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# Supplementary Materials for

## Covalently tethering siRNA to hydrogels for localized, controlled release and gene silencing

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**This PDF file includes:**

**Table S1. Sequence of the synthesized oligonucleotides.**

| siRNA strand | Sequence |
|---|---|
| GFP, AS | AfUGAfUAfUAGAfCGfUfUGfUGGfCfpsfUpsG |
| GFP, S | 5SS-GmCmCAmCAAmCGmUmCmUAmUAmUmCAmU-chol |
| Luc, AS | mCmUmUAmCGmCmUGAGmUAmCmUmUmCGA-chol |
| Luc, S | fUfCGAAGfUAfCfUfCAGfCGfUAAGpsfUpsG |
| fU and fC represent 2'-fluoro uridine and cytidine<br>mU and mC represent 2'-O-methyl uridine and cytidine<br>A and G represent 2' hydroxyl adenosine and guanosine.<br>A 5' thiol is indicated by 5SS-<br>3' cholesterol is indicated by -chol. | |

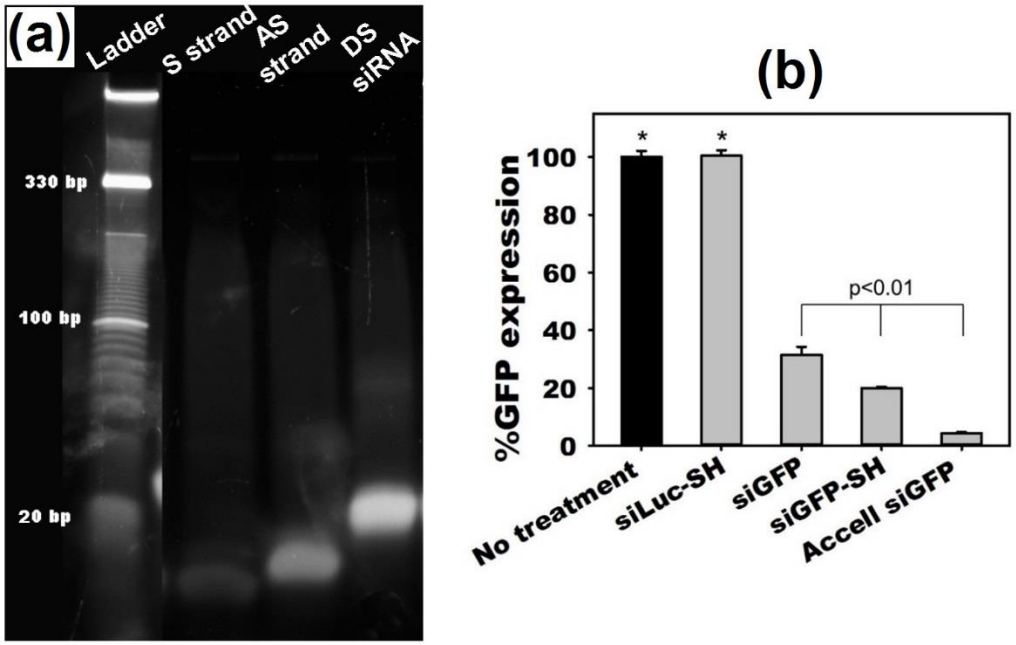


**Fig. S1. Annealing and gene silencing ability of synthesized siRNA.** (**a**) Acrylamide gel comparing single-stranded to annealed double-stranded siRNA-SH (S: sense strand; AS: antisense, DS: annealed double-stranded RNA) with single band for annealed double-stranded siRNA-SH, indicating successful annealing. (**b**) Cellular GFP silencing ability of synthesized siGFP and siGFP-SH in comparison with commercial Accell siGFP (Dharmacon) (N=3) after 2 days of treatment (* $p<0.01$ compared to all siGFP groups). When the cells were cultured with synthesized non-targeting siLuc-SH, cellular GFP expression was observed to be similar to those cultured with media only ("No treatment"). In contrast, cellular GFP expression was significantly decreased when treated with synthesized or commercialized siGFP, indicating the bioactivity of synthesized siGFP-SH, although to a lower extent at this concentration compared with commercial Accell siGFP.

