Inflammation Responsive Logic Gate Nanoparticles for the Delivery of Proteins

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Supporting Information

ABSTRACT: Oxidative stress and reduced pH are important stimuli targets for intracellular delivery and for delivery to diseased tissue. However, there is a dearth of materials able to deliver bioactive agents selectively under these conditions. We employed our recently developed dual response strategy to build a polymeric nanoparticle that degrades upon exposure to two stimuli in tandem. Our polythioether ketal based nanoparticles undergo two chemical transformations; the first is the oxidation of the thioether groups along the polymer backbone of the nanoparticles upon exposure to reactive oxygen species (ROS). This transformation switches the polymeric backbone from hydrophobic to hydrophilic and thus allows, in mildly acidic environments, the rapid acid-catalyzed degradation of the ketal groups along the polymer backbone. Dynamic light scattering and payload release studies showed full particle degradation only in conditions that combined both oxidative stress and acidity, and these conditions led to higher release of encapsulated protein within 24 h. Nanoparticles in neutral pH and under oxidative conditions showed small molecule release and swelling of otherwise intact nanoparticles. Notably, cellular studies show absence of toxicity and efficient uptake of nanoparticles by macrophages followed by cytoplasmic release of ovalbumin. Future work will apply this system to inflammatory diseases.

INTRODUCTION

Inflammation, especially chronic, relates to various diseases including cancerous and cardiovascular. Although there are numerous reports of “smart” nanoparticles that rapidly and selectively respond to different disease-specific stimuli, there is a dearth of nanoparticles capable of specifically targeting inflammation.1,2 Furthermore, nanoparticles capable of cytoplasmic delivery are particularly challenging and this remains a major hurdle for effective therapeutic delivery.3,4 In achieving increased cytoplasmic delivery, burst, fragmenting nanoparticles hold promise, as they can lead to elevated osmotic pressure within the endosome leading to endosomal escape of the nanoparticles payload.5–7 Important biomarkers of diseased tissue that have been successfully used to trigger nanoparticle degradation include reduced extracellular pH,8 thermal responsiveness,9 reductive microenvironments,10 and oxidative stress.11 These nanoparticles respond to a single disease stimulus. Furthermore, control over the degradation kinetics, and thus, the ON/OFF state of the system has been difficult to achieve. For example, polyketals have gained prominence as the choice of drug delivery nanoparticles owing to their rapid pH response.12,13 Nanoparticles formulated from these polyketals degrade at acidic pH into acetone and other benign molecules, rapidly releasing the payload within to the cytoplasm.14 However, a key drawback of these systems is that as hydrophobic polymeric nanoparticles their degradation rates are very slow (many days)12,13,15 in acidic pH (pH 6–5) because of reduced water influx. Although a hydrophobic backbone is advantageous in formulating stable nanoparticles at physiological conditions, a hydrophilic backbone is needed to produce fast release in targeted tissue. On the other hand, a hydrophilic backbone often results in nanoparticles that are not stable in pH 7.4 for longer than a few hours.16 Recently, we introduced logic gate nanoparticles that use dual response mechanisms to impart stability to the “OFF” state while maintaining a rapid degradation or a sharp “ON” state.17 We demonstrated the ability of such particles to deliver their payload to the cytoplasm because of the rapid fragmentation of the nanoparticle.18 Here, we apply this strategy to physiological cues of inflammatory diseases.19,20 Our new nanoparticles composed of a dual stimuli and response polymeric backbone are stable in healthy physiological conditions, yet they are able to rapidly fragment in subcellular and diseased conditions. Rapid fragmentation is important for cytoplasmic release of the bioactive payload.

It is known that polyketals degrade via surface hydrolysis while polysulfides degrade via bulk erosion.21,22 Herein, we designed a novel polythioether ketal by incorporating a thioether moiety in our polymer backbone; the thioether acts as a solubility switch that turns the polymer more hydrophilic when it becomes oxidized from a thioether into a sulfone. Sulfones are inherently

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Figure 1. Degradation mechanism of polythioether ketal. Hydrogen peroxide and acidic pH stimulate the degradation of the polymeric nanoparticles in tandem.

more hydrophilic because of a strong dipole due to the presence of the sulfur oxygen double bond; this strong dipole leads to an increased affinity for water, resulting in an accelerated rate of hydrolysis of the ketal groups along the backbone. This allowed our polymers sharper, more rapid degradation kinetics in comparison to conventional polyketals (Figure 1). Various other polysulfides have been used to successfully formulate oxidation sensitive vesicles1 and nanoparticles,20 with such nanoparticles also being used to selectively target dendritic cells in vivo.21

The synthesized polythioether ketal nanoparticles were prepared to target the oxidative stress areas of many diseases including tumors,22 atherosclerosis,23 and causes of aging due to mitochondrial damage.24 During these abnormalities, reactive oxygen species (ROS) are formed when oxygen undergoes a partial one-electron reduction to super oxide anion and subsequently forms hydrogen peroxide, hypochlorite peroxynitrite, and other oxidants.25 Herein, the formulated nanoparticles that are triggered by both ROS and low pH (typical of inflamed tissue), function akin to an “AND” logic gate in circuits. We hope that our design will lead to nanoparticles that will better target diseased tissue for drug delivery or diagnostics.

