Vesicle Self-Assembly

International Edition: DOI: 10.1002/anie.201502100
German Edition: DOI: 10.1002/ange.201502100

Aqueous Self-Assembly of Purely Hydrophilic Block Copolymers into Giant Vesicles**

Sarah M. Brosnan, Helmut Schlaad,* and Markus Antonietti*

Abstract: *Self-assembly of macromolecules is fundamental to life itself, and historically, these systems have been primitively mimicked by the development of amphiphilic systems, driven by the hydrophobic effect. Herein, we demonstrate that self-assembly of purely hydrophilic systems can be readily achieved with similar ease and success. We have synthesized double hydrophilic block copolymers from polysaccharides and poly(ethylene oxide) or poly(sarcosine) to yield high molar mass diblock copolymers through oxime chemistry. These hydrophilic materials can easily assemble into nanosized (< 500 nm) and microsized (> 5 μm) polymeric vesicles depending on concentration and diblock composition. Because of the solely hydrophilic nature of these materials, we expect them to be extraordinarily water permeable systems that would be well suited for use as cellular mimics.*

Self-assembly is critical to life itself. The most prominent example is the cellular membrane, which relies on the formation of a phospholipid bilayer. Scientists have worked with synthetic self-assembled membranes (liposomes) for decades; within the past twenty years, polymersomes have been developed as robust alternatives to liposomes, affording greater synthetic diversity.[1–9] These systems are thought to be, necessarily, amphiphilic—made from hydrophilic–hydrophobic block copolymers. In water, the hydrophobic segments self-assemble into aggregates.[10] This property leads to well-defined structures, but the resulting materials are always constrained by the limitations imposed by the requirement of amphiphilicity, while the hydrophobic membrane creates a (wanted or unwanted) barrier to the transport of hydrophilic substances. The self-assembly of small molecules and polymers, however, into supramolecular structures is not necessarily restricted to the hydrophobic effect but can be driven by several phenomena (e.g., van der Waals, ionic, and hydrogen bonding).[11–13]

An interesting example of non-amphiphilic phase separation is two-phase aqueous separation, which is used to purify and separate amino acids, peptides, and cells.[13] Highly water-soluble macromolecules are, under appropriate conditions, immiscible, and this principle, to our knowledge, has not been utilized for self-assembly of defined aggregate structures. The thermodynamic impetus driving their separation is competition for water; that is, the chemical potential of water (or osmotic pressure) must be equal in all communicating sub-phases; consequently, the polymer concentrations in solution have to differ. This de-mixing generates a water-in-water emulsion (an recent example of which is given by Nguyen et al.),[14] suggesting that self-assembly could occur if the different polymers were covalently attached to each other. The resulting water-in-water-in-water systems (implying that the membrane itself would also be mostly composed of water) would effectively contain separate but adjacent aqueous phases between which free transport would be possible. We hypothesize that the resulting self-assembly mechanism, driven by competition for water, could extend the capability for self-assembly beyond the standard amphiphilic structures.

We are not the first to put forth this idea, as there are a few examples of aggregation, or the organization, of purely hydrophilic materials, particularly concerning double hydrophilic block copolymers.[15–18] One of the first reports of such behavior was by Taubert et al., who demonstrated that double hydrophilic block copolymers can form lyotropic mesophases in pure water, which they could easily visualize by polarization microscopy. They suggested that the mesophases were just layers of water stacked on top of each other, separated only by the diblocks.[17] Blanazs and co-workers also used this phase separation of double hydrophilic block copolymers that is driven by differing abilities to absorb water, which thus creates a difference in the osmotic pressure that can only be equalized by separation. They reported that aqueous solutions (or rather viscous gels) of poly(ethylene glycol)-*block*-poly(2-(methacryloyloxy)ethyl phosphorylcholine) can organize into ordered phases. They observed formation lamellar and hexagonally packed structures, at concentrations greater than 40%, which they confirmed by small- and wide-angle X-ray scattering.[15] These examples wonderfully illustrate that the concept of hydrophilic phase separation into organized structures is sound, but no controlled micellar or vesicle-like aggregates were reported at any concentration in either case.

