ACCOUNTS of chemical research

Multifunctional Nanogels for siRNA Delivery

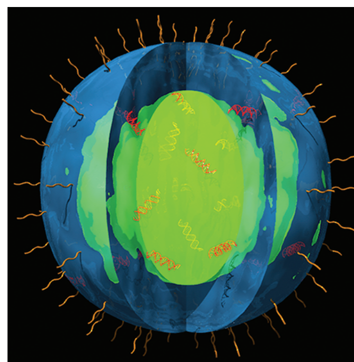
MICHAEL H. SMITH AND L. ANDREW LYON*

School of Chemistry and Biochemistry and the Petit Institute for Bioengineering & Bioscience, Georgia Institute of Technology, Atlanta, Georgia 30332-0400, United States

RECEIVED ON AUGUST 22, 2011

CONSPECTUS

The application of RNA interference to treat disease is an important yet challenging concept in modern medicine. In particular, small interfering RNA (siRNA) have shown tremendous promise in the treatment of cancer. However, siRNA show poor pharmacological properties, which presents a major hurdle for effective disease treatment especially through intravenous delivery routes. In response to these shortcomings, a variety of nanoparticle carriers have emerged, which are designed to encapsulate, protect, and transport siRNA into diseased cells. To be effective as carrier vehicles, nanoparticles must overcome a series of biological hurdles throughout the course of delivery. As a result, one promising approach to siRNA carriers is dynamic, versatile nanoparticles that can perform several in vivo functions.



Over the last several years, our research group has investigated hydrogel nanoparticles (nanogels) as candidate delivery vehicles for therapeutics, including siRNA. Throughout the course of our research, we have developed higher order architectures composed entirely of hydrogel components, where several different hydrogel chemistries may be isolated in unique compartments of a single construct. In this Account, we summarize a subset of our experiences in the design and application of nanogels in the context of drug delivery, summarizing the relevant characteristics for these materials as delivery vehicles for siRNA.

Through the layering of multiple, orthogonal chemistries in a nanogel structure, we can impart multiple functions to the materials. We consider nanogels as a platform technology, where each functional element of the particle may be independently tuned to optimize the particle for the desired application. For instance, we can modify the shell compartment of a vehicle for cell-specific targeting or evasion of the innate immune system, whereas other compartments may incorporate fluorescent probes or regulate the encapsulation and release of macromolecular therapeutics.

Proof-of-principle experiments have demonstrated the utility of multifunctional nanogels. For example, using a simple core/shell nanogel architecture, we have recently reported the delivery of siRNA to chemosensitize drug resistant ovarian cancer cells. Ongoing efforts have resulted in several advanced hydrogel structures, including biodegradable nanogels and multicompartment spheres. In parallel, our research group has studied other properties of the nanogels, including their behavior in confined environments and their ability to translocate through small pores.

Introduction

Over the past several years, our group has focused attention on the topic of hydrogel particles (microgels and nanogels). Although the first synthesis of aqueous microgels was reported decades ago, research continues to reveal intriguing properties of these materials. We have explored a number of advanced particles, including tumor-targeted nanogels,[1−3] bioresponsive microlenses,[4] and nonfouling biomedical device coatings.[5] Among these applications, we have recently investigated nanogels as carriers for RNA interference (RNAi) in cancer therapy. This Account reviews our laboratory's experience in nanogel-based drug delivery vectors, discussing key properties that enabled previous efforts in small interfering RNA (siRNA) delivery to ovarian cancer cells. This Account also discusses key particle characteristics and advanced architectures that will motivate future drug delivery efforts.

Nanogels for siRNA Delivery

RNA interference by small interfering RNA (siRNA) is an enabling technology for post-transcriptional gene silencing. Using siRNA, one can potently knock down genes in a sequence-specific fashion. As a result, gene silencing via siRNA has become a powerful research tool for investigations

Published on the Web 12/19/2011 www.pubs.acs.org/accounts
10.1021/ar200216f © 2011 American Chemical Society

of gene function and in the development of new disease therapies,[6] with a number of siRNA-based therapeutics having already made progress in clinical trials.[7] However, the delivery of siRNAs into cells remains as one of the greatest challenges for therapy development. SiRNAs are negatively charged, hydrophilic molecules that are unable to penetrate cell membranes on their own, are rapidly degraded by endogenous enzymes, and are recognized by the innate immune system. Thus, siRNAs require a delivery vehicle to accomplish efficient and effective transfection, with several technologies having emerged to enhance tissue-targeted delivery.[8] Delivery may be accomplished through two routes of administration: 1) localized siRNA delivery, where therapies are administered directly into the tissue of interest, or 2) systemic delivery, where formulations are administered into the bloodstream. Although localized delivery has the benefit of enhancing bioavailability and reducing adverse effects, many tissues can only be reached through the systemic route. Intravenous delivery is challenging, usually involving circulating nanoparticle carriers that must protect siRNA from degradation by serum nucleases, resist recognition by the immune system, and show tissue-specific uptake via cell targeting.

In the context of cancer therapy, several colloidal drug carriers have been proposed to improve siRNA tumor localization and bioavailability, while reducing toxicity.[9] In particular, we and others have recognized nanogels as a promising new class of drug delivery vehicles.[10−12] Composed of hydrophilic polymer chains that are lightly cross-linked together, nanogels have a high degree of porosity that permits the encapsulation of macromolecular therapeutics, while the high water content suggests biocompatibility. The dimensions of the nanogels may be tuned to the size range appropriate for passive tumor targeting via enhanced permeability and retention (EPR),[13] whereas their surfaces may be functionalized with targeting molecules for cell-specific uptake.[14] Notably, the importance of mechanical flexibility in biomaterials has also been emphasized in recent years; the mechanical softness of nanogels could positively impact cellular uptake and biodistribution.[15−17] In addition to these features, nanogels may also be composed of stimuli-responsive polymers, yielding colloids that are responsive (e.g., by swelling/deswelling transitions) to their local environment. For drug delivery applications, such changes in hydrogel swelling may be an effective means to drive the release of internalized solutes.[18] Additionally, reorganization of the polymer network often results in dramatic changes in the surface chemistry or energy,[19] which may be useful feature for cellular therapy.[3] A variety of stimuli-responsive particles have already been demonstrated (e.g to pH, light, macromolecules) with perhaps the most commonly investigated stimulus being temperature.[20]