MATERIALS AND METHODS

Materials. Potassium monohydrogen phosphate (K₂HPO₄) and potassium dihydrogen phosphate (KH₂PO₄) were purchased from Alfa Aesar Organics (Ward Hill, MA). Dichloromethane (DCM, methylene chloride) was purchased from Fisher Scientific (Hampton, NH). Nile red and poly(vinyl alcohol) (PVA) (MW 30–70k) were purchased from Sigma Chemical Co. (St. Louis, MO). 2,2’-Thiodiethanol was purchased Sigma Chemical Co. (St. Louis, MO). 4-Nitrophenylchloroformate was purchased from Acros organics (Belgium). Poly(vinyl alcohol) Alexa Fluor 594 was purchased from Invitrogen. All reagents were purchased from commercial sources and were used without further purification unless otherwise stated.

Synthesis of the Polymer. As shown in Figure 2, 2,2’-thiodiethanol (compound 1, 3.66 g, 0.03 mol) was taken with 4-nitrophenylchloroformate (compound 2, 13.3 g, 0.064 mol) in a dry, round-bottom flask with 125 mL of dry dichloromethane and stirred under a nitrogen atmosphere. To this, triethylamine (30 mL, 0.21 mol) in 25 mL of dry dichloromethane was added dropwise over 30 min at room temperature. The reaction was subsequently stirred for 4 h. At the end of the reaction, the reaction mixture was rotavaped, diluted with 250 mL of dichloromethane, and extracted from 2 × 100 mL of 1% HCl. The organic layer was dried over MgSO₄ and rotavaped. The resulting crude solid was recrystallized from ethyl acetate three times to yield 8.0 g (0.017 mol, 60% yield) of a white solid (MP 138.6–140.1 °C).

1H NMR (500 MHz, chloroform-d) δ 8.28 (d, J = 7.5 Hz, 1H), 7.38 (d, J = 7.5 Hz, 1H), 4.47 (dd, J = 6.3, 5.3 Hz, 1H), 2.97 (dd, J = 6.4, 5.2 Hz, 1H). 13C NMR (126 MHz, chloroform-d) δ 155.46, 152.52, 145.61, 121.91, 67.84, 30.79. HR-ESI-FT-MS (Orbit-Trap-MS) C18 H16 N2 O10 S Na. Mass Measured: 475.0420, Theo. Mass: 475.0418 δ (ppm) 0.4.

Polymer synthesis was completed by preparing diamine (compound 4, Figure 2) as per the literature.25 Diamine (0.518 g, 3.2 mmol) and carbonate (compound 3, Figure 2) (1.446 g, 3.2 mmol) were taken with 2 mL of dry dichloromethane in an 8 mL vial with a Teflon cap and purged with nitrogen. To this, 1 mL of triethylamine was added via syringe, and the contents were stirred for 4 days at room temperature. The contents were precipitated into diethyl ether and the waxy yellow solid obtained was subsequently purified on a gel column packed with lipophilic sephadex (LH-20) using dichloromethane as the eluent, to obtain a pale yellow solid in 70% yield.

1H NMR (500 MHz, chloroform-d) δ 5.42 (s, 1H), 4.21 (t, J = 6.4 Hz, 2H), 3.45 (t, J = 5.2 Hz, 2H), 3.30 (d, J = 5.3 Hz, 2H), 2.77 (t, J = 6.5 Hz, 2H), 1.32 (s, 3H). 13C NMR (126 MHz, chloroform-d) δ 156.42, 100.32, 64.24, 64.21, 59.93, 41.20, 31.34, 24.93, 24.90. Molecular weight: Obtained via GPC against polystyrene standards using DMP(0.01% LiBr) as the eluent. Mn = 59 800 Da, Mn = 34 100, PDI = 1.75.

