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Gadolinium-Hydrogel-Lipid Hybrid Nanoparticles Provide 'Off-On-Off' MRI Signals for Non-Invasive Thermometry

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ABSTRACT

Novel metal-chelating temperature-responsive ultrafine hydrogel nanoparticles were synthesized and loaded into a solid lipid nanoparticle carrier with various melting points. The temperature sensitive contrast agent was engineered with two consecutive 'off-on' and 'on-off' thermal transitions, which delimit a window within which thermotherapy can be applied. An 'off-on' step transition in contrast enhancement was detected at the melting point of the lipid matrix and an 'on-off' step transition is present at the lower critical solution temperature (LCST) of the copolymer hydrogel. A single composite gadolinium-hydrogel-lipid hybrid nanoparticle (Gd-HLN), as formulated here, can thus serve to indicate the onset of non-ablative thermotherapy (42-43^{o}C) and to delineate the transition from non-ablative to ablative temperatures (>55^{o}C). Since both the melting point of the lipid matrix and the LCST of the hydrogel are tunable in terms of temperature of phase transition, the intelligent multiparticle relaxation-enhancing system can be engineered to designate the temperature window specific to the goal of the individual thermotherapy.

INTRODUCTION

Locoregional treatment of solid tumors has been realized by various physical means, including hyperthermia therapy. The ability to precisely detect the temperature of the tissue in real time is paramount to both effective treatment and the prevention of inappropriate normal tissue death. In thermotherapy for cancer treatment, the tissue temperature is controlled to 42-43^{o}C (non-ablative) in order to maintain therapeutically effective conditions but prevent widespread tissue necrosis, which occur at higher temperatures (ablative). As the tissue temperature dictates the type of thermotherapy (ablative vs. non-ablative) and thus very different therapeutic outcomes, the precise control of tissue temperature is crucial.

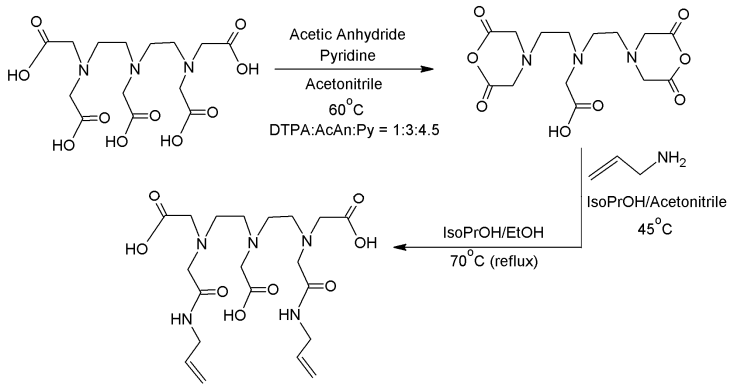
Due to its non-invasiveness and multidimensional imaging capability, magnetic resonance imaging (MRI) has been pursued as an alternative to surgically invasive and potentially infectious two-dimensional probe thermometry. Existing carrier-free temperature-sensitive contrast agents have failed to provide reliable temperature data due to low signal-to-noise ratio, masking any meaningful thermometric data, and the dependence of accurate interpretation of relaxivity data on tissue concentrations of the contrast agent, which are difficult to accurately obtain *in vivo* [1, 2]. While temperature sensitive liposomes (TSLs) have been reported to overcome these limitations, a single formulation was unable to provide a real-time measurement of the therapeutic temperature window that demarcates the onset of non-ablative thermotherapy and differentiates between non-ablative and ablative temperatures [1, 3]. In

addition, the TSLs are only able to undergo thermal transitions at temperatures equal to or below 43ºC [3].

By synthesizing gadolinium-chelating temperature-responsive ultrafine hydrogel nanoparticles and loading them into a solid lipid nanoparticle carrier, a temperature sensitive contrast agent was engineered with consecutive temperature-dependent 'off-on' and 'on-off' step transitions in MR contrast enhancement. In this manner, this system delimits a tunable window within which thermotherapy can be monitored in real-time.

