Photoinduced Protein Delivery

DOI: 10.1002/ange.201308174

Wavelength-Controlled Photocleavage for the Orthogonal and Sequential Release of Multiple Proteins**

*Malar A. Azagarsamy and Kristi S. Anseth**

On-demand manipulation of protein release to alter cellular phenotypes in real time is of great interest to the fields of drug delivery, tissue engineering, and regenerative medicine.[1–3] For such therapeutic and research ventures, hydrogels, owing to their high water content and mechanical stability, have emerged as suitable polymeric materials not only to localize proteins but also to control their release rate on-site.[4,5] Protein release from hydrogels is typically achieved either through diffusion or by stimuli. Diffusion-controlled mechanisms rely upon control of the hydrogel mesh size, which needs to be preprogrammed during synthesis; as a result, limited regulation of release is only possible afterward. To complement this strategy, stimuli (e.g., temperature, pH value, light and proteins)[6,7] sensitive materials have evolved and become attractive protein-delivery systems, as they offer opportunities to regulate molecular release using a specific stimulus. Stimuli controlled mechanisms applied to hydrogels are typically based on the degradation/swelling of hydrogel networks, in which noncovalently sequestered proteins are released in response to increased pore size/decross-linking of networks. Such noncovalent approaches do not require chemical modification of proteins and have the potential to precisely control the release of single protein molecules. However, applying such approaches to control the release of multiple proteins is quite complex and often requires either multiple gels or microspheres for encapsulation.[8–12] Thus, a number of studies aimed at directing cellular processes or disease regulation would benefit from the delivery of more than one protein and often necessitates their delivery in varied doses at different time points.[8–11] Herein, we present an approach that allows precise control over the release of multiple proteins from a single hydrogel depot using an external light.

Light-triggered molecular cleavage has received widespread interest in recent years, for activation of so-called "caged" biomolecular entities,[13–15] alteration of material properties,[16,17] and to control therapeutic release in real time.[18,19] Furthermore, user-defined time and spatial location of photocleavage reactions offer unique opportunities to control material properties more precisely than other classical stimuli.[14] However, many of these approaches rely on a single photocleavable unit, and thus, provide limited opportunities to control different material properties independently. Specific examples of wavelength-selective molecular activation by orthogonally functional units were introduced by Bochet,[20] and are now emerging as powerful strategies in controlling different properties in a sequential manner. Towards this, del Campo et al. first demonstrated the utility of such wavelength-selective photocleavable concepts by the spatial immobilization of multiple particles/fluorophores;[21] where they initially utilized 3,5-dimethoxy benzoin esters and nitrobenzyl derivatives,[21] a combination of functionalities coined by Bochet as "orthogonal units". Later nitrobenzyl and coumarin derivatives were explored as a new combination of orthogonal units,[22] which have been widely exploited for sequential uncaging of bioactive units for orderly regulation of biological actions.[23–27] More recently, sequential photoactivation of biomolecular ligands for spatio–temporal patterning of multiple proteins in a 3D gel matrix has also been reported.[28–30] Herein, we present two distinctive photocleavable units, based on: 1) nitrobenzyl ether (NB) and 2) coumarin methylester (CM), that can be selectively cleaved at different wavelengths of light and then harness their wavelength-dependent photodegradable characteristics to regulate the release of multiple proteins at different times. Specifically, proteins were covalently conjugated to hydrogel networks through photodegradable units[31–34] and the control over their release was achieved by simply varying the wavelength of light, the intensity and the time of light exposure.

The structural formula of NB, **1** and CM, **2** (See Supporting Information for syntheses of **1** and **2**) utilized in this work are shown in Figure 1 a. Note that both **1** and **2** have an azide functionality for subsequent functionalization of these molecules using click chemistry, and furthermore, the azide functionality of the coumarin molecule **2** is conjugated to the core ring structure. Under irradiation with UV/Vis light, nitrobenzyl derivatives, such as **1**, cleave to produce nitrosoacetophenone **3** as a byproduct,[16] while the coumarin methylester yields the corresponding coumarin methanol **4**.[35,36] Prior to utilizing these molecules for protein release, we first studied the photodegradation kinetics of **1** and **2** (2.5 mM) by separately irradiating them to both at 365 nm (10 mW cm^{-2}) and 405 nm (10 mW cm^{-2}), and analyzing the exposed solutions of **1** and **2** by reverse-phase HPLC. Before exposing to light, both **1** and **2** exhibited single peaks in the chromatogram, but exposure to 365 and 405 nm light resulted in the formation of distinguishable new peaks corresponding