However, more recently, Casse et al. confirmed, in dilute solutions of a poly(ethylene glycol)-*block*-poly(2-methyl-2-

[*] Dr. S. M. Brosnan, Prof. M. Antonietti
Department of Colloid Chemistry
Max Planck Institute of Colloids and Interfaces
Research Campus Golm, 14424 Potsdam (Germany)
E-mail: sarah.brosnan@mpikg.mpg.de
Prof. H. Schlaad
Institute of Chemistry
University of Potsdam
Karl-Liebknecht-Strasse 24-25, 14476 Potsdam (Germany)
E-mail: schlaad@uni-potsdam.de

[**] This work was supported by National Science Foundation (NSF) Postdoctoral Research Fellowship in Biology under Grant No. DBI-1308104 and the Max Planck Society. We would like to thank Marlies Gräwert (GPC), Antje Völkel (AUC), Charlotte Vacogne (cryo-SEM), Heike Runge (cryo-SEM), and Ina Dambowsky (movies) for their assistance with this work.

Supporting information for this article is available on the WWW under http://dx.doi.org/10.1002/anie.201502100.

oxazoline) copolymer, the presence of a very few, small swollen spherical aggregates.[16] They determined that only a small fraction of polymer in solution was able to form their hydrated aggregates, which had characteristic features indicating vesicle formation, but overall it was difficult to confirm the exact nature of the aggregates owing to their low overall number and size.

We suspect that a careful choice of polymer system should provide a new pathway for the robust aggregation of hydrophilic systems into vesicle-like structures (ideally suitable as cellular mimics). To test this hypothesis, we synthesized three different double hydrophilic block copolymers such that both blocks were hydrophilic, non-ionic, and immiscible when the solutions of the homopolymers are mixed (Figure 1 A). Inspired by traditional two-phase aqueous systems, we synthesized dextran-*block*-poly(ethylene oxide) (Dex-PEO), pullulan-*block*-poly(ethylene oxide) (Pul-PEO), and dextran-*block*-poly(sarcosine) (Dex-PSar). The block lengths were approximately equal and in the higher molecular weight range (ca. 20 kg mol^{-1}, yielding diblocks that were approximately 40 kg mol^{-1}, Table S1–3) to increase their immiscibility and, thus, hopefully, drive self-assembly.[19]

These block copolymers were conveniently synthesized by taking advantage of the equilibrium state of one polysaccharide terminus as an aldehyde end group that can easily react under mild conditions (a citric acid solution with a pH of 3 in a 1:1 mixture with dimethylsulfoxide) with a hydroxy amine end group of either PEO or PSar (both mono-functionalized, Figure 1 B).[20–26] This coupling approach yields a double hydrophilic block copolymer linked by a hydrolytically stable oxime bond, the presence of which can easily be detected by ^{1}H NMR spectroscopy and whose apparent molar mass was determined by size exclusion chromatography (SEC; Figure S1–S10 and Graphs S1–S3 in the Supporting Information). To confirm the formation of diblocks, in addition to the standard polymer characterization methods, analytical ultracentrifugation (AUC) was utilized (Figure S11 and S12).[27] This measurement is important because, at this time, we only want to study the self-assembly of these systems alone, not the mixtures of the block copolymers with the homopolymers. Although we cannot rule out the presence of homopolymers as a result of the general difficulty of purifying saccharide-based materials, it is clear from NMR spectroscopy and GPC that homopolymer contamination is overall rather low (< 15 %).

We initially approached the self-assembly of double hydrophilic block copolymers as if these were amphiphilic systems, working at very low concentrations and with the Dex-PEO diblock copolymer (as it is the more well-known diblock). We utilized both direct dissolution at low concentrations (0.1, 0.5, and 1.0 %) and electroformation methods[28] (at a final concentration of roughly 0.5 %) to attempt the formation of diverse structures. Dynamic light scattering (DLS) was first utilized to confirm the presence of aggregates and a genuine and distinct difference between homopolymers, homopolymer mixtures, and diblock copolymers (Figure S14 and Graph S4). Direct dissolution required long wait times to see any aggregation (likely a consequence of the high molar mass of the system), but we did observe defined aggregates by DLS after 7 days (Figure 1 C). The aggregates exhibited hydrodynamic radii (R_h) of approximately 250 nm for the lowest concentrations and greater than 700 nm for the highest concentration. In the electroformation experiments, small aggregates were formed immediately in all samples. Analysis of the direct dissolution solutions by static light scattering (SLS) also suggested that some concentrations produced vesicle-like structures (Figure S13). The radius of gyration (R_g) determined for the particles in solution ranged between