Nanoparticle Formulation. In o/w procedure, 25 mg of the synthesized polymer was dissolved in 2.5 mL DCM. Then,
500 μg Nile red were added, for preparing Nile red containing particles. DCM was added to 50 mL of phosphate buffer (pH 8) containing 1% PVA, and the mixture was stirred at 1000 rpm for 5 min to prepare an emulsion. Further emulsification was achieved using a high-pressure homogenizer (Microfluidic 110PS, USA) at 23 000 psi for three cycles. The nanoparticle suspension was stirred at 1000 rpm using a magnetic stirrer to evaporate the DCM. Nile red containing nanoparticles were filtered through 1 μm filter to remove the insoluble Nile red aggregate, and a concentrated mode tangential flow filtration system with 500 kDa Pellicon XL cassettes (Millipore, USA) was used to remove the PVA and any unencapsulated material. The nanoparticle suspension was concentrated to 10 mL and washed twice.26 Protein was encapsulated into the nanoparticles using the w/o/w emulsion method. Briefly, 2 mg Ovalbumin Alexa Fluor 594 was dissolved in 0.2 mL of PBS buffer and subsequently emulsified with 5 mL of DCM containing 100 mg polymer using probe sonication (stabilized using 2% Span 80) at amplitude of 40% for 5 min (1/8 inch tip, Misonix S-4000, USA). The primary emulsion was added to 50 mL of 1% PVA in buffer (pH 8) under stirring at 1000 rpm, and the secondary emulsion was produced. Additional emulsification was achieved using the high-pressure homogenizer at 23 000 Psi for two cycles and tangential flow filtration was accomplished as previously described. Finally, 5% Trehalose was added to the particles before lyophilization. The lyophilized particles were suspended in aqueous media at different pHs and H2O2 levels for further evaluation of stability or release.

The encapsulation efficiencies of the particles were determined by incubating them at acidic pH in presence of 100 mM H2O2 until no further particles are detected by the DLS and the detected fluorescence reaches its maximum.

Dynamic Light Scattering Measurements of the Nanoparticles. Nanoparticles were suspended in a phosphate buffer pH 7.4 or 5 in the presence or absence 100 mM H2O2, with stability determined by monitoring their size for 24 h via dynamic light scattering (DLS) using a Zetasizer-ZS (fixed attenuator of 7, Malvern Worcestershire, UK).

Effect of pH and H2O2 on Nile Red Release. Nile red was encapsulated into the nanoparticles as a sensor of changes in the hydrophobic—hydrophilic character of the surrounding environment. Nile red fluoresces in the hydrophobic environment inside the nanoparticles and quenches once released into the aqueous release media; this quenching was measured as an indicator of Nile red release into an aqueous medium. Nanoparticle powder was dispersed in PB pH 7.4 or 5 in the presence or absence 100 mM H2O2, with stability determined by monitoring their size for 24 h via dynamic light scattering (DLS) using a Zetasizer-ZS (fixed attenuator of 7, Malvern Worcestershire, UK).

Cell Toxicity of Nanoparticles. The cytotoxicity of nanoparticles was investigated using a 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyldtetrazolium bromide (MTT) reduction assay. RAW 264.7 macrophage cells were seeded at a density of 15 000 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 corresponded to the varying amounts of polymer contained in them (0–300 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 FlexStation microplate reader (Molecular Devices, Inc., Sunnyvale, CA, USA). Cell viability was obtained by comparing the absorbance of nanoparticle-treated cells to that of control cells not treated with particles.

Cellular Internalization of Ovalbumin-Containing Nanoparticles. The uptake of ovalbumin Alexa Fluor 594-loaded nanoparticles was studied in RAW264.7 cells using a fluorescent microscope. Cells were plated on CultureWell Chambered Coverglass slides (Invitrogen Corporation, Carlsbad, CA, USA) at a density of 10 000 cells per well for 24 h followed by treatment with polythioether or PLGA nanoparticles. The nanoparticle payload contained a final concentration of approximately 10 μg/mL ovalbumin Alexa Fluor594. After 8 h at 37 °C, the cells were washed with PBS before mounting and staining with DAPI.

RESULTS AND DISCUSSION

Infected tissues are frequently characterized by a decreased pH and the presence of reactive oxygen species (ROS).17–19 In order to simulate these conditions, particle behavior was tested at acidic pH and in the presence of H2O2, which is a prevalent reactive oxygen species. Accordingly, nanoparticles were subjected to different physiologically relevant conditions: pH 7.4 (pH of healthy tissue), pH 7.4/100 mM H2O2 (presence of ROS), acidic pH, and acidic pH/100 mM H2O2. Their behavior was
monitored via DLS and by measuring the release of Nile red and ovalbumin.

The hydrophobic nature of the polymer permitted the formulation of the nanoparticles using single- or double-emulsion techniques. This in turn allowed the encapsulation of different payloads with different hydrophobic/hydrophilic characteristics like Nile red and ovalbumin (Figure 3).

Monitoring the behavior of the nanoparticles using DLS (Figure 4) showed that only nanoparticles dispersed in pH 5 in the presence of H₂O₂ degraded within 24 h, while the particles dispersed in pH 5 alone remained detectable throughout this time period. Thus, the presence of both stimuli caused an accelerated degradation of the nanoparticles. This is further evidenced by the observed increase in particle size upon incubation in H₂O₂ at pH 7.4. This swelling is due to the backbone becoming oxidized, resulting in the hydration of the particles.