[*] Dr. M. A. Azagarsamy, Prof. K. S. Anseth
Department of Chemical & Biological Engineering
Howard Hughes Medical institute and the BioFrontiers Institute
University of Colorado at Boulder
596 UCB Boulder, CO 80303 (USA)
E-mail: Kristi.anseth@colorado.edu

[**] We thank the National Science Foundation (DM 1006711) and the Howard Hughes Medical Institute for funding. We would also like to thank Dr. Daniel Alge for discussion.

Supporting information for this article is available on the WWW under http://dx.doi.org/10.1002/anie.201308174.

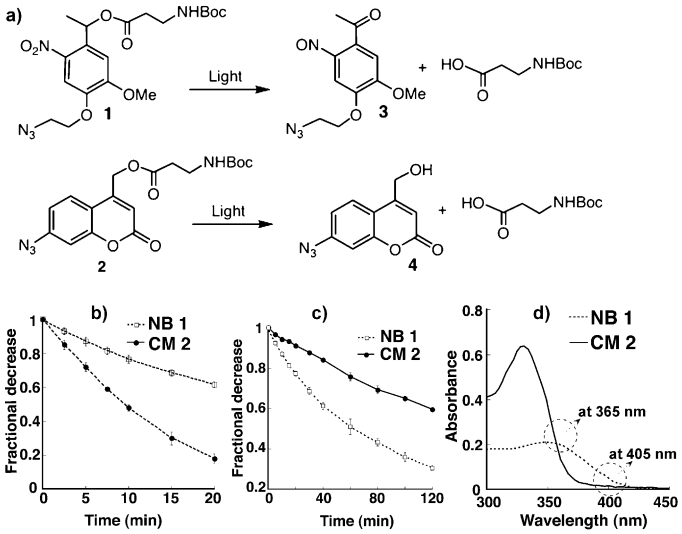


Figure 1. a) Compounds **1** and **2** and their corresponding photocleaved products **3** and **4**. Comparative photodegradation of **1** and **2** at b) 365 nm and c) 405 nm for different times of light exposure. d) Absorbance spectra of **1** (0.4 mM) and **2** (0.4 mM) dissolved in acetonitrile/PBS mixture (8:2).

to the degraded products **3** and **4** (see Supporting Information for HPLC chromatograms). The relative photodegradation of **1** and **2** at different times of exposure to 365 nm light is shown in Figure 1b, in which **2** shows a very high degradation rate compared to its nitrobenzyl counterpart **1**. The kinetic constant of degradation (k), determined from the slope of semi-logarithmic plot of Figure 1b (Table 1) (See Supporting Information for calculation details and corresponding semi-logarithmic plots), showed that k of **2** was four times higher than that of **1** at 365 nm. Conversely, to our surprise, when exposed to 405 nm (Figure 1c), the degradation trend reversed, **1** exhibited efficient degradation and its k value was found to be an order of magnitude higher than that of **2** (Table 1). The results clearly suggested that **1** and **2** undergo wavelength selective cleavage, that is, at 365 nm light, **2** degrades faster than **1** and vice versa at 405 nm. To better understand these differences, we calculated the molar extinction coefficient at the degrading wavelength (ε) of **1** and **2** and their quantum yield of degradation (ϕ) (Table 1 and Figure 1d), as these parameters directly correlate with k.[17] Even though the molar extinction coefficient of **1** at 365 nm is twice as high as **2**, we expect that the enhanced degradation of **2** at 365 nm is due to its higher quantum yield of degradation (ϕ). At the same time, almost an order of magnitude lower ε of **2** over **1** at longer wavelengths (i.e., at 405 nm) drastically decreases its degradation kinetics and ascribed the relative higher degradation kinetics of **1** over **2** at 405 nm. In summary, it appears that both the quantum yield of degradation and molar extinction coefficient play critical roles in the degradation behaviors of **1** and **2**.

Table 1: Degradation kinetic constant (k), molar extinction coefficient (ε), and quantum yield of degradation (ϕ). (for error limits see Supporting Information.)

