cumulative release of rhodamine from MPs with different compositions as a function of time is presented as the % of the maximum released at pH 5.0 (10% PPAA, 384 h), normalized to allow direct comparison across all groups for both pH values.

At acidic pH, below the pK_a of the carboxylate moiety of PPAA (pH ~6.7), all MP blends demonstrated an initial rapid release phase within 96 hours (Figure 5A). The 10% PPAA/PLGA blend demonstrated the fastest and highest cumulative rhodamine release at pH 5.0 over the time tested, with the rate and amount of release directly proportional to PPAA content among the different blends. Although loading efficiency was statistically equivalent, at the final time point investigated, MPs composed of PLGA alone (0% PPAA) released only 35% of the highest amount measured (the 10% PPAA group), while 1% PPAA MPs released 55% and 3% PPAA released 70% of the highest released amount measured. Because the carboxyl groups of PPAA will be primarily in a protonated (charge neutral) form at pH 5.0, the composition-dependent release in acidic conditions may be due to a reduction in electrostatic forces between positively charged rhodamine and the negatively charged carrier PLGA (carboxyl pKa ~3.5) [38].

At pH 7.0, introduction of PPAA into the MP blend also demonstrated higher rhodamine release levels compared to PLGA alone and were relatively linear throughout the time of analysis (Figure 5B). In this case, at pH above its pK_a 6.7, PPAA is in a more deprotonated and negatively charged form, which may increase intra-particle hydration and swelling, and could explain the higher release from the PPAA blends compared to PLGA alone. With increasing PPAA concentration, the amount of rhodamine released also increased. At the final time point, MPs composed of PLGA alone (0% PPAA) released only ~25% of the 10% PPAA group at pH 5.0, while 1% PPAA MPs released ~48%, 3% PPAA released ~60%, and 10% PPAA at pH 7.0 released ~84% of the highest released amount measured (10% PPAA at pH 5.0). Altogether, these results indicate that incorporating PPAA into the PLGA MP matrix is destabilizing, increasing the rate of release with a modest pH dependent effect. Environmentally responsive drug delivery vehicles are of broad interest, and these PPAA/PLGA blend characteristics may prove useful for improved cargo delivery in acidic environments such as endolysosomes and solid tumor microenvironments [15]. However, the

primary intent of PPAA incorporation into PLGA blends is to capitalize on the membrane destabilization properties PPAA, described below.

Cytotoxicity of PPAA/PLGA blend MPs

To investigate the biocompatibility of PPAA/PLGA blend MPs, a widely used method for determining cell viability was employed, based upon the measurement of release of the intracellular enzyme lactate dehydrogenase (LDH). LDH is released from non-viable cells with compromised membrane integrity [39]. Chinese hamster ovary (CHO) epithelial-like cells were incubated with a MP concentrations ranging from 5-50 MPs/cell for 24 h to investigate dose dependent toxicities of the MP blends. LDH release from cells treated with Triton X–100 as a positive control for toxicity was 21-fold higher than the basal background LDH activity in media without cells. LDH values for all treatment groups were less than 10% of the positive control. The percent cell viability value for each of the MP preparations was normalized to Triton X–100 treated cells, which represents 0% viable cells (Figure 6). No significant differences in viability were measured between any of the MP-treated and untreated control cells (i.e., “0” particles/cell). This finding indicates that the blended MP formulations do not result in appreciable cell membrane damage and are biocompatible. Furthermore, examination via phase contrast microscopy indicated that the MP treated cells were healthy with no apparent membrane blebbing (a distinctive feature of apoptotic cells). This finding corroborates that PPAA/PLGA blended MPs cause no significant cell toxicity under these conditions. These results are in agreement with others who have reported that particulate formulations containing PLGA have negligible toxicity *in vitro* [40].

pH-dependent membrane disruption of PPAA/PLGA blend MPs

The functional capability of PPAA/PLGA MPs to disrupt cellular membranes in a pH-dependent fashion via PPAA release is of primary interest, and was investigated by an *ex vivo* red blood cell (RBC) hemolysis assay [41]. The assay measures the amount of hemoglobin released due to RBC membrane disruption by measuring optical density at 405 nm, the peak absorption wavelength of hemoglobin. MPs formulated with PLGA alone demonstrated no hemolysis at any of the pH values examined, whereas pure PPAA demonstrated the highest membrane disruptive potential at pH values below pH 7.4 (Figure 7). PPAA/PLGA blends showed dose-dependent significant increases in RBC hemolysis below the pK_a of the carboxylate moiety of PPAA ($pK_a \sim 6.7$), with the 10% PPAA demonstrating the highest amount of hemolysis. At pH 5.6, representing lysosomal pH, the highest amount of hemoglobin release was observed for each of the three MP blends. Due to reduction in the immediate availability of PPAA by incorporation into the PLGA matrix, the apparent membrane disruptive potential was reduced compared to free PPAA polymer, as seen by the shift in pH dependent hemolysis to more acidic pH values. At physiological pH of 7.4, hemoglobin release for all MPs was near the background level, indicating that RBC membrane disruption was minimal. Control data is in agreement with the pH-dependent membrane disruptive potential of PPAA alone demonstrated previously [25]. Together, these data successfully demonstrate PLGA MPs as a matrix for the incorporation and release of PPAA as a pH-dependent membrane-disrupting agent.

Dendritic cell endosomal disruption by PPAA/PLGA MPs

Using previously described methods [42,43] the ability of PPAA/PLGA MPs to promote release of encapsulated material from intracellular endolysosomal compartments was examined in dendritic cells (DCs) (Figure 8). DCs are phagocytic cells in the mammalian immune system with high endocytic activity. In order to track uptake into and release from endosomes, DCs were incubated with the membrane impermeable fluorescent calcein with and without blended MPs. As a positive control for complete cytosolic distribution, the membrane permeable form of calcein, acetoxy methyl ester of calcein (calcein AM), was also used. The DCs incubated with calcein AM showed fluorescence distributed throughout the cell (Figure 8A). DCs incubated with membrane impermeable calcein alone evidenced a punctate pattern of fluorophore localization within endo-lysosomal compartments (Figure 8B). When membrane impermeable calcein was co-incubated with PLGA MPs, fluorescence distribution was slightly more diffuse in comparison to membrane impermeable calcein alone (Figure 8C). In contrast, the extent of cytosolic distribution dramatically increased with the incorporation of PPAA (10%) into MPs (Figure 8D).