EXPERIMENT

All chemicals were of analytical grade (Sigma-Aldrich Canada) and used without further purification unless otherwise indicated. Fatty acid esters used were synthesized as previously described [4], and the structures of the esters were confirmed by ^{1}H and ^{13}C-NMR using a Varian Mercury 400 spectrometer (Varian Inc., CA, USA). *N,N'*-Bisallylamidodiethylenetriamine-*N,N',N''*-triacetic acid (BADTTA) was synthesized from the amidation of the bisanhydride of diethylenetriaminepentacetic acid (DTPA) with allylamine (Scheme 1). DTPA bisanhydride was prepared as previously described [5]. The structure of BADTTA was confirmed by ^{1}H and ^{13}C-NMR in DMSO-d_6 using a Varian Mercury 400 spectrometer (Varian Inc., CA, USA). The pK_as of the carboxylic acids of BADTTA were determined by analysis of potentiometric titration curves (0.1M KOH at I=0.1mM KCl) using CurTiPot© software (Gutz, I., University of Sao Paolo, Brasil). A complexometric competition binding assay was performed to measure the binding constant of Gd to BADTTA, using xylenol orange as the competitive colorimetric indicator. The binding constant was calculated from the generated data using PSEQUAD software [6]. Ultrafine hydrogel nanoparticles were synthesized by a reverse emulsion-polymerization technique as previously described [7]. Particle size was determined by photon correlation spectroscopy (NICOMP 380 ZLS particle sizer, PSS-NICOMP, CA, USA) and was confirmed by transmission electron microscopy (Hitachi H7000, 75kV, uranyl acetate counterstain). The lower critical solution temperature (LCST) of the acrylamide-co-N-isopropylacrylamide hydrogel nanoparticles was determined by heating the sample in a differential scanning calorimeter using a TA Instruments DSC 2010 (TA Instruments, DE, USA,



Scheme 1. The synthesis of BADTTA involved a two-step procedure, the first being the dehydration of DTPA to form the bisanhydride, which has been adopted as a common scheme for the symmetric functionalization of DTPA [8, 9]. The second step was the amidation of DTPA bisanhydride by allylamine, resulting in the symmetrical addition of reactive ethylene groups to form BADTTA.

70°C at a rate of 3°C/min). The capacity for the hydrogels to bind Gd was assayed spectrophotometrically by competition binding to xylenol orange. To assess the utility of the Gd-loaded hydrogel nanoparticles as a contrast agent, the relaxivity (r_1) was calculated from total loaded Gd, and was measured using a 1.5T GE Signa Scanner (General Electric, WI, USA, 2D inversion recovery) below (20°C) and above the LCST of the hydrogel particles. Gd-HLN were formulated by the hot emulsion-sonication technique [7]. The size of Gd-HLN was measured by photon correlation spectroscopy, and the Gd loading was assayed by inductively coupled plasma atomic absorption spectroscopy using an Optima 7300 ICP AES (Perkin Elmer, MA, USA). The magnetic resonance contrast enhancement provided by the Gd-HLN was measured as a function of temperature. Aliquots of the hydrogel-lipid hybrid nanoparticle suspension were heated to a given temperature for 10 minutes, vortex mixed, then let cool to room temperature. The T_1-weighted relaxivity of the solutions were then measured by 2D inversion recovery.

