Published in final edited form as:

Mol Pharm. 2012 December 3; 9(12): 3506–3514. doi:10.1021/mp300338s.

Intracellular delivery and trafficking dynamics of a lymphoma-targeting antibody-polymer conjugate

Geoffrey Y. Berguig¹, Anthony J. Convertine¹, Julie Shi¹, Maria Corinna Palanca-Wessels^{2,3}, Craig L. Duvall^{1,4}, Suzie H. Pun¹, Oliver W. Press^{2,3}, and Patrick S. Stayton^{1,*}

¹Department of Bioengineering, University of Washington, Seattle, WA

²Fred Hutchinson Cancer Research Center, Seattle, WA

³Department of Medicine, University of Washington, Seattle, WA

Abstract

Ratiometric fluorescence and cellular fractionation studies were employed to characterize the intracellular trafficking dynamics of antibody-poly(propylacrylic acid) (PPAA) conjugates in CD22+ RAMOS-AW cells. The HD39 monoclonal antibody (mAb) directs CD22-dependent, receptor-mediated uptake in human B-cell lymphoma cells where it is rapidly trafficked to the lysosomal compartment. To characterize the intracellular-releasing dynamics of the polymer-mAb conjugates, HD39-streptavidin (HD39/SA) was dual-labeled with pH-insensitive Alex Fluor 488 and pH-sensitive pHrodo fluorophores. The subcellular pH-distribution of the HD39/SA-polymer conjugates were quantified as a function of time by live-cell fluorescence microscopy, and the average intracellular pH values experienced by the conjugates were also characterized as a function of time by flow cytometry. PPAA was shown to strongly alter the intracellular trafficking kinetics compared to HD39/SA alone or HD39/SA conjugates with a control polymer, poly(methacrylic acid) (PMAA). Subcellular trafficking studies revealed that after 6 hours only 11% of the HD39/SA-PPAA conjugates had been trafficked to acidic lysosomal compartments with values at or below pH 5.6. In contrast the average intracellular pH of HD39/SA alone dropped from pH 6.7 ± 0.2 at 1 hour to pH 5.6 ± 0.5 after 3 hours and pH 4.7 ± 0.6 after 6 hours. Conjugation of the control PMAA to HD39/SA showed an average pH drop similar to HD39/SA. Subcellular fractionation studies with tritium-labeled HD39/SA demonstrated that after 6 hours, 89% of HD39/SA was associated with endosomes (Rab5+) and lysosomes (Lamp2+), while 45% of HD39/SA-PPAA was translocated to the cytosol (lactate dehydrogenase+). These results demonstrate the endosomal-releasing properties of PPAA with antibody-polymer conjugates and detail their intracellular trafficking dynamics and subcellular compartmental distributions over time.

Keywords

intracellular trafficking; endosomal escape; pH-responsive polymer; anti-CD22 antibody; ratiometric fluorescence; subcellular fractionation; B-cell lymphoma

*Patrick S. Stayton, Box 355061, University of Washington, Seattle, WA 98195-1721, Tel: (206) 685-8148, stayton@u.washington.edu.

⁴Current Address: Department of Biomedical Engineering, Vanderbilt University

All work was completed in Seattle, WA, USA

INTRODUCTION

Non-Hodgkin lymphomas are typically treated with chemotherapy regimens¹ in combination with anti-CD20 monoclonal antibodies (mAb), although resistance to chemotherapy underscores the need for additional targeted therapeutic strategies²⁻⁴. Radiolabeled mAbs have also been employed to selectively target radioactivity directly to tumor cells^{5,6}, however eventual relapse in many patients again underscores the need for new therapeutics. Antibody-drug conjugates (ADCs) can improve the delivery of potent molecules to tumor lesions while limiting toxicities associated with non-targeted chemotoxins^{4,6-9}. ADCs constructed with biologic drugs (e.g. siRNA, DNA, peptides, and proteins) that require cytosolic delivery may be directed to a wide variety of oncogenic targets that are otherwise considered “undruggable” by small molecules. However, delivery of these molecules past the endosomal/lysosomal pathway to intracellular microenvironments (i.e., in the cytoplasm) remains a significant challenge. Following receptor-mediated endocytosis, internalized biologic drugs are typically delivered to acidified, early endosomes (pH ~6.6)^{10,11}. Biologic contents that are not recycled or destined for a distinct cellular compartment are trafficked to late endosomes (pH ~5.8) and eventually lysosomes (pH 4.3) for degradation¹²⁻¹⁴.

Our group has developed synthetic polymers including poly(propylacrylic acid) (PPAA) that display pH-sensitive, membrane-destabilizing activities that are especially well-tuned for intracellular delivery¹⁵. Incorporation of PPAA with biologic drugs have demonstrated enhanced therapeutic activity^{14,16-18}. The intracellular trafficking kinetics of PPAA conjugates has yet to be evaluated and could help evolve more efficacious drug delivery systems. Fluorescence imaging has been shown to be a powerful tool for following the intracellular trafficking of biomacromolecules. For example, Massignani *et al.* used subcellular imaging techniques to measure the trafficking kinetics of polymersomes from endocytosis to endosomal trafficking and cytosolic release¹⁹. In another study, Lee *et al.* employed ratiometric fluorescence techniques to characterize the pH of folate-driven trafficking through endosomes. The intracellular pH was quantified via confocal microscopy of live cells treated with folate modified with pH-sensitive or pH-insensitive fluorophores^{20,21}. Akinc and Langer used a similar methodology to measure the environmental pH of non-viral vectors for DNA delivery. Rather than microscopic imaging, the average environmental pH of DNA over a larger population of cells was quantitated by flow cytometry²²⁻²⁴.

In this work quantitative ratiometric fluorescence microscopy was combined with flow cytometry in order to study the intracellular trafficking dynamics of an anti-CD22 internalizing mAb with an endosomal-releasing polymer (Figure 1). CD22 receptor ligation results in rapid endocytosis of the mAbs followed by lysosomal trafficking. Ratiometric fluorescence studies employing live cell fluorescence microscopy and flow cytometry were used to quantify the trafficking kinetics of the mAb conjugates. The results were correlated with subcellular fractionation measurements that directly measured quantities of translocated mAb conjugates versus controls^{25,26}. These studies shed new mechanistic insight into the activity of endosomal releasing, pH-responsive polymer carriers.

