Published in final edited form as:
Bioconjug Chem. 2009 February ; 20(2): 241–248. doi:10.1021/bc800317a.

Antigen Delivery with Poly(Propylacrylic Acid) Conjugation Enhances MHC-1 Presentation and T-Cell Activation

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Abstract

While many infectious diseases are controlled by vaccine strategies, important limitations continue to motivate the development of better antigen delivery systems. This study focuses on the use of a pH-sensitive polymeric carrier based on poly(propylacrylic acid) (PPAA) to address the need for more potent CD8 cytotoxic T-cell (CTL) responses. An MHC-1/CD8 CTL cell model system with ovalbumin as the protein antigen was used to test whether PPAA could enhance the delivery of ovalbumin into the MHC-1 display pathway. Ovalbumin was conjugated to poly(propylacrylic acid-co-pyridyldisulfide acrylate) (PPAA-PDSA) by disulfide exchange to make reversible conjugates that could be reduced by the glutathione redox system in the cytosol of antigen presenting cells. The PPAA-PDSA ovalbumin conjugates displayed the pH-sensitive membrane disruptive properties of the parent polymer as determined by their hemolysis activities (sharply active at the endosomal pH values of 6–6.5). The polymer-ovalbumin conjugates exhibited strong 22-fold increases in the MHC-1 presentation and ovalbumin-specific CTL activation compared to free ovalbumin. No CTL activation was observed with control conjugates of ovalbumin and poly(methylacrylic acid) (PMAA) that do not display membrane disruptive activies, suggesting it is the membrane destabilizing properties of the polymer that result in increased MHC-1 display and CTL activation. Further mechanistic studies quantitated the time course of stable intracellular localization of radiolabeled conjugates. 52% of initially internalized PPAA-conjugated ovalbumin remained in the cells after 4 hrs, compared to less than 10% of ovalbumin or PMAA-ovalbumin. These results showing enhanced cytosolic delivery and MHC-1 presentation for the PPAA-antigen conjugates suggest that they warrant future characterization as a CD8-enhancing vaccine delivery system.

Introduction

Vaccines currently play an important role in medicine, but many pathogens remain resistant to vaccine development and therapeutic vaccines against cancer have faced considerable efficacy challenges. One of the most general challenges in the vaccine field is generating a robust and efficacious CD8 cytotoxic T-lymphocyte response, even though many potentially suitable antigens against tumors and infectious agents have been identified(1–3). $CD8^+$ cytotoxic T-lymphocytes (CTLs) are able to directly kill diseased cells and are critical to effective immunotherapy(4,5) while $CD4^+$ helper T-lymphocytes also play an important role in a sustained immune response(6,7). Helper T-lymphocytes are activated upon recognition of antigens presented by major histocompatibility complex class 2 (MHC-2) proteins. This pathway is accessed by exogenous antigens that are processed and combined with MHC-2 molecules within modified endosomal compartments of the APC. CTLs are activated by recognition of antigenic peptides displayed by MHC-1 proteins that are accessed primarily via the cytoplasm, although class I cross-presentation of antigens present in vesicular

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compartments can occur to some degree, especially in the case of larger particulate substances taken up by phagocytosis (8–10).

Exogenous proteins enter APCs through macropinocytosis and are often degraded, sequestered, or exocytosed in the endosomal-lysosomal pathway without gaining access to the cytoplasm(11–13). Enhancing the escape of therapeutic protein antigens from the compartments in which they are endocytosed could potentially increase the efficacy of protein vaccines through increased activation of CTLs(14,15). Several groups have explored the use of pH-sensitive polymer-based delivery systems that take advantage of the decreased pH in the endosome. Carrier particles containing acid-degradable linkages, such as acetal(14–16), hydrozone(17,18), or ketal(19) bonds have been developed to degrade more rapidly in the acidic endosomal environment to trigger release of their cargo. Block ionmer complexes have been developed that increase in positive charge at endosomal pH, which could potentially result in endosomal membrane interaction(20). A popular approach has utilized cationic polymer systems, which enhance endosomal release via the “proton sponge effect”(21–25). Additionally, particles formed from poly(D,L-lactic-co-glycolic acid) (PLGA) have been widely investigated for antigen delivery and have resulted in increasing MHC1 presentation of encapsulated antigens(26–28). While some endosomal escape is observed for this system (27), a mechanism for cytosolic delivery is not well established.

Our group has developed synthetic polymers based on alkyl(acrylic acid) monomers that display pH-sensitive, membrane destabilizing activities (29–36). In the ionized state the polymers are inactive at membrane destabilization, but at the decreased pH levels typical of the endosome (pH 5.5–6.5) the carboxyl groups become protonated and the polymers transition to a state that is membrane destabilizing. This mechanism mimics the pH-dependent membrane disruptive activity of pathogenic proteins such as hemaglutinin (37,38) and diphtheria toxin (39,40), and of synthetic fusogenic peptides such as GALA (41). The mechanistic utility of pathogenic proteins has demonstrated the importance of endosomal release in MHC1 presentation and CTL activation(42–46), but corresponding biological responses to the pathogenic proteins has limited their clinical translation.

Here we report the characterization of a pH-responsive, membrane-destabilizing polymeric carrier for cytosolic protein antigen delivery in a model MHC-1 presentation system. This model utilizes CD8+ T-cells that is activated upon T-cell receptor ligation to an MHC-1/ovalbumin peptide complex displayed from a model RAW macrophage cell(14,47). The poly (propylacrylic acid-co-pyridyldisulfide acrylate) (PPAA-PDSA) polymer was designed to conjugate ovalbumin by disulfide exchange between thiolated protein and the PDSA monomers. The resulting protein-polymer disulfide bond can be reduced by the cytosolic glutathione reduction system to leave the free antigen for subsequent proteolytic processing. Results showing a strong enhancement of CTL activation suggest that the intracellular pharmacokinetic step of vesicular release is a limiting barrier to subsequent protein processing and MHC-1 presentation.

Materials and Methods

Polymer Synthesis

All chemicals and reagents were ACS grade purchased from Sigma-Aldrich, St. Louis, MO, and used without further purification unless otherwise noted. For the synthesis of the PPAA-PDSA polymer, 0.007 mol propylacrylic acid (PAA) (Gateway Chemical Technology, St. Louis, MO), 0.00011 mol PDSA(33), and 0.000056 mol free-radical initiator azobisisobutyronitrile (AIBN, purified by recrystallization from methanol) were combined in a 5ml flask and degassed by 4 rounds of freeze-vacuum-thaw then reacted at 60°C for 24 hours. The polymer was dissolved in 3 ml dimethyl formamide (DMF) and purified by 3 rounds of

precipitation in 500 ml diethyl ether. Poly(methacrylic acid-co-PDSA) (PMAA-PDSA) was synthesized for use as a control polymer. It was formed by reversible addition fragmentation chain transfer polymerization (RAFT) by combining 0.016 mol methacrylic acid (MAA) (purified by distillation), 0.00022 mol PDSA, 0.000052 mol chain transfer agent 4-cyanopentanoic acid dithiobenzoate (CTP)(48), 0.000052 mol initiator 2,2′-Azobis (2,4-dimethyl valeronitrile) (V-65, Wako Chemicals USA, Richmond VA) and 3 ml DMF in a 5 ml round bottom flask. The reaction was degassed by purging with N_2 for 20 min then polymerized at 40°C for 24 hours. 6 ml DMF was added and the polymer was purified by 3 rounds of precipitation in 1 L diethyl ether.