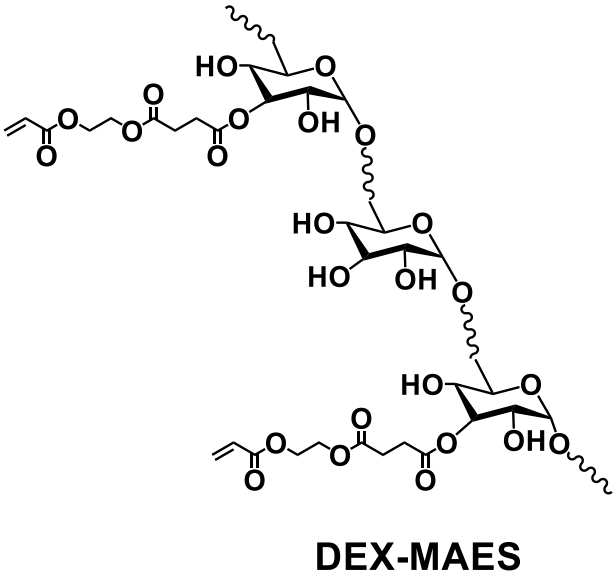


**Fig. S2. Chemical structure of DEX-MAES.**

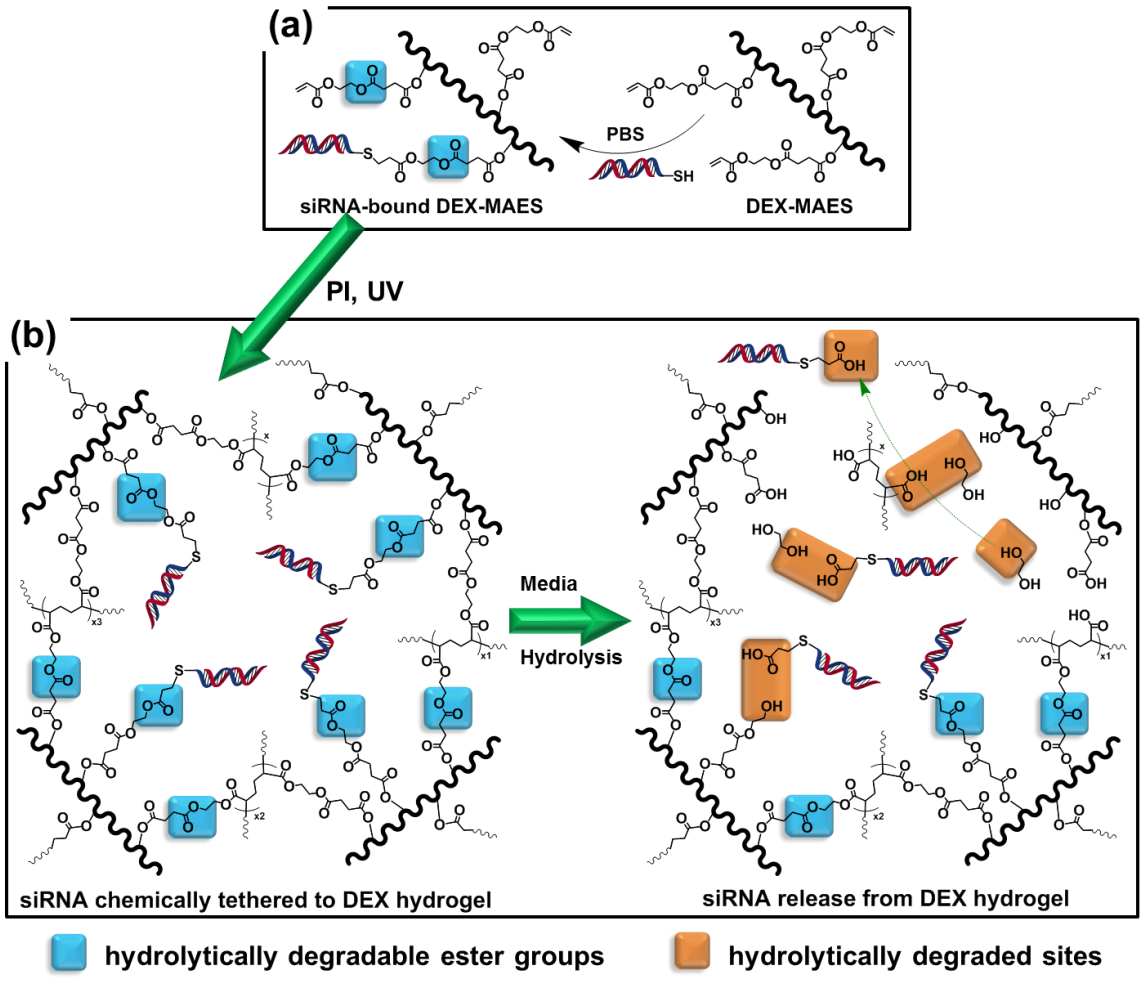


**Fig. S3. Schematic illustration of siRNA conjugation to hydrogels via Michael-addition reaction and its release.** (**a**) siRNA conjugation to DEX-MAES and (**b**) hydrogel formation via photocrosslinking and siRNA release upon hydrolytic degradation.

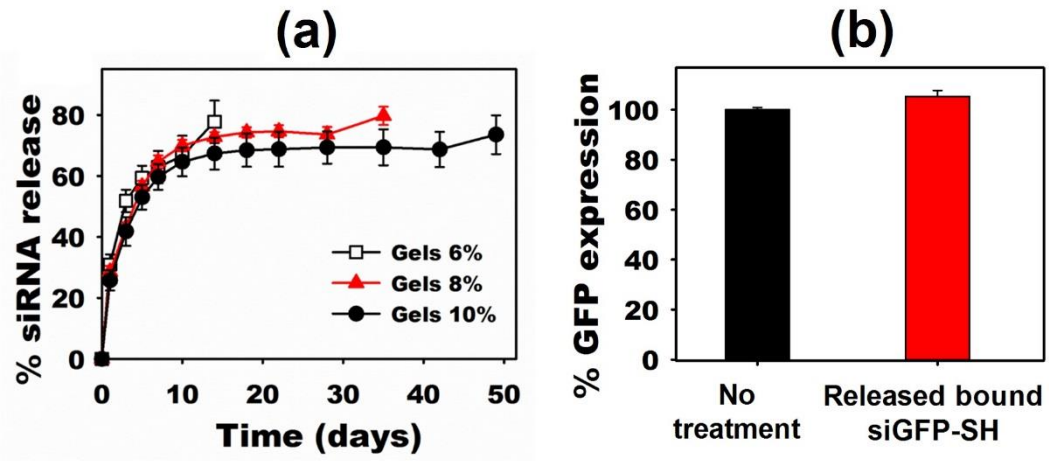


**Fig. S4. Effect of hydrogel concentration on the tethered siRNA release behavior and bioactivity of released siRNA.** (**a**) Release profiles of bound siGFP-SH from 6, 8, 10% (w/w) DEX hydrogels (10 μg RNA/50 μL gel). The hydrogels were completely degraded at the last time point of release experiment for each experimental condition, which were 14, 35 and 49 days for 6, 8 and 10% gels, respectively. Changing polymer hydrogel concentration between 6, 8 and 10 % had little effect on siRNA release, indicating that the release of tethered siRNA was governed via hydrolysis of ester linkages between DEX and siRNA and was independent of potential differences in hydrogel pore size or bulk degradation. (**b**) Bioactivity of released siGFP from Michael-addition tethered hydrogels without an additional transfection reagent. Released siGFP did not silence GFP in the GFP-expressing HeLa cells because it contained -COOH groups generated via hydrolytic degradation of ester linkages between the siRNA and DEX.