When Nile Red is encapsulated in the particles, it acts as a fluorescent probe of hydrophobicity and release. In the presence of H₂O₂, oxidation of the polymer backbone occurs, rendering the nanoparticles more hydrophilic. This causes quenching of encapsulated Nile red upon its release from the nanoparticles (Z-average = 200 nm). This hydrophilic change occurred in both neutral and acidic conditions and depended entirely on whether the particles were incubated in hydrogen peroxide or not (Figure 5). Also, Nile red was minimally quenched at pH 5 due to the slow degradation of the particle via surface erosion. However, more quenching is observed when both conditions are operational. Finally, in order to confirm that our polymer degrades under the said conditions, we performed degradation and analyzed via ¹H NMR (Figure 6 and Figure 7). Here, as expected, we did not see a difference in polyketal hydrolysis rates because the polymer is completely dissolved without the need of oxidation. The control that we observed in the rates of particle degradation is due to the nanoparticle architecture, which is lost when the polymer is dissolved. We subsequently tested this systemic control in releasing ovalbumin as a model protein.

Figure 8 shows that ovalbumin release was initiated by oxidation of the polymer, or more accurately due to swelling of the nanoparticles. Meanwhile, higher release was observed at acidic pH in the presence of hydrogen peroxide due to
degradation of the nanoparticles. Importantly, the nanoparticles showed excellent stability at pH 7.4. Furthermore, the full degradation of our new nanoparticles system is important as small fragments are more readily excreted.\textsuperscript{30}

We evaluated the cytotoxicity of polythioether ketal nanoparticles in cells using the MTT assay using RAW264.7 cell line. The RAW264.7 macrophage cell line is a great model system for studying immune response and can produce relatively higher levels of endogenous ROS. Additionally, these cells are able to take up cells very readily without the need of cell penetrating peptides.

RAW 264.7 cells were incubated with various amounts of nanoparticles for 20 h. Figure 9 illustrates the comparison of cytotoxicity between cells treated with increasing concentrations of polythioether ketal polymer. There was no significant cytotoxicity observed with the cells incubated with up 300 \( \mu \)g/mL of polymer (\( p = 0.294 \)).

Finally, we analyzed the cellular uptake of nanoparticles and release of cargo within cells by comparing polythioether ketal and PLGA nanoparticles containing equal amounts of fluorescently labeled ovalbumin (Figure 10). Nanoparticles were added to RAW 264.7 cells and evaluated by fluorescence microscopy. Polythioether ketal nanoparticles were able to efficiently deliver protein within RAW264.7 macrophage cells that have an \( \mathrm{H}_2\mathrm{O}_2 \) concentration of 2 \( \mu \mathrm{M} \).\textsuperscript{33}

Cells treated with polythioether ketal nanoparticles show thorough diffusion of ovalbumin throughout the cytoplasm and the nucleus, indicating that labeled ovalbumin was released within the cell. This is in contrast to slow-degrading PLGA nanoparticles that show punctuated spots and less diffused ovalbumin throughout the cell.

\section*{CONCLUSION}

We designed a polymeric nanoparticle with two stimuli responsive moieties in its backbone, and which undergoes programmed degradation when stimulated by inflammatory cues, ROS, and acidic pH, respectively. This system functions akin to an “AND” Logic Gate. Upon stimulation by ROS, the polymer becomes hydrophilic, followed by ketal hydrolysis promoted at an acidic pH. We observed release of small hydrophobic molecules, Nile red, in acidic pH and an increase in polymer hydrophilicity upon oxidation. Higher release of encapsulated ovalbumin was observed in the presence of both hydrogen peroxide and acidic pH. We found that the nanoparticles were readily taken up by macrophage cells and that ovalbumin release from the nanoparticles into the cytoplasm was in agreement with the degradation behavior of the nanoparticles as shown in the DLS measurements. We hypothesize that these nanoparticles can differentiate among the conditions of our target areas from others. This should ensure the release of the payload in specific diseased regions such as inflammatory or tumor sites and allows for intracellular delivery. These studies suggest that our nanoparticles can be applied to the delivery of diagnostics and therapeutics to sites of inflammation. Thus, our current work focuses on the application of these nanoparticles in targeting theranostics to vulnerable plaque versus stable plaque.

\section*{ASSOCIATED CONTENT}

\subsection*{Supporting Information.} \textsuperscript{1}H NMR of compound 3, \textsuperscript{13}C NMR of compound 3, HR-ESI-FT-MS (Orbit-Trap-MS) of compound 3, \textsuperscript{1}H NMR of poly 5, \textsuperscript{13}C NMR of poly 5, GPC trace
of Poly 5 calibrated against polystyrene standards. This material is available free of charge via the Internet at http://pubs.acs.org.

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■ REFERENCES