Polymer compositions were determined by H^1-NMR using a Bruker AVance 300MHz instrument and deuterated dimethyl sulfoxide (DMSO-d6, Fisher Chemical, Pittsburgh, PA). The characteristic ^{1}H-NMR (DMSO-d_6) polymer peaks were: 4.0 ppm (2H, O-CH_2-R, PDSA), 7.2 ppm (1H, aromatic, PDSA), 7.8 ppm (2H, aromatic, PDSA), 8.5 ppm (1H, aromatic, PDSA), 12.1 ppm (1H, -COOH, PAAc/MAAc). PDSA content was determined both by NMR and by the absorbance at 343 nm of pyridine-2-thione released from the polymer following reduction with excess of dithiothreitol (DTT). The polymer molecular weights were determined by GPC using a Viscotek VE2001 sample module and VE3580 refractive index detector (Viscotek, Houston, TX), and Tosoh TSK-GEL α-3000 and α-4000 columns (Tosoh Biosciences, Montgomeryville, PA). Polymer molecular weights were determined based on poly(methacrylic acid) (PMMA) standards using HPLC-grade DMF with 0.1% lithium bromide as the mobile phase. The pKa of the PPAA-PDSA was determined by acid/base titration of 50 mg polymer in distilled, deionized water, starting at pH 12.5 and titrating with 0.1 N HCl.

Polymer-Protein Conjugation and Characterization

Conjugation was performed via disulfide exchange between the PDSA component of the polymer and free thiols introduced onto ovalbumin by reaction with Traut's reagent (2-iminothiolane, Pierce Biotechnology, Rockford, IL). 10 mg ovalbumin was mixed with a 10x molar excess of Traut's reagent in conjugation buffer (0.1M phosphate buffer, pH 7.8, 0.15M NaCl, 5mM EDTA) for 1 hour at room temperature. The reaction mixture was purified using a PD-10 desalting column containing Sephadex G-25 (MWCO 5kD, GE Healthcare, Piscataway, NJ) and the degree of modification was estimated by Ellman's assay (Pierce Biotechnology, Rockford, IL). A 2.5x molar excess of polymer, either PPAA-PDSA or PMAA-PDSA, was immediately added to the modified protein and allowed to react 2 hours at room temperature in conjugation buffer. The degree of conjugation was estimated by measuring the absorbance at 343nm (A_{343}) of the pyridine-2-thione group released from PDSA upon disulfide exchange, and the conjugate was purified on a PD-10 column and lyophilized for storage.

Radioactively labeled PPAA-PDSA and PMAA-PDSA - ovalbumin conjugates were formed for use in the cellular uptake and exocytosis experiments. The procedure was identical to that listed above, with the following exceptions. Ovalbumin was mixed with a 20-fold molar excess of Traut's reagent. A 3x molar excess of ^{14}C-iodoacetamide (MP Biomedical, Solon, OH), was then added to react with some of the introduced thiol groups. After reacting for 1 hour, the 2.5X molar excess of polymer was added to form the conjugate.

The molecular weight distribution was determined by GPC (Viscotek VE2001 sample module, VE3580 RI Detector, Waters Corp. ultrahydrogel columns) in 0.1 M sodium phosphate buffer, pH 8 using poly(ethylene oxide) (PEO) standards (Polysciences, Inc., Warrington, PA). The final weight percent of ovalbumin in the conjugates was determined by the BCA protein assay (Pierce Biotechnology, Rockford, IL), using an ovalbumin standard curve. SDS-PAGE (polyacrylamide gel electrophoresis) was performed to detect any unreacted ovalbumin in the conjugate solution. Precast Tris-HCl polyacrylamide gels, 4–20% gradient, sample and running

buffer, and protein standards were purchased from Bio-Rad, Hercules, CA. Based on the results of the BCA assay, conjugates were loaded onto the gels in the appropriate amounts to give 10ug of ovalbumin per lane. Gels were run for 45 min at 100mA, then visualized using Coomassie staining (Bio-Rad, Hercules, CA).

Red Blood Cell Hemolysis

The pH-dependent membrane-disruptive ability of the polymers and polymer-protein conjugates was estimated using a red blood cell hemolysis assay, described previously(29). Briefly, red blood cells were isolated and added to polymer and conjugate solutions (normalized to equivalent polymer amounts) of varying concentrations in 0.1M phosphate buffer at pH values of 5.8, 6.6, and 7.4. The degree of RBC membrane disruption (% hemolysis) was quantified by measuring the absorbance at 541 nm of the hemoglobin released into the solution by lysed cells, in comparison with complete lysis by Triton X-100 detergent. Sample concentrations were all normalized to contain 5μg/ml of polymer, and samples were performed in triplicate with error reported as +/− one standard deviation.

