Bioactive Surfaces

DOI: 10.1002/anie.201101581

Bio-orthogonal "Double-Click" Chemistry Based on Multifunctional Coatings

*Xiaopei Deng, Christian Friedmann, and Joerg Lahann**

As a consequence of recent progress in biotechnology, regenerative medicine, and developments concerning medical implants, an increasing need for precise and flexible conjugation methods has emerged.[1] For the immobilization of biomolecules, chemical reactions with high specificity towards the molecule of interest, mild reaction conditions compatible with physiological milieu, and rapid as well as quantitative conversion are essential.[2] If defined immobilization of two or more biomolecules on the same surface in controlled ratios is required, the individual reactions not only need to be orthogonal with respect to ongoing biological events, but also with respect to each other.[3] This prerequisite puts major constraints on the type of chemical reactions that can be exploited for bio-orthogonal immobilization. The development of bio-orthogonal reaction schemes has been heavily influenced by the concept of "click" chemistry, which was first introduced by Sharpless and co-workers in 2001.[4] As the archetypal example of click chemistry, the copper(I)-catalyzed Huisgen 1,3-dipolar cycloaddition of azides and terminal alkynes (CuAAC) has since been widely used as a surface-modification strategy.[5] CuAAC is a highly efficient reaction under mild conditions, with complete regioselectivity for the 1,4-triazole product.[6] Triazoles are stable linkers that are resistant to hydrolysis, oxidation, or reduction.[6b] Initial work was conducted on model surfaces, such as gold[7] and silicon,[8] but was recently extended to a wide range of different substrates.[9]

Chemical vapor deposition (CVD) polymerization[10] is a versatile coating process that effectively decouples the surface chemistry from the bulk composition. The CVD polymerization of functionalized [2.2]paracyclophanes can result in functionalized poly(*p*-xylylene) coatings with a wide range of different groups including active esters,[11] aldehydes,[12] ketones,[13] or anhydrides.[14] These coatings have been used for the immobilization of proteins,[15] peptides,[13] DNA,[16] and cells.[17] CVD coatings can be conformally deposited on a broad range of materials with different geometry[10] and are useful for applications including functional electrically conductive polymer films,[18] polymer gradients,[19] protein-resistant surfaces,[20] solventless adhesive bonding,[21] 3D photoresists,[22] and polymer/carbon nanotube composites.[23]

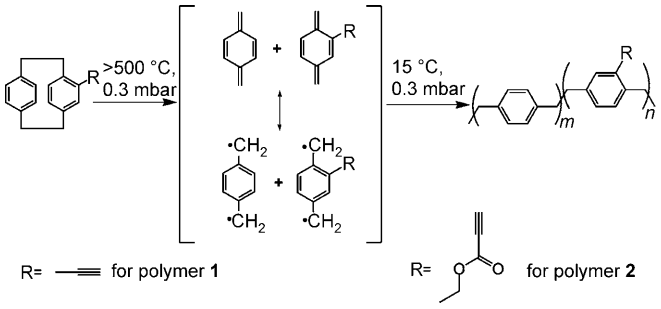
The CVD process has been successfully applied for the deposition of alkyne-functionalized polymers on a range of different substrates and can even support micro- and nanopatterning by CuAAC.[8b,9] Despite the success of CuAAC, the requirement of a potentially cytotoxic copper catalyst may limit its biomedical applications.[24] To develop Cu-free click chemistry, alkynes were activated by applying ring-strain, incorporating an electron-withdrawing group, or both.[24a] Bertozzi and co-workers conducted extensive studies on the synthesis of cyclooctyne derivatives for copper-free azide–alkyne cycloadditions.[25] They successfully improved the cyclooctyne reactivity by introducing electron-withdrawing fluorine atoms and used the copper-free click reactions for selective modifications of biomolecules and living cells.[25] Boons and co-workers achieved a similar rate enhancement by fusing two aryl rings to the cyclooctyne scaffold.[26] The strain-promoted cycloaddition of functionalized cyclooctynes to azides is an efficient reaction, but their challenging synthesis has prevented them from being more widely investigated and applied to bioimmobilization.[24a]

