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Functionalization of Gold Nanoparticles (GNPSs) using the Isolate, Functionalize, and Release (ISOFURE) Methodology

David Spencer, Hariharasudhan D. Chirra, J. Zach Hilt

Introduction

The unique chemical and physical properties of gold nanoparticles (GNPs) render them as an effective tool for various biomedical applications. GNPs, as such, are inert and can be easily functionalized with a wide variety of polymers and biomolecules using gold-thiol chemistry. The various properties of GNPs are mostly size dependent, whereas their stability is primarily dependent on the surface property of the functionalized nanoparticles (such as charge, steric hindrance, etc.).

Agglomeration is a major issue in the functionalization of most nanoparticles, and can limit their use in biomedical applications. [1] Therefore, researchers employ various stabilizing methods to prevent agglomeration, such as charged capping agents and neutral steric groups. However, these processes limit the available surface area for further functionalization/loading, thereby reducing the effectiveness of the functionalized nanoparticle. In addition, the plethora of strategies available for obtaining stabilized nanoparticles have issues associated with the stabilizing agents (e.g., aggregation in the presence of salts or changes in pH, solubility in aqueous medium, exchange with serum and plasma proteins, lack of *in vivo* suitability and/or stability, etc.) that make their translation for bioapplications difficult.

In this paper, a novel strategy to perform solution based chemistries in a stabilized matrix to eliminate agglomeration issues during the intermediate steps involved in the functionalization of nanoparticles is reported. The methodology, in short, deals with the isolation, functionalization, and release (ISOFURE) of nanoparticles/nanocarriers using a composite of the nanoparticles/nanocarriers entrapped in a flexible polymer matrix. This system eradicates the need to use stabilizing reagents and eliminates the need for various purification steps usually needed during synthesis (e.g., centrifugation), which can also lead to additional irreversible agglomeration. The swelling properties of biodegradable hydrogels in different solvents and their degradation in an aqueous medium were harnessed as the ISOFURE polymer system to prevent nanoparticle agglomeration issues. Herein, two biodegradable hydrogel matrices were synthesized to make nanocomposites. First a biodegradable hydrogel matrix was synthesized in the presence of a gold nanoparticle solution, and then a biodegradable hydrogel matrix in which gold nanoparticles were precipitated in-situ was synthesized. Polymer growth over the composites was carried out using atom transfer radical polymerization (ATRP) and stable particles were released via degradation (Fig 1).

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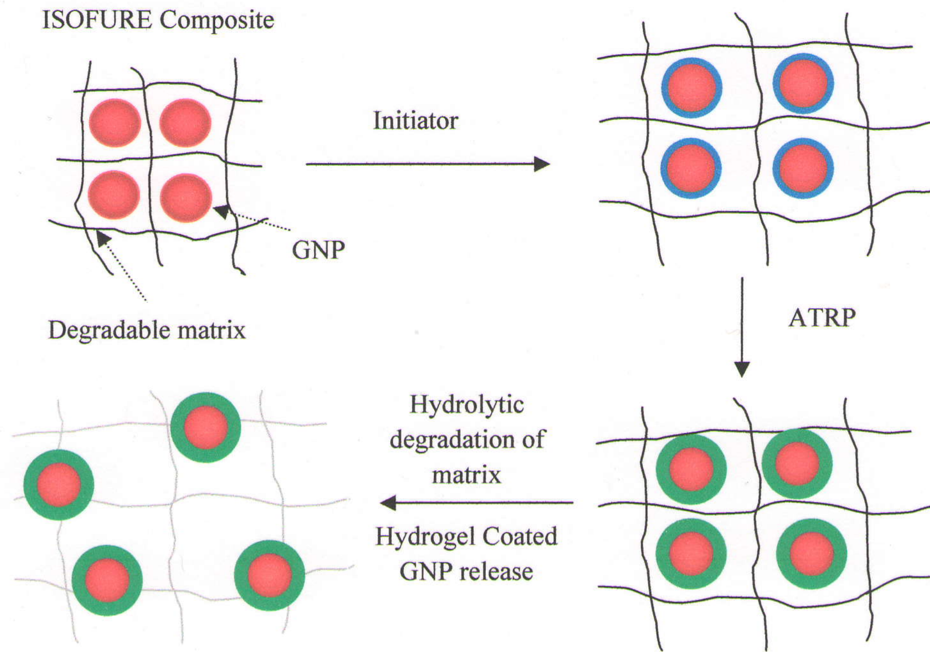

Figure 1. Schematic of the steps involved in ATRP of ISOFURE composites.

Background

Gold nanoparticles are relatively non-toxic, non oxidative/inert, easy to synthesize with core sizes ranging from 1-100nm, and are easy to functionalize using gold-thiol chemistry. Functionalized GNPs are used for a variety of biomedical applications, and have been studied extensively for various in-vivo therapeutic applications. Among these applications are diagnostic applications such as biosensors and therapeutic applications such as drug delivery and hyperthermia. Specifically, GNPs have become of interest as a platform for delivery of pharmaceuticals and biomolecules to specific targets.[2-4]

Hydrogels are 3-dimensional hydrophilic polymer systems that have a high affinity for water or physiological fluids and swell in a medium. Functional groups along the polymeric backbone can be tailored to make hydrogels respond to environmental stimuli (pH, ionic strength, temperature, pressure, etc.) and can result in significant changes in the equilibrium swelling ratio. Applications of hydrogels include contact lenses, sutures, dental materials, materials for artificial skin, linings for artificial hearts, and a matrix for tissue engineering.[5-6]

Biodegradable hydrogels are the set of hydrogels that will degrade under physiological conditions. Therefore, biodegradable hydrogels are of great interest for in vivo applications because of their potential to be used as temporary scaffold for tissue engineering and drug delivery matrices, without the need of additional steps to remove the matrix after application. [7] Biodegradable Poly(β-amino esters) (PBAEs) are polymeric networks that are characterized by hydrolytic degradation at their ester bonds. PBAEs can be tailored for desired mechanical and degradation properties, ranging from complete degradation in a few hours to limited degradation over several months. Applications of PBAEs include scaffolds for tissue regeneration and treatment of cancer by hyperthermia.[8-9]

Atom Transfer Radical Polymerization (ATRP) is of interest in research because it allows for controlled nanometer level surface initiated growth, and can be used with a wide variety of monomers on most surfaces with ease. Depending on the catalyst used, ATRP can be carried out at relatively low temperatures and is relatively tolerant of water and oxygen. ATRP can be used to coat nanoparticles with a temperature sensitive poly (N-isopropyl acrylamide) PNIPAAm hydrogel network. PNIPAAm has a lower critical solution temperature (LCST) of 32°C, and exhibits a noticeable decrease in size as temperature is increased through the LCST. The temperature response through the LCST can be followed with Dynamic Light Scattering (DLS) to verify the presence of a hydrogel coating over the surface of a nanoparticle. [2, 10]

Materials and Methods:

Materials

Chloroauric acid ($HAuCl_4$), trisodium citrate, isobutyl amine (IBA), ammonium persulfate (APS), tetramethylethylene diamine (TEMED), N-isopropyl acrylamide (NIPAAm), copper bromide, 2,2'-dipyridyl, and copper powder of size less than 425 μm were purchased from Sigma. Poly(ethylene glycol)400 diacrylate (PEG400DA) and poly(ethylene glycol) 600 dimethacrylate (PEG600DMA) were purchased from Polysciences, Inc.