MHC- I Antigen Presentation and CTL Activation Assay

The ability of the polymer to increase cytoplasmic delivery and subsequent MHC class I antigen presentation was evaluated using the *lacZ* antigen presentation assay(14,47). This assay utilizes a specialized *LacZ* B3Z CTL hybridoma. These CTLs produce β-galactosidase upon recognition of the ovalbumin class I antigenic epitope SIINFEKL complexed with the MHC class I molecule H-2K^b, present on RAW 309.1 CR macrophages. Therefore, a measure of β-galactosidase activity can be used to determine the degree to which delivered ovalbumin is presented as a class I antigen. All tissue culture reagents were purchased from Invitrogen Corp, Carlsbad, CA, unless otherwise noted. RAW 309.1 CR macrophages (ATCC, Manassas, VA) were cultured in 90% DMEM with D-Glucose and L-glutamine, 10% fetal bovine serum (FBS), supplemented with 100U/ml penicillin/100μg/ml streptomycin. B3Z CTLs were a gift from Dr. Nilabh Shastri, UC Berkeley. They were cultured in 90% RPMI medium with D-Glucose and L-glutamine, 10% FBS, supplemented with 100U/ml penicillin/100μg/ml streptomycin, 50μM 2-mercaptoethanol (Sigma-Aldrich, St. Louis, MO), and 1mM sodium pyruvate. For the assay, RAW cells were plated at 5×10^4 cells per well in a 96-well plate and grown overnight. PPAA-ovalbumin conjugates and control samples were added to the cells and incubated 6 hrs in DMEM with 5% FBS. Three sample concentrations were tested: 50μg/ml, 100μg/ml, and 150μg/ml (samples were normalized to represent equivalent ovalbumin concentrations). RAW cells were rinsed with DMEM, then 1×10^5 B3Z cells per well were added and incubated 16 hrs. Cells were rinsed with phosphate buffered saline then 100μl lysis buffer [100 μM mercaptoethanol (Sigma-Aldrich, St. Louis, MO), 9 mM $MgCl_2$ (Sigma-Aldrich, St. Louis, MO), and 0.15 mM chlorophenol red β-D- galactoside (EMD Biosciences, San Diego, CA) in PBS] was added. After 4 hrs the absorbance of released chlorophenol red was measured at 595 nm. All samples were evaluated in triplicate and errors are reported as +/− one standard deviation. Maximum possible β-galactosidase production was determined by chemically stimulating the B3Z cells in media containing 3.15 μM ionomycin (Sigma-Aldrich, St. Louis, MO) and 10ng/ml phorbol 12-myristate 13-acetate (Sigma-Aldrich, St. Louis, MO) for 4 hours before rinsing and adding the lysis buffer.

Cellular Uptake and Exocytosis of ^{14}C-Ovalbumin and Ovalbumin-Polymer Conjugates

The cellular uptake and exocytosis of ^{14}C-labeled ovalbumin and the PPAA and PMAA ovalbumin conjugates was studied in RAW 309.1 CR macrophages, using a procedure similar to that described by Besterman et al(49). For measurement of the cellular accumulation of ^{14}C ovalbumin over time, RAW macrophages were plated in a 48-well plate at 75,000 cells/well and allowed to grow overnight. Either ovalbumin, PPAA-ovalbumin conjugate, PMAA-

ovalbumin conjugate, or a PPAA and ovalbumin physical mixture was added to the cells at a concentration of 50μg/ml of ovalbumin. Samples were incubated for either 15 min, 30 min, 1 hr, or 2 hrs. The cells were then washed 2X with PBS and lysed using 1% Triton X-100 in water. Radioactivity in the cell media, PBS wash, and cell lysate was measured using a Beckman-Coulter LS 6500 liquid scintillation counter. EcoScint scintillation fluid was obtained from National Diagnostics, Atlanta, GA. Uptake of ^{14}C-ovalbumin is presented as the % radioactivity present in the cell lysate compared to the total radioactivity delivered. The experiment was performed with a minimum of n=3.

To measure the exocytosis of ^{14}C-ovalbumin, RAW macrophages were again plated at 75,000 cells/well in a 48-well plate and allowed to grow overnight. Either ovalbumin, PPAA-ovalbumin conjugate, PMAA-ovalbumin conjugate, or a PPAA and ovalbumin physical mixture was added to the cells at a concentration of 50μg/ml of ovalbumin. Samples were incubated for either 1 min or 15 min, then uninternalized conjugate was removed and cells were washed 2X with media. Fresh media was added to the cells at the following timpoints: 5 min, 10 min, 20 min, 30 min, 1 hr, 2 hr, and 4 hr. The reappearance of ^{14}C-ovalbumin into the supernatant was measured at each timepoint. After 4 hrs, cells were lysed with 1% Triton X-100 and the radioactivity in the lysate was measured. The amount of ovalbumin internalized after the 1 min or 15 min incubation time was determined as a percentage of the total delivered. The amount of ovalbumin exocytosed at each timepoint, as well as the amount remaining in the cells after 4 hrs, was then determined as a percentage of the total internalized. The experiments were performed with a minimum of n=3.

In vitro Cytotoxicity of Polymers and Conjugates

The cytotoxicity of the PPAA and PMAA polymers and conjugates was determined for both the RAW and B3Z cell lines used in the MHC-1 presentation assay. Cytotoxicity was evaluated using the LDH (lactate dehydrogenase) assay (Roche Applied Sciences, Indianapolis, IN). This assay allows colorimetric measurement of LDH activity in the supernatant, which correlates to the proportion of dead or damaged cells. Cells were plated at 5×10^4 cells/well in their normal culture media and polymer and conjugate samples were added to concentrations up to 300 μg/ml then incubated for 24 hrs. Cells were centrifuged at 250 g for 10 min, then 100 μl supernatant was removed and combined with 100 μl LDH reagent. The absorbance at 490 nm (reference 650 nm) was recorded every 5 min for 30 min and cell survival was determined by comparing to untreated cells and cells lysed with 1% Triton X-100 in water. Each sample was evaluated in triplicate and errors are reported as the standard error of the mean (SEM.)

Statistical Anaylsis

Statistical testing was performed on all data using analysis of variance (ANOVA). The least significant difference (LSD) method was subsequently used for comparison of specific groups in the ANOVA analysis.

Results and Discussion

Synthesis and Characterization of Polymers and Conjugates

The conjugation scheme for attaching ovalbumin to PPAA-PDSA is shown in Figure 1. The conjugates are formed through disulfide exchange of the ovalbumin thiol with the PDSA group in the polymer, and are designed to be reduced by glutathione in the cell cytoplasm, freeing the protein for processing and MHC-1 presentation. The membrane-disruptive PPAA-PDSA polymer was synthesized by free radical polymerization and resulted in a polymer with 3 mol % PDSA and M_w=26 kD, M_n=10 kD, and PDI=2.6, based on GPC analysis using PMMA standards. The pKa of this polymer was determined by acid/base titration to be 6.8, which is in the range of the early endosome. A non membrane-disruptive polymer, PMAA-PDSA, of

similar size (M_w=34 kD, M_n=12 kD, PDI=2.8) containing 2 mol % PDSA was synthesized as a control. Methacrylic acid is less hydrophobic than propylacrylic acid in the protonated form and does not cause any membrane disruption in the RBC hemolysis assay.

The degree of thiolation of the ovalbumin used to form the polymer-ovalbumin conjugates was determined by Ellman's assay to be 15% of the total available lysines. The ovalbumin conjugates formed from both the PPAA-PDSA and PMAA-PDSA polymers were found by BCA assay to contain 37 weight% ovalbumin and 63 weight% polymer, or 1.7 μg polymer per μg protein, and GPC analysis gave M_w=200 kD, M_n=45 kD, PDI=4.4 for the PPAA-PDSA-ovalbumin conjugate and M_w=130 kD, M_n=57 kD, PDI=2.3 for the PMAA-PDSA-ovalbumin conjugate. The ^{14}C-lableled conjugates used for the cellular internalization studies were of similar sizes: M_w=140 kD, M_n=40 kD, PDI=3.5 for the PPAA conjugate and M_w=120 kD, M_n=50 kD, PDI=2.4 for the PMAA conjugate. The degree of thiolation of the ovalbumin used to form these conjugates was determined by Ellman's assay to be 30%. The radiolabeled PPAA and PMAA conjugates were found to be 34 and 40 wt.% ovalbumin, or 1.9 μg polymer per μg protein and 1.5 μg polymer per μg protein, respectively. SDS-PAGE was performed to assure that free protein did not remain in the conjugate mixtures, as it could interfere with interpretation of the MHC-1 presentation assay results.