Herein, we report a synthetically straightforward approach towards reactive coatings for copper-free 1,3-dipolar cycloadditions and demonstrate a bio-orthogonal reaction scheme based on two sequential click reactions. Our approach is based on CVD polymerization of appropriately functionalized [2.2]paracylophanes. The development of a CVD coating that presents alkyne groups capable of copper-free click chemistry poses a number of challenges: 1) The reactive groups must readily react with azide groups at room temperature in benign solvents (e.g., water); 2) The functional groups have to be compatible with the processing conditions during CVD polymerization without decomposition or side reactions; 3) The precursors should be accessible by straightforward synthesis. Herein, we chose to synthesize [2.2]paracyclophane-4-methyl propiolate, which provides an electron-deficient alkynyl group for the spontaneous reaction with azide groups even in the absence of a catalyst. Neighboring electron-withdrawing groups are known to increase the reactivity of alkyne groups.[24a] Functional moieties such as sulfonyl and carbonyl groups were investigated in different studies.[27] Applications of electron-deficient alkyne moieties include DNA modification, gold nanoparticle functionalization, or hydrogel crosslinking.[27b,28]

As shown in Scheme 1, [2.2]paracyclophane-4-methyl propiolate was sublimed at 100 °C and 0.3 mbar and then subjected to thermal pyrolysis at 510 °C in vacuum to generate

[*] X. Deng, Dr. C. Friedmann, Prof. Dr. J. Lahann
Departments of Chemical Engineering, Materials Science and Engineering, Macromolecular Science and Engineering and Biomedical Engineering, University of Michigan
Ann Arbor, 48109 (USA)
and
Institute of Functional Interfaces, Karlsruhe Institute of Technology
Hermann-von-Helmholtz-Platz 1
76344 Eggenstein-Leopoldshafen (Germany)
Fax: (+1) 734-764-7453
E-mail: lahann@umich.edu

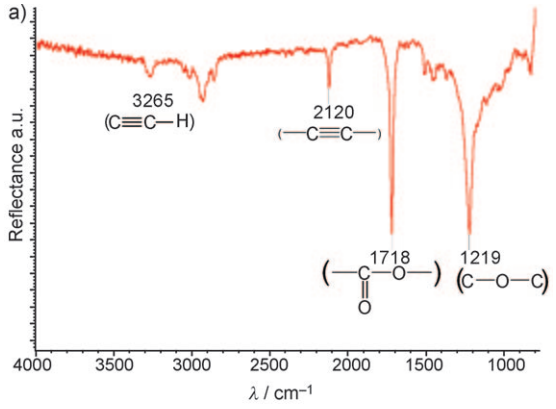
Supporting information for this article is available on the WWW under http://dx.doi.org/10.1002/anie.201101581.



Scheme 1. CVD polymerization process of polymers with non-activated (**1**) and activated alkyne groups (**2**).

reactive species that spontaneously polymerized upon adhesion onto the cooled substrate, which was maintained at 15 °C. This procedure resulted in a homogenous polymer film that was stable in aqueous solutions and organic solvents such as ethanol, acetone, dichloromethane, or chloroform.

FTIR spectroscopy and X-ray photoelectron spectroscopy (XPS) were used to assess the chemical structure of this newly synthesized polymer film and confirmed that the resulting polymer was indeed poly[(*p*-xylylene-4-methyl propiolate)-*co*-*p*-xylylene] (**2**, Figure 1). The FTIR spectrum clearly shows C–H stretching bands at 3265 cm^{-1} and –C≡C– stretching bands at 2120 cm^{-1}, which are characteristic of the terminal alkyne groups. The carbonyl C=O stretching band at 1718 cm^{-1} and C–O stretching band at 1219 cm^{-1} indicate the presence of ester bonds. No signs of decomposition or side reactions were observed. XPS was used to further confirm the chemical composition of the polymer films. From the XPS survey spectrum, the atomic ratios of C1s and O1s were found to be 90.9% (calcd: 90.9%) and 9.1% (calcd: 9.1%), respectively. The calculated values based on the structure of the starting material [2.2]paracyclophane-4-methyl propiolate were consistent with the experimental results shown in Figure 1b. This observation confirmed that polymer **2** was successfully synthesized by CVD polymerization.



b)

	Binding energy [eV]	Experimental [%]	Calculated [%]
C-C/H	285.0	73.7	77.5
C-C=O	285.8	4.3	4.5
C-O	286.8	5.5	4.5
O-C=O	289.2	4.3	4.5
π→π*	291.3	3.1	-
O-C=O	532.6	4.7	4.5
O-C=O	534.0	4.4	4.5

Figure 1. a) FTIR spectrum for poly[(*p*-xylylene-4-methyl-propiolate)-*co*-*p*-xylylene] (**2**); b) chemical composition of polymer **2** determined by XPS. Experimental values are compared with calculated values. Spectra are shown in the Supporting Information, Figure S1.