Synthesis of Monodispered Gold Nanoparticles

The Turkevich method for the reduction of gold salts was used to prepare monodispered gold nanoparticles. [11] A 1mM aqueous solution of $HAuCl_4$ was boiled while stirring. To this solution, 3mM trisodium citrate dissolved in water was added to reduce the $HAuCl_4$ to produce GNPs.

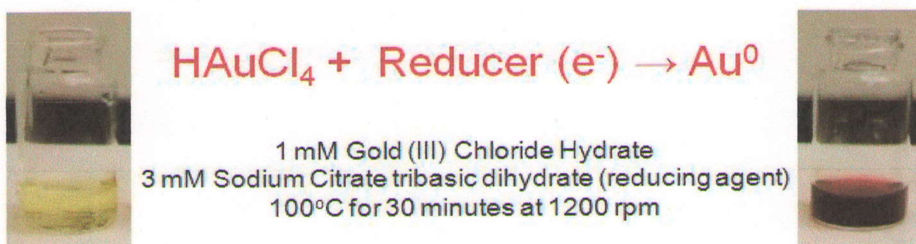

Figure 2. Schematic of the reduction of gold salt to gold nanoparticles.

Synthesis of H6 macromer

The macromer used for synthesizing the biodegradable hydrogel matrix was synthesized in accordance to a previous paper. [9] The system chosen for these studies was a 1.2:1 molar ratio of PEG400DA (represent by 'H' in the macromer library) to isobutyl amine (represented by '6' in the macromer library). The acrylate was added to a round bottom flask with a stirrer. To this, the amine was added and the mixture was reacted at 85°C for 48 hours. After 48 hours, the macromer was cooled to room temperature and stored at 4°C.

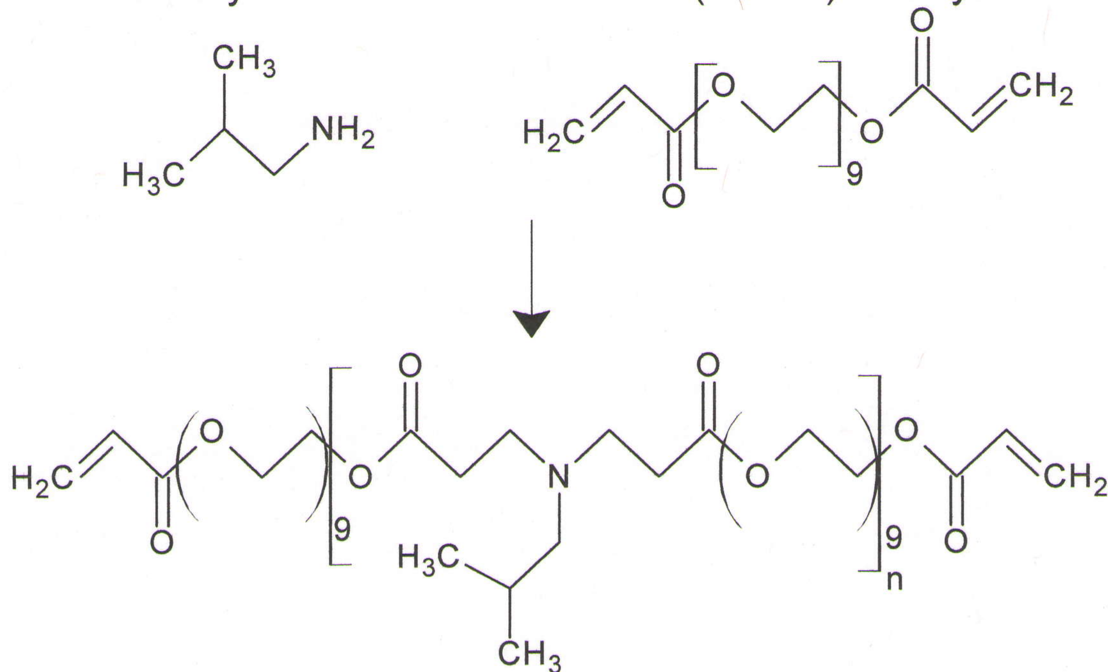

Figure 3. Schematic of the synthesis of H6 macromer.

Synthesis of H6 hydrogel

The biodegradable H6 hydrogels were synthesized using free radical redox polymerization. The reaction set-up consisted of glass plates that were separated using a 1.5mm Teflon spacer, sealed with parafilm around the edges and clipped with binder clips. The H6 macromer was mixed with 50 wt % DMSO. To this, 0.75 wt % TEMED was added and mixed using a vortex. To this solution, 1.5 wt % APS dissolved in 3 wt % de-ionized water was added and mixed using a vortex for approximately 15 seconds, and was then transferred to the glass plates using a pipette. The top of the glass plate assembly was sealed, and the free radical polymerization was allowed to take place for 24 hours at room temperature. After 24 hours, the hydrogel was removed from the glass plates, washed in DMSO for approximately 15 minutes, and vacuum dried.

Synthesis of degradable ISOFURE composite

The ISOFURE GNP composites were synthesized in a similar manner to the H6 hydrogels. Aqueous GNPs were concentrated by centrifugation at 12,500 rpm for 10 minutes using an Accuspin centrifuge (Fisher Scientific). The supernatant was removed from the concentrate, and the particles were suspended in DMSO. Using the GNPs suspended in DMSO in place of the DMSO, the ISOFURE GNP composite was synthesized in accordance to the procedure outlined for the H6 hydrogel.

Materials and Methods:

Materials

Chloroauric acid ($HAuCl_4$), trisodium citrate, isobutyl amine (IBA), ammonium persulfate (APS), tetramethylethylene diamine (TEMED), N-isopropyl acrylamide (NIPAAm), copper bromide, 2,2'-dipyridyl, and copper powder of size less than 425 μm were purchased from Sigma. Poly(ethylene glycol)400 diacrylate (PEG400DA) and poly(ethylene glycol) 600 dimethacrylate (PEG600DMA) were purchased from Polysciences, Inc.

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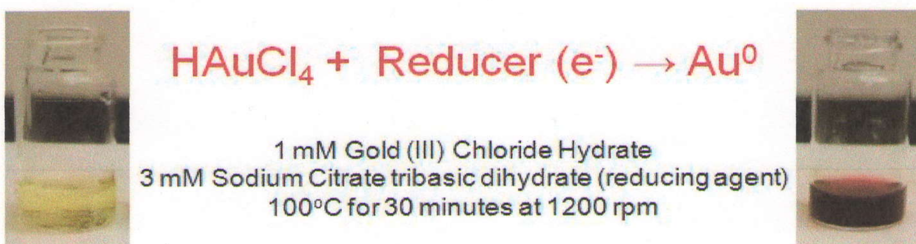

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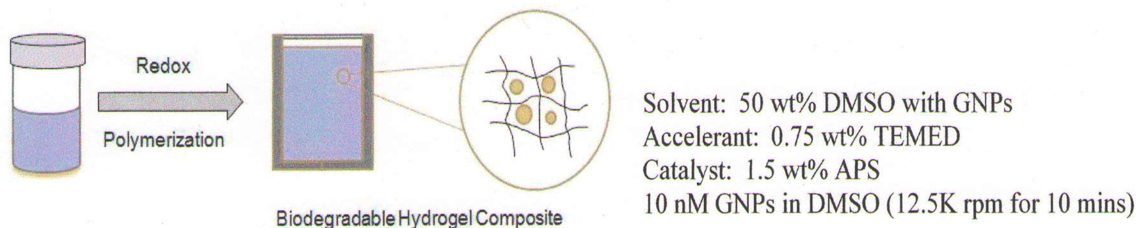

Figure 4. Schematic of the synthesis of ISOFURE composites.

