Peptidomimetics

DOI: 10.1002/ange.201104624

Protein Transduction Domain Mimics: The Role of Aromatic Functionality**

*Abhigyan Som, Anika Reuter, and Gregory N. Tew**

Cell-penetrating peptides (CPPs), or protein transduction domains (PTDs), are a special class of membrane-active proteins that can cross the cell membrane with unusual efficiency.[1] They have attracted considerable attention because of their ability to readily cross biological membranes, in spite of their highly charged nature.[2] While the exact mechanism of this transport remains under intense investigation, energy-independent pathways are known.[2a,3] Perhaps the clearest example is the ability of CPPs, and their synthetic mimics, to cross model phospholipid bilayer vesicle membranes.[4] One suggested mechanism implies that, in fact, CPPs like polyarginine (pR) need assistance to cross the membrane.[1d,4a–c,5] It suggests that hydrophobic counterions complex around the guanidinium-rich backbone, thus "coating" the highly cationic structure with lipophilic moieties. This process has been termed "activation", in which the lipophilic anion acts as an activator. In a series of detailed studies it was shown that aromatic activators outperform aliphatic ones.[4a–c,5] For example, sodium 4-(pyren-1-yl)butane-1-sulfonate gave an EC_{50} (effective concentration to obtain 50% activity) of 6.7 μM whereas the value for sodium dodecane-1-sulfonate was 16 μM.[5] Among other activators studied, the larger aromatic counterion, coronene, was not better than pyrene; however, a fullerene analogue was surprisingly effective.[5] While this work beautifully demonstrated the role of various counterions for pR activation, it was not clear if this better activation was due to general hydrophobicity or to the aromatic nature of these activators.

There is good reason to think that aromatic functional groups may play a special role, beyond their general hydrophobicity. It is well recognized that membrane proteins are enriched in aromatic amino acids at the membrane surface.[6] Their central hydrophobic core, composed mostly of aliphatic residues, is flanked on both sides by "aromatic belts".[6a,7] Although this belt is predominantly composed of tryptophan and tyrosine, as opposed to phenylalanine, it was shown that aromatic residues, including *N*-methylindole, have favorable free energies of insertion into the bilayer interface.[6b,8] This rules out a dominant effect of hydrogen bonding.[7] It was suggested that the flat-rigid shape, π-electronic structure, and associated quadrupolar moments provide unique and highly favorable interactions with the bilayer interface.[6b] Specific interactions that have been proposed include π-cation, electrostatic, dipole–dipole, and entropic factors related to bilayer perturbation.[6,7,9] Even HIV-TAT, the original protein that initiated the field of small PTDs, requires tryptophan (Trp_{11}) for translocation.[10] Moreover, an oligoarginine consisting of seven arginine residues with a C-terminal tryptophan (R_7W) and a $TAT_{48–60}$ peptide with residue 59 substituted with a tryptophan ($TAT_{48–60}P59W$) exhibit cellular internalization through energy-independent pathways.[3b,11] Another classical CPP, penetratin (Pen), contains two tryptophan residues. Substitution of tryptophan by phenylalanine (Pen2W2F) did not significantly impact cell uptake.[11] Among the aromatic amino acids, phenylalanine has the unique ability to partition at the interface and in the membrane core.[9f] In fact, aromatic residues, especially phenylalanine, are most effective at anchoring proteins in the membrane due to their "special ability" to form and stabilize essential interactions with the polar elements of the bilayer.[12] As a result, aromatic functionality could be a critical element facilitating the interactions between CPPs and the bilayer during transduction.

In the past few years, we and others have reported polymers designed to mimic the transduction activity of PTDs.[4d,13] More recently, we demonstrated that these protein transduction domain mimics (PTDMs) have "self-activation" properties when hydrophobic alkyl side chains were built into the copolymers.[14] Here, a new series of PTDMs was designed to determine if an aromatic functionality provides better transduction efficiency than aliphatic ones, at the same relative hydrophobicity. Given the importance of aromatic amino acids in membrane proteins and their interactions with the bilayer, it was proposed that aromatic side chains would make better activators, given equal relative hydrophobicity. Although aromatic groups have been studied in peptide-based CPPs,[3b,11,15] demonstration of the importance of aromatic functionality in these synthetic analogues is critical to establishing them as appropriate mimics, or PTDMs. By using reversed-phase HPLC to determine side-chain hydrophobicity and EC_{50} values in a classic transduction experiment, it is demonstrated here that it was possible to differentiate between side-chain hydrophobicity and aromaticity.