Red Blood Cell Hemolysis

Red blood cell hemolysis assays were performed to evaluate the pH-dependent membrane disruptive activity of the polymers and polymer-protein conjugates (50). Hemolysis assays were conducted at three pH values (7.4, 6.6, and 5.8) in order to approximate physiological conditions and the conditions found in early and late endosomes. Conjugate concentrations were adjusted to contain 5μg/ml of polymer. The results of this assay are detailed in Figure 2. As expected, the PPAA-PDSA polymer and conjugates were considerably more hemolytic at the lower pH characteristic of the endosome (pH 5.8). The PPAA-PDSA polymer shows slightly higher hemolyis at physiological pH than does a PPAA homopolymer of the same size, which consistently gives less than 10% hemolysis. This increased hemolyis at pH 7.4 is likely due to the hydrophobicity added by the PDSA monomer, especially the pyridyl group, which is removed upon conjugation. The increased hemolysis at pH 7.4 has not resulted in increased cytotoxicity at the concentrations and incubation times used. The PPAA ovalbumin conjugates have similar hemolysis profiles to the PPAA-PDSA polymer alone, indicating that the polymer retains its hemolytic activity when attached to the 40 kD ovalbumin protein.

MHC-I Antigen Presentation and CTL Activation Assay

The ability of the polymer to enhance delivery of protein antigen into the MHC class I pathway was evaluated using the *LacZ* class I presentation assay (14,47). The PPAA-PDSA-ovalbumin conjugate resulted in a strong 22-fold increase in MHC-I presentation and CTL activation compared to free ovalbumin (p=0.0001), as shown in Figure 3. The ovalbumin must be chemically attached to the PPAA in order to be effectively delivered into the MHC-1 pathway, as physical mixtures showed only background CTL activation levels (p=0.20 compared to free ovalbumin). The PMAA-ovalbumin conjugates did not significantly enhance CTL activation over delivery of free ovalbumin (p=0.72.) These results correlate the membrane destabilizing activity of the polymer to the level of MHC-1 pathway and CTL activation, and suggest that the increase in presentation is not solely the result of increased cellular uptake due to the larger size of the conjugate compared to free ovalbumin. Furthermore, PPAA conjugation increases the CTL activation in a dose-dependent manner, whereas higher concentrations of control samples do not result in significant increases in CTL activation ($p<0.2$) (Figure 3b). Increasing the amount of PPAA in the conjugate further exaggerates this effect: when the weight ratio of polymer:protein is doubled from 1.7 to 3.2 μg polymer per μg protein, CTL activation increases, becoming maximized at a lower ovalbumin concentration. While direct comparisons to other

similar antigen delivery systems are not possible due to the different carrier architectures (soluble vs. particulate) and differences in experimental conditions and data presentation, the PPAA conjugates perform similarly to those reported previously with the B3Z CTL activation assay. Acid-degradable particles used to deliver ovalbumin have resulted in maximal A_{595} values (the measure of CTL activation) in the range of 0.25–0.4(14,16), which is similar to that reported in our study, and PLGA particles have been shown to provide a 5-fold increase in CTL activation over delivery of ovalbumin alone(26).

Cellular Uptake and Exocytosis of ^{14}C-Ovalbumin and Ovalbumin-Polymer Conjugates

In order to further explore the mechanism of PPAA action, the cellular uptake and internalization of radiolabled ovalbumin was studied in RAW macrophages. It was found that the major effect of PPAA was to reduce the amount of ovalbumin recycled back out of the cell after initial internalization. The samples were incubated with RAW macrophages for 1 minute, and fresh media was applied after washing of the cells at regular time intervals from 5 min to 4 hrs. The amount of radioactivity that reappeared in the supernatant at each time interval was measured, as well as the radioactivity remaining in the cells. This method is similar to methods previously employed for the study of exocytosis of radiolabeled sucrose in guinea pig alveolar macrophages(49), hapten-protein conjugates in murine macrophages(51), and PLGA nanoparticles in vascular smooth muscle cells(52). The initial amount of ^{14}C-ovalbumin taken up by the cells was statistically similar for all the sample groups (p=0.66), and was approximately 0.8% of the total amount delivered (Figure 4a, left panel). However, less PPAA-conjugated ovalbumin was exocytosed compared to the control groups, which is detailed in the exocytosis profiles shown in Figure 4a, right panel. This resulted in the greater and more stable accumulation of the PPAA-ovalbumin inside the cells, even after 4 hours of exocytosis. 52% of the internalized PPAA-ovalbumin was found inside the cells at four hours, whereas less than 10% of the ovalbumin remained for the free ovalbumin, the physical mixture, and the non membrane-active PMAA conjugate.

Similar exocytosis trends are found when the initial incubation was extended to 15 min (Figure 4b). At this uptake time, the amount of PPAA-conjugated ovalbumin initially inside the cell was already considerably greater than the control groups (left panel). When normalized to the initial amount taken up, the exocytosis profiles were similar to those for the 1 min uptake (right panel). Furthermore, between 15 min and 2 hrs, the amount of intracellular PPAA-ovalbumin increased from 0.75% of the total amount delivered to 4.8%, whereas the control groups remain within experimental error of the initial values (p>0.09) (Figure 5). These results support the hypothesis that PPAA-ovalbumin accumulates in the cell as a result of endosomal escape to the cytosol with an accompanying decrease in exocytosis. It can be noted that for all samples, reappearance of the radiolabled ovalbumin in the supernatant was observed as early as 5 min after the cells were washed, and the majority of exocytosis occured in the first 30 min, a timescale which is in accordance with previous exocytosis studies(49, 51, 52).

In vitro Cytotoxicity of Polymers and Polymer-Ovalbumin Conjugates

The cytotoxicity of the PPAA-PDSA and PMAA-PDSA polymers and conjugates was tested in both the RAW macrophages and B3Z T-cells. Concentrations up to 300 μg/ml, twice the concentration used in the MHC-I presentation assay, were tested using the LDH assay. Cell survival was calculated by comparing the polymer-treated cells with untreated cells and with cells lysed with 1% Triton X-100. It can be seen in Figure 6 that none of the PPAA-PDSA or PMAA-PDSA polymers or their ovablumin conjugates induce toxicity in either cell type at this concentration.

