Multiresponse Strategies To Modulate Burst Degradation and Release from Nanoparticles

Jagadis Sankaranarayanan,[†] Enas A. Mahmoud,[†] Gloria Kim,[†] José M. Morachis,[†] and Adah Almutairi[†,‡,*]

[†]Pharmaceutical Sciences and [‡]Materials Science and Engineering, University of California at San Diego, La Jolla, California 92093-0657

ABSTRACT Logic gate nanoparticles, where two chemical transformations take place one after the other, were successfully formulated from a newly synthesized random *co*-polymer. This polymer, poly([2,2′-(propane-2,2-diylbis(oxy))bis(ethane-2,1-diyl) diacrylate]-*co*-[hexane-1,6-diyl diacrylate]-4,4′ trimethylene dipiperidine), (poly-β-aminoester ketal-2) contains two pH responsive moieties within its backbone. As nanoparticles they function akin to an AND logic gate. The β-aminoester backbone moiety provides a pH triggered solubility switch, only when this switch is "ON" does the ketal moiety also turn "ON" to undergo rapid acid catalyzed hydrolysis. These AND logic gate polymeric nanoparticles were prepared using an oil in water emulsion method. Their degradation in the pH range of 7.4−5 was monitored by dynamic light scattering and showed excellent stability at pH 7.4 and rapid degradation at pH 5. Our results indicate that the prepared logic gate nanoparticles may prove valuable in delivering therapeutics and diagnostics to cells and diseased tissue.

KEYWORDS: logic gate · nanoparticles · burst release · dual pH response polymers · poly-β-aminoester ketal

Nanoscale carriers capable of a controlled and rapid triggered response to physiological events such as changes in extracellular pH, temperature and reactive oxygen species are particularly useful in the delivery of therapeutics and diagnostics to diseased cells and tissue.[1−8] Such carriers can maximize therapeutic efficacy and minimize undesirable side effects by decreasing their dosage.[9] In addition, encapsulation of therapeutics and imaging agents protects them from harsh *in vivo* conditions that lead to proteolytic degradation,[10] sequestration, and renal clearance. Encapsulation within biodegradable polymers is one strategy by which bioactives are delivered to diseased cells *via* endosome to cytosol release.[11−13] Cytosolic delivery is particularly challenging and is a major hurdle for effective therapeutic delivery.[14,15] Burst-degrading drug delivery systems hold promise in achieving increased cytosolic release through elevated osmotic pressure within the endosomes.[16,17] To this effect, hydrogels utilizing ketal cross-links were developed

and are promising; however, their payloads are limited to large water-soluble macromolecules and the catalytic nature of their degradation leads to significant degradation at physiological pH over time.[18] Similarly hydrophobic polyketals are also promising classes of polymeric biomaterials as they can encapsulate both hydrophobic and hydrophilic payloads, but as nanoparticles they no longer undergo rapid acid catalyzed hydrolysis unless fully hydrated.[19]

Formulation of nanoparticles from polymers requires them to have a hydrophobic character. This, however dramatically slows down their hydrolysis degradation kinetics. Here degradation would occur slowly by a surface erosion mechanism.[19−22] This leads to a catch 22 situation, and we hypothesized that formulating nanoparticles from polymers with a hydrophilic pH switch can both ensure the stability of the nanoparticles in physiological pH and still achieve the desired rapid catalytic degradation in acidic conditions.

We hypothesized that developing a system that has two or more pH response mechanisms to a single triggering event would more finely tune the response to pH stimuli. Among pH sensitive polymers which are able to switch from hydrophobic to hydrophilic, poly-β-amino esters, (PbAE), are widely studied because of their excellent tunability[23−27] and ease of preparation.[26,28] Hydrophobic nanoparticles made of PbAE undergo protonation of the amine backbone upon decreasing the pH, leading to immediate *dissolution* in aqueous solutions.[29−32]
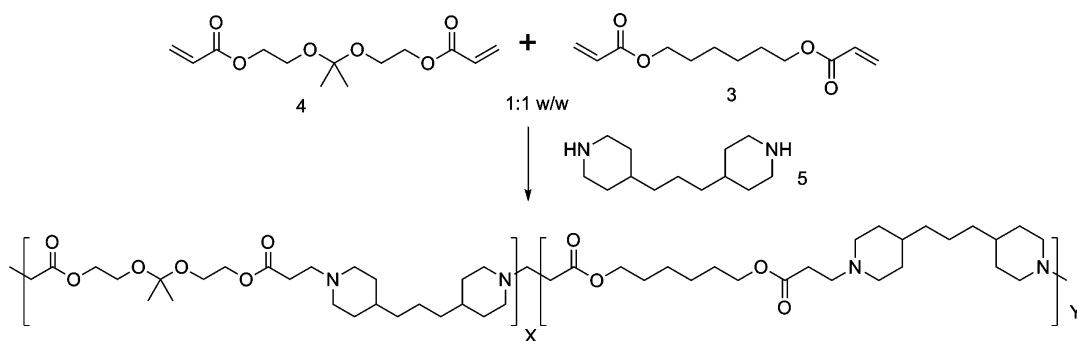
In the present work we developed dual pH responsive random *co*-polymer poly([2,2′-(propane-2,2-diylbis(oxy))bis(ethane-2,1-diyl) diacrylate]-*co*-[hexane-1,6-diyl diacrylate]-