EXPERIMENTAL SECTION

Materials

Spectra/Pro Molecular Porous membrane tubing (MWCO 6-8,000 Dalton) was purchased from Spectrum Laboratories (Houston, TX). Alexa Fluor 488 carboxylic acid, succinimide ester (AF488) and pHrodo, succinimide ester (pHrodo) was purchased from Molecular Probes (Eugene, OR). Bio-Spin 30 Chromatography Columns, pre-packed with Bio-Gel

P-30 gel in saline-sodium citrate (SSC) buffer (40,000 MW limit, 732-6006-lot 400030949) were purchased from Bio-Rad (Hercules, CA). 2-(4-hydroxyphenolazo)benzoic acid (HABA) was purchased from Sigma-Aldrich (St. Louis, MO). Proactive biotin-coated microspheres (10.14 μm diameter, CP10N-lot 9310) were purchased from Bangs Laboratories (Fishers, IN). Propylacrylic acid (PAA) was synthesized as described previously²⁷. Methacrylic acid (MAA) (Sigma-Aldrich) was vacuum distilled prior to use. ³H-N-Succinimidyl propionate was purchased from American Radiolabeled Chemicals, Inc (St. Louis, MO). The HD39 mAb was produced by injecting hybridomas into pristine-primed mice to generate ascites and purified as previously described²⁸. HD39 was then conjugated to streptavidin via an SMCC heterobifunctional linker to form covalent chemical conjugates using previously described methods²⁹. The purified HD39/SA conjugate contained 1.2 SA per mAb.

Ramos-AW Cell Culture

Ramos-AW cells were obtained from the European Collection of Cell Cultures (ECACC, Salisbury, UK). Cell cultures were maintained in log phase growth in RPMI 1640 medium containing L-glutamine, 25 mM HEPES, supplemented with 1% penicillin-streptomycin (GIBCO) and 10% fetal bovine serum (FBS, Invitrogen) at 37 °C and 5% CO₂.

Synthesis of Biotinylated Polymers

RAFT (radical addition-fragmentation chain transfer) polymerization of PAA and MAA was conducted in DMF and DMSO, respectively, under anhydrous conditions for 24 hours at 60 °C using biotin-ECT (4-Cyano-4-(ethylsulfanylthiocarbonyl) sulfanylpentanoic acid)³⁰ as the chain transfer agent (CTA) and azobisisobutyronitrile (AIBN) as the radical initiator (Scheme 1). The initial monomer to CTA ratio ($[M]_0/[CTA]_0$), and initial CTA to initiator ratios ($[CTA]_0/[I]_0$) for the polymerization of PAA and MAA were 150 to 1 and 1 to 1 respectively. The resultant polymers were isolated by precipitation in diethyl ether. The precipitated polymers were then redissolved in DMF and reprecipitated into ether (x 4). The dry polymers were redissolved in DMSO, added to 0.5 M Na₂(CO₃)₂, pH 8.7 at a 10-fold volume dilution, and dialyzed in dH₂O using dialysis tubing (MWCO 6-8,000 Da) to remove monomer and solvent. Gel permeation chromatography (GPC) was used to determine molecular weights and polydispersities (M_w/M_n , PDI) of PPAA and PMAA using Tosoh SEC TSK-GEL α -3000 and α -4000 columns (Tosoh Bioscience, Montgomeryville, PA) connected in series to an Agilent 1200 Series Liquid Chromatography System (Santa Clara, CA) and Wyatt Technology miniDAWN TREOS, 3 angle MALS light scattering instrument and Optilab rEX, refractive index detector (Santa Barbara, CA). HPLC-grade DMF containing 0.1 wt.% LiBr at 60 °C was used as the mobile phase at a flow rate of 1 ml/min.

Antibody-Polymer Conjugation

A HABA binding assay, modified from Green³¹, was used to determine the molar excess of PPAA or PMAA chains to achieve 4 polymer chains per HD39/SA. HABA was dissolved in 10 mM sodium hydroxide buffer, pH 12 at 2.65 mM and added to SA or HD39/SA in sodium phosphate buffer, pH 7.4 at 40-fold molar excess to occupy all biotin-binding sites. Biotin, PPAA, or PMAA in PBS was added to the HABA solution at a range of concentrations. The final SA or HD39/SA solution at 6.6 μM was measured on a plate reader. A biotin standard curve was made to correlate A_{500nm} with biotin-binding events and then used to quantify polymer-binding events. To verify complexation of PPAA or PMAA with HD39/SA, a gel retardation assay was performed with 10 μg of streptavidin or 2.56 μg of HD39/SA. Samples were loaded into a Bio-Rad Ready Gel containing 4–15% Tris-HCl using a 5X-loading buffer that contained 310 mM Tris HCl, 50% glycerol and 1 $\mu\text{g}/\text{ml}$ of bromophenol blue. The running buffer contained 30 g/L Tris Base, 144 g/L glycine, 10 g/L

SDS in dH₂O. Samples were run at a constant 125 V for approximately 1 hour and stained using Gel-Code Blue.

Preparation of Dual-Fluorescently Labeled HD39/SA Conjugates

For ratiometric fluorescence studies, HD39/SA was dual-labeled with amine reactive, pH-insensitive Alex Fluor 488, succinimydyl ester (AF488), and pH-sensitive pHrodo, succinimydyl ester. For dual-labeling, AF488 and pHrodo were dissolved in DMSO at 1 mg/ml and added to the reaction mixture at a 5-fold molar excess to 6.08 μ M HD39/SA in a 0.1 M sodium bicarbonate buffer, pH 8.5. The reaction was performed at room temperature for 1 hour followed by purification in micro-spin columns. For 95% recovery of the antibody conjugate with micro-spin columns, SSC buffer was removed from the prepacked gel, and rinsed with 0.5% BSA in PBS and 10% acetonitrile in PBS. Spin-columns were then equilibrated with 0.5 mg/mL NaN₃ in PBS, pH 7.4. Each rinse consisted of 1.1 mL buffer volume and centrifugation at 1000 g for 4 min. Dual-labeled HD39/SA was purified from unreacted fluorophore using the pretreated spin columns. The final concentration of HD39/SA and degree of labeling was determined spectrophotometrically by applying absorbance measurements to a formula provided by the manufacturer. Extinction coefficients for HD39/SA (380,000 M⁻¹ cm⁻¹) and pHrodo (39,600 M⁻¹ cm⁻¹) were measured experimentally.