After verification of the chemical composition of polymer **2**, we tested the chemical reactivity of polymer **2** towards azide groups under copper-free conditions and compared it to the previously reported poly[4-ethynyl-*p*-xylylene-*co*-*p*-xylylene] (**1**);[8b] reactions were carried out by microcontact printing (μCP; Figure 2). μCP is a commonly used method to

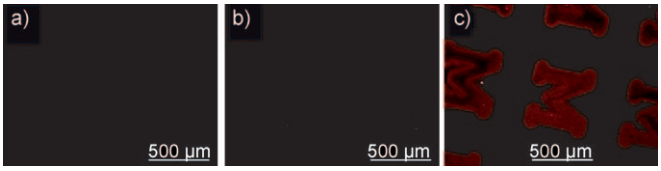


Figure 2. Chemical reactivity comparison of polymers **1** and **2** under copper-free condition using μCP for 3 h at room temperature. CVD polymer coatings on samples: a) poly(*p*-xylylene) (no reactive functional group, R = H in Scheme 1); b) polymer **1** (with nonactivated alkyne groups); c) polymer **2** (with activated alkyne groups). After CVD coating, the same treatment was applied to samples all 3 samples: μCP of biotin–PEG–azide followed by TRITC–streptavidin solution incubation. Scale bars represent 500 μm.

generate micro-scale patterns and has been used in the past to modify functionalized poly(*p*-xylylene)s.[8b,11,15] By using μCP, high local concentrations of reagents in the contact area can be achieved that can lead to significantly increased reaction rates.[29] Rozkiewics et al. reported printing of acetylene groups onto azide-functionalized self-assembled monolayers without the use of a copper catalyst. The microcontact printing approach further enables the selective reaction of defined surface areas embedded in a background of unreacted material; this unreacted material can be used as internal control in subsequent immobilization steps. Herein, a water-based solution of biotin–PEG–azide was used as ink to print onto three different surfaces: polymer **2**, polymer **1,** and unfunctionalized poly(*p*-xylylene). Poly(*p*-xylylene) has the same backbone as coatings **1** and **2**, but does not contain reactive functional groups (R = H in Scheme 1). This polymer was used as control compound to assess nonspecific adsorption onto the surfaces. Biotin was immobilized on polymer **2** through the triazole linker formed under copper-free conditions in water. Tetramethylrhodamine-5(6)-isothiocyanate (TRITC) conjugated streptavidin was then used to visualize the patterns.[30,31] In Figure 2, fluorescence patterns that are indicative of immobilized TRITC–streptavidin bound to biotin are only observed on polymer **2**, but not on polymer **1** or the unfunctionalized polymer. This result verifies that under copper-free conditions at room temperature, polymer **2**

successfully reacted with biotin–PEG–azide, while polymer **1** and poly(*p*-xylylene) showed no reaction.