*Address correspondence to aalmutairi@ucsd.edu.

poly β–aminoester ketal-2

Scheme 1. Synthesis of random *co*-poly-β-aminoester ketal-2.

4,4′-trimethylene dipiperidine) (poly-β-aminoester ketal-2) that utilizes both mechanisms described in the previous paragraphs (Scheme 1) to undergo degradation. In response to a single triggering event of a decrease in pH, the amine backbone undergoes a sharp hydrophobic−hydrophilic switch. This leads to an increase in uptake of water (*bulk dissolution*) and hence an increase in ketal hydrolysis (*surface degradation*). We reasoned that the second degradation step should proceed by both surface and bulk erosion simultaneously. The degradation and release profile of this newly developed system has promise in exhibiting increased cytosolic release (Figure 1).[33]

Nanoparticles formulated using the poly-β-aminoester ketal-2 become hydrophilic at mildly acidic pH 6.5−5.0 and in turn lead to accelerated hydrolysis of the ketal moieties. The pronounced effect of a hydrophilic−hydrophobic balance is evidenced by the fact that our degradation times are significantly faster than that obtained for other hy-

drophobic polyketals.[21,22] Furthermore, our dual pH response design showed better stability at physiological pH (7.4) than other hydrophilic polyketals,[34] while maintaining the desired rapid degradation at acidic pH.

RESULTS AND DISCUSSION

To test our hypothesis of creating a dual pH response system we initially synthesized poly-β-aminoester ketal-1 shown in Scheme 2.

Nanoparticles formulated from poly-β-aminoester ketal-1 were found to dissolve rapidly at pH 7.4 owing to the increased charge density of the polymer backbone. While the pK_a of the polymer is expected to be similar to the ones calculated in the literature,[35,36] the solubility switch of the polymer depends on a hydrophilic−hydrophobic balance between the hydrophilic protonated amines and hydrophobic alkyl backbone. We hypothesized that the presence of the ketal group on every monomer unit makes the backbone more hydrophilic and results in a more rapidly soluble
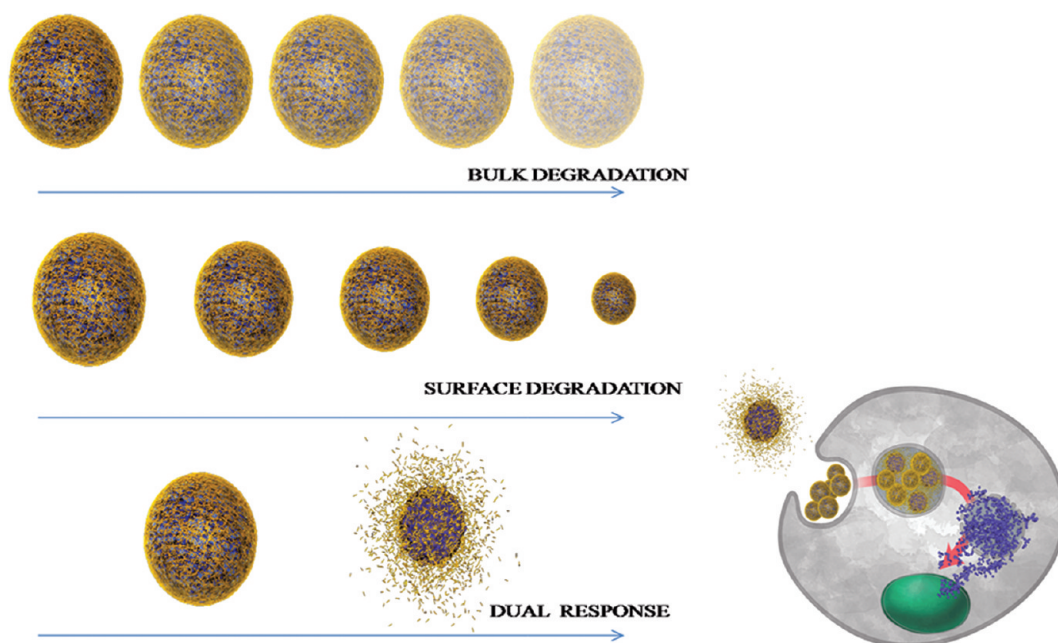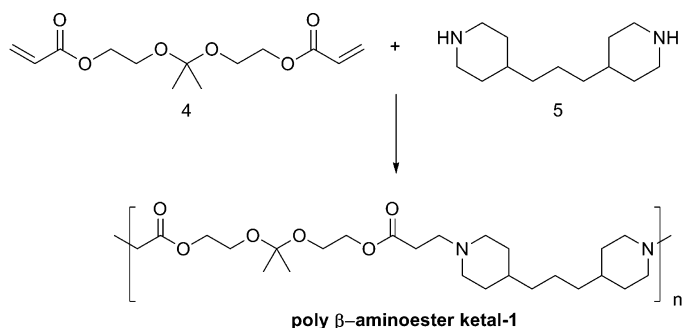