Characterization of Ratiometric Fluorescence by Flow Cytometry

The relationship between pH and ratiometric fluorescence of the dual-labeled HD39/SA conjugate was characterized by fluorescence microscopy. HD39/SA was mixed with biotin coated polystyrene beads and imaged with a fluorescence microscope. Briefly, dual-labeled HD39/SA was incubated at 25 nM with 10⁶ beads/mL for 1 h then aliquoted and washed 2X in pH buffers (4.6–7.4). pH buffers were made by mixing 0.2 M phosphate buffer (monobasic, pH 5.0) with 0.1 M citric acid (pH 4.0) or 0.2 M phosphate buffer (dibasic, pH 8.0). The change in ratiometric fluorescence with pH buffers was measured by flow cytometry and fluorescence microscopy. For flow cytometry, gating was performed with unlabeled beads. For microscopy, beads were added to chamber slides and imaged with a mercury lamp and a 100X objective using the following filter sets: 480/40, (EX) and 535/50 nm (EM) for AF488, and 560/40 nm (EX) and 630/75 (EM) for pHrodo (Chroma 49000 Series, Rockingham, VT). The ratio of pHrodo to AF488 as a function of pH was analyzed as described below.

Administration of Dual-Labeled Conjugates and pH Calibration

Dual-labeled HD39/SA-PPAA and HD39/SA-PMAA conjugates were formulated as previously described. 10⁶ cells/ml in media were incubated with 25 nM HD39/SA, HD39/SA-PMAA or HD39/SA-PPAA for 1 h at 37°C. Cells were pelleted and washed with chilled modified PBS containing CaCl₂ (0.1 g/L), MgCl₂ (0.1 g/L) and 0.5% BSA, then resuspended in fresh media at the same concentration and incubated at 37 °C. Cells were collected 1, 3 and 6 hours after treatment with 150,000 cells per sample, then resuspended in 250 μ L of modified PBS for flow cytometry or 500 μ L for fluorescence microscopy, including an untreated cell sample. To develop a pH calibration curve for each timepoint of the ratiometric studies, cells were incubated in pH-clamping buffers ranging from pH 4.5 to 7.4. pH-clamping buffers were created by mixing 50 mM MES (pH 6.0) with 50 mM citric acid (pH 4.5) or 50 mM HEPES (pH 7.4) containing KCl (120 mM), NaCl (20 mM), CaCl₂ (1 mM), MgCl₂ (1 mM), and the ionophores nigericin (10 μ M), monensin (1 μ M), and valinomycin (10 μ M)^{32,33}. Cells were incubated for 15 min at 4 °C then analyzed by flow cytometry or fluorescence microscopy.

Flow Cytometry: Acquisition and Analysis

The environmental pH exhibited by trafficked HD39/SA conjugates was measured by ratiometric fluorescence using a flow cytometer (Becton Dickinson LSR II Cell Analyzer). Cells were excited at 488 and 532 nm and fluorescence emission was collected at 510 and 575 nm, respectively. For accurate measurements, cells were gated by FSC-A and SSC as well as FSC-H and FSC-W with 10,000 gated events per sample. Post-acquisition analysis of ratiometric fluorescence was performed using FlowJo, flow cytometry analysis software (Tree Star, Oregon). For each timepoint, gating of untreated cells was applied to all treatments. For each cell event, pHrodo signal was divided by AF488 signal after subtracting the median autofluorescence from untreated cells to obtain a ratiometric fluorescence value. A pH calibration curve was made to convert ratio values to pH (Supplemental Figure 4A). pH clamped cells were analyzed in the same manner and their median ratio values were plotted against pH. A linear regression curve was fit to the data and used to convert ratiometric fluorescence values to environmental pH.

Fluorescence Microscopy: Acquisition and Analysis

The subcellular compartmental pH distribution of HD39/SA conjugates was measured by ratiometric fluorescence using live-cell microscopy. After treatment, cells were collected and added to Lab-Tek II Chambered Coverglass Slides (NUNC, Rochester, NY). Chamber slides were placed on a Live-Cell Fluorescence Microscope (Nikon Ti-E) equipped with an environmental control chamber. Cells were imaged with a mercury lamp and a 100X objective using the following filter sets: 480/40, (EX) and 535/50 nm (EM) for AF488, and 560/40 nm (EX) and 630/75 (EM) for pHrodo (Chroma 49000 Series, Rockingham, VT). 3 image stacks per treatment with a minimum of 4 cells per image were collected for each timepoint. Image stacks were deconvolved using object-based measurement software, Volocity (Perkin Elmer) to remove out-of focus fluorescence and identify conjugate containing compartments. For deconvolution, calculated point spread functions were applied to the green and red channels with 25 iterations to reach a near 100% confidence interval. Green and red deconvolved channels were thresholded and their overlapping voxels were identified as regions of interest. All touching voxels within a cell were defined as a compartment and a ratiometric algorithm was used to measure each compartment's average ratiometric fluorescence from the original green and red channels with background subtraction. Ratios were converted to pH using the pH-calibration curves and the compartmental pH values were plotted as a histogram to evaluate the compartmental pH distribution. Calibration curves were made for each timepoint by imaging and analyzing pH-clamped cells in the same manner (Supplemental Figure 4B). The average ratiometric fluorescence of compartments was plotted against pH and fit with a linear regression curve to obtain a relationship between ratiometric fluorescence and compartmental pH.

Preparation of Tritium-Labeled HD39/SA

For fractionation studies, HD39/SA was radiolabeled with tritium and measured by scintillation counting. 6.2 mg of HD39/SA at 2 mg/ml in 50 mM sodium borate, pH 8, 50 mM NaCl was reacted with 1mCi ^3H -N-succinimidyl propionate for 1 h at 25 °C. A PD-10 desalting column was used for PBS buffer exchange and removal of unreacted radiolabel. Additionally, [^3H]HD39/SA was concentrated with an Amicon Ultra-4 centrifugal filter unit with 30,000 NMWL and measured by A_{280} measurements. The specific reactivity of [^3H]HD39/SA was measured on a scintillation counter with ULTIMA GOLD (Perkin Elmer, Waltham, MA) scintillation fluid.