We then prepared microengineered surfaces that contained either polymer **1** or polymer **2** in different surface areas (Figure 3). We hypothesized that such an approach would enable a head-to-head comparison of the activated versus nonactivated polymer coatings under otherwise identical reaction conditions. This approach was carried out by first coating the entire substrate with one layer of polymer **1** and then depositing a second layer of polymer **2** onto selected surface areas using the previously developed vapor-assisted micropatterning in replica structures (VAMPIR) technique.[20a,32] The only exception is shown in Figure 3c, where the coating sequence was reversed. The difference in thickness between the two polymer layers for all microengineered samples used in this study was measured to be 3–4 nm by imaging ellipsometry. An example of a representative thickness measurement is shown in the Supporting Information (Figure S2). To ensure that the fluorescence contrast does not stem from possible auto-fluorescence of the polymers, the sample shown in Figure 3a1 and a2 was never in contact with any azide compound, but was exposed to deionized water and then TRITC–streptavidin solution only. The microengineered surface did not show any fluorescence signal (Figure 3a2), hence confirming that auto-fluorescence of the polymers was negligible and thus would not interfere with the bioconjugation study. The contrast in the bright-field image (Figure 3a1) was caused by differences in thickness between the polymer layers and confirmed that the surface was successfully microengineered. Figure 3b and c show fluorescence images of two different surfaces that were modified to present inverse polymer patterns, which were achieved by simply reversing the coating sequence. Independent of the sequence of deposition, the results were the same, that is, only polymer **2** reacted with biotin–PEG–azide, while polymer **1** did not reveal any significant fluorescence signal. Note that the reaction time for solution-based reaction was 12 h and thus longer than for the μCP experiments, which were allowed to proceed for 3 h only. Even under prolonged exposure times, the selectivity was high and biotin–azide molecules were confined to areas that present propiolate groups. We observed that the copper-free reaction for polymer **2** started within seconds, as we could already observe initial contrast in the fluorescence signal after printing for 10 seconds (see the Supporting Information, Figure S3).

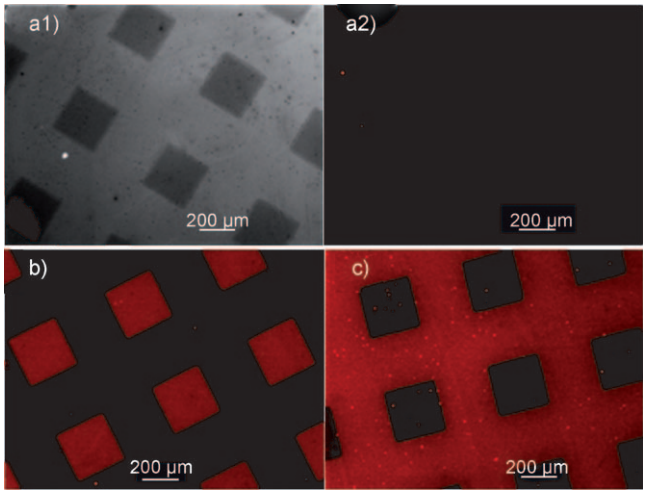


Figure 3. Chemical reactivity comparison of polymer **1** and **2** under copper-free conditions in solution reactions at room temperature. Samples coated by a 2-step CVD process using the VAMPIR technique resulted in surfaces with different polymer coatings inside and outside the squares. a1), a2), b) polymer **2** inside the squares and polymer **1** outside as shown in Figure 4, c) the reverse pattern, where polymer **1** is inside the squares. a1) bright field image of sample incubated in deionized water and then TRITC–streptavidin, a2) same sample as in (a1) but under red channel. b) and c) samples incubated in biotin–PEG–azide water solution and then TRITC–streptavidin solution. Scale bars represent 200 μm.

Finally, we took advantage of the difference in reactivity between polymers **1** and **2** and developed a cascade of bioorthogonal reactions.[3a] We devised a 2-step "click" chemistry procedure for sequential immobilization of different molecules on separate areas of the same surface (Figure 4). The cascade was put into effect by azide–alkyne reactions on the surface, first without and then with Cu catalyst (only in this order). Using microengineered substrates similar to the ones used above, the electron-deficient alkyne groups were first reacted inside the squares with Oregon 488 azide in deionized water at room temperature. Under these conditions, the nonactivated alkyne groups that were located in the remaining areas did not react. In a second reaction step, we used CuAAC to immobilize biotin–PEG–azide moieties on the remaining background. Subsequent self-assembly of a TRITC–streptavidin allowed for visualization of the selective surface modification. Figure 4b shows the overlay image of green and red channels, clearly with two different molecules immobilized on separate defined areas of the same surface. No cross-reaction of different areas was observed, thus indicating a high degree of selectivity as well as complete conversion during the copper-free reaction of polymer **2** (first reaction step).

A potential limitation of this work is that the copper-free reaction has always to occur first. Reversal of the reaction sequence is not possible in this specific example. However, the newly synthesized CVD coating with activated alkyne groups could also be used in conjunction with other bioorthogonal reactions that have been previously shown to be compatible with CVD coatings.[11–14] Ultimately, the "double-click" approach proposed herein as well as other bioorthogonal immobilization strategies[3a] will likely find applications in areas, in which controlled immobilization of multiple ligands is needed.

