Enhanced Cell Penetration of Acid-Degradable Particles Functionalized with Cell-Penetrating Peptides

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Biopharmaceuticals, such as proteins and DNA, have demonstrated their potential to prevent and cure diseases. The success of such therapeutic agents hinges upon their ability to cross complex barriers in the body and reach their target intact. In order to reap the full benefits of these therapeutic agents, a delivery vehicle capable of delivering cargo to all cell types, both phagocytic and non-phagocytic, is needed. This article presents the synthesis and evaluation of a microparticle delivery vehicle capable of cell penetration and sub-cellular triggered release of an encapsulated payload. pH-sensitive polyacrylamide particles functionalized with a polyarginine cell-penetrating peptide (CPP) were synthesized. The incorporation of a CPP into the microparticles led to efficient uptake by non-phagocytic cells in culture. In addition, the CPP-modified particles showed no cytotoxic effects at concentrations used in this study. The results suggest that these particles may provide a vehicle for the successful delivery of therapeutic agents to various cell types.

INTRODUCTION

In addition to small-molecule based drugs, biopharmaceuticals, such as proteins and DNA, have tremendous potential to prevent and cure diseases (*1–3*). Currently there are over 400 biotechnology medicines in development for the treatment of over 100 diseases, including medications to treat or help prevent multiple sclerosis, hepatitis, breast cancer, and diabetes (*4*). The widespread success of such therapeutic agents hinges upon their ability to cross complex barriers in the body and to reach their target intact. Exogenous therapeutic agents are notoriously unstable in the harsh *in vivo* environment due to proteolytic degradation, sequestration, and renal clearance. These factors limit their use as therapeutic agents. However, encapsulation strategies have successfully mitigated some of these stability and delivery issues by providing protection from physical and chemical damage (*5–11*). Recent advances in polymer synthesis have allowed formulations capable of encapsulation and controlled release of macromolecular therapeutic agents, thereby increasing their stability and ultimately their bioavailability (*12–15*). In addition, by incorporating certain ligands or compounds into the polymer backbone, it is possible to enhance delivery and targeting of the vehicle (*16, 17*). A recent delivery strategy involves the use of cell-penetrating peptides to enhance cellular uptake of cargo by non-phagocytic cells (*18*). Herein, we show the integration of ligands for cell penetration and sub-cellular triggered release in a single delivery vehicle.

Biodegradable polymers have been extensively investigated as potential carriers for biopharmaceuticals (*19–21*). Delivery of a therapeutic agent via encapsulation in particles incorporating programmed release mechanisms, such as acid-degradable crosslinks, allows for their controlled release at select targets, such as inflammatory tissues, tumors, and cells of the immune system. This selective delivery through triggered release mechanisms enables enhanced therapeutic efficiency, allowing lower doses of the therapeutic agent to be used, with less toxicity (*22–27*). Recently, we described the synthesis of polyacrylamide microparticles that incorporated acid-labile crosslinks into the polymer scaffold, as well as targeting groups on the surface (*28–31*). These acid-degradable particles were capable of encapsulating and delivering a protein antigen to phagocytic cells of the immune system, specifically macrophages and dendritic cells. These cell types specialize in taking up foreign matter and trafficking it to acidic sub-cellular lysosomal compartments. After the particles are phagocytosed in this manner, they degrade rapidly in the acidic lysosome, causing the protein payload to be released into the cytoplasm (*29, 30, 32*). This is hypothesized to occur through the destabilization of the lysosomal membrane as a result of the sudden increase in osmotic pressure, due to particle swelling and degradation (*33*).

