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Photoresponsive DNA-Crosslinked Hydrogels for Controllable Release and Cancer Therapy

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Abstract

We have developed a photoresponsive DNA-crosslinked hydrogel which can be photoregulated by two wavelengths with a reversible sol-gel conversion. This photoinduced conversion can be further utilized for precisely controllable encapsulation and release of multiple loads. Specifically, photosensitive azobenzene moieties are incorporated into DNA strands as crosslinkers, such that their hybridization to complementary DNAs responds differently to different wavelengths of light. Based on the rheology variation of hydrogels, it is possible to utilize this material for storing and releasing molecules and nanoparticles. To prove the concept, three different materials, fluorescein, horseradish peroxidase and gold nanoparticles, were encapsulated inside the gel at 450 nm and then released by photons at 350 nm. Further experiments were carried out to deliver the chemotherapy drug doxorubicin in a similar manner *in vitro*. Our results show a net release rate of 65% within 10 minutes, and the released drug maintained its therapeutic effect. This hydrogel system provides a promising platform for drug delivery in targeted therapy and in biotechnological applications.

1. Introduction

Not only have hydrogels been explored extensively as building materials in complex functional devices,[1] tissue growth, [2] and pharmaceutical carriers,[3] they have also been developed with a biosensing function to respond only upon exposure to external stimuli, such as temperature changes,[4] photons,[5] ions,[6] proteins[7] and DNA.[8] Hydrogels that undergo such physicochemical changes upon the application of stimuli are of great interest for biomedical research, drug development and clinical applications.[9] Particularly, the application of light has been reported to induce morphological and rheology changes of polymers and gels. [5] To date, however, only a few studies have reported the use of photon energy to control the molecular release of loads from hydrogels. [10] Nonetheless, it has been shown that the use of light energy to drive hydrogel gel-sol conversion can be easily and effectively performed with photons of different wavelengths. Moreover, photon-initiated response can induce precisely localized changes in physical and chemical properties with excellent spatial resolution. Photon energy is also a clean energy source, and it can be applied in otherwise inaccessible environments by the easy transportation of light through optical fibers and waveguides. In addition, photons with longer wavelengths can be directly introduced for faster and deeper penetration through biological samples, including human

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tissue. Thus, the ability to use photons to control the release of loads from hydrogels has many potential applications in basic research, controlled release of drugs and clinical practice.

Currently, both mono-component materials, such as polymer chains or pure DNA, [11] and hybrid materials, such as the combination of DNA or peptides with polymer conjugates, have been used to build functional hydrogels. [12] The hybrid hydrogels demonstrate the advantages of diversified controllability and multifunctional properties due to the feasible molecular engineering of each component. Here we report the design of a new type of DNA-crosslinked polymer hybrid hydrogel with reversible photocontrollability. These hydrogels are functionalized by the incorporation of azobenzene moieties (Azo-s) into a DNA crosslinker. Upon UV-Vis light irradiation, azobenzene isomerizes between the *trans*- and *cis*- states and determines the possibility of hybridization between azobenzene-incorporated DNA and its complementary strand. Based on this phenomenon, we engineered DNA and polymer strands to develop a novel type of reversible photoregulated hydrogel, and utilized sol-gel conversion to encapsulate and release different loads. The Azo- incorporated DNA crosslinkers are critical not only for constructing the hydrogels, but also for the photosensitivity, photocontrollability and other physical properties. Besides the photoregulated reversible sol-gel conversion, we have tested the encapsulation and release capabilities using small molecules, proteins and even nanoparticles, and the results demonstrated stable and controlled releasing processes. In order to investigate this biomaterial for biomedical applications, the biocompatibility was first tested for cell viability. Subsequently, the DNA-crosslinked hydrogel was used as a drug carrier by encapsulating the cancer drug doxorubicin to test the toxicity of the released drug to cancer cells. These findings proved that the DNA-crosslinked hydrogel is a promising biomaterial for delivering multiple types of pharmaceuticals and reagents of interest with excellent localization and controllability.

2. Results and Discussion

2.1. Construction of Photocontrollable Hydrogel

As shown in Figure 1, the hydrogels are composed of three major components: two DNA-polymer conjugates and a DNA linker. The modified DNA linker was prepared following the previous method of synthesizing and incorporating azobenzene phosphoramidite (Azo-) onto a DNA backbone.[13] UV light (~350 nm) can photoisomerize the azobenzene moieties to the *cis*-state, while visible light (~450 nm) can switch the configuration back to the *trans*- state.[14] The *cis*- state inhibits the hybridization between Azo-incorporated DNA and its complementary DNA strand (cDNA), while the *trans*- state stabilizes the hybridization, and both processes are reversible by exchanging irradiation light. Hence, photons can be used to control the isomerization of Azo- and, in consequence, regulate the hybridization between two complementary strands. In fact, a key factor in the design depends on the use of Azo- DNA crosslinker, such that the hydrogel can be reversibly controlled by visible and UV light to encapsulate and release loads.