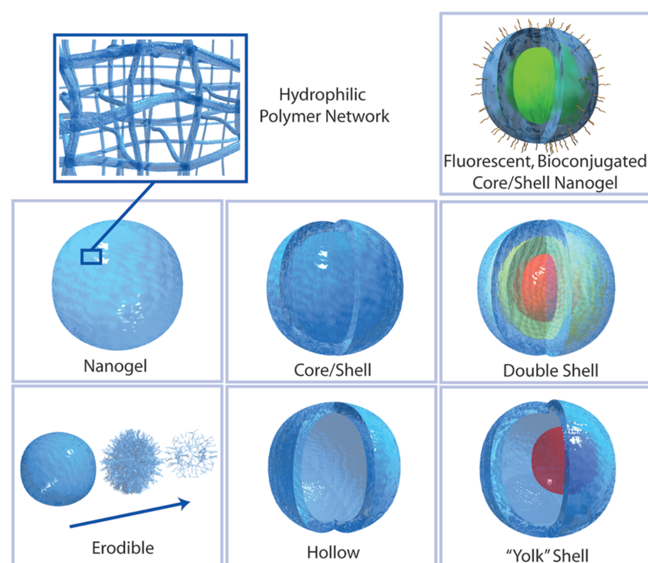


FIGURE 1. Summary of higher-order hydrogel particles reported in recent years.

Multicompartment Particles

At the onset of our research program in 1999, others had demonstrated the utility of adding hydrogel shells to "hard" nanoparticles (e.g., silica[21] and gold[22]). The resultant particles showed properties governed by the chemistry of the added polymer. Through hydrogel shell addition, chemical functionalities could be localized in the particle periphery,[23] stimuli-responsive characteristics could be imparted,[24] or the stability of the particles could be enhanced.[25] Inspired by those results, our research group was the first to explore the synthesis of core/shell particle architectures composed entirely of hydrogel.[26] Such core/shell microgels showed intriguing properties, wherein different stimuli-responsive components and chemical functionalities could be imparted into different compartments of a single particle. Since our first report of core/shell microgels in 2000, we have investigated a number of higher order architectures, each demonstrating interesting features in the context of drug delivery. Those particles include multi-shelled structures,[27] erodible particles,[28−30] hollow microgel capsules,[31] and "yolk" shell spheres (Figure 1).[27]

Although we are actively investigating a number of these architectures for general drug encapsulation and release properties, we have made progress toward siRNA delivery with core/shell nanogels in particular. In addition to core/shell nanogels, we report recently elucidated properties of nanogels

that we consider enabling for drug delivery applications, including erodible particles and nanogels that can pass through small orifices, such as those found in biological tissues.

Microgel and Nanogel Synthesis

To synthesize microgels, we typically employ free radical precipitation polymerization. This method exploits the thermally triggered collapse of growing polymer chains to self-assemble the microgel,[26] often resulting in narrow particle size distributions at high yield. For instance, poly (*N*-isopropylacrylamide) (pNIPAm) undergoes an abrupt coil-to-globule transition at ~31 °C, defined as the lower critical solution temperature (LCST) of the polymer.[32] Typical syntheses are performed by dissolving the monomer (e.g., NIPAm), other comonomers, and a cross-linking agent (e.g., *N,N*′-methylenebis(acrylamide), BIS) in water. The reactants are subsequently heated to a temperature between 60 and 70 °C (above the LCST). After purging the solution with N_2, the polymerization is initiated by addition of initiator (e.g., ammonium or potassium persulfate, APS/KPS). At these reaction temperatures, persulfates thermally decompose to form sulfate radicals that initiate polymerization. The sulfate radicals attack the NIPAm monomer, which then undergoes chain growth via radical propagation. At a critical chain length (~10 monomer units), the polymer collapses to form a globular particle. The particles then grow in mass by the capture of oligoradicals, monomer addition, or aggregation with other nuclei.[19]

To produce core/shell microgels, we described a "seed and feed" method, using a two step reaction strategy.[26] In the first step, core particles are synthesized using the method described above. Once a core is synthesized, a hydrogel shell with the desired composition and properties is added. In a typical reaction, the core particles are heated to a temperature above the LCST of the polymer (commonly 70 °C) and a monomer solution is added to make up the shell composition. The mixture is purged with N_2, and subsequently initiated via the addition of persulfate (APS/KPS) and reacted for several hours. Collapsed microgel cores are hydrophobic under these reaction conditions (above the LCST of the polymer), which promotes the capture of any oligomers formed in solution. It is important to note that the "seed and feed" method requires all oligomers formed in the reaction to precipitate on preformed core particles, otherwise homonucleation of the shell polymer may occur where a second population of microgels is generated. To prevent the homonucleation of shell polymer, optimization is required with respect to the concentration of core particles, initiator, surfactant, and shell monomer. To achieve small particle sizes desired for intravenous application (≤100 nm in diameter), syntheses are performed with stabilizing agents added to the system, such as ionic surfactants. Stabilizing components prevent hydrophobic nuclei fusion during precipitation polymerization, thereby promoting particle growth mainly by oligomer or monomer addition.[19]

Nanogels for Cellular Delivery

Our earliest research involving microgels/nanogels for cellular delivery investigated several fundamental aspects of the particles themselves, including their ability to target and become internalized by a simple cancer cell line (KB cells). We chose folic acid as an initial targeting ligand, since it has a high affinity for the folate receptor expressed in a wide range of tumor types (over 90% of ovarian carcinomas),[33] and the KB cell line.[34] Folic acid has been used for the targeted delivery of a number of compounds to cancer cells, where conjugated molecules are imbibed by cells via receptor-mediated endocytosis.[35] In our initial investigation, nanogels composed of pNIPAm were studied. To achieve cellular targeting core/shell nanogels were synthesized wherein the core was fluorescently labeled to enable particle tracking, and amine functionalities were imparted into the shell compartment for subsequent folic acid conjugation.[3]

