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PAPER

Dextran based photodegradable hydrogels formed *via* a Michael addition†

Ke Peng,[a] Itsuro Tomatsu,[a] Bram van den Broek,[b] Chao Cui,[c] Alexander V. Korobko,[d] John van Noort,[b] Annemarie H. Meijer,[c] Herman P. Spaink[c] and Alexander Kros*[a]

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A photodegradable, covalently crosslinked hydrogel system has been constructed from the biocompatible polymers dextran and poly(ethylene glycol) using the acrylate–thiol Michael addition as the crosslinking method. Light sensitivity of the hydrogel was introduced by placing a non-toxic photolabile *o*-nitrobenzyl moiety in between dextran backbone and acrylate group. Hydrogels were prepared under physiological conditions without the need of any additional reagents by mixing solutions of dextran functionalized with acrylate-modified *o*-nitrobenzyl moieties (Dex-AN) and dithiolated poly(ethylene glycol) (DSPEG). The degradation of the hydrogels due to UV irradiation was investigated with scanning electron microscopy, infrared and UV-vis spectroscopy. Using green fluorescent protein (GFP) as a model protein, light triggered protein release from the obtained gel matrices was investigated in different forms. Furthermore, photodegradation of the hydrogel *via* two photon excitation was also examined using focused pulsed near infrared (NIR) laser beam as a light source.

Introduction

Hydrogel materials are widely used in a diverse range of applications,[1] especially for drug delivery, biosensing, and tissue engineering, due to their excellent biocompatibility and capability as a container for fragile bioactive molecules such as proteins.[2,3] Recently we have developed hydrogel systems composed of the biocompatible polymers, dextran and poly (ethylene glycol) (PEG), which were crosslinked *in situ* to form hydrogels.[4,5] The excellent biocompatibility of the obtained gel was demonstrated *in vivo* using a zebrafish embryo assay.[5,6]

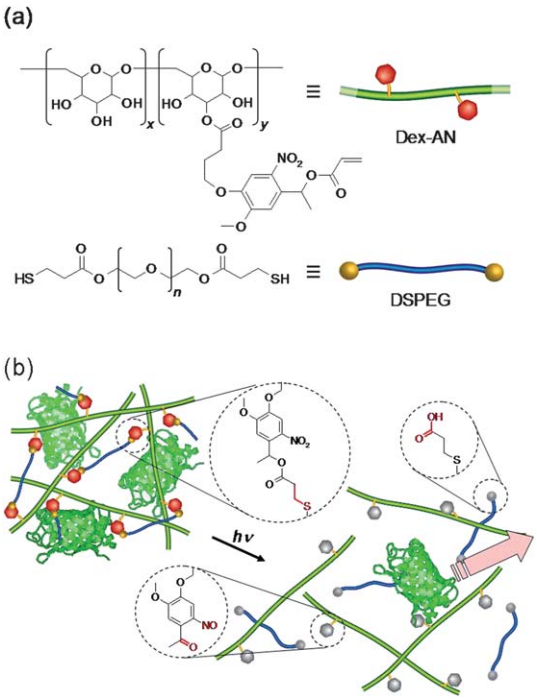
One of the main issues in current biomaterial science is to control the properties of hydrogels (*e.g.* swelling rate, permeability and mechanical strength) with external stimuli such as pH, temperature, and light.[7–11] For example, these stimuli-responsive hydrogels are beneficial for an efficient drug delivery system.[12] Light is an ideal stimulus to manipulate the hydrogel, which is a remote stimulus that can be controlled spatially and temporally with great ease and convenience.[13–15] Moreover, the light irradiation except deep-UV does not have a harmful effect on various bioactive compounds including most of the proteins.[16–18] Therefore, light sensitive hydrogels are attracting great attention and a variety of systems are being developed.[19–27]

Recently, Anseth *et al.* developed a series of covalently crosslinked photodegradable hydrogels and demonstrated that the migration of stem cells in the hydrogel network can be controlled *via* photodegradation.[28,29] For this they used free radical co-polymerizations of water-soluble acrylate monomers and photodegradable difunctionalized crosslinkers. Inspired by their work, we have introduced light sensitivity to our *in situ* forming hydrogel systems.[4,5] With our method, hydrogels having similar properties can be obtained from biocompatible polymers and prepared under physiological conditions without the need of any additional reagents (*e.g.* radical initiators or catalysts, which are potentially toxic) and no side-products are formed. Therefore, we believe that this strategy for fabrication of covalently crosslinked hydrogels is promising for future biomedical applications (Scheme 1).[30–32]