Synthesis of degradable in-situ ISOFURE composite

In-situ ISOFURE GNP composites were prepared by immersing an H6 hydrogel in $HAuCl_4$ dissolved in DMSO for 12 hours. Surprisingly, reduction occurred without the addition of a reducing agent, yielding a gold nanoparticle composite hydrogel (IS-ISOFURE composite).

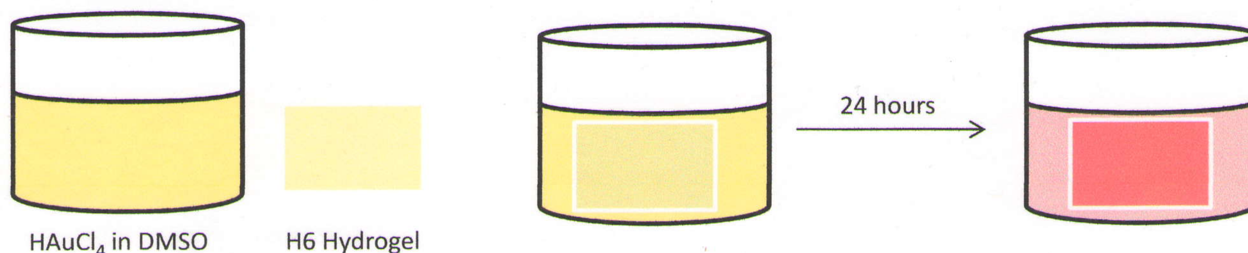

Figure 5. Schematic of the synthesis of in-situ ISOFURE composites.

ATRP of ISOFURE composites

To the ISOFURE GNP composites, a 10mM stock solution of (Br-Ini) in ethanol was added to a final concentration of 1.5mM. After 24 hours, the composites were washed in ethanol, air dried for one hour, and used for ATRP.

Surface initiated ATRP was used to prepare the temperature responsive hydrogel shells. First, nitrogen was bubbled through a 20.5mL: 0.5mL methanol to water solution for 30 minutes. To that solution, a 90:10 molar ratio of hydrogel ingredients made up of 22.5 mM of monomer N-isopropyl acrylamide (NIPAAm), 2.5 mM crosslinker poly(ethylene glycol) 600 dimethacrylate (PEG600DMA), 0.2 mM copper bromide catalyst, 0.6 mM of ligand 2,2'-dipyridyl, and approximately 0.4 mg of copper powder of size less than 425 μm were added.

After the selected reaction time, the composites were removed and washed in DMSO for 30 minutes. The washed gels were then packed into a 12,000 molecular weight cut-off dialysis membrane for degradation. Dialysis was done for 72 hours, changing the sink every 12 hours to ensure complete degradation and removal of degraded products. The solution remaining in the dialysis membrane was used for characterization.

A control sample was prepared using solution based GNPs (S-GNPs) not entrapped by a hydrogel matrix. To the S-GNPs, 10mM Br-Ini stock in ethanol was added to a final concentration of 1.5mM and mixed for 24hrs. After 24hrs, the solution was dialyzed against water to remove excess initiator or ethanol. The resulting solution was centrifuged at 10,000rpm for seven minutes, the supernantant was removed, and the concentrate was suspended in anhydrous ethanol. This solution was then used for ATRP using the same procedure as the ISOFURE composites. To stop the ATRP reaction after the allotted time air was bubbled into the mixture, and then the solution was ultra-filtered. The resulting concentrate was dialyzed against water, and the solution remaining in the dialysis tubing was used for characterization.

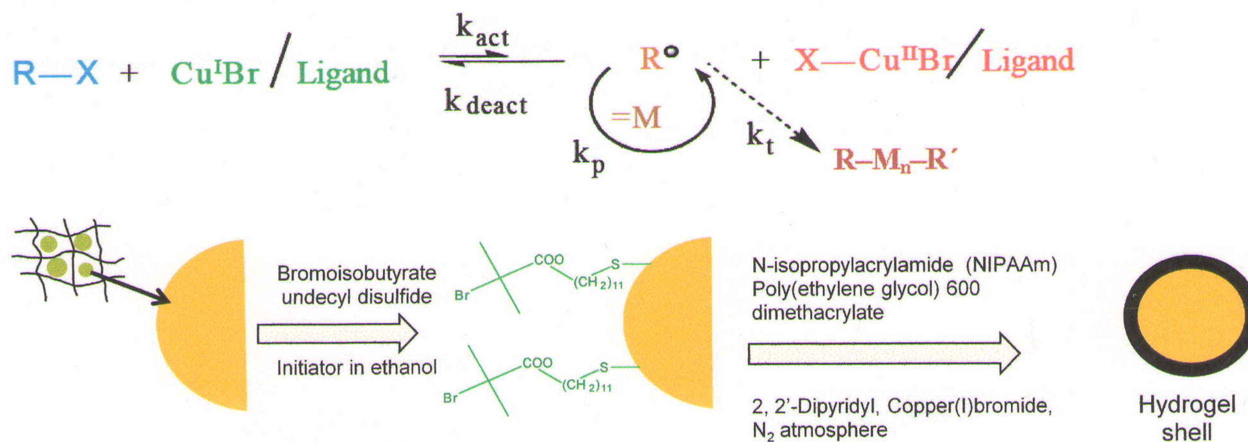

Figure 6. Schematic of ATRP reaction.

Results and Discussion

Characterization of ISOFURE system properties

Swelling and degradation studies were carried out in different solvents in order to determine the properties of the biodegradable hydrogel. First, preliminary swelling studies were conducted in DMSO, ethanol, acetone, and methanol. In the study, H6 hydrogels were cut into disks and weighed in a small petri dish. The different solvents were added to the petri dishes, and the hydrogels were allowed to swell for four hours. After four hours, excess solvent was removed and the hydrogels were weighed. The percent swelling was found using the initial and final weights of the gel.

The H6 system used for these experiments swelled the most in DMSO, followed by ethanol, and then methanol. Figure 7 shows the swelling studies conducted for DMSO and methanol. In this study, 4mL of solvent was added to 7mm H6 disks for 15min, 30min, 45min, 1hr, 1.5hrs, 2hrs, 2.5hrs, and 3hrs. After the allotted period of time, the solvent was removed, and the final mass

was taken (DMSO samples dried for 1 minute, methanol samples dried for 3 minutes). Studies were done in triplicate.