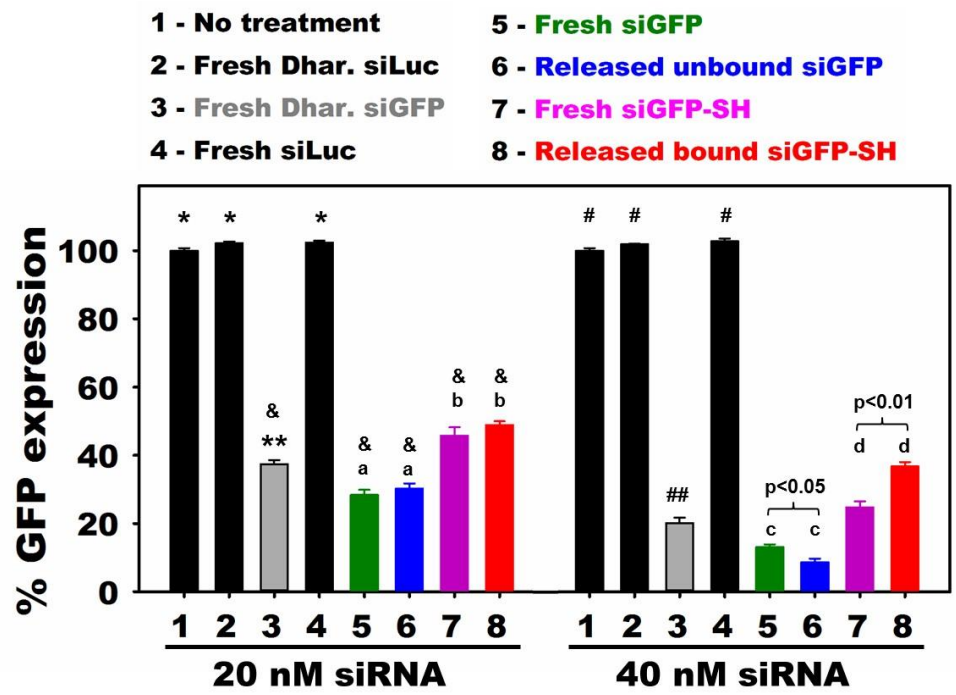


**Fig. S5. Lipofectamine-mediated bioactivity of released siRNA from siRNA-tethered hydrogels.** GFP expression of HeLa cells after 2 days transfected with fresh and released siGFP from Michael-addition tethered hydrogels complexed with Lipofectamine transfection reagent at the final siRNA concentration of 20 and 40 nM ($^{*,**,\#,\#\#}$ $p<0.01$ compared to groups that do not share these symbols and used the same siRNA concentration; $^{\&}$ $p<0.01$ compared to corresponding groups that used 40 nM siRNA; $^{a}$ $p<0.001$ compared to $^{b}$,; $^{c}$ $p<0.001$ compared to $^{d}$). For transfection, the released siRNA was complexed with Lipofectamine 2000 (Thermofisher Scientific) at a 1:2 ratio of siRNA:Lipofectamine (w/w) for 30 min at ambient conditions and then used to transfect monolayer cultured HeLa cells. 16 h post-seeding, cells in wells of 24-well plates were cultured with 500 µL of serum-free DMEM-HG containing siRNA/Lipofectamine complexes (final concentration of 20 or 40 nM siRNA) for 6 h, followed by replacement of the media with DMEM-HG containing 5% FBS. The cells were cultured for an additional 42 h prior to quantifying GFP expression using a flow cytometer (N=3). HeLa cells treated with DMEM-HG only ("No treatment") were considered to express 100% GFP and all other groups were normalized to this group. While "Fresh Dhar. siLuc" and fresh unbound siLuc ("Fresh siLuc"), two negative controls, did not affect GFP expression, the "Fresh Dhar. siGFP" positive control (40 nM) reduced GFP expression to 18.2%. Since a transfection reagent was used, cells treated with 40 nM of fresh unbound siGFP ("Fresh siGFP"), "Released unbound siGFP", "Fresh siGFP-SH" and "Released bound siGFP-SH" inhibited GFP expression to 13.1, 8.7%, 24.1 and 38.0%, respectively. These results indicate that when complexed with a transfection reagent, the released siGFP exhibits high bioactivity via silencing GFP expressing of HeLa cells cultured in monolayer. The "Fresh siGFP-SH" or "Released bound siGFP" did, however, silence cellular GFP expression less effectively than the "Fresh siGFP" or "Released unbound siGFP", which is likely due to the thiol modification. When HeLa cells were transfected with 20 nM siRNA, a similar trend was also observed but with significantly decreased siGFP knockdown extent compared to when 40 nM was used.

