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PAPER

A multi-photoresponsive supramolecular hydrogel with dual-color fluorescence and dual-modal photodynamic action

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We have developed an engineered supramolecular hydrogel formed, in the absence of any toxic solvents or reagents, by the self-assembly of four different components: a poly-β-cyclodextrin polymer, a hydrophobically modified dextran, a commercial zinc phthalocyanine and a tailored nitric oxide photodonor. The formation of this supramolecular assembly is based on a "lock-and-key" mechanism in which the alkyl side chains of the modified dextran form inclusion complexes with the cyclodextrin cavities of the poly-β-cyclodextrin polymer. The multivalent character of the interactions between the all components ensures the stability of the hydrogel and the negligible leaching of the photoactive components from the gel network under physiological conditions, even in the absence of protective coating agents. The combination of steady-state and time-resolved spectroscopic techniques together with photoamperometric measurements shows that the two chromo-fluorogenic components do not interfere with each other while being enclosed in their supramolecular matrix and can thus be operated in parallel under control of light stimuli. Specifically, excitation with visible light results in the red and green fluorescence emission typical of the two photoactive centers and in their capability to generate singlet oxygen and nitric oxide, two cytotoxic species playing a key role in photodynamic cancer and bacterial therapies.

Introduction

Hydrogels are three-dimensional hydrophilic polymer networks which can be prepared by exploiting the formation of either covalent bonds or physical interactions such as hydrophobic, electrostatic or hydrogen bonding.[1] The presence of the network confers to hydrogels integrity and good mechanical properties. Besides, the high water content and low surface tension make hydrogels highly flexible and biocompatible materials and, as a consequence, very suited for a variety of applications.[2] In this scenario, hydrogels that respond to external light stimuli are particularly intriguing and have emerged as "smart" materials, especially for biomedical delivery applications.[3] Light is a highly orthogonal external stimulus and in view of its easy manipulation, in terms of intensity, wavelength, duration and localization, represents the most elegant trigger for the release of therapeutic agents with an exquisite spatiotemporal control.[4] In addition, light triggering is "biofriendly", provides fast reaction rates and offers the great benefit of not affecting physiological parameters such as temperature, pH and ionic strength, which is a fundamental requisite for biomedical applications. Photoresponsive character can be imposed on hydrogels with the covalent introduction of appropriate chromophores in the individual macromolecular components.[5] Alternatively, photoresponsive groups can be encapsulated noncovalently in the hydrophobic compartments of the hydrogel.[6] In both cases, the optical transparency of hydrogel scaffolds permits the facile irradiation of the photoactive species entangled therein.

Singlet oxygen ($^1O_2$) and nitric oxide (NO) represent two of the main cytotoxic species that can be produced upon light excitation of suitable photosensitive compounds. In particular, $^1O_2$ is the actual active agent in photodynamic cancer and bacterial therapy (PDT)[7] and is photogenerated by energy transfer between the lowest triplet state of photosensitizers such as porphyrins or phthalocyanines, and molecular oxygen.[8] NO has recently come to the limelight not only for its pivotal role in the maintenance and regulation of vital functions[9] but also for its promising anticancer activity and antibacterial activity.[10] This inorganic free radical can be precisely delivered by means of suitable NO photodonors.[11] $^1O_2$ and NO share some common key features such as small size and absence of charge, capability to attack biological substrates of different nature (*i.e.,* lipids, proteins, and DNA), absence of multidrug resistance and confinement of their action to short distance. In this view the combination of $^1O_2$ with NO represents in principle an ideal strategy in view of bimodal treatments.[12] The validity of this approach is withnessed by a number of multifunctional photoactivable nanoconstructs reported by us and others in recent years.[11d,13] While hydrogels photogenerating either $^1O_2$[14] or NO[15] have been recently achieved, no examples of hydrogel photoreleasing simultaneously these two cytotoxic species are known to date. The visualization of the photoactive chromophores encapsulated in the hydrogel through fluorescence techniques represents an indispensable requisite in view of the non invasive monotoring of the gel localization and the image-guided phototherapy.[16] However, only a few examples of gel applications in this field have been reported so far.[17] On the basis of these considerations, the