Figure 1. Different release profiles from nanoparticles result in different cellular response.

Scheme 2. Synthesis of poly-β-aminoester ketal-1.

nanoparticle at pH 7.4. To improve on our original design we synthesized poly-β-aminoester ketal-2 in which we incorporated a more hydrophobic spacer in order to increase the hydrophobicity of the backbone and thereby improve the stability of the nanoparticles at pH 7.4.

Poly-β-aminoester ketal-2 was synthesized as shown in Scheme 1 *via* Michael-type addition of bis(secondary amine) monomers to diacrylate ester and diacrylate ester ketal monomers in a 2:1:1 mixture. The resulting poly-β-aminoester ketal-2 was then formulated using emulsion techniques into logic gate nanoparticles. TEM (transmission electron microscope) images of the nanoparticles formulated (Supporting Information, Figure 5) show diameters of 100−150 nm while their average hydrodynamic radius was ∼300 nm by dynamic light scattering, DLS (Supporting Information, Figure 6).

Influence of pH on Nanoparticles Size. The nanoparticles were monitored for 24 h by DLS, using a Zetasizer−ZS (Malvern, U.K). DLS measures hydrodynamic diameter and charge of particles. Figure 2 shows that the nanoparticles remain stable at pH 7.4 over a period of 24 h
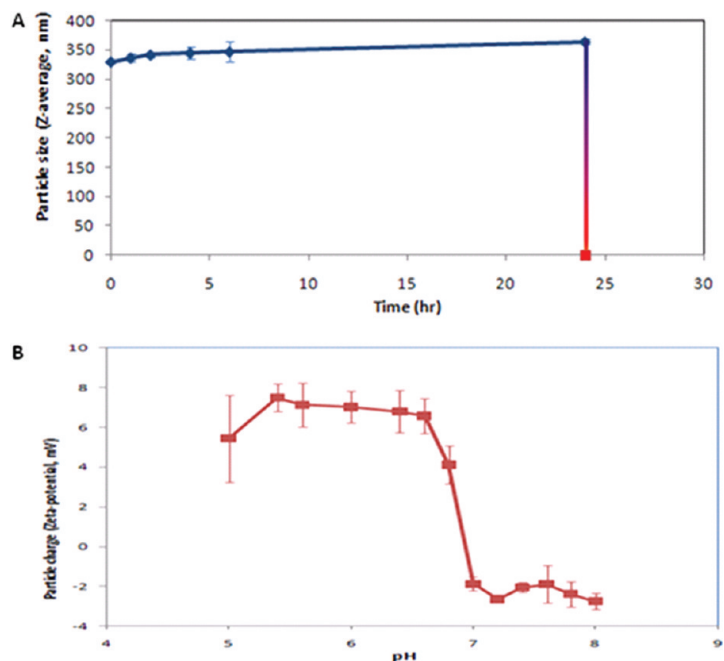

