

5R21EB006499-02 Final Status Report: “Cancer Targeting with Multi-Responsive Core/Shell Microgels”

A. Specific Aims

The original grant proposal contained two specific aims. These aims remain unchanged at this point in the program. The overarching goals of the program relate to the development of multi-responsive/functional hydrogel nanoparticles for cancer cell targeting. This is to be accomplished in the following specific aims:

Aim 1: To utilize doxorubicin-loaded, folate-modified, degradable core/shell microgels to target cells *in vitro* to specifically evade P-glycoprotein based multi-drug resistance. This aim will demonstrate that the core/shell microgel construct is effective in cellular targeting, that the designed degradation pathways enable cytosolic delivery of an anti-tumor agent (doxorubicin), and that the construct enables the evasion of multi-drug resistance that arises from P-glycoprotein overexpression.

Aim 2: To develop multi-functional core/shell microgels that “display” folate in response to specific matrix metalloproteinase (MMP) activity. Taking advantage of elevated MMP activity proximal to tumor growth, these microgels will have “stealth” properties that allow for folate display, cell surface binding, and endocytosis only after exposure to elevated MMP activity. This will further reduce the potential for binding and uptake of the vehicles by healthy tissues.

Entry	Core composition	Shell composition	Total size (radius)
1	pNIPAm-AFA	pNIPAM-APMA	52 nm
2	pNIPAm-AFA	pNIPAM-PEG-APMA	49 nm
3	pNIPMAM-AFA	pNIPMAM-APMA	55 nm
4	pNIPMAM-AFA	pNIPMAM-PEG-APMA	55 nm

B. Studies and Results

(1) Core/shell nanogel synthesis

In order to investigate the impact of different nanogels on cellular uptake, we have optimized the syntheses of ~100-nm diameter core/shell nanogels composed of various polymers and characterized them by light scattering techniques. The table below summarizes the different particles prepared, where pNIPAm is poly(*N*-isopropylacrylamide), pNIPMAM is poly(*N*-isopropylmethacrylamide), AFA is acryloylfluorescein (fluorescent co-monomer), PEG is a 575MW poly(ethyleneglycol diacrylate) cross-linker, and

APMA is aminopropyl methacrylamide (amine sidechain co-monomer). All nanogels have subsequently been modified with folic acid for cell targeting studies. Folic acid loading has been quantified spectrophotometrically.

As described in the original proposal, the PEG cross-linkers are being added to resist non-specific adsorption of protein. These particles will allow for *in vitro* comparisons to be made between the two types of particles.

(2) Degradable cross-linkers

The synthesis of degradable particles was a key aspect of the original proposal. A series of ketal-based cross-linkers were originally proposed, but it was found early on that these cross-linkers were not well behaved in our core/shell nanogel syntheses. We have subsequently focused on two main cross-linker types. The first is *N*-(methacryloyloxy)methacrylamide, which is shown in **Figure 1**. Whereas the literature suggests that the exotic “Lossen Rearrangement” is associated with its cleavage, we have recently shown that the cross-linker erodes hydrolytically. We have prepared the cross-linker in our labs, and have prepared both nanogels and macroscopic gels containing this cross-linker. The nanogel erosion data are extremely encouraging.

N-(methacryloyloxy)methacrylamide

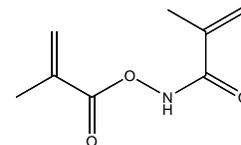


Figure 1. A novel degradable cross-linker, which decomposes via the Lossen rearrangement.

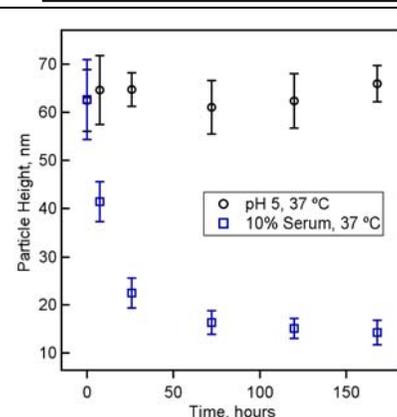


Figure 2. DMHA cross-linked nanogel degradation as determined by AFM imaging of nanogel films as a function of solution conditions (pH 5 vs. serum containing culture medium).