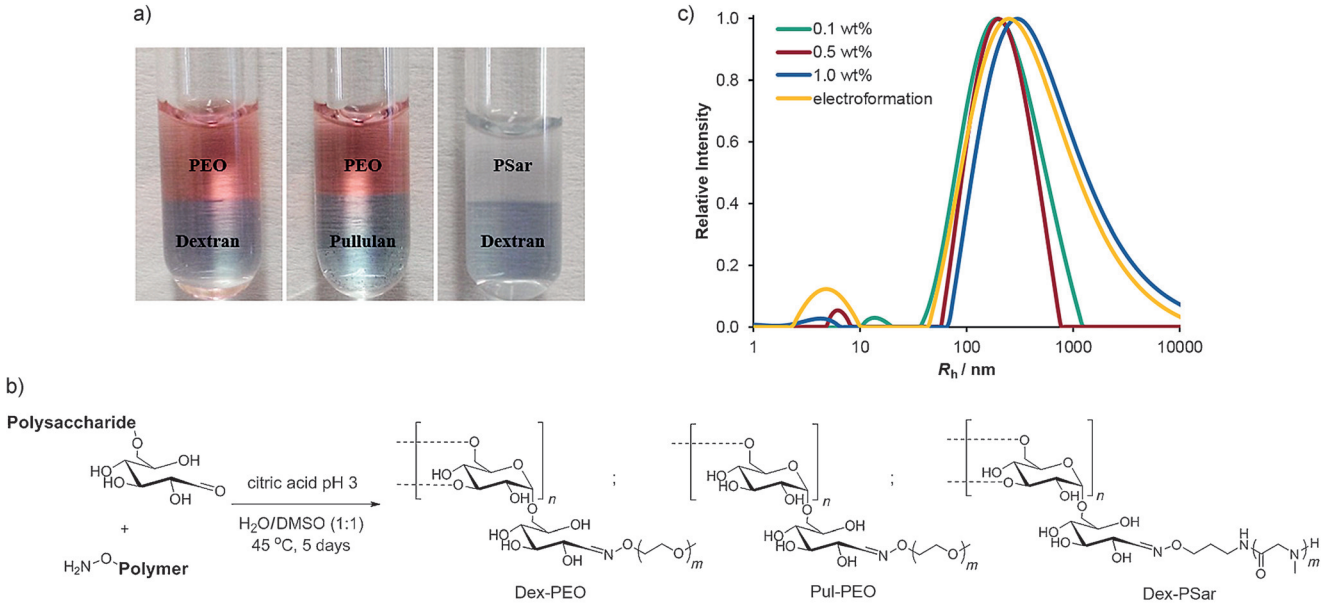

Figure 1. Self-assembly of double hydrophilic block copolymers. a) Two-phase aqueous separation occurs easily between the homopolymers dextran, pullulan, poly(ethylene oxide), and poly(sarcosine) (dye was added to aid visualization). b) Synthesis of the double hydrophilic block copolymers by coupling the hydroxy-amine modified poly(ethylene oxide) or poly(sarcosine) with dextran or pullulan. c) DLS of the direct dissolution at different concentrations and electroformation experiments shows the presence of aggregates (with the Dex-PEO diblock copolymer).

195 and 289 nm; thus, for at least the 0.5% solution, the characteristic R_g/R_h ratio is approximately 0.93, which suggests a hollow-sphere structure.[29]

To more conclusively demonstrate that we do indeed have self-assembled aggregates with a vesicle-like structure, we utilized electron microscopy. Typically, (cryogenic) transmission electron microscopy (TEM) is used as a more definitive method to confirm the structure of such aggregates; however, our system is mainly composed of water. Even if we had perfect bilayer structures, the membrane would still be swollen with water, which means that ordinary TEM (especially after drying) would only give information about the dried polymer—not the swollen aggregates. Cryogenic scanning electron microscopy, used more often in biological systems, provided far greater utility, as we could easily visualize our system, specifically Dex-PEO (Figure 2). In all cases, we were able to see aggregates similar to the size determined by DLS and observed that these aggregates were indeed self-assembled polymer vesicles—not solid particles or gels. The nature of the membrane itself is currently unknown but a focus of research. The aggregates shown, as expected, seem to have a much thicker membrane (10–30 nm) than standard liposomal or cell membranes; however, because they are not amphiphilic in nature, they should be not classified as polymersomes. Rather, it may be more appropriate to call these aggregates "aquanelles" (meaning 'little waters'). Herein, we demonstrate a robust and definitive system that illustrates that double hydrophilic block copolymers can indeed self-assemble in a similar fashion to amphiphilic systems that result in vesicle-like structures.