The size of these particles (ranging from 200 nm to 1 μm in diameter) makes them well-suited for uptake by phagocytic cells, such as macrophages and dendritic cells. Thus, these particles have demonstrated great success at delivering a protected payload to cells of the immune system (*31, 34*). However, the majority of cells in the human body are non-phagocytic, meaning that they are not efficient at ingesting foreign matter and particulates. As a result, access to the lysosomal delivery pathway in non-phagocytic cells is limited, making it difficult to reap the full benefits of therapeutic delivery systems, such as the hydrogel system described above. Thus, one of the most important challenges facing the use of these particles to deliver therapeutic agents is overcoming their lack of uptake by non-phagocytic cells. This problem has motivated work toward making new delivery vehicles capable of encapsulation, cell membrane penetration, and programmed release. Our aim is to design a delivery vehicle that possesses these three properties. In order to create a universal delivery vehicle which would allow for the delivery of cargo to non-phagocytic cells, we have modified our existing delivery system for phagocytic cells by incorporating a cell-penetrating peptide into our pH-sensitive particles.

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Cell membranes act as protective barriers for the cell, only allowing compounds within a narrow range of molecular size, polarity, and charge to enter. Overcoming the barrier of the cell membrane to deliver membrane-impermeable cargos often requires harsh methods such as electroporation or liposomal transfection. These methods are limited to *in vitro* applications and often cause unwanted cellular effects, such as high cytotoxicity (*35*). A more recently developed delivery strategy involves the use of cell-penetrating peptides. Cell-penetrating peptides (CPPs) are peptides with up to 30 amino acids with the ability to translocate across cell membranes of various cell types (*36*). CPPs have been used to deliver a range of cargos including proteins, DNA, antisense peptide nucleic acids, small-molecule drugs, liposomes, and nanoparticles into the cell both *in vitro* and *in vivo* (*37*) without disturbing the stability of the cell membrane and with low cytotoxic effects. CPPs offer several appealing properties as delivery agents including applicability to all cell types, no apparent size constraint of the cargo, and seemingly no immunogenic or inflammatory properties (*36*). CPPs consist of a diverse group of peptides derived from such sources as HIV-Tat, the third helix of the homeodomain of Antennapedia, VP22 herpes virus protein, and other synthetic peptides (*38*), including various arginine-rich sequences (*39–44*). One common feature among these CPPs is the high number of cationic residues, such as arginine. In one study, it was found that the HIV-Tat sequence could be replaced with a simple nonamer of arginine (*45*), suggesting that the guanidinium headgroup of arginine is the essential component of this sequence's ability to transport cargos into the cell (*41, 46, 47*). In addition to the guanidine headgroup, other factors such as the number of arginine residues (*47, 48*) and the length of the side chain (*41*) were found to be important factors in the control of translocation.

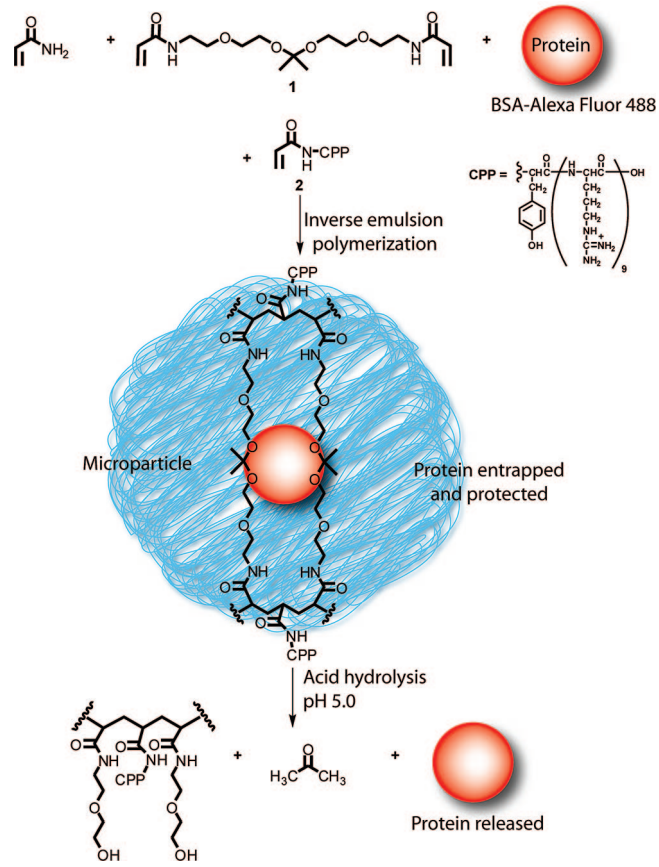
In this report, we describe the synthesis and evaluation of a new delivery vehicle for biopharmaceuticals. A cell-penetrating peptide (CPP) consisting of nine arginine residues was incorporated into acid-degradable polyacrylamide particles to enhance cellular uptake by non-phagocytic cells. Incorporation of the CPP was achieved by copolymerizing a functional monomer **2** with an acid-degradable crosslinker **1** and acrylamide (Scheme 1). The cell uptake of these modified particles in two epithelial cell lines was then investigated.