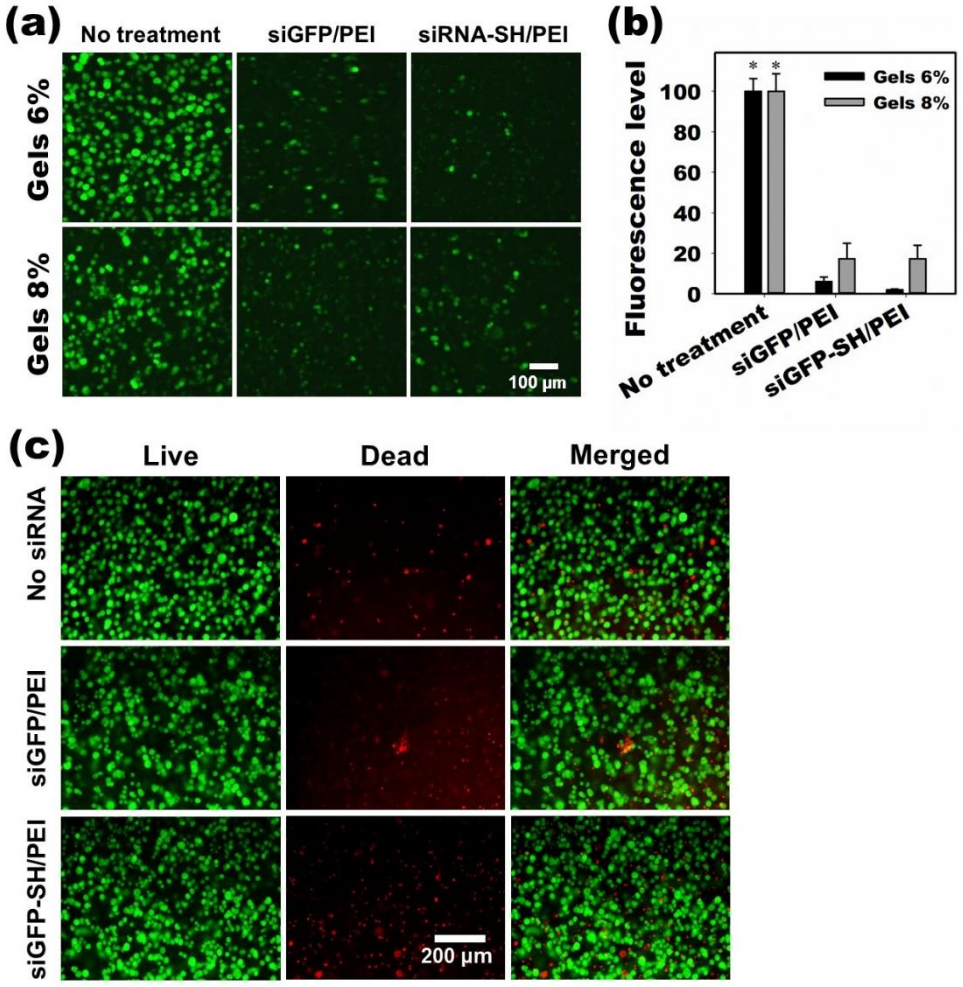


**Fig. S6. Transfection reagent-mediated bioactivity of siRNA loaded or tethered in the hydrogels.** (**a**) Confocal images showing GFP signal of deGFP HeLa cells encapsulated within 6 and 8% (w/w) DEX hydrogels after 2 (gels 6%) and 3 (gels 8%) days of culture. (**b**) Fluorescence level quantification of the confocal images (* $p<0.001$ compared to groups do not share the symbol). (**c**) Photomicrographs showing live/dead (green/red) staining of HeLa cells encapsulated within 6% DEX photocrosslinked hydrogels containing no siRNA, or siGFP/PEI or siGFP-SH/PEI complexes after 2 days of culture. To perform the experiment, siGFP or covalently tetherable siGFP-SH was mixed with DEX-MAES solution (12%, w/w) in PBS (pH 8.0) overnight to allow conjugation followed by complexation with polyethyleneimine (PEI, branched, 25 kDa, Sigma) solution in water (1 mg/mL) and PBS (pH 7.4) at an N/P ratio of 10/1 for 30 min at room temperature (final 40 μg/mL siRNA and 6 or 8% (w/w) of DEX-MAES. The mixtures were then mixed with deGFP HeLa cells ($10\times10^6$ cells/mL) followed by application of UV light to crosslink the hydrogels. The cells-embedded hydrogels were cultured in DMEM-HG with 10% FBS for 2 (6% gels) or 3 days (8% gels) followed by examining the cellular GFP signal using a confocal microscope (LSM510, Zeiss, Jena, Germany) and