VIP Protein Manipulation

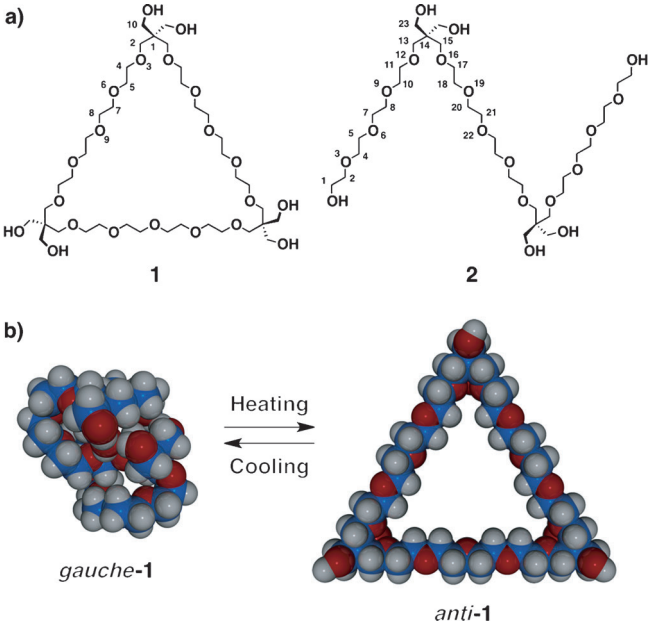
DOI: 10.1002/ange.201206563

A Structured Monodisperse PEG for the Effective Suppression of Protein Aggregation**

Takahiro Muraoka, Kota Adachi, Mihoko Ui, Shunichi Kawasaki, Nabanita Sadhukhan, Haruki Obara, Hidehito Tochio, Masahiro Shirakawa, and Kazushi Kinbara**

Poly(ethylene glycols) (PEGs) are popular polyethers that have been used in wide-ranging fields including chemical biology, medical chemistry,[1] and materials science.[2] In protein engineering, owing to their high water solubility, low toxicity, and low antigenicity, PEGs have been used for enhancing refolding, assisting crystallization, increasing water solubility, and prolonging the blood circulation time of proteins. In relation to these applications, the introduction of PEGs by covalent-bond formation, that is, PEGylation,[1] is an important method for improving the stability of proteins. However, chemistry of pure PEG molecules remains unexplored, as most conventional PEGs are polydisperse.[3] For example, it was not until very recently that the synthesis of a discrete PEG 44-mer ($M = 1956$) was accomplished.[3b] Moreover, PEGs are linear polymers and their structural diversity is limited, although recent studies have indicated that the properties of PEGs and their analogues change drastically depending on the molecular weight and topology.[1a,k]

Herein, we propose a practical approach to the formation of PEGs with higher dimension structures having topological diversity by using monodisperse oligoethylene glycol and pentaerythritol (PE) as a junction unit. PE has four hydroxy groups, and therefore allows multiple PEGs to be connected with the introduction of minimal structural difference from an ethylene glycol unit; the resulting structure has hydroxy groups at the vertex, like the termini of linear PEGs. As the first example of structured monodisperse PEGs, we synthesized trianglular PEG analogue **1**, ($M = 883$) which consists of three tetraethylene glycol (TEG) and three PE units (Scheme 1 a).[4] Interestingly, **1** undergoes dehydration, which is associated with conformational switching between



Scheme 1. a) Molecular structures of structured monodisperse PEGs **1** and **2**. b) A schematic representation of the heat-responsive conformational change of **1**. Left and right structures are molecular models *gauche*-**1** and *anti*-**1**, respectively, where all the ethylene units adopt either *gauche* or *anti* conformation (C blue, O red, and H white). The structures were optimized by a molecular mechanics calculation with Merck molecular force field (MMFF) by using Spartan′08.