In the current system, a hydrogel was formed by mixing two solutions: dextran functionalized with acrylate-modified *o*-nitrobenzyl moieties (Dex-AN) and dithiolated poly(ethylene glycol) (DSPEG) *via* a Michael addition between the acrylate and thiol groups. The photolabile *o*-nitrobenzyl moiety was introduced between the dextran and PEG backbones, thus other than unconnected polymers, no small-molecule fragments are generated after photodegradation, which decreases the risk of toxic side effects. Furthermore, the biocompatibility of the

[a]*Leiden Institute of Chemistry, Leiden University, P.O. Box 9502, 2300 RA Leiden, The Netherlands. E-mail: a.kros@chem.leidenuniv.nl; Fax: +31 71527 4397; Tel: +31 71 527 4234*
[b]*Physics of Life Processes, Leiden Institute of Physics, Leiden University, Niels Bohrweg 2, 2333 CA Leiden, the Netherlands*
[c]*Institute of Biology, Leiden University, P.O. Box 9502, 2300 RA Leiden, The Netherlands*
[d]*Nano Structured Materials, TU Delft, Julianalaan 136, 2628 BL Delft, The Netherlands*

† Electronic supplementary information (ESI) available: Movies of the microscopic hydrogel degradation under UV irradiation, and movements of quantum dots in the hydrogel matrix before and after photodegradation *via* two-photon excitation. See DOI: 10.1039/c1sm05291h



Scheme 1 Chemical structures of the components of *in situ* forming photodegradable hydrogel: dextran functionalized with acrylate-modified *o*-nitrobenzyl moieties (Dex-AN) and dithiolated poly(ethylene glycol) (DSPEG). Upon mixing, these underwent Michael addition between acrylate and thiol groups to form a light sensitive hydrogel. Upon the irradiation, the hydrogel was decomposed due to the cleavage of the photolabile moiety, which triggered the release of the protein (GFP) entrapped in the polymer network.

o-nitrobenzyl moiety has been proven; both the residues before and after photodegradation and the degradation process are inert for fragile biomacromolecules including proteins.[33–36] Herein we report the preparation of both macroscale and microscale hydrogels and hydrogel degradations upon UV irradiation (365 nm). As a possible application of these hydrogels, the capability of protein storage and light triggered release using green fluorescent protein (GFP) as a model protein were also examined. Upon the light-induced degradation of the hydrogel matrix, the entrapped proteins were released from the matrix and migrated into the media. Furthermore, besides with UV light, photodegradation by two-photon excitation using near infrared (NIR) light was tested to broaden the applicable field of the obtained hydrogel system in future.

Results and discussion

Synthesis of Dex-AN and DSPEG

Dex-AN was prepared by a conventional carbodiimide mediated esterification of the hydroxyl groups of dextran (20 kDa) with 4-(4-(1-(acryloyloxy)ethyl)-2-methoxy-5-nitro-phenoxy)butanoic acid (AN).[28,37] The obtained Dex-AN was characterized by ^{1}H NMR, as shown in Fig. 1, besides signals attributed to dextran, signals at 7.6, 7.1, 6.4–6.0, 4.1 and 3.9 ppm due to the protons from the AN group were observed which indicated the functionalization of dextran with AN. The AN derivatization was

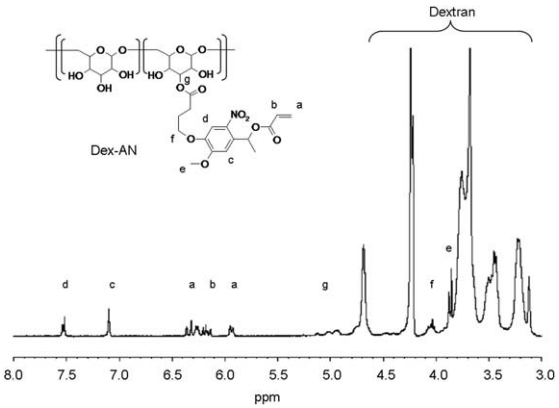


Fig. 1 ^{1}H NMR spectrum of Dex-AN (DMSO-d_6 containing 5% D_2O). The chemical structure shows the substitution of AN at C-3 position of glucose unit as a possible structure.

