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(–)-Riboflavin (vitamin B2) and flavin mononucleotide as visible light photo initiators in the thiol–ene polymerisation of PEG-based hydrogels†

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The photoinitiators used in light mediated hydrogelation have been limited due to cytotoxicity and solubility issues. Here we report the use of riboflavin and flavin mononucleotide as nontoxic, naturally occurring photoinitiators for the thiol–ene hydrogelation of functionalised poly(ethylene glycol). High Resolution Magic Angle Spinning (HRMAS) NMR spectroscopy and oscillatory rheometry were used to probe the efficiency of these initiators for this hydrogel system. The gelation and reaction progression could easily be manipulated using different initiator concentrations and light intensities.

Characterised by their ability to uptake large amounts of water, hydrogels are networks of crosslinked hydrophilic polymers that have found increasing use in the biomedical field.[1] Poly(ethylene glycol)(PEG) is one of the most widely used materials for synthetic hydrogels. Its non-reactivity and biocompatibility combined with its ease of tuning its chemical and physical properties make PEG a highly versatile scaffold for biomimetic materials, with applications in tissue engineering and drug delivery.[2–5]

Traditionally PEG-based hydrogels were prepared by radical-chain-growth polymerization of di(meth)acrylate monomers to yield low cytotoxic hydrogels with heterogeneous networks of poly(meth)acrylate chains and long PEG crosslinks.[6,7] Radical step growth polymerisation of PEG hydrogels was established by Fairbanks and Anseth,[8] where they employed a thiol–ene photo reaction, a well-established chemical reaction scheme in the field of click chemistry,[5,9] to afford the hydrogel by clicking together norbornene functionalised PEG and cysteine containing peptides. This polymerisation method has the added benefits of being extremely rapid and easily tuneable in its network structure and gelation kinetics as well as possessing mild and orthogonal reaction conditions.[10] As such, the use of the thiol–ene click reaction, along with other click reactions such as tetrazine–alkene[11] and tetrazole–alkene[12] have gained traction in the synthesis of PEG-based hydrogels. More focus has been on the thiol–ene reaction due to its versatility in bio conjugation as well as its synthetic accessibility, however one of the many challenges in the design and synthesis of photo initiated hydrogels is the cytocompatibility of the photo initiator and cell death caused by exposure to UV light. Although the rapid reaction times of thiol–ene reactions renders the biosafety concern associated with UV exposure negligible,[13] it would nevertheless be desirable to conduct these experiments in the visible region. The visible region does not only offer the advantage that it is more cytocompatible, but it can also enable deeper tissue penetration as the depth of penetration is increasing with increasing wave length.[14] Currently, there are few cytocompatible photo initiators, with UV active Igracure-2959 and lithium phosphanate being the main cytocompatible initiators employed in photo-mediated thiol–ene hydrogelation.[3,4,10,15]

In the last few years, recent literature[16,17] has expanded photo-mediated thiol–ene hydrogelation into the visible light spectrum, with Eosin Y employed as a highly efficient sole initiator of PEG-based hydrogels under visible light (400–700 nm). Although studies have shown eosin Y is cytocompatible over a short time period,[17] it is classified as a class 3 carcinogen,[18] and its effects long term and *in vivo* are not known. Riboflavin, also known as vitamin B2, is naturally occurring in the body, non-toxic and absorbs light strongly in between 330–470 nm, making it an attractive alternative to the current synthetic photo initiators. Riboflavin can generate superoxide radicals ($O_2^{\bullet-}$), which can subsequently initiate a range of reactions.[19,20] Initially reported as a photo initiator for acrylamide gels,[21] riboflavin, and its bioactive, water soluble form Flavin mononucleotide (FMN), have found use as photo initiators for radical chain growth (meth)acrylate systems[22,23] as well as photo-crosslinking silk fibroin.[24] However, as far as we know it hasn't been explored as an initiator for thiol–ene click reaction.