Cytosolic distribution of calcein for each treatment was quantified and plotted (Figure 9). The positive control (calcein AM, which is membrane permeable) demonstrated ~80% cytosolic distribution, while DCs incubated with calcein alone (membrane impermeable; negative control) showed the area of fluorescence spread ~5% of the cell area. When PLGA MPs were co-incubated with calcein, the average fluorescence distribution within the cytosol increased to ~20% of the spread area. Notably, when calcein was incubated with 10% PPAA/PLGA MPs, the cytosolic distribution increased to ~70% of the spread area, amounting to a greater than three-fold increase in endosomal escape. All groups were significantly different from each other by ANOVA ($P \geq 0.05$).

While the mode of endolysosomal escape was not examined here, previous work has demonstrated the mechanism by which PPAA enables endosomal escape; the protonation of PPAA carboxylate ions into deionized/hydrophobic carboxylic acid groups at lower pH values encountered during endolysosomal trafficking causes PPAA to partition into endosomal membranes, disorganizing their lamellar structure and releasing internalized material [16,44–46].

Conclusion

We have demonstrated that incorporating PPAA into PPAA/PLGA blend MPs yields a biocompatible drug delivery platform for the triggered cytosolic delivery of a small molecule payload. Incorporation of PPAA endowed the blended MPs with pH-dependent membrane disruptive activity that facilitates escape from endolysosomal compartments following internalization. Furthermore, PPAA incorporation resulted in a modest pH-dependent drug release effect. The blended MP formulations were found to be biocompatible across a range of relevant doses, indicating that they are amenable to non-toxic drug delivery. Unlike drug-loaded polyplexes formed with PPAA alone, the PPAA/PLGA MPs are colloidally stable. As a result, blended MPs have a reduced probability of aggregation in the presence of physiologic salts and serum, thereby opening up the possibility for systemic administration. The advantages of this blended PPAA/PLGA MP platform include tunable degradation and

release kinetics, which can be controlled by varying lactide and glycolide content and by doping in different amounts of PPAA. Furthermore, the cytosolic delivery capability of PPAA/PLGA MPs is greatly enhanced over that of MPs comprised of PLGA alone. Therefore, incorporation of the pH-responsive, membrane-disruptive PPAA into a PLGA MP matrix shows promise as an easily produced and tunable drug delivery vehicle for cytosolic delivery of intracellular acting therapeutics such as DNA, siRNA, proteins, peptides, and small molecule drugs.

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References

1. Danhier F, Ansorena E, Silva JM, Coco R, Le Breton A, Préat V. PLGA-based nanoparticles: an overview of biomedical applications. J Control Release Off J Control Release Soc. 2012; 161:505–522. DOI: 10.1016/j.jconrel.2012.01.043
2. Rao JP, Geckeler KE. Polymer nanoparticles: Preparation techniques and size-control parameters. Prog Polym Sci. 2011; 36:887–913. DOI: 10.1016/j.progpolymsci.2011.01.001
3. Mao S, Xu J, Cai C, Germershaus O, Schaper A, Kissel T. Effect of WOW process parameters on morphology and burst release of FITC-dextran loaded PLGA microspheres. Int J Pharm. 2007; 334:137–148. DOI: 10.1016/j.ijpharm.2006.10.036 [PubMed: 17196348]
4. Bilati U, Allémann E, Doelker E. Development of a nanoprecipitation method intended for the entrapment of hydrophilic drugs into nanoparticles. Eur J Pharm Sci Off J Eur Fed Pharm Sci. 2005; 24:67–75. DOI: 10.1016/j.ejps.2004.09.011
5. Freiberg S, Zhu XX. Polymer microspheres for controlled drug release. Int J Pharm. 2004; 282:1–18. DOI: 10.1016/j.ijpharm.2004.04.013 [PubMed: 15336378]
6. Goddard JM, Hotchkiss JH. Polymer surface modification for the attachment of bioactive compounds. Prog Polym Sci. 2007; 32:698–725. DOI: 10.1016/j.progpolymsci.2007.04.002
7. Lewis JS, Zaveri TD, Crooks CP 2nd, Keselowsky BG. Microparticle surface modifications targeting dendritic cells for non-activating applications. Biomaterials. 2012; 33:7221–7232. DOI: 10.1016/j.biomaterials.2012.06.049 [PubMed: 22796161]
8. Duvvuri S, Gaurav Janoria K, Mitra AK. Effect of polymer blending on the release of ganciclovir from PLGA microspheres. Pharm Res. 2006; 23:215–223. DOI: 10.1007/s11095-005-9042-6 [PubMed: 16320000]
9. Cao X, Schoichet MS. Delivering neuroactive molecules from biodegradable microspheres for application in central nervous system disorders. Biomaterials. 1999; 20:329–339. [PubMed: 10048405]
10. Marrache S, Dhar S. Engineering of blended nanoparticle platform for delivery of mitochondria-acting therapeutics. Proc Natl Acad Sci U S A. 2012; 109:16288–16293. DOI: 10.1073/pnas.1210096109 [PubMed: 22991470]
11. Balashanmugam MV, Nagarethinam S, Jagani H, Josyula VR, Alrohaimi A, Udupa N. Preparation and Characterization of Novel PBAE/PLGA Polymer Blend Microparticles for DNA Vaccine Delivery. Sci World J. 2014; 2014doi: 10.1155/2014/385135
12. Mi FL, Shyu SS, Lin YM, Wu YB, Peng CK, Tsai YH. Chitin/PLGA blend microspheres as a biodegradable drug delivery system: a new delivery system for protein. Biomaterials. 2003; 24:5023–5036. [PubMed: 14559016]
13. Samdancioglu S, Calis S, Sumnu M, Atilla Hincal A. Formulation and in vitro evaluation of bisphosphonate loaded microspheres for implantation in osteolysis. Drug Dev Ind Pharm. 2006; 32:473–481. DOI: 10.1080/03639040500528871 [PubMed: 16638686]

14. Murthy N, Campbell J, Fausto N, Hoffman AS, Stayton PS. Bioinspired pH-responsive polymers for the intracellular delivery of biomolecular drugs. Bioconjug Chem. 2003; 14:412–419. DOI: 10.1021/bc020056d [PubMed: 12643752]

15. Lynn D, Amiji M, Langer R. pH-responsive polymer microspheres: Rapid release of encapsulated material within the range of intracellular pH. Angew Chem Int EdAngew Chem Int Ed. 2001; 40:1707–1710.