Atomic force microscopy of substrate-supported, degradable nanogels show erosion rates (particle height decreases) commensurate with typical circulation/deposition times for nanoparticles (**Figure 2**). Importantly, we have been able to incorporate this cross-linker into the appropriate core/shell architectures; a scheme of the synthetic method is shown in **Figure 3**.

(3) Orthogonal chemoligation on nanogels

We have developed a simple, 1-pot method for the synthesis of azido-functionalized microgels that is completely compatible with all of the core/shell syntheses developed by the Lyon group over the last decade. The microgel synthetic scheme is shown in **Figure 4**. As a preliminary test of this particle's utility in 'click' chemistries, we performed Cu-catalyzed 3-2 dipolar cycloaddition between the microgel-localized azido groups and the alkyne moiety on propargylfluorescein thiourea. Following purification by centrifugation and resuspension in water, a dilute solution of the particles was dried on a coverslip for microscopic observation. **Figure 4** shows a representative fluorescence micrograph of these particles illustrating the very bright, microgel localized fluorescence. This is clear indication of the success of the coupling reaction; we are currently exploring the efficiency of this reaction in the coupling of alkyne-modified peptides.

(4) Cellular uptake studies

Finally, we have explored the uptake of non-degradable, folate-modified, core/shell nanogels in various KB cell lines. Preliminary results indicate that the pNIPAM-based nanogels perform somewhat better in vitro than the pNIPAM-based counterparts. This is largely due to some aggregation of the latter. In preliminary studies, pNIPAM-based nanogels functionalized with folic acid show ~2-fold greater uptake than the cationic, folate(-) control particles. We are currently optimizing the syntheses with respect to folate loading so as to increase the degree of differential uptake.

We have also investigated the generality of the construct by using a peptide-based targeting approach. We enlisted the assistance of Prof. John McDonald's group here at GT for the sake of expedience, and because of their experience with the 'YSA' peptide (YSAYPDSVPMMS, a peptide-mimetic of ephrinA1).

In this architecture, the fluorescent core enables tracking and imaging via confocal fluorescence microscopy, whereas the YSA-labeled shell enables targeting to the EphA2 receptor. The EphA2 receptor is overexpressed

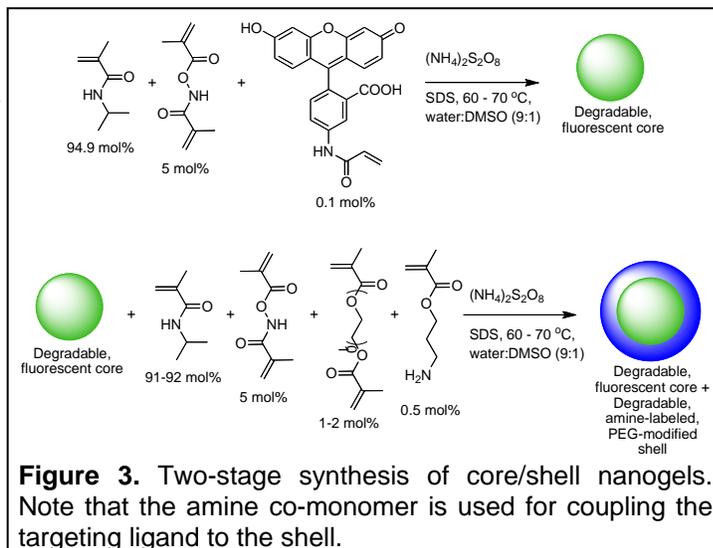


Figure 3. Two-stage synthesis of core/shell nanogels. Note that the amine co-monomer is used for coupling the targeting ligand to the shell.