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In this contribution, we report the use of riboflavin and FMN (Fig. 1B) as the sole initiators of visible light mediated thiol–ene hydrogelation. For this gelation system, a multi-arm PEG macromer functionalised with norbornene moieties and dithiothreitol (DTT) were employed as the hydrogel scaffold and cross-linker respectively (Fig. 1C). Unlike in previous studies employing riboflavin, which require the use of a co-initiator such as L-arginine[25] or triethanolamine,[22,23] we found that the excitation of riboflavin with blue light (455 nm) was sufficient to initiate polymerisation. This eliminates the need to add potentially harmful substances to the reaction mixture. Mechanistically, riboflavin (or FMN) is excited by exposure to light that causes hydrogen abstraction from the sulfhydryl groups on the DTT. The resulting thiyl radical then propagates rapidly across the alkene group on the norbornene moiety forming a carbon centred radical which then abstracts another hydrogen from a thiol, creating a thioether linkage and regenerating the thiyl radical (Fig. 1A). Using this reaction scheme, we examined the effects of light intensity and initiator concentration on the rate and mechanical properties of the resulting hydrogels.

We first examined the step-growth nature of the thiol–ene reaction quantitatively using high resolution magic angle spinning ^{1}H NMR spectroscopy. Evidence of a thiol–ene reaction should be seen through the disappearance of the alkene peaks of the norbornene moieties and the appearance of an α-proton thioether peak (ESI, Fig. S1†). For this investigation, 40 μL inserts were filled with the hydrogel precursor solution containing 7 wt% PEG-4-NB and a stoichiometrically balanced amount of DTT and 1.3 mM of riboflavin in distilled water. These inserts were irradiated under blue light at 27 mW cm^{-2} over a period of 1200 s and ^{1}H NMR spectrum was recorded at different time intervals. The disappearance of the norbornene alkene peaks at 5.6–6 ppm clearly indicated the consumption of the alkene (ESI, Fig. S1†), however the appearance of the α-proton thioether peak could not be distinguished due to overlap of the chemical shifts from the reacted norbornene. A HSQC spectrum was obtained from a fully reacted sample to verify the thiol–ene reaction mechanism. Fig. S2 in the ESI† shows the characteristic α-proton thioether peak at 2.5 ppm. Another study[8] was able to confirm this mechanism by showing the appearance of the thioether peak in the proton spectrum. However, potential reasons why we were getting chemical shift overlap was due to using a different thiol cross linker and the complex splitting pattern associated with the norbornene systems and using a mixture of the *endo* and *exo* isomers.

As such, the reaction progression was monitored only by the disappearance of the alkene protons. The reaction was found to progress rapidly with 57% of the norbornene reacted for the riboflavin system and 58% for the FMN system within 30 s (Fig. 2A). Since Eosin Y is the main visible light initiator for this type of system and absorbs at 455 nm, a comparison against riboflavin and FMN was conducted. The Eosin Y