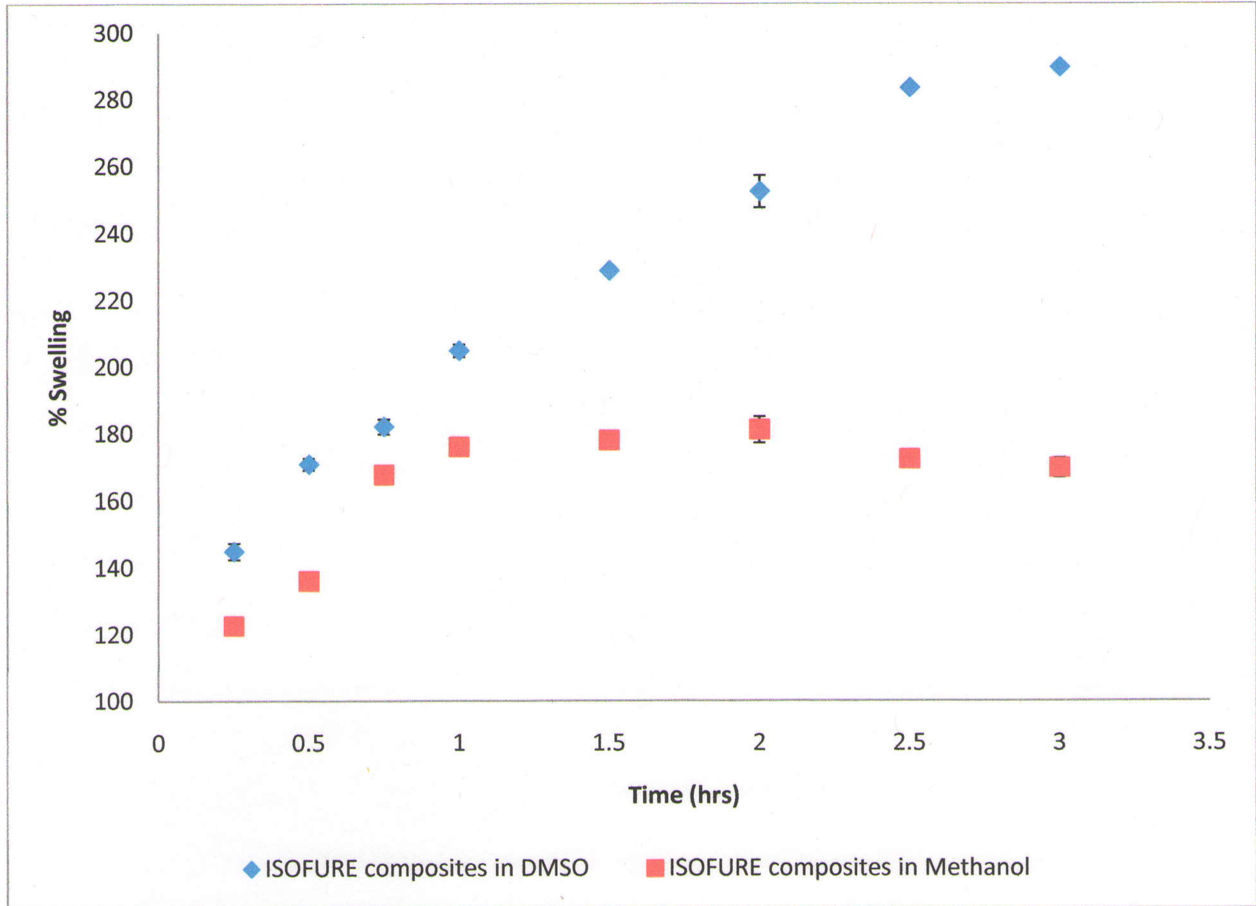

Figure 7. ISOFURE GNP composite swelling studies in DMSO and methanol

Degradation studies were conducted in a similar manner to the swelling studies. H6 hydrogels were cut into 7mm disks and massed in small petri dishes. 4mL of water was added to each dish for the following time intervals: 15min, 30min, 45min, 1hr, 1.5hrs, 2hrs, 2.5hrs, 3hrs, 3.5hrs, 4hrs, 6hrs, and 6.5hrs. After the allotted time, excess water was removed from the petri dishes and the remaining gel was frozen. The samples were allowed to freeze for 24hrs, and were dried using a freeze drier for 24-48hrs. Studies were done in triplicate.

Additional degradation studies were carried out to compare the degradation kinetics of H6 hydrogels to ISOFURE GNP composites (Fig 8). The same degradation procedure was used in this study.

