Nanostructures

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Light-Triggered Bioactivity in Three Dimensions**

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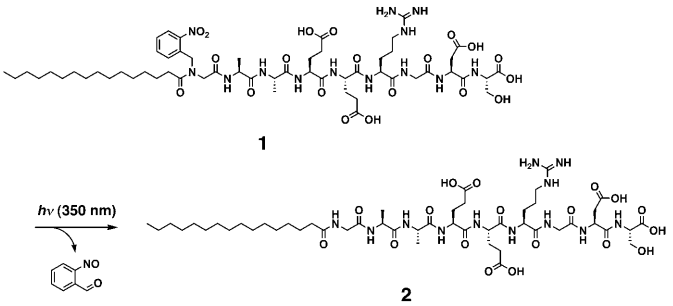
Hydrogelators that undergo stimuli-responsive sol–gel transitions have attracted attention as biomaterials because of their potential applications, for example as sophisticated cell culture substrates, drug carriers, microvalves, and microactuators.[1,2] Among the various stimuli possible, light is unique because it allows both spatial and temporal control of a specific reaction without requiring physical contact. Many photochemical sol–gel transitions have been reported, mostly in polymers but also in low-molecular-weight peptides.[1,3] The importance of peptide systems is their potential biocompatibility and the opportunity for researchers to molecularly design bioactive functions.[4,5] The advent of two-dimensional self-assembling systems and patterning technologies has generated some examples of stimulus-driven bioactivity on surfaces.[6] Using a peptide amphiphile (PA) containing the fibronectin epitope Arg-Gly-Asp-Ser (RGDS) for cell adhesion,[7] we demonstrate here light-triggered enhancement of bioactivity in a three-dimensional system.

PAs are known as highly versatile molecules that self-assemble into nanostructures such as spherical micelles, fibers, and helices through hydrogen-bond formation and hydrophobic collapse.[4,5,8–10] Our laboratory first reported on PAs that form high-aspect-ratio nanofibers and therefore gels at very low concentrations.[4] These specific PAs self-assemble into nanofibers as a result of their β-sheet peptide domains, and their self-assembly can be triggered by charge screening through changes in pH or the addition of salts. Molecular changes in the β-sheet domains can disrupt nanofiber formation. For example, Hartgerink and co-workers reported the formation of spherical micelles and not fibers as a result of the N-methylation on the amide nitrogen closest to the alkyl tail of a PA.[9] Our laboratory also reported recently the formation of quadruple helices in PAs containing a photolabile 2-nitrobenzyl group in the β-sheet domain.[10] These quadruple helices in turn dissociate into single nonhelical fibrils in response to light.

In this work, we have synthesized PA molecule **1** containing both the photocleavable 2-nitrobenzyl group as well as the bioactive epitope Arg-Gly-Asp-Ser (RGDS) (Scheme 1). Based on the previous work, photoirradiation



Scheme 1. Upon irradiation of **1** the 2-nitrobenzyl group is cleaved to afford **2**.

at 350 nm cleaves the 2-nitrobenzyl group to afford **2**.[10,11] The synthesis and characterization of compound **1** followed the procedures of our previous paper.[10] An authentic sample of compound **2** was also prepared separately for comparative purposes ($\mathbf{2}^{aut}$). To test the gelation ability of these two molecules, we dissolved **1** or $\mathbf{2}^{aut}$ (4.0×10^{-3} M) in 0.10 M aqueous $CaCl_2$ at 25 °C. Interestingly, in contrast to the quadruple-helix-forming PA which also generates nanofibers and gels, **1** remains as a clear solution under self-assembling conditions (Figure 1a). We believe this is the combined effect of nitrobenzyl substitution and use of a weaker β-sheet-forming sequence in **1** relative to that used previously (Gly-Ala-Ala-Glu-Glu (GA_2E_2) vs. Gly-Val-Val-Val-Ala-Ala-Ala-Glu-Glu-Glu ($GV_3A_3E_3$), respectively). However $\mathbf{2}^{aut}$ under the same self-assembling condition generates a transparent gel implying the formation of high-aspect-ratio nanofibers (Figure S1a in the Supporting Information). In fact, transmission electron microscope (TEM) images of these two molecules under self-assembling conditions reveal the formation of nanospheres in **1** and nanofibers in $\mathbf{2}^{aut}$ (Figure 2a and Figure S1b in the Supporting Information). We therefore expected, and verified experimentally, that **1** undergoes light-triggered gelation in the presence of charge-screening salts (Figure 1b). Furthermore, TEM reveals a change from spheres (12 ± 2) nm in diameter to fibers with aspect ratios exceeding 1000 as a result of irradiation (Figure 2). The diameter of the fibers is equal to (11 ± 1) nm (twice the