Cellular Fractionation

Cytosolic and endosomal/lysosomal fractions containing HD39/SA conjugates were separated by cellular homogenization and fractionation. Briefly, hot antibody was spiked into cold antibody at a molar ratio of 1:2.5 and complexed with PPAA as previously described. 3×10^7 cells were treated with 25 nM [^3H]HD39/SA or [^3H]HD39/SA-PPAA for 1 h pulse and 5 h chase. 10 μL of media containing cells and conjugate were collected at the beginning of the pulse for scintillation counting. After treatment, cells were washed with cold PBS and TES buffer (1 mM triethanolamine, 1 mM EDTA, 0.25 M sucrose, pH 7.4, and protease inhibitors). Cells were resuspended in 1 mL of TES buffer at 4 °C and homogenized with 15–20 strokes of a syringe with a 26 $\frac{1}{2}$ GA needle. After confirming 80% cell homogenization, lysate was centrifuged at $250 \times g$ for 5 min to sediment nuclei and unbroken cells. 400 μL of 20% OptiPrep (Sigma, D1556) was layered under the post nuclear supernatant (PNS), which was then centrifuged for 1 h at $150,000 \times g$ using a swinging bucket rotor (TLS-55) to separate vesicular compartments. Afterwards, 5 fractions (200 μL) were collected from the top of the sample and 10 μL of each fraction were resuspended in scintillation fluid to measure tritium on a scintillation counter. Untreated cells were also fractionated as described above to analyze the cytosolic, endosomal, and lysosomal content within each fraction. For analysis, fractions were run by SDS-PAGE as previously described and transferred to a PVDF membrane via conventional protocol using transfer buffer (12 mM Tris-base, 100 mM glycine, 20% MeOH, 1% (w/v) SDS) for 1.5 hour at 100 V on ice. Membrane was incubated with blocking buffer (Thermo Scientific, PI-37536) for 1 hour at room temperature, washed 10 min in TBS-T (x 3), then incubated with primary antibody (1:2000 dilution in blocking buffer) overnight at 4 °C. Membranes were washed 10 min in TBS-T (x 3) and probed with secondary antibody (HRP goat anti-mouse, diluted 1:100,000 in blocking buffer) in blocking buffer for 1 hour at room temperature. Membrane was washed 10 min in TBS-T (x 3), then incubated for 5 min with premixed chemiluminescence substrate (Pierce, West Femto) at room temperature. Excess substrate was drained and the membrane was developed for 5 min on a Kodak Imager (Rochester, NY). Western blotting with anti-Rab5 and anti-Lamp2 antibodies in untreated cells was used to measure endosome and lysosome activity³⁴ and lactate dehydrogenase assay (Roche) was used to measure cytosolic activity.

RESULTS

Polymer Synthesis

Biotin-ECT was employed as the RAFT CTA for the polymerization of PPAA and PMAA as shown in Scheme 1. For both PAA and MAA the initial monomer to CTA ($[\text{M}]_0/[\text{CTA}]_0$) and CTA to initiator ($[\text{CTA}]_0/[\text{I}]_0$) were 150:1 and 1:1 respectively. The average molecular weights for the resultant PPAA and PMAA were 10.9 and 10.7 kDa, with polydispersity indices of 1.8 and 1.18, respectively. PMAA was employed as a negative control polymer because of its structural similarity to PPAA but lack of endosomolytic properties^{35,36}.

Antibody-Polymer Complexation

A single biotin functional group at the alpha terminus of the polymer was incorporated at time of synthesis for facile conjugation to HD39/SA. Antibody-polymer conjugation through the biotin-streptavidin linkage was measured using the HABA assay (Supplemental Figure 1A). A molar excess of 12 PPAA chains yielded an average 3.5 polymers bound per SA or HD39/SA. A gel retardation assay was used to verify protein-polymer conjugation, as observed by the disappearance of the free SA band or a shift in the HD39/SA band after polymer conjugation (Supplemental Figure 1B).

Calibration of Ratiometric Fluorescence to a Standard pH Curve

The HD39/SA was dual-labeled with pH-insensitive AF488 and pH-sensitive pHrodo to develop a pH probe for reporting the intracellular pH environment of trafficking protein-polymer conjugates. The dual-labeled HD39/SA conjugate contained 3.2 AF488 and 3.5 pHrodo fluorophores per conjugate (Supplemental Figure 2). To standardize the ratiometric fluorescence as a function of pH, conjugates were incubated with biotinylated polystyrene beads in a range of pH buffers and analyzed by flow cytometry. The fluorescence histograms in Supplemental Figure 3 demonstrate that AF488 fluorescence is independent of pH but pHrodo fluorescence is inversely proportional to pH. The ratio of pHrodo to AF488 signal was plotted as a function of pH (Figure 2) demonstrating that ratiometric fluorescence decreases linearly from pH 5 to 7. These results validate the combination of AF488 and pHrodo for intracellular measurements in a broad but physiologically relevant pH range. Ratiometric fluorescence values measured by fluorescence microscopy and flow cytometry were converted to pH using a pH standard curve made with pH-clamped cells. For each timepoint, HD39/SA treated cells were resuspended in a range of clamping buffer from pH 4.5 to 7.4 and the average ratiometric fluorescence value was plotted as a function of pH (Supplemental Figure 4). For the three treatments, ratiometric fluorescence values were converted to compartmental pH (microscopy) or average intracellular pH (flow cytometry) using linear regression curve from the pH-standard plots.

