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An iron oxide nanoparticle-based magnetic resonance method to monitor release kinetics from polymeric particles with high resolution

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Abstract

A new method to precisely monitor rapid release kinetics from polymeric particles using super paramagnetic iron oxide nanoparticles, specifically by measuring spin-spin relaxation time (T_2), is reported. Previously, we have published the formulation of logic gate particles from an acid-sensitive poly- β -aminoester ketal-2 polymer. Here, a series of poly- β -aminoester ketal-2 polymers with varying hydrophobicities were synthesized and used to formulate particles. We attempted to characterize particle release kinetics via fluorescence of encapsulated Nile red to determine whether the chemistry designed into the polymers could finely tune the release kinetics in the minutes time regime; however, standard fluorescence-based techniques did not differentiate the release kinetics among our new series of polymeric particles. Thus, a new method based on encapsulation of iron oxide nanoparticles was developed, which enabled us to resolve the release kinetics of our particles. Moreover, the kinetics matched the relative hydrophobicity order determined by octanol-water partition coefficient. To the best of our knowledge, this method provides the highest resolution of release kinetics to date.

Keywords

Iron oxide nanoparticles; magnetic resonance; monitoring release; rapidly degradable particles; ketals; Logic Gate particles

INTRODUCTION

Polymeric nanoparticles promise to vastly improve the efficacy of therapeutics by allowing delivery of poorly water-soluble drugs, fragile proteins and other bioactives, enhancing circulation time, increasing delivery to target tissues, and releasing drugs in a more controlled manner.^{1–10} The clinical potential of the various nanoparticle platforms yet developed, including nanoemulsions, micelles, dendrimers and hydrogels,^{1–3,6} is determined by their release kinetics,^{11–13} as drugs and biopharmaceuticals have to be delivered in precise

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Author Contributions

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Supporting Information. Methods details for DLS, TEM, measurement of T_2 relaxation rate vs. iron concentration, and data for characterization of Nile red-encapsulating particles, size distribution of SPION-encapsulating particles, and contact angle. This material is available free of charge via the Internet at <http://pubs.acs.org>.

amounts at precise times to achieve a therapeutic effect. Current FDA-approved nanoformulation materials are polyester-based, such as poly(d,l-lactide-co-glycolide) (PLGA) and poly(l-lactic acid) (PLA)^{14,15}, and release their contents in the range of hours to days. However, many medical applications require release of bioactives in the range of minutes to hours.^{16,17} Furthermore, the acidic byproducts of polyester degradation have been known to destabilize some protein payloads.¹⁸ To address this need, ketal-based materials have recently been developed; in mildly acidic conditions they degrade into nontoxic byproducts and release their contents over minutes to days, depending on the hydrophobicity of the polyketal.^{10,16,17,19} In an effort to increase the shelf life of rapidly degradable polyketal based nanoparticles, our group developed a ketal-based polymeric nanoparticle which degrades through an 'AND' Logic Gate mechanism: disease associated conditions such as mild acidity or oxidative stress first promote solubilization, which then allows acid catalyzed hydrolysis. Nanoparticles made from this polymer provide unprecedented stability in neutral conditions, yet maintain their rapid degradation in conditions that characterize disease and the subcellular environment.^{10,20}

Here, we hoped to modulate and fine-tune the release kinetics of Logic Gate nanoparticles within the range of minutes to hours by varying the hydrophobicity of the poly- β -aminoester ketal-2 backbone. To do so, we synthesized five types of poly- β -aminoester ketal-2: polymer AB has a four-carbon (butane) spacer in the diacrylate; polymer ANP, neopentyl group; ACH, cyclohexane; AH, hexane; and AN, nonane (Scheme 1). We hypothesize that as the hydrophobicity of the polymer increases, the local concentration of water around the pH-labile ketal group will be lower, resulting in slower degradation of the polymers.^{10,16,17,19} In order to test our hypothesis, we formulated particles using the polymers and compared their release kinetics.