Figure 2. (A) Influence of pH on particle size (Z-average) and (B) particle charge (zeta-potential) of poly-β-aminoester ketal-2 nanoparticles.

without a significant change in size for the first 4 h at pH 7.4 ($p > 0.05$). Subsequently we note a slight but significant increase between 6 and 24 h ($p = 0.044$ and 0.002, respectively). This increase in diameter reflects the hydration process of the polymeric nanoparticle due to partial protonation of the amino groups ($pK_a \approx 6.7$)[35,36] at this pH (also see Figure 2). Changing the pH to 5 (pH of many cellular subcompartments) caused a sudden or burst degradation of the nanoparticles. The drop we see in particle size is very dramatic compared to the nanoparticle systems previously published in the literature.[21,22]

Influence of pH on Nanoparticle Charge. We also measured the zeta potential of the nanoparticles at different pH (Figure 2) to detect the protonation of these polymeric nanoparticles with decrease in pH. Zeta potential measurements using DLS gives us the charge on these dual response nanoparticles as a function of pH and thus can help elucidate the degradation mechanism. Poly-β-aminoester ketal-2 nanoparticles were prepared as described and washed using water. A 100 μL suspension of nanoparticles was dispersed in different phosphate buffers with different pH values. We note that the charge on the particle increases with decrease in the pH confirming that the amines on the polymer backbone are progressively protonated and the degree of protonation increases dramatically around pH 7−6 which corresponds to the pK_a of the backbone.

Polymer Degradation Studies by GPC and NMR. Polymer degradation studies were performed in order to observe the degradation products and molecular weights of the fragments. NMR studies were carried out by dissolving the polymer in pH 5 phosphate buffer and recording spectra at various time intervals. We found that acetone peaks appeared immediately and grew until about 2 h. We continued to record NMR spectra (Supporting Information, Figure 7) for a two week period and observed no further degradation of the backbone. A 100 mg portion of the polymer was also separately incubated at 37 °C in pH 5 phosphate buffer. Samples were withdrawn at various time points, lyophilized, and analyzed *via* GPC. The GPC traces show that the polymer fragments into smaller fragments at this pH (Supporting Information, Figure 8). On the basis of these findings, this new hydrophobic polymeric nanoparticle is stable at pH 7.4; however, upon decreasing the pH the tertiary amines along the polymer backbone become protonated and the polymer becomes more hydrophilic. This results in an increased uptake of water followed by acid catalyzed hydrolysis of the ketal groups along the polymeric backbone (Scheme 3). The ease of degradation of these polymeric nanoparticles is a significant advantage over other systems especially in gene delivery applications as it could minimize the cytotoxicity of the carrier in contrast to other more toxic polyamine systems.[37]
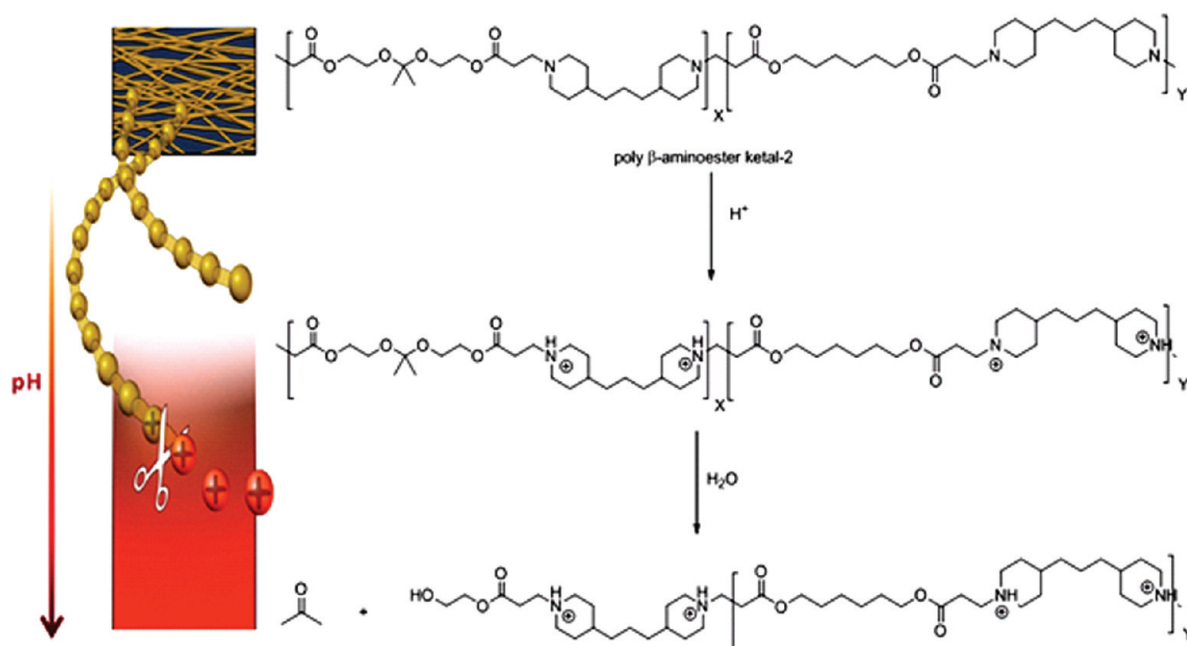
Scheme 3. Mechanism of degradation of the polymer backbone of poly-β-aminoester ketal-2.