Time-Dependent Dynamics of Subcellular Trafficking

The subcellular pH of HD39/SA-localized compartments was measured by live-cell ratiometric fluorescence microscopy. Representative images of the subcellular compartmental pH are illustrated in Figure 3. The ratiometric fluorescence value for each three dimensional pixel called a voxel, was converted to pH and a color map was used to visualize the pH of each compartment, denoted by the legend at the bottom right. The average pH of each compartment in 10–15 cells per treatment was plotted as a histogram in Figure 5. After 1 hour, subcellular compartments for all three conjugates ranged from pH 6.2 to 7.6 (Figure 3). After 3 hours, the compartmental pH of HD39/SA and HD39/SA-PMAA ranged broadly from pH 4.0 to 6.8, indicating endosomal/lysosomal trafficking. After 6 hours, 92% of HD39/SA-containing compartments were trafficked below pH 5.6 and 90% of HD39/SA-PMAA-containing compartments were trafficked below pH 6.0 (Figure 4C). In comparison, 92% of the HD39/SA-PPAA compartments showed pH values between pH 6.4 and 7.6 (Figure 4B). HD39/SA-PPAA exhibited a bimodal distribution after 6 hours with 11% of compartments between pH 4.8 and 6.0 and 89% of compartments greater than pH 6.0. Shown in Figure 4D is the fraction of each treatment displaying pH values above those typically encountered in early endosomes as a function of time. After 1 hour, 75% of HD39/SA, 53% of HD39/SA-PMAA, and 85% of HD39/SA-PPAA compartments were above pH 6.6. By 3 hours less than 7% of HD39/SA or HD39/SA-PMAA remained while 72% of HD39/SA-PPAA was still present above pH 6.6. After 6 hours, no HD39/SA or HD39/SA-PMAA compartments remained above pH 6.6 but 45% of HD39/SA-PPAA still remained above this threshold. These findings combined with cell fractionation studies (vide infra) suggest that a significant fraction of HD39/SA-PPAA exists in a combination of early endosomal and cytosolic locations.

Time-Dependent Dynamics of Trafficking by Flow Cytometry

The time-dependent dynamics of the HD39 intracellular trafficking was also evaluated by flow cytometry in RAMOS-AW cells, a model B-cell lymphoma cell line that expresses CD22. The intracellular pH of HD39/SA, HD39/SA-PMAA and HD39/SA-PPAA averaged over each cell was defined as the ratio of pHrodo to AF488 signal measured by flow cytometry over the course of 6 hours (Figure 5). After 1 hour, the intracellular pH was 6.7 ± 0.2 for HD39/SA, 6.45 ± 0.25 for HD39/SA-PMAA, and 6.75 ± 1.5 for HD39/SA-PPAA.

These results are consistent with receptor-mediated uptake of the conjugates into early endosomal compartments. After 3 hours, the intracellular pH dropped to 5.6 ± 0.50 for HD39/SA and 5.45 ± 0.55 for HD39/SA-PMAA, while the HD39/SA-PPAA conjugates had an average intracellular pH of 6.5 ± 0.20 . After 6 hours the intracellular pH of HD39/SA and HD39/SA-PMAA were 4.65 ± 0.55 and 4.9 ± 0.50 , characteristic of lysosomal pH. Significantly, the average intracellular pH of the HD39/SA-PPAA conjugates was determined to be 6.25 ± 0.25 with a narrow distribution compared to the other conjugates.

Subcellular Fractionation Localization of Antibody-Polymer Conjugates

Cellular fractionation was used to separate cytosolic from vesicular compartments³⁷ and measure the percent of radiolabeled-HD39/SA conjugates in each fraction. Cytosolic fractions were identified by a lactate dehydrogenase (LDH) assay that measures the cytosolically active enzyme LDH. Vesicular fractions were measured by immunoblotting using anti-Rab5 antibodies to identify endosomes and anti-Lamp2 antibodies to identify late endosomes and lysosomes. Scanned immunoblots were quantified by ImageJ (NIH) to measure the percentage of Rab5 and Lamp2 activity in each fraction relative to the total signal (Figure 6a). LDH (cytosol) was distributed between fraction 1 (25%), 2 (28%), and 3 (32%). Rab5 (endosome) was most active in fraction 3 (38%) and 4 (42%), and Lamp2 activity (late endosome/lysosome) was found in fraction 4 (75%) and 5 (25%) (Figure 6b). Fraction 3 was positive for LDH and Rab5 because it was the boundary layer between the supernatant and denser media used to separate vesicles. The percent of tritium-labeled HD39/SA and HD39/SA-PPAA in each fraction of the 5 fractions was quantified and plotted in Figure 6c. HD39/SA was mostly found in three fractions with 8% in fraction 2 (LDH+), 62% in 3 (LDH+, Rab5+), and 20% in 4 (Rab5+, Lamp2+). In contrast, the HD39/SA-PPAA was found at the highest level (40%) in the cytosolic fraction 2, with 28% in the mixed fraction 3 (LDH+, Rab5+), and only 22% in 4 (Rab5+, Lamp2+). These results are consistent with the conclusion that PPAA mediates endosomal escape of the HD39 mAb.

DISCUSSION

Bioinformatic studies propose that 75% to 80% of all disease targets reside within the intracellular space³⁸. Evading lysosomal trafficking to gain access to intracellular targets remains a delivery challenge for biologic drugs^{39–41}. Carriers that provide endosomal-releasing activities are being investigated to address these barriers^{42–44}. In the current work, pH-responsive poly(propylacrylic acid) (PPAA) and the control poly(methacrylic acid) (PMAA) were synthesized with a biotin-functional RAFT CTA for complexation with mAb-streptavidin bioconjugates. Ratiometric fluorescence and fractionation studies revealed that PPAA significantly increased cytosolic transport and altered the natural trafficking fate of HD39/SA out of endosomes and lysosomes. PMAA, a polymer with similar structure but lacking endosomolytic activity had no significant effect on the HD39/SA trafficking dynamics.

Shan and Press first described the internalization and metabolic degradation of CD22 when ligated to anti-CD22 and hypothesized that the acidic compartments were likely lysosomes⁴⁵. Anti-CD22 mAb trafficking to endosomal and lysosomal compartments was also confirmed by Carnahan *et al*⁴⁶. We used ratiometric fluorescence and fractionation techniques to characterize the time-dependent trafficking dynamics and found that HD39/SA was compartmentalized at pH 6.7 at 1 hour and trafficked through endosomes to lysosomes between pH 4.0 to 5.2 after 6 hours. Incorporation of PPAA had a strong effect on the subcellular pH distribution of HD39/SA over time. By 3 hours, the average intracellular pH of HD39/SA-PPAA was 6.5 and 75% of HD39/SA-PPAA compartments were above pH 6.6. After 6 hours, the average intracellular pH decreased to 6.2 and a bimodal distribution

of subcellular compartments was observed with 45% of compartments above pH 6.6. This bimodality indicates that a fraction of conjugates escape from endosomes while the remainder is eventually trafficked to lysosomes. The use of more potent pH-responsive polymers could increase the amount of conjugate that escapes endosomes and improve cytosolic delivery efficiency^{14–16}.