16. Stayton PS, El-Sayed MEH, Murthy N, Bulmus V, Lackey C, Cheung C, Hoffman AS. "Smart" delivery systems for biomolecular therapeutics. Orthod Craniofac Res. 2005; 8:219–225. DOI: 10.1111/j.1601-6343.2005.00336.x [PubMed: 16022724]

17. Kyriakides TR, Cheung CY, Murthy N, Bornstein P, Stayton PS, Hoffman AS. pH-Sensitive polymers that enhance intracellular drug delivery in vivo. J Controlled Release. 2002; 78:295–303. DOI: 10.1016/S0168-3659(01)00504-1

18. Nelson CE, Kintzing JR, Hanna A, Shannon JM, Gupta MK, Duvall CL. Balancing cationic and hydrophobic content of PEGylated siRNA polyplexes enhances endosome escape, stability, blood circulation time, and bioactivity in vivo. ACS Nano. 2013; 7:8870–8880. DOI: 10.1021/nn403325f [PubMed: 24041122]

19. Cabiaux V. pH-sensitive toxins: interactions with membrane bilayers and application to drug delivery. Adv Drug Deliv Rev. 2004; 56:987–997. DOI: 10.1016/j.addr.2003.10.044 [PubMed: 15066756]

20. Goletz TJ, Klimpel KR, Leppla SH, Keith JM, Berzofsky JA. Delivery of antigens to the MHC class I pathway using bacterial toxins. Hum Immunol. 1997; 54:129–136. [PubMed: 9297531]

21. Bulmus V, Woodward M, Lin L, Murthy N, Stayton P, Hoffman A. A new pH-responsive and glutathione-reactive, endosomal membrane-disruptive polymeric carrier for intracellular delivery of biomolecular drugs. J Control Release Off J Control Release Soc. 2003; 93:105–120.

22. Lackey CA, Murthy N, Press OW, Tirrell DA, Hoffman AS, Stayton PS. Hemolytic activity of pH-responsive polymer-streptavidin bioconjugates. Bioconjug Chem. 1999; 10:401–405. DOI: 10.1021/bc980109k [PubMed: 10346870]

23. Flanary S, Hoffman AS, Stayton PS. Antigen Delivery with Poly(Propylacrylic Acid) Conjugation Enhances MHC-1 Presentation and T-Cell Activation. Bioconjug Chem. 2009; 20:241–248. DOI: 10.1021/bc800317a [PubMed: 19125614]

24. Convertine AJ, Benoit DSW, Duvall CL, Hoffman AS, Stayton PS. Development of a novel endosomolytic diblock copolymer for siRNA delivery. J Control Release Off J Control Release Soc. 2009; 133:221–229. DOI: 10.1016/j.jconrel.2008.10.004

25. Evans BC, Hocking KM, Kilchrist KV, Wise ES, Brophy CM, Duvall CL. Endosomolytic Nano-Polyplex Platform Technology for Cytosolic Peptide Delivery To Inhibit Pathological Vasoconstriction. ACS Nano. 2015; 9:5893–5907. DOI: 10.1021/acsnano.5b00491 [PubMed: 26004140]

26. Evans BC, Hocking KM, Osgood MJ, Voskresensky I, Dmowska J, Kilchrist KV, Brophy CM, Duvall CL. MK2 inhibitory peptide delivered in nanopolyplexes prevents vascular graft intimal hyperplasia. Sci Transl Med. 2015; 7:291ra95.doi: 10.1126/scitranslmed.aaa4549

27. Hoffman AS. Stimuli-responsive polymers: biomedical applications and challenges for clinical translation. Adv Drug Deliv Rev. 2013; 65:10–16. DOI: 10.1016/j.addr.2012.11.004 [PubMed: 23246762]

28. Ferrito M, Tirrell DA. Poly2-ethylacrylic acid. Macromol Synth. 1992; 11:59–62.

29. Fernando AN, Fernando LP, Fukuda Y, Kaplan AP. Assembly, activation, and signaling by kinin-forming proteins on human vascular smooth muscle cells. Am J Physiol Heart Circ Physiol. 2005; 289:H251–257. DOI: 10.1152/ajpheart.00206.2004 [PubMed: 15961376]

30. Acharya AP, Carstens MR, Lewis JS, Dolgova N, Xia CQ, Clare-Salzler MJ, Keselowsky BG. A cell-based microarray to investigate combinatorial effects of microparticle-encapsulated adjuvants on dendritic cell activation. J Mater Chem B Mater Biol Med. 2016; 4:1672–1685. DOI: 10.1039/C5TB01754H [PubMed: 26985393]

31. Lewis JS, Dolgova NV, Zhang Y, Xia CQ, Wasserfall CH, Atkinson MA, Clare-Salzler MJ, Keselowsky BG. A combination dual-sized microparticle system modulates dendritic cells and

prevents type 1 diabetes in prediabetic NOD mice. Clin Immunol Orlando Fla. 2015; 160:90–102. DOI: 10.1016/j.clim.2015.03.023