Received: March 3, 2011
Published online: June 7, 2011

Keywords: biomaterials · click chemistry · coatings · polymers · surfaces

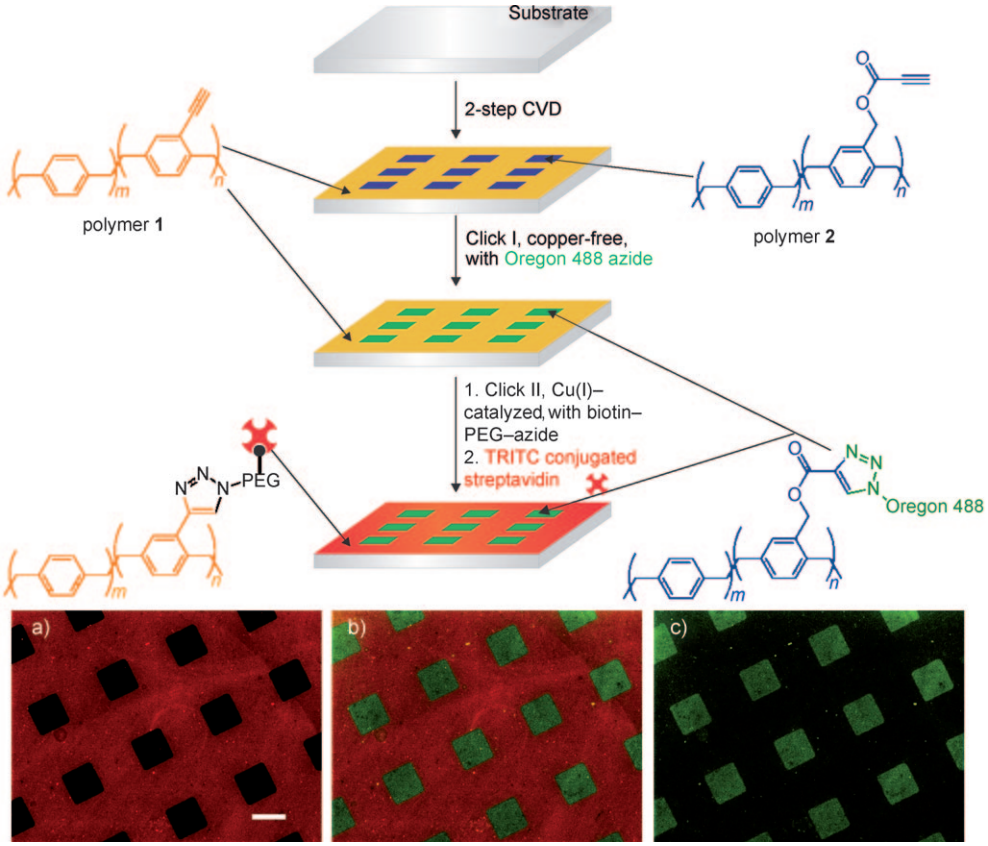


Figure 4. Scheme of two-step click reactions on surface coated with different polymers in different selected areas. The goal is to achieve sequential immobilization of molecules on defined areas of the same surface by utilizing the different reactivity of activated and nonactivated alkynyl groups towards azide groups. a), b), c) Fluorescence images of samples prepared exactly as shown in the schematic representation. b) overlay image of green and red channels shown in (a) and (c). Scale bar represents 200 μm.

[1] a) B. D. Ratner, S. J. Bryant, *Annu. Rev. Biomed. Eng.* **2004**, *6*, 41–75; b) D. G. Castner, B. D. Ratner, *Surf. Sci.* **2002**, *500*, 28–60.

[2] E. M. Sletten, C. R. Bertozzi, *Angew. Chem.* **2009**, *121*, 7108–7133; *Angew. Chem. Int. Ed.* **2009**, *48*, 6974–6998.

[3] a) J. C. Jewett, C. R. Bertozzi, *Chem. Soc. Rev.* **2010**, *39*, 1272–1279; b) Y. Elkasabi, H. Y. Chen, J. Lahann, *Adv. Mater.* **2006**, *18*, 1521–1526.