There were two intriguing features of the cellular uptake. First, the majority of internalized particles appeared to lie outside of the endosomes, where the green fluorescence signal from nanogels was uncorrelated with the red fluorescence signal of an endosomal indicator dye. This was a striking result since endosomal escape is considered an important criterion for effective cellular delivery. In fact, the majority of particles designed for intracellular delivery must utilize a secondary trigger to promote endosomal release.[36,37] Despite their lack of any purposely designed endosomal escape mechanism, our particles showed efficient delivery into the cytosol. Second, the fluorescence images showed little difference when cells were incubated at 27 °C versus 37 °C. As described earlier, pNIPAm microgels undergo an entropically driven transition from a swollen state to a deswollen state above 31 °C due to the LCST properties of the polymer, with the particles being more hydrophobic above the LCST. However, this temperature switch had a significant impact on the cell toxicity, with a strong temperature dependence on viability being observed. A marked decrease is viability was observed when cells were incubated at 37 °C, which was attributed to an increase in particle hydrophobicity and a loss of intracellular

colloidal stability.[23] This result suggests that a simple temperature switch may be employed to induce cell death following particle uptake, perhaps enabling nonpharmacological antitumor activity. Despite this result, we did not consider the temperature range used (i.e., 27–37 °C) particularly useful for practical drug delivery applications and chose instead to focus on the properties of nanogels as carriers instead of their intrinsic (switchable) cytotoxicity.

In the domain of targeted chemotherapies, we found small interfering RNA (siRNA) an attractive candidate for particle-mediated delivery.[38] In light of our previous efforts in nanogel delivery,[3] we sought particles that would remain swollen at physiological temperatures (i.e., 37 °C). In 2009, Blackburn et al. introduced a new class of core/shell nanogels (~100 nm in diameter) using a modified precipitation polymerization approach.[39] Instead of pNIPAm, nanogels were composed of poly(*N*-isopropylmethacrylamide) (pNIPMAm), which remains water swollen at physiological temperatures (LCST ≅ 44 °C). Hence, pNIPMAm microgels can still be synthesized by precipitation polymerization but have an LCST that is distinct from physiological temperatures. Similar to our previous work, fluorescence was incorporated into the core of the particles to permit particle tracking by confocal fluorescence microscopy, whereas primary amine groups were localized in the nanogel shell for subsequent bioconjugation reactions.[1] Instead of folic acid, the nanogels were labeled with a 12 amino acid peptide sequence (YSAYPDSVPMMS). The peptide mimics the ligand ephrin-A1, which interacts strongly with the EphA2 receptor. This receptor is overexpressed in ~75% of ovarian cancers and is associated with increased metastasis and decreased survival.[40]

To encapsulate siRNA, we used an approach that exploits the superabsorbent properties nanogels. Following peptide conjugation, nanogels were freeze-dried to form a hygroscopic, low density powder. A concentrated solution of siRNA was added to the dried nanogels, using a solution volume that is completely imbibed via particle swelling in the medium. We termed this method "breathing-in"; the approach results in high encapsulation payloads (16 μg siRNA/mg polymer) and efficiencies (93 ± 1%) (Figure 2).[1] The method has the advantage of being a convenient, simple, and effective means to entrap siRNA within the nanogel structure. For instance, in vitro release studies revealed that nanogels typically leak a modest fraction of the siRNA within the first 12 h at 37 °C in serum-containing PBS, and retain a large fraction of siRNA out to 35 h. The level of retention observed (~67% after 12 h) suggests efficient

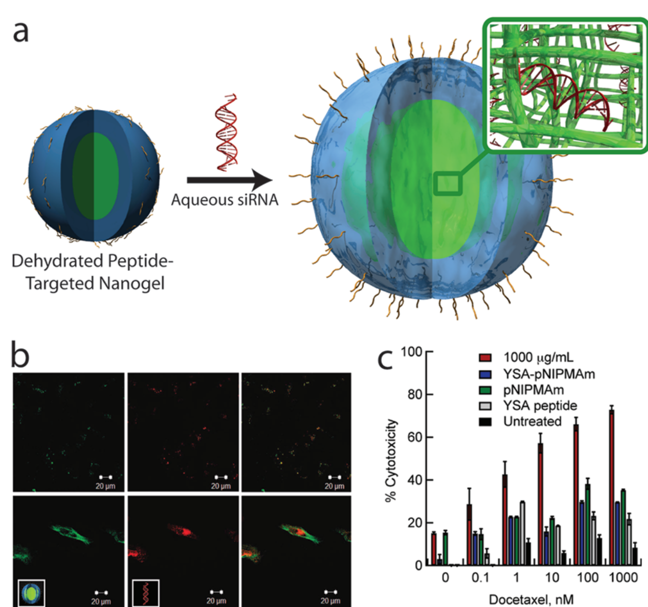


FIGURE 2. (a) Noncovalent encapsulation of siRNA using "breathing-in". (b) Confocal microscopy of Hey cells following exposure to fluorescent siRNA-loaded and YSA-conjugated nanogels. Uptake was compared after 1 h incubation with ephrin-A1 (top) and with nanogel administration alone (bottom). Fluorescence channels are represented for fluorescein (green) and siRNA (red). (c) Chemosensitization of Hey cells to docetaxel after exposure to YSA-targeted, EGFR siRNA loaded nanogels. Controls: unloaded YSA-nanogels (YSA-pNIPMAm), unloaded pNIPMAm nanogels (pNIPMAm), YSA peptide alone (YSA Peptide), and untreated cells (Untreated). Cytoxicity was assessed after incubation for 48 h. Panel (b) reprinted with permission from ref 1. Copyright 2009 American Chemical Society. Panel (c) reproduced from ref 2, Copyright BioMed Central.

entrapment of the oligonucleotide within the nanogel network, with this time scale commensurate with the time needed for extravasation into a tumor by EPR.[13]

Similar to our previous work,[3] the uptake of targeted nanogels by ovarian cancer cells was determined via confocal fluorescence microscopy, monitoring the green fluorescence of the cores. In addition, the nanogels were loaded with red-fluorescent siRNA (siGLO), enabling visualization of siRNA. Internalization was higher in cells expressing the targeted receptor (EphA2), whereas decreased particle fluorescence was observed in cells with limited receptor expression, suggesting good specificity for the peptide-targeting strategy.[1] Overlay of the green (nanogel) and red (siGLO) channels suggested that the nanogel and the siRNA were colocalized in the targeted cells (Figure 2b). To establish the mechanism of targeting, we investigated the internalization properties of the EphA2 receptor: the receptor binds the ligand ephrin-A1, resulting in internalization and degradation of the receptor–ligand complex.[41] When ephrin was introduced to the cells prior to incubation with nanogels, the amount of particle uptake was greatly diminished compared

to untreated cells (Figure 2b). This result suggests that nanogel uptake was primarily driven by EphA2 binding.