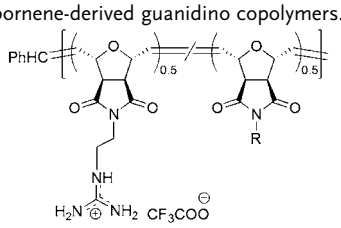
As shown in Table 1, a series of new PTDM polymers was prepared by ring-opening metathesis polymerization (see the Supporting Information for detailed synthesis and characterization of monomers and polymers). Reversed-phase HPLC, commonly used to evaluate relative hydrophobicity,[16] was

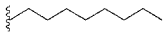
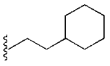
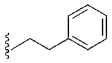
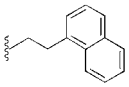
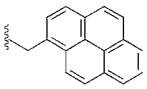
[*] Dr. A. Som, A. Reuter, Prof. G. N. Tew
Polymer Science & Engineering Department
University of Massachusetts
120 Governors Drive, Amherst, MA 01003 (USA)
E-mail: tew@mail.pse.umass.edu
Homepage: http://www.pse.umass.edu/gtew/index.html

[**] Generous support was primarily provided by the NSF (CHE-0910963). Dr. Semra Colak is acknowledged for her contribution in octyl monomer synthesis.

Supporting information for this article is available on the WWW under http://dx.doi.org/10.1002/anie.201104624.

Table 1: Oxanorbornene-derived guanidino copolymers.[a]



Polymer	R	DP	M_n	M_w/M_n
GOc		40	14900	1.08
GCy		34	12200	1.04
GPh		30	10700	1.05
GNp		30	11600	1.04
GPy		27	11100	1.07

[a] Degree of polymerization (DP), apparent molecular weight (M_n), and polydispersity index (M_w/M_n) were calculated from GPC. The repeat units are Cy cyclohexyl, G guanidino, Np naphthyl, Oc octyl, Ph phenyl, and Py pyrenyl.

performed on each nonpolar monomer. Using a C8 column in 100% acetonitrile (isocratic), the chromatograms of all five nonpolar monomers were obtained as shown in Figure 1.

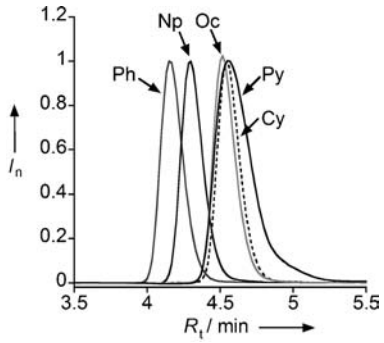


Figure 1. Retention time (R_t) on a reversed-phase C8 HPLC column (under isocratic conditions, 100% acetonitrile) of the corresponding hydrophobic monomers that were copolymerized with the guanidine monomers. I_n, normalized intensity. Individual R_t [min] of the monomers: Ph 4.15, Np 4.27, Oc 4.50, Cy 4.55, Py 4.57.

Pyrene has been commonly used as an activator of pR,[2a,4a–c,5] so its retention time (R_t) was of particular interest. In this study, its R_t was 4.57 min while the aliphatic monomers containing eight carbon atoms yielded similar R_t values of 4.55 (Cy) and 4.50 min (Oc). As a result, these three monomers have similar relative hydrophobicities. In contrast, the other two aromatic monomers, Np and Ph, are less hydrophobic with R_t values of 4.27 and 4.15 min, respectively. This series of monomers spans a range of relative hydrophobicities and therefore enables the deconvolution of hydrophobicity and aromaticity in transduction activity.

Transport activities for these novel PTDMs were determined using the standard biophysical assay that is well documented in the CPP literature.[2f,4d,5,14] Specifically, 5(6)-carboxyfluorescein (CF) was used as a fluorescent probe in egg yolk phosphatidylcholine large unilamellar vesicles (EYPC-LUVs). The activity of these transporters increased with increasing polymer concentration at a constant vesicle concentration as detected by CF emission intensity, thereby yielding plots of fluorescence intensity versus polymer concentration (Supporting Information, Figures S1 and S2). Fitting the Hill equation $[Y \propto (c/EC_{50})^n]$ to these data for each individual polymer revealed a nonlinear dependence of the fractional fluorescence intensity Y on the polymer concentration c, which is classical behavior demonstrated by CPPs.[4a–d,5,14] This analysis gave Y_{max} (maximal CF release relative to complete release by Triton X-100), EC_{50} (effective polymer concentration needed to reach $Y_{max}/2$), and the Hill coefficient n (see Table 2). For direct comparison it is worth mentioning that the CPP polyarginine hydrochloride was inactive under these conditions, a known fact since pR needs counterions for activation.