Conclusions

In this study, a pH-sensitive, membrane-disruptive polymer, poly(propylacrylic acid) (PPAA), was synthesized and used to enhance the cytoplasmic delivery and MHC-I presentation of a model protein antigen, ovalbumin. The protein was derivatized to have a thiol group, which was reacted with the pendant PDSA disulfide bond on the polymer to yield a polymer-protein conjugate linked by a disulfide bond. This polymer-S-S-protein linkage was designed to allow release of the protein by glutathione reduction in the cytoplasm. The polymer exhibited low toxicity *in vitro* and retained its membrane-disruptive capabilities after attachment to the hydrophilic protein, as indicated by a red blood cell hemolysis assay. Conjugation to the PPAA polymer was shown to result in increased intracellular accumulation and decreased exocytosis of ovalbumin, which corresponded to significantly enhanced MHC-I presentation of ovalbumin and subsequent CTL activation. This effect is attributed to destabilization of the endosomal membrane by PPAA in the low pH environment of the endosome, and is in accordance with previous studies investigating the destabilizing action of PPAA(32,33,53). This system shows promise for protein vaccine strategies against cancer and viruses, and is also applicable to any technique requiring improved delivery of a protein cargo to the cytoplasm of a cell.

Acknowledgments

This project was supported by funding from the National Institutes of Health (grant R01EB2991-01) and the Whitaker Foundation. We also thank Nilabh Shastri (UC Berkeley) for the B3Z hybridoma cells, which were essential to this study.

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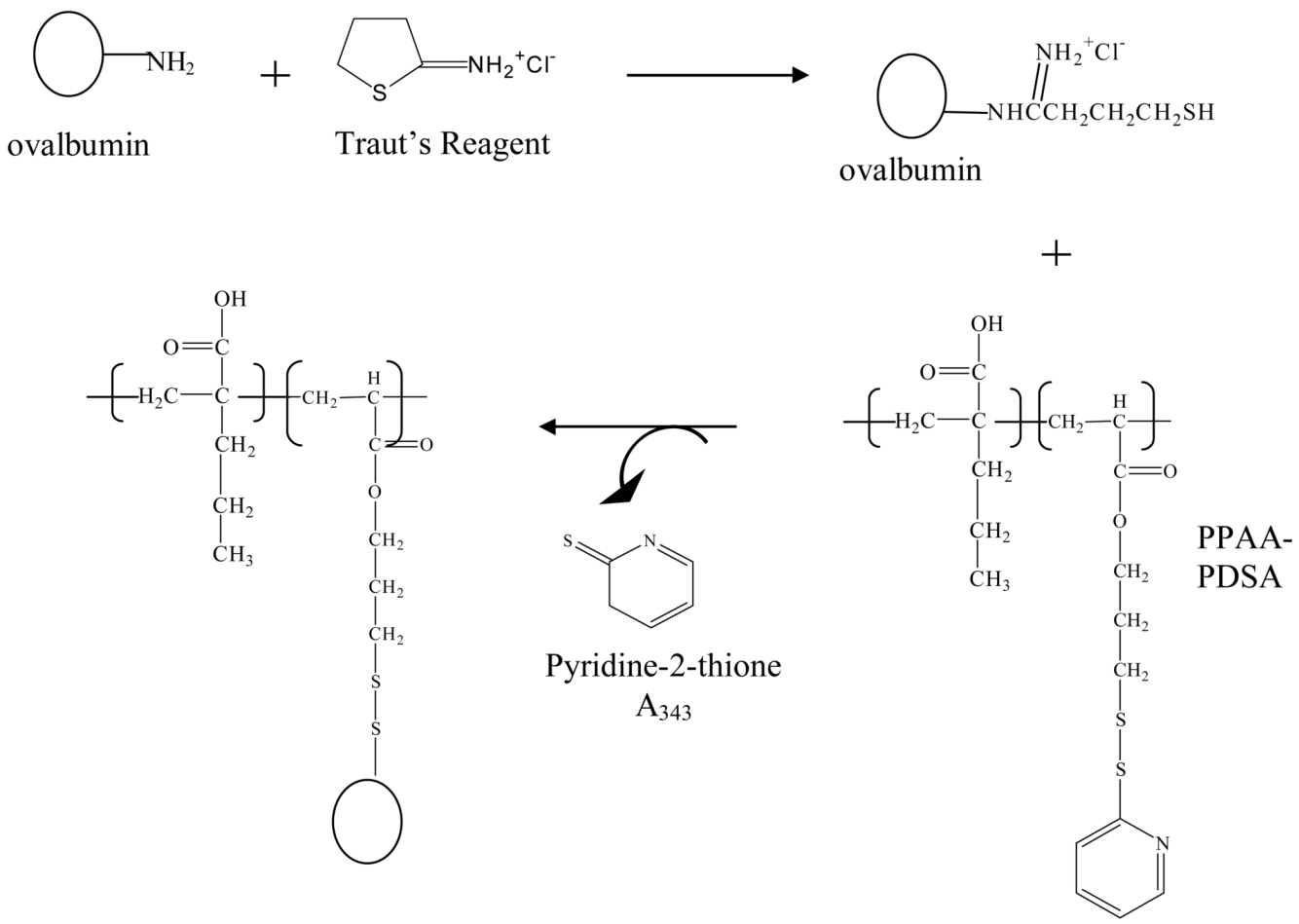

Figure 1.

Schematic of chemistry for conjugation of ovalbumin to the PDSA moiety of the PPAA-PDSA polymer. The protein is first modified using Traut's reagent to convert primary amines to thiols, then the polymer is added and a disulfide exchange reaction occurs, releasing pyridine-2-thione. Reaction progress can be monitored by measuring A_{343} of released pyridine-2-thione. PPAA and PMAA conjugates consisting of 1.7 μg polymer per μg ovalbumin were formed.