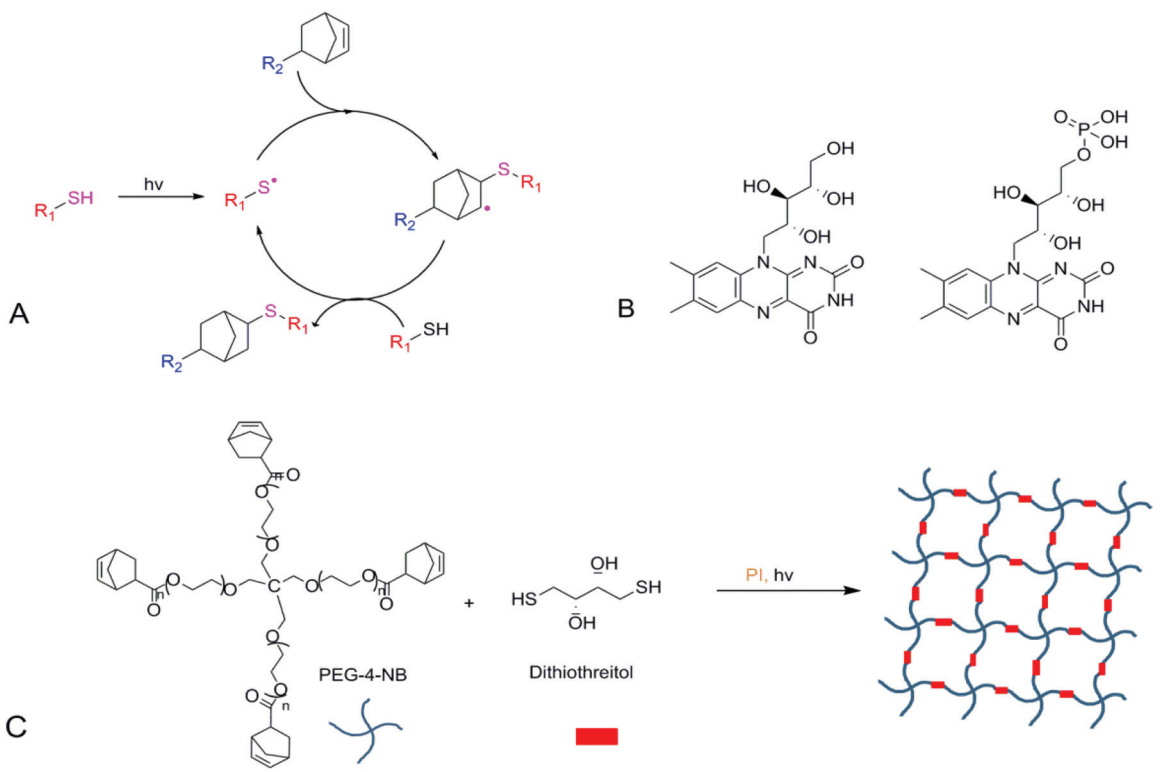

Fig. 1 (A) Schematic of the thiol–ene reaction mechanism. (B) The photo initiators used in this study; riboflavin (left) and FMN (right). (C) Schematic of the hydrogelation using PEG-4-NB and DTT.

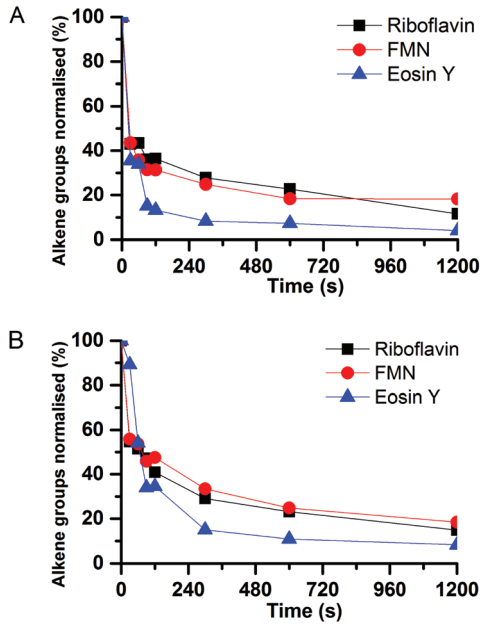

Fig. 2 HRMAS data showing the amount of alkene functional groups present. (A) Initiator concentration 1.3 mM. (B) Initiator concentration 0.26 mM.

showed faster conversion of the norbornene moieties, with 66% conversion after 30 s. The use of Eosin Y as the initiator also resulted in a higher final conversion of 95% compared to riboflavin and FMN, that displayed 89% and 82% conversion respectively.

The effect of a lower initiator concentration on the reaction progression was next evaluated. Here a more significant difference in the reaction rates occurred, with the use of riboflavin and FMN resulting in slower initial conversion after 30 s of 45% and 46% (Fig. 2B). A much slower conversion was seen with Eosin Y at 30 s with 11%. Although a further investigation is required to elucidate the underlying reason, the probable cause for the slower reaction rate is the excitation wavelength is at the edge of Eosin's absorption, resulting in decreased photon efficiency. What is of interest however, is the system using Eosin again shows the highest conversion (93%) after 20 min. This is due to the photo-degradation of riboflavin and FMN under blue light. Here, the riboflavin is degraded through the reduction of the isoalloxazine ring by donated electrons from the ribityl side chain.[26,27] Fig. S3 in the ESI† shows the UV-visible spectrum of FMN in water after irradiation under blue light at various time intervals.

To investigate the effect these initiators have on the gelation, *in situ* rheometry was used to determine gelation kinetics. The evolution of the modulus was monitored during *in situ* polymerisation with the gelation point defined as the time when the storage modulus (G') exceeds the loss modulus (G'').[28] From Fig. 3A, the visible light mediated reaction using both riboflavin and FMN at 1.3 mM both achieved gelation rapidly within 30 s. The use of riboflavin resulted in a slightly slower gelation point of 31 s, compared to that of FMN which