Subcellular fractionation studies with radiolabeled mAb-polymer conjugates were used to confirm the interpretation of the pH trafficking dynamic studies. After 6 hours, 89% of HD39/SA was localized within endosome- and lysosome-associated fractions 3–5. Conjugation of PPAA to HD39/SA was shown to increase the mAb-SA quantity in fraction 2 (cytosolic) by 34%. While HD39/SA and HD39/SA-PPAA were both present in the endosomal and lysosomal fractions, only 27% of the PPAA conjugates were ultimately trafficked to lysosomes (fraction 4 and 5) over the studied time frame. The findings are consistent with previous therapeutic studies showing that PPAA-based polymer-peptide conjugates could escape endosomes and exhibit high cell-killing activity⁴⁷. The quantitative mechanistic information that describe the antibody-polymer trafficking dynamics shed light onto the fate and distribution of compartmental environments experienced by the mAb. Such information can be used to screen carrier activities and potentially to optimize polymer compositions for endosomal-releasing activities and pH-profiles.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

The authors would like to thank James Lai and Matthew Manganiello for their insightful scientific discussions and experimental support. We are grateful to the NIH (Grants No. R01EB002991 and 2K12CA076930-11), Washington State Life Sciences Discovery Fund (Grant No. 2496490 to the Center for Intracellular Delivery of Biologics), Lymphoma Research Foundation (Fellowship Award to M.C.P-W), Wayne D. Kuni & Joan E. Kuni Foundation and the 3725 Fund of the Oregon Community Foundation (Kuni Scholar Award to M.C.P-W) for funding this project. G.Y.B. and J.S. are Graduate Research Fellows of the National Science Foundation.

References

1. Magrath I, Janus C, Edwards B, Spiegel R, Jaffe E, Berard C, Miliauskas J, Morris K, Barnwell R. An Effective Therapy for Both Undifferentiated (Including Burkitts) Lymphomas and Lymphoblastic Lymphomas in Children and Young-Adults. *Blood*. 1984; 63:1102–1111. [PubMed: 6546890]
2. Hainsworth JD, Litchy S, Burris HA, Scullin DC, Corso SW, Yardley DA, Morrissey L, Greco FA. Rituximab as first-line and maintenance therapy for patients with indolent non-hodgkin's lymphoma. *J Clin Oncol*. 2002; 20:4261–4267. [PubMed: 12377971]
3. Wang J, Press OW, Lindgren CG, Greenberg P, Riddell S, Qian X, Laugen C, Raubitschek A, Forman SJ, Jensen MC. Cellular immunotherapy for follicular lymphoma using genetically modified CD20-specific CD8+ cytotoxic T lymphocytes. *Mol Ther*. 2004; 9:577–586. [PubMed: 15093188]
4. Polson AG, Calemine-Fenaux J, Chan P, Chang W, Christensen E, Clark S, de Sauvage FJ, Eaton D, Elkins K, Elliott JM, Frantz G, Fujii RN, Gray A, Harden K, Ingle GS, Kljavin NM, Koeppen H, Nelson C, Prabhu S, Raab H, Ross S, Slaga DS, Stephan JP, Scales SJ, Spencer SD, Vandlen R, Wranik B, Yu SF, Zheng B, Ebens A. Antibody-drug conjugates for the treatment of non-Hodgkin's lymphoma: target and linker-drug selection. *Cancer Res*. 2009; 69:2358–2364. [PubMed: 19258515]
5. de Santes K, Slamon D, Anderson SK, Shepard M, Fendly B, Maneval D, Press O. Radiolabeled antibody targeting of the HER-2/neu oncoprotein. *Cancer Res*. 1992; 52:1916–1923. [PubMed: 1348016]