cell viability. The gels were imaged every 5 μm from their bottom for 100 μm, and then the images were projected into a single 3D image (N=3 gels/group) (a). HeLa cells in hydrogels without siRNA ("No treatment") expressed strong GFP signal, whereas the hydrogels with siGFP/PEI or siGFP-SH/PEI down-regulated GFP expression. Relative fluorescence intensity of 1 z-slice image from each gel 95 μm from their bottom was quantified using image J software (N=3 gels/group), and normalized to the "No treatment"

group, which was assigned a value of 100% (b). To demonstrate that the reduced GFP expression resulted from gene silencing and not from dead cells, viability of incorporated HeLa cells was assessed using a live/dead assay containing fluorescein diacetate (FDA, Sigma) and ethidium bromide (EB, Fisher) (c). Cells-embedded hydrogels in wells of 24-well plates containing 0.5 mL PBS (pH 7.4) were stained with 20 µL of a PBS mixture of 5 µg/mL FDA and 50 µg/mL EB for 5 min at room temperature, followed by imaging with a fluorescence microscope (ECLIPSE TE 300; Nikon, Tokyo, Japan) equipped with a digital camera (Retiga-SRV; Qimaging, Burnaby, BC, Canada). More green cells than red cells in both hydrogels with and without siRNA/PEI complexes were observed, indicating cytocompatibility of hydrogel constructs and that GFP silencing was induced via siGFP transfection.