further confirmed by the presence of small peaks around 5.0 ppm (peak g) due to the peak shift of glucosidic protons of the anhydroglucose units upon esterification. The degree of substitution (the number of substitutes per 100 anhydroglucose units) was determined to be 10 according to the ratio between the signal at 7.1 ppm corresponding to AC and the dextran anomeric proton at 4.7 ppm. The DSPEG was synthesized as reported previously and the Ellman test showed a thiol functionality of 90%.[38]

Macroscale hydrogel formation

Hydrogels were obtained by mixing the same amounts of solutions of Dex-AN (200 mg mL^{-1}) and DSPEG (120 mg mL^{-1}) in phosphate buffered saline (PBS). Upon mixing the solutions, gelation occurred within 60 min at 37 °C. The ratio between acrylate and thiol groups was 1 : 1 and the concentrations of the polymers were 9 wt% and 5 wt% for Dex-AN and DSPEG, respectively.

The mechanical property of the obtained hydrogel was characterized with rheometry; the dependences of storage (G') and loss (G'') moduli on angular frequency (ω) were measured. As shown in Fig. 2, after mixing of Dex-AN and DSPEG, it showed a dominant of G' than G'' in the frequency range measured. This observation and nearly parallel behavior of G' and G'' at low

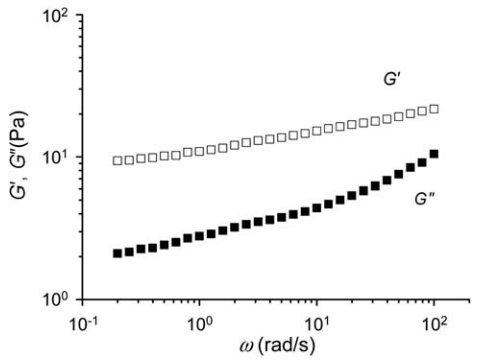


Fig. 2 Dynamic moduli of the hydrogel obtained by mixing of solutions of Dex-AN (30 mg in 150 μL PBS) and DSPEG (18 mg in 150 μL PBS).

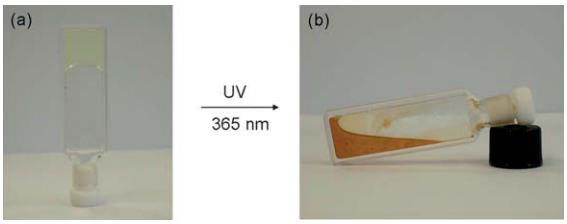


Fig. 3 Photographs of a mixture of 50 μL of Dex-AN (200 mg mL^{-1}) and 50 μL of DSPEG (120 mg mL^{-1}) in PBS: taken after mixing (a) and after irradiation with UV light for 72 hours (b).

frequencies indicate that DSPEG crosslinked Dex-AN *via* a thiol–acrylate reaction resulted in the formation of hydrogel.

Photodegradation of the macroscale hydrogel

The photodegradation of the freshly prepared hydrogel (100 μL, 1 cm × 1 cm × 1 mm) was shown in Fig. 3. After UV irradiation of 72 hours, the gel was degraded to a fluidic solution due to the hydrolysis of the ester group in the *o*-nitrobenzyl moiety resulting in the dissociation of the polymer network (Scheme 1).

To investigate the photodegradation of the macroscale hydrogel, lyophilized samples of the freshly prepared and degraded hydrogels were subjected to scanning electron microscopy (SEM). As shown in Fig. 4a freshly prepared hydrogels showed a regular microporous structure, indicating the existence of a well extended homogeneous three-dimensional polymer network before removing the water molecules. In contrast, dense amorphous solids were observed from the photodegraded hydrogel (Fig. 4b), which is similar to the one obtained from a dextran or PEG solution in the absence of crosslinking.