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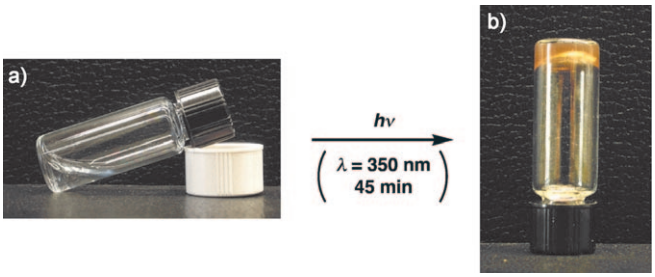

Figure 1. Photographs of **1** a) before and b) after photoirradiation (350 nm, 250 W, 45 min, 25 °C) at a concentration of 4.0×10^{-3} M in 0.10 M aq. $CaCl_2$ at 25 °C.

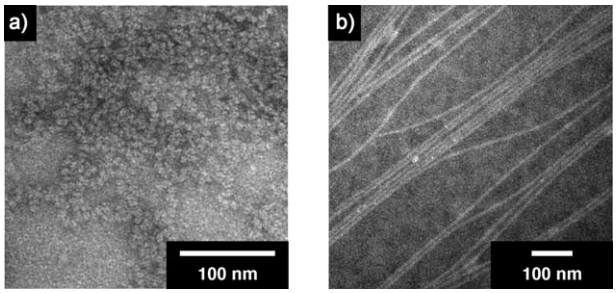

Figure 2. TEM images of structures formed by deposition of 4.0×10^{-4} M solutions of a) **1** and b) **2** in water followed by air-drying on the substrate.

extended molecular length, 5.1 nm), indicating that they are the well-known cylindrical nanostructures formed by PAs.

We characterized the photoreaction in the absence of $CaCl_2$ in order to maintain samples in the liquid state. UV/Vis spectroscopy showed an intensity decrease of the absorption band at 266 nm and an increase of the absorption band around 325 nm with isosbestic points at 236 and 284 nm. This indicates that the photocleavage of the 2-nitrobenzyl group occurs after irradiation (Figure 3 a).[10,11] In ESI mass spectra, the signals corresponding to **2** were clearly observed after the photoirradiation (Figure S2 in the Supporting Information), and analytical HPLC showed 98% conversion from **1** to **2** (Figure S3 in the Supporting Information). The circular dichroism (CD) spectrum of **1** revealed only a slight Cotton

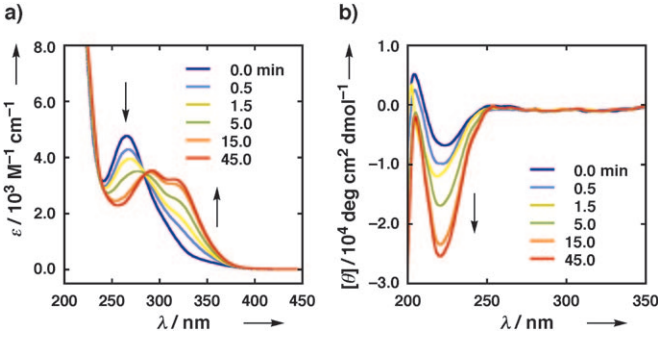

Figure 3. Changes in the a) UV/Vis and b) CD spectra of **1** (4.0×10^{-3} M) in water upon 350 nm irradiation at 25 °C. Arrows indicate the direction of the spectral changes.