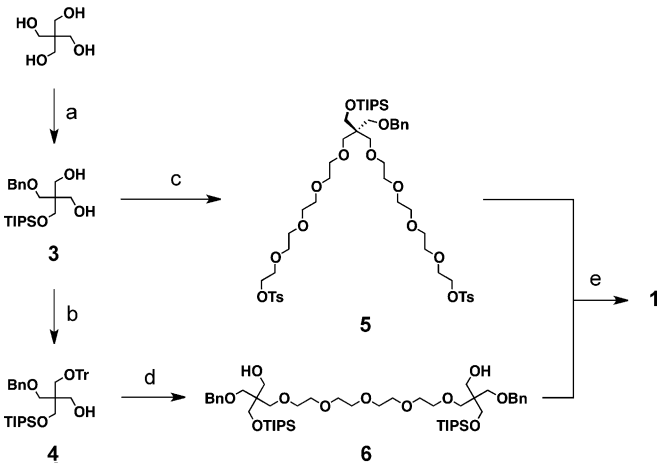
the *gauche* and *anti* forms (Scheme 1 b), at lower temperature than the corresponding linear version, **2** (Scheme 1 a). This unique feature could be applied for the manipulation of proteins,[5] that is, **1** exhibits the ability to suppress protein thermal aggregation, unlike **2** or PEG-1000, which is a conventional linear PEG of a similar molecular weight ($M_w = 993$).

1 was synthesized from PE by the procedure shown in Scheme 2. First, three hydroxy groups of PE were protected selectively (**3** and **4**) and the residual free hydroxy groups were coupled with tosylated TEGs to afford ditosylate **5** and diol **6**. Williamson ether synthesis between **5** and **6** and subsequent stepwise cleavage of triisopropyl (TIPS) and benzyl (Bn) groups successfully yielded compound **1**.[6] Similarly, **2** was also synthesized by Williamson ether syn-

[*] Dr. T. Muraoka, K. Adachi, Dr. M. Ui, S. Kawasaki, Dr. N. Sadhukhan, H. Obara, Prof. K. Kinbara
Institute of Multidisciplinary Research for Advanced Materials
Tohoku University, 2-1-1, Katahira, Aoba-ku, Sendai (Japan)
E-mail: kinbara@tagen.tohoku.ac.jp
Homepage: http://www.tagen.tohoku.ac.jp/labo/kinbara/index.html
Dr. H. Tochio, Prof. M. Shirakawa
Department of Molecular Engineering
Graduate School of Engineering, Kyoto University
Kyoto-Daigaku Katsura, Nishikyo-ku, Kyoto (Japan)

[**] We thank Prof. K. Tsumoto (University of Tokyo) for fruitful discussions. This work was partially supported by the Ministry of Education, Science, Sports and Culture (Japan), Grants-in-Aid for Young Scientists S (21675003 for K.K.), Scientific Research on Innovative Areas "Spying minority in biological phenomena (No. 3306)" (23115003 for K.K.), and Exploratory Research (24655112 for T.M.) and the Management Expenses Grants for National Universities Corporations from the Ministry of Education, Culture, Sports, Science and Technology of Japan (MEXT).

Supporting information for this article is available on the WWW under http://dx.doi.org/10.1002/anie.201206563.



Scheme 2. Synthesis of **1**. a) 1) $CH_3C(OEt)_3$, $TsOH{\cdot}H_2O$, toluene; 2) BnBr, KOH, DMSO; 3) HCl (aq.), MeOH, then Na_2CO_3; 4) TIPSCl, imidazole, DMF, 41% over 4 steps. b) TrCl, Et_3N, DMAP, CH_2Cl_2, quant. yield. c) 1) NaH, $TsO(CH_2CH_2O)_4PMB$, THF; 2) DDQ, CH_2Cl_2, water; 3) TsCl, Et_3N, CH_2Cl_2, 60% over 3 steps. d) 1) NaH, $TsO(CH_2CH_2O)_4Ts$, THF; 2) $ZnBr_2$, CH_2Cl_2, MeOH, 54% over 2 steps. e) 1) NaH, THF; 2) TBAF, THF; 3) H_2, Pd/C, EtOH, 32% over 3 steps. See the Supporting Information for the details. Bn = benzyl, DMAP = 4-dimethylaminopyridine, DMF = *N*,*N*′-dimethylformamide, DMSO = dimethyl sulfoxide, TBAF = tetra-*n*-butylammonium fluoride, TIPS = triisopropylsilyl, Tr = trityl, Ts = *p*-toluenesulfonyl.

thesis between the PE and TEG units. **1** and **2** are colorless oils, and readily dissolve in water at room temperature.