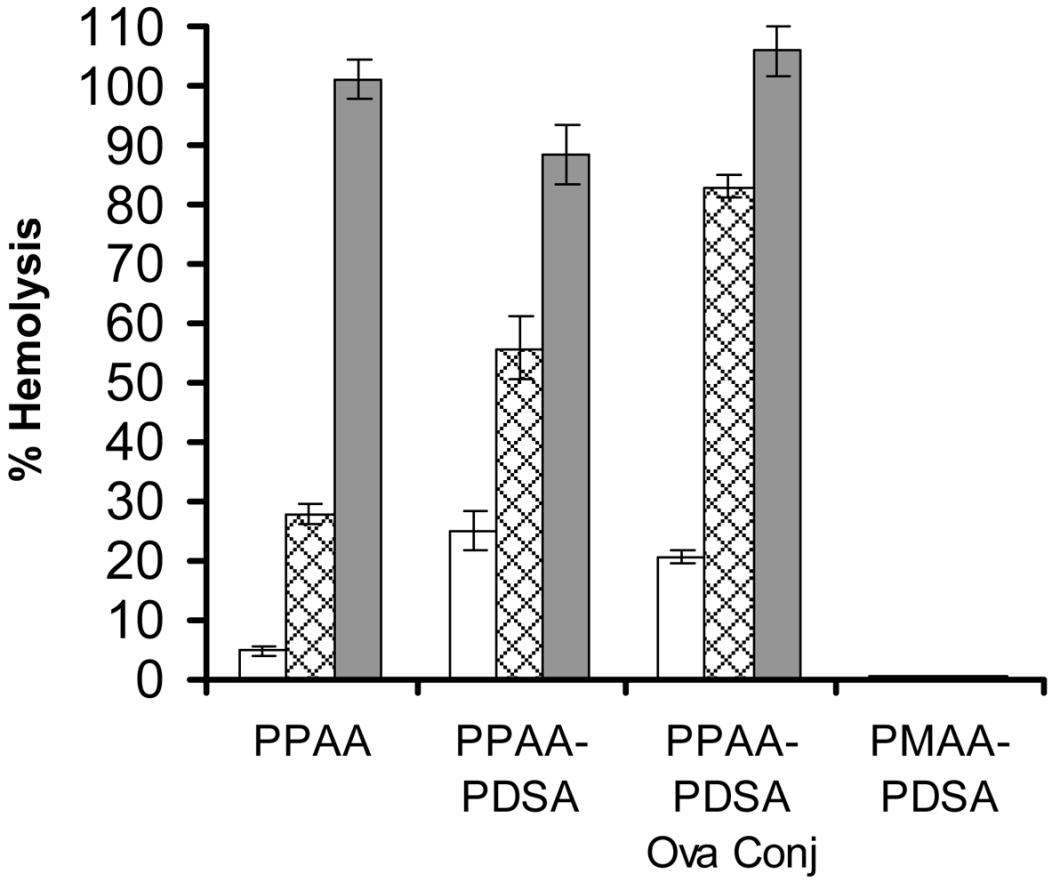

Figure 2.

pH-dependent hemolysis properties of PPAA polymers and ovalbumin conjugates. Red blood cells were isolated and added to polymer and conjugate solutions (polymer concentrations = 5μg/ml) in 0.1M phosphate buffer at pH values of 7.4 (□), 6.6 (x), and 5.8 (■). Hemolysis is reported as a percentage of complete lysis by Triton X-100. PPAA-PDSA shows high membrane-disruptive activity at pH 5.8 but considerably lower membrane disruption at pH 7.4. The polymer retains its hemolysis capabilities after conjugation to the hydrophilic protein ovalbumin. PMAA-PDSA, however, does not lyse red blood cells at any pH due to the decreased hydrophobicity of its methyl side chain compared with the propyl side chain of PPAA.

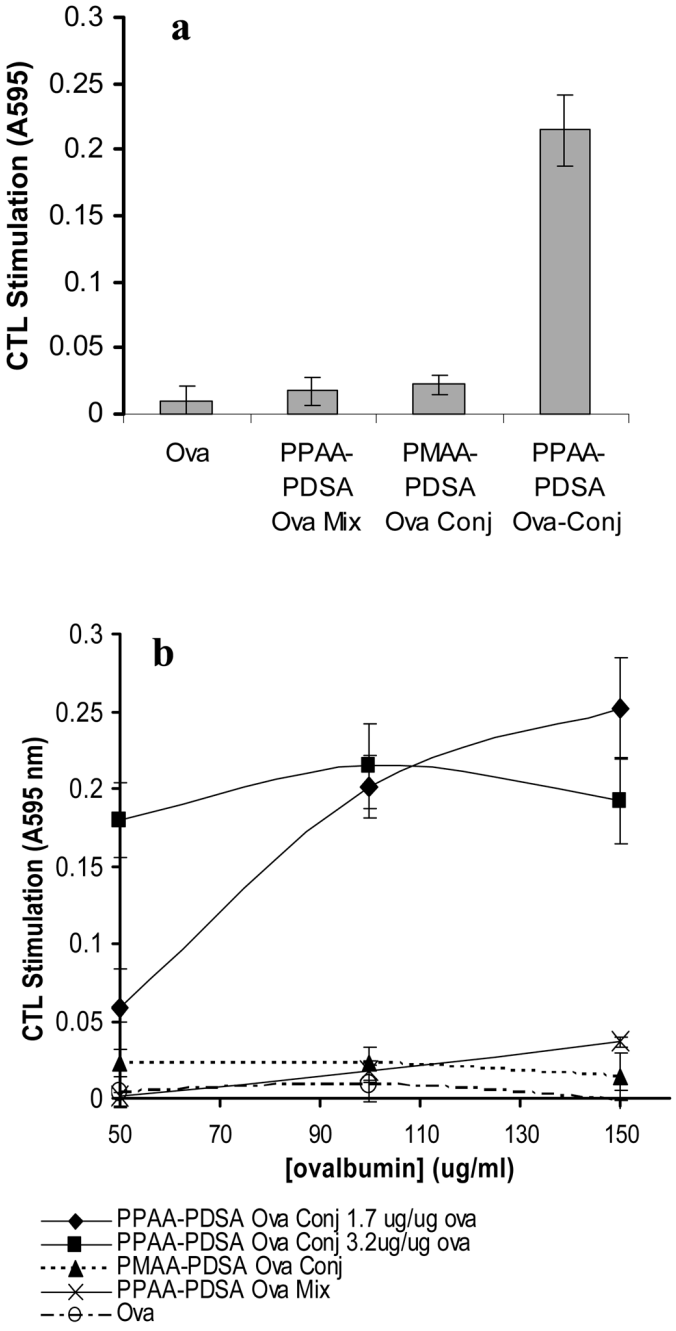

Figure 3.

CTL activation/MHC-I presentation of PPAA-ovalbumin conjugates. Samples were incubated with RAW macrophages for 6 hrs then removed and B3Z T-cells were added for 16 hrs. Cells were rinsed and incubated 4 hrs with lysis buffer containing chlorophenol red β-D- galactoside, then absorbance of released chlorophenol red was measured at 595 nm. Samples were evaluated in triplicate and errors are reported as +/− one standard deviation. For reference, the maximum possible β-galactosidase production was determined by chemically stimulating the B3Z cells using PMA/ionomycin for 4 hrs, which gave an A_{595} of 0.56. a.) Ovalbumin oncentration=100μg/ml. The PPAA-ovalbumin conjugate shows significantly greater CTL activation than do any of the control samples (p<0.0005). This is likely due to the endosomal

disruption provided by PPAA, which allows the protein to more efficiently access the MHC-I pathway in the cytoplasm. However, PMAA conjugation results in low CTL activation, similar to that for free ovalbumin or a PPAA ovalbumin physical mixture (p=0.2). This suggests that the increase in MHC-1 presentation provided by PPAA is due to its endosomal disruptive properties rather than solely to increased uptake due to its larger size compared to free ovalbumin. b) The PPAA-ovalbumin conjugates enhance CTL stimulation is a dose-dependent manner. When the polymer ratio is increased from 1.7 ug/ug ova to 3.2 ug/ug ova, maximal CTL activation is reached at a lower ovalbumin concentration.