6. Milenic DE, Brady ED, Brechbiel MW. Antibody-targeted radiation cancer therapy. *Nature Reviews Drug Discovery*. 2004; 3:488–499.
7. Wu AM, Senter PD. Arming antibodies: prospects and challenges for immunoconjugates. *Nat Biotechnol*. 2005; 23:1137–1146. [PubMed: 16151407]
8. Schrama D, Reisfeld R. Antibody targeted drugs as cancer therapeutics. *Nature Reviews Drug Discovery*. 2006
9. Foyil KV, Bartlett NL. Anti-CD30 Antibodies for Hodgkin lymphoma. *Curr Hematol Malig Rep*. 2010; 5:140–147. [PubMed: 20446121]
10. Al-Awqati Q. Proton-translocating ATPases. *Annu Rev Cell Biol*. 1986; 2:179–199. [PubMed: 2435311]
11. Tycko B, Maxfield FR. Rapid acidification of endocytic vesicles containing alpha 2-macroglobulin. *Cell*. 1982; 28:643–651. [PubMed: 6176331]
12. Nilsson C, Kagedal K, Johansson U, Ollinger K. Analysis of cytosolic and lysosomal pH in apoptotic cells by flow cytometry. *Methods in Cell Science*. 2004; 25:185–194. [PubMed: 15801164]
13. Bevan AP, Drake PG, Bergeron JJ, Posner BI. Intracellular signal transduction: The role of endosomes. *Trends Endocrinol Metab*. 1996; 7:13–21. [PubMed: 18406720]
14. Convertine AJ, Benoit DSW, Duvall CL, Hoffman AS, Stayton PS. Development of a novel endosomolytic diblock copolymer for siRNA delivery. *Journal of Controlled Release*. 2009; 133:221–229. [PubMed: 18973780]
15. 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]
16. El-Sayed MEH, Hoffman AS, Stayton PS. Rational design of composition and activity correlations for pH-sensitive and glutathione-reactive polymer therapeutics. *Journal of controlled release: official journal of the Controlled Release Society*. 2005; 101:47–58. [PubMed: 15588893]
17. Albarran B, To R, Stayton P. 1111. TAT-Streptavidin: A Novel Drug Delivery Vector for the Intracellular Uptake of Macromolecular Cargo. *Mol Ther*. 2005
18. Pack D, Hoffman A, Pun S, Stayton P. Design and development of polymers for gene delivery. *Nature Reviews Drug Discovery*. 2005
19. Massignani, M.; Canton, I.; Patikarnmonthon, N. Cellular delivery of antibodies: effective targeted subcellular imaging and new therapeutic tool. 2010.
20. Yang J, Chen H, Vlahov IR, Cheng JX, Low PS. Characterization of the pH of Folate Receptor-Containing Endosomes and the Rate of Hydrolysis of Internalized Acid-Labile Folate-Drug Conjugates. *Journal of Pharmacology and Experimental Therapeutics*. 2007; 321:462–468. [PubMed: 17289839]
21. Lee R, Wang S, Low P. Measurement of endosome pH following folate receptor-mediated endocytosis. *Bba-Mol Cell Res*. 1996; 1312:237–242.
22. Akinc A. Measuring the pH environment of DNA delivered using nonviral vectors: implications for lysosomal trafficking. *Biotechnol Bioeng*. 2002
23. Akinc A, Thomas M, Klibanov AM, Langer R. Exploring polyethylenimine-mediated DNA transfection and the proton sponge hypothesis. *J Gene Med*. 2005; 7:657–663. [PubMed: 15543529]
24. Bayer N, Schober D, Prchla E, Murphy RF, Blaas D, Fuchs R. Effect of bafilomycin A1 and nocodazole on endocytic transport in HeLa cells: implications for viral uncoating and infection. *J Virol*. 1998; 72:9645–9655. [PubMed: 9811698]
25. Philipp Seib F, Jones AT, Duncan R. Establishment of subcellular fractionation techniques to monitor the intracellular fate of polymer therapeutics I. Differential centrifugation fractionation B16F10 cells and use to study the intracellular fate of HPMA copolymer–doxorubicin. *Journal of Drug Targeting*. 2006; 14:375–390. [PubMed: 17092838]
26. Manunta M, Izzo L, Duncan R, Jones AT. Establishment of subcellular fractionation techniques to monitor the intracellular fate of polymer therapeutics II. Identification of endosomal and lysosomal compartments in HepG2 cells combining single-step subcellular fractionation with fluorescent imaging. *Journal of Drug Targeting*. 2007; 15:37–50. [PubMed: 17365272]

27. Kim J, Tirrell DA. Synthesis of Well-Defined Poly(2-ethylacrylic acid). *Macromolecules*. 1999; 32:945–948.
28. Pagel JM, Pantelias A, Hedin N, Wilbur S, Saganic L, Lin Y, Axworthy D, Hamlin DK, Wilbur DS, Gopal AK, Press OW. Evaluation of CD20, CD22, and HLA-DR targeting for radioimmunotherapy of B-cell lymphomas. *Cancer Res*. 2007; 67:5921–5928. [PubMed: 17575162]
29. Hylarides M, Mallett R. A robust method for the preparation and purification of antibody/streptavidin conjugates. *Bioconjugate Chem*. 2001
30. Palanca-Wessels MC, Convertine AJ, Cutler-Strom R, Booth GC, Lee F, Berguig GY, Stayton PS, Press OW. Anti-CD22 Antibody Targeting of pH-responsive Micelles Enhances Small Interfering RNA Delivery and Gene Silencing in Lymphoma Cells. *Mol Ther*. 2011
31. GREEN N. A Spectrophotometric Assay for Avidin and Biotin Based on Binding of Dyes by Avidin. *The Biochemical journal*. 1965; 94:C23.
32. Ng LL, DUDLEY C. Intracellular pH clamping of human leucocytes: a technique for determination of cellular buffering power and Na⁺/H⁺ antiport characteristics. *Clin Sci*. 1989; 77:417–423. [PubMed: 2805600]
33. Deriy LV, Gomez EA, Zhang G, Beacham DW, Hopson JA, Gallan AJ, Shevchenko PD, Bindokas VP, Nelson DJ. Disease-causing Mutations in the Cystic Fibrosis Transmembrane Conductance Regulator Determine the Functional Responses of Alveolar Macrophages. *Journal of Biological Chemistry*. 2009; 284:35926–35938. [PubMed: 19837664]
34. Chin LS. Hrs Interacts with Sorting Nexin 1 and Regulates Degradation of Epidermal Growth Factor Receptor. *Journal of Biological Chemistry*. 2000; 276:7069–7078. [PubMed: 11110793]
35. 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. [PubMed: 19125614]
36. 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. *The Biochemical journal*. 2003; 372:65–75. [PubMed: 12583812]
37. Bartz R, Fan H, Zhang J, Innocent N, Cherrin C, Beck SC, Pei Y, Momose A, Jadhav V, Tellers DM, Meng F, Crocker LS, Sepp-Lorenzino L, Barnett SF. Effective siRNA delivery and target mRNA degradation using an amphipathic peptide to facilitate pH-dependent endosomal escape. *The Biochemical journal*. 2011; 435:475–487. [PubMed: 21265735]
38. Verdine GL, Walensky LD. The challenge of drugging undruggable targets in cancer: lessons learned from targeting BCL-2 family members. *Clin Cancer Res*. 2007; 13:7264–7270. [PubMed: 18094406]
39. Varkouhi AK, Scholte M, Storm G, Haisma HJ. Endosomal escape pathways for delivery of biologicals. *Journal of Controlled Release*. 2011; 151:220–228. [PubMed: 21078351]
40. Dinh A, Pangarkar C, Theofanous T, Mitragotri S. Understanding Intracellular Transport Processes Pertinent to Synthetic Gene Delivery via Stochastic Simulations and Sensitivity Analyses. *Biophysical Journal*. 2007; 92:831–846. [PubMed: 17085500]
41. Elde NC, Malik HS. The evolutionary conundrum of pathogen mimicry. *Nature Reviews Microbiology*. 2009; 7:787–797.
42. Wong S, Pelet J, Putnam D. Polymer systems for gene delivery—Past, present, and future. *Progress in Polymer Science*. 2007
43. Little SR, Kohane DS. Polymers for intracellular delivery of nucleic acids. *J Mater Chem*. 2008; 18:832.
44. 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. [PubMed: 12643752]
45. Shan D. Constitutive endocytosis and degradation of CD22 by human B cells. *The Journal of Immunology*. 1995
46. Carnahan J, Wang P, Kendall R, Chen C, Hu S, Boone T, Juan T, Talvenheimo J, Montestruque S, Sun J, Elliott G, Thomas J, Ferbas J, Kern B, Briddell R, Leonard JP, Cesano A. Epratuzumab, a

- humanized monoclonal antibody targeting CD22: characterization of in vitro properties. Clin Cancer Res. 2003; 9:3982S–90S. [PubMed: 14506197]
47. Duvall CL, Convertine AJ, Benoit DSW, Hoffman AS, Stayton PS. Intracellular delivery of a proapoptotic peptide via conjugation to a RAFT synthesized endosomolytic polymer. Mol Pharm. 2010; 7:468–476. [PubMed: 19968323]

Figure 1.