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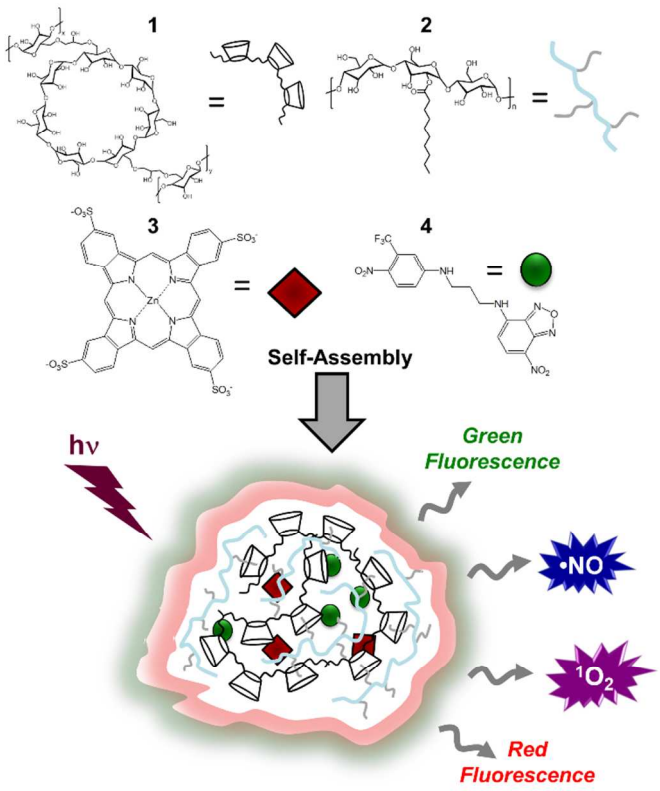
creation of a photoresponsive hydrogel combining dual therapeutic release with dual fluorescence imaging capacity is a very challenging objective to pursue.

Although research has mainly focused on macroscopic hydrogels, there is now an upsurge of interest in hydrogels confined to micro- and nanoscopic space regime (micro- and nanogels).[8] In this context, soft self-assembled hydrogels containing cyclodextrins have shown potential application as injectable matrix for the sustained delivery of poorly soluble drugs.[18b] Thus, a procedure has been recently developed to obtain supramolecular micro- and nanogels in the absence of any toxic solvents or reagents by the self-assembling of two hydrosoluble polymers: a poly-β-cyclodextrin polymer (**1**) and a dextran modified with alkyl side chains (**2**) (Scheme 1).[19] Depending on the operating conditions, either micro- and nanogels could be formed.[19] The formation of these supramolecular structures is based on a "lock-and-key" mechanism in which practically all the dextran alkyl side chains are included into some β-cyclodextrin (β-CD) cavities of polymer **1**, leaving most of them still available for further complexation. This offers the possibility to include additional guests such as conventional and photoactivated drugs,[19,15a] and contrast agents,[20] leading to functional hydrogels with great potential in drug delivery and diagnostic applications. Moreover, the low toxicity of the hydrogels after intramuscular administration in rabbits makes them promising devices for local delivery of drugs.[18b] Besides, we have very recently shown that polymer **1** is able to co-encapsulate both the phthalocyanine **3** and the tailored NO photodonor **4** (Scheme 1), leading to water soluble nanoparticles (NPs) exhibiting a dual color red and green fluorescence and able to photogenerate simultaneously $^1O_2$ or NO inducing amplified cancer cells death.[21] Encouraged by these findings we combine herein the above strategic approaches and demonstrate that a multi-photoresponsive supramolecular hydrogel exhibiting dual color fluorescence and dual photodynamic properties can be easily achieved by the self-assembling of all the components illustrated in Scheme 1. This paper deals with the preparation and spectroscopic and photochemical characterization of the hydrogel while the photobiological activity will be reported separately in due course.



Scheme 1. Schematic representation of the multi-photoresponsive supramolecular hydrogel and molecular structures of the compounds used in this work.