32. Desai MP, Labhasetwar V, Walter E, Levy RJ, Amidon GL. The mechanism of uptake of biodegradable microparticles in Caco-2 cells is size dependent. Pharm Res. 1997; 14:1568–1573. [PubMed: 9434276]
33. Jung GY, Na YE, Park MS, Park CS, Myung PK. Preparation of sustained release microparticles with improved initial release property. Arch Pharm Res. 2009; 32:359–365. DOI: 10.1007/s12272-009-1308-9 [PubMed: 19387579]
34. Lewis JS, Roche C, Zhang Y, Brusko TM, Wasserfall CH, Atkinson M, Clare-Salzler MJ, Keselowsky BG. Combinatorial delivery of immunosuppressive factors to dendritic cells using dual-sized microspheres. J Mater Chem B Mater Biol Med. 2014; 2:2562–2574. DOI: 10.1039/C3TB21460E [PubMed: 24778809]
35. Kandel PK, Fernando LP, Ackroyd PC, Christensen KA. Incorporating functionalized polyethylene glycol lipids into reprecipitated conjugated polymer nanoparticles for bioconjugation and targeted labeling of cells. Nanoscale. 2011; 3:1037–1045. DOI: 10.1039/c0nr00746c [PubMed: 21152603]
36. Govender T, Stolnik S, Garnett MC, Illum L, Davis SS. PLGA nanoparticles prepared by nanoprecipitation: drug loading and release studies of a water soluble drug. J Controlled Release. 1999; 57:171–185. DOI: 10.1016/S0168-3659(98)00116-3
37. Makadia HK, Siegel SJ. Poly Lactic-co-Glycolic Acid (PLGA) as Biodegradable Controlled Drug Delivery Carrier. Polymers. 2011; 3:1377–1397. DOI: 10.3390/polym3031377 [PubMed: 22577513]
38. Liu Y, Ghassemi AH, Hennink WE, Schwendeman SP. The microclimate pH in poly(D,L-lactide-co-hydroxymethyl glycolide) microspheres during biodegradation. Biomaterials. 2012; 33doi: 10.1016/j.biomaterials.2012.06.013
39. Lappalainen K, Jääskeläinen I, Syrjänen K, Urtti A, Syrjänen S. Comparison of cell proliferation and toxicity assays using two cationic liposomes. Pharm Res. 1994; 11:1127–1131. [PubMed: 7971713]
40. Nkabinde LA, Shoba-Zikhali LNN, Semete-Makokotlela B, Kalombo L, Swai H. Poly(D,L-lactide-co-glycolide) nanoparticles: Uptake by epithelial cells and cytotoxicity. EXPRESS Polym Lett. 2014; 8:197–206.
41. Evans BC, Nelson CE, Yu SS, Beavers KR, Kim AJ, Li H, Nelson HM, Giorgio TD, Duvall CL. Ex vivo red blood cell hemolysis assay for the evaluation of pH-responsive endosomolytic agents for cytosolic delivery of biomacromolecular drugs. J Vis Exp JoVE. 2013; :e50166.doi: 10.3791/50166 [PubMed: 23524982]
42. Hu Y, Litwin T, Nagaraja AR, Kwong B, Katz J, Watson N, Irvine DJ. Cytosolic delivery of membrane-impermeable molecules in dendritic cells using pH-responsive core-shell nanoparticles. Nano Lett. 2007; 7:3056–3064. DOI: 10.1021/nl071542i [PubMed: 17887715]
43. Jones RA, Cheung CY, Black FE, Zia JK, Stayton PS, Hoffman AS, Wilson MR. Poly(2-alkylacrylic acid) polymers deliver molecules to the cytosol by pH-sensitive disruption of endosomal vesicles. Biochem J. 2003; 372:65–75. DOI: 10.1042/BJ20021945 [PubMed: 12583812]
44. Thomas JL, Barton SW, Tirrell DA. Membrane solubilization by a hydrophobic polyelectrolyte: surface activity and membrane binding. Biophys J. 1994; 67:1101–1106. DOI: 10.1016/S0006-3495(94)80575-2 [PubMed: 7811920]
45. Lackey CA, Press OW, Hoffman AS, Stayton PS. A biomimetic pH-responsive polymer directs endosomal release and intracellular delivery of an endocytosed antibody complex. Bioconjug Chem. 2002; 13:996–1001. [PubMed: 12236781]
46. Kyriakides TR, Cheung CY, Murthy N, Bornstein P, Stayton PS, Hoffman AS. pH-sensitive polymers that enhance intracellular drug delivery in vivo. J Control Release Off J Control Release Soc. 2002; 78:295–303.

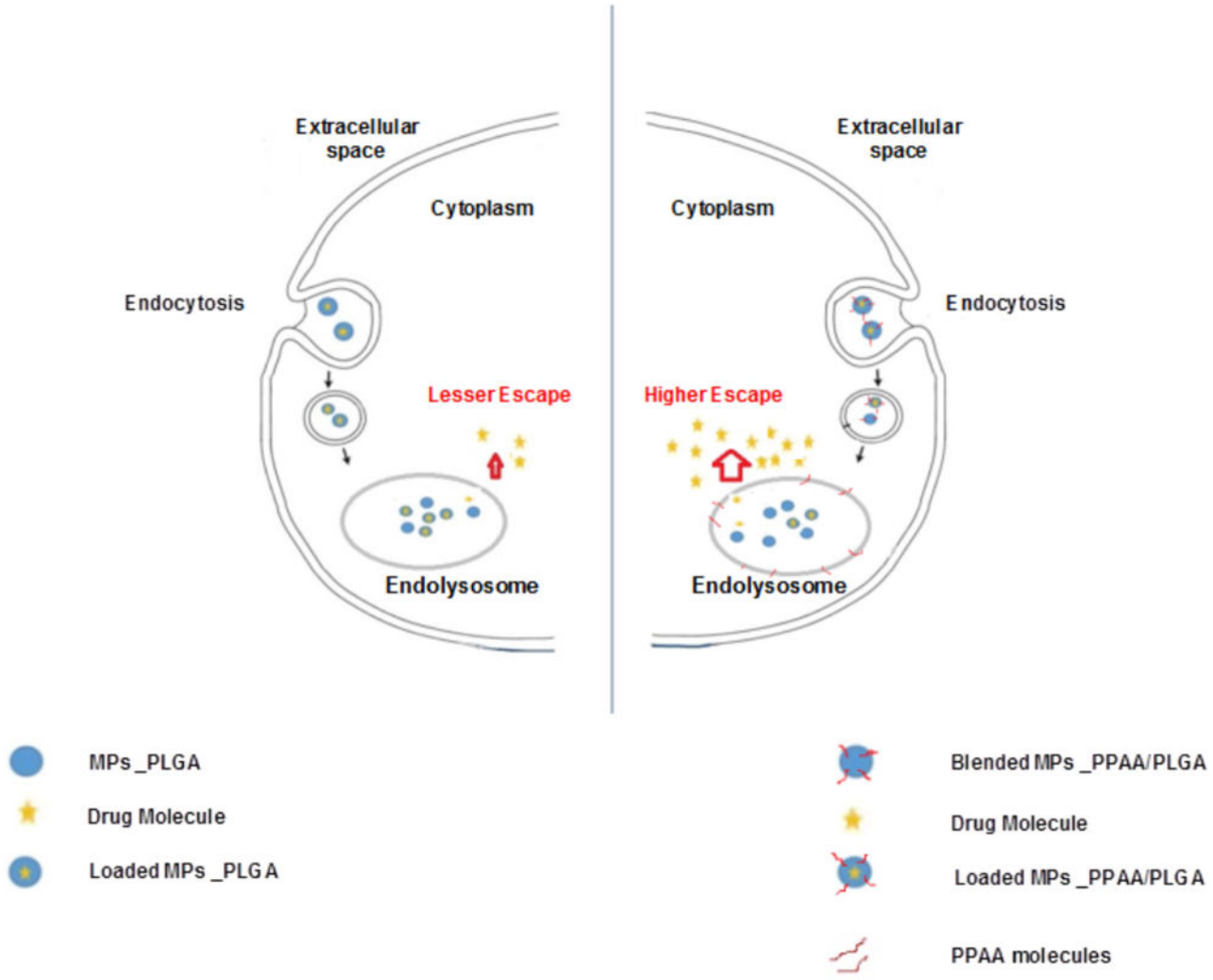

Figure 1.