effect at 220 nm indicating the presence of β-sheet structure.[12] However, upon irradiation, the CD signature for β-sheet formation continuously increased. Based on our previous work, this change in CD spectra is consistent with the gelation after irradiation for 45 min that was observed in the presence of $CaCl_2$ (Figure 3 b). In the IR spectra, the amide A bands for $\mathbf{2}^{aut}$ and photoirradiated **1** are observed at 3279 and 3280 cm^{-1}, respectively (Figure S4 in the Supporting Information). This indicates the formation in these two samples of intermolecular β-sheet structures.[13]

Given the observed self-assembly behavior of **1**, we investigated its potential for light-triggered bioactivity in a three-dimensional system. NIH/3T3 mouse embryonic fibroblasts were cultured in T-75 flasks and treated with trypsin. The cells were then cultured in three dimensions by mixing them with **1** at a concentration of 7.9×10^{-3} M and Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10% fetal bovine serum (FBS) and 0.10 M $CaCl_2$ and seeded in a 24-well tissue-culture-treated plate at a density of 4×10^5 cells per well. One plate was irradiated for 90 min to convert **1** into **2** and allow gelation to occur, while the other was kept in the dark to avoid gelation. Cell viability was visualized by fluorescence microscopy using the acetoxymethyl ester of calcein (calcein AM) to stain living cells in green and ethidium homodimer 1 to stain dead cells in red. The cells were also analyzed by flow cytometry on a Guava MiniCyte. As shown in Figure 4 a, most cells in the gels of **2** were stained green, which indicates the PA and the by-products of the photoreaction were not toxic to the cells and that cell proliferation was normal after the irradiation (by flow cytometry the cells were 92% viable in all cases). We studied focal adhesions formed by the cells in **1** and **2** by monitoring vinculin (a cytoskeletal protein found in focal adhesion plaques).[14,15] Real-time reverse-transcription polymerase chain reaction (RT-PCR) revealed that the mRNA expression levels of vinculin in a straight DMEM medium with and without 90 min photoirradiation were comparable (Figure 4 b, bars 1 and 2). When PA **1** was included in the mixture and the sample was kept in the dark, the expression level slightly increased (1.2 times, Figure 4 b, bar 3) compared to the case in pure media (Figure 4 b, bar 1). In great contrast, the level significantly increased (1.8 times) after light-triggered gelation of **1** (Figure 4 b, bar 4), which was comparable to expression of cells cultured in gels of $\mathbf{2}^{aut}$. Additionally, the level of protein expression characterized by Western blotting was found to be enhanced significantly (Figure 4 c). Based on the absorbance ratio of the bands, the expression of vinculin in **2** was estimated to be 1.9 times larger than that in **1**. These results indicate that light-triggered gelation of **1** increases its bioactivity.

In order to study the biological effect of gelation separately from the epitope, we synthesized compound **3** containing Arg-Gly-Glu-Ser (RGES) instead of RGDS of **1**. PA **3** underwent a light-triggered gelation similar to that of **1**. The mRNA expression levels of vinculin in the mixture of DMEM and **3** before and after photoirradiation were comparable to media controls and **1** (Figure 4 b, bars 1, 3, 6 and 7). In addition, cell experiments similar to those described above were performed using three different non-bioactive