Experimental

Chemicals

Poly-β-cyclodextrin polymer (**1**) was prepared by crosslinking β-cyclodextrin (β-CD) with epichlorohydrin (EP), under strong alkaline conditions, following a described method.[22]The β-CD content in **1** was 70% w/w, according to the ^{1}H NMR spectra. The average molar mass of polymer **1** was 7×10^5 g/mol, as determined by size exclusion chromatography using pullulan standards. Dextran bearing lauryl side chains **2** was prepared by grafting lauroyl chloride to the dextran polymer and subsequently purified by precipitation and dialysis, according to a previously described procedure.[19] The substitution yield of MD was determined according to the ^{1}H NMR spectra and was found to be 4.3%, in agreement with the amount of lauroyl chloride introduced in the reaction mixture. The macrocyclic zinc phthalocyaninetetrasulfonate **3** was purchased from Frontier Scientific and used without further purification. The tailored NO photodonor **4** was synthesized according to recently reported procedure.[21] Phosphate buffer (10 mM, pH 7.4) was prepared with biological grade reagents and all solutions were prepared with nanopure water (18 MW).Unless stated otherwise, all other chemicals were obtained from commercial sources and used as received. All solvents used were spectrophotometric grade.

Samples and gel preparation

Solution of **1** was prepared by stirring 1h at R.T. 75 mg mL^{-1} of **1** in phosphate buffer (PB) at pH 7.4. Solution of **3** in the presence of **1** was prepared by stirring for 7 h at room temperature a mixture obtained by adding 10 µl of a stock solution of **3** in PB to 2.99 mL of the solution of **1**. Solution of **4** in the presence of **1** was obtained as follows: compound **4** was dissolved in methanol and slowly evaporated to form a thin film. This film was then hydrated with an aqueous solution of **1** in PB. The mixture was stirred over night at RT. Solution of both **3** and **4** in the presence of **1** (solution **A**) was obtained as follows: the film of **4** was hydrated with an aqueous solution **3** in the presence of **1**. The mixture was stirred over night at RT. Solution of **2** was prepared by stirring overnight 75 mg mL^{-1} of the modified dextran **2** in PB (solution **B**). The supramolecular gel was prepared by simply pouring slowly (to avoid the formation of bubbles) at room temperature 2 mL of solution **A** to 1 mL of solution **B** and stirring the mixture with a magnetic stirrer for 2 min. The magnet was then removed and the gel was kept in the dark for stabilization for 30 min. After the removal of the supernatant the gel was ready for use.

Instrumentation

Steady state absorption, emission and irradiation. UV/Vis absorption and fluorescence spectra were recorded with a Jasco V 650 spectrophotometer and Fluorolog-2 (Model, F-111) spectrofluorimeter, respectively. The reference sample for the absorption spectra was a phosphate buffer (10 mM, pH 7.4) solution. Irradiation to detect NO release and $^1O_2$ was performed in a quartz cell (1 cm path length, 3 mL capacity) by using the monochromatic radiations at 420 nm and 680 nm, respectively, of the same spectrofluorimeter as the light sources.

Laser flash photolysis. All of the samples were excited with the second harmonic of Nd–YAG Continuum Surelite II–10 laser (532 nm, 6 ns FWHM). The excited solutions were analyzed with a Luzchem Research mLFP–111 apparatus with an orthogonal pump/probe configuration. The probe source was a ceramic xenon lamp coupled to quartz fiber-optic cables. The laser pulse and the mLFP–111 system were synchronized by a Tektronix TDS 3032 digitizer, operating in pre-trigger mode. The signals from a compact Hamamatsu photomultiplier were initially captured by the digitizer and then transferred to a personal computer, controlled by Luzchem Research software operating in the National Instruments LabView 5.1 environment. The sample temperature was 295 ± 2 K. The energy of the laser pulse was measured at each shot with a SPHD25 Scientech pyroelectric meter. The measurements in solution were performed in a 1 cm path length quartz cell. The measurements on the hydrogel were performed placing the gel between two quartz slides (1 cm x 3 cm) and placing the sample at 45° with respect to both the excitation and probe beams.

Nitric oxide detection. NO release was measured with a World Precision Instrument, ISO-NO meter, equipped with a data acquisition system, and based on direct amperometric detection of NO with short response time (< 5 s) and sensitivity range 1 nM–20 μM. The analog signal was digitalized with a four-channel recording system and transferred to a computer. The sensor was accurately calibrated by mixing standard solutions of $NaNO_2$ with 0.1 M H_2SO_4 and 0.1 M KI according to the reaction:

$$4H^+ + 2I^- + 2NO_2^- \rightarrow 2H_2O + 2NO + I_2$$

NO measurements were carried out with the electrode positioned outside the light path in order to avoid NO signal artifacts due to photoelectric interference on the ISO-NO electrode.