EXPERIMENTAL PROCEDURES

General Methods. All reagents were purchased from commercial sources and were used without further purification unless otherwise stated. The cell-penetrating peptide (acrylamide-Y-$(R)_9$-COOH) was purchased from Applied Peptech Suzhou. Acryloyl chloride was distilled prior to use. Triethylamine and tetrahydrofuran (THF) were dried by passing through two columns of neutral alumina within a commercial solvent purification apparatus. Combined organic layers after extractions were dried over $MgSO_4$, which was removed by filtration. Solvents were removed under reduced pressure using a rotary evaporator. Reactions requiring anhydrous conditions were performed in flame-dried vessels and under a positive pressure of dry nitrogen. ^{1}H NMR spectra were recorded at 300 or 400 MHz on a Bruker spectrometer. $CDCl_3$ was passed through a plug of basic alumina prior to recording NMR spectra of acid-sensitive samples. To prevent acid-catalyzed hydrolysis of acetal-containing compounds during chromatographic separation, 1% triethylamine was added to the solvents used for elution. Absorbance was measured using a microplate reader (Molecular Dynamics). Particles were imaged using a scanning electron microscope (S5000, Hitachi) after sputter-coating with a 35 Å platinum film.

Compound **1** was prepared following a literature procedure (*28*).

Scheme 1. Synthesis and Subsequent Degradation of CPP-Modified Microparticles



Synthesis of CPP-Modified Microparticles. Microparticles were prepared via an inverse emulsion free radical polymerization (*29, 30*). The organic phase consisted of 3% (w/v) of 3:1 (w/w) Span 80/Tween 80 in hexanes. The aqueous phase was prepared by dissolving cell-penetrating peptide monomer **2** (2.13 mg, 1.3×10^{-6} mol) in 250 μL of phosphate buffered saline (pH 8.0) containing 2 mg/mL BSA-Alexa Fluor 488 conjugate. The pH of this solution was adjusted by the addition of 0.1 M NaOH until the solution had a pH of approximately 8. Acrylamide (85 mg, 1.20 mmol) and acid-labile crosslinker **1** (37 mg, 0.10 mmol, 8 mol %) were dissolved in the aqueous phase. A 50% (w/v) ammonium persulfate solution (10 μL) was added to the aqueous phase. The aqueous phase was then combined with 2.5 mL of the organic phase. The emulsion was prepared by sonicating the solution in a water bath at rt for 30 cycles (ca. 1 s each) in rapid succession using a Branson 450 Sonifier with a ½ in flat tip, an output setting of 2, and a duty cycle of 40%. Polymerization was initiated by the addition of 20 μL of *N*,*N*,*N*′,*N*′-tetramethylethylenediamine (TMEDA) and continued for 10 min at room temperature with constant stirring. The particles were recovered by centrifugation at 1380*g* (rt) for 10 min using 5 μm PVDF spin filters (Ultrafree-CL Durapore, Millipore). The particles were washed with hexanes (2 × 2 mL) and acetone (3 × 2 mL), centrifuging after each wash. After collecting the particles, residual acetone was removed by drying under high vacuum overnight.

Non-degradable CPP-Modified Particles. Non-degradable particles were prepared following the method described above using *N*,*N*′-methylene-bis-acrylamide as the crosslinker. The aqueous phase consisted of acrylamide (103 mg, 1.45 mmol), *N*,*N*′-methylene-bis-acrylamide (19.0 mg, 0.12 mmol, 8 mol %), and cell-penetrating peptide (2.62 mg, 0.1 mol %) dissolved in 250 μL of phosphate buffered saline (pH 8.0) containing 2 mg/mL BSA-Alexa Fluor 488 conjugate.