Unfortunately, the most commonly used methods for determining release kinetics from particles were not able to differentiate between our five polymers despite their different chemistries. In order to accurately measure release from our new nanoparticles, we developed a real-time method that depends entirely on whether cargo is encapsulated. We measured the transverse relaxation time (T_2) of 10 nm super paramagnetic iron oxide (Fe_3O_4) nanoparticles (SPIONs) encapsulated in our polymeric particles. SPIONs are frequently studied as magnetic resonance imaging (MRI) contrast agents, as they can shorten the transverse relaxation time (T_2) of the surrounding water and enhance negative contrast.^{21–24} A solution of nanoparticles encapsulating SPIONs would have a low T_2 value; as the particles start to degrade, the hydrophobic SPIONs are released and precipitate out of solution²¹, resulting in an increase in T_2 . Because their sizes are similar to typical proteins (diameter ~3nm to 10nm)²⁵, the release kinetics of SPIONs can indicate the time required to release proteins.

In this study, we show that magnetic resonance measurements using SPIONs precisely resolve the differences in release kinetics of the five different particles, which is not possible by Nile red release, a commonly-used method. With real-time kinetic monitoring capacity, this method also reduces the time and effort in sample collection and handling.

MATERIALS AND METHODS

Materials

4,4-Trimethylenedipiperidine and iron oxide nanoparticles (10nm, 5mg/mL in toluene) were 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), D-(+)-Trehalose dihydrate 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-2

The polymers were synthesized as described previously (Scheme 1).^{10,26} In brief, diacrylate (5 mmol) and diacrylic ketal (4.2 mmol) were dissolved in 1 mL of DCM followed by addition of 1 mL of TEA. While the reactants were stirred, dipiperidine (10 mmol) was added to the vial, which was then sealed. The reaction was purged with nitrogen gas and stirred at room temperature for 4 days. The solvent was then evaporated, and the crude polymer was dissolved in 10 mL of DCM. The polymer was purified by precipitation into 2 $\times$ 200 mL hexane to yield 360 mg of the polymer. The polymer was collected and dried under vacuum prior to analysis.

Formulation of nanoparticles

For encapsulation of Nile red, 100 μ L of dye (1 mg/mL in chloroform) was added to a 2 mL solution of each polymer at 30 mg/mL in chloroform in a glass vial. For encapsulation of iron oxide nanoparticles, 200 μ L of iron oxide nanoparticles (10nm, 5mg/mL in toluene) was added to each polymer solution. 8 mL of 3% polyvinyl alcohol (PVA) in potassium phosphate buffer (pH 8.4) was added to the polymer solution. The solution was sonicated using a 1/8 inch tip sonicator (Misonix S-4000) at about 9.6 W for 5 min. Particle solutions were stirred overnight, allowing the chloroform to evaporate. The particle solution was washed with phosphate buffer (pH 8.4) by tangential flow filtration through 500 kDa Pellicon XL cassettes (Millipore) to remove PVA and insoluble Nile red. 5% (w/v) of trehalose dihydrate was then added to the particle solution, which was then vortexed for 20 s, frozen by liquid nitrogen and lyophilized. The original concentration of the particles was determined by dividing the resulting lyophilized mass by the initial solution volume. The original Fe concentration of the iron oxide-containing nanoparticle solution was determined by inductively coupled plasma atomic emission spectroscopy (ICP-AES).