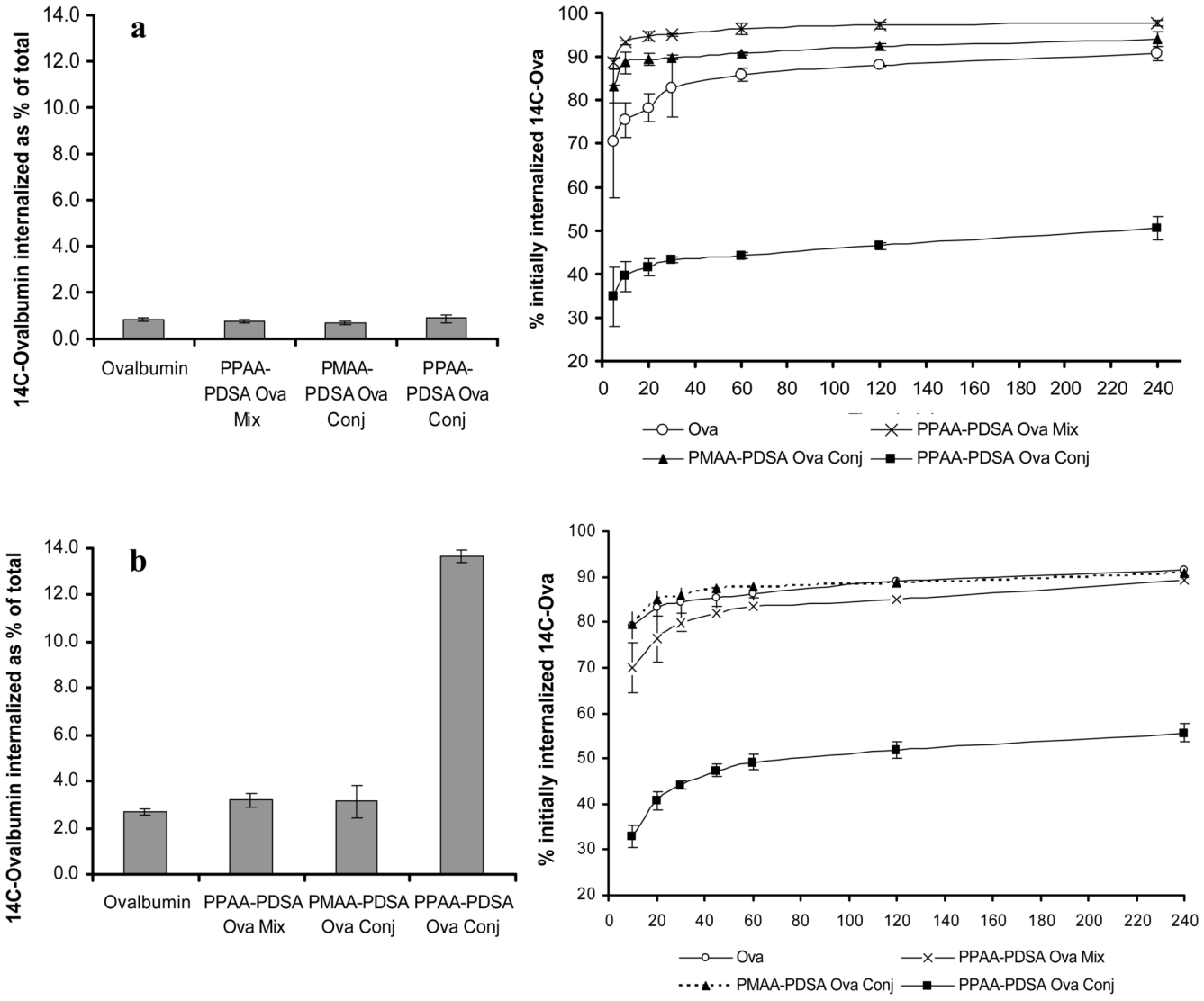

Figure 4.

Exocytosis of ^{14}C-ovalbumin after **a**) 1 min uptake time and **b**) 15min uptake time. Samples were incubated with RAW macrophages and un-internalized conjugate was removed. Fresh media was added to the cells at various time intervals and the reappearance of ^{14}C-ovalbumin into the supernatant was measured. After 4 hrs, cells were lysed with 1% Triton X-100 and the radioactivity in the lysate was measured. Samples were evaluated with a minimum of n=3. The amount of ovalbumin initially internalized was determined as a percentage of the total delivered (left-side panels). It can be seen that the amount of ovalbumin taken into the cells in 1 min is similar for all samples (p=0.66) giving a uniform starting point for the exocytosis measurements. However, the preferential accumulation of PPAA-ovalbumin is already noticeable at the 15-min uptake time. The amount of ovalbumin exocytosed at each timepoint was then calculated as a percentage of the amount initially internalized (right-side panels). Most exocytosis occured in the first 30 minutes, and exocytosis rates were similar for all samples. However, the amount of ovalbumin that remained in the cells and was not exocytosed was greatly enhanced for PPAA conjugates. This effect is likely due to the ability of the polymer to disrupt the endosomal membrane and deliver the ovalbumin to the cytosol before exocytosis can occur.