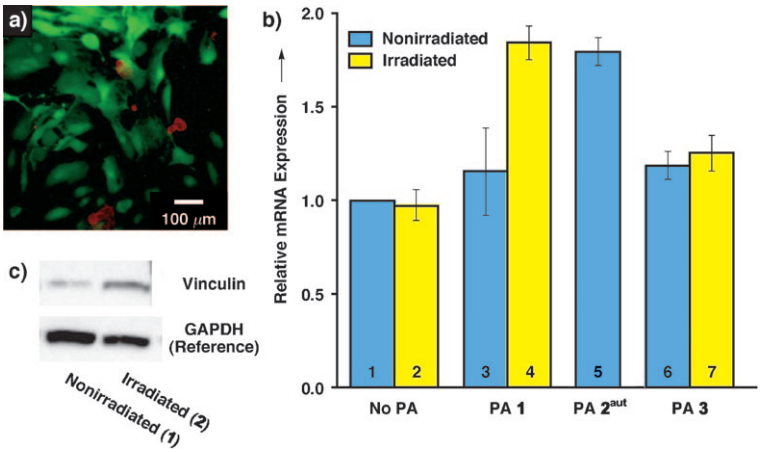

Figure 4. a) Fluorescence micrograph of NIH/3T3 mouse embryonic fibroblasts cultured in **2** obtained by photoirradiation of **1**. Calcein AM and ethidium homodimer 1 were utilized to stain living and dead cells in green and red, respectively. b) RT-PCR analyses of vinculin in DMEM without PA (bars 1 and 2), a mixture of DMEM and PA **1** (bars 3 and 4) before (light blue) and after (yellow) the photoirradiation, PA $\mathbf{2}^{aut}$ (bar 5) and PA **3** (bars 6 and 7) before and after the photoirradiation. c) Western blot analysis of the expression level of vinculin in PAs **1** (nonirradiated) and **2** (irradiated). Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) was used as the housekeeping gene.

gels: gelatin, agarose, and a PA (palmitoyl-Val-Val-Val-Ala-Ala-Ala-Glu-Glu-Glu). We found that the vinculin expression levels in these three systems were also comparable to those found in media alone as well as **1** (Figure 4b, bars 1, 3, 5–7 and Figure S5 in the Supporting Information). These results demonstrate that the origin of bioactivity is not the encapsulation of cells by the gels.[16] Instead, our observations suggest the bioactivity has its origin in the presence of epitope-bearing nanofibers in the extracellular space. We also learn from our results that at comparable epitope densities, nanospheres are not nearly as effective as nanofibers at cell signaling (Figure 4b, bars 3 and 4, Figure 4c). Finally, the model system described in this paper offers a pathway to light-triggered patterning of bioactivity in three dimensions.

We conclude that an RGD-containing peptide amphiphile bearing a photoresponsive 2-nitrobenzyl group undergoes a sol-to-gel transition in response to light. This small structural change can induce a significant change in the supramolecular structure from nanospheres to nanofibers, affecting the response of cells surrounded by the nanostructures. Our results offer a model to develop biomaterials that exhibit light-triggered bioactivity in three dimensions.

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[1] a) J. Edahiro, K. Sumaru, Y. Tada, K. Ohi, T. Takagi, M. Kameda, T. Shinbo, T. Kanamori, Y. Yoshimi, *Biomacromolecules* **2005**, *6*, 970; b) S. R. Sershen, G. A. Mensing, M. Ng, N. J. Halas, D. J. Beebe, J. L. West, *Adv. Mater.* **2005**, *17*, 1366; c) K. Takada, T. Miyazaki, N. Tanaka, T. Tatsuma, *Chem. Commun.* **2006**, 2024; d) M. S. Hahn, L. J. Taite, J. J. Moon, M. C. Rowland, K. A. Ruffino, J. L. West, *Biomaterials* **2006**, *27*, 2519; e) S. Matsumoto, S. Yamaguchi, A. Wada, T. Matsui, M. Ikeda, I. Hamachi, *Chem. Commun.* **2008**, 1545; f) S. Matsumoto, S. Yamaguchi, S. Ueno, H. Komatsu, M. Ikeda, K. Ishizuka, Y. Iko, K. V. Tabata, H. Aoki, S. Ito, H. Noji, I. Hamachi, *Chem. Eur. J.* **2008**, *14*, 3977.

