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Localized and Sustained Delivery of Silencing RNA from Macroscopic Biopolymer Hydrogels

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RNA interference (RNAi) is a powerful gene silencing mechanism which inhibits gene expression at the post-transcriptional level by the targeted destruction of specific mRNA molecules.[1] RNAi has the potential to revolutionize disease treatment and aid in the functional repair of damaged tissue by decreasing the expression of specific proteins, for example in cancer therapeutics by inhibiting promoters of angiogenic processes[2] or regenerative medicine by inhibiting proteins that negatively impact the healing process or by altering stem cell differentiation pathways.[3−5] However, effective delivery of short interfering RNA (siRNA) to target cells *in vivo* remains a significant challenge to realizing its full therapeutic potential[6,7] because it is highly prone to degradation by ubiquitous RNases,[7−10] targeting and retention of the siRNA at a specific location is problematic,[11] and the silencing effect often only lasts a few days in rapidly dividing cells.[12] Here, we present a new paradigm whereby three-dimensional (3-D) macroscopic, biopolymer scaffolds are utilized to retain siRNA locally and release it in a sustained manner to prolong the effect directly at the site of interest. Three different biodegradable, injectable hydrogel systems were used for localized, sustained delivery of siRNA: calcium crosslinked alginate, photocrosslinked alginate, and collagen. The released siRNA remained bioactive and able to silence protein expression in cells surrounding the hydrogels. Cells incorporated into these hydrogels with the siRNA also exhibited sustained gene knockdown within this 3-D microenvironment. This promising new class of injectable biopolymer-based siRNA delivery systems capable of achieving localized and sustained gene silencing to both host and transplanted cells may have great utility in tissue engineering and therapeutic medicine.

siRNA can be delivered to cells either directly or by plasmid DNA that encodes for the siRNA molecule of interest.[10] Delivery of siRNA itself offers the advantage of not requiring transport to the nucleus for expression, as it functions directly in the cytoplasm. This highly conserved process of RNAi in eukaryotic cells utilizes intrinsic cellular machinery to degrade mRNAs that are complementary to short (<30 nucleotides) siRNA.[12] Current approaches for delivery of siRNA *in vivo* include the direct injection of siRNA in saline, incorporation into liposomes, chemical conjugation with molecular entities to aid in targeting or stabilization of the molecule, complexation with positively charged peptides or polymers to form nanoparticles, or encapsulation within polymeric nano- or microspheres.[13] However, since most of these techniques form nanoscale complexes with the siRNA, they can be rapidly dispersed and, therefore, must rely on either targeting for uptake only by the desired cell population or delivery of high concentrations to allow sufficient dosage to reach the site of interest during a brief therapeutic window. In this work, several macroscopic, biodegradable hydrogel systems that are capable of localized, sustained delivery of siRNA to surrounding cells and transplanted cells within the material were employed (Figure 1). To our knowledge, this is the first report of injectable, biodegradable biopolymer constructs for the controlled delivery of siRNA, permitting long-term inhibition of gene expression at a specific site.

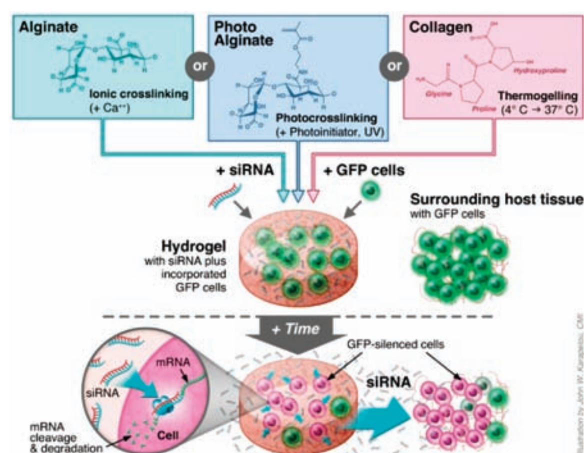

Figure 1. Schematic of hydrogel formation for delivery of siRNA and subsequent inhibition of gene expression in incorporated and neighboring cells. Biomaterial solutions of alginate, photo alginate, or collagen are mixed with siRNA and GFP-positive cells, and hydrogels are then formed by crosslinking, photocrosslinking, or thermogelling, respectively. The siRNA diffuses through the hydrogel to affect incorporated cells, and it is also released from the hydrogel to locally affect surrounding cells that are part of the host tissue.