Singlet oxygen assay

For the detection of this species we used the well-known spectrophotometric method based on the bleaching of the *N,N*-dimethyl-4-nitrosoaniline (RNO) in the presence of imidazole.[23] Basically, photogenerated $^1O_2$ reacts with imidazole to form a transannular peroxide which bleaches the RNO at its absorption maximum (λ_{max} = 440 nm). The degree of bleaching is directly proportional to the production of $^1O_2$. A solution containing *N,N*-dimethyl-4-nitrosoaniline (RNO, 47 μM) and imidazole (10 mM) was prepared in PBS (50 mM, pH=7.4). This solution was poured over the gel and left to equilibrate for 30 min. After that the sample was irradiated at different times and the absorption at 440 nm was recorded.

Results and discussion

Polymer **1** is the host component for the preparation of the supramolecular hydrogel and consists of β-CD units interconnected using epichlorohydrin to form glyceryl cross-linked β-CD polymer. This polymer is highly soluble in water where it exists under the form of NPs of ca. 25 nm in diameter.[20] Due to the presence of different hydrophobic nanodomains these NPs are able to entrap a variety of guests[21,24] with enhanced stability constants and payloads as compared with the unmodified β-CD. Compounds **3** and **4** are the two chromo-fluorogenic guests chosen to impose multi-photoresponsive character to the hydrogel. For sake of clarity, we consider it useful to briefly recall the logical design behind the choice of these guests as active species. The phthalocyanine **3** is a well-known red photoemitter and an effective $^1O_2$ photogenerator.[25] Despite this photosensitizer is soluble in aqueous solution, where it exhibits absorption bands with maxima at 335 and 635 nm (***a*** in Fig. 1A), the formation of water soluble aggregates[26] makes it photodynamically inactive. However, in the presence of polymer

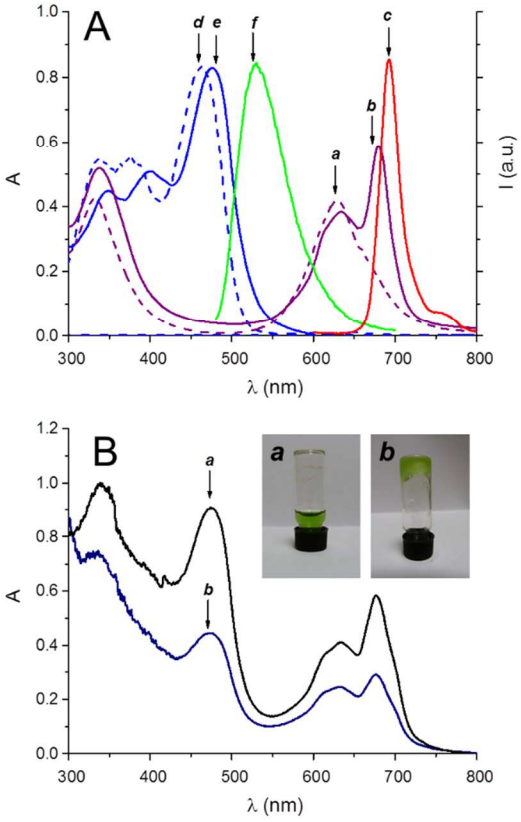


Fig. 1 (**A**) Absorption spectra of an aqueous solution of **3** (***a***) and a methanol solution of **4** (***d***) and aqueous dispersion of **1** in the presence of **3** (***b***) and **4** (***e***). Fluorescence emission spectra of an aqueous dispersion of **1** in the presence of **3** (***c***) and **4** (***f***), recorded at λ_{exc} = 470 and 650 nm, respectively. [**1**] = 75 mg mL^{-1}; [**3**] = 11 μM; [**4**] = 45 μM. (Phosphate buffer 10 mM, pH 7.4, 25°C). (**B**) Absorption spectra of an aqueous dispersion of **1** in the presence of **3** + **4** (***a***). Absorption spectrum of the hydrogel obtained after adding an aqueous solution of the dextran **2** to the aqueous dispersion of **1** in the presence of **3** + **4** (***b***). The inset shows the pictures taken before (***a***) and after (***b***) mixing the two solutions and removing the supernatant. [**1**] = 75 mg mL^{-1}; [**2**] = 75 mg mL^{-1} [**3**] = 11 μM; [**4**] = 45 μM. Phosphate buffer 10 mM, pH 7.4, 25°C.