Conceptual schematic comparing endocytosis-based delivery of microparticle-loaded encapsulate via poly(lactid-co-glycolic acid) microparticle (PLGA MPs; left) versus poly(propylacrylic acid)/poly(lacticco- glycolic acid) blend microparticles (PPAA/PLGA MPs; right) for pH-dependent endosomal membrane disruption and cytosolic delivery. The width of the red arrow indicates the released amount.

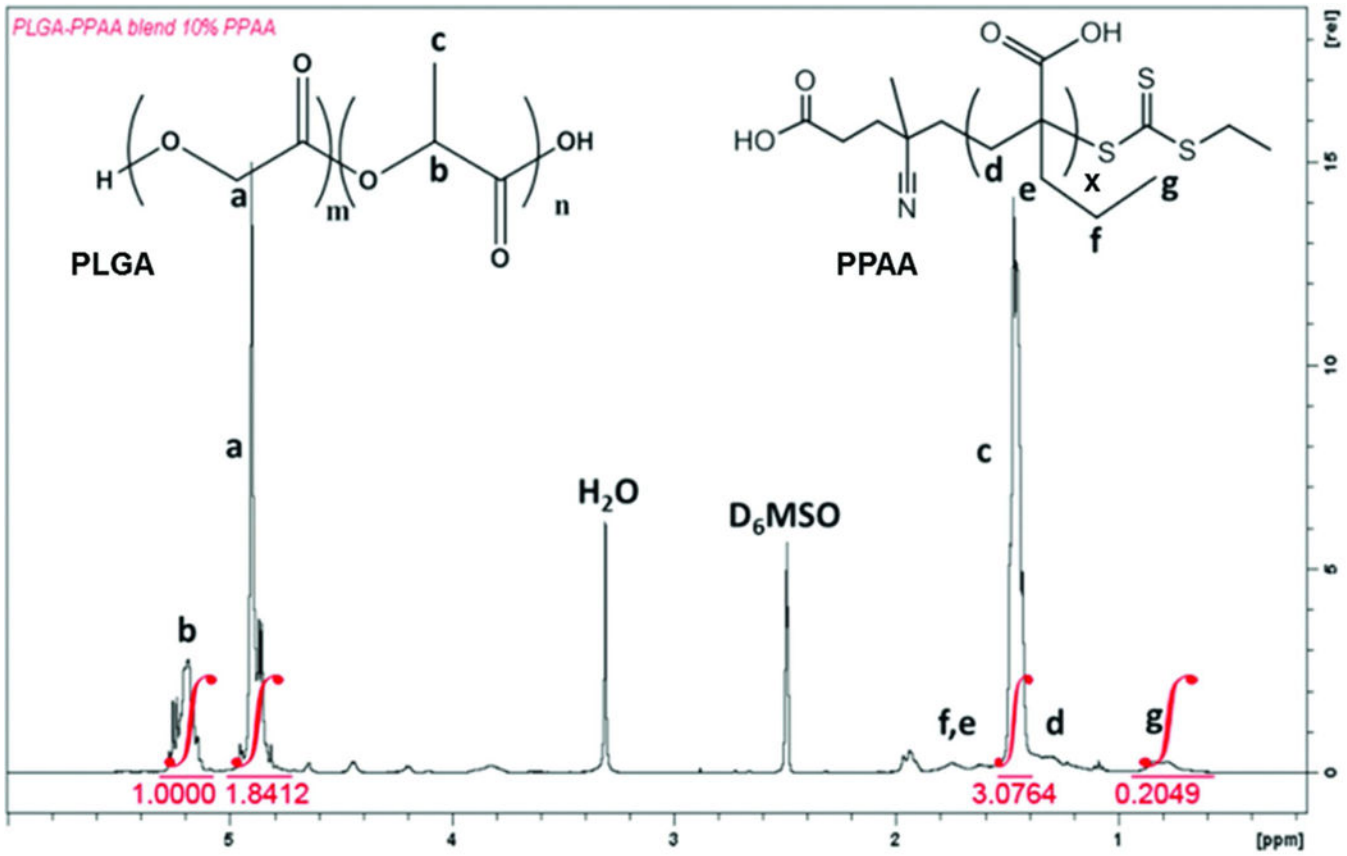

Figure 2.

Representative H1 NMR spectra for 10% polypropylacrylic/polylactic-co-glycolic acid (PPAA/PLGA) blend MPs in D6MSO.The chemical shift values (δ) for the methyl group of PPAA (CH2-CH2-CH3, -peak g), the methyl group of the lactic acid unit of PLGA (C=O-CH(CH3)-O, peak c), and the alkyl group of the glycolic acid unit of PLGA (C=O-CH2-O, peak a) were integrated to determine MP composition. The 4- cyanopentanoic acid and ethyl trithiocarbonate moieties pendant to the propylacrylic acid polymer constitute the ethyl cyanovaleric acid trithiocarbonate chain transfer agent used in RAFT polymerization of PPAA are also shown in the structural diagram of PPAA.