Measurement of Nile red fluorescence

Lyophilized particles were resuspended in milliQ H₂O at one-fourth their original concentration. The particle solution was vortexed for 10 s and pipetted repeatedly for 3 min. The solution was then filtered through an Acrodisc CR 13 mm Syringe Filter (0.45 μ m PTFE membrane, Pall Life Science). 330 μ L of the particle solution was added to 2.97 mL of pH 7.2 potassium phosphate buffer in a disposable polymethacrylate fluorimeter cuvette (Sigma-Aldrich) and incubated at 37°C. Emission spectra were acquired by excitation at 556 nm over time (three per nanoparticle type). Because fluorescence intensity decreases immediately at pH 7.2 for the less hydrophobic particles such as AB and ANP, the initial time point of the fluorescence release at pH 7.2 was obtained by taking the emission spectra of particles (330 μ L) in pH 8.4 potassium phosphate buffer (2.97 mL).

Dynamic light scattering (DLS)

Size and polydispersity of nanoparticles encapsulating iron oxide or Nile red were measured using a Zetasizer (Malvern, U.K). Experimental details provided in supporting information.

Transmission electron microscopy (TEM)

Nanoparticles were imaged using a FEI Tecnai Spirit at 200 kV (details described in supporting information)

Measurement of change in spin-spin relaxation time (T_2)

Lyophilized particles were resuspended in milliQ H₂O at their original concentrations. 200 μ L of particle solution and 200 μ L of 100 mM potassium phosphate buffer (pH 6.4, pH 7.3) were incubated at 37°C separately for 10 min. At time = 0, the particle solution was then added to the buffer (resulting in pH 6.8 or 7.4); 200 μ L of the resulting solution was added to an NMR tube and placed in the relaxometer (Aspect M2 relaxometer with 1T field (Aspect Imaging, Shoham, Israel) with a 60 mm diameter whole body coil) for continuous measurement of T_2 over 13 h. Scan parameters: recycle delay = 10 s, 10000 data point for fit, 90° – 180° pulse separation (τ) = 1. To resolve the release kinetics between ACH-SPION and AN-SPION, the same T_2 measurement was done with incubating the particle at pH 4.2 over 13 h. Data was normalized to the first time point for each particle type.

RESULTS AND DISCUSSION

By tuning their degradation rates, the clinical potential of polyketals can be widely expanded. To test our hypothesis that kinetics of release of the polymeric particles can be controlled by changing the hydrophobicity of the polymer, we synthesized a series of poly- β -aminoester ketal-2 of varying hydrophobicity via Michael-type addition of bis(secondary amine) monomers to diacrylate ketals and diacrylate ester monomers (used to modulate the hydrophobicity) in a 2:1:1 mixture (Scheme 1). All the diacrylate esters are commercially available, and diacrylate ester ketals can be synthesized from commercially-available reagents.²⁶ This 2-step synthesis, which does not require purification by columns, makes this polymer series an even more attractive type of biomaterial.

It is expected that polymer AB, with the shortest straight chain (4-carbon) as spacer in the diacrylate, should be the least hydrophobic. The branching of ANP's neopentyl group increases bulkiness and thus makes it slightly more hydrophobic than AB. AH should be more hydrophobic than ANP because it has a longer hydrocarbon chain (6-carbon) as spacer. Although the length of spacer in ACH is approximately the same as that of AH, cyclization in the cyclohexane spacer should increase the polymer's hydrophobicity. The most hydrophobic polymer, AN, has the longest hydrocarbon chain (9-carbon). In fact, the anticipated hydrophobicity order is supported by the octanol-water partition coefficient of a series of poly(β -amino ester)s with diacrylates AB, ANP, AH, ACH and AN, but without the acid-sensitive diacrylic ketal.²⁷ Although our polymers are degradable, due to the ketal groups, the relative hydrophobicity among the polymers should be the same. The octanol-water partition coefficient represents the ratio of the solubility of a compound in octanol to its solubility in water; the larger the logP value, the more hydrophobic a molecule is. LogP values for these polymers were: AB: 1.9; ANP: 2.2; AH: 2.9; ACH: 3; AN: 4.3. This result matches the hydrophobicity order we anticipated for our polymer.