1 a significant amount of **3** (more than 50%) is entrapped as the photoactive monomeric species, as confirmed by the appearance of its typical absorption band at λ_{max} = 680 nm[26] (**b** in Fig. 1A) accompanied by the return of the characteristic red fluorescence (**c** in Fig. 1A). The molecular conjugate **4** integrates a nitroaniline derivative as suitable NO photodonor[27] and a 4-amino-7-nitrobenzofurazan (ANBF) moiety, a well-known fluorophore emitting in the green region[28] joined together by an alkyl spacer. This component is well soluble in organic solvents (**d** in Fig. 1A) but poorly soluble aqueous medium. However, it readily dissolves in water in the presence of polymer **1** as confirmed by the absorption maxima at 400 and 480 nm typical for the nitroaniline[27] and ANBF,[28] chromophores, respectively (**e** in Fig. 1A) and the characteristic green fluorescence of the ANBF luminophore (**f** in Fig. 1A). The two photoresponsive guests **3** and **4** were deliberately chosen to conserve independent photobehavior when co-encapsulated within the polymer NPs of **1** (see below). In fact, the green emission of **4** does not significantly superimpose the absorption region of **3**, making a Fluorescence Resonance Energy Transfer quenching mechanism between **4** and **3** not feasible due to the negligible J overlap. Besides, an energy transfer between **3** and **4** is thermodynamically endergonic since the singlet state of **3** is lower than that of **4**. This allows, in principle, to both chromophores to "ignore" each other once localized in the same compartment and to be operated in parallel under light inputs.

The absorption spectrum of both the photoactive guests in the presence of NPs of **1** (***a*** in Fig. 1B) matches very well the profile obtained by summing the spectra of the NPs loaded with the single components, **3** or **4**, with an experimental error below 10%. This confirms their co-entrapment within the NPs and the absence of relevant interactions with each other in the ground state. The mixing at room temperature of the polymer NPs solution of **1** co-encapsulating **3** and **4** with that of the modified dextran **2** led to the instantaneous formation of the hydrogel (see experimental). The loaded gel presents a viscoelastic behavior (see inset Fig. 1B), according to what previously reported.[17b] The absorption spectrum recording after removing the supernatant solution (***b*** in Fig. 1B) shows clearly the appearance of the typical absorption features of the phthalocyanine **3** (above 600 nm) and the tailored NO photodonor **4** (below 500 nm) and thus unequivocally demonstrates the successful encapsulation of the photoactive guest components in the gel matrix. Furthermore, the very similar spectral shape, the maxima position and the relative intensities with respect to those observed before the addition of the modified dextran **2** (compare spectra **a** and **b** in Fig. 1B), suggest that the gel formation does not induce either any significant rearrangement (*i.e.* displacement/aggregation) and only minor changes in the molar ratio of the components **3** and **4**. This finding accounts for the absence of relevant interactions between the chromophoric units with each other, in the ground state even in the gel matrix.

For a potential use of the hydrogel in biomedical applications an important pre-requisite is that the guest components remain entangled in the polymer matrix. To this end, the stability of the as-prepared hydrogel was tested by leaving it immersed in a buffer solution at pH 7.4 at room temperature and recording the absorbance at the diagnostic wavelengths of 680 and 470 nm (absorption maxima of the two chromophoric components) of withdrawing 1 mL aliquots of the supernatant solution and, for comparison of the gel phase, at regular time intervals. Fig. 2 shows that negligible leaching was found after 24 hours suggesting that of both the photoactive guests are very tightly bound within the polymeric network. This remarkable stability is probably be due to the multivalent character of the interactions between the all components and represents a great advantage with respect to other polymeric gels incorporating functional guests in a non-covalent fashion. In these cases, considerable leaching of the photoactive centers from the polymer to the soak media was in fact observed, requiring additional coating of the gel polymer with polyurethane walls.[29]