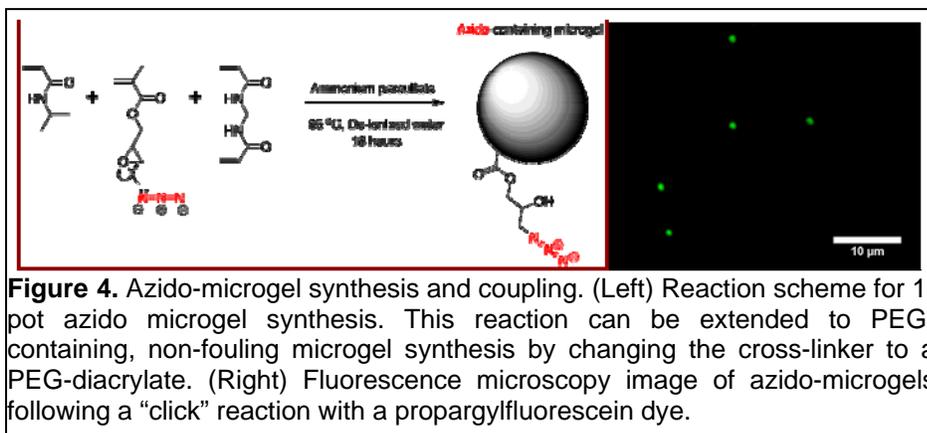


Figure 4. Azido-microgel synthesis and coupling. (Left) Reaction scheme for 1-pot azido microgel synthesis. This reaction can be extended to PEG-containing, non-fouling microgel synthesis by changing the cross-linker to a PEG-diacrylate. (Right) Fluorescence microscopy image of azido-microgels following a "click" reaction with a propargylfluorescein dye.

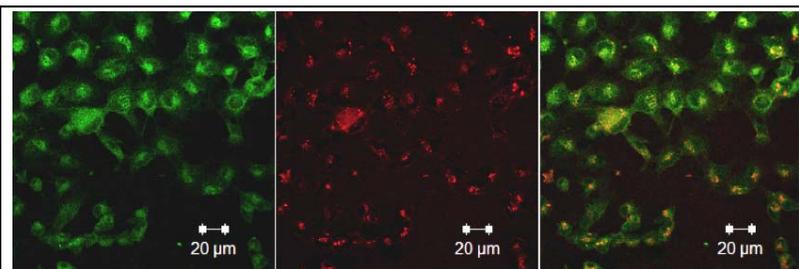


Figure 5. Confocal microscopy images of Hey cells following a 4-hour incubation with YSA-targeted, fluorescent pNIPAM core/shell nanogels loaded with siRNA. (left) Particle fluorescence, (middle) siRNA fluorescence, (right) overlaid images.

on the surface of many ovarian carcinomas and also in neovasculature associated with tumors, making it very attractive for ovarian tumor targeting applications. To evaluate the efficacy of peptide-mediated targeting, we evaluated targeting to Hey cells (an ovarian cancer cell line). The Hey cells were incubated with either unconjugated nanogels or nanogels conjugated with the YSA peptide. The peptide-conjugated nanogels were preferentially taken up by the cells over non-targeted nanogels, as observed by fluorescence microscopy. The McDonald group is also very interested in the therapeutic potential of siRNA in cancer delivery. We used our nanogels to load siRNA and deliver it to Hey cells via peptide targeting. The excellent preliminary results shown in **Figure 5** illustrate the potential of nanogels for targeted delivery of therapeutics.

C. Significance

This work is significant in multiple ways. From the basic science perspective, it represents by far the most complex hydrogel nanoparticle architectures prepared to date. This work is cutting-edge with respect to the topological design of nanoparticles for biomedical applications. From the perspective of human health, these materials represent the next step in nanotechnology's contributions to healthcare. While targeted drug delivery vehicles have existed (in the laboratory) for some time, rationally designed architectures such as these should make a significant impact as the field moves forward to clinical relevance.

Publications.

1. Singh, N.; Lyon, L. A., Synthesis of multifunctional nanogels using a protected macromonomer approach. *Colloid Polym. Sci.* **2008**, *286* (8-9), 1061-1069.
2. Blackburn, W. H.; Lyon, L. A., Size-controlled synthesis of monodisperse core/shell nanogels. *Colloid Polym. Sci.* **2008**, *286* (5), 563-569.

Three additional papers are currently in preparation.