A photoresponsive Azo-incorporated DNA crosslinker (ADL), which is 24-base DNA strand with 11 Azo-insertions, can crosslink two complementary DNA strands, each one precisely hybridized to one half of the ADL. Each of these two DNA sequences is tethered with a polymer chain to form a comb-shaped DNA-polymer conjugate. Two DNA polyacrylamide conjugates (DPC-A, DPC-B) were synthesized individually by photo-initiated polymerization of 5' acrydite-modified oligonucleotide monomer mixed with acrylamide (4%, w/v); the molar ratio of two repeating units is 1:200. The DNA-polymer conjugates will not be further characterized here, because this article focuses mainly on the

photoresponsive behavior and how sol-gel conversion can drive encapsulation and release functions.

The two ADL half segments are complementary to each of the 12-base DNA pieces from DPC-A and DPC-B, and the 12-base DNA strands are linked to the polyacrylamide backbone by a four-T-base spacer. The photoisomerization of Azo- on the DNA linker upon light irradiation was characterized by absorption spectroscopy. The maximum number of Azo- moieties were incorporated into the ADL to optimize photoregulation efficiency. The synthesized and purified ADL is water soluble and displays a yellow color in buffer solution. In the presence of the ADL linker, the crosslinking of two polymer chains between ADL and complementary strands from DPC-A and DPC-B immediately takes place and yields a yellow-colored hydrogel. Because of the unevenly distributed concentration when mixing the components at room temperature, incomplete hybridization can result from the *cis*- state Azo-. To maximize the crosslinking event, a pretreatment of annealing (10 minutes in a 50 °C water bath) and visible light irradiation (5 minutes, 450 nm) was always performed. This pre-treatment process utilized the melting of DNA duplex structure at the high temperature to homogeneously mix each component in solution and re-hybridize the complementary DNA strands as the temperature slowly decreases. This thermal behavior of the hydrogels will be further discussed below. These construction steps result in a design allowing a reversible association/dissociation phenomenon between ADL and DNA-polymer conjugates under visible/UV light. Therefore, the ADL-crosslinked hydrogel is expected to undergo phase transformation between gel and sol states, such that it can be engineered into a convenient carrier for controlled release (Fig. 1a).

2.2. Reversible Sol-gel Conversion

The sol-gel conversion and its reversibility were investigated through repeated UV and visible light irradiations. The ADL-crosslinked hydrogel sample was prepared by directly mixing ADL, DPC-A and DPC-B in stoichiometric amounts with 3 mM concentration based on DNA quantity. At this concentration, a yellow and robust hydrogel was obtained. The initially formed 3 mM hydrogel could be further diluted to other concentrations by annealing at 50 °C with buffer. These different hydrogel concentrations were used to investigate concentration-dependent properties, such as photosensitivity and efficiency of encapsulation and release. The reversible photoconversion was demonstrated with a 300 μ M ADL hydrogel, which was first irradiated by visible light and then by either UV or visible light. A portable UV lamp (350 nm) was used for the UV light source, and a 60W table lamp with a 450 nm filter was used as the visible light source. Both light sources were optimized from several candidates, and they did not induce crosslinking or destruction of DNAs or polymers. The 350 nm UV light irradiation initiated an observable melting behavior of hydrogel after 2 minutes (Fig. 2a, top row). The irradiation process lasted for approximately 20 minutes before the gel completely dissolved as a liquid. The melted gel could be rapidly re-gelled by 450 nm visible light irradiation within 2 minutes. This gel-sol conversion could be repeated for at least ten times without noticeable loss of conversion ability and rate. By contrast, the same hydrogel did not display such a melting progress under continuous visible light irradiation, indicating the ineffectiveness of visible light for the gel-to-sol conversion (Fig. 2a, bottom row).

The control experiment was carried out on a 300 μ M hydrogel crosslinked with a plain DNA linker (DL) (Fig. 2b), with a sequence identical to ADL, but without any Azo- moiety. The DL crosslinked the polymer chains, DPC-A and DPC-B, in the same manner as the ADL hydrogels and produced a colorless and robust gel. Compared to the rapid melting of ADL crosslinked hydrogel, UV irradiation for up to 20 minutes on the DL-crosslinked hydrogel induced only a very slight tilting by the effect of gravity (Figure 2b). It is the inert response to UV light of this plain DNA-crosslinked hydrogel that validates the influence of the Azo-

moiety, which is the key mechanism underlying the photocontrollability and reversibility of the hydrogel. Aside from the effect of gravity, it is also possible that both ADL- and DL-constructed hydrogels could absorb a small amount of visible light energy and thereby induce gel deformation. However, even though both phenomena could potentially influence the slow melting of hydrogels, they are otherwise insignificant when compared to the application of photon energy. Since DNA hybridization is sensitive to temperature, the light sources were placed 5 cm away from the gel, and the temperatures on all gel samples were always monitored with ± 2 °C variation to prevent thermal effects. Such precautions were also taken for all photo-induced gel-sol conversions to maintain a stable temperature.