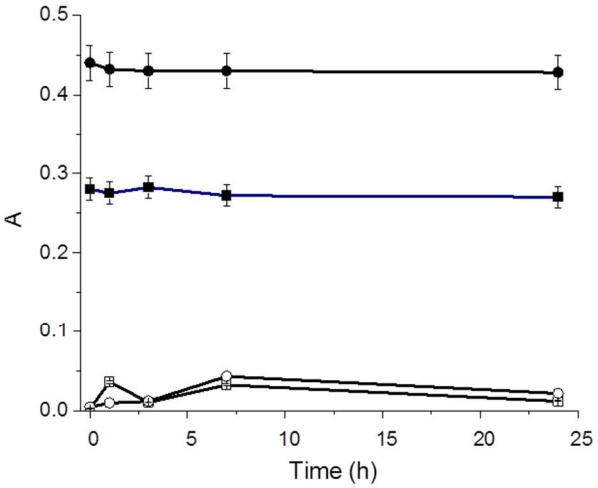


Fig. 2 Absorbance values, recorded at 680 nm (squares) and 470 nm (circles), as a function of the incubation time for the supernatant solution (open symbols) and the gel phase (solid symbols). Cell pathlength = 1 cm. Phosphate buffer 10 mM, pH 7.4, 25°C.

The multi-photoresponsive character of the hydrogel was demonstrated by the combination of fluorescence and photochemical experiments. The two fluorogenic guests retain their luminescence behavior when entangled in the hydrogel making it dual-color fluorescent. In fact, the selective excitation at 680 and 480 nm results in the characteristic red and green fluorescence arising from **3** and **4** (Fig. 3).

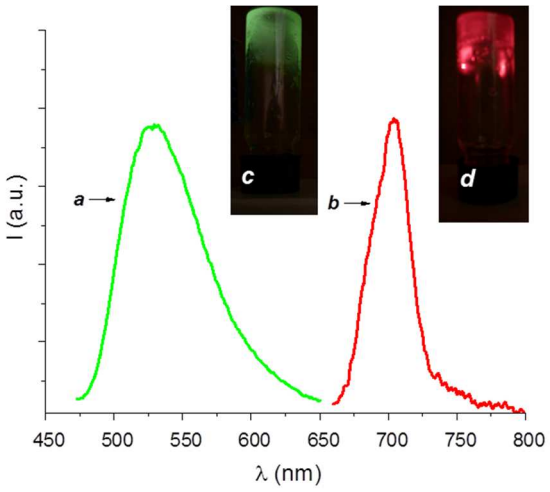


Fig. 3 Fluorescence emission spectra and actual images of the hydrogel recorded at λ_{exc} = 470 (***a*** and ***c***) and 650 nm (***b*** and ***d***), respectively.

The fluorescence quantum yields Φ_f were estimated to be ca. 0.03 and 0.01, respectively. These values are similar to those observed for the single fluorophores encapsulated in the polymer NPs of **1** before the addition of the dextran **2** to form the gel.[21] Moreover, both the spectral shape and position of the emission maxima are also very similar to those observed before the formation of the gel. These findings provide indication that the role of the modified dextran **2** is exclusively to "freeze" the photophysical behavior observed in solution and to move it into the gel phase, without altering the photoluminescence features of the individual guests.

The capability of the hydrogel to release NO under visible light stimuli was demonstrated by the direct and real-time monitoring of this transient species using an ultrasensitive NO electrode, which directly detects NO with nM concentration sensitivity by an amperometric technique.[30] The NO electrode was immersed into the supernatant water phase whereas the gel was placed at the bottom of a quartz vial and irradiated with visible light therein. In this way, the electrode is expected to detect the NO directly once it diffuses from the gel to the water phase. The results illustrated in Fig. 4 provide unambiguous evidence that the supramolecular gel is stable in the dark but supplies NO upon illumination with λ_{exc} = 420 nm. The release process is strictly dependent on the external light inputs, as confirmed by the NO photodelivery which promptly stops as the light is turned off and restarts as the illumination is turned on again. A control experiment also demonstrates that no release of NO is detected upon illumination at λ_{exc} = 500 nm, an excitation wavelength out of the region of absorption of the NO photodonor (see Fig. 4).