[2] a) L. E. Bromberg, E. S. Ron, *Adv. Drug Delivery Rev.* **1998**, *31*, 197; b) S. Kiyonaka, K. Sugiyasu, S. Shinkai, I. Hamachi, *J. Am. Chem. Soc.* **2002**, *124*, 10954; c) K. J. C. van Bommel, M. C. A. Stuart, B. L. Feringa, J. van Esch, *Org. Biomol. Chem.* **2005**, *3*, 2917; d) K. N. Plunkett, K. L. Berkowski, J. S. Moore, *Biomacromolecules* **2005**, *6*, 632; e) W. Weng, J. B. Beck, A. M. Jamieson, S. J. Rowan, *J. Am. Chem. Soc.* **2006**, *128*, 11663; f) B. Wei, I. Cheng, K. Q. Luo, Y. Mi, *Angew. Chem.* **2008**, *120*, 337; *Angew. Chem. Int. Ed.* **2008**, *47*, 331; g) W. Deng, H. Yamaguchi, Y. Takashima, A. Harada, *Chem. Asian J.* **2008**, *3*, 687; h) A. K. Das, R. Collins, R. V. Ulijn, *Small* **2008**, *4*, 279.

[3] a) J. H. Collier, B.-H. Hu, J. W. Ruberti, J. Zhang, P. Shum, D. H. Thompson, P. B. J. Messersmith, *J. Am. Chem. Soc.* **2001**, *123*, 9463; b) L. A. Haines, K. Rajagopal, B. Ozbas, D. A. Salick, D. J. Pochan, J. P. Schneider, *J. Am. Chem. Soc.* **2005**, *127*, 17025; c) J. T. Meijer, M. J. A. G. Henckens, I. J. Minten, D. W. P. M. Löwik, J. C. M. van Hest, *Soft Matter* **2007**, *3*, 1135.

[4] a) J. D. Hartgerink, E. Beniash, S. I. Stupp, *Science* **2001**, *294*, 1684; b) J. D. Hartgerink, E. Beniash, S. I. Stupp, *Proc. Natl. Acad. Sci. USA* **2002**, *99*, 5133; c) H.-A. Klok, J. J. Hwang, J. D. Hartgerink, S. I. Stupp, *Macromolecules* **2002**, *35*, 6101; d) G. A. Silva, C. Czeisler, K. L. Niece, E. Beniash, D. A. Harrington, J. A. Kessler, S. I. Stupp, *Science* **2004**, *303*, 1352; e) E. Beniash, J. D. Hartgerink, H. Storrie, J. C. Stendahl, S. I. Stupp, *Acta Biomater.* **2005**, *1*, 387; f) M. O. Guler, S. Soukasene, J. F. Hulvat, S. I. Stupp, *Nano Lett.* **2005**, *5*, 249.

[5] a) H.-W. Jun, V. Yuwono, S. E. Paramonov, J. D. Hartgerink, *Adv. Mater.* **2005**, *17*, 2612; b) V. Jayawarna, M. Ali, T. A. Jowitt, A. F. Miller, A. Saiani, J. E. Gough, R. V. Ulijn, *Adv. Mater.* **2006**, *18*, 611; c) J. D. Hartgerink, *Nat. Nanotechnol.* **2006**, *1*, 166; d) D. Stroumpoulis, H. Zhang, L. Rubalcava, J. Gliem, M. Tirrell, *Langmuir* **2007**, *23*, 3849.