Figure 2c displays the SEM images of hydrogels prepared using DL and ADL. These images demonstrate that both types of materials have a regular cellular structure with macropore size of approximately 10 μm . However, the microstructures of these two types of hydrogels appears different. The DL-crosslinked hydrogel has a tangled network with more diversified aperture size and thinner reticular structure. In comparison, the ADL- crosslinked hydrogel appears to have a parallel tube-like structure with regular aperture size. Since the images were obtained after visible light irradiation to ensure that all the azobenzene moieties were in the *trans*-form, it seems that *trans*- Azo- can induce regulated hybridization, as well as a more uniform and regularly tube-like cellular structure.

By its very nature, the robustness of hydrogels will decrease with lower density of crosslinked scaffold. Previous synthesized DNA-crosslinked hydrogels have generally required a DNA concentration above 1 mM to maintain a well-defined gel state and biofunction.[12e] However, we have already clearly demonstrated the effective conversion of both sol and gel states at the much lower concentration of 300 μM . In addition, the hydrogels could be diluted to 50 μM , while keeping enough rigidity to maintain a gel-like morphology (the hydrogel required 1 minute to flow from top to bottom in a 1 mL inverted microtube). On the one hand, it should be noted that a loosely crosslinked hydrogel network can cause low-concentration hydrogels to deform rapidly, which may damage the encapsulating capability, but high-concentration hydrogels may require a longer time for gel-to-sol conversion and thus reduce the release rate. As a consequence, there is a need to balance the gel and sol states equally, so that gel rigidity and conversion rate may both be optimized in order to further develop their photocontrollable load carrying function. In fact, this balance was achieved by simply diluting the 3 mM prototype hydrogel with buffers to different hydrogel concentrations.

2.3. Photocontrollable Encapsulation and Release

To demonstrate the photon-triggered release of loads from the photosensitive ADL-crosslinked hydrogels, a series of ADL-crosslinked hydrogels with different concentrations was prepared to explore their controllability. To investigate this property, the effects of hydrogel concentration relative to encapsulation and release of different loads were studied, leaving other conditions unchanged. The entire initial doping process can be achieved by simply mixing the loads with sol state hydrogels by either heating or UV light irradiation while stirring, followed by cooling and visible light irradiation for loading the gel.

Encapsulation capability relies on gel stability and immobility, which, in turn, are related to the hydrogel matrix. Since crosslinking character is determined only by DNA hybridization between the crosslinker and the DNA segments from two different polymer chains, as opposed to random crosslinking among polymer-based hydrogels, it is possible to estimate some physical properties of this gel matrix. To accomplish this, the term “cage” was defined to represent the hydrogel network pore size, thereby enabling an estimate of individual particle entrapment by its physical size and interaction with the gel matrix. Such cage size is generally difficult to obtain from calculations in normal polymer-based hydrogels, as a result

of the wide distribution of molecular sizes and structures and the difficulty of treating the crosslinking mathematically. However, in DNA-based hydrogels, the crosslinking results from precisely synthesized DNA chain lengths on a regulated 2-D hydrogel structure, which then allows meaningful calculations. In the present case, two neighboring DPC polymer chains are assumed to extend on one plane and along the same direction, crosslinked by an intermediate ADL strand (Fig. S1). By modeling the chemical bond length of the cage structure, the calculated cage size along the polymer chain between two neighbor DNA branches is 49.25 nm, and the distance between two parallel neighbor polymer chains is 7.33 nm. Because of the softness of these linkages, we expected the hydrogel to have a possible circular structure 36.02 nm in diameter with maximum encapsulation capability and minimum interior stress. Actually, the concentration of each component determines the real cage size, due to partial crosslinking at low concentration or irregular crosslinking at high concentration (Fig. S2). In the situation of homogeneously isometric mix of 3 mM hydrogel, the average hydrogel cage size is approximately 10.1 nm in diameter. The much smaller cage size compared with the modeling result at this concentration illustrates a high density packing situation. According to the same calculation, 300, 100 and 50 μ M hydrogels have actual cage sizes of 17.42, 25.12 and 31.65 nm, respectively. Apparently, the 50 μ M hydrogel best matches the ideal regular packing condition. However, the physical encapsulation and release processes are also related to other conditions which will determine the optimum concentration. Lowering the concentration of hydrogel by direct dilution can still maintain regularity by first increasing regular crosslinking and then decreasing it by swelling. Noticeably, although we have applied pre-treatment and concentration adjustment to the hydrogels, irregular crosslinking among multiple polymer chains is inevitable in this system.

In order to study the capability and efficiency of hydrogels for controlled release, three hydrogel concentrations (300, 100, 50 μ M) were prepared and pre-loaded with the following: small molecule fluorescein (<1 nm), bioactive Horseradish Peroxidase (HRP) enzyme ($\approx$ 6 nm), and gold nanoparticles (NPs) (13 nm) (Fig. 3a), representing a diverse set of pharmaceutical candidates: small molecule drugs, chemo- and phototherapeutic reagents, and protein-based nanomedicines, respectively.