Table 2: EC_{50}, Y_{max}, and Hill coefficient n of the copolymers' transduction activity.

Polymer[a]	EC_{50} [nM][b]	Y_{max}[c]	n
GOc (50:50)	11.4 ± 2.8	0.80 ± 0.02	1.7 ± 0.05
GCy (50:50)	9.7 ± 0.9	0.80 ± 0.03	2.6 ± 1.0
GPh (50:50)	4.3 ± 0.1	0.96 ± 0.01	1.1 ± 0.1
GNp (50:50)	3.8 ± 0.6	0.84 ± 0.04	1.4 ± 0.2
GNp (80:20)	7.8 ± 1.8	0.88 ± 0.02	1.2 ± 0.2
GNp (96:04)	73.0 ± 0.9	0.91 ± 0.03	0.9 ± 0.1
GPy (50:50)	6.1 ± 0.2	0.81 ± 0.01	2.8 ± 0.3

[a] The molar ratios between guanidino (G) repeat units and the hydrophobic repeat units are given in parentheses. [b] EC_{50}: effective polymer concentration needed to reach $Y_{max}/2$. [c] Y_{max}: maximal CF release relative to complete release by Triton X-100. Each data point was collected in three independent experiments.

Figure 2a is a plot of $1/EC_{50}$ versus $1/R_t$ for GOc, GCy, GPh, GNp, and GPy. The data were plotted in this way to give the most efficient transporter the highest value as it relates to effective concentration. Since lower EC_{50} values are said to be more active, $1/EC_{50}$ directly provides the largest value for the best transporter. Similarly, it would be ideal to limit the hydrophobicity of the transporters while maintaining efficient transport activity; thus, $1/R_t$ was plotted since the retention time is larger for more hydrophobic monomers. Figure 2a shows that while GOc, GCy, and GPy have similar $1/R_t$ values, GPy is a more effective transporter (higher $1/EC_{50}$). In fact, it is approximately 1.5 to 2.0 times more active than GOc or GCy, despite the similar relative hydrophobicities of their corresponding nonpolar monomers. This activity difference is similar to that previously reported for pyrene (EC_{50}, 6.7 and 9.3 μM) versus alkyl activators (EC_{50}, 16 and 19 μM),[5a] which suggests that aromatic functionality may indeed have a special role in PTD(M) transduction.

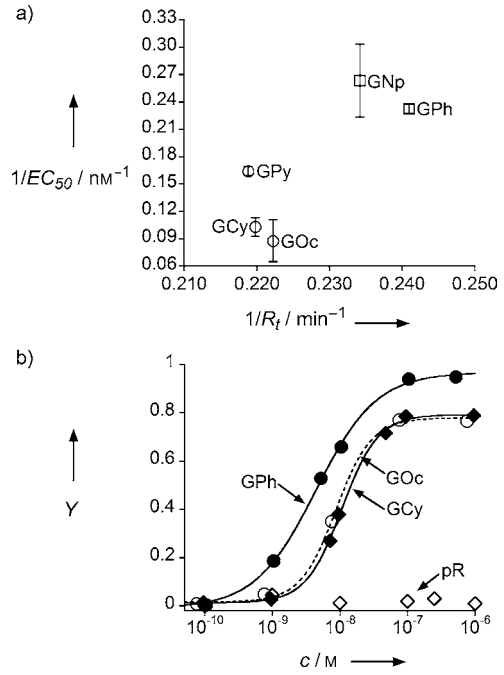


Figure 2. a) Plot of $1/EC_{50}$ (for the PTDM copolymer) versus $1/R_t$ (for the corresponding monomers) for GOc, GCy, GPh, GNp, and GPy. Data represent mean ± standard deviation (s.d.) from three independent experiments. b) Concentration (*c*)-dependent activity of copolymers GOc (○), GCy (◆), GPh (●), and pR (◇) in EYPC⊃CF vesicles with fit to the Hill equation. Transmembrane activity *Y* defines the fractional fluorescence intensity at 800 s.