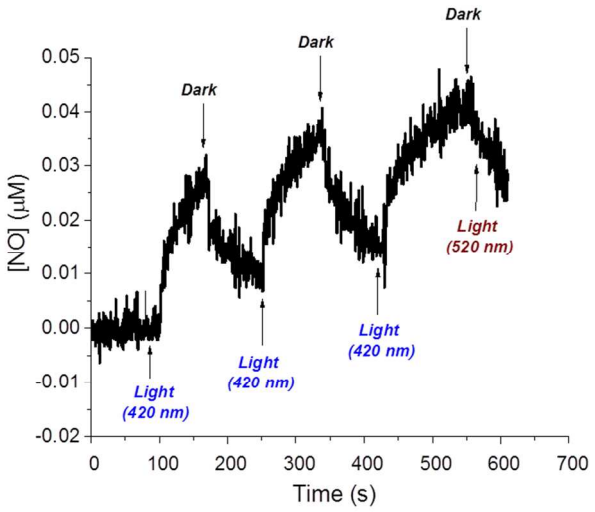


Fig. 4 NO release profile observed upon alternate period of irradiation of the supramolecular hydrogel at 25°C.

The excited triplet state of the phthalocyanines is the key transient intermediate for the photosensitization of $^1O_2$ and its effective generation is thus crucial for the photodynamic action.[7,8] Fig. 5A shows the transient absorption spectral changes observed with elapsing time after 532 nm laser excitation of the hydrogel. They account for the formation of a single transient species with maximum at ca. 530 nm. As shown by the linear dependence of the △A on the laser intensity (inset Fig. 5A) this transient species is generated through a mono-photonic process, and decays monoexponentially with a time-constant of ca. 20 μs (Fig. 5B). According to literature, this species can be safely assigned to the lowest excited triplet state of the phthalocyanine **3**.[31]

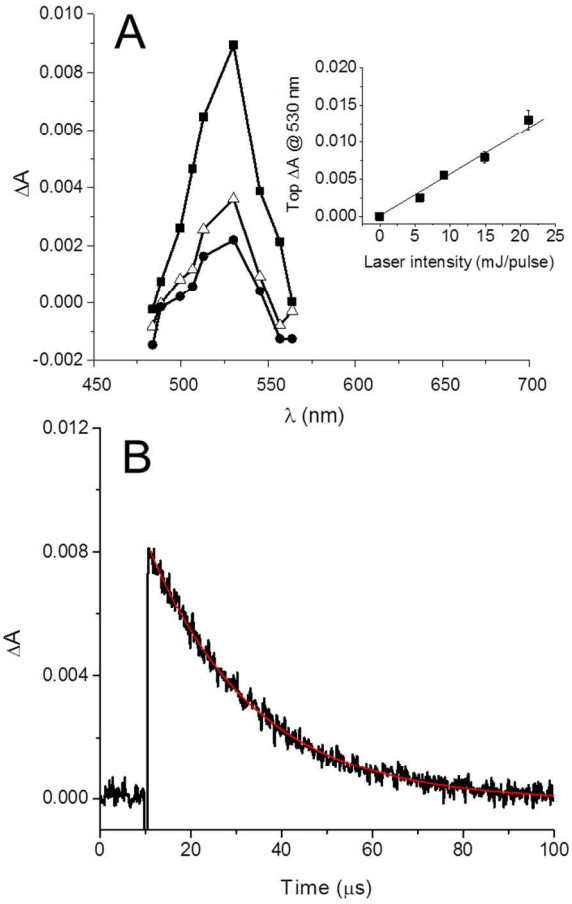


Fig. 5 (**A**) Transient absorption spectra observed (■) 1 μs, (△) 15 μs and (●) 30 μs after 532 nm laser excitation (E_{532} ~ 10 mJ/pulse, pulse with ca. 6 ns) of the supramolecular hydrogel in air-equilibrated conditions. The inset shows the laser intensity dependence of the △A monitored at 530 nm and taken 0.1 μs after the laser pulse. (**B**) Decay trace monitored at 530 nm and the related first-order fitting.