Our SEM analysis showed particles large enough to be viewed by optical microscopy, which suggested that we could make even larger assembly structures. For liposomes and amphiphilic polymersomes, electroformation is the technique typically used to create well-defined giant vesicles. However, we were not able, at realistic concentrations, to achieve giant aggregates by electroformation, and our best results yielded only a few approximately 1 μm particles. Additional attempts with direct dissolution methods (i.e., thin films, longer wait times) also did not result in larger or greater numbers of aggregates. With hindsight, this is not surprising as electroformation relies on the buildup of an electric field over the lipid or polymer film, which would be more difficult in our solely aqueous system.[30]

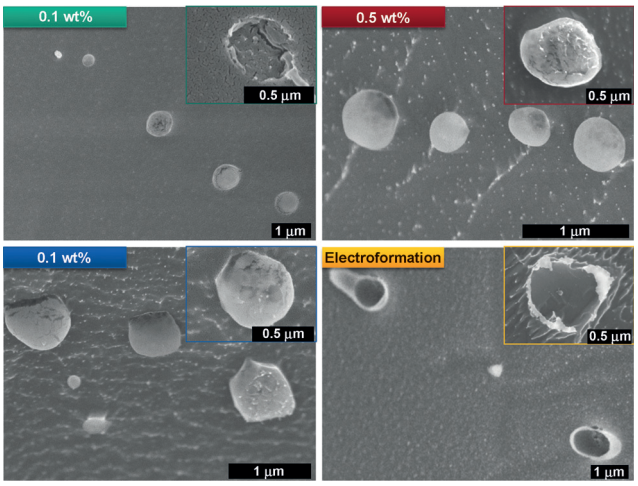

Figure 2. Cryogenic scanning electron microscopy of the direct dissolution and electroformation solutions of Dex-PEO. Insets: magnified images of the aggregates. Scale bars are 1 μm for the main images and 0.5 μm for the insets.

The path to more organized systems is, however, straightforward. In our system, unlike an amphiphilic system, water will not be excluded from the interlayer by the hydrophilicity of the component polymers, thus leading to microphase separation and organized aggregation (amphiphilic systems). However, if higher polymer concentrations of our double hydrophilic block copolymers are utilized, we can draw on the osmotic pull from the more dilute phase to potential create microphase separation in solution. If we consider that polysaccharides can strongly bind over 10 water molecules per repeat unit,[31] while PEO can only bind around 3 water molecules per repeat unit (the hydration of PSar is not known but presumably similar to PEO),[32] then the osmotic pressure can only balance out by microphase formation in solution. For our system, this means that membrane stability will be dependent on the effective concentration of the less-hydrated block (in this case, PEO or PSar). At low concentrations (<1%), where we see only a few small aggregates, the polymer concentration within the membrane is too low (ca. 5%) to provide significant viscoelasticity and stability, as the effective concentration of the PEO block remains comparatively low, and the membranes are ruptured by electrical or mechanical stress.

With this in mind, we explored self-assembly at higher concentrations, which should have a profound effect on the membrane stability of the system. The results of this change are quite impressive (Figure 3). Simply by raising the concentration above 10 wt%, we were easily able to achieve giant polymer vesicles with sizes between 2 and 20 μm. For the present double hydrophilic block copolymers, we found the upper concentration limit to be around 25 wt% before the system became too viscous for processing, while at 10 wt% and below only smaller, disperse aggregates could be found. This required overall concentration is not unlike the macromolecular crowding (and organization) observed in real cells.[33] Our results were not unique to the Dex-PEO double hydrophilic block copolymers, and similar complex giant aggregates could also be formed from Pul-PEO and Dex-PSar. These giant polymer vesicle solutions were stable for many days (>7) and could even be frozen and reformed (though the individual vesicles were not likely to survive the freezing process, so they are reformed upon warming to room temperature). We also are very nicely able to observe fusion, bursting, and encapsulation behavior that is typical of vesicle (not gel) structures (Movies S1 and S2, and Figures S15, S16).