Further support for this hypothesis comes from comparing the values (EC_{50} and hydrophobicity) for GPh to the others in Figure 2a. GPh is the least hydrophobic (larger $1/R_t$) yet it is the most active (higher $1/EC_{50}$). This is consistent with phenylalanine's unique ability to partition at the membrane interface and in the membrane core.[9f] Although GNp is more hydrophobic than GPh, they show similar activities within the $1/EC_{50}$ error level. Figure 2b shows the Hill plots for GOc, GCy, and GPh which yields their respective EC_{50} values of (11.4 ± 2.8), (9.7 ± 0.9), and (4.3 ± 0.1) nM. This comparison is particularly interesting since all three nonpolar monomers contain a total of eight carbon atoms. In addition, both GPh and GCy contain cyclic rings and, in fact, represent the closest possible structural analogues. While the aromatic group was expected to be less hydrophobic,[17] it clearly demonstrates that transduction activity is not solely dominated by hydrophobicity, but rather that aromaticity plays a crucial role. It also shows that the large pyrene ring is not essential and that smaller, more protein-like aromatic groups can effectively promote transduction in these PTDMs.

To further examine the role of aromatic size on transduction activity for this system, copolymers containing Np were prepared. The 50:50 copolymer provided a similar EC_{50} value ((3.8 ± 0.6) nM, see Figure 3 and Table 2) to those of the other aromatic-containing polymers. Given the similarity in values among all three aromatic-containing polymers, the molar content of Np was lowered to understand whether or not a "threshold" of aromatic content was needed for activity. As Figure 3 shows, the activity of GNp decreased with

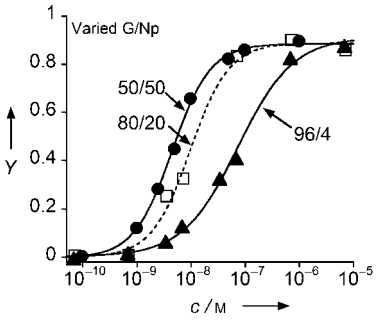


Figure 3. Hill plot of GNp copolymers with different G to Np repeat unit ratios (50:50, 80:20, and 96:4) in EYPC⊃CF vesicles with fit to the Hill equation.

decreasing molar content of Np, which suggests that no threshold was present. These data indicate that when more Np is present in the polymer it is more effective at transduction, although there is most likely an upper limit, at least because of the solubility of the polymer.

Table 2 summarizes the Hill parameters for these polymers and shows that they all have similar Y_{max} values and Hill coefficients n (around 2), which suggests poor cooperativity and supporting transduction.[4d] However, no detailed understanding of the mechanism is available at this time, and other factors such as polymer conformation and aggregation cannot be conclusively ruled out. To compare "activators" of varying EC_{50} values and total fractional transport activity, the activator efficiency E was calculated based on the exponential relationship between Y_{max} and EC_{50}.[5a] The same arbitrary scaling factor that was previously used to calibrate E between 0 and 10[5a] was also used here to determine E values for these covalently activated PTDMs (see the Supporting Information, Table S3). For GPh, E was found to be 25 or 2.5 times larger than the value for the highly active fullerene analogue and five times better than for pyrene butyrate. These covalent PTDMs have both low EC_{50} and high Y_{max} values, features previously suggested for the perfect activator.[5a] This is markedly different from the supramolecular activators in which more potent activators (lowest EC_{50} values) also had low Y_{max} values.[5] The fact that these covalently activated PTDMs are more effective than the supramolecular analogues (pR–activator) is not necessarily surprising, since covalent attachment eliminates the binding equilibrium between pR and the activator. A previously reported homopolymer of the guanidine monomer was activated with pyrene butyrate with an EC_{50} of 70 μM,[4d] whereas in this study a very low EC_{50} value (6.1 nM) was achieved with GPy. The best activators most likely also have solubility limitations since they are significantly hydrophobic. At the same time, the ability to design PTDMs that are significantly more active than classical CPPs is extremely encouraging.