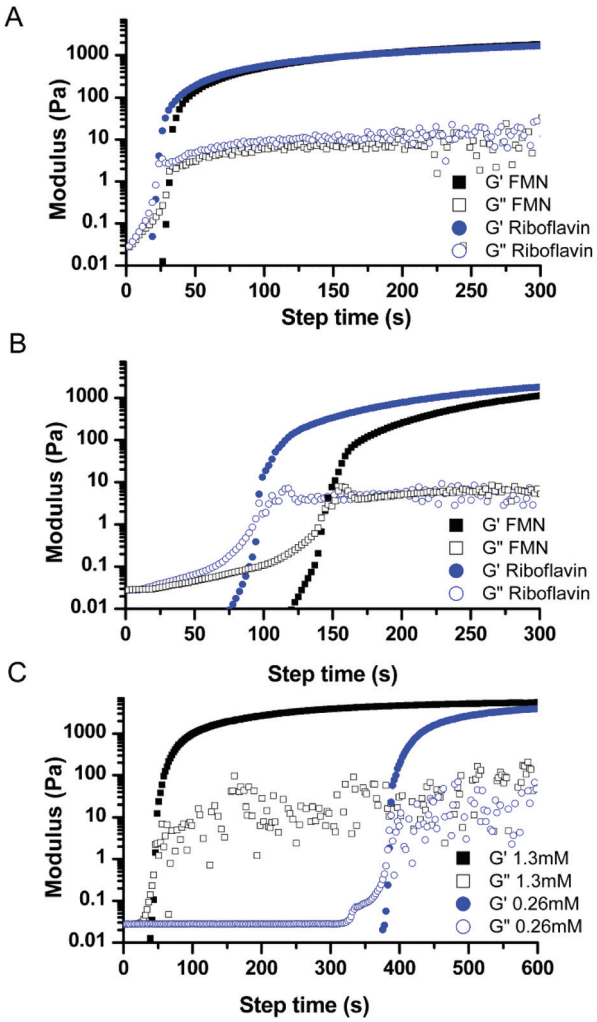

Fig. 3 Time sweeps showing effect initiator concentration has on gel point at a light intensity of 27 mW cm^{-2}.(A) [I] = 1.3 mM. (B) [I] = 0.26 mM. (C) Time sweep using Eosin Y as the initiator.

reached the gel point at 25 s. The gelation point using Eosin Y was also assessed and found to be much slower at 96 s (Fig. 3C).

We also demonstrated the effect a much lower concentration of initiator has on the gelation kinetics. As expected, a lower initiator concentration resulted in delayed gelation of 48 s for the riboflavin system (Fig. 3B). Unexpectedly, the gelation of the system using FMN was much slower at 150 s. One proposed reason for this difference is that at the higher concentration there is more aggregation of the riboflavin possibly resulting in lower initiation efficiency, hence at the lower concentration, the riboflavin is soluble resulting in the faster gelation time and/or in combination with the slightly lower extinction coefficient observed for FMN. Eosin Y exhibited an extremely slow gelation time of 387 s (Fig. 3C). We expected that the gelation points of the hydrogels initiated by Eosin Y would be delayed due the wavelength of irradiated light only being small in eosin Y's absorption. Interestingly though, the final storage moduli of the eosin Y hydrogels were much higher

(6700 Pa) than those obtained from the riboflavin and FMN systems (both approx. 3600 Pa). The reason for this result is more crosslinking within the Eosin Y hydrogels. The NMR results also indicate that more alkene groups have been converted in these systems indicating that Eosin Y is a more efficient initiator.

To further manipulate the hydrogel kinetics, the effect of different light intensities was explored. Here we evaluated the effectiveness a light intensity of 68 mW cm^{-2} and 7 mW cm^{-2} had on the gelation of the synthesised hydrogels. Using an initiator concentration of 0.26 mM, the highest light intensity displayed a gelation point of 54 s and 68 s for the riboflavin and FMN systems respectively (Fig. 4A). The riboflavin system displayed a slower gelation time compared to the 27 mW cm^{-2} sample, although, they are similar and within error range. Irradiating at 7 mW cm^{-2} resulted in slower gelation times for both riboflavin (157 s) and FMN (184 s). At a higher initiator concentration of 1.3 mM, less variance in the gelation time in relation to light intensity was observed. At the lower light intensity, the system using riboflavin gelled around 42 s and 64 s for the FMN system (Fig. 4B). With the higher light intensity, gelation rapidly occurred around 15 s when using either initiator.

