ChemComm

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Enzyme-controllable delivery of nitric oxide from a molecular hydrogel†

Cite this: *Chem. Commun.*, 2013, **49**, 9173

Received 25th July 2013,
Accepted 5th August 2013

DOI: 10.1039/c3cc45666h

www.rsc.org/chemcomm

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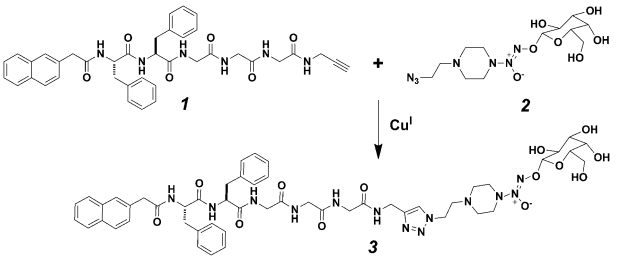
A β-galactosidase-responsive molecular hydrogelator of a nitric oxide (NO) donor can release NO in a controllable manner to improve wound healing.

NO, as a diatomic free radical produced in the human body, plays vital roles in cardiovascular, respiratory, nervous, and immune systems.[1] It has been demonstrated to act as a functional platelet aggregation inhibitor, a neurotransmitter, and an antimicrobial and antitumor agent.[2] Though NO has been demonstrated to be an important molecule, it is unstable under physiological conditions. What's more, its concentration should be precisely regulated for its normal and correct biological functions. Higher concentrations of NO than its normal biological levels will lead to undesirable outcomes such as toxicity to cells. Therefore, NO donors,[3] such as organic compounds, metal complexes, dendrimers, and micelles, have been developed to store and deliver NO in a controllable manner (*e.g.* light irradiation, pH and temperature change, enzymes).[4,5] The development of these NO donors helps to understand the biological function of NO and may lead to novel therapeutic agents for the treatment of NO-involved diseases.

Biological systems use enzyme and signal transduction machinery to regulate the level of NO. Inspired by such principles in nature, caged NO molecules with enzyme-responsive properties have been reported.[5,6] The NO released from caged NO molecules can be controlled by using different concentrations of enzymes. However, most of enzyme-responsive caged NO molecules are free molecules that will diffuse easily to the whole body. Local delivery of enzyme-responsive caged NO molecules will be a more precise way that may eliminate undesirable side effects in the treatment of many diseases, such as tumor inhibition and wound healing. In this study, we combine a β-galactosidase-responsive caged NO molecule and a self-assembling short peptide to produce a molecular hydrogelator. The resulting hydrogel can sustainedly release NO molecule by the addition of β-galactosidase and therefore assist the wound healing in mice models.

Molecular hydrogels are formed by the self-assembly of low-molecular weight molecules.[7] They have shown great potential for the controlled release of bioactive molecules,[8] imaging important biological molecules,[9] and regenerative medicine.[10] In this study, we opt to combine a sugar caged NO donor (**2** in Scheme 1) and a short peptide of Nap-FFGGG to generate a hydrogelator that can form hydrogels for local delivery of NO. The synthetic route for the designed gelator is shown in Scheme 1. The Cu(I) catalyzed a click reaction between an alkyne containing a short peptide derivative (**1** in Scheme 1) and an azide containing a caged NO donor leading to the formation of the possible gelator (**3** in Scheme 1).

After the synthesis and purification using reverse phase high performance liquid chromatography, the gelation ability of **3** was tested using the inverted tube method. A transparent hydrogel (inset image on the left bottom corner of Fig. 1A) could form within 2 minutes upon cooling the hot phosphate buffered saline (PBS) solution (pH = 7.4) containing 0.5 wt% of **3** to ambient temperature (25 °C). The hydrogel was stable for at least one month. A rheometer was used to characterize the mechanical properties of the hydrogel at 37 °C. Both values of storage moduli (G') and loss moduli (G'') were independent of



Scheme 1 Synthetic route of the hydrogelator containing a caged NO donor.

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† Electronic supplementary information (ESI) available: Synthesis and characterization, hydrogel preparation, HE staining, details experimental procedure in the animal study. See DOI: 10.1039/c3cc45666h

‡ The authors pay equal contributions to this work.