Using the lyophilized samples of the freshly prepared and degraded hydrogels, infrared spectroscopy (FT-IR) was also performed (Fig. 5). FT-IR analysis revealed only a marked difference after irradiation in the band at 1520 cm^{-1}, which is ascribed to the asymmetric stretch of NO_2. This indicates the reduction of the NO_2 to NO groups which is expected upon the degradation of the *o*-nitrobenzyl moiety. The integrity of the dextran and PEG polymers was not affected upon the irradiation as expected (see Fig. 5).[39]

The degradation of the *o*-nitrobenzyl moiety was also monitored by UV-vis spectroscopy at diluted conditions as shown in Fig. 6. The absorbance maximum at 350 nm decreased upon UV irradiation, while the intensity around 280 and 430 nm increased

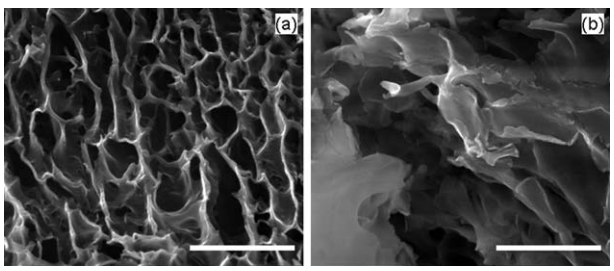


Fig. 4 SEM images of the freeze-dried hydrogel prepared from Dex-AN and DSPEG in pure water: before (a) and after UV light irradiation (b). Scale bars represent 5 μm.

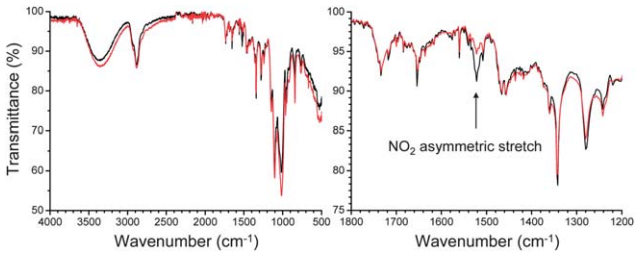


Fig. 5 FT-IR spectra of the freeze-dried samples of the hydrogel (black line) and the photodegradated hydrogel (red line). In the enlarged spectra (right), a decrease of a signal ascribable to NO_2 asymmetric stretch can be seen.[39]

with isosbestic points at 301 and 387 nm and the reaction reached a steady state after 120 min.[40,41]

Protein release from the macroscale hydrogel

Our previous hydrogel system showed excellent biocompatibility *in vivo*,[5] and we have now introduced photoresponsivity into the hydrogels, thus we believe that the current system can be beneficial for the future biomedical applications, for example, as a photoresponsive protein carrier. Since hydrogels has a structural similarity with hydrated body tissues and allows to retain the proteins in the protective 3-D network and prevents them from decomposition and denaturation, hydrogels can be an ideal material.[2,42] Moreover stimuli responsive hydrogels hold the potential to transport entrapped protein to the target site and to trigger the release by a stimulus at the desired point and time are drawing more attentions.[10] Furthermore as a number of attractive therapeutic proteins have been developed recently against a broad range of diseases such as cancers, autoimmune diseases and metabolic disorders,[43] development of containers that can handle proteins becomes more important.

The capability of protein storage and light triggered release from the obtained hydrogels were examined using green fluorescent protein (GFP) as a model protein. The GFP carrying macroscale gel (~10 μL) was placed in the bottom of a 1 cm^2 cuvette with 1 mL of fresh PBS and the fluorescence intensities of the solution part were measured as a function of time (Fig. 7). Under the UV light irradiation, the emission intensities of GFP in PBS began to increase immediately, indicating a fast start of

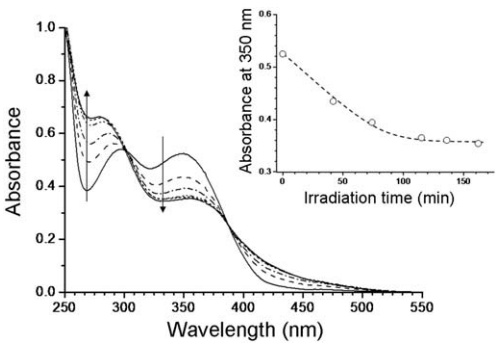


Fig. 6 UV-vis spectra measured at diluted condition after different irradiation times (0–160 min). Inset: the obtained absorbance at 350 nm was plotted as a function of irradiation time.