Unmodified Microparticles. Unmodified degradable and non-degradable microparticles encapsulating BSA-Alexa Fluor 488 conjugate were prepared in a similar fashion as described above omitting monomer **2**. For the degradable particles, the aqueous phase consisted of acrylamide (87 mg, 1.22 mmol) and crosslinker **1** (38 mg, 0.11 mmol, 8 mol %) dissolved in 250 μL of phosphate buffered saline (pH 8.0) containing 2 mg/mL BSA-Alexa Fluor 488 conjugate. For the non-degradable particles, the monomer composition contained acrylamide (105 mg, 1.48 mmol) and *N*,*N*′-methylene-bis-acrylamide (20 mg, 0.13 mmol, 8 mol %) dissolved in 250 μL of phosphate buffered saline (pH 8.0) containing 2 mg/mL BSA-Alexa Fluor 488 conjugate. It was unnecessary to adjust the pH of the aqueous phase in these cases.

CPP Quantification. CPP quantification was performed using a Micro BCA Protein Assay Kit (Pierce Biotechnology, Rockford, IL). The particles were dispersed in 300 mM acetic acid buffer pH 5.0 at a concentration of 5 mg/mL and incubated at 37 °C overnight to degrade the particles. The solution was then analyzed for the concentration of peptide using the kit according to the manufacturer's instructions, and the fluorescence of the resulting solution was quantified on a microplate reader. A standard curve was prepared using known concentrations of peptide in acidic buffer (pH 5.0, 300 mM acetic acid).

Cell Culture. A lung epithelial cell line derived from transformed normal human airway epithelial cells (BEAS-2B, CRL-9609, American type Tissue Culture Collection, Manassas, VA) was used for the majority of cell-based studies. Confirmatory experiments were also conducted in an alternative epithelial cell line (HeLa, obtained from ATCC, catalog no. CCL-2). With both lines, cells were grown overnight to 50% confluence on four-well LabTek plastic chamber slides using Optimem (Invitrogen) media supplemented with 5% FBS and penicillin/streptomycin (approx 50 μg/mL final). Microparticles were added to the media when the cells were 50–60% confluent.

Cell Uptake Studies. Microparticles were sonicated for two 2 s pulses just prior to incubation with cells using concentrations ranging from 15 μg/mL to 500 μg/mL in a 500 μL volume. Cells were incubated with microparticles for 2 h then washed three times in PBS and allowed to incubate an additional 24 h (HeLa cells) or simply allowed to incubate for 2 or 24 h (BEAS-2B cells). At the end of the incubation period, cells were then fixed and imaged. All studies were conducted in duplicate using 2–3 different preparations of microparticles.

Confocal Microscopy. After incubation, chamber slides were washed with PBS and fixed in 4% paraformaldehyde for 15 min at rt. Following additional PBS washes, Alexa Fluor 568 conjugated phalloidin (1:80, Molecular Probes no. A12380) was added to each well and incubated 30 min at rt to label filamentous actin. Chambers were then removed from the slides, and slides were washed again in PBS with a final rinse in water. Following shaking to remove excess water, cells were covered with mounting media (Vectashield, Vector Laboratories, Burlingame, CA) containing 4′,6-diamidino-2-phenylindole (DAPI) to stain intracellular DNA, coverslipped, and sealed with Cytoseal-60. Cells were examined by indirect fluorescent microscopy using an Olympus BX51 and/or confocal microscopy using a Zeiss 510 LSM laser scanning fluorescent confocal microscope. Cells not treated with microparticles and a well with microparticles but no cells were also prepared to measure background fluorescence.