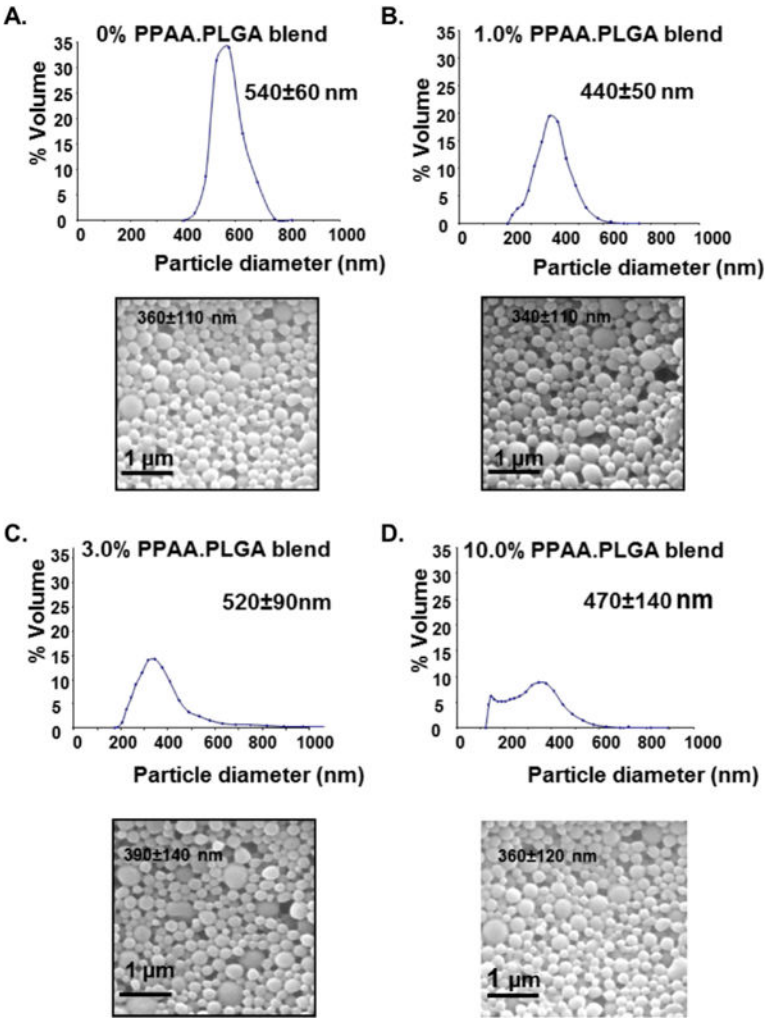

Figure 3.

Size distribution of microparticles (MPs) by dynamic light scattering analysis (DLS, upper panels) and scanning electron microscopy (SEM, lower panels). The data are presented as the frequency (% volume) as a function of particle size for A.) 0% PPAA; B.) 1.0% PPAA; C.) 3.0% PPAA; D.) 10% PPAA blended MPs. Representative SEM scans for each MP formulation demonstrating agreement with DLS results. For each MP preparation, at least 75 individual particles in 3 separate SEM scans were analyzed for diameter using Image J software. Scale bars represents 1 μm at a magnification of 1000×.

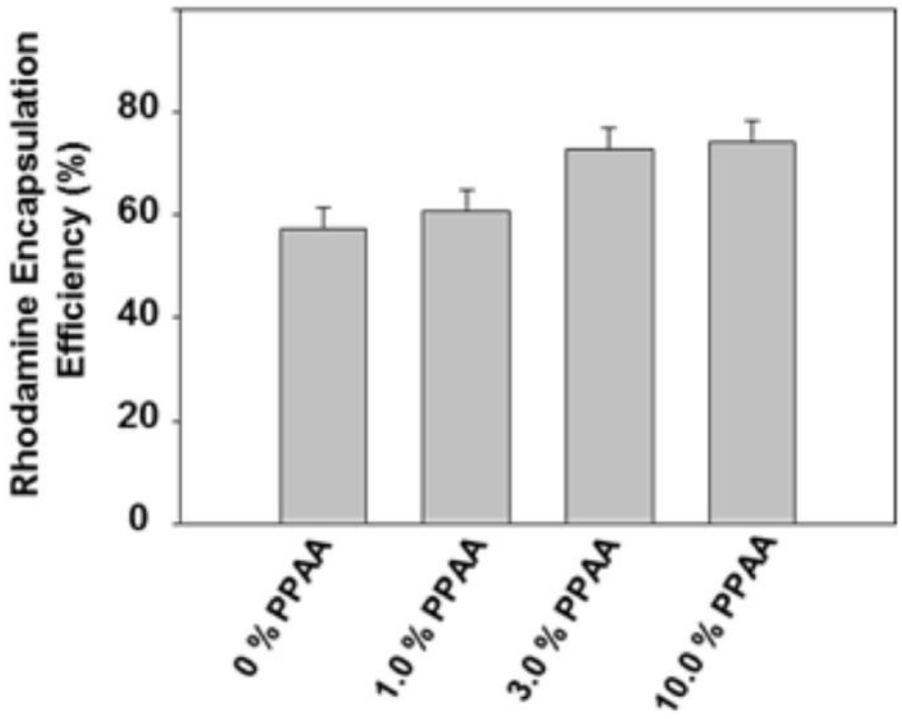

Figure 4.
Rhodamine loading efficiency for the PPAA/PLGA MP blends do not vary significantly ($P \geq 0.05$). Average and standard deviation values for each sample are plotted.

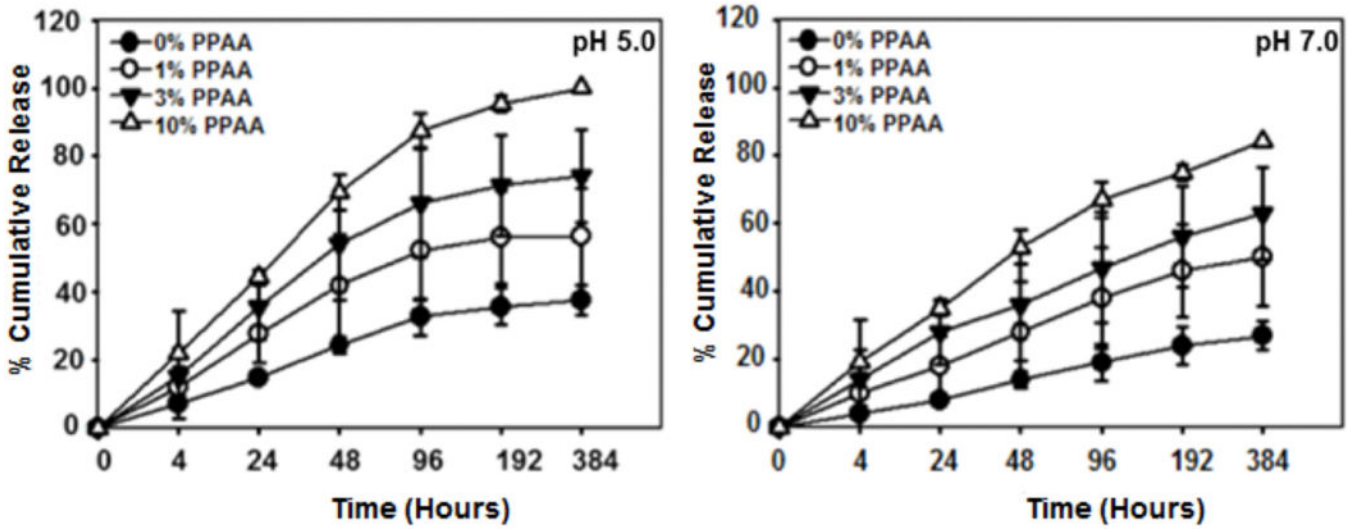

Figure 5.