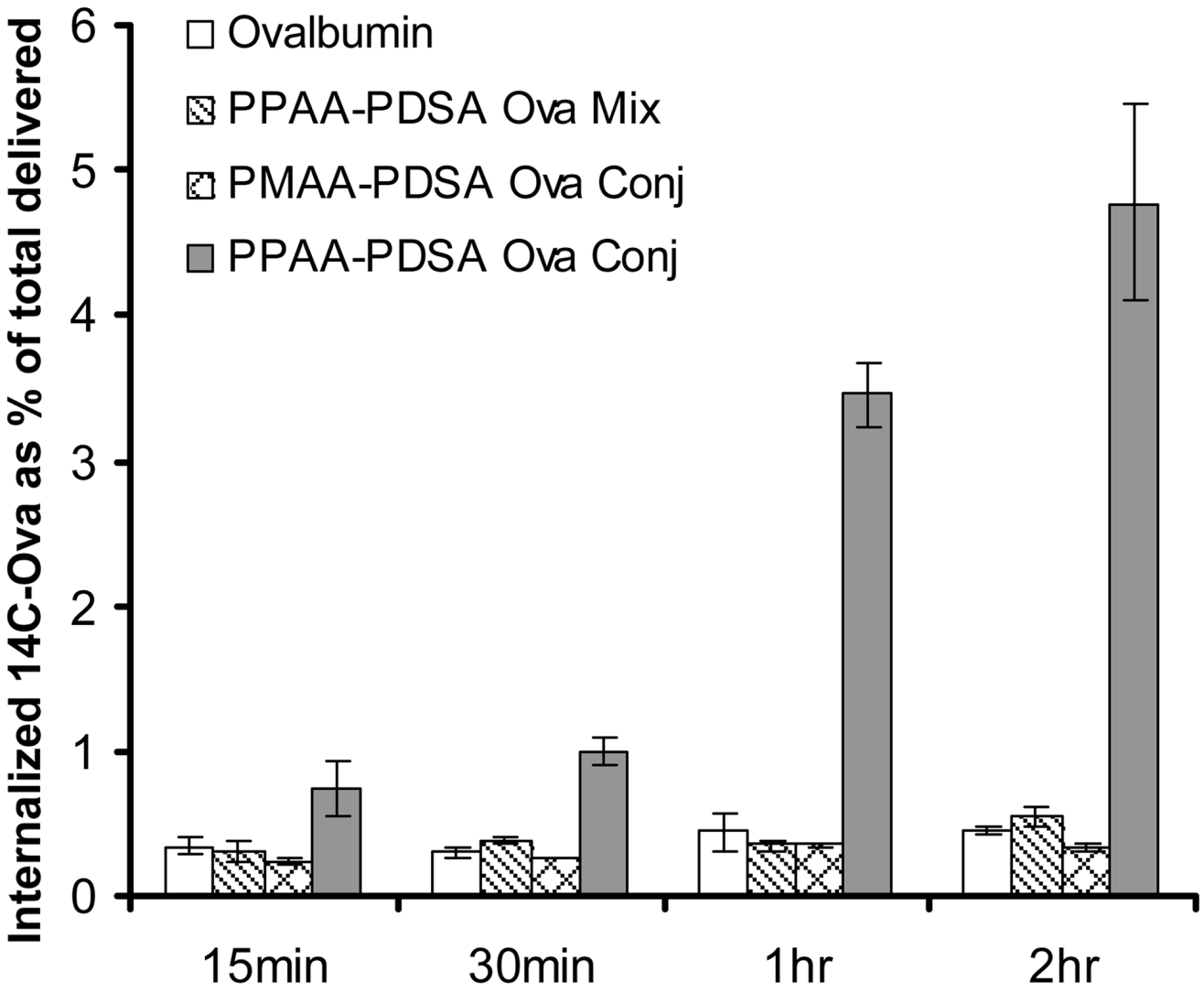

Figure 5.

Uptake of ^{14}C-Ovalbumin-PPAA increases over time. RAW macrophages were incubated with samples at a concentration of 50μg/ml of ovalbumin. The cells were then washed with PBS and lysed using 1% Triton X-100. Radioactivity in the cell media, PBS wash, and cell lysate was measured, and uptake of ^{14}C-ovalbumin was calculated as the % radioactivity present in the cell lysate compared to the total radioactivity delivered. Experiments were performed in triplicate and error is expressed as +/− one standard deviation. It can be seen that PPAA-conjugated ovalbumin continually accumulates significantly in the cell, whereas the control sample levels remain fairly constant (p>0.09). This is likely due to the ability of PPAA-ovalbumin to escape the endosome before being exocytosed.

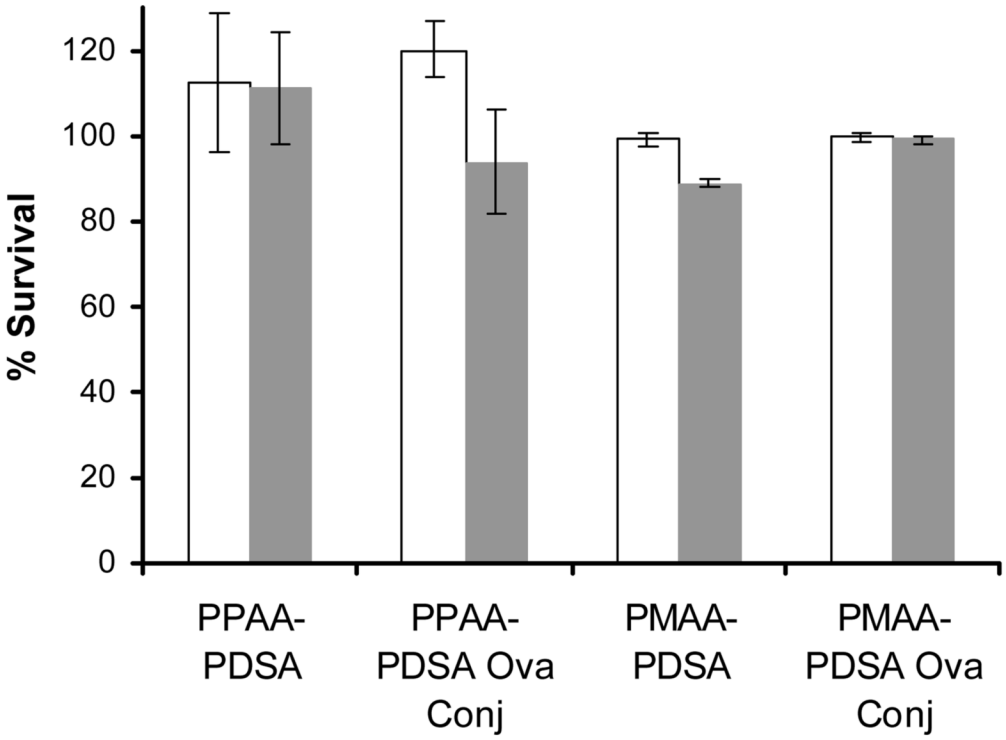

Figure 6.

Cytotoxicity of PPAA and PMAA polymers and conjugates. Cytotoxicity was determined for both RAW and B3Z cells using the LDH assay. Samples were added to cells for 24 hrs at a concentration of 300 μg/ml polymer, twice the highest concentration used in the MHC-1 presentation assay. The cell supernatant was then combined with LDH reagent and the absorbance at 490 nm was recorded. Percent survival = $1-[(A_{490}$ of sample $- A_{490}$ of untreated cells control)/(A_{490} of TritonX control $- A_{490}$ of untreated cells control)] × 100%. Samples were evaluated in triplicate and error is expressed as +/− SEM. It can be seen that excessive toxicity was not observed for any of the samples.