Influence of pH on Nile Red Release from Poly-β-aminoester Ketal-2 Nanoparticles (Figure 3). Nile red, a nonpolar probe, fluorescent in hydrophobic environments was encapsulated in our dual response nanoparticles in order to investigate the ability of these dual response nanoparticles to release a small hydrophobic molecule, as a model pharmaceutical.

Nanoparticles containing Nile Red were prepared and the fluorescence of Nile Red poly-β-aminoester ketal-2 nanoparticles was measured at pH 7.4 and 5 (Figure 3). Upon changing the pH from 7.4 to 5 we observed a decrease in fluorescence intensity coupled with a red shift in the fluorescence peak. These results are indicative of Nile Red release immediately upon decreasing the pH to 5.

Cytoxicity and Cellular Internalization Studies of the Poly-β-aminoester Ketal-2 Nanoparticles. We evaluated the cytotoxicity of poly-β-aminoester ketal-2 nanoparticles in cells by a MTT assay. RAW 264.7 cells were incubated with various amounts of nanoparticles for 20 h. Figure 4 illustrates the comparison of cytotoxicity between cells treated with increasing concentrations of poly-β-aminoester ketal-2 with respect to polymer and control cells. There was no significant cytotoxicity observed until we reached high concentrations of polymer (beyond 11.1 μg/mL).

Finally, the uptake of our nanoparticles by RAW 264.7 macrophages was evaluated by fluorescence microscopy. We observed more diffuse fluorescence from our dual pH responsive polymeric nanoparticles
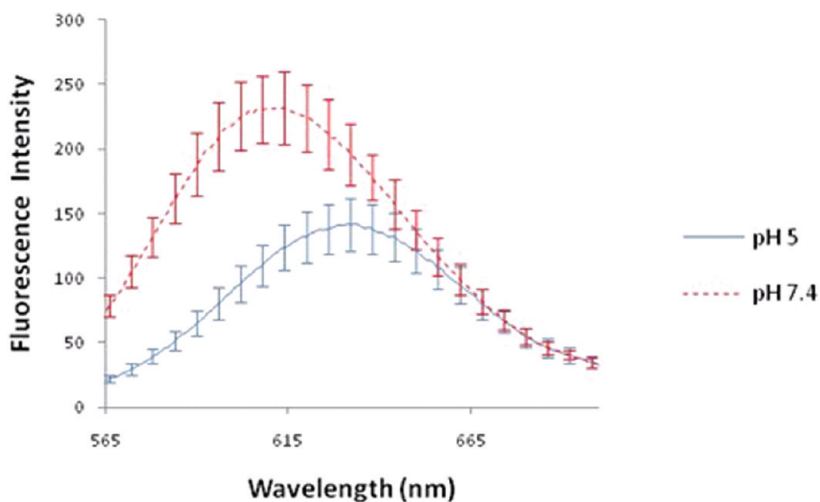