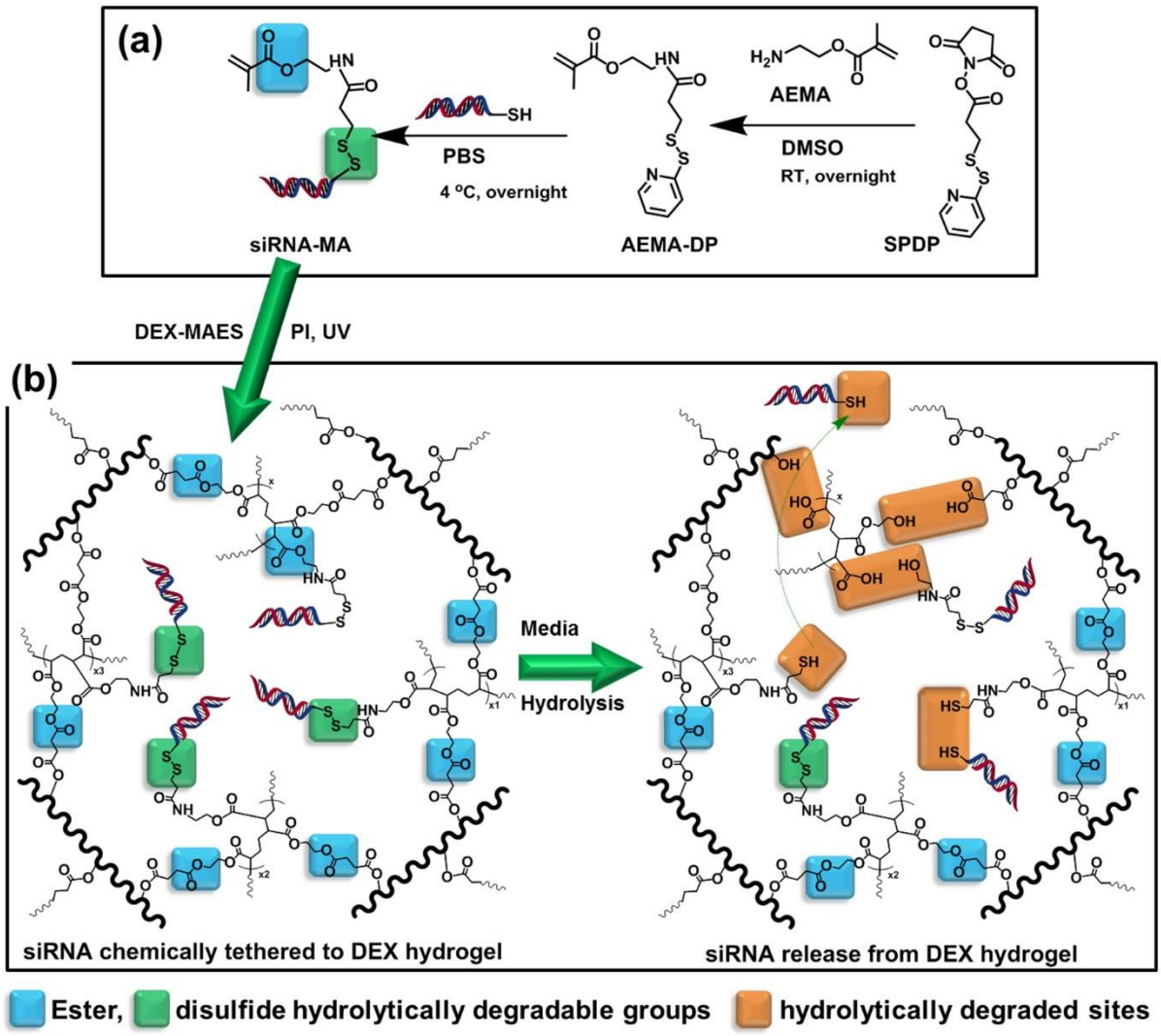


**Fig. S7. Schematic illustration of siRNA conjugation to hydrogels via photoconjugation and its release.** (**a**) siRNA-MA synthesis and (**b**) conjugation of siRNA-MA to DEX-MAES via photo-conjugation, and siRNA release upon hydrolytic degradation of ester and/or disulfide bonds within DEX hydrogels.