PPAA/PLGA MP blends demonstrate pH- and composition dependent rhodamine release kinetics. Cumulative release of rhodamine from MPs with different compositions as a function of time at A) pH 5.0 (acidic) and B) pH 7.0 (neutral) is presented as the % of the maximum released at pH 5.0 (10% PPAA, 384h).

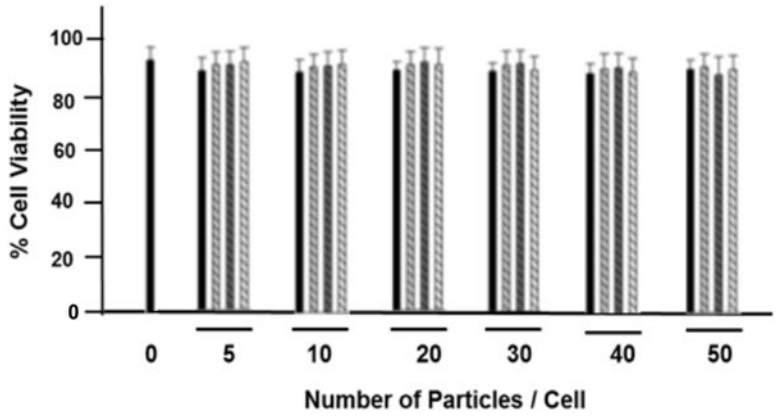

Figure 6.

PPAA/PLGA MPs are not toxic to CHO epithelial cells at 24 h in cell culture media. The percent cell viability for each of the MP preparation at increasing particle number to cell ratios are plotted. X-axis labels: 0 (no particles); 0%PPAA; 1%PPAA; 3%PPAA; 10%PPAA.

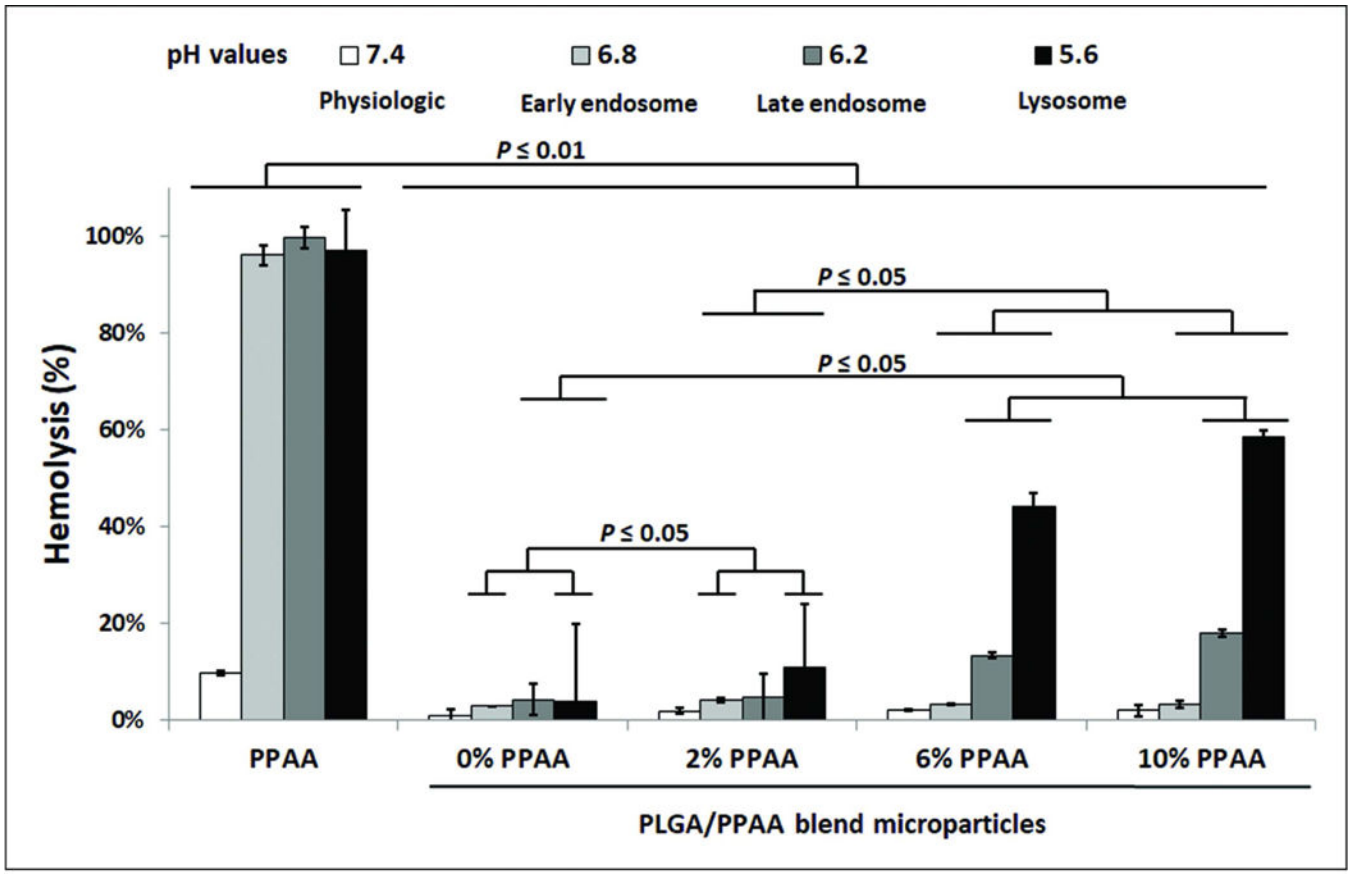

Figure 7.

pH-dependent membrane disruptive activity of PPAA/PLGA blended MPs. Incorporation of PPAA within the PLGA MPs shows dose dependent membrane disruption leading to release of hemoglobin from human red blood cells. The effect is directly proportional to the amount of PPAA incorporated into the MPs. The data represent four technical replicates.