It is known that in water linear PEGs undergo a heat-responsive conformational change, whereby C–C bonds in the *gauche* form at room temperature change into the *anti* form at high temperatures.[7] Infrared (IR) spectroscopic analysis of a neat sample of **1** at 25 °C showed absorption bands at 1458 and 1352 cm^{-1}, which are characteristic to the CH_2 vibration of the ethylene glycol units in *gauche* conformation (see the Supporting Information, Figure S2, blue line).[7f] Upon being heated to 120 °C, **1** displayed an intensified absorption band at 1328 cm^{-1}, with a relatively intensified band at 1473 cm^{-1} (see the Supporting Information, Figure S2, red line), thus suggesting that the ethylene glycol units change the conformation from *gauche* to *anti* form.[7f] This conformational change is also indicated by variable temperature ^{13}C nuclear magnetic resonance (VT ^{13}C NMR) spectroscopy, in which a downfield shift of all the peaks in the spectrum of **1** in D_2O (1.6 $mg\,mL^{-1}$) occurs at elevated temperatures (see the Supporting Information, Figure S3).[7b]

With respect to the conformation at the junction between PE and TEG, variable temperature heteronuclear multiple-bond correlation (VT HMBC) experiments also indicate that rotation around C–O bonds occurs. In the HMBC measurement, ^1H and ^{13}C atoms in a *gauche* relationship display either no cross peak or a weak cross peak because of the small $^3J_{CH}$ value.[8] Indeed, at 25 °C, a cross peak between ^1H at C4 and ^{13}C at C2 was too weak to be observed, however a cross peak appeared upon heating the sample up to 60 °C (see the Supporting Information, Figure S4).[9] Hence, this marked alteration of the cross peak intensity suggests that the torsion angles around the C–O bonds between the PE and TEG units (C2 and O3 atoms) change from an *anti* to a *gauche* relationship upon increasing the temperature. These spectroscopic studies indicate that dynamic changes in the conformation of **1** are triggered by heating.

As is the case with **1**, **2** shows downfield shift of the ^{13}C NMR signals upon an increase in temperature from 20 °C to 80 °C, thus suggesting the *gauche*-to-*anti* conformational change of the ethylene glycol units (see the Supporting Information, Figure S5). As such *gauche*-to-*anti* conformational change is known to increase the hydrophobicity of PEGs,[7a–e] we then investigated their hydrophobicity in terms of dehydration. Hydrated PEGs are known to exhibit a linear relationship between $1/\eta$ and T_1/T, where η and T represent the viscosity of water and temperature, respectively, since the mobility of a PEG chain is governed mainly by the viscosity of the solvent (Figure 1).[7a] Actually, **2** shows an almost linear

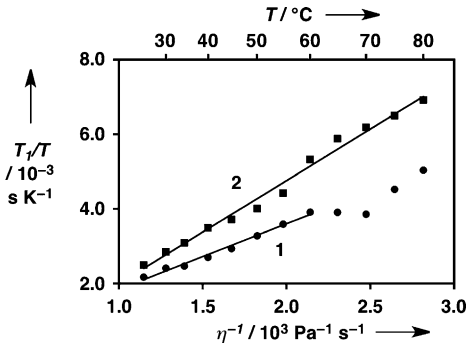


Figure 1. ^1H NMR T_1/T values of **1** (circles) and **2** (squares) in D_2O plotted as a function of the reciprocal of water viscosity (η) and temperature (T). T_1 values were measured for ^1H NMR signals corresponding to ^1H at C5, C7, and C8 of **1** and C2, C4, C5, C7, C8, C10, C18, C20, and C21 of **2**.