DISCUSSION

Synthesis And Characterization of the Novel Chelator-Cross Linker

In order to stably load gadolinium into the hydrogel nanoparticles and, importantly, ensure its retention in the hydrogel nanoparticles and prevent the systemic accumulation of Gd, a novel bifunctional molecule was synthesized. The commonly employed heavy metal chelator DTPA was functionalized with two ethylene groups, one at each end of the molecule. With this modification, the resultant chelator BADTTA was endowed with cross-linking functionality (Scheme 1). The structure of BADTTA was confirmed with ^{1}H- and ^{13}C-NMR spectroscopy, and potentiometric titration of the free carboxylic acid moieties in the molecule. While the parent molecule DTPA has five titratable acids, as expected, BADTTA had only three titratable acid groups (pK_a= 9.55, 4.40, 2.66), further validating the formation of two amide bonds between DTPA bisanhydride and allylamine. It has been shown that the sum of the pK_a of heavy metal chelators shows a direct correlation to the strength of heavy metal binding ability [9]. Since BADTTA is a novel molecule with unique pK_a values, the strength of binding of Gd by BADTTA was determined through competition with the metal-chelating dye xylenol orange. The binding of Gd by BADTTA was expectedly less strong than by DTPA, however the log K of 16.4 was in line with that predicted by the sum of the pK_as [9].

Synthesis And Characterization of Gadolinium-Chelating, Temperature-Responsive Hydrogel Nanoparticles

Copolymer hydrogel nanoparticles were synthesized by a reverse emulsion technique as previously described [10] (Figure 1). The hydrogel particles, as synthesized herein, are on the order of 10 to 12 nm in diameter, and circular in shape. The novelty of these small hydrogel particles comes from the use of BADTTA as cross-linker, imparting metal chelating functionality to the hydrogels, making them amenable for use as $_2$MRI contrast agents. By competing with xylenol orange for Gd, the metal loading capacity of the hydrogel particles was determined to be 32.5 μM Gd/μL particle suspension. However, this value can be tailored by varying the degree of hydrogel cross-linking. Once loaded with Gd, the ability of the hydrogels to enhance the T_1-weighted relaxivity of water protons was assessed at 20°C and found to be 12.4 Hz/mM, a 3-fold enhancement of MR contrast signal relative to an equal concentration of

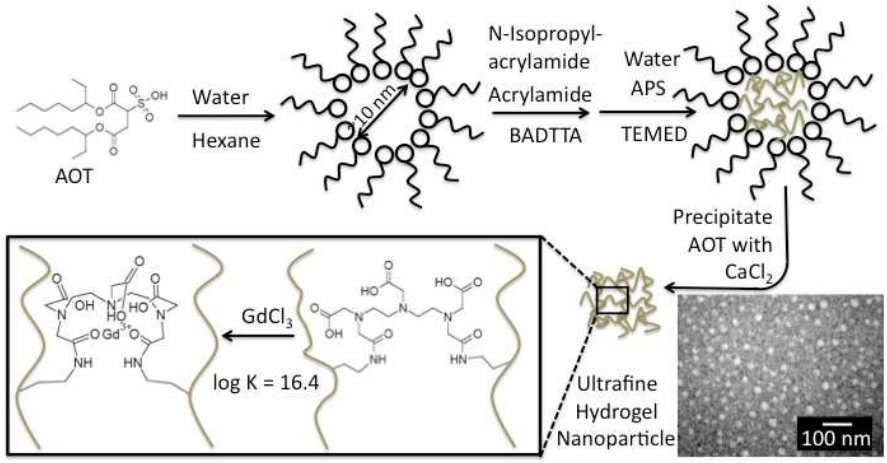


Figure 1. Synthesis of hydrogel nanoparticles with gadolinium-chelating ability. Polymerization was carried out in AOT reverse micelles. After removal of the AOT by precipitation with CaCl, hydrogel nanoparticles were able to be loaded with gadolinium (Gd). *Inset:* a transmission electron micrograph of the particles. AOT: dioctyl sulfosuccinate, APS: ammonium persulfate, TEMED: *N,N,N',N'*-Tetramethylethylenediamine.

DTPA-Gd. The mechanism of enhanced relaxivity is not yet known, but may relate to the enhanced relaxivity observed with macromolecular conjugates of Gd [11].