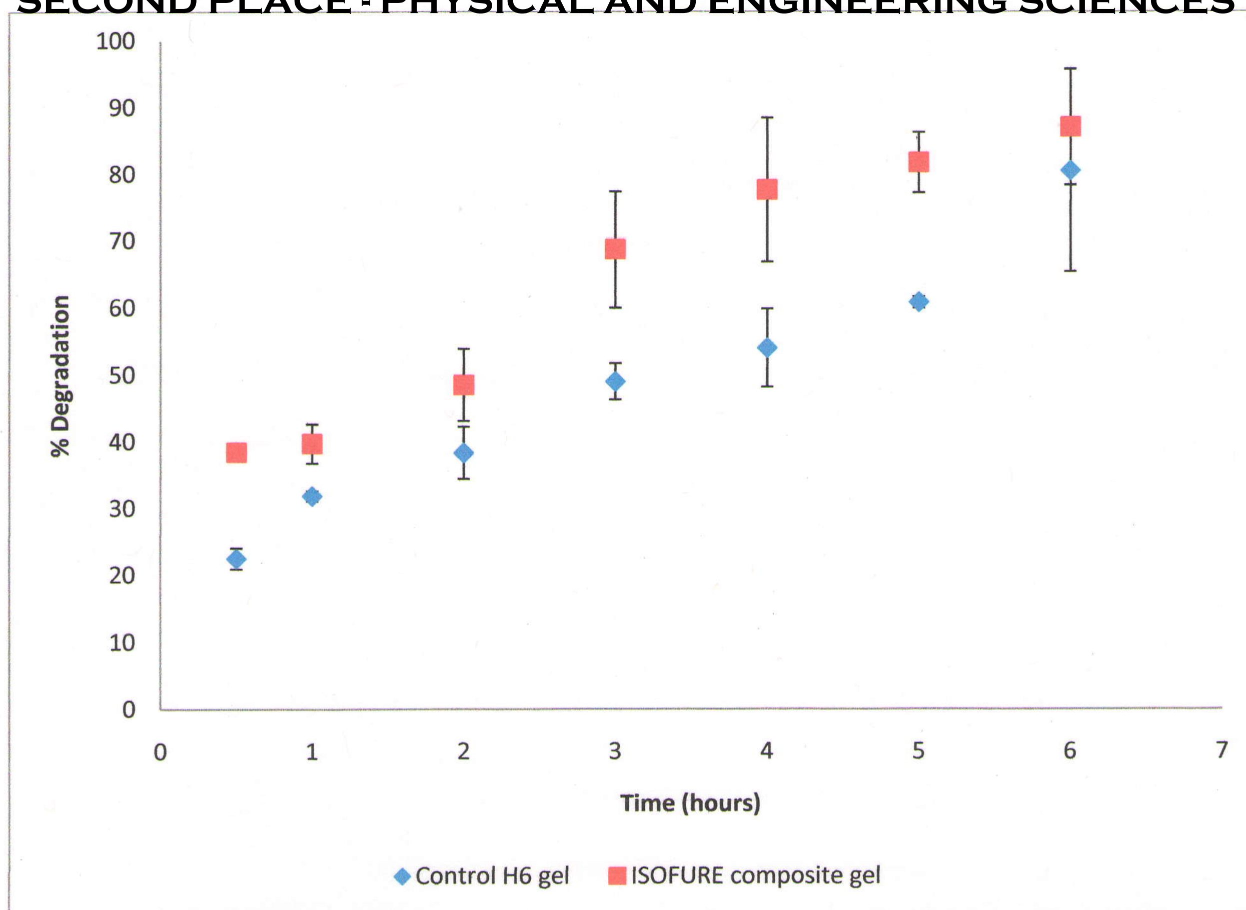

Figure 8. Control H6 hydrogel vs. ISOFURE composite degradation study

Degradation studies were also conducted at different pHs to determine if acidic or basic environments led to increased degradation rates. However, during the study significant deposits were made on the samples by the acidic and basic buffers and the results were inconclusive.

The swelling studies were used to pick the solvents for each step of the ATRP reaction. Ethanol was used for the initiator because the H6 hydrogels exhibited moderate swelling in ethanol. Methanol was used for ATRP because the H6 hydrogels swelled to a lesser extent, inhibiting particles from escaping the hydrogel matrix. The H6 hydrogels were then washed in DMSO to ensure only particles well entrapped in the matrix remained and to remove excess reagents.

The degradation studies demonstrated that the H6 system used for these studies would fully degrade in 8 hrs. To ensure complete degradation and removal of degraded products by the dialysis membrane, the hydrogels were degraded in water for 72 hours.

ATRP of ISOFURE composites

Atom transfer radical polymerization was chosen as the proof of concept reaction to show the ability of the ISOFURE methodology to functionalize gold nanoparticles without the need of stabilizing agents.

Characterization of ISOFURE composites

UV-Vis spectroscopy of the ISOFURE GNP composites during each step of ATRP is shown in Figure 9. To take the scans, the hydrogels were pressed between two glass plates and clipped on the ends. The scan of the ISOFURE GNP composites shows a surface Plasmon resonance (SPR) peak of 525nm. The initiator solution and the initiator coated ISOFURE composite did not show any peaks. The ATRP ISOFURE GNP composites showed two SPR peaks at 520nm and 673nm. The 520nm peak is consistent with the presence of gold nanoparticles. The 673nm peak shifted depending on the length of the ATRP reaction time and was a result of the growth of the hydrogel over the surface of the particle and not particle aggregation.

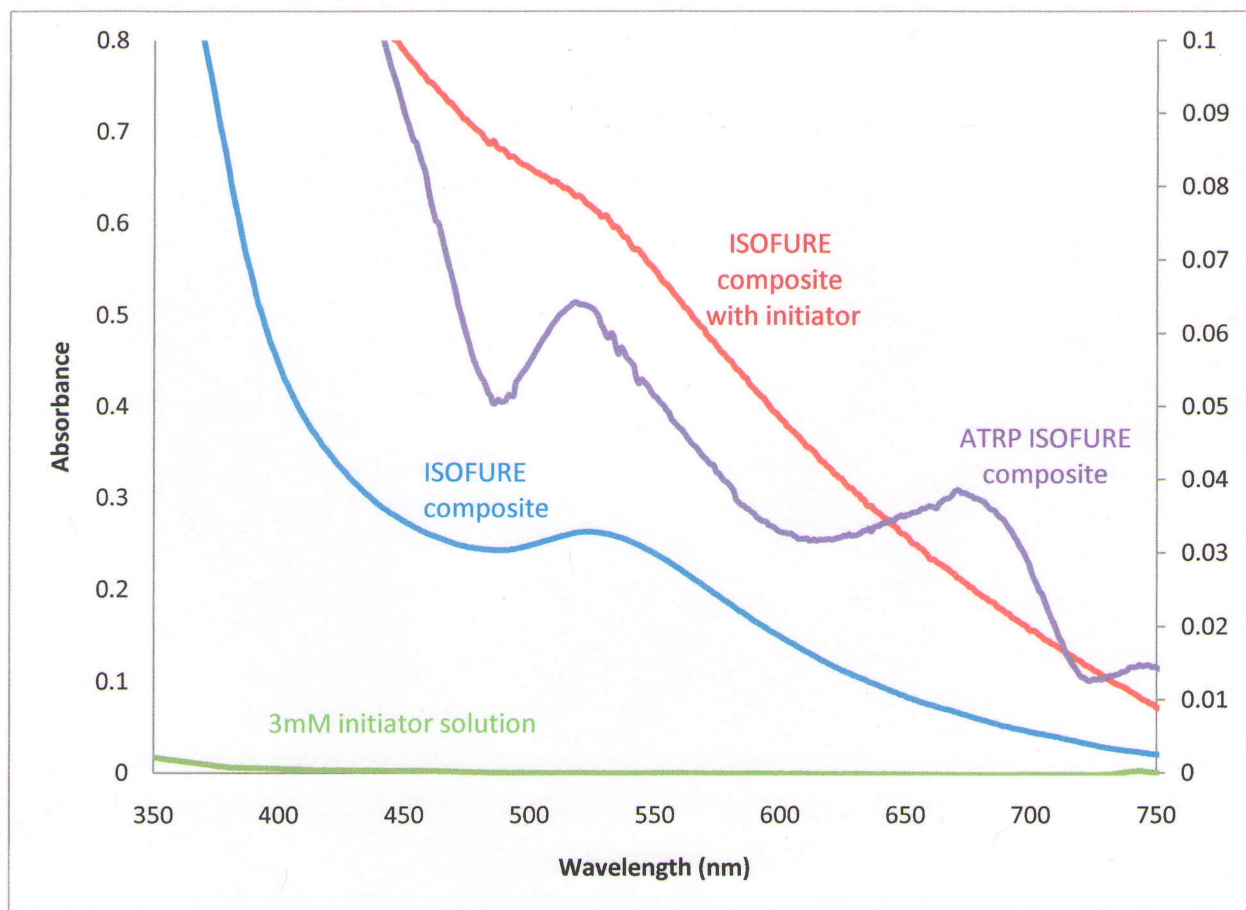

Figure 9. UV-Vis scan of each step during ATRP.

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Temperature response behavior of ATRP ISOFURE GNP composites

ATRP was carried out on the ISOFURE GNP composites for 8, 16, and 24hr periods. The temperature response profiles for the three time periods were followed with DLS from 20°C to 60°C. At the LCST (32°C), there was a notable decrease in particle diameter, representative of the collapse of the ATRP grown hydrogel shell on the surface of the gold nanoparticles. The temperature response profiles for the three reaction times were similar, with particle size increasing with reaction time (Fig 10).