Using HPLC to determine the relative hydrophobicity of various side chains, it was possible to demonstrate the improved transport activity of aromatic functionality. This provides guidance for building molecules that more favorably interact with the membrane while reducing the overall hydrophobicity. Understanding the broader goals of how macromolecules (synthetic or natural) interact with the

biological membrane is critically important. At the same time, learning to program synthetic polymers with natural protein-like activity remains an incredibly important task of modern macromolecular chemistry. Many fundamental questions remain but these new synthetic PTDMs appear to be useful tools for studying macromolecule–membrane interactions.

Received: July 5, 2011
Revised: September 6, 2011
Published online: December 13, 2011

Keywords: aromaticity · cell-penetrating peptides · hydrophobic effect · peptidomimetics · transduction

[1] a) P. Guterstam, F. Madani, H. Hirose, T. Takeuchi, S. Futaki, S. El Andaloussi, A. Graslund, U. Langel, *Biochim. Biophys. Acta Biomembr.* **2009**, *1788*, 2509 – 2517; b) I. Nakase, T. Takeuchi, G. Tanaka, S. Futaki, *Adv. Drug Delivery Rev.* **2008**, *60*, 598 – 607; c) S. Futaki, *Adv. Drug Delivery Rev.* **2005**, *57*, 547 – 558; d) J. B. Rothbard, T. C. Jessop, R. S. Lewis, B. A. Murray, P. A. Wender, *J. Am. Chem. Soc.* **2004**, *126*, 9506 – 9507; e) P. A. Wender, D. J. Mitchell, K. Pattabiraman, E. T. Pelkey, L. Steinman, J. B. Rothbard, *Proc. Natl. Acad. Sci. USA* **2000**, *97*, 13003 – 13008; f) P. S. Kabouridis, *Trends Biotechnol.* **2003**, *21*, 498 – 503.

[2] a) T. Takeuchi, M. Kosuge, A. Tadokoro, Y. Sugiura, M. Nishi, M. Kawata, N. Sakai, S. Matile, S. Futaki, *ACS Chem. Biol.* **2006**, *1*, 299 – 303; b) T. Hitz, R. Iten, J. Gardiner, K. Namoto, P. Walde, D. Seebach, *Biochemistry* **2006**, *45*, 5817 – 5829; c) P. A. Wender, W. C. Galliher, E. A. Goun, L. R. Jones, T. H. Pillow, *Adv. Drug Delivery Rev.* **2008**, *60*, 452 – 472; d) E. A. Goun, T. H. Pillow, L. R. Jones, J. B. Rothbard, P. A. Wender, *ChemBioChem* **2006**, *7*, 1497 – 1515; e) K. Melikov, L. Chernomordik, *Cell. Mol. Life Sci.* **2005**, *62*, 2739 – 2749; f) S. M. Fuchs, R. T. Raines, *Biochemistry* **2004**, *43*, 2438 – 2444; g) D. J. Mitchell, D. T. Kim, L. Steinman, C. G. Fathman, J. B. Rothbard, *J. Pept. Res.* **2000**, *56*, 318 – 325.

[3] a) T. Suzuki, S. Futaki, M. Niwa, S. Tanaka, K. Ueda, Y. Sugiura, *J. Biol. Chem.* **2002**, *277*, 2437 – 2443; b) P. E. G. Thorén, D. Persson, P. Isakson, M. Goksor, A. Onfelt, B. Norden, *Biochem. Biophys. Res. Commun.* **2003**, *307*, 100 – 107; c) J. L. Zaro, W. C. Shen, *Exp. Cell Res.* **2005**, *307*, 164 – 173.

[4] a) N. Sakai, S. Matile, *J. Am. Chem. Soc.* **2003**, *125*, 14348 – 14356; b) N. Sakai, S. Futaki, S. Matile, *Soft Matter* **2006**, *2*, 636 – 641; c) N. Sakai, T. Takeuchi, S. Futaki, S. Matile, *ChemBioChem* **2005**, *6*, 114 – 122; d) A. Hennig, G. J. Gabriel, G. N. Tew, S. Matile, *J. Am. Chem. Soc.* **2008**, *130*, 10338 – 10344; e) A. Pantos, I. Tsogas, C. A. Paleos, *Biochim. Biophys. Acta Biomembr.* **2008**, *1778*, 811 – 823.

[5] a) M. Nishihara, F. Perret, T. Takeuchi, S. Futaki, A. N. Lazar, A. W. Coleman, N. Sakai, S. Matile, *Org. Biomol. Chem.* **2005**, *3*, 1659 – 1669; b) F. Perret, M. Nishihara, T. Takeuchi, S. Futaki, A. N. Lazar, A. W. Coleman, N. Sakai, S. Matile, *J. Am. Chem. Soc.* **2005**, *127*, 1114 – 1115.