The small-sized fluorescein molecule is a commonly used material for labeling and tracking because of its observable bright orange color and detectable strong fluorescence. More importantly, the size and physical properties of fluorescein are very similar to those of many chemotherapy drugs. For the fluorescein-loaded hydrogel, there was no observable color diffusion from gel to surrounding buffer solution after more than 30 minutes of visible light irradiation at room temperature (25 $^{\circ}$ C). After applying UV light, however, the hydrogel mixture started to melt, and fluorescein molecules were observed to rapidly diffuse into the buffer solution (Fig. 3b), illustrating the dissolving process of the hydrogel. This dissolving was not caused by a strong absorption of UV light, as confirmed by a control experiment in which fluorescein dissolved with the DL-crosslinked hydrogel had no response to either visible or UV light. It is interesting to observe the stable encapsulation and induced release, since the fluorescein molecule is much smaller than the calculated cage size. We believe that one main factor underlying this phenomenon is the inert mobility of fluorescein molecules in the large hydrogel pocket without an external driving force for self-diffusion, which significantly prolongs the retention time of the trapped molecules. Two other hydrogels with concentrations of 100 and 50 μ M were tested under the same conditions, and the diffusion processes seemed faster with uncontrollable leaking as a consequence of the larger hydrogel cage.

Next, 13 nm water-soluble BSA-modified gold NPs were used to investigate the entrapment/release capability of the hydrogels with a large sized load. In order to specifically study the

relationship between hydrogel concentration and doped loads, three different hydrogels were prepared based on DNA concentrations, 300 μM , 100 μM and 50 μM , and each was used to encapsulate 500 nM gold NPs. A small portion of such NP-loaded gel mixture was placed on the bottom of a quartz microcell (Fig. 3c, insert) with buffer solution on top. When irradiated with UV light, the hydrogel started to melt, and the trapped gold NPs were released to the top buffer solution and quantitatively monitored by the strong gold NP absorption at 520 nm at each interval (Fig. 3c). The hydrogels were initially irradiated with thirty minutes of visible light to evaluate the leaking effect before applying UV light.

The absorption curves show that the UV light dissolved all hydrogels rapidly after 1 minute, and gold NPs were released to buffer solution for absorption measurement. Both 300 μM and 100 μM hydrogels could steadily encapsulate NPs without leaking, while the 50 μM hydrogel seemed to be unable to enclose the particles tightly. The UV light dissolved the hydrogels rapidly after 1 minute, and gold NPs were released to buffer solution. The absorption curves also demonstrate different release rates of gold NPs under UV light irradiation at different gel concentrations. All hydrogels seem to have an initial bursting release period, followed by gradual slowing. For the 300 μM hydrogel, the release rate was comparably slow and lasted for more than 15 minutes before reaching a plateau with an average rate of $1.96 \pm 0.19 \times 10^{-4}$ nmole/min. The 100 μM hydrogel seemed to have a higher rate of release upon UV irradiation. The average release rate was $3.95 \pm 0.36 \times 10^{-4}$ nmole/min, before reaching a plateau at 15 minutes. Despite a serious problem in uncontrollable leaking, the evenly diluted 50 μM hydrogel turned out to be the fastest to reach the plateau with a release rate of $6.88 \pm 0.70 \times 10^{-4}$ nmole/min in 5 minutes. This difference among the three hydrogels clearly demonstrated concentration-dependent encapsulation and release capability under light irradiation. It seems that the 100 μM hydrogel has the best overall balance of stable containment and rapid release rate, properties which actually correlate well with the stability of gel and sol states in hydrogels.

Besides the release rate, the net amount of gold NPs released from each concentrated hydrogel is also an important factor in evaluating delivery capability. The 300 μM hydrogel displays high resistance to releasing gold NPs by UV illumination, and only 38.1% of NPs were released after 30 minutes of visible and UV light, while the 100 and 50 μM hydrogels could release up to 66.9% and 48.4 %, respectively, during the same period. The 100 μM DL-crosslinked hydrogel displayed weak response to both visible and UV light on both release rate and amount (less than 10% overall release). Similarly, although the size of gold NPs is also smaller than that of the theoretically calculated cage size for an ideally crosslinked hydrogel, small NPs are retained inside the hydrogel matrix stably. This can be explained in two ways. First, the well-packed hydrogel structure will slow down the diffusion of small sized particles. Second, it is very likely that the physical interaction between the cage skeleton and the trapped particles can account for the retention of NPs. This interaction occurs irrespective of particle size, but it is significantly influenced by the physiochemical characteristics of both hydrogel and particles.