Compound	k_{365} [$\times10^{-3}$ s^{-1}]	k_{405} [$\times10^{-3}$ s^{-1}]	ε_{365} [cm^{-1} M^{-1}]	ε_{405} [cm^{-1} M^{-1}]	ϕ_{365}	ϕ_{405}
NB **1**	4	2	4437	935	0.16	0.11
CM **2**	13	0.7	2183	150	0.57	0.15

We explored the utility of photodegradable units **1** and **2** for selective, light-controlled release of model compounds, for which we first utilized small-molecular-weight dyes. Rhodamine B isothiocyanate and fluorescein *N*-hydroxysuccinimidyl ester were conjugated to nitrobenzyl azide **1** and coumarin azide **2**, respectively, to afford the corresponding rhodamine-tethered nitrobenzyl azide **5** and fluorescein-tethered coumarin azide **6** (Figure 2a). The dye-conjugated photodegradable azides **5** and **6** were then covalently bound into poly(ethylene glycol) (PEG) hydrogels by copper-free, strain-promoted azide–alkyne click (SPAAC);[37] this hydrogel formulation was selected as it does not require any additional reagents or initiators that might lessen bioactivity of proteins during encapsulation. 4-armed PEG tetra-dibenzocyclooctyne (PEG-DBCO) **7** and 4-armed PEG tetraazide (PEG-N_3) **8** were used as

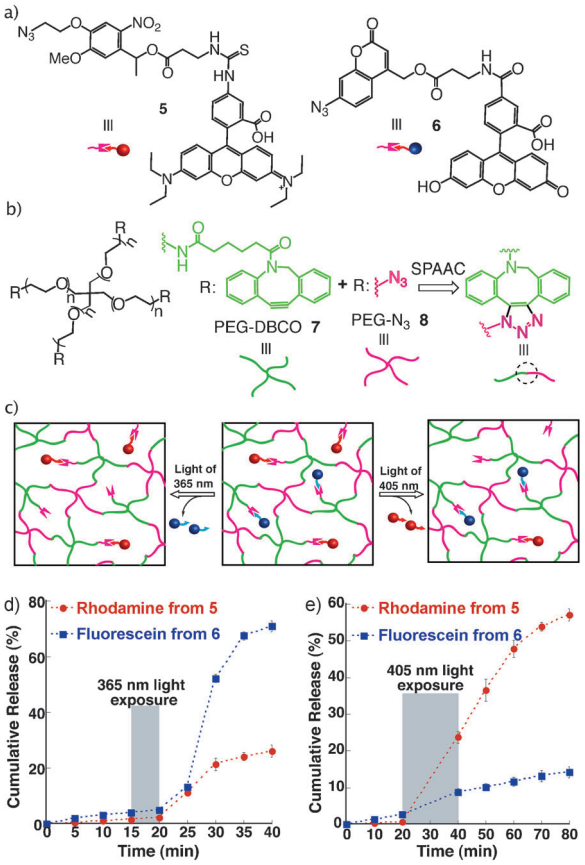
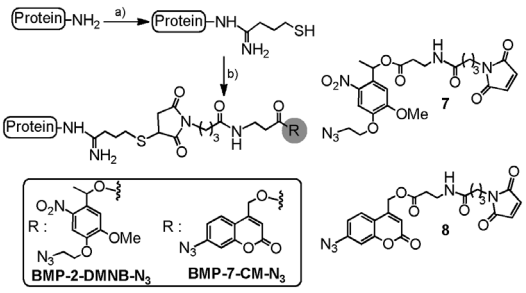


Figure 2. a) Rhodamine B tethered nitrobenzyl azide **5** and fluorescein-tethered coumarin azide **6**; b) PEG polymeric precursors that produce SPACC hydrogel networks: 4-arm PEG-tetra DBCO **7** and 4-arm PEG-tetraazide **8**; c) Schematic representation of light-wavelength-regulated selective release of dye molecules from hydrogel networks; Dye-release upon exposure to light sources of d) 365 nm (10 mW cm^{-2}) for 5 min and e) 405 nm (10 mW cm^{-2}) for 20 min. Blue-gray bars indicate the light exposure.

precursors to form the hydrogel (Figure 2b). In this case, a slight molar excess of PEG-DBCO **7** over PEG-N_3 **8** was used to allow subsequent covalent tethering of azides **5** and **6**.