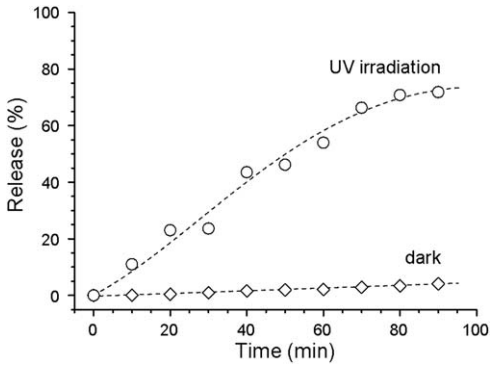


Fig. 7 Release of green fluorescent protein (GFP) from the macroscale hydrogel. The samples were placed under UV light (circle) and dark (diamond), respectively.

the release. The release of GFP can be continued; ~50% of the loaded proteins were released in 60 min under UV irradiation and reached *ca.* 70% in 90 min. In contrast, no significant increase in the release of GFP into the surrounding solution was observed without UV light irradiation even after 90 minutes (*ca.* 5%). Therefore we conclude that the release of GFP was in a UV light responsive manner (Scheme 1b).

Microscale hydrogel preparation and degradation

Since the degradation of the macroscopic hydrogel requires long time UV exposure, microscale hydrogel particles were prepared using PDMS mold in order to increase the speed of degradation.[44]

Sub-millimetre scale hydrogel blocks with a volume of approximately 5 nL were prepared and submersed in a PBS solution (100 μL). The photodegradation of the hydrogel was then studied with optical microscopy. The complete degradation of a hydrogel particle was observed within 30 minutes of irradiation, while in the absence of radiation no changes were observed (see Movie 1 in the ESI†).

Furthermore, GFP loaded microscale hydrogel blocks were also prepared by mixing a Dex-AN solution containing GFP and a DSPEG solution. The release from the hydrogel was monitored using fluorescence microscopy. When the gel was kept in the dark, the shape of the fluorescent block was not changing within 30 minutes (Fig. 8), showing that the GFP was firmly encapsulated in the hydrogel matrix. In contrast, UV exposure (365 nm) of the hydrogel led to a rapid decrease in fluorescence indicative of the loss of GFP. After 30 min of irradiation, the hydrogel disappeared completely, suggesting that the polymer network was eroded and the GFP was liberated into the surrounding medium. The decrease of fluorescence of the GFP-loaded gels was plotted as a function of time. Under irradiation (circle), the fluorescence decreased significantly in the first 10 minutes as most of the GFP was released concurrently with the erosion of the polymer network. In the control experiment without UV irradiation (diamond) only a small decrease was observed in the first 6 min presumably due to the loss of loosely bound GFP on or near the surface of the hydrogel, after which the fluorescence stabilized showing that the encapsulated GFP remained inside the hydrogel matrix. It should be noted here that 10 minutes of irradiation with UV light is feasible for experiments in living zebrafish embryos.[45–47] Thus we believe that the current system can also be applied for *in vivo* use in zebrafish embryos and other transparent animals by adjusting the size of the hydrogel.

To exclude photo-bleaching of the GFP as a possible cause for the observed fluorescence decrease, the stability of GFP under UV irradiation was examined. As shown in Fig. 9, the fluorescent intensity of the GFP solution was preserved after 30 min of UV irradiation. Thus the observed fluorescence decrease in Fig. 8 was due to the decrease in the concentration of GFP (*i.e.* degradation of the hydrogel) and not to photo-bleaching of the GFP itself.

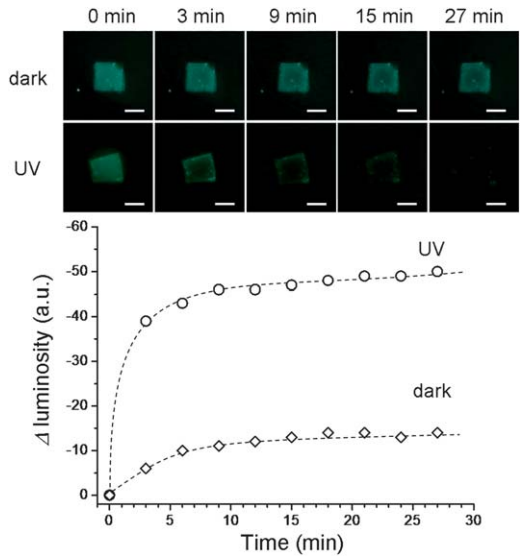


Fig. 8 Release of GFP from the gel matrices upon the irradiation with UV light. Pictures show representative states of the gel matrices for both samples kept in the dark and under UV irradiation taken with fluorescence microscope. Scale bars represent 200 μm. The difference of the luminosities of the gels from the original state is calculated from the pictures and plotted as a function of time.