At the time of our initial siRNA delivery efforts, our collaborators and we considered chemosensitization a promising approach to cancer therapy. In particular, numerous reports had demonstrated that the knockdown of epidermal growth factor receptor (EGFR) is clinically significant in the treatment of drug-resistant carcinomas,[42] increasing the sensitivity of cells to traditional chemotherapeutics. Overexpression of EGFR had been characterized in several solid tumors, with particularly high levels expressed in ovarian cancer. RNA interference was shown as an effective approach toward chemosensitization,[43] while we considered nanogels as a promising platform to deliver a range of siRNAs for those clinical applications.

Expanding upon previous studies of nanogel-mediated transfection, we reported the delivery of siRNA targeting EGFR.[1,2] The previously developed nanogel architecture[1] was evaluated for its ability to target and deliver therapeutic siRNAs to ovarian cancer cells. For a typical delivery experiment, nanogels are loaded with siRNA via the "breathing-in" method and administered to cells. After 4 h incubation, unincorporated nanogels were removed by washing and replacing the medium. To test the time course of EGFR knockdown, the expression of the receptor was monitored at distinct time points in Hey cells by immunoblot following cell lysis.[2] The delivery resulted in a decrease in EGFR expression, with the greatest knockdown occurring at 48 h, and receptor re-expression beginning after approximately 72 h. The appropriate dosage of the particles was determined subsequently through 10-fold dilutions of the siRNA-loaded nanogels and assessing EGFR levels after 48 h of knockdown. The mean percent expression was reduced to ~65% with as little as 10 μg/mL nanogel.[2] To assess chemosensitization via the nanogel-mediated delivery, Hey cells were incubated with EGFR siRNA-loaded nanogels for 48 h, treated with decreasing concentrations of docetaxel, and assayed for cytotoxicity (Figure 2c).[2]

After siRNA-loaded nanogel treatment, we noticed an increase in the sensitivity of Hey cells to docetaxel, increasing chemosensitization by almost 8-fold over untreated cells (Figure 2c). It is interesting to note that nanogel control (without siRNA) appeared to increase chemosensitization. Thus, the total therapeutic effect is a function of the activity of the nanogel alone and the loaded siRNA. Effective chemosensitization of the Hey cells was observed at extremely low docetaxel concentrations ($\geq$1 nM). Furthermore, the nanogel delivery was demonstrated to be cell specific. When YSA-targeted nanogels were delivered to cells with reduced EphA2 expression (SK-OV-3), EGFR levels were not decreased in the cell line and the nanogels appeared to have no chemosensitization effects.[2] The results showed that nanogels are capable of loading siRNA, stabilizing the molecule in serum-containing media, and delivering active siRNA to the cytosol of specific cells to reduce EGFR expression.[1,2] Reducing EGFR expression in this fashion was an effective approach for inducing chemosensitivity in vitro.

Erodible Nanogels and Microgels

In the pursuit of nanogels as potential siRNA carriers, bioaccumulation and nonspecific organ localization is an ongoing concern. Ideally, nanoparticles would be capable of clearance following drug administration, reducing toxicity caused by particle accumulation. For a number of drug delivery devices, the renal clearance pathway is considered the most efficient means of elimination in comparison to others (e.g., uptake by hepatocytes and biliary excretion). Clearance is governed by several physicochemical properties of the particles, including their molar mass, dimensions, hydrophobicity, and surface charge. The kidneys can excrete particles smaller than 8 nm, whereas the liver and spleen are capable of capturing particles larger than ~200 nm.[44] Degradation of nanogels into low molar mass components may therefore improve their clearance via the renal filtration pathway. Alternatively, erosion may also serve as means to modulate drug release via network decomposition.[45]

Imparting erodible properties into nanogels is a challenge when stimuli-responsivity and degradability is sought within a single particle architecture. We and others have demonstrated that cross-link scission is a versatile means to impart erodible properties to the spheres, where erosion rate and mechanism may be tuned by the choice of cross-linking agent.[28−30,46] We have focused our attention on two different classes of degradable particles: (1) those that erode under physiologic conditions over long time periods and (2) those that demonstrate triggered decomposition at rapid rates in response to a stimulus. Whereas slow erosion may enable clearance of the drug delivery particle, triggered erosion enables the release of encapsulated therapeutic agents in a stimuli-specific fashion. Erosion is likely to influence a number of characteristics for the particles used in drug delivery, including the network diffusivity and drug release rates, and the stability of the particles in the medium (as a result of size and topology changes).

To impart hydrolytic degradation into our hydrogels, we employed the cross-linker *N*,*O*-(dimethacryloyl) hydroxylamine (DMHA). The DMHA cross-linker has been successfully used in several different classes of hydrogel biomaterials,[47] permitting network decomposition under physiologic conditions (i.e., pH 7.4, 37 °C), while showing low in vivo toxicity.[48] Nanogels were synthesized using a similar precipitation polymerization approach as demonstrated for siRNA delivery vehicles,[1,2] yielding particles that displayed both pH and temperature-dependent erosion,[29] with faster decomposition being observed at neutral to basic pH and at elevated temperatures.

In contrast to the slow erosion imparted by the DMHA cross-linker, we have also demonstrated the rapid, triggered decomposition of particles by incorporating a chemically labile cross-linker into the microgel network, (1,2-dihydroxyethylene)bisacrylamide (DHEA);[28,31] the DHEA cross-linker contains a vicinal diol that can be cleaved by periodate addition. Although we are currently investigating more physiologically relevant cross-linkers for this function, the periodate-induced cleavage of DHEA may serve as an effective model for investigating structure–function relationships in these degradable colloids. In a recent report, we investigated the erosion of particles composed of two thermoresponsive polymers, pNIPAm and poly(*N*-isopropylmethacylamide) (pNIPMAm). Using Multiangle light scattering (MALS), the network decomposition of both particles was monitored in real-time, revealing distinct differences between the pNIPAm and pNIPMAm particles (Figure 3). For those experiments, the microgels and periodate were simultaneously delivered to the MALS flow cell via a controlled mixing device. The particle molar mass and radius was then monitored in situ. Microgels composed of pNIPMAm-DHEA swelled early in the erosion, followed by decay into smaller spheres and eventually to linear chains with poorly defined angular scattering functions. The observables described in that work were suggestive of a particle homogeneous in density and with uniform connectivity. As erosion proceeded, a decrease in network connectivity caused the microgel to swell. After sufficient reaction time, the microgels dissolved into a collection of oligomers with low scattering cross sections (Figure 3, top).[28] Very different erosion characteristics were observed for pNIPAm-DHEA particles. In contrast to the uniform connectivity observed for pNIPMAm microgels, the pNIPAm particles instead showed a radial distribution of connectivity, with the greatest polymer density present in the core. As erosion proceeded, mass loss was favored from the particle exterior, eventually proceeding toward the interior. Thus, instead of swelling in response to periodate, pNIPAm particles showed an immediate decrease in radius and mass upon erosion (Figure 3, bottom). Kinetic differences were also observed in the erosion as a result of the heterogeneous distribution of the cross-linker in pNIPAm microgels.