To demonstrate this concept, a reporter gene stably transfected in HEK293 cells, a rapidly proliferating cell line, was used. The reporter gene was destabilized GFP (deGFP) which has a half-life of ~2 h, compared to enhanced GFP (eGFP) which has a half-life of ~24 h.[14] As many proteins in the dynamic *in vivo* environment do not exhibit long half-lives, deGFP was chosen as a "proof of principle" target gene representative of potential knockdown when silencing a gene for a therapeutic application. When the siRNA was applied exogenously in tissue culture media to these stably transfected HEK293 cells in a monolayer at three different concentrations of siRNA, deGFP expression was significantly decreased at 24 and 48 h post-transfection (Figure 2). After 48 h, the expression of deGFP has been silenced almost entirely at higher concentrations of siRNA. This shows the powerful ability of siRNA to inhibit protein expression and verifies the use of deGFP as a reporter gene to assay knockdown in this time frame with this cell line.

The siRNA was uniformly distributed and encapsulated into three biopolymer hydrogel systems for sustained release: calcium crosslinked alginate, photocrosslinked alginate, and collagen. All

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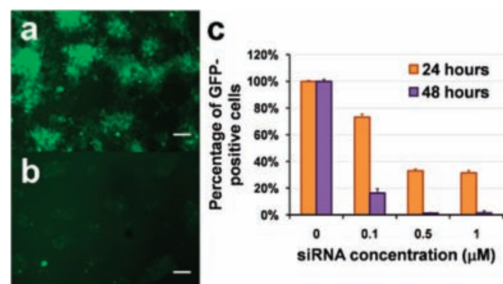

Figure 2. HEK293 cells treated with siRNA. (a,b) Fluorescence photomicrographs 48 h post-treatment. (a) No siRNA. (b) 1 μM siRNA. Scale bars represent 100 μm. (c) Quantification by flow cytometry analysis of GFP expression at 24 and 48 h post-transfection with three concentrations of siRNA, normalized to no treatment control group.

three systems are crosslinked to form hydrogels by different mechanisms. Alginate is a polysaccharide composed of α-L-guluronic acid and β-D-mannuronic acid units; the guluronic acid residues can be cross-linked with divalent cations such as calcium. This method for cross-linking is gentle for cells and reversible in the presence of calcium chelators. However, it can be difficult to modulate the mechanical properties, swelling, and degradation of these hydrogels.[15] To enhance control over the aforementioned properties, our group recently engineered a photocrosslinkable alginate system that exhibits more controlled degradation by hydrolysis of ester linkages.[16] The photocrosslinking occurs following brief exposure to UV light in the presence of a photoinitiator, which has been shown to have minimal effect on the viability of cells. The third natural biopolymer examined was a commercially available acid-solubilized collagen, which is a liquid at 4 °C and forms a hydrogel at 37 °C after adjustment to neutral pH. Collagen is a native extracellular matrix molecule to which cells can naturally adhere, and the gentle gelling process allows cells and bioactive factors to be easily incorporated.

The same amount of siRNA was incorporated into each hydrogel, and release from all three systems was quantified over the course of 2 weeks. Each material exhibited a different release profile (Figure 3a,b). siRNA was released in a sustained manner for ∼1 week from the alginates and ∼2 weeks from the collagen. The photocrosslinked alginate released the highest total amount of siRNA, followed by the calcium crosslinked alginate, then the collagen. Alginate is a highly negatively charged polysaccharide, so the possibility of retaining more siRNA (also negatively charged) within the hydrogel and delaying its release by the addition of positively charged polymers was examined. Indeed, the addition of either chitosan or polyethylenimine (PEI) to the calcium crosslinked alginate hydrogels delayed the release of the siRNA into the surrounding media (Figure 3a) likely due to electrostatic interactions between the nucleotides and positively charged polymers. The mechanism of siRNA release over the time period examined is likely a combination of diffusion through the biopolymer pores as well as biopolymer degradation.