In conclusion, we have demonstrated for the first time the robust self-assembly of purely double hydrophilic block copolymers into vesicle-like aggregates. By taking care to choose polymers that are immiscible with each other in water (in this case, polysaccharides, poly(ethylene oxide), and poly(sarcosine)) we were able to demonstrate that it is possible to form nano- and microsized polymer vesicles or "biomimetic" complex structures in pure water solutions,

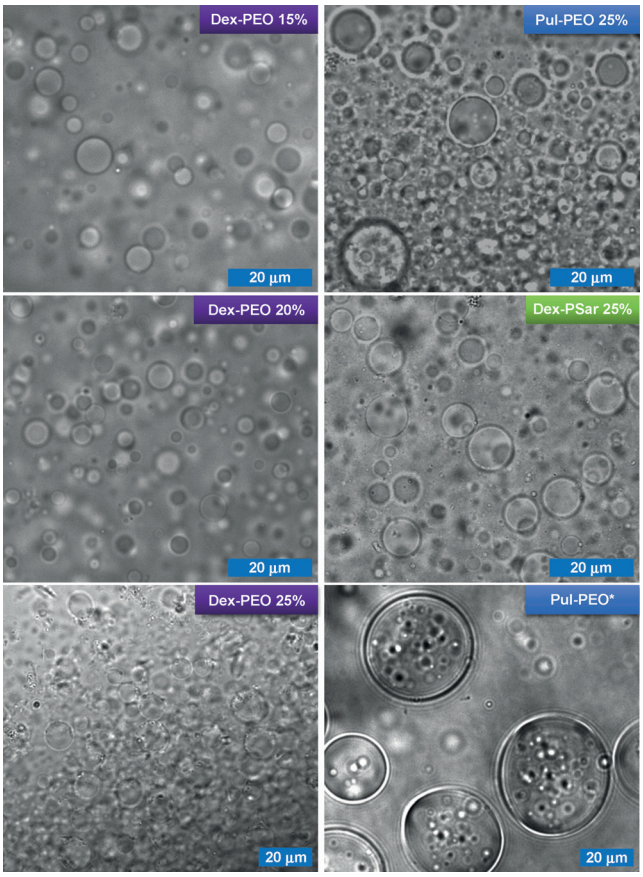

Figure 3. Optical microscopy images of the giant double hydrophilic polymer vesicles or "aquanelles". Left set of images show structures made by the Dex-PEO at different concentrations (from 25 to 15%). Right set of images show that these aquanelles can also be formed from the Pul-PEO (top-right) and Dex-PSar (middle-right), and that, in solution, these vesicles can be reformed easily after being frozen (bottom-right image, Pul-PEO*).

which we call "aquanelles", where all subphases are also aqueous. The resulting aggregates are expected to provide a new tool that will be applicable to cases where separation in aqueous compartments on the micro- and nanoscale is relevant (e.g., for delivery, as nanoreactors, or to build up enzyme cascades of mutually incompatible reactants). Aquanelles are presumably also well suited to be used as model cells and organelles because they are likely not only to have highly permeable membranes but also to be strongly controlled by internal compartmentalization, known in biological cells as the macromolecular crowding effect.

Keywords: block copolymers · polymersomes · polysaccharides · self-assembly · vesicles

How to cite: *Angew. Chem. Int. Ed.* **2015**, *54*, 9715–9718
Angew. Chem. **2015**, *127*, 9851–9855