Cytotoxicity Assay. Possible cytotoxicity associated with cell microparticle uptake was assessed by measuring the release of lactic dehydrogenase (LDH) into cell media using a commercially available kit according to the manufacturer's instructions (Roche Laboratories). BEAS-2B cells were cultured in 100 mm dishes with DMEM/10% FBS to approximately 80% confluence. Cells were washed and then incubated for 24 h in fresh media containing 500 μg/mL non-degradable microparticles, 10 mM H_2O_2 (ensuring cell death, thereby serving as a positive control), or no treatment. Testing was done in duplicate. After incubation, media was recovered, centrifuged, and LDH concentrations were measured in 1 mL of supernatant.

RESULTS AND DISCUSSION

Preparation and Characterization of Microparticles Containing a Cell-Penetrating Peptide. The pH sensitivity of our microparticulate delivery system is imparted by an acetal-containing crosslinker **1** (Scheme 1). This dimethyl acetal crosslinker is relatively stable to hydrolysis under physiological conditions (pH 7.4) but degrades rapidly under the acidic conditions typically found in lysosomes (pH 5.0–5.5) (*28*). Incorporation of oligo(ethylene glycol) substituents enhances the solubility of the crosslinker in aqueous solution, an essential characteristic for the inverse emulsion polymerization technique used to prepare the particles. The inverse emulsion process allows for the encapsulation of hydrophilic molecules such as protein and DNA in the particles. Hydrolysis of crosslinker **1** produces acetone, a relatively nontoxic metabolic intermediate of fatty acid oxidation.

Particles containing a CPP were prepared using a 10 amino acid sequence that was modified on the N-terminus with an acrylamide group, **2** (Scheme 1). This acrylamide moiety enables the direct incorporation of the peptide into the particle during its preparation. The sequence selected for use in the particles was acrylamide-Y-$(R)_9$-COOH because nona-arginine has been shown to be the most effective cell-penetrating peptide known today that is composed of natural L-amino acid residues (*41, 49*). Nona-arginine (R_9) was shown to be 20-fold more efficient than HIV-$Tat_{49–57}$ at cellular uptake (*41*). A tyrosine residue was also incorporated into the peptide sequence to allow for radiolabeling of the peptide for future *in vivo* studies.

Microparticles encapsulating bovine serum albumin-Alexa Fluor 488 (BSA-Alexa Fluor 488), a model protein conjugated with a fluorescent tracer, and including CPP were successfully prepared and characterized to investigate the possibility of enhancing cellular uptake of the particles by non-phagocytic cells. The particles were prepared using an inverse emulsion polymerization technique similar to one described previously (*30*), forming protein-loaded polymer particles incorporating CPP. In this process, CPP and BSA-Alexa Fluor 488 conjugate were dissolved in an aqueous buffer along with acrylamide, acid-sensitive crosslinker **1**, and ammonium persulfate. The aqueous phase was then dispersed as droplets in an organic phase consisting of hexanes and a mixture of two surfactants. Free-radical polymerization was initiated by the addition of ammonium persulfate and TMEDA, resulting in cross-linked polymer particles decorated with cell-penetrating peptide throughout the particles but most importantly at the surface. A schematic representation of the synthesis and subsequent degradation of these particles is depicted in Scheme 1. Exposure of the cross-linked particles to an acidic environment leads to rapid hydrolysis of the acetal linkages. Degradation of the polymer particles thus results in the release of BSA-Alexa Fluor 488, acetone, and the CPP-containing polymer backbone.

The isolated particles analyzed in the dry state using scanning electron microscopy (Figure 1) had sizes ranging from 0.2 to 1 μm. Peptide incorporation was measured by degrading a sample of particles under acidic conditions and analyzing the concentration of peptide using a bicinchoninic acid (BCA) assay (*50*). Using this method, the peptide content in the particles was found to be 0.3% by mass.