In order to effectively load the hydrogel-bound Gd into the hydrophobic fatty acid core of the lipid nanoparticle, the hydrogel must possess hydrophobic tendencies. However, for the Gd to alter the relaxivity of water protons and result in enhanced MR contrast, the hydrogel must also be hydrophilic once released from the lipid nanoparticle matrix. To this end, the hydrogel was synthesized from a copolymer of a temperature-sensitive monomer, N-isopropylacrylamide (NIPAM), with a non-responsive monomer acrylamide (AM). NIPAM polymers and hydrogels are well known and switch from an extended, hydrophilic state to a more compact hydrophobic state at temperatures above 32°C. When copolymerized with temperature insensitive monomers (AM) the temperature of hydrophilic-to-hydrophobic transition, called LCST, can be raised with increasing amounts of AM. When heated above its LCST, the relaxivity of the Gd-loaded hydrogel nanoparticle decreased 33% (to 8.6 Hz/mM), which could be the result of dehydration of the hydrogel core due to polymer chain collapse.

Formulation and Characterization of Gadolinim-Hydrogel-Lipid Hybrid Nanoparticles

In order to formulate lipid nanoparticles with matrices of various and controlled melting temperatures, a high melting point lipid (eicosanoate) was esterified with methanolic or ethanolic HCl as previously described [4]. The structures of esterified eciosanoates were confirmed with ^{1}H- and ^{13}C-NMR spectroscopy in $CDCl_3$. The appearance of proton signals at 3.76 ppm (s, 3H) and at 1.28 ppm (t, 3H), 4.12 ppm (q, 2H), and the shift of the carbonyl carbon signal from 180.0 ppm to 174 ppm indicate the esterification of eicosanoic acid with methanol and ethanol, respectively. Methyl- and ethyleicosanoate allowed for lipid nanoparticles to be formulated with melting temperatures of 42 and 46°C, respectively. The hot emulsion-sonication method was used to formulate the Gd-HLN [7]. Since the formulation temperature was chosen to be greater than the LCST of the hydrogel, the hydrogel nanoparticle, in its hydrophobic state, would

partition into the hydrophobic environment of the molten fatty acid, allowing for its encapsulation upon cooling and lipid solidification. The Gd-HLN were of about 109±47 nm in diameter. ICP AES was employed to determine the mass of Gd present in the formulated HLN. The loading efficiency of this formulation was found to be 40.8% w/w. The size and loading efficiency did not vary significantly with the type of fatty acid esters used.

Functional Characterization of Gadolinium-Hydrogel-Lipid Hybrid Nanoparticles for Non-Invasive Magnetic Resonance Thermometry

The encapsulation of Gd-loaded hydrogel nanoparticles within larger solid lipid nanoparticles has demonstrated an 'off-on-off' MR contrast enhancement in response to the temperature of the microenvironment of the particles. By aliquoting portions of the Gd-HLN and heating them to specific temperatures, this dual response was quantified by MR imaging. The initial 'off-on' response was shown to be governed by the melting temperature of the solid lipid matrix (Figure 2). As the melting temperature of the lipid increased, the 'off-on' transition temperature also increased. When the melting temperature of the lipid fell above the LCST of the hydrogel nanoparticle (e.g. 55°C), the 'off-on' response was less apparent. The 'on-off' response was observed at the LCST of the hydrogels. This has demonstrated that as the local temperature is raised above the melting temperature of the lipid, the hydrogel particle is released to the aqueous environment as it is in its hydrophilic conformation, enhancing MR contrast. . Since the initial 'off-on' response is due to the melting of formulated lipid nanoparticles, the thermometry imparted by this 'particle-in-a-particle' system is irreversible; a single 'off-on-off' cycle is afforded as the initial solid lipid nanoparticle will not spontaneously reform. Figure 3 illustrates the mechanism of the 'off-on-off' MR signal change as the temperature is increased to beyond the melting point of the lipid and then above the LCST of the hydrogel.