We tested the light-triggered release capabilities of **5** and **6** by separately incorporating them into different hydrogels and exposing to 365 nm light (10 mW cm^{-2}) for 10 min. A sudden increase in fluorescence observed within 10 min in both cases clearly indicated the release of covalently tethered dye molecules into solution. Then, to investigate orthogonal release, both **5** and **6** were introduced together at equal concentrations into a single hydrogel and exposed to both 365 and 405 nm light sources separately (Figure 2c). After 5 min of irradiation with 10 mW cm^{-2} of 365 nm light (Figure 2d), almost 70% of coumarin's fluorescein was observed to be released from the gel in a period of just 20 min after exposure, while the release of nitrobenzyl's rhodamine was only about 26%, presumably because of the enhanced cleavage rate of coumarin methyl esters over nitrobenzyl ethers. However, under the same conditions, when gels were exposed to 10 mW cm^{-2} of 405 nm for 20 min (Figure 2e), the release of rhodamine (60%) was significantly higher than that of fluorescein (10%), indicating not only an inversion in the cleavage trend, but also the higher photocleavage rate of nitrobenzyl over coumarin at 405 nm. Two points are noteworthy here: 1) The dye-release trend is in agreement with the cleavage data quantified in solution, photocleavage studies of **1** and **2** (Figure 1); 2) Conversion of the conjugated azide of coumarin **2** into its corresponding triazole did not result in a significant change in its degradation behavior. Overall, these results demonstrated the feasibility for the photocleavable units **1** and **2** as orthogonal units to independently control the release of dual therapeutic agents after gel formation.

The orthogonal dye-release results then prompted us to further investigate the effectiveness of **1** and **2** in regulating the release of two different proteins of biological relevance. For this purpose, we selected isoforms of the bone morphogenetic proteins (BMPs): BMP-2 and BMP-7, which are key regulatory proteins closely involved in the cascade of events occur during bone regeneration. Studies have shown that delivering BMP-2 and BMP-7 in combination or in a sequential manner results in improved osteogenic differentiation of mesenchymal stem cells (MSCs).[10,38] Herein, BMPs were covalently modified with photocleavable azides to enable their attachment to SPACC hydrogels and later to trigger their release upon exposure to light of pre-selected wavelengths. BMP modification was achieved upon treating the amine functionality of the proteins with Traut's reagent to produce an extended free thiol and then exposing the resultant thiol to maleimide containing photocleavable azides **7** and **8** (Scheme 1). The resulting modified proteins BMP-2-NB-N3 (5 ng) and BMP-7-CM-N3 (5 ng) were then introduced together into the hydrogel by SPAAC. After leaching out untethered protein molecules for 3 days, the hydrogels were separately exposed to light of 365 (5 mW cm^{-2}) and 405 nm (5 mW cm^{-2}) at day 4, and analyzed using enzyme-linked immunosorbent assay (ELISA) to monitor the BMP release kinetics. In hydrogels exposed to 365 nm light for either 2, 4, or 6 min independently (Figure 3a), we observed significantly increased release of BMP-7, that is, tethered through coumarin, over nitrobenzyl tethered BMP-2. As expected, the amount of protein release increased steadily with increasing light exposure, but in case of BMP-7, the trend continued only up to 4 min. No significant increase in cumulative release was observed upon further light exposure, suggesting that optimal light exposures can be tailored to achieve maximum release of BMP-7 while minimizing BMP-2 release. The release data obtained for hydrogels exposed to 405 nm light for various



Scheme 1. Incorporation of photocleavable azides onto BMP-2 and BMP-7 proteins: a) Traut's reagent, pH: 7.0, Phosphate buffer (PBS), room temperature; b) **7** (for BMP-2) or **8** (for BMP-7), PBS, pH: 7.0, room temperature.