The released siRNA was tested for bioactivity to ensure its incorporation and subsequent release from these hydrogels at 37 °C, and its exposure to UV in the photocrosslinked hydrogels did not affect its ability to silence protein expression. HEK293 cells cultured in a monolayer were exposed to siRNA released from hydrogels suspended over the cells in transwell permeable supports. The GFP expression of these cells was measured after 3 and 6 days of culturing in the presence of calcium crosslinked alginate, photocrosslinked alginate, and collagen hydrogels containing siRNA. All three materials delivered siRNA sufficient to silence GFP expression to less than 20% of the no treatment control samples (Figure 3c). Furthermore, the effect was more pronounced at day 6 compared to day 3. To ensure bioactivity of the siRNA, cells were cultured in serum-free media for 3 days followed by culture in 1% serum for the next 3 days, and by day 6 the cells started to show some effects of serum deprivation. Notably, the no treatment controls have a large standard deviation in their level of GFP expression, as some cells seemed to be shutting down production of the GFP in the absence of serum, as has been previously documented.[14] However, even when normalized to the no treatment controls, the gene knockdown is significant in the treated samples, indicating that GFP is being silenced by the siRNA even under these serum deprivation conditions. The GFP silencing of these cells due to the RNAi is clearly seen in a histogram from representative samples measured at day 3 using flow cytometry (Figure 3d). All cell populations exposed to siRNA released from any of the hydrogels exhibit decreased fluorescence as compared to the cells not treated with siRNA. The released siRNA retains its bioactivity and the effect is still strong even at 6 days after starting the release.

One strong advantage to the use of hydrogels as biopolymer scaffolds is that they are injectable, so delivery to the site of interest is minimally invasive. Additionally, due to their hydrophilic nature and high gas permeability, which permits easy transport of nutrients and oxygen and removal of waste products, it is possible to incorporate cells within the scaffold to provide an additional population of transplanted cells to increase the therapeutic effect, especially for applications in regenerative medicine. While delivery of genetic material to transplanted cells for upregulation of specific genes has been shown to enhance the quality and rate of tissue formation in tissue engineering strategies,[17] downregulation of gene expression at the post-transcriptional level via siRNA delivery is also a powerful approach to regulate the function of both mature cells and stem cells and ultimately enhance the formation of new tissue. Therefore, the ability of cells incorporated within the hydrogels to uptake siRNA incorporated within the same hydrogels was examined. Two materials with very different siRNA release profiles were used: photocrosslinked alginate and collagen. The

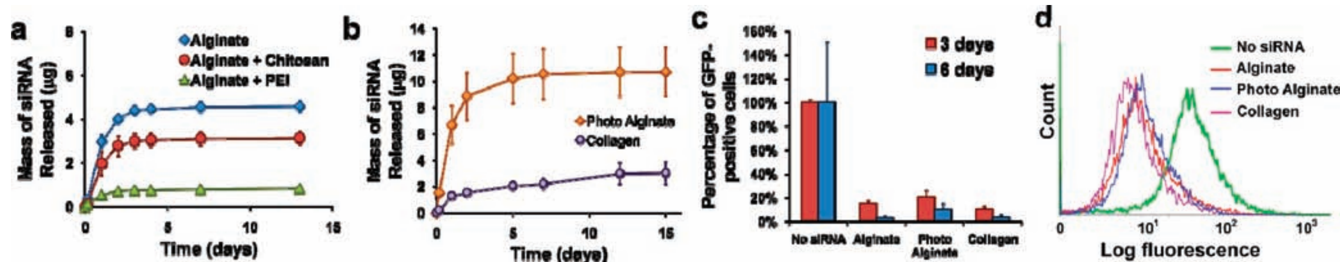

Figure 3. Release and bioactivity of siRNA from hydrogels. (a,b) Cumulative release of siRNA from hydrogels. (a) Calcium crosslinked alginate with or without the addition of polycationic chitosan or PEI to modulate release. (b) Photocrosslinked alginate and collagen. (c,d) Bioactivity of released siRNA. (c) Percentage of GFP-positive HEK293 cells after 3 and 6 days, normalized to no treatment controls. (d) Flow cytometry histograms of samples demonstrate GFP silencing at day 3.

HEK293 cells were incorporated into the hydrogels, and three conditions were examined: (1) cells in the hydrogels without siRNA; (2) cells in siRNA-containing hydrogels; and (3) cells in hydrogels without siRNA incorporated but that were exposed to an equal amount of siRNA supplied exogenously in the surrounding media for the first 24 h only. The cells incorporated into the hydrogels without siRNA exhibited strong GFP expression after both 3 and 6 days in culture (Figure 4). In contrast, cells that were coincorporated with siRNA in the hydrogels showed significant knockdown at day 3. By day 6, the GFP expression had increased in the cell population within the photocrosslinked alginate. However, the cells in the collagen continued to display substantial GFP knockdown at day 6 compared to both the control samples and the samples supplied with exogenous siRNA. This correlates well with the release profiles of siRNA from these hydrogels, as the collagen exhibited more sustained release compared to the photocrosslinked alginate.