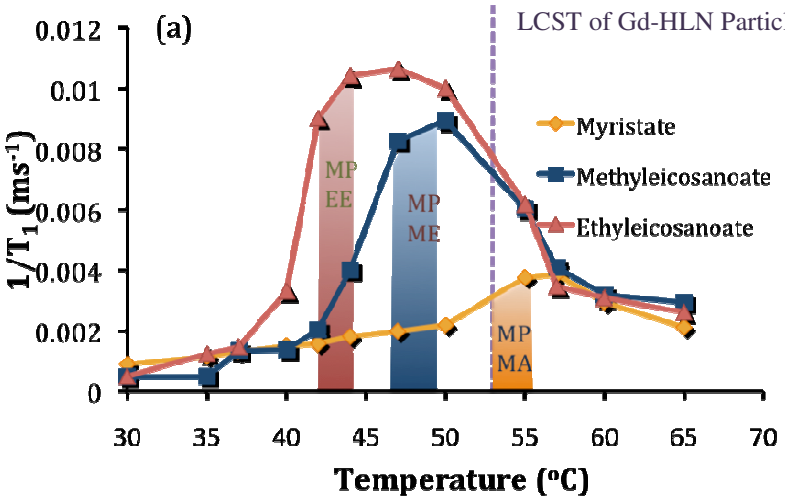


Figure 2. Tunability of the 'off-on-off' contrast enhancement of Gadolinium-Hydrogel-Lipid Hybrid Nanoparticles. Heating of aliquots of Gd-HLN prepared with various fatty acids and subsequent MR imaging revealed that the 'off-on' transition is dependent on the fatty acid melting point (columns), while the 'on-off' transition is dependent on the LCST of the hydrogel nanoparticle. Line plot indicates MR contrast signal of Gd-HLN solution. EE=ethyleicosanoate, ME=methyleicosanoate, MA=myristate. MP=melting point.

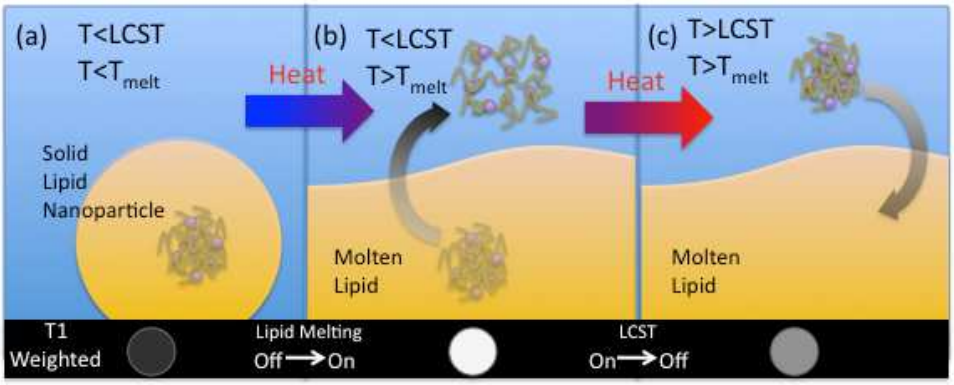


Figure 3. 'Off-on-off' transitions of Gadolinium-Hydrogel-Lipid Hybrid Nanoparticle. (a) Little contrast enhancement is observed when the gadolinium-loaded hydrogel nanoparticle is encapsulated in the solid lipid matrix. (b) Upon heating above the melting temperature (T_{melt}) of the lipid, the hydrogel leaves the molten lipid in its hydrophilic state, interacting with water to enhance the MR contrast signal. (c) Upon further heating above its LCST, the hydrogel collapses and becomes hydrophobic, partitioning back to the molten lipid to decrease the contrast enhancement.

CONCLUSIONS

Herein we present the creation of a non-invasive thermometric contrast agent for MR-guided thermotherapy that can delimit a specified temperature window. By adjusting the lipid used for the solid lipid nanoparticle carrier and the monomer ratio of the Gd-containing hydrogel ultrafine nanoparticles, the 'off-on' transition and the 'on-off' transition can be tailored, respectively. By defining a temperature window non-invasively, this contrast agent can prevent extraneous tissue damage while ensuring the attainment of therapeutic tissue temperatures during thermotherapy.

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