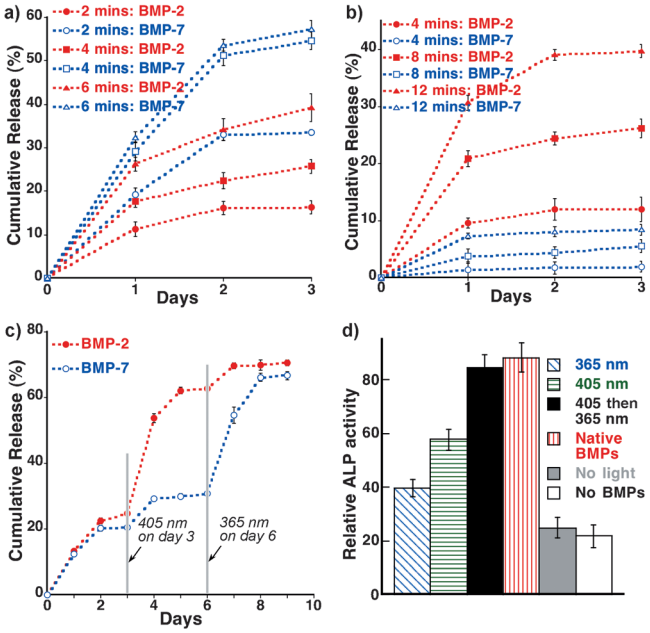


Figure 3. Daily release of BMP-2 and BMP-7 upon exposure to light of a) 365 nm (5 mW cm^{-2}) and b) 405 nm (5 mW cm^{-2}) for varied times; c) Sequential release of BMP-2 and BMP-7 upon exposing to both 405 and 365 nm light but at different times; d) Relative ALP activity for hMSCs seeded on hydrogels that contained both BMP-2 and BMP-7 in equal amounts (10 ng mL^{-1}) and exposed to 365 nm (for 6 min) and 405 nm (for 12 min) separately and also to both but sequentially, that is, 405 nm first for 12 min and then 365 nm for 6 min (see text for details); native BMPS: cells seeded on PEG hydrogels and exposed to BMP-2 and BMP-7 (10 ng mL^{-1}) but at day 1 and day 4, respectively; no light: not exposed to any light but gel contained both BMPs; no BMPs: cells seeded on hydrogel that did not contain any proteins.

times (Figure 3b) led to the expected opposite trend with increased BMP-2 release compared to BMP-7 (as observed with the model compound release studies, Figure 2d and e).

We then sought to investigate the possibility of releasing both proteins from a single gel, but in a manner that allowed their sequential and independent release. To achieve this, hydrogels were first exposed to 405 nm for 12 min at day 3, which resulted in a substantial and predominant release of BMP-2, and then exposed to 365 nm for 6 min at day 6 to trigger the release of the still-tethered BMP-7. Figure 3c shows the entire daily release of both the proteins from before exposure to light of 405 and 365 nm to trigger their cleavage and release from the gel. This result clearly illustrates that the second exposure to light (365 nm), results in extensive release of BMP-7 (ca. 40%), suggesting the requisite of higher energy light for cleavage of coumarin molecules and the resulting release of tethered BMP-7. Given that the majority of the BMP-2 (ca. 62%) was released during the first exposure to light (405 nm), the second exposure at 365 nm resulted in just 10% release of additional BMP-2. Taken together, the amount of recovered BMP-2 and BMP-7 was in the range of about 68–72%. Overall, the release results clearly demonstrated the sequential release of BMP-2 and BMP-7 by an ordered exposure to light of different wavelengths.

We tested the bioactivity of released BMPs by evaluating their influence on osteogenic differentiation of human MSCs (hMSCs), as measured by their alkaline phosphatase (ALP) activity at day 8. We observed that 1) neither light exposure nor covalent modification of the BMPs significantly affected their bioactivity, 2) hMSCs exposed to photoreleased BMP-2 and BMP-7 led to elevated levels of ALP compared to control cells that were not exposed to either BMPs, and 3) BMP-2 appeared to influence ALP activity more as compared to BMP-7 (Supporting Information). Then, to test the activity of sequential release of BMPs, BMP-2 and BMP-7 were first separately conjugated into different hydrogel formulations, and hMSCs were seeded on top of the gel. After allowing the cells to adhere to the gel surface, the gels were exposed to 365 nm light (5 mW cm^{-2}) for 10 min. When analyzed at day 8 (see Figure S4 in the Supporting Information), hMSC ALP activity was increased for photoreleased gels, as compared to controls (i.e., not exposed to light), but it was also comparable to the cellular responses obtained for native BMPs delivered in a soluble form. These results suggest that BMPs released by light indeed retain a bioactive nature that can locally influence cellular function.