Unmodified microparticles free of CPP were also prepared using the same inverse emulsion method but omitting monomer

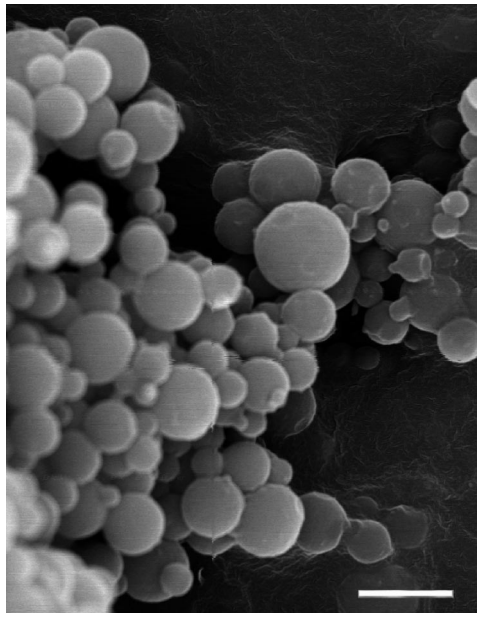


Figure 1. SEM image of CPP-modified microparticles. Scale bar = 1 μm.

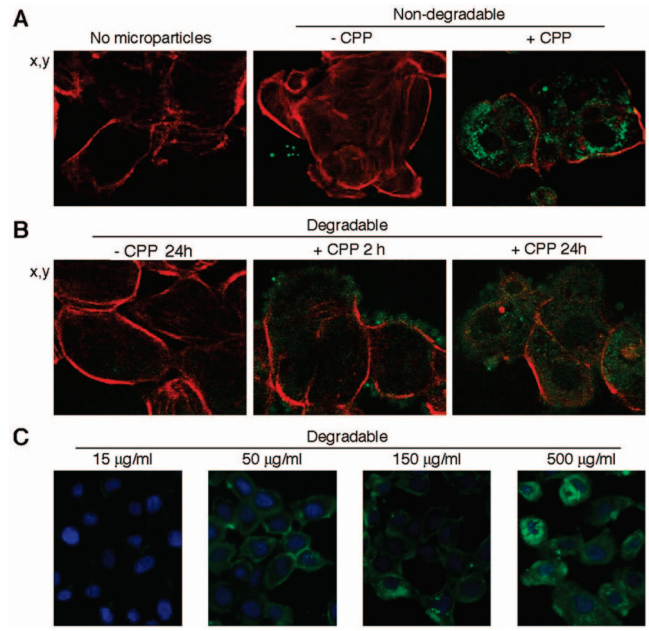


Figure 2. Representative confocal microscope images of BEAS-2B cells, taken at midcell level, 24 h after incubation with microparticles. Cell membranes and cytoskeleton are stained red by phalloidin. Panel A: The cellular accumulation of CPP-modified microparticles 500 μg/mL is markedly greater compared with cells incubated with particles without CPP modification. Panel B: A time dependency is seen when cells are incubated with microparticles at room temperature. At 2 h, most particles appear to be adherent on the cell surface. By 24 h, intracellular accumulation has increased markedly. Note also that the overall fluorescence appears to be more diffuse and less intense than with nondegradable particles. Panel C: Increasing fluorescence is seen intracellularly as microparticle concentration is increased in the medium.

2. These particles contain the same mole percent of cross-linking acetal as the functionalized particles described above. Non-degradable microparticles both with and without cell-penetrating peptide were also prepared as described above using *N*,*N*′-methylene-bis-acrylamide as the cross-linker instead of monomer **1**. SEM examination of these particles showed that they were similar in size and shape to the degradable CPP-modified particles (data not shown).

Cellular Uptake of CPP-Modified Microparticles by Non-Phagocytic Cells. The effect of the CPP modification of the microparticles on their interaction with BEAS-2B epithelial cells is shown in Figure 2. In the absence of microparticle incubation, cell membranes and elements of the intracellular Actin skeleton are clearly evident by phalloidin staining. As expected, no fluorescence from the Alexa Fluor 488 is seen, even at high magnification. When cells were incubated with unmodified non-degradable microparticles (panel A), midcell images obtained by confocal microscopy showed little fluorescence—only visible under high magnification—despite 24 h of incubation time. In contrast, the intracellular accumulation of CPP-modified non-degradable microparticles over the 24 h incubation period was readily evident.