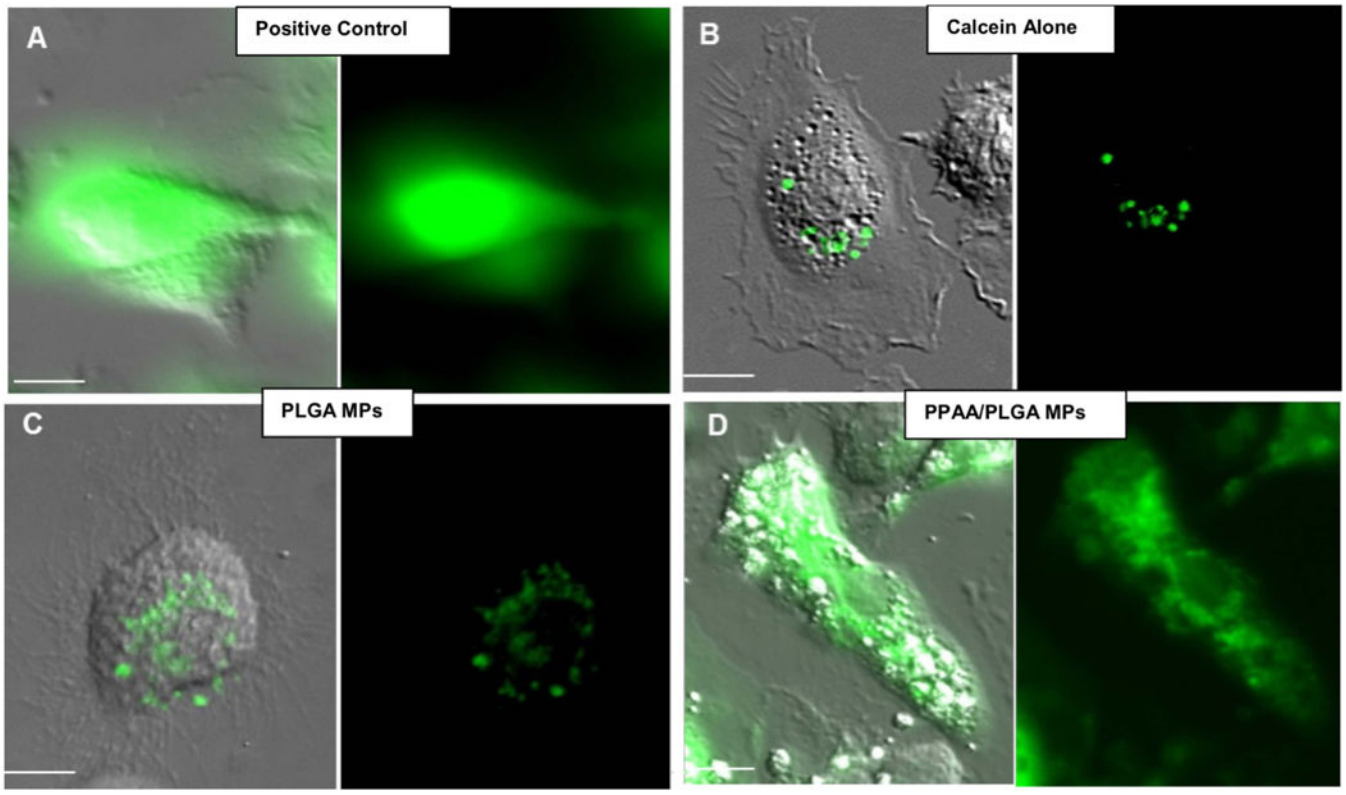

Figure 8.

PPAA/PLGA blended microparticles (MPs) disrupt dendritic cell endolysosomes to a greater extent than PLGA MPs. Co-incubation of dendritic cells 2 h with PPAA/PLGA MPs and membrane impermeable calcein leads to wider cytosolic distribution of calcein fluorescence due to PPAA-mediated release from endosomal compartments. A) Positive control of membrane permeable fluorescent calcein AM. B) Negative control of calcein alone, without MPs; C) Calcein and PLGA MPs, D) Calcein and 10% PPAA/PLGA MPs. Scale bars represent 10 μm.

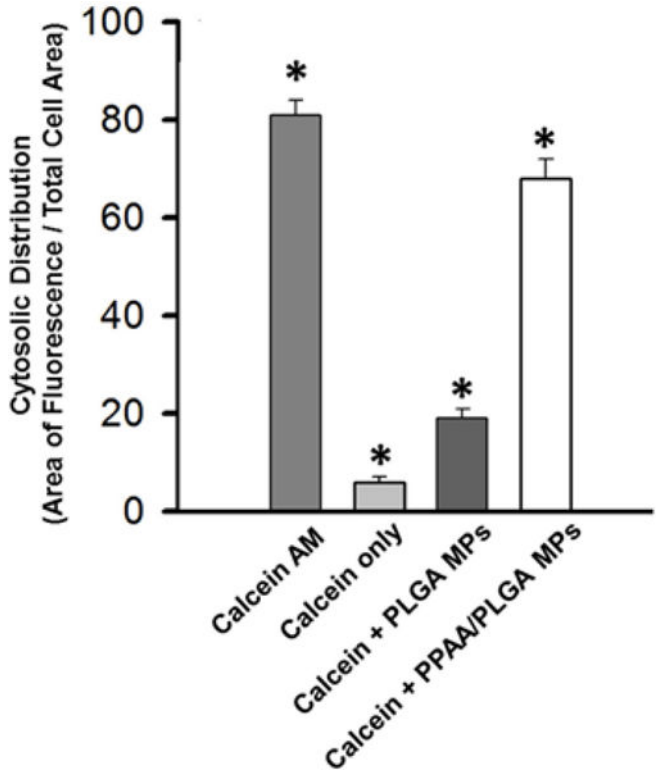

Figure 9.

Quantitative fluorescence microscopic image analysis of calcein released into the cytosol from vesicular compartments upon co-incubation of dendritic cells 2 h with microparticles (MPs). Cytosolic localization is greatly increased by the blend, 10% PPAA/PLGA MPs, compared to PLGA MPs. The ratio of fluorescent area over total cell area is plotted for each treatment. Symbol * denotes significant difference between all groups ($p \leq 0.05$).