[1] B. M. Discher, Y.-Y. Won, D. S. Ege, J. C.-M. Lee, F. S. Bates, D. E. Discher, D. A. Hammer, *Science* **1999**, *284*, 1143–1146.
[2] D. E. Discher, F. Ahmed, *Annu. Rev. Biomed. Eng.* **2006**, *8*, 323–341.
[3] K. Letchford, H. Burt, *Eur. J. Pharm. Biopharm.* **2007**, *65*, 259–269.
[4] D. M. Vriezema, M. Comellas Aragonès, J. A. A. W. Elemans, J. J. L. M. Cornelissen, A. E. Rowan, R. J. M. Nolte, *Chem. Rev.* **2005**, *105*, 1445–1489.
[5] G. N. Grover, H. D. Maynard, *Curr. Opin. Chem. Biol.* **2010**, *14*, 818–827.
[6] J. Sun, X. Chen, C. Deng, H. Yu, Z. Xie, X. Jing, *Langmuir* **2007**, *23*, 8308–8315.
[7] L. Zhang, A. Eisenberg, *J. Am. Chem. Soc.* **1996**, *118*, 3168–3181.
[8] L. Zhang, A. Eisenberg, *Science* **1995**, *268*, 1728–1731.
[9] L. Luo, A. Eisenberg, *J. Am. Chem. Soc.* **2001**, *123*, 1012–1013.
[10] M. Antonietti, S. Förster, *Adv. Mater.* **2003**, *15*, 1323–1333.
[11] C. F. J. Faul, M. Antonietti, *Adv. Mater.* **2003**, *15*, 673–683.
[12] P. J. Flory, *J. Chem. Phys.* **1942**, *10*, 51.
[13] *Partitioning in Aqueous Two-Phase System: Theory, Methods, Uses, And Applications To Biotechnology* (Eds.: H. Walter, D. E. Brooks, D. Fisher), Academic Press, San Diego, **1985**.
[14] B. T. Nguyen, W. Wang, B. R. Saunders, L. Benyahia, T. Nicolai, *Langmuir* **2015**, *31*, 3605–3611.
[15] A. Blanazs, N. J. Warren, A. L. Lewis, S. P. Armes, A. J. Ryan, *Soft Matter* **2011**, *7*, 6399.
[16] O. Casse, A. Shkilnyy, J. Linders, C. Mayer, D. Häussinger, A. Völkel, A. F. Thünemann, R. Dimova, H. Cölfen, W. Meier, et al., *Macromolecules* **2012**, *45*, 4772–4777.
[17] A. Taubert, E. Furrer, W. Meier, *Chem. Commun.* **2004**, 2170–2171.
[18] C. Valverde Serrano, Dissertation *Self-Assembly Behavior in Hydrophilic Block Copolymers*, Universität Potsdam, **2011**.
[19] S. Jain, F. S. Bates, *Science* **2003**, *300*, 460–464.
[20] R. Novoa-Carballal, A. H. E. Müller, *Chem. Commun.* **2012**, *48*, 3781–3783.
[21] T. L. Schlick, Z. Ding, E. W. Kovacs, M. B. Francis, *J. Am. Chem. Soc.* **2005**, *127*, 3718–3723.
[22] J. Aggen, P. Fatheree, M. S. Linsell, D. D. Long, D. Marquess, E. J. Moran, M. B. Nodwell, S. D. Turner, *Cross-Linked Glycopeptide-Cephalosporin Antibiotics*, **2003**, WO2003031449A2.
[23] Z. Miao, J. Liu, T. Norman, R. Driver, *Compositions Containing, Methods Involving, and Uses of Non-Natural Amino Acids and Polypeptides*, **2006**, WO2006069246A3.
[24] J. W. Robinson, C. Secker, S. Weidner, H. Schlaad, *Macromolecules* **2013**, *46*, 580–587.
[25] L. Ilić, K. Jeremić, S. Jovanović, *Eur. Polym. J.* **1991**, *27*, 1227–1229.
[26] K. Jeremić, L. Ilić, S. Jovanović, *Eur. Polym. J.* **1985**, *21*, 537–540.
[27] P. Schuck, *Biophys. J.* **2000**, *78*, 1606–1619.
[28] Y. Li, R. Lipowsky, R. Dimova, *Proc. Natl. Acad. Sci. USA* **2011**, *108*, 4731–4736.
[29] J. P. Patterson, M. P. Robin, C. Chassenieux, O. Colombani, R. K. O'Reilly, *Chem. Soc. Rev.* **2014**, *43*, 2412–2425.
[30] M. I. Angelova, D. S. Dimitrov, *Faraday Discuss. Chem. Soc.* **1986**, *81*, 303.
[31] J. Hunger, A. Bernecker, H. J. Bakker, M. Bonn, R. P. Richter, *Biophys. J.* **2012**, *103*, L10–2.
[32] T. P. Smart, O. O. Mykhaylyk, A. J. Ryan, G. Battaglia, *Soft Matter* **2009**, *5*, 3607–3610.
[33] G. J. Pielak, *Proc. Natl. Acad. Sci. USA* **2005**, *102*, 5901–5902.

Received: March 5, 2015
Revised: May 21, 2015
Published online: July 3, 2015