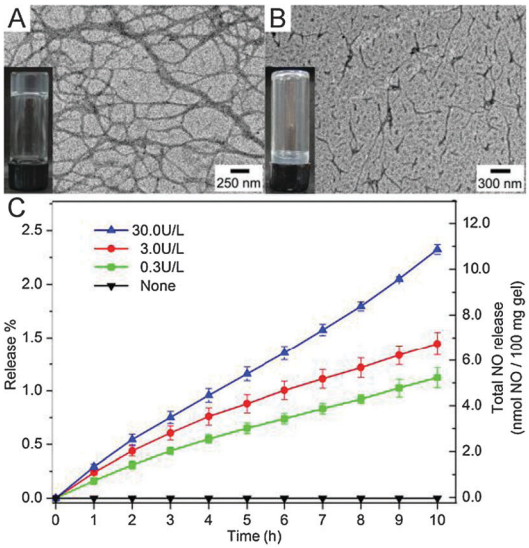

Fig. 1 TEM images of hydrogel containing 0.5 wt% of **3** 0 h and (B) 24 h after the addition of β-galactosidase (3000.0 U L^{-1}) (insets: optical images of the gel and solution). (C) The release profile of NO from gel containing 0.5 wt% of **3** with the addition of different concentrations of β-galactosidase. For results using higher concentrations of enzyme (20, 200, 2000 U L^{-1}), please see Fig. S-6 (ESI†).

the frequency at the range of 0.1 to 100 rad s^{-1} (Fig. S-5, ESI†). The G' value was about an order of magnitude greater than the G'' value (Fig. S-5, ESI†), indicating the formation of a true hydrogel.[11] We also used transmission electron microscopy (TEM) to reveal the micro-structure in the gel. Results in Fig. 1A indicated that the gel was constructed by entangled long nano-fibers with diameters of 30–80 nm.

We then tested the enzyme-responsive property of the hydrogel and studied the NO release behavior from the gel. The enzyme β-galactosidase was used to remove the protective galactose from the NO donor and trigger the release of NO from **3** (Scheme S-3, ESI†). A gel to sol phase transition (inset image on left bottom corner of Fig. 1B) was observed 24 hours after the addition of β-galactosidase. TEM images showed that the original long fibers in the gel (longer than 2.5 μm, in Fig. 1A) changed to short fibers in the resulting solution (30 to 600 nm, in Fig. 1B). These observations clearly indicated the enzyme-responsiveness of the gel.

In order to evaluate the controllable NO releasing ability of the hydrogel, 50 μL of PBS solution containing different amounts of β-galactosidase was placed on top of 50 μL of gel (0.5 wt% of **3**). The amount of released NO in the upper solution was measured every 1 hour using a classic Griess reaction Nitric oxide assay. The results of accumulation of released amounts of NO from the gel were determined (Fig. 1C and Fig. S-6, ESI†). During the 10 hours of experimental time, the proportion of total released NO was tunable, ranging from 1.1% to 17.8% by varying the amount of enzyme added to the gel. The releasing rate remained constant without the burst release phenomenon during the whole experimental procedure. Meanwhile, no NO molecule could be detected when PBS without enzyme was added on top of the gel. These results clearly demonstrated that, by adjusting the concentration of β-galactosidase in the system, such a molecular hydrogel possessed the ability of continually releasing NO at controllable rates. This property was vital in NO therapies because the effective therapeutic dose of NO might vary greatly and needed to be precisely controlled.[12]

The MTT assay of NIH 3T3 mouse fibroblast cells indicated that more than 90% of 3T3 cells were alive after being cultured in the medium containing **3** at concentrations lower than 800 μM for 24 hours (Fig. S-7, ESI†). These observations indicated the compatibility of **3** with 3T3 cells at these concentrations. One of the vital biomedical applications of NO is wound healing. NO is believed to assist wound repair by enhancing re-epithelializiation, increasing collagen synthesis, and promoting angiogenesis by inducing vascular endothelial growth factor expression.[13] The wound healing promoting ability of our molecular hydrogels was investigated in C_{57} mice and the results including areas of wounds and density of small new-born blood vessels were assessed.

Animals were separated into five groups and submitted to excisional wounds on the dorsal surface on the first day. Since gels of **3** would change to solutions gradually upon the treatment with β-galactosidase and hydrogel could prevent contamination of environmental microbe, we used a mixed component gel for the evaluation. 20 μL of the mixed component hydrogel (0.6 wt% of **3** (120 nmol) and 1.0 wt% of Nap-FF (432 nmol)) were applied on the wound bed in the NO Gel + GAL and NO Gel groups. During the first 4 days, β-galactosidase was added daily in the NO Gel + GAL group, while the wounds in the NO Gel group were treated with only the mixed component gel without β-galactosidase. The wound in the Nap-FF group was topically treated with 20 μL of the Nap-FF hydrogel (1.0 wt%) without **3** for the same period. The other two control groups were treated with PBS only (PBS group) and caged NO donor (Free NO + GAL group: PBS solution containing 0.2 wt% (120 nmol) of **2**, with daily β-galactosidase treatment as well), respectively. The wound area on the first day (Day 0) and seven days after treatment (Day 7) was measured. The wound area at Day 0 was set to be 100% and the wound area reduction at Day 7 was calculated for all groups. As shown in Fig. 2B, the reduction of wound area was 81.9, 64.6, 62.1, 60.6, and 57.9% for NO Gel + GAL, NO Gel, Free NO + GAL, Nap-FF, and PBS groups, respectively. The results clearly indicated that, compared with other groups, the wound areas were dramatically reduced in the NO Gel + GAL group (Fig. 2B), which indicated the importance of NO molecules in the wound healing. The wound area reduction was significantly greater in the NO Gel + GAL group (81.9%, Fig. 2A) than that in NO Gel group (17.3% greater than NO Gel group (P = 0.012)), indicating that the additional β-galactosidase could catalyze the release of NO at higher levels. As for the Free NO + GAL group, it exhibited a similar wound healing effect on the PBS control group probably due to the fast clearance of **2** by rapid diffusion to the whole body of mice. These results also indicated the importance of hydrogel formulation for topical treatments.