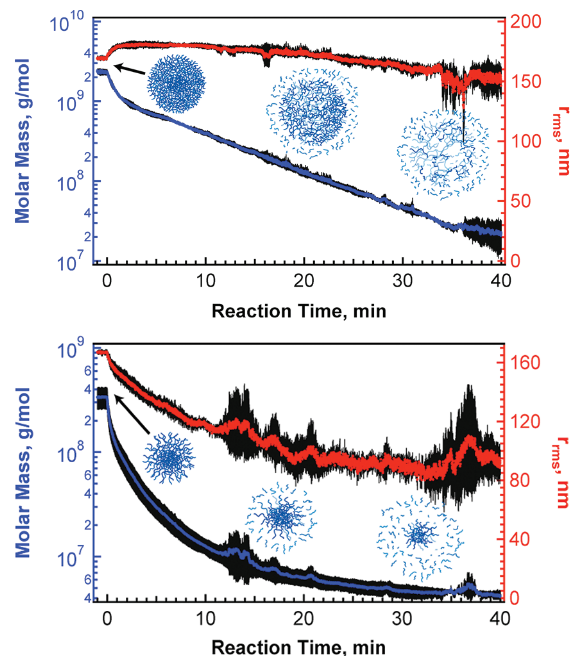


FIGURE 3. Comparison of the erosion behavior for (top) pNIPMAm-DHEA and (bottom) pNIPAm-DHEA microgels, monitored in real-time via MALS. The changes in particle topology over the course of erosion are graphically depicted (blue spheres). Reprinted with permission from ref 28. Copyright 2011 American Chemical Society.

Importantly, the erosion products from pNIPAm and pNIPMAm microgels were different. Whereas pNIPMAm microgels decayed into linear chains, the products of pNIPAm erosion continued to show angular-dependent scattering, indicating the presence of particles despite cleavage of the DHEA cross-linker. We attributed the stability of those particles to the presence of nondegradable cross-linking sites within the networks as a byproduct of precipitation polymerization. NIPAm is prone to undergo chain transfer reactions during precipitation polymerizations, forming noncleavable cross-links in the resulting polymer network.[49] This result suggested that alternative strategies would be needed to form completely degradable pNIPAm microgels, perhaps by reducing the parasitic chain transfer.

Recently, we have demonstrated an alternative precipitation polymerization approach that limits chain transfer, while enabling particle formation at dramatically lower temperatures (between 37 and 45 °C).[50] At those temperatures, the decomposition of persulfates (i.e., APS) is slow,

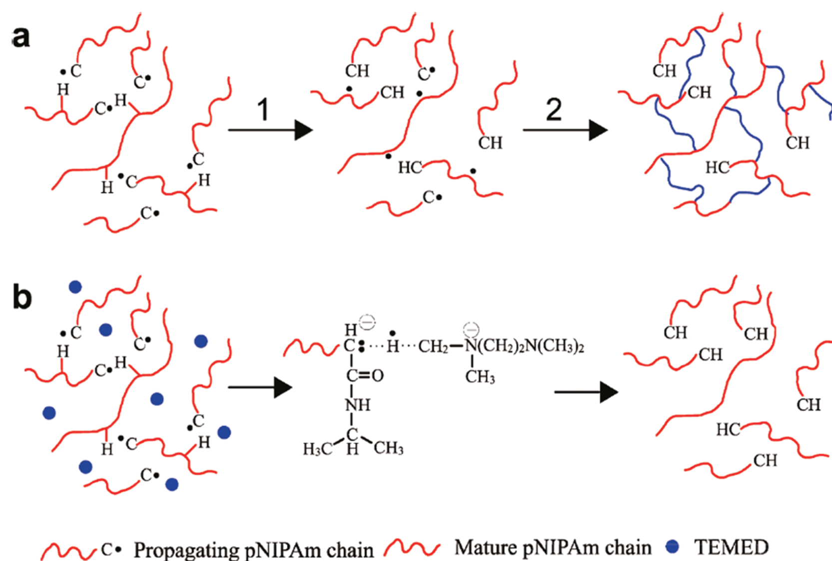


FIGURE 4. Schematic depiction of the self-cross-linking process for pNIPAm. (a) Step 1: Chain transfer of polymer chains. Step 2: Monomer addition, chain termination, network formation. (b) TEMED participates in chain transfer, limiting branching and a cross-linked network. Reprinted with permission from ref 50. Copyright 2011 American Chemical Society.

resulting in a lower radical yield, slower monomer conversion, and poorly defined and unstable microgel nuclei. As a result, colloidal stability is hindered, resulting in extensive coagulation and polydispersity in such syntheses. However, we have shown that the introduced tetramethylethylenediamine (TEMED), a catalyst for the decomposition of persulfates, increased the radical yield at low temperatures. Interestingly, the self-cross-linking of pNIPAm appeared to be less favored under redox initiating conditions. Electron-rich chain transfer agents such as triethylamine (similar to TEMED) show higher reactivity with electron-accepting NIPAm. The pNIPAm chain is more likely to prefer chain-transfer with TEMED over other propagating chains (Figure 4). Through elimination of parasitic chain transfer, particles appeared to undergo complete decomposition upon scission of the DHEA cross-linker via periodate.[50]

Nanogels in Confined Environments

Particle size is considered a critical design parameter for in vivo performance, affecting cellular uptake and other processes such as lymphatic drainage, extravasation, and kidney filtration. However, the mechanical properties of biomaterials (i.e., rigidity) also play a significant role in their activity. For instance, the softness of interfaces influences mechanotransduction and cell proliferation.[51,52] Other reports have illustrated the role of rigidity at much smaller scales, affecting processes such as phagocytosis and endocytosis,[15] and hydrogel circulation.[16] Thus, biological events that show a nanoparticle size-dependence are also likely governed by the mechanical flexibility of the particle.