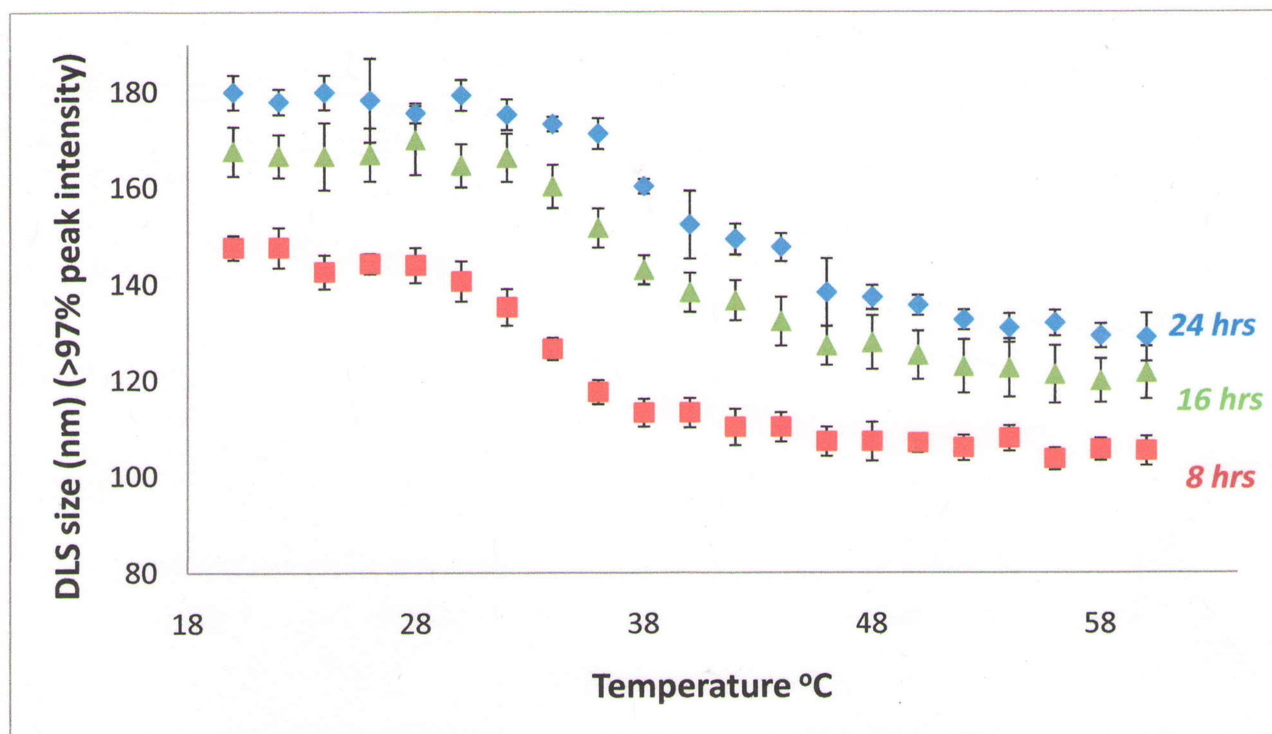

Figure 10. Temperature Response of 8, 16, and 24hr H6 composite ATRP samples

The 24hr ATRP S-GNPs had a very similar temperature response profile to the 24hr ATRP ISOFURE GNPs (Fig 11), while no temperature response was shown for initiator coated particles. The ISOFURE system particles were smaller than the solution based particles but it is likely that some agglomeration was still present.

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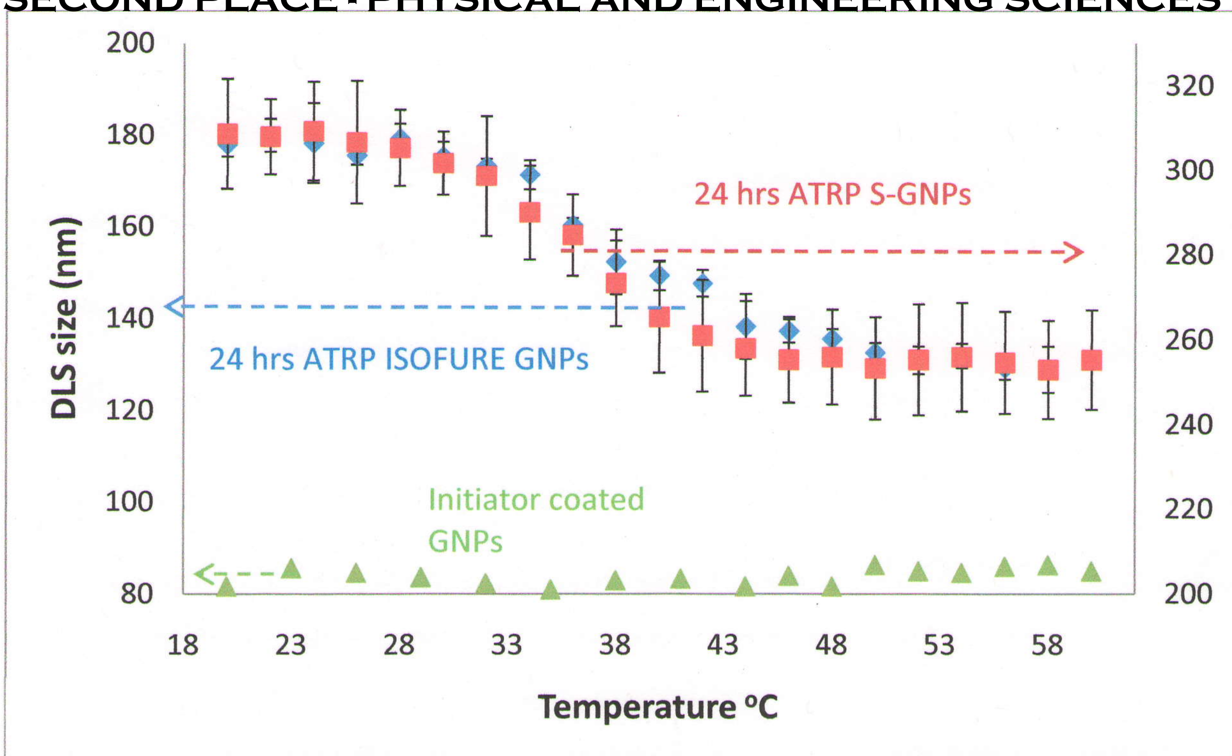

Figure 11. Comparison of ATRP ISOFURE GNPs vs. ATRP S-GNPs

During the temperature response analysis, a difference in the stability of the particles was noticed. In order to quantify the stability of the particles in solution, UV-Vis analysis was used to measure the absorbance of the particles over a 12 hr time period at 530nm (SPR peak for gold). As shown in Figure 12, the ISOFURE GNPs remained stable over the 12 hour period of time, while the S-GNPs settled. The matrix present in the ISOFURE composite system inhibits interaction between particles, and thereby enhances the stability of the ATRP particles.