Much research has been done to elucidate the mechanism by which cell-penetrating peptides mediate the transport of various cargoes across the cell membrane. The punctate appearance of microparticles within the cells shown in Figure 2 is consistent with endosomally mediated uptake. Studies by others suggest that uptake is facilitated by positively charged arginine residues (including those present in the CPP used in this study) forming bidentate hydrogen bonds with negatively charged phosphates, sulfates, and carboxylates on the cell surface (*49, 51*).

These results can be contrasted with those observed after cells were incubated with acid-degradable microparticles (panel B, Figure 2). In the absence of the CPP modification, only low levels of fluorescence were seen intracellularly, even after 24 h of incubation (again, most evident at high magnification). With CPP modification, however, high levels of fluorescence are obvious. After just 2 h incubation, microparticles are present both intracellularly as well as clustered on the cell surface. A similar pattern was seen when HeLa cells were incubated with acid-degradable CPP-modified microparticles (data not shown). This association with the cell membrane is probably due to the hydrogen bonding ability of the guanidine headgroup of arginine and the highly cationic nature of the cell-penetrating peptide. After 24 h incubation, the intracellular accumulation of the microparticles is even greater. Again, a similar phenomenon was observed when HeLa cells were incubated with these microparticles. Furthermore, in addition to a time dependency, the accumulation of the CPP-modified microparticles was also concentration-dependent (panel C, Figure 2).

Note, however, that the intensity of fluorescence is more diffuse and less intense than when cells were incubated with the non-degradable particles (compare panel B with panel A). We interpret this difference in appearance to be compatible with time-dependent intracellular microparticle degradation and release of the encapsulated protein. As mentioned above, acid-sensitive crosslinker **1** is chemically stable at pH 7.4, but rapidly hydrolyzes in the acidic environment (pH 5.0) of the lysosome. This hydrolysis is believed to result in an increase in osmotic pressure, leading to lysosomal disruption, thus providing delivery of the encapsulated cargo to the cytoplasm (*28–30*). These results are thus suggestive of release of the encapsulated BSA-Alexa Fluor 488 conjugate into the cytoplasm of the cell.

Importantly, there was no evidence of any cytotoxicity associated with intracellular accumulation of either degradable or non-degradable microparticles. Cells appeared viable with normal cell shape and morphology when viewed microscopically, and DAPI staining showed no evidence of nuclear fragmentation indicative of apoptosis. Furthermore, LDH concentrations in the media of cells incubated with non-degradable particles (with or without CPP modification) were <100 IU/mL. The LDH concentration in cells incubated without micro-

particles was also <100 IU/mL, whereas LDH levels were 4 times higher in cells treated with 10 mM H_2O_2 as a positive control.

CPP modification would be expected to be effective in any tissue. Thus, widespread intracellular accumulation of microparticles would be expected after intravenous administration *in vivo*. For some applications, this may be advantageous, but it is detrimental in others due to unwanted toxicities. It is possible that the microparticles could be further modified with highly specific tissue-targeting moieties that would result in reduced uptake in nontargeted tissues. However, with the lungs, a very attractive delivery strategy is to administer the microparticles intratracheally (e.g., as an aerosol). In this case, cellular uptake would likely remain confined to the lung epithelium, and indeed, it was for this reason that we chose a lung epithelial cell line as the primary target for these studies. Future studies will determine if CPP-modified microparticles will accumulate in lung epithelium after intratracheal delivery *in vivo*.

CONCLUSIONS

A microparticle delivery vehicle encapsulating a fluorescently labeled model protein and containing a cell-penetrating peptide was successfully prepared. *In vitro* studies demonstrated that this delivery vehicle is effective at promoting particle uptake in non-phagocytic epithelial cells. Thus, incorporation of a cell-penetrating peptide expands the potential applications of this delivery vehicle for use in non-phagocytic, as well as phagocytic, cell lines. In addition, by incorporating an acid-degradable crosslinker, the particles are capable of releasing the encapsulated payload upon uptake by these cells. Future studies will explore the feasibility of using these particles to deliver plasmid DNA, peptide nucleic acids, or small molecule therapeutic cargoes to lung epithelial cells to treat acute and chronic lung disease.

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