Figure 3. Significant red and hypochromic shifts ($p < 0.001$ and $p = 0.010$, respectively) were observed in the fluorescence absorption spectra of Nile Red poly-β-aminoester ketal-1 nanoparticle suspensions upon changing the pH from 7.4 to 5. These shifts indicate the presence of Nile Red in a hydrophobic environment such as within the nanoparticles which suddenly changes upon decreasing the pH to a more hydrophilic environment.
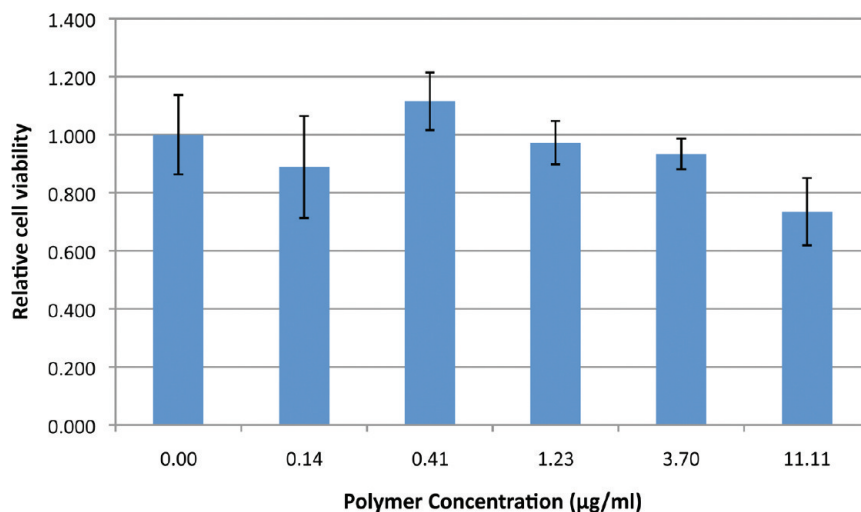
Figure 4. Cytotoxicity of poly-β-aminoester ketal-2 nanoparticles at different concentrations in RAW 264.7 macrophage cells.

encapsulating labeled BSA, compared with poly-lactic-co-glycolic acid (PLGA) encapsulated fluorescent BSA where fluorescence was more punctuate (Figure 5). We reasoned from these images that the uptake of poly-β-aminoester ketal-2 nanoparticles and release of fluorescent BSA is enhanced when compared to PLGA nanoparticles (Figure 5).

CONCLUSIONS

We have shown that two pH response moieties, a pH solubility switch and a pH labile group can be incorporated into the backbone of polymers which can then be formulated into dual responsive nanoparticles encapsulating small hydrophobic molecules and larger protein payloads. Our dual pH response moieties function like a logic gate to fine-tune the degradation of and

release from these nanoparticles. The nanoparticle formulations were stable for 24 h in healthy physiological pH, and upon reducing the pH to endosomal levels, pH 5, these dual responsive nanoparticles underwent a rapid and dramatic degradation followed by concomitant release of their payloads. We have shown that their degradation mechanism begins with the protonation of the tertiary amines along the backbone that then switch the polymeric nanoparticle from hydrophobic to hydrophilic. The increased uptake of water under acidic conditions now allows the ketal groups to rapidly hydrolyze to give a bulk degradation profile. In acidic pH, these polymeric nanoparticles degrade into innocuous fragments, namely diols and acetone. Furthermore, the degradation byproduct, acetone, is anti-inflammatory, alleviating concerns of an inflammatory
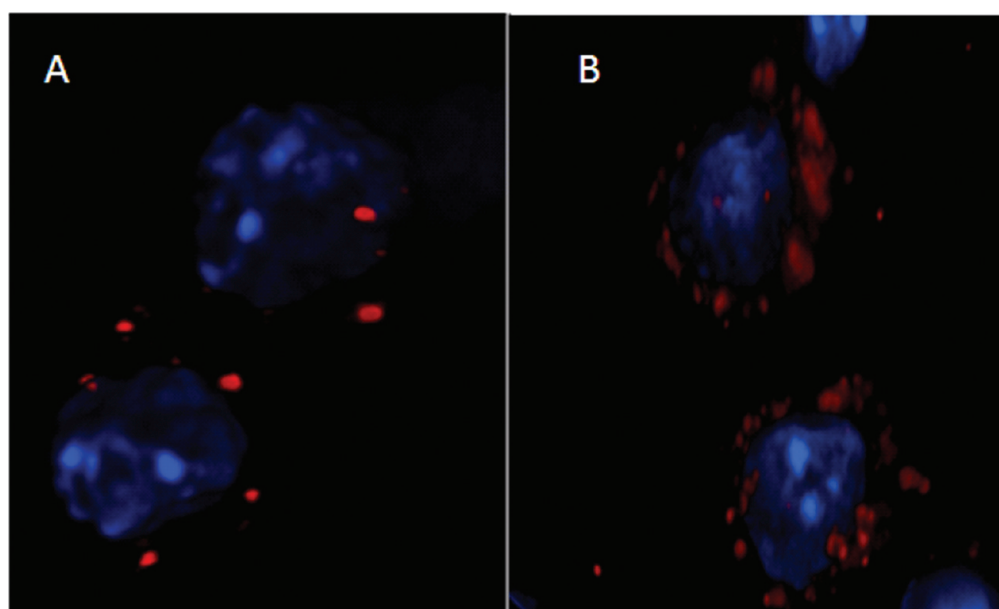