We then investigated the effect of hydrogels on angiogenesis in wounded skin by measuring the expression of vWF in the wound area (Fig. S-8 and S-10, ESI†) and the border area (Fig. S-9, ESI†). As shown in Fig. S-8 (ESI†), there were about

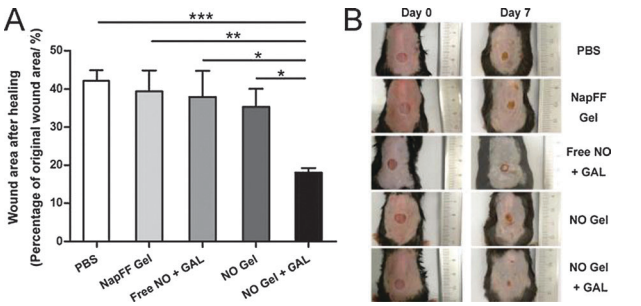

Fig. 2 (A) Percentage of wound area left in different groups at Day 7 compared to the original wound area (mean ± SEM) at Day 0. (B) Photographs of wounds in animals treated with PBS, NapFF (hydrogel containing 1.0 wt% NapFF), Free NO + GAL (solution containing 0.2 wt% of **2** with daily addition of 1.5×10^{-4} U β-galactosidase), NO Gel (hydrogel containing 1.0 wt% NapFF and 0.6 wt% **3** without the addition of β-galactosidase) and NO Gel + GAL (hydrogel containing 1.0 wt% NapFF and 0.6 wt% **3** with the addition of 1.5×10^{-4} U β-galactosidase each day). Significance levels were set to: $^{*}p < 0.05$, $^{**}p < 0.01$, and $^{***}p < 0.001$.

31.0, 21.2, 12.3, 14.1 and 14.6 micro-vessels per HPF in the wound area for NO Gel + GAL, NO Gel, Free NO + GAL, Nap-FF and PBS groups, respectively. The results indicated that there was significantly more neo-vascularization in the NO Gel + GAL group compared to other groups, including the control PBS group ($P = 0.003$), Nap-FF group ($P = 0.011$), Free NO + Gal group ($P = 0.001$), and NO Gel group ($P = 0.016$), as determined by number of stained micro-vessels per HPF (200×; 10 fields) (Fig. S-8A–E, ESI†). There were no significant differences among the PBS, Nap-FF, and NO Gel groups, suggesting that hydrogels in these groups failed to promote angiogenesis in the wound area. In the border area of the wound, the NO Gel + GAL, NO Gel, and free NO + GAL groups displayed similar vascularization densities (20.8, 17.9, and 19.8, respectively), which were higher than those in both PBS and NapFF groups (11.1 and 14.1, respectively). It was mostly likely that NO donors traveled to the border area and released NO molecules by enzymes (β-glycosidase or other digestion enzymes) in blood. Combined with the observation of the wound area in different groups, the results demonstrated that the vascularizations in both wound area and border area of the wound were crucial to the wound healing. These results also indicated that the combination therapy in the NO Gel + GAL group could promote angiogenesis in both wound and border areas, which was conducive to the wound healing process.

In summary, we have reported on the first example of a molecular hydrogelator with the enzyme-controllable NO release property. This hydrogelator consisted of a naphthalene-capped short peptide and a caged NO molecule. The addition of β-glycosidase could remove the sugar capping group on the caged NO molecule, leading to the release of NO in a controllable way. The release rate of NO was constant and could be tuned *via* adjusting the concentration of β-galactosidase added to the hydrogel. The hydrogel could be applied for topical treatment of wound because it could promote angiogenesis in the wound bed, thus accelerating the wound healing process. The production of hydrogel formulation could minimize fast diffusion and clearance of bioactive molecules. We therefore believed that our hydrogel system had great potential for local controllable delivery of NO for regenerative medicine and tissue engineering.

This work is supported by National Basic Research Program of China (2011CB964903) and NSFC (31070856, 81220108015, 81171478, and 81000680).

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