Finally, to test the ability to sequentially deliver proteins, the impact of sequential release of BMP-2 and BMP-7 was tested by first seeding hMSCs on gel that contained both growth factors covalently bound. The cell-laden gels were first exposed to 405 nm light for 12 min at day 1, and after allowing the hMSCs to interact with the first protein, BMP-2, for three days, we again exposed the gel to 365 nm light for 6 min at day 4 to trigger the release of the second protein, BMP-7. These cellular responses were analyzed at day 8 (Figure 3d) and show that: 1) ALP activity in hMSCs exposed to both 405 and 365 nm, but in a sequential manner, was greater than that of cells exposed to only 405 nm, which largely releases BMP-2 or 365 nm light, which mostly trigger the release of BMP-7; 2) the cellular activity observed during sequential exposure was comparable to that exposed to native BMP-2 and BMP-7 at day 1 and day 4, respectively. Collectively, these results clearly suggested that differentiation of hMSCs can be manipulated by sequential release of BMP-2 then BMP-7 by user-defined exposure to 405 and 365 nm light.

In summary, we have synthesized and studied a new combination of selectively photocleavable nitrobenzyl- and coumarin-based molecular entities using light of different wavelengths. Our study has shown that the coumarin azides **2** cleave better than nitrobenzyls **1** with 365 nm light, but the cleavage of nitrobenzyl **1** is more efficient than coumarin **2** at 405 nm. We demonstrated the potential utility of the wavelength-dependent photocleavable characteristics of these molecular units to selectively release dye/protein (BMP-2 and BMP-7) molecules of any choice from a single hydrogel depot after gel formation. Selective protein release was further used to stimulate sequential signaling of hMSCs at different times by systematically switching the light from one wavelength to another. Such orthogonally programmed multiple protein release should allow researchers to tune the release kinetics of multiple proteins at any time from preloaded protein depots. We expect that this work should provide basic insight for strategies to synthesize and engineer new materials for controlled delivery of more than one protein with various release patterns and profiles and ultimately have implications in approaches to design materials for applications in the delivery of protein therapeutics related to tissue regeneration, wound healing, and disease treatment.

Received: September 17, 2013
Published online: October 31, 2013

Keywords: dyes/pigments · hydrogels · photocontrolled release · proteins · protein delivery

[1] N. A. Peppas, R. Langer, *Science* **1994**, *263*, 1715.
[2] S. D. Putney, P. A. Burke, *Nat. Biotechnol.* **1998**, *16*, 153.
[3] K. Y. Lee, M. C. Peters, K. W. Anderson, D. J. Mooney, *Nature* **2000**, *408*, 998.
[4] S. P. Baldwin, W. M. Saltzman, *Adv. Drug Delivery Rev.* **1998**, *33*, 71.
[5] R. Censi, P. Di Martino, T. Vermonden, W. E. Hennink, *J. Controlled Release* **2012**, *161*, 680.
[6] N. Murthy, M. C. Xu, S. Schuck, J. Kunisawa, N. Shastri, J. M. J. Frechet, *Proc. Natl. Acad. Sci. USA* **2003**, *100*, 4995.
[7] M. A. Azagarsamy, P. Sokkalingam, S. Thayumanavan, *J. Am. Chem. Soc.* **2009**, *131*, 14184.
[8] T. P. Richardson, M. C. Peters, A. B. Ennett, D. J. Mooney, *Nat. Biotechnol.* **2001**, *19*, 1029.
[9] J. Sohier, T. J. H. Vlugt, N. Cabrol, C. Van Blitterswijk, K. de Groot, J. M. Bezemer, *J. Controlled Release* **2006**, *111*, 95.
[10] F. Buket Basmanav, G. T. Kose, V. Hasirci, *Biomaterials* **2008**, *29*, 4195.
[11] F. M. Chen, M. Zhang, Z. F. Wu, *Biomaterials* **2010**, *31*, 6279.
[12] D. R. Griffin, A. M. Kasko, *J. Am. Chem. Soc.* **2012**, *134*, 13103.
[13] S. Petersen, J. M. Alonso, A. Specht, P. Duodu, M. Goeldner, A. del Campo, *Angew. Chem.* **2008**, *120*, 3236; *Angew. Chem. Int. Ed.* **2008**, *47*, 3192.