[6] a) J. A. Killian, G. von Heijne, *Trends Biochem. Sci.* **2000**, *25*, 429 – 434; b) S. H. White, W. C. Wimley, *Annu. Rev. Biophys. Biomol. Struct.* **1999**, *28*, 319 – 365.

[7] W. M. Yau, W. C. Wimley, K. Gawrisch, S. H. White, *Biochemistry* **1998**, *37*, 14713 – 14718.

[8] W. C. Wimley, S. H. White, *Nat. Struct. Biol.* **1996**, *3*, 842 – 848.

[9] a) A. Arbuzova, L. B. Wang, J. Y. Wang, G. Hangyas-Mihalyne, D. Murray, B. Honig, S. McLaughlin, *Biochemistry* **2000**, *39*, 10330 – 10339; b) M. M. Gromiha, *Biophys. Chem.* **2003**, *103*, 251 – 258; c) M. M. Gromiha, M. Suwa, *Int. J. Biol. Macromol.* **2005**, *35*, 55 – 62; d) W. J. Haynes, X. L. Zhou, Z. W. Su, S. H. Loukin, Y. Saimi, C. Kung, *FEBS Lett.* **2008**, *582*, 1514 – 1518; e) S. Mall, R. Broadbridge, R. P. Sharma, A. G. Lee, J. M. East, *Biochemistry* **2000**, *39*, 2071 – 2078; f) D. Sengupta, J. C. Smith, G. M. Ullmann, *Biochim. Biophys. Acta Biomembr.* **2008**, *1778*, 2234 – 2243; g) E. Strandberg, S. Morein, D. T. S. Rijkers, R. M. J. Liskamp, P. C. A. van der Wel, J. A. Killian, *Biochemistry* **2002**, *41*, 7190 – 7198; h) P. Braun, G. von Heijne, *Biochemistry* **1999**, *38*, 9778 – 9782.

[10] H. Yezid, K. Konate, S. Debaisieux, A. Bonhoure, B. Beaumelle, *J. Biol. Chem.* **2009**, *284*, 22736 – 22746.

[11] C. E. B. Caesar, E. K. Esbjorner, P. Lincoln, B. Norden, *Biochemistry* **2006**, *45*, 7682 – 7692.

[12] C. T. K. Yuen, A. R. Davidson, C. M. Deber, *Biochemistry* **2000**, *39*, 16155 – 16162.

[13] a) C. B. Cooley, B. M. Trantow, F. Nederberg, M. K. Kiesewetter, J. L. Hedrick, R. M. Waymouth, P. A. Wender, *J. Am. Chem. Soc.* **2009**, *131*, 16401 – 16403; b) E. M. Kolonko, J. K. Pontrello, S. L. Mangold, L. L. Kiessling, *J. Am. Chem. Soc.* **2009**, *131*, 7327 – 7333; c) E. M. Kolonko, L. L. Kiessling, *J. Am. Chem. Soc.* **2008**, *130*, 5626 – 5627.

[14] A. Som, A. O. Tezgel, G. J. Gabriel, G. N. Tew, *Angew. Chem.* **2011**, *123*, 6271 – 6274; *Angew. Chem. Int. Ed.* **2011**, *50*, 6147 – 6150.

[15] a) K. L. Horton, K. M. Stewart, S. B. Fonseca, Q. Guo, S. O. Kelley, *Chem. Biol.* **2008**, *15*, 375 – 382; b) J. R. Maiolo, M. Ferrer, E. A. Ottinger, *Biochim. Biophys. Acta Biomembr.* **2005**, *1712*, 161 – 172.

[16] a) H. D. Thaker, F. Sgolastra, D. Clements, R. W. Scott, G. N. Tew, *J. Med. Chem.* **2011**, *54*, 2241 – 2254; b) G. J. Gabriel, J. G. Pool, A. Som, J. M. Dabkowski, E. B. Coughlin, M. Muthukurnar, G. N. Tew, *Langmuir* **2008**, *24*, 12489 – 12495.

[17] R. Talhout, J. B. F. N. Engberts, *Org. Biomol. Chem.* **2004**, *2*, 3071 – 3074.