[4] H. C. Kolb, M. G. Finn, K. B. Sharpless, *Angew. Chem.* **2001**, *113*, 2056–2075; *Angew. Chem. Int. Ed.* **2001**, *40*, 2004–2021.

[5] J. A. Johnson, M. G. Finn, J. T. Koberstein, N. J. Turro, *Macromol. Rapid Commun.* **2008**, *29*, 1052–1072.

[6] a) C. W. Tornøe, C. Christensen, M. Meldal, *J. Org. Chem.* **2002**, *67*, 3057–3064; b) V. V. Rostovtsev, L. G. Green, V. V. Fokin, K. B. Sharpless, *Angew. Chem.* **2002**, *114*, 2708–2711; *Angew. Chem. Int. Ed.* **2002**, *41*, 2596–2599.

[7] a) J. P. Collman, N. K. Devaraj, T. P. A. Eberspacher, C. E. D. Chidsey, *Langmuir* **2006**, *22*, 2457–2464; b) J. L. Brennan, N. S. Hatzakis, T. R. Tshikhudo, N. Dirvianskyte, V. Razumas, S. Patkar, J. Vind, A. Svendsen, R. J. M. Nolte, A. E. Rowan, M. Brust, *Bioconjugate Chem.* **2006**, *17*, 1373–1375.

[8] a) S. Ciampi, T. Bocking, K. A. Kilian, M. James, J. B. Harper, J. J. Gooding, *Langmuir* **2007**, *23*, 9320–9329; b) H. Nandivada, H. Y. Chen, L. Bondarenko, J. Lahann, *Angew. Chem.* **2006**, *118*, 3438–3441; *Angew. Chem. Int. Ed.* **2006**, *45*, 3360–3363.

[9] a) S. G. Im, B. S. Kim, L. H. Lee, W. E. Tenhaeff, P. T. Hammond, K. K. Gleason, *Macromol. Rapid Commun.* **2008**, *29*, 1648–2654; b) H. Y. Chen, M. Hirtz, X. P. Deng, T. Laue, H. Fuchs, J. Lahann, *J. Am. Chem. Soc.* **2011**, *132*, 18023–18025.

[10] M. E. Alf, A. Asatekin, M. C. Barr, S. H. Baxamusa, H. Chelawat, G. Ozaydin-Ince, C. D. Petruczok, R. Sreenivasan, W. E. Tenhaeff, N. J. Trujillo, S. Vaddiraju, J. J. Xu, K. K. Gleason, *Adv. Mater.* **2010**, *22*, 1993–2027.

[11] J. Lahann, I. S. Choi, J. Lee, K. F. Jenson, R. Langer, *Angew. Chem.* **2001**, *113*, 3273–3276; *Angew. Chem. Int. Ed.* **2001**, *40*, 3166–3169.

[12] H. Nandivada, H. Y. Chen, J. Lahann, *Macromol. Rapid Commun.* **2005**, *26*, 1794–1799.

[13] Y. Elkasabi, M. Yoshida, H. Nandivada, H. Y. Chen, J. Lahann, *Macromol. Rapid Commun.* **2008**, *29*, 855–870.

[14] J. Lahann, D. Klee, H. Hocker, *Macromol. Rapid Commun.* **1998**, *19*, 441–444.

[15] Y. Elkasabi, H. Nandivada, H. Y. Chen, S. Bhaskar, J. D'Arcy, L. Bondarenko, J. Lahann, *Chem. Vap. Deposition* **2009**, *15*, 142–149.

[16] S. Thévenet, H. Y. Chen, J. Lahann, F. Stellacci, *Adv. Mater.* **2007**, *19*, 4333–4337.

[17] J. Lahann, M. Balcells, T. Rodon, J. Lee, I. S. Choi, K. F. Jensen, R. Langer, *Langmuir* **2002**, *18*, 3632–3638.

[18] a) N. J. Trujillo, M. C. Barr, S. G. Im, K. K. Gleason, *J. Mater. Chem.* **2010**, *20*, 3968–3972; b) J. P. Lock, S. G. Im, K. K. Gleason, *Macromolecules* **2006**, *39*, 5326–5329.

[19] Y. Elkasabi, J. Lahann, *Macromol. Rapid Commun.* **2009**, *30*, 57–63.