correlation between T_1/T and $1/\eta$ in the temperature range between 20 and 80 °C, thus indicating that **2** is hydrated below 80 °C. In contrast, although T_1/T values of **1** showed a linear correlation with $1/\eta$ below 60 °C, this correlation did not occur above 60 °C, thus strongly indicating that dehydration of **1** occurs around 60 °C. As well as **2**, conventional linear PEGs are known to dehydrate over 80 °C.[7a] Thus, the low dehydration temperature of **1** is likely due to a distinct effect of structuralization into the 2D trianglular geometry.

The unique thermo response of **1**, whereby the hydrophobicity is increased at a rather lower temperature than for linear PEGs inspired the following hypothesis. Namely, after dehydration at high temperatures, **1** is able to interact with thermally unfolded proteins, which have more exposed hydrophobic residues on the surface than the folded ones, and then is able to prevent their aggregation. This is a practically important issue for the manipulation of therapeutic proteins.

When chicken-egg-white lysozyme was dissolved in phosphate-buffered saline (PBS, pH 7.4; 3.0 $mg\,mL^{-1}$) in the absence and presence of **1**, **2**, or PEG-1000 (30 $mg\,mL^{-1}$), all the resulting mixtures afforded clear solutions at 20 °C

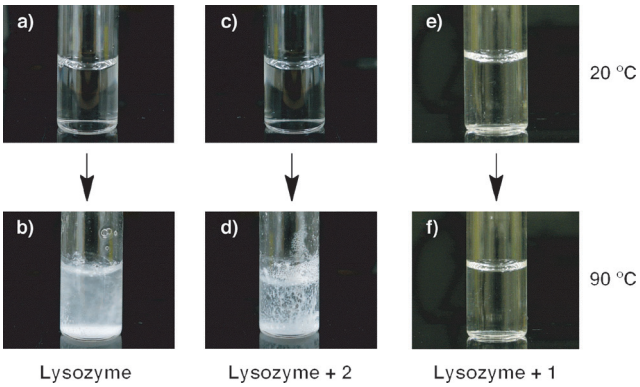


Figure 2. Photographs of lysozyme in PBS (3.0 mg mL^{-1}) at 20 °C (top) and 90 °C (bottom). a and b) no additive, c and d) with **2** (30 mg mL^{-1}), and e and f) with **1** (30 mg mL^{-1}).

(Figure 2). Upon heating the sample without any additives up to 90 °C, white precipitates appeared owing to thermal aggregation of denatured lysozyme (Figure 2 b). The samples containing either **2** or PEG-1000 also gave similar precipitates shortly after heating (Figure 2 d, and the Supporting Information, Figure S6). In sharp contrast, the lysozyme solution containing **1** remained colorless and transparent, even after incubation at 90 °C for 30 min (Figure 2 f). These simple experiments clearly demonstrate that only **1** effectively suppresses thermal aggregation of lysozyme.

The enzymatic activity of lysozyme after heat treatment were investigated to evaluate the ability of the additives to suppress thermal aggregation (Figure 3). Lysozyme in PBS

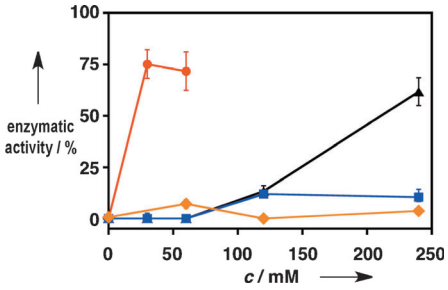


Figure 3. Enzymatic activity of lysozyme (3.0 mg mL^{-1} = 0.21 mM) after heating (98 °C, 30 min) with different concentrations of additives (**1** red; **2** orange; L-arginine hydrochloride black; PEG-1000 blue).