The small-molecule fluorescein dye and large-sized gold NPs are generally structurally stable and will not change activity while entrapped in most transporting methods or materials. However, invasive carriers, which need chemical binding or strong physical adsorption, may cause the functional structure of biopolymers to be irreversibly altered with resulting damage to their bioactivity. Therefore, the hydrogels were used to deliver HRP as a bioactive drug model. HRP enzyme has both bioactivity and substrate specificity and is widely used as a preferred enzymatic label.[15] The presence of HRP enzyme can be demonstrated by quantitatively monitoring chemiluminescence from substrate oxidation. Similar to the visible/UV irradiation applied to gold NPs, the HRP-loaded hydrogels were examined with kinetic release, and the luminescence profiles were recorded by

spectrophotometer and further converted to HRP amount released into the buffer solution (Fig. 3d). All three ADL-crosslinked hydrogels with different densities have typical UV switch-on releasing profiles. The 100 μM hydrogel had the best performance in releasing HRP in this case, and the diluted 50 μM hydrogel had the fastest release rate. The calculated results demonstrated a net release of 46.7%, 59.2% and 56.0% of active HRP after 60 minutes of UV irradiation released from 300 μM , 100 μM and 50 μM ADL hydrogels, respectively, and 4.8% for the control 100 μM DL hydrogel. These results strongly validated the successful delivery of a bioactive protein by these photocontrollable hydrogels. The activity of the enzyme molecules was confirmed by the enzymatic reaction. In comparison, the 100 μM hydrogel still has the best balance between enzyme storage under visible light and rapid release under UV light. Without the azobenzene moiety, the 100 μM DL-crosslinked hydrogel could only store the enzymes with a slight response to the light irradiations.

2.4. Thermodynamic Behavior

Besides the photocontrollability imparted by the photosensitive Azo- moiety for multiple-load release, these DNA-crosslinked hydrogels may have a similar reversible thermodynamic melting property independent of light energy. That is, the melting property itself could also be regarded as a gel-to-sol conversion event, independent of Azo- moiety and external light energy, and defined only by DNA sequences. We therefore investigated the thermodynamic behavior of three ADL hydrogels, 300 μM , 100 μM , 50 μM , and one 100 μM DL hydrogel for gold NPs encapsulation and release as a function of temperature (Fig. 4). As expected, all DNA-crosslinked polymer hydrogels had melting profiles very similar to those of typical pure DNA duplexes without polymer backbone. These thermodynamic results are consistent with those of photocontrolled release based on hydrogel concentration dependency. Since this thermo-response property of ADL-crosslinked hydrogels is independent of Azo- modification, it actually provides an additional factor for controlling the release of loads. These results strongly suggest that the DL-crosslinked hydrogel can also be used for controllable encapsulation and release by the thermodynamic method.

2.5. Biocompatibility

Biocompatibility of the hydrogels was investigated by incubating hydrogel samples with cells. Different concentrations of DNA linkers and hydrogels were mixed with human leukemia CEM cells, and cell proliferation was monitored by counting living cells and screening cell apoptosis at different stages from 0 to 72 hours for both ADL crosslinker and ADL-crosslinked hydrogels (Fig. S3). The toxicities of ADL crosslinker and ADL-crosslinked hydrogels, as measured by their concentration dependencies, are different. The ADL crosslinker seems have a continuous inhibition to cell viability as concentration increases (Fig. S3a). However, hydrogels from 100 to 1000 μM exhibit less inhibition. Specifically, at an ADL concentration higher than 100 μM and after more than 48 hours, the living cell population significantly decreased from 90% to 60%. For lower concentrations or shorter periods, the population of living cells was maintained above the 85% level. For the cytotoxicity of ADL-crosslinked hydrogels, the reduced toxicity of hydrogels at higher concentrations is most likely a result of the high density of hydrogels preventing access of the cells to the hydrogel matrix (Fig S3b). These results indicate that the azobenzene-crosslinked hydrogels have limited toxicity on cells as long as the hydrogel concentration is lower than 1 mM and within 72 hours' incubation time. Therefore, hydrogels should be safe for *in vivo* use under certain concentration limitations.

2.6. Photocontrollable Drug Delivery for Cancer Therapy

By its photocontrollable release and biocompatibility properties, the DNA-crosslinked hydrogel demonstrated that it is capable of carrying and delivering a variety of particles and bioactive enzymes. We were therefore motivated to test the efficacy of this photoresponsive hydrogel as a therapeutic modality for the treatment of cancer. To accomplish this, an anticancer drug, doxorubicin, was selected for chemotherapy study using the photocontrollable hydrogel as a carrier, following the same strategy as above for the encapsulation and release of fluorescein molecules. A 100 μ M hydrogel was homogeneously mixed with 10 mg/mL of doxorubicin (Dox) in the sol state. The hydrogel was then irradiated with visible light to reform the gel and it was loaded with either buffer (control) solution or cell medium at the top position. Once having loaded the doxorubicin-encapsulated hydrogel, drug release was quantitatively measured in the dark, and with visible and UV irradiation, respectively, to evaluate overall photocontrollability of drug release *in vitro* (Fig. 5a). Under dark conditions, the 100 μ M hydrogel system had an approximately 5.0% inherent leaking ratio within 20 minutes. Irradiation with visible light for 20 minutes could induce another 4.1% net release, both mainly due to the self-diffusion of this small drug molecule within the matrix. In particular, the drug molecules close to the surface region will have a faster rate of escape at the early stages. Finally, as expected, UV irradiation triggered an immediate and rapid release of the drug into the solution. Consistent with our expectation, the release under UV light seems to have had a faster rate during the first ten minutes, which then slowed to reach a final plateau. The net amount of drug released to the top solution within 20 minutes of UV light irradiation was approximately 65.1%.