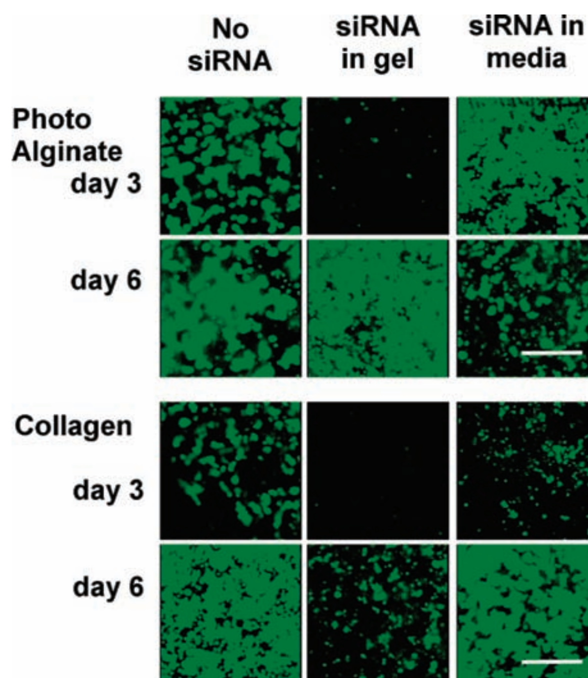

Figure 4. Confocal fluorescent micrographs of cells cultured in 3-D hydrogels. Cells were exposed to no siRNA, siRNA only in the hydrogels, or siRNA present in the media for only the first 24 h of the experiment. The hydrogels examined were photo-cross-linked alginate and collagen, at days 3 and 6. Substantial GFP knockdown is seen at day 3 in both hydrogels with siRNA incorporated. The cells in collagen with incorporated siRNA still exhibit decreased expression of GFP at day 6. Scale bars represent 200 μm.

It is important to consider the effect of exogenously supplied siRNA at the same total amount as incorporated into the hydrogels, but only for the first 24 h. The cells encapsulated within the photocrosslinked alginate were unaffected by the exogenous supplementation of siRNA, as the GFP expression appeared similar to the controls. In contrast, the cells within collagen did exhibit GFP silencing at day 3, although not to as great of an extent as those exposed to the hydrogel-incorporated siRNA. However, the silencing from exogenous supplementation did not last until day 6, where the GFP expression returned to levels as strong as the no treatment control cells. This demonstrates the importance of sustained delivery of siRNA as opposed to a single dosage at one time point.

The ability to deliver siRNA in a sustained manner from several biodegradable hydrogel systems to both incorporated and surrounding cells is shown. The choice of biopolymer hydrogel strongly influences the delivery rate of the siRNA. The released siRNA remains highly bioactive, and cells exposed to these gels exhibit significant gene silencing even after 6 days of release. Moreover, the siRNA incorporated into these hydrogels is also available for uptake by cells that are coencapsulated in the hydrogels. Although all three systems were able to silence gene expression from released siRNA at least 6 days after fabrication, the cells incorporated into the photocrosslinked alginate showed GFP knockdown only at day 3, in contrast to the collagen hydrogels which still exhibited silencing at day 6. This indicates that the appropriate hydrogel system can be selected based on the desired effect to transplanted cells and surrounding host cells. Since the siRNA diffuses out from the photocrosslinked alginate hydrogels more rapidly, it is not available later for the encapsulated cells. However, this material would offer a much more potent effect to the surrounding host cells, as a higher fraction of incorporated siRNA was ultimately released as compared to the collagen. In contrast, the collagen hydrogel would allow for a more sustained effect on both surrounding host cells and transplanted cells, although a lower fraction of incorporated siRNA would be released to the surrounding host cells in the short term. The specific application for which RNA interference will be used should be examined to determine the most beneficial delivery regimen.

This new delivery method for siRNA offers the ability to achieve sustained release locally at the site of interest. The hydrogels are injectable and therefore can be administered in a minimally invasive manner. Furthermore, a population of cells can be incorporated within these hydrogels, allowing dual use for siRNA delivery and cell transplantation. This represents an exciting new opportunity for further development in RNAi therapeutics.

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Supporting Information Available: Detailed materials and methods. This information is available free of charge via the Internet at http://pubs.acs.org.

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