Two-photon hydrogel degradation

It has been shown that *o*-nitrobenzyl moieties can not only be photocleaved by 365 nm UV light, but also by two-photon

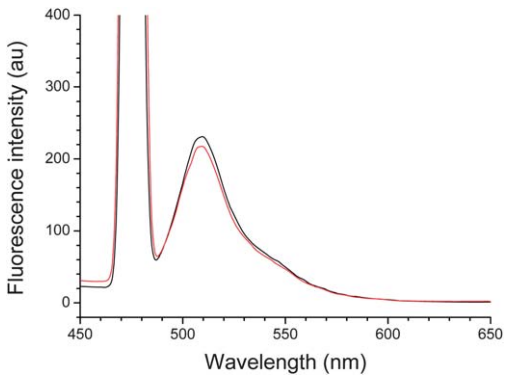


Fig. 9 Fluorescence spectra of a GFP solution (2 μg mL^{-1}) in PBS measured before (black line) and after 30 min of UV-irradiation (red line). The GFP fluorescence did not change significantly by UV irradiation (*ca.* 95% of the emission at 509 nm).

excitation.[48,49] Here, a focused pulsed near infrared (NIR) laser beam (740 nm) is used to trigger simultaneous absorption of two photons. Since cells and tissues are more transparent to near infrared light than UV light,[50] protein release from hydrogels by two-photon degradation holds broader application in the biomedical field than that by UV exposure.[28,51] Furthermore two-photon excitation only occurs at the focal point which can proceed photodegradation locally. Thus it is beneficial to obtain three dimensional pattern in the matrix, which will be important for the next generation of tissue engineering.[52,53]

In order to test whether the hydrogel can also be degraded by two-photon excitation using NIR light, we prepared a hydrogel containing quantum dots and illuminated in a multifocal laser scanning system.[55] Fluorescence images of the illuminated area (25 × 25 μm) were observed with a low power 532 nm solid state laser before and after the two-photon irradiation. At the start of the experiment all quantum dots were immobilized in the gel matrix (see Movie 2 in the ESI†). After 5 minutes of two-photon irradiation many quantum dots were clearly diffusing within the irradiated area (see Movie 3 in the ESI†) indicating erosion of the polymer network. In control experiments with CW mode and with our previous hydrogel[5] which was not photoreactive, no change in quantum dot movement was observed after 5 minutes of NIR irradiation. Thus we conclude that indeed the polymer network can be eroded by a two-photon process.

Conclusions

In summary, we have introduced light responsiveness in an *in situ* forming, covalently crosslinked, hydrogel system composed of the biocompatible polymers dextran and poly(ethylene glycol). The current system which is functionalized with a photolabile *o*-nitrobenzyl moiety can form a hydrogel under physiological conditions without the need of any additional reagents. Macroscale and microscale hydrogels were prepared and their photodegradations were investigated. Using the photolysis of the obtained hydrogel, light triggered release of proteins from the gel matrices was tested with green fluorescent protein as a model protein. Furthermore, photodegradation of the hydrogel *via* two photon excitation was examined using focused pulsed NIR laser beam. It is envisaged that the obtained hydrogel can be used for the delivery of bioactive molecules and also for various other applications such as cell culturing because of the unique combination of the properties of the current system, *i.e.* biocompatibility, convenient gel formation and photoresponsivity.

Experimental

Materials

N,*N*′-Dicyclohexylcarbodiimide (DCC), *p*-toluenesulfonic acid monohydrate, 3-mercaptopropionic acid (Fluka), 4-hydroxy-3-methoxyacetophenone, 4-(dimethylamino)pyridine, ethyl 4-bromobutyrate, acryloyl chloride, dithiothreitol (DTT) (Aldrich), and polyethylene glycol (PEG, $M_w = 2000$) (Merck) were used as received. 4-(Dimethylamino)pyridinium 4-toluenesulfonate (DPTS) was synthesized from 4-(dimethylamino)pyridine and *p*-toluenesulfonic acid monohydrate and recrystallized from toluene. Dextran ($M_w = 20\,000$, Pharmacia Fine Chemicals) was dried in the vacuum oven for several days before use.