[20] a) X. W. Jiang, H. Y. Chen, G. Galvan, M. Yoshida, J. Lahann, *Adv. Funct. Mater.* **2008**, *18*, 27–35; b) S. H. Baxamusa, K. K. Gleason, *Adv. Funct. Mater.* **2009**, *19*, 3489–3496.

[21] a) H. Y. Chen, A. A. McClelland, Z. Chen, J. Lahann, *Anal. Chem.* **2008**, *80*, 4119–4124; b) S. G. Im, K. W. Bong, C. H. Lee, P. S. Doyle, K. K. Gleason, *Lab Chip* **2009**, *9*, 411–416.

[22] H. Y. Chen, J. M. Rouillard, E. Gulari, J. Lahann, *Proc. Natl. Acad. Sci. USA* **2007**, *104*, 11173–11178.

[23] a) S. Tawfick, X. P. Deng, A. J. Hart, J. Lahann, *Phys. Chem. Chem. Phys.* **2010**, *12*, 4446–4451; b) K. K. S. Lau, J. Bico, K. B. K. Teo, M. Chhowalla, G. A. J. Amaratunga, W. I. Milne, G. H. McKinley, K. K. Gleason, *Nano Lett.* **2003**, *3*, 1701–1705.

[24] a) A. J. Inglis, C. Barner-Kowollik, *Macromol. Rapid Commun.* **2010**, *31*, 1247–1266; b) M. F. Debets, C. W. J. van der Doelen, F. P. J. T. Rutjes, F. L. van Delft, *ChemBioChem* **2010**, *11*, 1168–1184.

[25] a) E. M. Sletten, H. Nakamura, J. C. Jewett, C. R. Bertozzi, *J. Am. Chem. Soc.* **2010**, *132*, 11799 – 11805; b) N. J. Agard, J. A. Prescher, C. R. Bertozzi, *J. Am. Chem. Soc.* **2004**, *126*, 15046 – 15047; c) J. M. Baskin, J. A. Prescher, S. T. Laughlin, N. J. Agard, P. V. Chang, I. A. Miller, A. Lo, J. A. Codelli, C. R. Bertozzi, *Proc. Natl. Acad. Sci. USA* **2007**, *104*, 16793 – 16797.

[26] X. H. Ning, J. Guo, M. A. Wolfert, G. J. Boons, *Angew. Chem.* **2008**, *120*, 2285 – 2287; *Angew. Chem. Int. Ed.* **2008**, *47*, 2253 – 2255.

[27] a) S. G. Gouin, J. Kovensky, *Synlett* **2009**, 1409 – 1412; b) Z. M. Li, T. S. Seo, J. Y. Ju, *Tetrahedron Lett.* **2004**, *45*, 3143 – 3146.

[28] a) W. Limapichat, A. Basu, *J. Colloid Interface Sci.* **2008**, *318*, 140 – 144; b) M. Clark, P. Kiser, *Polym. Int.* **2009**, *58*, 1190 – 1195.

[29] a) D. I. Rozkiewicz, D. Janczewski, W. Verboom, B. J. Ravoo, D. N. Reinhoudt, *Angew. Chem.* **2006**, *118*, 5418 – 5422; *Angew. Chem. Int. Ed.* **2006**, *45*, 5292 – 5296; b) T. P. Sullivan, M. L. van Poll, P. Y. W. Dankers, W. T. S. Huck, *Angew. Chem.* **2004**, *116*, 4286 – 4289; *Angew. Chem. Int. Ed.* **2004**, *43*, 4190 – 4193.

[30] P. C. Weber, D. H. Ohlendorf, J. J. Wendoloski, F. R. Salemme, *Science* **1989**, *243*, 85 – 88.

[31] a) T. Sano, C. L. Smith, C. R. Cantor, *Science* **1992**, *258*, 120 – 122; b) J. Spinke, M. Liley, F. J. Schmitt, H. J. Guder, L. Angermaier, W. Knoll, *J. Chem. Phys.* **1993**, *99*, 7012 – 7019; c) M. Howarth, K. Takao, Y. Hayashi, A. Y. Ting, *Proc. Natl. Acad. Sci. USA* **2005**, *102*, 7583 – 7588.

[32] H. Y. Chen, J. Lahann, *Adv. Mater.* **2007**, *19*, 3801 – 3808.