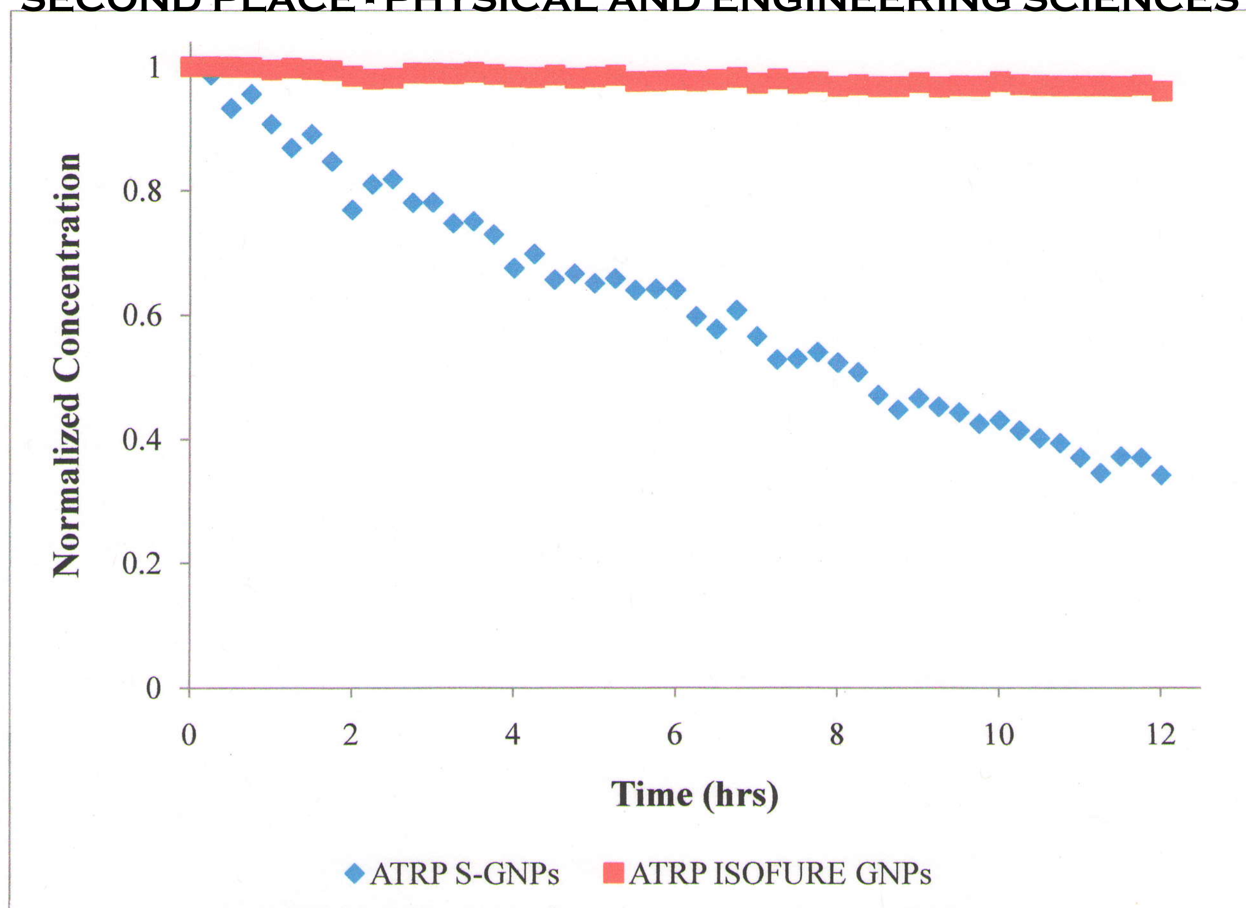

Figure 12. Stability of ATRP S-GNPs vs. ATRP ISOFURE GNPs

In-situ ISOFURE ATRP

ATRP was carried out over IS-ISOFURE GNP composites and ISOFURE GNP composites for 6, 12, and 18 hours so that a direct comparison between the two methodologies could be observed. The temperature response profiles for the two systems were similar, with the ISOFURE GNPs yielding larger particles than the IS ISOFURE GNPs (Fig 13-15). This result supports the hypothesis that some agglomeration occurs during the centrifugation of aqueous GNPs to their suspension in DMSO.