In 2010, Hendrickson and Lyon demonstrated microgel translocation through cylindrical pores under pressures and size-scales relevant to renal filtration.[53] In that work, a track-etch membrane modeled the pores of the renal system (~8 nm pores associated with the glomerular endothelial gaps) and it was shown that microgels and nanogels were capable of passage through pores nearly 10-fold smaller than the microgels themselves under hydrostatic pressures relevant for renal filtration. We attributed this remarkable phenomenon to the extreme softness and the conformational flexibility of the polymers. The kinetics and deformation dynamics of microgel translocation have more recently been elucidated through the use of resistive pulse analysis techniques in collaboration with White and co-workers (Figure 5).[54,55]

As described earlier, our research group has focused on multiple approaches to enhance particle clearance following drug delivery. For instance, a number of degradable architectures were reported to show decomposition into much small constituents.[28−30] These erosion events, combined with the extreme softness of the material itself, make the particles potential candidates for excretion via the renal route. In addition to clearance, we anticipate the softness of the particles may enhance particle performance in other biological environments (e.g., extravasation by the enhanced permeability and retention effect, or perhaps increased tumor penetration).

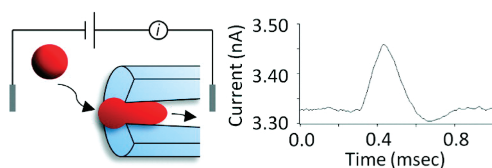


FIGURE 5. Schematic depiction of microgel flux through glass nanopore. Microgel passage is driven by an applied pressure, whereas passage events are monitored via changes in pore conductance caused by electrolyte displacement. Reprinted with permission from ref 54. Copyright 2011 American Chemical Society.

Conclusions and Future Directions

Although we discussed several useful features of nanogels in this Account, numerous challenges persist in optimizing the vectors for in vivo siRNA delivery:

siRNA Protection and Release. Nanogels must guard siRNA from inactivation during circulation (e.g., by clearance, aggregation, degradation) while preventing premature release. Although the hydrophilic nanogel network has shown promise for siRNA protection, the release profile imparted by the gel network will require optimization to suit specific tumor phenotypes and physiological conditions. Furthermore, stimuli-responsive characteristics may be engineered into these materials for cell-specific, triggered release events.

Cell Targeting and Transfection. In vivo transfection will require optimization for both passive and active targeting mechanisms. In addition, the endosomal escape mechanism previously reported for folate-targeted microgels requires elucidation.

Stealth. Recognition of particles by phagocytes remains an ongoing challenge for a variety of synthetic siRNA vectors. Whereas nanogels are hydrophilic and have shown low levels of cytotoxicity, the properties of the nanogel periphery will need optimization (e.g., via poly(ethylene glycol) incorporation) to enhance tumor accumulation via EPR while avoiding clearance by immune recognition.

Tissue Penetration. The siRNA carrier must be capable of passage within confined environments in vivo, including the porous vasculature of tumors and the dense extracellular matrix of the target tissue. Our previous nanogel pore translocation experiments showed that particle softness likely enables nanogel mobility in confined areas. We anticipate that ongoing research via the resistive pulse analysis technique will yield additional insight into those properties and how to control particle penetration into dense tissues.

Clearance and Toxicity. In order to permit repeated administration of the vector, while limiting toxicity, it is common to impart biodegradability. We have found cross-link scission to be a convenient means to enable erosion, which may assist in nanogel clearance while reducing off-target effects after repeated delivery. Additionally, nanogels with triggered erosion may enable tissue-specific release in future particle formulations, where network decomposition in response to cell-specific signal would provide greater specificity toward the siRNA release.

The number of features required in siRNA vectors is large, as illustrated by the (by no means comprehensive) list above. However, we consider multifunctional nanogels likely candidates to meet those demands. The potential for further synthetic diversity in such constructs, along with their favorable mechanical properties and the ability to create complex, multicompartment vehicles should provide the tools needed to create particles tailored for clinical use in vivo.

This work was partially supported by the National Institutes of Health (1 R01 GM088291-01). Additional funding for MHS was provided by U.S. Department of Education GAANN awards, the Georgia Tech Center for Drug Design, Development and Delivery, and the Georgia Tech TI:GER program.

BIOGRAPHICAL INFORMATION

L. Andrew Lyon is a Professor and Associate Chair in the School of Chemistry & Biochemistry at Georgia Tech. He received his B.A. in Chemistry from Rutgers College and Ph.D. in Physical Chemistry from Northwestern U. After postdoctoral research at the Penn State, he joined the GT faculty in 1999. He has been honored as a Beckman Young Investigator, Sloan Fellow, Dreyfus Teacher-Scholar, and Fresenius awardee. Dr. Lyon serves as an Associate Editor for the journal *Colloid and Polymer Science* and on the Scientific Advisory Council for the Arnold and Mabel Beckman Foundation.

Michael H. Smith is a Ph.D. Candidate at the School of Chemistry and Biochemistry, Georgia Institute of Technology. He obtained a B.A. in Chemistry from Wheaton College (MA) in 2007, with a minor in Biology. His current research interests include soft colloids for the therapeutic delivery of macromolecular therapeutics.

REFERENCES

1 Blackburn, W. H.; Dickerson, E. B.; Smith, M. H.; McDonald, J. F.; Lyon, L. A. Peptide-Functionalized Nanogels for Targeted siRNA Delivery. *Bioconjugate Chem.* **2009**, *20* (5), 960–968.

2 Dickerson, E.; Blackburn, W.; Smith, M.; Kapa, L.; Lyon, L.; McDonald, J. Chemosensitization of cancer cells by siRNA using targeted nanogel delivery. *BMC Cancer* **2010**, *10* (1), 1–11.

3 Nayak, S.; Lee, H.; Chmielewski, J.; Lyon, L. A. Folate-Mediated Cell Targeting and Cytotoxicity Using Thermoresponsive Microgels. *J. Am. Chem. Soc.* **2004**, *126* (33), 10258–10259.