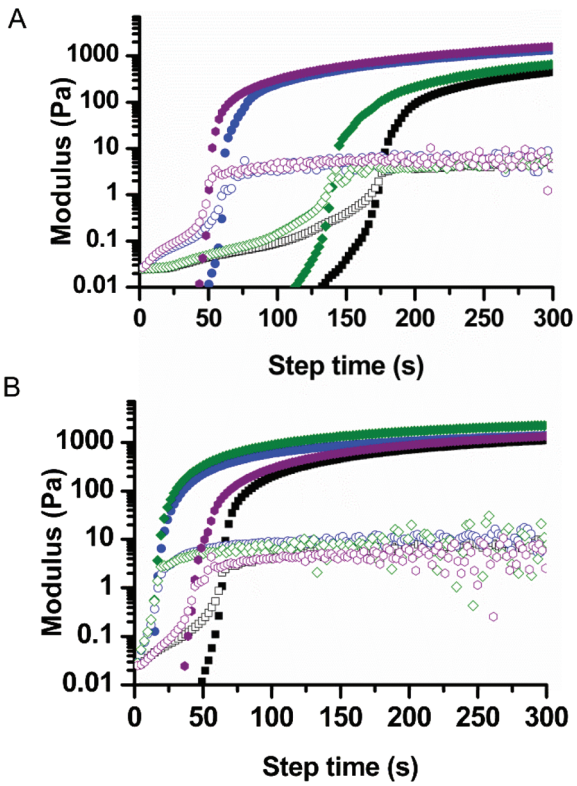

Fig. 4 Time sweeps showing effect of different light intensities at different concentrations and initiators. Values for G' are coloured and G'' is the shape outline. Riboflavin at 7 mW cm^{-2} (purple), riboflavin at 68 mW cm^{-2} (green), FMN at 7 mW cm^{-2} (black), riboflavin at 68 mW cm^{-2} (blue), (A) initiator concentration 0.26 mM (B) initiator concentration 1.3 mM.

Table 1 Equilibrium volume swelling ratio, average molecular weight between crosslinks and mesh size of hydrogels prepared using different initiators and light intensities. N = 3

Initiator	c (mM)	E_e (mW cm^{-2})	Q_v	$\bar{M}_c$	ξ (nm)
Riboflavin	0.26	9	16.8	4061	14.8 ± 0.3
		27	19.9	4282	15.6 ± 0.4
		68	18	4162	14.9 ± 0.4
	1.3	9	18.4	4186	15.0 ± 0.1
		27	18.1	4141	14.8 ± 0.9
		68	16.7	4046	14.3 ± 0.9
FMN	0.26	9	17.4	4108	14.6 ± 0.5
		27	17.6	4130	14.7 ± 0.1
		68	17.9	4150	14.8 ± 0.2
	1.3	9	17.4	4110	14.6 ± 0.3
		27	17.3	4103	14.6 ± 0.3
		68	19.2	4246	15.3 ± 0.4
Eosin Y	0.26	27	16.7	4050	14.8 ± 0.4
	1.3	27	18.7	4204	14.8 ± 0.6

The mesh sizes of the resulting hydrogels were determined using the Flory–Rehner equation at equilibrium swelling. The swelling ratio was determined after 72 h of incubation in water and was calculated from the mass at equilibrium swelling. The Flory–Rehner equation was then used to determine the average molecular weight between crosslinks and the mesh size was determined by using the root mean square ene-to-end distance of the polymer in the unperturbed state as described by Canal and Peppas[29] (see ESI† for the formula and calculations of mesh size). It was found that none of hydrogels displayed significant mesh size differences based on the concentration of initiator used (Table 1), with all hydrogels displaying a mesh size between 14.2–15.6 nm, which is consistent as all hydrogels have the same macromer content. A degree of non-ideality is observed in this system, as can be seen from the low average molecular weight between crosslinks, due to intramolecular crosslinking on the PEG macromers.

In conclusion, this communication has presented the use of riboflavin and FMN as efficient photo initiators for thiol–ene hydrogelation. Even though riboflavin and FMN do undergo rapid photodegradation, it has been shown that the use of these initiators provides fast and efficient gelation and that the time of gelation can be controlled by initiator concentration and light intensity. In comparison to Eosin Y, a known visible light initiator, there does not appear to be a significant difference in the performance. The key advantages, however, in employing riboflavin and FMN, is the non-toxicity associated with these naturally occurring compounds as well as the use of visible light, all of which overcome the biosafety concerns associated with UV mediated gelation and the cytotoxicity of current photo initiators.

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