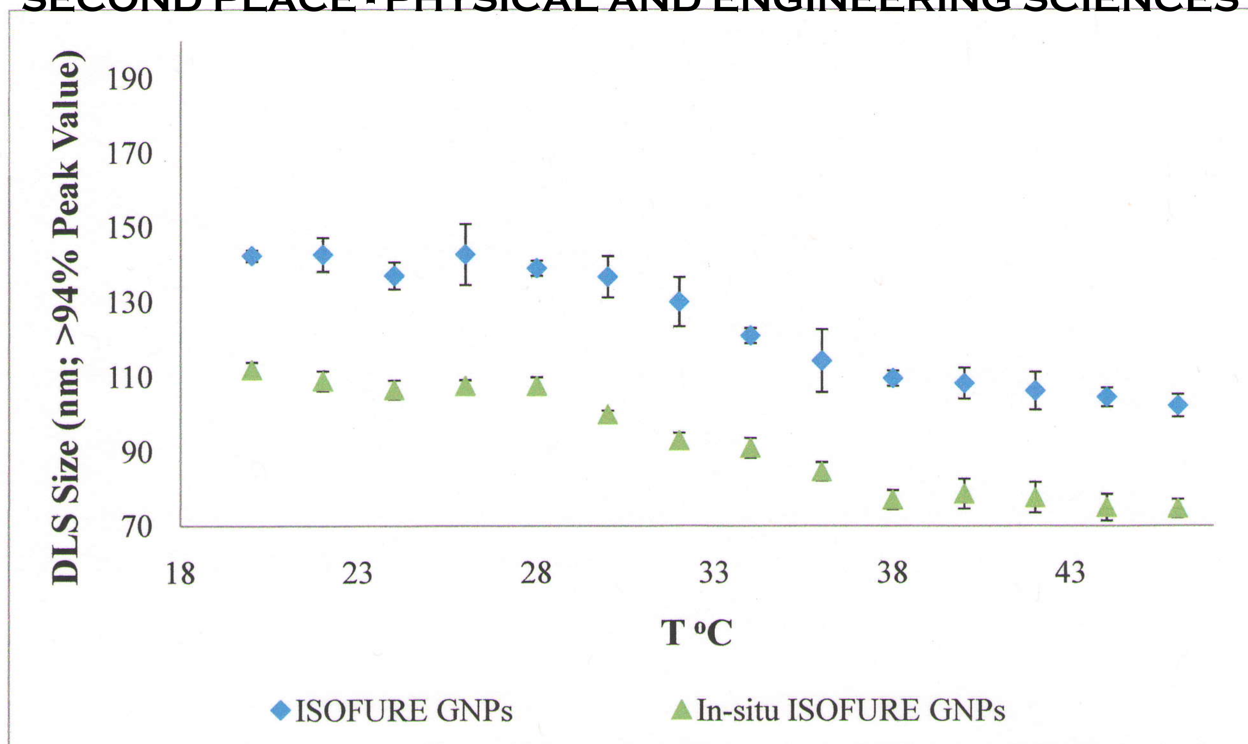

Figure 13. 6 hr temperature response

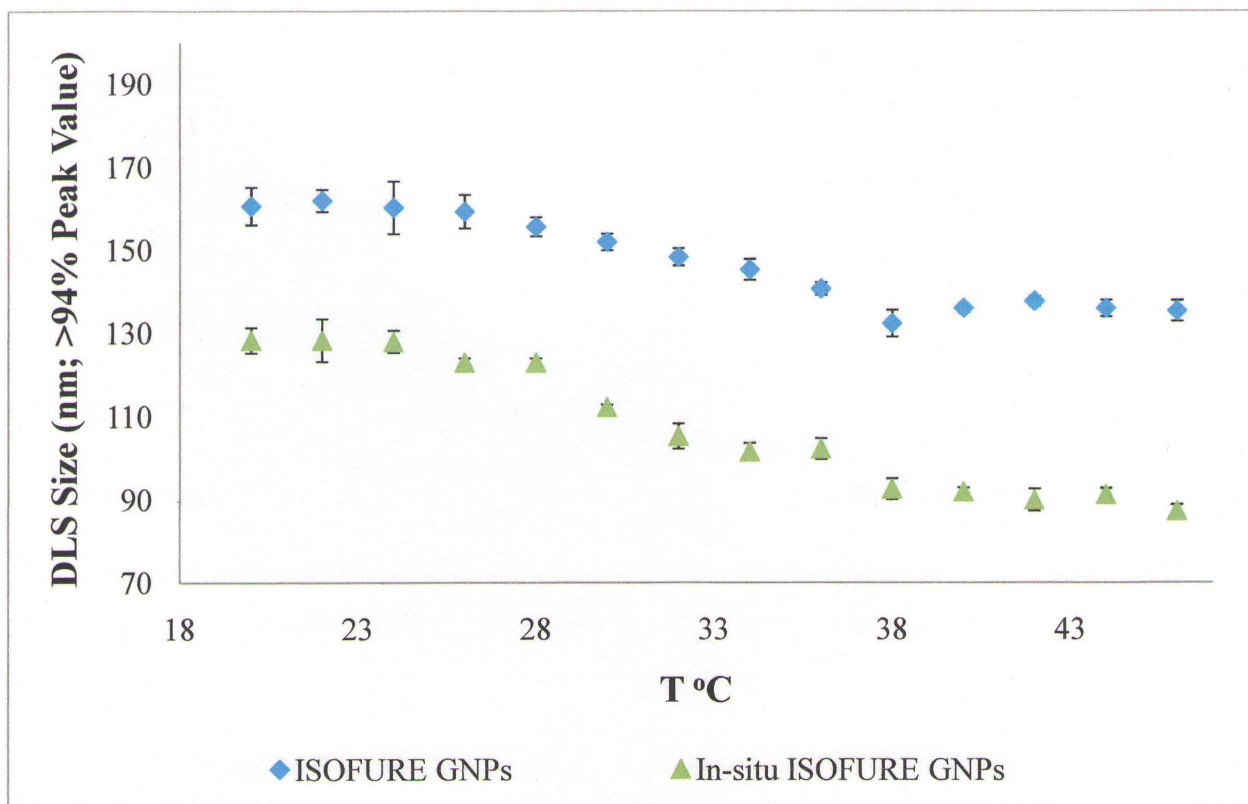

Figure 14. 12 hr temperature response

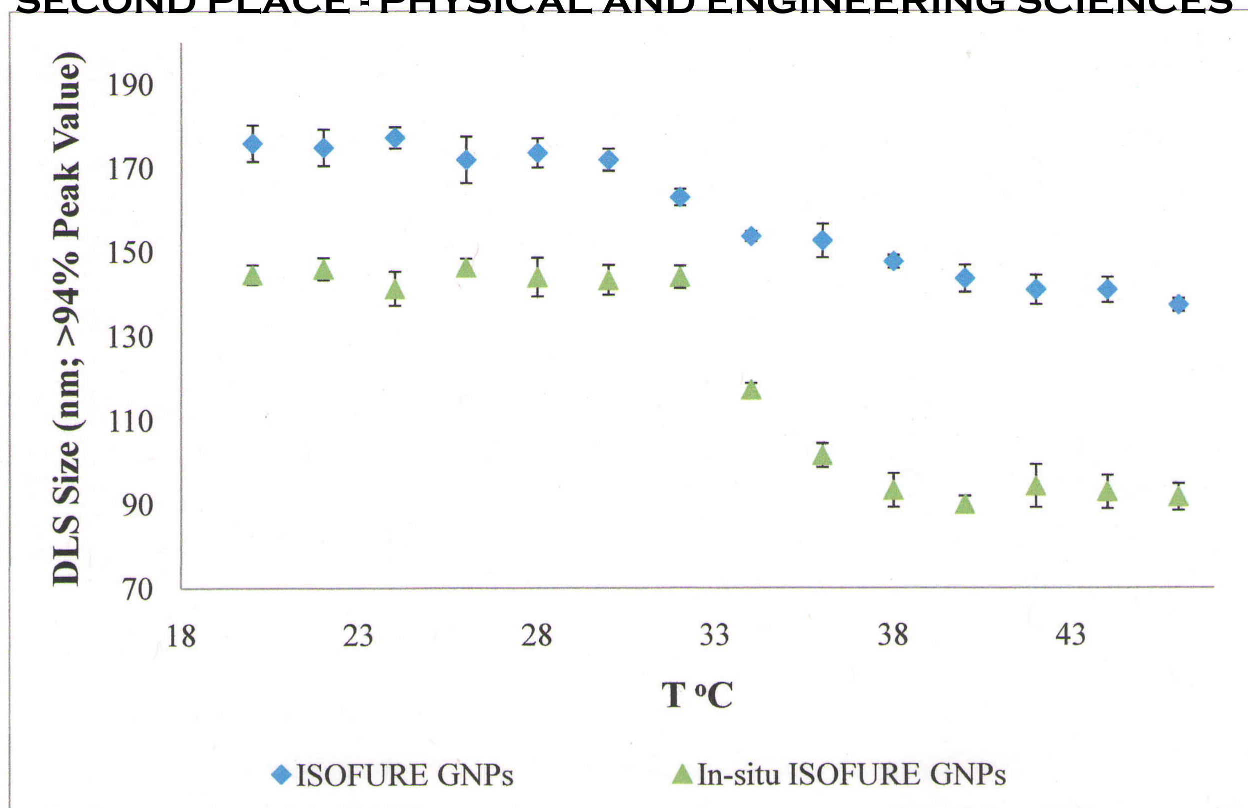

Figure 15. 18 hr temperature response

Conclusion:

Herein, a proof of concept for using ISOFURE systems to increase stability and loading of nanoparticles was demonstrated. GNP encompassed degradable PBAE hydrogels were synthesized by either adding GNPs to the redox polymerizing macromer solution or by in-situ precipitation of GNPs inside the hydrogel matrix. UV-Vis spectroscopy demonstrated the presence of stable in-situ precipitated particles. ATRP was successfully carried out over different GNPs and DLS of the particles at increasing temperatures showed a temperature response. Aging studies showed that the ISOFURE system yielded higher stability than that of the solution based GNPs.

Future Work

Future work includes demonstrating the enhanced loading of nanoparticles using a biomolecular reaction, and extending the ISOFURE methodology to iron oxide nanoparticles. Additional future work includes finding a PBAE hydrogel system that will further reduce agglomeration.

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