4 Hendrickson, G. R.; Lyon, L. A. Bioresponsive hydrogels for sensing applications. *Soft Matter* **2009**, *5* (1), 29–35.

5 Bridges, A. W.; Singh, N.; Burns, K. L.; Babensee, J. E.; Andrew Lyon, L.; García, A. J. Reduced acute inflammatory responses to microgel conformal coatings. *Biomaterials* **2008**, *29* (35), 4605–4615.

6 Kurreck, J. RNA Interference: From Basic Research to Therapeutic Applications. *Angew. Chem., Int. Ed.* **2009**, *48* (8), 1378–1398.

7 Kim, D. H.; Rossi, J. J. Strategies for silencing human disease using RNA interference. *Nat. Rev. Genet.* **2007**, *8* (3), 173–184.

8 Whitehead, K. A.; Langer, R.; Anderson, D. G. Knocking down barriers: advances in siRNA delivery. *Nat. Rev. Drug Discovery* **2009**, *8* (2), 129–138.

9 Brannon-Peppas, L.; Blanchette, J. O. Nanoparticle and targeted systems for cancer therapy. *Adv. Drug Delivery Rev.* **2004**, *56* (11), 1649–1659.

10 Li, L.; Hoffman, R. M. The feasibility of targeted selective gene therapy of the hair follicle. *Nat. Med.* **1995**, *1* (7), 705–706.

11 Hendrickson, G. R.; Smith, M. H.; South, A. B.; Lyon, L. A. Design of Multiresponsive Hydrogel Particles and Assemblies. *Adv. Funct. Mater.* **2010**, *20* (11), 1697–1712.

12 Peppas, N. A.; Hilt, J. Z.; Khademhosseini, A.; Langer, R. Hydrogels in Biology and Medicine: From Molecular Principles to Bionanotechnology. *Adv. Mater.* **2006**, *18* (11), 1345–1360.

13 Maeda, H.; Wu, J.; Sawa, T.; Matsumura, Y.; Hori, K. Tumor vascular permeability and the EPR effect in macromolecular therapeutics: a review. *J. Controlled Release* **2000**, *65* (1−2), 271–284.

14 Byrne, J. D.; Betancourt, T.; Brannon-Peppas, L. Active targeting schemes for nanoparticle systems in cancer therapeutics. *Adv. Drug Delivery Rev.* **2008**, *60* (15), 1615–1626.

15 Beningo, K. A.; Wang, Y.-l. Fc-receptor-mediated phagocytosis is regulated by mechanical properties of the target. *J. Cell Sci.* **2002**, *115* (4), 849–856.

16 Merkel, T. J.; Jones, S. W.; Herlihy, K. P.; Kersey, F. R.; Shields, A. R.; Napier, M.; Luft, J. C.; Wu, H. L.; Zamboni, W. C.; Wang, A. Z.; Bear, J. E.; DeSimone, J. M. Using mechanobiological mimicry of red blood cells to extend circulation times of hydrogel microparticles. *Proc. Natl. Acad. Sci. U.S.A.* **2011**, *108* (2), 586–591.

17 Banquy, X.; Suarez, F.; Argaw, A.; Rabanel, J. M.; Grutter, P.; Bouchard, J. F.; Hildgen, P.; Giasson, S. Effect of mechanical properties of hydrogel nanoparticles on macrophage cell uptake. *Soft Matter* **2009**, *5* (20), 3984–3991.

18 Malmsten, M. Soft drug delivery systems. *Soft Matter* **2006**, *2* (9), 760–769.

19 Nayak, S.; Lyon, L. A. Soft Nanotechnology with Soft Nanoparticles. *Angew. Chem., Int. Ed.* **2005**, *44* (47), 7686–7708.

20 Lyon, L. A.; Meng, Z. Y.; Singh, N.; Sorrell, C. D.; John, A. S. Thermoresponsive microgel-based materials. *Chem. Soc. Rev.* **2009**, *38* (4), 865–874.

21 Zha, L. S.; Zhang, Y.; Yang, W. L.; Fu, S. K. Monodisperse Temperature-Sensitive Microcontainers. *Adv. Mater.* **2002**, *14* (15), 1090–1092.

22 Singh, N.; Lyon, L. A. Au Nanoparticle Templated Synthesis of pNIPAm Nanogels. *Chem. Mater.* **2007**, *19* (4), 719–726.

23 Duracher, D.; Sauzedde, F.; Elaissari, A.; Pichot, C.; Nabzar, L. Cationic amino-containing N-isopropyl-acrylamide-styrene copolymer particles: 2-surface and colloidal characteristics. *Colloid Polym. Sci.* **1998**, *276* (10), 920–929.

24 Matsuoka, H.; Fujimoto, K.; Kawaguchi, H. Stimuli-response of microsphere having poly (N-isopropylacrylamide) shell. *Polym. J. (Tokyo, Jpn.)* **1999**, *31* (11), 1139–1144.

25 Senff, H.; Richtering, W.; Norhausen, C.; Weiss, A.; Ballauff, M. Rheology of a Temperature Sensitive Core-Shell Latex. *Langmuir* **1998**, *15* (1), 102–106.

26 Jones, C. D.; Lyon, L. A. Synthesis and Characterization of Multiresponsive Core−Shell Microgels. *Macromolecules* **2000**, *33* (22), 8301–8306.

27 Hu, X.; Tong, Z.; Lyon, L. A. Multicompartment Core/Shell Microgels. *J. Am. Chem. Soc.* **2010**, *132* (33), 11470–11472.

28 Smith, M. H.; Herman, E. S.; Lyon, L. A. Network Deconstruction Reveals Network Structure in Responsive Microgels. *J. Phys. Chem. B* **2011**, *115* (14), 3761–3764.

29 Smith, M. H.; South, A. B.; Gaulding, J. C.; Lyon, L. A. Monitoring the Erosion of Hydrolytically-Degradable Nanogels via Multiangle Light Scattering Coupled to Asymmetrical Flow Field-Flow Fractionation. *Anal. Chem.* **2009**, *82* (2), 523–530.

30 South, A. B.; Lyon, L. A. Direct Observation of Microgel Erosion via in-Liquid Atomic Force Microscopy. *Chem. Mater.* **2010**, *22* (10), 3300–3306.