Nile red release

To measure release kinetics, we used our polymers to formulate nanoparticles encapsulating Nile red (NP-NR), a polarity probe. DLS measurements of hydrodynamic size and polydispersity, together with TEM images, confirmed the formation of nanoparticles with a narrow size distribution (Supp. Fig. 1). The fluorescence of Nile red, 9-diethylamino-5H-benzo[*a*]phenoxazine-5-one, a neutral small-molecule hydrophobic dye often considered a model drug for evaluation of release kinetics^{28–30}, is sensitive to the polarity of its environment.^{31,32} As Nile red is released from a hydrophobic particle into aqueous solution, an increase in polarity causes a red shift in the emission wavelength of Nile red coupled with a decrease in fluorescence intensity.^{31,32} Thus, by monitoring the emission intensity and wavelength over time, the rate of release from particles can be compared.

Emission spectra of NP-NR in buffer at pH 7.2 were taken over time

This pH was chosen because a lower pH (pH 6.8) would cause immediate hydration in all particles except the most hydrophobic, AN (Supp. Fig. 2). Therefore, conclusions concerning relative hydration rates cannot be drawn. The fluorescence intensity at pH 7.2 distinguishes three groups: AB releases fastest (60% decrease in fluorescence intensity within 1 minute), ANP and AH are intermediate (60% decrease in about 1h), and ACH and AN are slowest (60% decrease after 24 hours) (Figure 1a). The maximum emission wavelength ranks the polymers' hydration rates similarly, but can also distinguish ACH from AN: ACH shifted from 612nm to 625nm in 24 hours while AN did not shift at all (Figure 1b).

This result generally agrees with the hydrophobicity order, which predicts that AB, ANP and AH will be hydrated faster than ACH and AN. However, Nile red fluorescence does not clearly resolve the differences between the release rates of particles formulated from ANP compared with AH particles. There are several possible explanations; most importantly, NR release may reflect hydration rate (how fast water gets into the particle) rather than the actual release of Nile red. Protonation of the polymers prior to degradation increases hydration of NR, causing a red shift and a decrease in fluorescence intensity for Nile red.^{28–30,33–36} In addition, because of its small size, NR can leak out from the hydrophobic particle into the aqueous solution. Further, burst release of dye adsorbed to the surface of particles, and quenching may also introduce artifacts in release kinetics. Therefore, we sought a new method to compare the release kinetics of our polymer series.

Iron oxide particle release

We theorized that changes in spin-spin relaxation time (T_2) of iron oxide-encapsulating nanoparticles would accurately distinguish the release kinetics of our new polymers. A solution of iron oxide particle-loaded particles (NP-SPIONs) will have a low T_2 value. When the particles degrade, the hydrophobic SPIONs will be released into the aqueous solution and form a precipitate, which is outside of the detection window of the relaxometer. Therefore, T_2 of the solution will increase as particles release SPIONs, and the release kinetics of particles can be monitored continuously by measuring the T_2 relaxation time.

SPION-encapsulating particles were formulated through emulsion and characterized by DLS and TEM (Fig. 2). Because they are hydrophobic, SPIONs are efficiently encapsulated into our polymeric particles. DLS reveals that the nanoparticles range in diameter from 136 to 188 nm (Fig. 2b), and all five particles are quite monodisperse (Supp. Fig. 3). Though the hydrodynamic size measured by DLS is slightly larger than the size measured by TEM (probably a result of the hydration of particles in solution), TEM generally confirms sizes determined by DLS (Figure 2c).

The T_2 relaxation rate of a representative NP-SPION was also measured to confirm that the magnetic and relaxation properties were characteristic of SPION-encapsulating particles (Supp. Fig. 4). The T_2 relaxation time of the particle solution was measured at pH 7.4 and plotted as a function of iron concentration [Fe]; the slope of the linear regression yielded a T_2 relaxivity of $89.7 \pm 1.1 \text{ mM}^{-1} \text{ s}^{-1}$.