Intracellular trafficking of the HD39/SA-PPAA conjugate. Ligation of the anti-CD22 monoclonal antibody (HD39) to CD22 leads to receptor-mediated endocytosis. A portion of the conjugate is trafficked from endosomes to lysosomes while a second fraction is released into the cytosol via endosomal escape mediated by PPAA.

Figure 2.

The ratiometric fluorescence of dual-labeled HD39/SA is pH-dependent. HD39/SA was dual-labeled with pH-insensitive AF488 and pH-sensitive pHrodo, then coated onto biotinylated-polystyrene beads. HD39/SA-coated beads were resuspended in a range of pH buffers and the AF488 and pHrodo fluorescence intensity was measured at each pH. The median intensity of pHrodo divided by AF488 was defined as ratiometric fluorescence and plotted as a function of pH. A linear regression was fit to the data to demonstrate that the ratiometric fluorescence of the HD39/SA conjugates is pH dependent.

Figure 3.

Time-dependent dynamics of subcellular trafficking defined as a pH color map. RAMOS-AW cells were treated with HD39/SA, HD39/SA-PMAA, or HD39/SA-PPAA for 1 hour at 25 nM, washed in PBS, then resuspended and incubated in fresh media. At the indicated times, cells were resuspended in PBS, and transferred to chamber slides for deconvolution wide-field fluorescence microscopy. Cells were selected by scanning in brightfield to avoid fluorescence bias and locate 3 or more cells per viewing field. 10–15 cells were imaged per treatment and all were included in morphometric processing. Compartments were defined as touching voxels containing both green (AF488) and red (pHrodo) fluorescence with Volocity image analysis software. The ratiometric fluorescence of each voxel was defined by dividing pHrodo signal by AF488 signal. Ratiometric fluorescence was then converted to pH using standard curves made by pH clamping (see Supporting Information). The pH of each voxel was converted map defined by the key at the bottom right. Representative cell images for each timepoint and treatment are displayed above.

Figure 4.

Time-dependent subcellular pH distribution of conjugates obtained by ratiometric fluorescence microscopy. RAMOS-AW cells were treated with HD39/SA, HD39/SA-PMAA, or HD39/SA-PPAA at 25 nM for 1 hour, washed in PBS, then resuspended and incubated in fresh media. Cells were transferred to chamber slides at indicated timepoints and the ratiometric fluorescence of subcellular compartments was measured by deconvolution wide-field fluorescence microscopy (See Figure 3). Ratiometric fluorescence defined as pHrodo signal divided by AF488 signal was converted to pH for each voxel using standard curves made by pH clamping (See Supporting Information). The average pH of the voxels within a compartment was defined as the subcellular compartmental pH and plotted as a histogram following 1 h (A), 3 h (B), and 6 h (C) of trafficking. The percent of subcellular compartments above pH 6.6 is also defined (D).

Figure 5.

Time-dependent trafficking dynamics measured by average intracellular pH. RAMOS-AW cells were treated with HD39/SA (square), HD39/SA-PMAA (diamond), or HD39/SA-PPAA (circle) at 25 nM for 1 hour, washed in PBS then resuspended in fresh media and incubated for up to 5 hours. For each timepoint 1.5×10^5 cells were washed and resuspended in PBS on ice. Ratiometric fluorescence was measured for 10,000 cells per treatment per timepoint via flow cytometry by dividing the pHrodo fluorescence signal by AF488 signal. Ratiometric fluorescence was converted to average intracellular pH using pH standard curves made by pH clamping (see Supporting Information). Error bars represent a 50% confidence interval of the total cell population.

Figure 6.

Colocalization of radiolabeled conjugates with cytosolic, endosomal, and lysosomal markers. 30×10^6 RAMOS-AW cells were treated with [^3H]HD39/SA or [^3H]HD39/SA-PPAA at 25 nM for 1 hour, washed with PBS, then resuspended and incubated in fresh media for 5 hours. Cells were homogenized with 15–20 needle stroke and separated from nuclear fractions and non-homogenized cells. Cytosolic content was separated from vesicular content by ultracentrifugation and $5 \times 200 \mu\text{l}$ fractions were collected. (A) Untreated cell fractions were separated by SDS-PAGE, transferred to immunoblots and stained with anti-Rab5 (early and late endosomes) and anti-Lamp2 (late endosome and lysosome) antibodies. (B) ImageJ was used to measure antibody signal in each fraction and an LDH assay was used to measure cytosolic activity. (C) The amount of HD39/SA or HD39/SA-PPAA in each fraction was measured by radioactivity measurements and plotted as a percent of total intracellular content.

Biotin-ECT Chain Transfer Agent

R' = CH₃ for PMAA
C₃H₇ for PPAA

RAFT Polymerization of PAA and MAA with Biotin-ECT **Scheme 1.**

RAFT polymerization of PAA and MAA was conducted in DMF and DMSO, respectively, under anhydrous conditions for 24 hours at 60 °C using biotin-ECT as the chain-transfer agent (CTA) and azobisisobutyronitrile (AIBN) as the radical initiator. The initial monomer to CTA to initiator ratio ($[M]_0/[CTA]_0/[I]_0$) for the polymerization of PAA and MAA was 150 to 1 to 1. The average molecular weights for the resultant PPAA and PMAA were 10.9 and 10.7 kDa, with polydispersity indices of 1.8 and 1.18, respectively.