Synthesis

4-(4-(1-(Acryloyloxy)ethyl)-2-methoxy-5-nitrophenoxy)butanoic acid (AN) was prepared according to the procedure as reported.[28,37] Briefly, 4-hydroxy-3-methoxyacetophenone was alkylated with ethyl 4-bromobutyrate in DMF in the presence of K_2CO_3. Nitration was performed by treating with HNO_3. The ketone was reduced with $NaBH_4$ and followed by hydrolysis of the ethyl ester with 1 M NaOH. The desired compound AN was obtained from the reaction with acryloyl chloride in the presence of triethylamine. The product was purified by silica gel column chromatography. ^{1}H NMR (400 MHz, $CDCl_3$): δ 1.7 (3H, d, $J = 6.4$), 2.2 (2H, m), 2.6 (2H, t, $J = 7.2$), 3.9 (3H, s), 4.1 (2H, t, $J = 6.4$), 5.9 (1H, d, $J = 1.2$), 6.2 (1H, m), 6.4 (1H, d, $J = 1.2$), 6.5 (1H, q, $J_1 = 6.4$, $J_2 = 6.4$), 7.3 (1H, s), 7.6 (1H, s), 10.5 (1H, br); ^{13}C NMR (100 MHz, $CDCl_3$): δ 21.8, 23.8, 30.1, 56.1, 67.9, 68.4, 108.0, 108.8, 128.0, 131.3, 133.1, 139.5, 147.0, 153.9, 164.8, 178.8.

Dex-AN was prepared by an esterification of the hydroxyl groups of dextran with AN using the procedure that we reported previously.[4] 0.5 g of AN was dissolved in 10 mL of DMSO, followed by the addition of DCC (0.5 g) and DPTS (0.07 g). To the mixture, dextran (0.7 g) solution in DMSO (10 mL) was added and stirred for 36 hours at room temperature. After filtration, the filtrate was put into a dialysis tube (MWCO 3500) and dialyzed against DMSO and water. After lyophilization, 0.4 g of the product was recovered.

Dithiol functionalized poly(ethylene glycol) was prepared by following previously reported procedures.[5,54]

Macroscale hydrogel formation

Macroscale hydrogels were obtained by mixing solutions of Dex-AN and DSPEG. The typical procedure is as follows: 150 μL Dex-AN (20 mg) and 50 μL DSPEG (12 mg) in phosphate-buffered saline (PBS) solutions were mixed by vortexing (the ratio of acrylates to thiols was 1 : 1), and the resulted solution was incubated at 37 °C in the dark. The gelation time was determined to be 60 min by the vial tilting method. (When there was no flow of the sample within 5 seconds, it was regarded as a gel.[54])

Sample for visco-elastic measurements was prepared by mixing two solutions of Dex-AN (30 mg in 150 μL PBS) and DSPEG (18 mg in 150 μL PBS). To probe viscoelastic properties of the gel, a small sample of the hydrogel was analyzed using a TA Instruments AR-G2 rheometer with plate–plate geometry (plate of 40 mm in diameter and a 220 micron gap distance). Prior to the measurements, the strain–sweep tests were performed on the sample to determine the limits of the linear viscoelastic regime. Data acquisition started when steady state of the gel mechanical stress was reached, as indicated by normal forces. Frequency sweeps were done between 0.1 and 100 rad s^{-1} in the linear response regime at 37 °C.

Macroscale hydrogel degradation

A handheld UV lamp (100 W, Black-Ray) at the wavelength of 365 nm was used for all the light irradiation with a fixed distance of 30 cm between the sample and the lamp.

Scanning electron microscopy (SEM) was conducted on a Nova NanoSEM (FEI) with an accelerating voltage of 10 kV

and a spot size of 3.5. Sample was coated with carbon before measurements.

The FT-IR spectra were obtained using a Perkin Elmer Paragon 1000 FT-IR spectrometer.