[6] a) C. B. Herbert, T. L. McLernon, C. L. Hypolite, D. N. Adams, L. Pikus, C.-C. Huang, G. B. Fields, P. C. Letourneau, M. D. Distefano, W.-S. Hu, *Chem. Biol.* **1997**, *4*, 731; b) M. N. Yousaf, B. T. Houseman, M. Mrksich, *Proc. Natl. Acad. Sci. USA* **2001**, *98*, 5992; c) J. Nakanishi, Y. Kikuchi, T. Takarada, H. Nakayama, K. Yamaguchi, M. Maeda, *J. Am. Chem. Soc.* **2004**, *126*, 16314; d) A. L. DeMond, T. Starr, M. L. Dustin, J. T. Groves, *J. Am. Chem. Soc.* **2006**, *128*, 15354; e) Y. Ohmuro-Matsuyama, Y. Tatsu, *Angew. Chem.* **2008**, *120*, 7637; *Angew. Chem. Int. Ed.* **2008**, *47*, 7527.

[7] M. D. Pierschbacher, E. Ruoslahti, *Nature* **1984**, *309*, 30.

[8] a) D. W. P. M. Löwik, J. C. M. van Hest, *Chem. Soc. Rev.* **2004**, *33*, 234; b) R. Bitton, J. Schmidt, M. Biesalski, R. Tu, M. Tirrell, H. Bianco-Peled, *Langmuir* **2005**, *21*, 11888; c) H.-W. Jun, S. E. Paramonov, J. D. Hartgerink, *Soft Matter* **2006**, *2*, 177; d) E. Kokkoli, A. Mardilovich, A. Wedekind, E. L. Rexeisen, A. Garg, J. A. Craig, *Soft Matter* **2006**, *2*, 1015; e) J. R. Lu, X. B. Zhao, Z. M. Yaseen, *Curr. Opin. Colloid Interface Sci.* **2007**, *12*, 60; f) I. W. Hamley, *Angew. Chem.* **2007**, *119*, 8274; *Angew. Chem. Int. Ed.* **2007**, *46*, 8128; g) R. V. Ulijn, A. M. Smith, *Chem. Soc.*

Rev. **2008**, *37*, 664; h) S. Cavalli, A. Kros, *Adv. Mater.* **2008**, *20*, 627.
[9] S. E. Paramonov, H.-W. Jun, J. D. Hartgerink, *J. Am. Chem. Soc.* **2006**, *128*, 7291.
[10] T. Muraoka, H. Cui, S. I. Stupp, *J. Am. Chem. Soc.* **2008**, *130*, 2946.
[11] Y. Tatsu, T. Nishigaki, A. Darszon, N. Yumoto, *FEBS Lett.* **2002**, *525*, 20.
[12] a) M. E. Magar, *Biochemistry* **1968**, *7*, 617; b) N. J. Greenfield, G. D. Fasman, *Biochemistry* **1969**, *8*, 4108.
[13] The position of the amide A bands below 3300 cm^{-1} suggests the exclusion of bent or folded structures. a) B. B. Doyle, E. G. Bendit, E. R. Blout, *Biopolymers* **1975**, *14*, 937; b) C. Toniolo, M. Palumbo, *Biopolymers* **1977**, *16*, 219.
[14] a) B. Geiger, *Cell* **1979**, *18*, 193; b) B. M. Jockusch, M. Rüdiger, *Trends Cell Biol.* **1996**, *6*, 311; c) C. F. Deroanne, A. C. Colige, B. V. Nusgens, C. M. Lapiere, *Exp. Cell Res.* **1996**, *224*, 215; d) B. G. Keselowsky, A. J. García, *Biomaterials* **2005**, *26*, 413.
[15] Immunohistochemistry was attempted but nonspecific staining and what is perceived to be interference from the peptide amphiphile system prevented reproducible results.
[16] The stiffness of irradiated **1**, irradiated **3**, and palmitoyl-Val-Val-Val-Ala-Ala-Ala-Glu-Glu-Glu PA gels is comparable ((45.7 ± 7), (45.6 ± 2) and (45.0 ± 3) kPa, respectively). See the Supporting Information for the experimental details and rheology data.