The same Dox-loaded hydrogel was then applied to CEM cancer cells to study drug efficacy. Both DL-crosslinked and ADL-crosslinked hydrogel, with and without drug, were compared for photocontrollable chemotherapy (Fig. 5b). In each case, an aliquot of culture medium solution with cells was loaded on top of a hydrogel layer inside a cuvette. The hydrogel layer was individually irradiated with visible or UV light for 10 minutes. The top medium solution was removed from the cuvette followed by a cell viability study. Compared to the cell solutions without hydrogel, all DL hydrogels showed less than 10% increase in cell death rate, which was caused by the material toxicity and leaking of drug. The ADL hydrogels showed profiles similar to the DL hydrogels under visible light, indicating the marginal influence of the Azo- moiety on cell viability. However, under UV light irradiation, the ADL hydrogel melted and released the loaded Dox. This, in turn, induced a very high rate of cancer cell death, up to approximately 80%. These results clearly validate the sensitive photoresponse of the ADL-crosslinked hydrogel to UV light, with the resultant release of a large amount of active drug molecules and subsequent inhibition of cell proliferation.

3. Conclusion

In summary, we have designed and constructed a novel biomaterial, which demonstrates reversible UV/visible photocontrolled properties. The hydrogels are easy to synthesize and can be prepared with controllable sizes and structures. Different from chemically triggered hydrogels, they can be reversibly photoregulated by photons. This key property derives from photoisomerization of the azobenzene moiety on the DNA crosslinker, which triggers a sol-gel conversion. The ADL-crosslinked hydrogel system was shown to be a very efficient carrier for the encapsulation and delivery of small molecules, large-sized nanoparticles and bioactive enzymes. More importantly, this hydrogel-based carrier is biocompatible and has successfully carried and released the cancer drug doxorubicin, showing optimal photocontrollability and medicinal efficacy *in vitro*. By rational design of DNA crosslinker and DNA polymer conjugates, we could further apply this hydrogel system to a much wider

range of pharmaceutical carriers for precise spatially photocontrollable delivery. Moreover, the additional aspect of temperature control could drive a similar delivery as an independent factor. Although the penetration depth could limit the applications of this biomaterials in vivo whereas the UV light has difficulty to reach, the low dose of UV light needed to manipulate the transformation of the gel suggested solutions from optical fibers. Future investigation of multiple-factor and concatenate control of the hydrogels could extend this biomaterial to applications involving drug and gene delivery and tissue engineering.

4. Experimental Section

Materials

All the materials to synthesize acrydite phosphoramidite were purchased from Aldrich Chemical, Inc. The materials for DNA synthesis, including CPG columns and reagents for DNA modification and coupling, were purchased from Glen Research Co. CEM-CCRF cells (CCL-119 T-cell, human acute lymphoblastic leukemia) were obtained from ATCC. NB4 cells (acute promyelocytic leukemia) were obtained from the Department of Pathology at the University of Florida.

Synthesis of Azobenzene Phosphoramidite (Azo-)

The synthesis of Azo- followed the protocol from Asanuma et al. with minor modification [13]. The azobenzene phosphoramidite was obtained as an orange-red solid. ^1H NMR (CDCl_3): δ 8.00-6.79 (m, 22H), δ 6.62 (d, 1H), δ 4.48 (m, 1H), δ 4.39 (m, 1H), δ 4.21-4.10 (m, 2H), δ 3.77 (s, 6H), δ 3.57-3.34 (m, 4H), δ 2.76-2.72 (m, 2H), δ 1.30-1.25 (m, 15H). ^{31}P (CDCl_3): δ 149.