Samples for both FT-IR and SEM were prepared as follows: 150 μL Dex-AN (20 mg) and 50 μL DSPEG (12 mg) in water solutions were mixed by vortexing, and the resulted solution was incubated at 37 °C in the dark for 60 min and then kept in the dark overnight at room temperature. The formed gel was divided into 2 portions: one was freeze dried immediately and the other was kept under UV irradiation for 72 hours before freeze drying.

UV-vis spectra were recorded on a Cary 3 Bio UV-vis spectrometer. The sample was prepared as follows: Dex-AN (1.2 mg) was dissolved in 600 μL PBS and then a solution of DSPEG (0.6 mg) in 600 μL PBS was added. The mixture was vortexed and incubated at 37 °C in the dark for 60 min. The sample was then put under the UV irradiation.

Protein release from the macroscale hydrogel

Dex-AN (5 mg) and DSPEG (3 mg) were dissolved in 50 μL PBS containing green fluorescent protein (GFP, 0.1 μg μL^{-1}) and vortexed, the resulted solution was incubated at 37 °C in the dark overnight. The hydrogel (11 mg) was placed into a 1 cm quartz cuvette, centrifuged for 10 minutes to form a square at one of the bottom corners of the cuvette. After gentle washing with 1 mL PBS, 1 mL of fresh PBS was added. The released amount of GFP was monitored through the fluorescence from the solution part.

Fluorescence measurements were performed using a luminescence spectrometer LS50B (Perkin Elmer). All spectra were obtained at room temperature. Each spectrum was measured with the excitation and emission slits of 5 nm. The excitation wavelength was 475 nm.

The emission spectra were recorded every 10 min while the cuvette was shaking at 50 rpm. After the experiment, the cuvette was vortexed vigorously to make a homogeneous solution and check the fluorescence intensity of 100% release.

Microscale hydrogel preparation and degradation

An aliquot of the formed hydrogel was taken out and mounted onto a poly(dimethylsiloxane) (PDMS) mold (created by using a metal master square pillars of 300 × 300 × 50 μm). The obtained hydrogel piece was then taken out from the mold to a Petri dish and 100 μL of PBS was added on top of the gel. The degradation process of the hydrogel under UV irradiation was monitored by a Leica MZ16FA stereo fluorescent microscope using bright field. Movies were recorded every 4 min with a pause of 1 min between each. The movie shown in the ESI† is in a combined form of three succeeding runs and 10 times faster than the real-time recording rate.

Protein release from the microscale hydrogel

100 μL of GFP solution (0.1 μg μL^{-1}) was added to 50 μL Dex-AN (20 mg) in PBS solution, and then 50 μL PBS solution of DSPEG (12 mg) were added and vortexed. After incubation at 37 °C in the dark for 60 min, an aliquot of the formed hydrogel was taken out and mounted onto the PDMS mold. The obtained hydrogel piece was then taken out from the mold to a Petri dish and 100 μL of PBS was added on top of the gel. The release profile was monitored with the Leica MZ16FA stereo fluorescent microscope by taking picture of the hydrogel every 3 min. Luminosity of the gel was determined as the mode value of the central part of the gel (200 × 200 pixels, whole gel piece was typically about 400 × 400 pixels) after subtracting the background value.

Two-photon hydrogel degradation

In order to test whether, besides with 365 nm UV light, the hydrogel can also be degraded by two-photon excitation using NIR light, we use the following approach. A custom-build multifocal laser scanning two-photon microscope is used to illuminate a rectangular area of 25 × 25 μm in a hydrogel containing streptavidin-conjugated quantum dots (Qdot 565, Invitrogen). The wavelength of the laser was 780 nm. Since two-photon absorption depends on the square of the illumination intensity, only a thin slice of the hydrogel with thickness ~1 μm is expected to be degraded. A 3D scan through the sample showed that quantum dots were only moving a 1 μm thick slice (data not shown).

Fluorescence images of the same area were acquired using a low power 532 nm solid state laser (Cobolt Samba) before and after the two-photon irradiation. To exclude the argument that the local heating of the gel would cause the liberation of the quantum dots, we repeated the experiment with the laser set in CW mode. This way, the peak intensity is dramatically reduced to far below the two-photon absorption threshold, while the overall laser power remains the same.

Acknowledgements

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