Figure 5. Uptake of nanoparticles and loaded with fluorescent BSA by macrophage cells. Raw 264.7 macrophage cells were treated for 2 h with poly-β-aminoester ketal-2 or PLGA nanoparticles containing BSA-Alexa Fluor 594 (red) and stained with DAPI (blue).

response of acidic byproduct typical of traditional polyester biomaterials. Such rapid hydrolysis is known to increase the osmotic pressure inside subcellular compartments leading to cytoplasmic release of encapsulated payloads. This system seems to be a promising vehicle for the administration of hydrophilic and hydrophobic payloads into target areas of the human body. Continued efforts in designing optimal bioresponsive polymers would improve limitations in drug, diagnostic, and biopharmaceutical delivery. We are currently applying these novel systems to sense and image early stages of metabolic diseases and inflammation.

MATERIALS AND METHODS

Materials. 4,4′-Trimethylenedipiperidine was purchased from Aldrich Chemical Co. (Milwaukee, WI). Triethylamine (TEA), potassium hydrogen phosphate (K_2HPO_4) anhydrous, potassium dihydrogen phosphate (KH_2PO_4), and 1,6-hexanediol diacrylate were purchased from Alfa Aesar Organics (Ward Hill, MA). Dichloromethane (DCM, methylene chloride) was purchased from Fisher Scientific (Hampton, NH). Poly(vinyl alcohol) (PVA) (MW 30−70 k) was purchased from Sigma Chemical Co. (St. Louis, MO). All reagents were used without further purification unless otherwise stated.

Synthesis of Poly-β-aminoester Ketal-1 and -2 (Scheme 1). The polymers were prepared by Michael addition of the corresponding diacrylates with trimethyl dipiperidine. Typically acrylic ketal monomer **4** was synthesized from commercially available reagents as described in the literature.[38] In a vial, diacrylate **3** (5 mmol) and acrylicketal **4** (4.2 mmol) was dissolved in 1 mL of DCM followed by addition of 1 mL of TEA. A Teflon-coated stir bar was added to mix the reactants, and dipiperidine (10 mmol) was added to the vial which was then sealed with a Teflon-lined screw-cap, The reaction was purged with nitrogen gas. The reaction was stirred at room temperature for 4 days. The solvent was then evaporated, and the crude polymer was disolved in 10 mL of DCM. The polymer was purified by precipitating it into 2 × 200 mL hexane to yield 360 mg of the polymer. The polymer was collected and dried under vacuum prior to analysis.

Poly-β-aminoester Ketal-1. ^{1}H NMR (600 MHz, CDCl$_3$) δ 4.24−4.14 (m, 4H), 3.68−3.57 (m, 4H), 2.85 (d, J = 10.3 Hz, 4H), 2.65 (t, J = 7.5 Hz, 4H), 2.52 (t, J = 7.5 Hz, 4H), 1.93 (t, J = 9.9 Hz, 4H), 1.63 (d, J = 6.4 Hz, 4H), 1.35 (d, J = 9.2 Hz, 6H), 1.26 (d, J = 4.3 Hz, 2H), 1.17 (s, 10H).

^{13}C NMR (151 MHz, CDCl$_3$) δ 172.77, 100.36, 100.12, 63.92, 58.97, 54.00, 53.94, 36.86, 35.73, 32.48, 32.32, 24.90, 24.01.