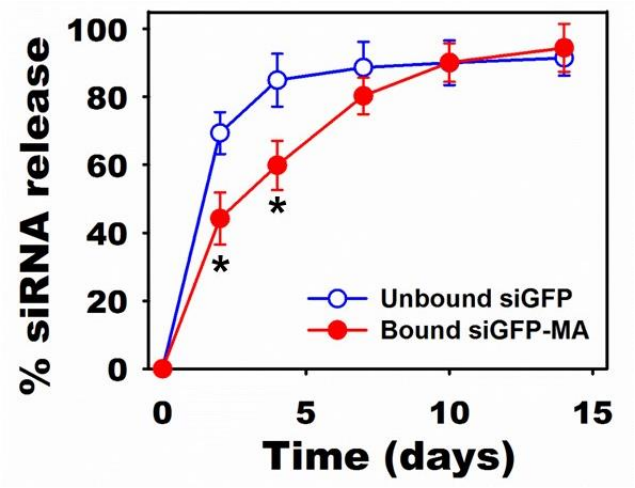


**Fig. S8. Additional release profiles of tethered siGFP from hydrogels with longer sampling intervals compared to Fig. 3A.** Release of siGFP (10 μg/50 μL gel) from 10% (w/w) DEX hydrogels into phenol red-free DMEM-HG showed significant delay in release of the "Bound siGFP" compared to "Unbound siGFP" groups (*$p<0.01$ compared with "Unbound siGFP" at the same time point).

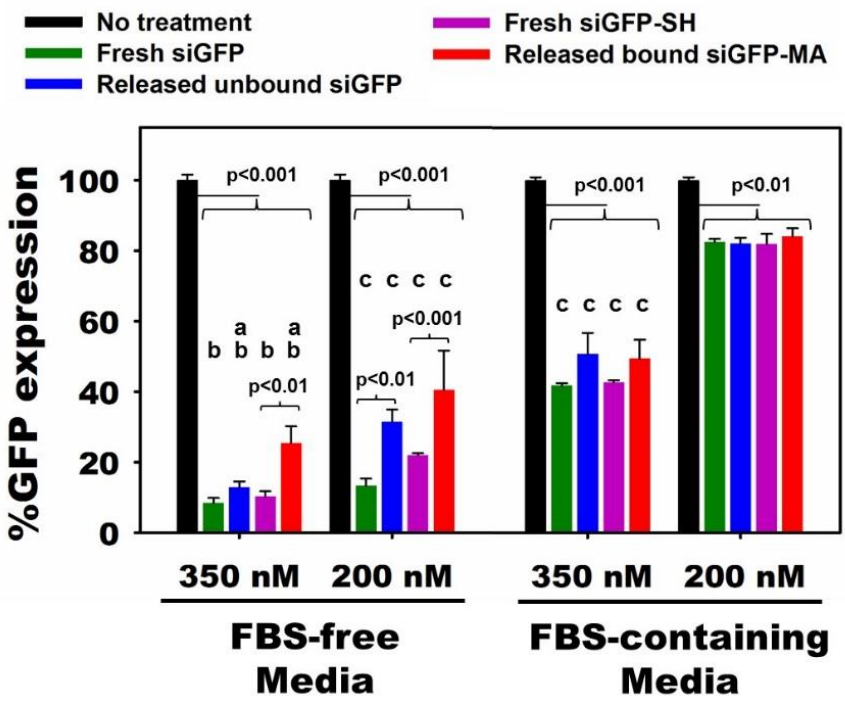


**Fig. S9. Additional bioactivity data of released siGFP (200 and 350 nM RNA treatment).** GFP expression of HeLa cells cultured with DMEM-HG containing **200 or 350 nM** of fresh or released siGFP (from 10% (w/w) DEX hydrogels) in the presence of 0% FBS ("FBS-free") or 2.5% (v/v) FBS. [a] $p<0.01$ compared to corresponding groups (color) in 200 nM/FBS-free media; [b] $p<0.001$ compared to corresponding groups (color) in 350 nM/FBS-containing media; [c] $p<0.01$ compared to corresponding color in 200 nM/FBS-containing media. A similar trend was also obtained, however, with significantly lower GFP expression reduction when the cells were transfected with a lower siRNA concentration (200 nM). The reduction in bioactivity was also observed when the transfection was performed in FBS-free media.