Synthesis of Acrydite Phosphoramidite

The title compound was synthesized by two steps. First, a mixture of 6-amino-1-hexanol (9.32 g, 0.08 mol) and triethylamine (TEA, 16.16 g, 0.16 mol) in 100 mL dichloromethane was cooled to 0 °C. Methacryloyl chloride (10 g, 0.0957 mol) was then added slowly, and the reaction was stirred at 0 °C for 2 hours, after which 100 mL water was added to quench the reaction. The organic layer was washed with 5% HCl and dried. After evaporating all of the solvent, the crude 6-hydroxyhexyl methacrylamide was used for the next step without further purification. Second, to a solution containing 6-hydroxyhexyl methacrylamide (2 g, 10.8 mmol) in anhydrous CH_3CN (40 mL) at 0 °C, N, N' Diisopropylethylamine (DIPEA) (3.9 g, 30.0 mmol) was added in 15 minutes. Then, 2-cyanoethyl diisopropyl chlorophosphoramidite (2.9 mL, 13 mmol) was added dropwise, and the reaction mixture was stirred at 0 °C for 5 h. After removing the solvent, the residue was dissolved in ethyl acetate, and the organic phase was washed with NaHCO_3 solution and NaCl solution and dried over anhydrous magnesium sulfate. The solvent was evaporated, and the residue was purified by column chromatography (ethyl acetate/hexane/triethylamine 40:60:3) and dried to afford the title compound (3.33 g, 8.64 mmol, 80%) as a colorless oil. ^1H NMR (CDCl_3): δ 5.92 (br, 1H), 5.63 (m, 1H), 5.27 (m, 1H), 3.86-3.72 (m, 2H), 3.66-3.49 (m, 4H), 3.30-3.23 (m, 2H), 2.61 (t, 2H), 1.92 (m, 3H), 1.58-1.50 (m, 4H), 1.37-1.32 (m, 4H), 1.17-1.13 (m, 12H). ^{13}C NMR (CDCl_3): δ 168.6, 140.4, 119.3, 118.0, 63.8, 63.6, 58.6, 58.3, 43.2, 43.1, 39.8, 31.3, 29.7, 26.8, 25.8, 24.9, 24.8, 24.7, 19.0. ^{31}P (CDCl_3): δ 148.

Synthesis of Azobenzene DNA Linker (ADL) and Normal DNA Linker (DL)

ADL was synthesized by using the DNA/RNA synthesizer ABI3400 (Applied Biosystems). The sequences of ADL and DL are 5'-AC*TC*AT*CT*GT*GA*AG*AG*AA*CC*TG*GG-3' and 5'-ACTCATCTGTGAAGAGAACC TGGG-3', respectively. Both crosslinkers have the same normal DNA sequence, and ADL contains 11 extra Azo- (*) moieties. The synthesis started with a 3'-DabcyI controlled-pore glass (CPG) column at

the 1 μ mol scale. A routine coupling program was used to couple the normal bases from the 3' end. After synthesis, the DNAs were cut and eluted from silica beads and chemically treated before being transferred to HPLC for purification. The final sample was dried and stored at -20°C for future use. The DL was prepared by the same method as that used for ADL by DNA/RNA synthesizer and HPLC.

Synthesis of DNA Polymer Conjugates

Acrydite phosphoramidite was dissolved in acetonitrile and loaded into the DNA synthesizer for two types of acrydite-modified oligonucleotides (Sequence A: 5'-Acrydite-TTTTTCACAGATGAGT-3'; Sequence B: 5'-Acrydite-TTTTCCCAGGTTCTCT-3'). The synthesized acrydite-modified oligonucleotide monomers were further purified by reverse HPLC and quantitatively characterized by absorption at 260 nm. DPC-A and -B were prepared separately at 3 mM DNA concentration. The stock solution contained 10 mM Tris buffer (pH 8.0), 50 mM NaCl, 10 mM MgCl_2 , 4% acrylamide, 1% MW Ciba IRGACURE 2959 and 3 mM DNA Sequence A or B. After mixing, UV light from a portable UV lamp (350 nm) was applied 5 cm away from this mixed solution for 18 min for copolymerization. DPC-A and -B were obtained with clear yellow color solutions. The degree of polymerization was estimated by GPC with numerical average weight of approximately 100,000.

Hydrogel Preparation

DNA linker (ADL or DL), DPC-A and DPC-B were mixed in stoichiometric DNA concentrations in Tris buffer (10 mM Tris (pH 8.0), 50 mM NaCl, 10 mM MgCl_2). Crosslinked hydrogels (yellow color) or DL-crosslinked hydrogels (colorless) were formed immediately after mixing. All hydrogels were treated for 10 minutes of incubation at 50°C before tests. Other concentrations of hydrogels were prepared with direct dilution with buffer solution followed with annealing and visible light irradiation.

Encapsulation and Release of Hydrogels

The temperature of loaded hydrogels was carefully controlled by positioning the light sources at a distance that would prevent direct heating. The temperature was monitored with maximum 5 degree variation, and thermal effects were negligible. Encapsulation of loads was the same for fluorescein and gold NPs. Both materials were mixed with hydrogels by incubation at 50°C for 10 minutes. The HRP enzyme was incubated at 40°C in order to maintain bioactivity. The release of fluorescein from hydrogels was monitored by observation and imaging by the CANON SD870 digital camera. To accomplish this, 200 μM fluorescein was homogeneously dissolved in 300 μM hydrogel. 5 μL of gel mixture was placed on a transparent plastic plate with an additional 100 μL of blank buffer. Visible and UV light were applied on top of the small reservoir, respectively.

The release of gold NPs was monitored by absorption spectroscopy. The instrumentation included a Cary Bio-300UV spectrometer (Varian) and a pair of micro-square quartz cells (Starna Cells, Inc.). The release curves of gold NPs were obtained by measuring the absorption at 520 nm. On both light-driven and thermal-driven release, 20 μL of hydrogels were placed on the bottom of the quartz cuvette and allowed to sit for 10 minutes. 80 μL Tris buffer was added on top, followed by 5 to 10 minutes of visible light irradiation by a 60 W lamp and 450 nm optical filters (Asahi Technoglass). Then, either light source or water bath was applied to the loaded cells, and they were immediately transferred to UV/Vis spectrometer for absorption measurement.