31 Nayak, S.; Gan, D.; Serpe, M.; Lyon, L. Hollow Thermoresponsive Microgels. *Small* **2005**, *1* (4), 416–421.

32 Schild, H. G. Poly(N-isopropylacrylamide): experiment, theory and application. *Prog. Polym. Sci.* **1992**, *17* (2), 163–249.

33 Sudimack, J.; Lee, R. J. Targeted drug delivery via the folate receptor. *Adv. Drug Delivery Rev.* **2000**, *41* (2), 147–162.

34 Ross, J. F.; Chaudhuri, P. K.; Ratnam, M. Differential regulation of folate receptor isoforms in normal and malignant tissues in vivo and in established cell lines. Physiologic and clinical implications. *Cancer* **1994**, *73* (9), 2432–2443.

35 Anderson, R.; Kamen, B.; Rothberg, K.; Lacey, S. Potocytosis: sequestration and transport of small molecules by caveolae. *Science* **1992**, *255* (5043), 410–411.

36 Duan, H.; Nie, S. Cell-Penetrating Quantum Dots Based on Multivalent and Endosome-Disrupting Surface Coatings. *J. Am. Chem. Soc.* **2007**, *129* (11), 3333–3338.

37 Kakudo, T.; Chaki, S.; Futaki, S.; Nakase, I.; Akaji, K.; Kawakami, T.; Maruyama, K.; Kamiya, H.; Harashima, H. Transferrin-Modified Liposomes Equipped with a pH-Sensitive Fusogenic Peptide: An Artificial Viral-like Delivery System†. *Biochemistry* **2004**, *43* (19), 5618–5628.

38 Sepp-Lorenzino, L.; Ruddy, M. K. Challenges and Opportunities for Local and Systemic Delivery of siRNA and Antisense Oligonucleotides. *Clin. Pharmacol. Ther* **2008**, *84* (5), 628–632.

39 Blackburn, W. H.; Lyon, L. A. Size-controlled synthesis of monodisperse core/shell nanogels. *Colloid Polym. Sci.* **2008**, *286* (5), 563–569.

40 Thaker, P. H.; Deavers, M.; Celestino, J.; Thornton, A.; Fletcher, M. S.; Landen, C. N.; Kinch, M. S.; Kiener, P. A.; Sood, A. K. EphA2 Expression Is Associated with Aggressive Features in Ovarian Carcinoma. *Clin. Cancer Res.* **2004**, *10* (15), 5145–5150.

41 Miao, H.; Burnett, E.; Kinch, M.; Simon, E.; Wang, B. Activation of EphA2 kinase suppresses integrin function and causes focal-adhesion-kinase dephosphorylation. *Nat. Cell Biol.* **2000**, *2* (2), 62–69.

42 Thaker, P. H.; Yazici, S.; Nilsson, M. B.; Yokoi, K.; Tsan, R. Z.; He, J.; Kim, S.-J.; Fidler, I. J.; Sood, A. K. Antivascular Therapy for Orthotopic Human Ovarian Carcinoma through Blockade of the Vascular Endothelial Growth Factor and Epidermal Growth Factor Receptors. *Clin. Cancer Res.* **2005**, *11* (13), 4923–4933.

43 Fan, Q.-W.; Weiss, W. A. RNA interference against a glioma-derived allele of EGFR induces blockade at G2M. *Oncogene* **2005**, *24* (5), 829–837.

44 Nel, A. E.; Madler, L.; Velegol, D.; Xia, T.; Hoek, E. M. V.; Somasundaran, P.; Klaessig, F.; Castranova, V.; Thompson, M. Understanding biophysicochemical interactions at the nano-bio interface. *Nat. Mater.* **2009**, *8* (7), 543–557.

45 Cohen, S.; Yoshioka, T.; Lucarelli, M.; Hwang, L. H.; Langer, R. Controlled Delivery Systems for Proteins Based on Poly(Lactic/Glycolic Acid) Microspheres. *Pharm. Res.* **1991**, *8* (6), 713–720.

46 Hennink, W. E.; van Nostrum, C. F. , Novel crosslinking methods to design hydrogels. *Adv. Drug Delivery Rev.* **2002**, *54* (1), 13–36.

47 Horak, D.; Kroupava, J.; Slouf, M.; Dvorak, P. Poly(2-hydroxyethyl methacrylate)-based slabs as a mouse embryonic stem cell support. *Biomaterials* **2004**, *25* (22), 5249–5260.

48 Pradny, M.; Michalek, J.; Lesny, P.; Hejcl, A.; Vacik, J.; Slouf, M.; Sykova, E. Macroporous hydrogels based on 2-hydroxyethyl methacrylate. Part 5: Hydrolytically degradable materials. *J. Mater. Sci.: Mater. Med.* **2006**, *17*, 1357–1364.

49 Gao, J.; Frisken, B. J. Influence of Reaction Conditions on the Synthesis of Self-Cross-Linked N-Isopropylacrylamide Microgels. *Langmuir* **2003**, *19* (13), 5217–5222.

50 Hu, X.; Tong, Z.; Lyon, L. A. Control of Poly(N-isopropylacrylamide) Microgel Network Structure by Precipitation Polymerization near the Lower Critical Solution Temperature. *Langmuir* **2011**, *27* (7), 4142–4148.

51 Engler, A. J.; Sen, S.; Sweeney, H. L.; Discher, D. E. Matrix Elasticity Directs Stem Cell Lineage Specification. *Cell* **2006**, *126* (4), 677–689.

52 Ingber, D. E. Cellular mechanotransduction: putting all the pieces together again. *The FASEB J.* **2006**, *20* (7), 811–827.

53 Hendrickson, G. R.; Lyon, L. A. Microgel Translocation through Pores under Confinement. *Angew. Chem., Int. Ed.* **2010**, *49* (12), 2193–2197.

54 Holden, D. A.; Hendrickson, G.; Lyon, L. A.; White, H. S. Resistive Pulse Analysis of Microgel Deformation During Nanopore Translocation. *J. Phys. Chem. C* **2011**, *115* (7), 2999–3004.

55 Holden, D. A.; Hendrickson, G. R.; Lan, W.-J.; Lyon, L. A.; White, H. S. Electrical signature of the deformation and dehydration of microgels during translocation through nanopores. *Soft Matter* **2011**, *7* (18), 8035–8040.