[14] C. Brieke, F. Rohrbach, A. Gottschalk, G. Mayer, A. Heckel, *Angew. Chem.* **2012**, *124*, 8572; *Angew. Chem. Int. Ed.* **2012**, *51*, 8446.
[15] J. X. Cui, V. San Miguel, A. del Campo, *Macromol. Rapid Commun.* **2013**, *34*, 310.
[16] A. M. Kloxin, A. M. Kasko, C. N. Salinas, K. S. Anseth, *Science* **2009**, *324*, 59.
[17] C. A. DeForest, K. S. Anseth, *Nat. Chem.* **2011**, *3*, 925.
[18] H. I. Lee, W. Wu, J. K. Oh, L. Mueller, G. Sherwood, L. Peteanu, T. Kowalewski, K. Matyjaszewski, *Angew. Chem.* **2007**, *119*, 2505; *Angew. Chem. Int. Ed.* **2007**, *46*, 2453.
[19] B. Yan, J. C. Boyer, D. Habault, N. R. Branda, Y. Zhao, *J. Am. Chem. Soc.* **2012**, *134*, 16558.
[20] C. G. Bochet, *Angew. Chem.* **2001**, *113*, 2140; *Angew. Chem. Int. Ed.* **2001**, *40*, 2071.
[21] A. del Campo, D. Boos, H. W. Spiess, U. Jonas, *Angew. Chem.* **2005**, *117*, 4785; *Angew. Chem. Int. Ed.* **2005**, *44*, 4707.
[22] P. Stegmaier, J. M. Alonso, A. del Campo, *Langmuir* **2008**, *24*, 11872.
[23] N. Kotzur, B. Briand, M. Beyermann, V. Hagen, *J. Am. Chem. Soc.* **2009**, *131*, 16927.
[24] S. Kantevari, M. Matsuzaki, Y. Kanemoto, H. Kasai, G. C. R. Ellis-Davies, *Nat. Methods* **2010**, *7*, 123.
[25] V. San Miguel, C. G. Bochet, A. del Campo, *J. Am. Chem. Soc.* **2011**, *133*, 5380.
[26] B. N. Goguen, A. Aemissegger, B. Imperiali, *J. Am. Chem. Soc.* **2011**, *133*, 11038.
[27] N. Fomina, C. L. McFearin, M. Sermsakdi, J. M. Morachis, A. Almutairi, *Macromolecules* **2011**, *44*, 8590.
[28] V. Gatterdam, T. Stoess, C. Menge, A. Heckel, R. Tampe, *Angew. Chem.* **2012**, *124*, 4027; *Angew. Chem. Int. Ed.* **2012**, *51*, 3960.
[29] N. Labòria, R. Wieneke, R. Tampe, *Angew. Chem.* **2013**, *125*, 880; *Angew. Chem. Int. Ed.* **2013**, *52*, 848.
[30] C. Grunwald, K. Schulze, A. Reichel, V. U. Weiss, D. Blaas, J. Piehler, K. H. Wiesmuller, R. Tampe, *Proc. Natl. Acad. Sci. USA* **2010**, *107*, 6146.
[31] K. Nakayama, T. Tachikawa, T. Majima, *Langmuir* **2008**, *24*, 6425.
[32] J. M. Alonso, A. Reichel, J. Piehler, A. del Campo, *Langmuir* **2008**, *24*, 448.
[33] M. Wirkner, J. M. Alonso, V. Maus, M. Salierno, T. T. Lee, A. J. Garcia, A. del Campo, *Adv. Mater.* **2011**, *23*, 3907.
[34] M. Álvarez, J. M. Alonso, O. Filevich, M. Bhagawati, R. Etchenique, J. Piehler, A. del Campo, *Langmuir* **2011**, *27*, 2789.
[35] Y. R. Zhao, Q. Zheng, K. Dakin, K. Xu, M. L. Martinez, W. H. Li, *J. Am. Chem. Soc.* **2004**, *126*, 4653.
[36] J. Babin, M. Pelletier, M. Lepage, J. F. Allard, D. Morris, Y. Zhao, *Angew. Chem.* **2009**, *121*, 3379; *Angew. Chem. Int. Ed.* **2009**, *48*, 3329.
[37] C. A. DeForest, B. D. Polizzotti, K. S. Anseth, *Nat. Mater.* **2009**, *8*, 659.
[38] H. W. Liu, C. H. Chen, C. L. Tsai, I. H. Lin, G. H. Hsiue, *Tissue Eng.* **2007**, *13*, 1113.