To monitor the release of SPION, the five NP-SPIONs were incubated at pH 7.4 and 6.8, and T_2 was measured continuously for 13 hours (Figure 4). At pH 6.8 (Fig. 3a), T_2 of AB increases fastest, reaching a plateau in about 7 h, followed by ANP and then AH, while T_2 of ACH and AN remain unchanged. To resolve the release kinetics of ACH and AN, the NP-SPIONs were incubated at pH 4.2 (Fig. 3c). These conditions reveal that ACH releases SPION faster. As a control, T_2 of the five NP-SPIONs were also measured at pH 7.4,

revealing no change in T_2 for all particles (Fig. 3b), which indicates that SPIONs are not released.

This method clearly resolves the release rates of the five particles: AB > ANP > AH > ACH > AN. SPION release rate agrees with the hydrophobicity order: as the hydrophobicity of the polymer increases, the rate of degradation of the polymer and release from the particle decreases.

CONCLUSIONS

We introduce a novel method for monitoring *in vitro* release of nanoscale cargo from polymeric particles, real-time measurement of T_2 relaxation time of encapsulated SPIONs, which offers many advantages over other commonly used methods. This method clearly resolves the release kinetics of closely related polyketals, while fluorescence of Nile red, a commonly used fluorescent model cargo, does not. Further, as SPIONs are of a similar size to proteins (10 nm), the measured release kinetics are likely proportional to those for proteins; we have previously shown that polyketal nanoparticles allow rapid release of protein cargo¹⁰.

While a few methods exist for real-time measurement of nanoparticle release kinetics^{37,38}, release is often measured by taking aliquots over time and isolating released drug or protein^{5,39–45}, usually through centrifugation.²⁰ However, because of their small size, particles may not be completely separated from the released cargo; long centrifugation may also change the time interval over which release is determined; in addition, centrifugation can induce drug or protein release from particles.⁴⁵ An alternative to centrifugation is dialysis⁴⁶; however, adsorption of released molecules to the membrane also introduces errors in measurement.^{5,45,46} In addition, commonly used methods to quantify release, such as fluorescence, radioactivity, high-performance liquid chromatography, and protein assay, often require labor-intensive sample preparation. As aliquots must be collected regularly, obtaining release data over a period of more than 20 hours is difficult. In our method, continuous measurements are taken by the relaxometer. Further, only 200 μ L of particle solution is required for T_2 measurement, which minimizes the amount of materials needed, and the relaxometer is relatively inexpensive and easy to operate.

Most importantly, T_2 measurement can clearly resolve the release kinetics of our polymers with varying hydrophobicities, which is not possible by conventional measurements. Together, this makes our method an attractive method for monitoring the release kinetics of nanoscale cargo from polymeric nanoparticles.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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ABBREVIATIONS

AB, ANP, AH, ACH, and AN	various poly- β -aminoester ketal-2 polymers (see Scheme 1)
SPION	super paramagnetic iron oxide nanoparticle
NR	Nile red
DLS	dynamic light scattering
TEM	transmission electron microscopy

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FIGURE 1.

Nile red release from the nanoparticles incubated at pH 7.2. Fluorescence emission spectra were taken over time at excitation wavelength, $\lambda=557$ nm; maximum emission intensity (A) and wavelength (B) is plotted as a function of time.

FIGURE 2.

(A) Formation of SPION-loaded nanoparticles (NP-SPION). (B) Hydrodynamic sizes of all five NP-SPIONs as measured by DLS. (C) Representative TEM image of SPION-loaded nanoparticles formulated with polymer AN.

FIGURE 3. Spin-spin relaxation time (T_2) over 13 hours for all five SPION-encapsulating particles at (A) pH 6.8, (B) pH 4.2, and (C) pH 7.4.

SCHEME 1.

Synthesis of random *co*-poly- β -aminoester ketal-2. Below the reaction scheme is the composition of each polymer in order of hydrophobicity.