Energy transfer from this triplet state to molecular oxygen is the process responsible for the formation of $^1O_2$. Photogeneration of $^1O_2$ from the hydrogel was demonstrated by means of the well-known spectrophotometric method based on the bleaching of the *N,N*-dimethyl-4-nitrosoaniline (RNO) in the presence of imidazole as a chemical trap for $^1O_2$.[24] Basically, the photogenerated $^1O_2$ reacts with imidazole to form a transannular peroxide which bleaches RNO at its absorption maximum (λ_{max} =440 nm). The degree of bleaching is directly proportional to the production of $^1O_2$. The results illustrated in Figure 6 show that selective excitation of the hydrogel at λ_{exc} = 680 nm (absorption maximum of the phthalocyanine monomeric form) leads to a significant bleaching at 440 nm as a function of irradiation time (Figure 6). Control experiments carried out either in the dark or

upon illumination in the absence of the hydrogel showed only negligible absorbance changes at 440 nm (Fig. 6), providing clear evidence for the $^1O_2$ photogeneration by the phthalocyanine **3** entangled in the gel matrix.

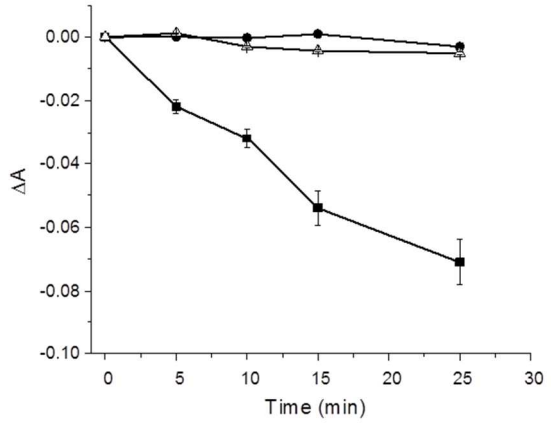


Fig. 6 Bleaching of the RNO absorption at 440 nm as a function of the time observed in the presence of the hydrogel in the dark (△) and upon irradiation with λ_{exc} = 680 nm (■), and in the absence of the hydrogel (●) upon irradiation with λ_{exc} = 680 nm. [RNO] = 47 μM; [imidazole] = 10 mM. Cell pathlength = 1 cm. Phosphate buffer 10 mM, pH 7.4, 25°C.

Conclusion

We have shown herein a simple "green" approach taking advantages of strong hydrophobic interactions between four *ad-hoc* chosen functional components to achieve a multi-photoresponsive gel platform. The co-entrapped species show an excellent stability in the gel matrix, which does not require any protective coating or shell to prevent the premature leaching. Despite their confined state, the photoactive guests can be operated independently under the exclusive control of visible light inputs. In fact, the hydrogel is stable in the dark but it is able to: i) release two powerful anticancer and antibacterial species, $^1O_2$ and NO and ii) simultaneously exhibit a dual-color red and green fluorescence under illumination with appropriate wavelengths. It should be noted that in contrast to non-photoresponsive compounds, the preservation of the photobehavior of independent components after their confinement in a restricted region of space is a "non-trivial" result. In most cases, the response to light of multiple photoactive units can in fact be considerably influenced, in both nature and efficiency, by the occurrence of competitive photoprocesses (*i.e.*, photoinduced energy and/or electron transfer, non-radiative deactivation, *etc.*), which preclude the final goal.[32] To our knowledge, this represents the first example of hydrogel showing the convergence of a dual-color fluorescence and dual modal photodynamic action. In this view, it represents an intriguing model system for potential image-guided phototherapeutic applications. In principle, irradiation with appropriate light sources allows the precise localization of the hydrogel implanted in a bioenvironment by means of its double emission and to activate controlled release of $^1O_2$, NO or both these active species. Further studies in this concern are currently underway in our laboratories and will be reported in due course.

Acknowledgements

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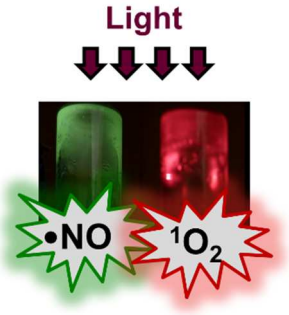

The self-assembly of four different components forms a supramolecular hydrogel exhibiting green and red fluorescence and releasing two powerful anticancer species upon illumination with visible light.