The release of HRP utilized the same set of microcells. In this case, the hydrogel was placed on the bottom of a small vial, and buffer was added on top. After each light irradiation, 1 μL

supernatant was transferred to a 2 mL vial and mixed with luminol and hydrogen peroxide in buffer and stirred for 10 minutes. The emission curves were obtained by measuring the chemiluminescence at 410 nm after 10 minutes with fluorospectrometer (Fluorolog-Tau-3, Jobin Yvon, Inc.). Since the luminescence intensity is proportional to the HRP concentration, the released HRP amount could be calculated from the chemiluminescence intensity emitted from oxidation reaction.

Biocompatibility of ADL and ADL Hydrogels

ADL and ADL hydrogels were prepared with different concentrations and mixed with the same amount of CEM cells. The cytotoxicity of each cell sample was calculated by counting cell proliferation at 0, 12, 24, 48 and 72 hours. The proliferation was obtained by counting living cells under the microscope. The distribution of cells at different stages was monitored by Vybrant Apoptosis Assay Kit #2 (Invitrogen) and flow cytometry (FACScan cytometer, Becton Dickinson Immunocytometry Systems).

Cell Viability Study

DL- and ADL-crosslinked hydrogels were loaded with doxorubicin (10 mg/mL) by mixing the drug with sol state hydrogels. The hydrogels were placed inside the bottom of a small quartz cuvette with cells on top (CEM cells, 20,000/well). After irradiation by visible/UV light (only on hydrogel portion), the cells were incubated for 48 hours before viability test. The cell samples were mixed with MTS Cell Proliferation Assay, and the living cells were calculated by monitoring the absorbance at 490 nm.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1. Mechanism and design of photocontrollable DNA-crosslinked hydrogels. (a) Azo-incorporated DNA linker can crosslink the DNA-polymer conjugates and form the hydrogel (a–b). The hydrogel can encapsulate loads in the sol state. The loaded hydrogel can be reversibly controlled by visible and UV light to release previously encapsulated loads. (b) Design of ADL- and DNA-polymer conjugates. The two 12- base DNA segments form DNA-polymer conjugates, and the 24-base ADL can hybridize under proper conditions and crosslink the polymer chains, resulting in a hydrogel.

Figure 2.

Photosensitivity of ADL- and DL-crosslinked hydrogels. (a) Reversible gel-sol conversion from UV-visible irradiations on a 300 μ M hydrogel. The gel began to respond at 2 minutes and totally melted after 20 minutes. The sol state can be rapidly re-gelled by visible light (top). The same hydrogel irradiated solely by visible light displayed slight tilting. (b) Response to continuous UV light of the 300 μ M DNA linker (DL, without Azo-) crosslinked hydrogel. (c) SEM images of the dried DNA hydrogels with insertions representing amplified gel structure. The left image is a DL-crosslinked hydrogel, and the right image is an ADL-crosslinked hydrogel.

Figure 3.

Controllable release of ADL-crosslinked hydrogels loaded with different materials. (a) Model of, and size/natural effect of, different types of loads on hydrogel controllable encapsulation and release. (b) UV irradiation of fluorescein-encapsulated ADL hydrogel (300 μ M), which started to release dye molecules after 2 minutes. (c) Controllable release of 500 nm 13 nm gold NPs mixed with three different concentrations of ADL hydrogels. The mixtures were each retained inside a quartz microcell with buffer solution on top and were irradiated by visible light and UV light. The absorption at 520 nm was monitored. The absorption values were normalized by setting pure buffer absorption as 0% and 100 nM NPs solution as 100% (The right top insertion is the setting of the microcell for absorption).

measurement.). (d) Controllable release of HRP enzyme encapsulated in hydrogels quantitatively calculated by catalyzing luminol oxidation. The reaction was monitored by chemiluminescence at 410nm. However, the timeline of this experiment is not comparable to that of the gold NPs because of the different measurement procedure applied. All experiments were repeated at least three times.

Figure 4.

Thermal release of gold NPs. Gold NPs (13 nm) were mixed with hydrogels at an initial concentration of 500 nM. All hydrogels were irradiated by visible light for 10 minutes at 20 °C before increasing the temperature. The absorption at 520 nm was monitored every 10 degrees. 100% absorbance was obtained by measuring 100 nM gold NPs in buffer solution. All experiments were repeated at least three times.

Figure 5. Controlled release of doxorubicin-loaded hydrogels

(a). Photocontrollable release of doxorubicin. The drug-loaded hydrogel was kept in the dark for 20 minutes, irradiated with visible light for 20 minutes, and then irradiated with UV light for another 20 minutes. (b) Corresponding CEM cell proliferation after treatment with Dox-loaded hydrogels under different photoirradiation conditions.