Molecular weight: Estimated by size exclusion chromatography against polystyrene standards in DMF/0.01% LiBr with a VWD (variable wavelength detector) at 250 nm. M_w = 33400, Mn = 13300, PDI = 2.52

Poly-β-aminoester Ketal-2. ^{1}H NMR (400 MHz, CDCl$_3$) δ 4.20 (m, 1.6H), 4.07 (t, J = 6.6 Hz, 2.3H), 3.64 (m, 1.6H), 2.87 (d, J = 9.8 Hz, 4H), 2.67 (t, J = 6.9 Hz, 4H), 2.52 (dd, J = 16.3, 8.4 Hz, 4H), 1.95 (s, 4H), 1.64 (s, 6H), 1.37 (s, 5H), 1.22−1.19 (br, m, 14H).

^{13}C NMR (126 MHz, chloroform-D) δ 172.87, 172.72, 100.31, 64.42, 63.88, 58.92, 54.03, 53.95, 53.87, 36.81, 35.69, 32.43, 32.27, 28.58, 25.65, 24.86, 23.96.

Molecular weight: Estimated by size exclusion chromatography against polystyrene standards in DMF/0.01% LiBr with a VWD (variable wavelength detector) at 250 nm. M_w = 6300, Mn = 2880, PDI = 2.18

Preparation of Dual-Responsive Nanoparticles. In a vial, 25 mg of the synthesized polymer was dissolved in 2.5 mL of DCM. The dissolved polymer was added to 50 mL of phosphate buffer (pH 8) containing 1% PVA. The mixture was stirred at 1000 rpm for 10 min to prepare an emulsion. Further emulsification was achieved using a high pressure homogenizer (Microfluidic 110PS, USA) at 23000 psi for three cycles. The nanoparticle suspension was stirred at 1000 rpm using a magnetic stirrer to evaporate the DCM. Concentrated mode tangential flow filtration system using 500 kDa Pellicon XL cassettes (Millipore, USA) was used to remove the PVA. The nanoparticle suspension was concentrated to 10 mL and washed two times using phosphate buffer (pH 8).[39] The pH of nanocapsule suspensions was reduced to 7.4 using KH_2PO_4 (6.5 w/v%). The pH was acidified to 5 using 0.1 N HCl. This

nanoparticles solution was used diluted and used to perform the degradation and zeta potential experiments.

Nile Red Containing Poly-β-aminoester Ketal-2 Nanoparticles. For this study nanoparticles were prepared as described earlier and by the addition of 500 μg Nile Red in the DCM used during the single emulsion preparation step. This nanoparticles solution was diluted with buffer and used as such for the performing the degradation experiments using Nile Red.

BSA-AlexaFlour Containing Poly-β-aminoester Ketal-2 Nanoparticles. Nanoparticles were prepared using double emulsion method water/oil/water (W/O/W). Briefly, 5 mg of BSA Alexa fluor 594 was dissolved in 0.5 mL of PBS buffer and emulsified with 5 mL of DCM containing 100 mg of polymer (poly-β-aminoester ketal-2 or PLGA) using magnetic stirring. The primary emulsion was added to 50 mL of 1% PVA in PBS pH 8, and the secondary emulsion was produced. Subsequently further emulsification was achieved similar to before. This nanoparticles solution was diluted and used as such for the fluorescence microscopy.

Cell Toxicity of Nanoparticles. The cytotoxicity of nanoparticles was investigated using a 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) reduction assay. RAW 264.7 macrophage cells were seeded at a density of 15000 cells/well in a 96-well plate and incubated for 24 h to reach ~60% confluency. Cells were treated with various amounts of nanoparticles that correspond to different amounts of polymer content in them (0.14−11.4 μg/mL) and incubated for 20 h. To each well was added 10 μL of MTT solution, and the wells were incubated for 3 h. Dimethyl sulfoxide (DMSO, 100 μL) was added to cells to dissolve the resulting formazan crystals. After 20 min of incubation, the absorbance at 570 nm was measured using a microplate reader (Flexstation, Molecular Device Co.). Cell viability was obtained by comparing the absorbance of nanoparticles-treated cells to that of control cells not treated with particles.

Cellular Internalization of Nanoparticles. The uptake of Alexa Fluor-594-BSA-loaded nanoparticles was studied in RAW264.7 cells using a fluorescent microscope. Cells were plated on 12 mm cover slides at a density of 10 000 cells per well for 24 h followed by treatment with poly-βaminoester ketal-2 or PLGA nanoparticles. The payload in the nanaparticles contained a final concentration of approximately 50ug/mL Alexa Fluor-594-BSA. After 2 h at 37 °C, the cells were washed three times with PBS before mounting and staining with DAPI

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