(3.0 mg mL^{-1} = 0.21 mM) containing no additives completely lost its activity after incubation at 98 °C for 30 min (Figure 3, 0 mM). In contrast, nearly 80 % enzymatic activity was retained even after such harsh thermal treatment when **1** (30 mM) was present (Figure 3, red line). Interestingly, the presence of **2** barely helped lysozyme retain enzymatic activity (Figure 3; orange line), thus clearly demonstrating that the topology of PEGs drastically influences their properties. Also the presence of PEG-1000 barely improved the outcome (Figure 3, blue line). Notably, the capability of **1** to suppress thermal aggregation is significantly higher than that of L-arginine, which is known to be a high-performance aggregation suppressor (Figure 3, black line), and its ester derivative (Figure S7).[10]

The effect of **1** on the higher-order structures of lysozyme were investigated by circular dichroism (CD) and VT ^{1}H NMR spectroscopy. Upon being heated from 20 °C to 90 °C, for a PBS solution of lysozyme containing no additive there was a significant decrease in the intensity of CD bands around 210 nm and 225 nm, corresponding to the secondary structures (β sheet and α helix, respectively); these signals did not recover, but in fact decreased after cooling to 20 °C (Figure 4 a). In contrast, in the presence of **1**, the intensity of

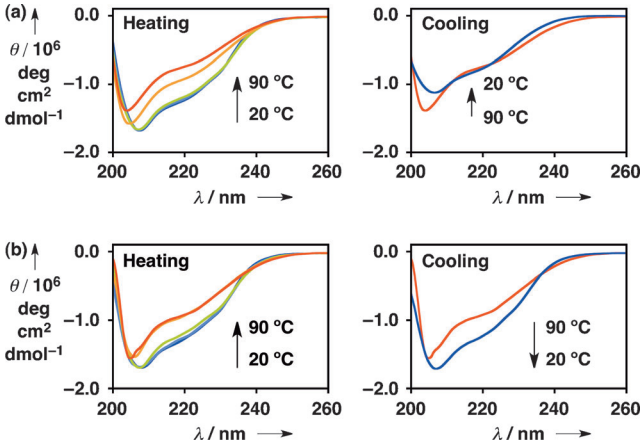


Figure 4. CD spectra of lysozyme (0.5 mg mL^{-1} = 0.035 mM) in PBS buffer with a) no additive and b) **1** (25 mg mL^{-1} = 28 mM). Arrows indicate the directions of spectral changes upon heating (left; 20 °C (blue), 40 °C (pale blue), 60 °C (dark yellow), 80 °C (orange), to 90 °C (red)) and cooling (right; 90 °C to 20 °C).

the CD bands at 90 °C was higher than that in the absence of **1**, and the signal intensity was mostly recovered after cooling to 20 °C (Figure 4 b). These CD spectral changes are consistent with the changes in enzymatic activity of lysozyme after heating (Figure 3). It should be mentioned that the CD signals of **1** were recovered upon repeating the heat treatment cycles (see the Supporting Information, Figure S8).

We investigated the conformational states of lysozyme during heating in more detail by using VT ^{1}H NMR spectroscopy. It is known that the protons of folded proteins exhibit a wide range of chemical shifts owing to the anisotropic magnetic fields of proximal aromatic or carbonyl groups, whereas denatured proteins exhibit chemical shifts of protons in a rather narrower range (δ = 8.5 to 0.8 ppm), such as short linear peptides adopting a random coil conformation.[11] In fact, a PBS solution of lysozyme (1.0 mg mL^{-1} = 0.070 mM) showed signals of protons from δ = 11 to −2 ppm at 40 °C (see the Supporting Information, Figure S9).[12] Upon heating to 70 °C, the peak intensities decreased in the magnetic field regions downfield of δ = 8 ppm and upfield of δ = 0.6 ppm, and further heating up to 80 °C resulted in the disappearance of peaks in those regions. At 80 °C, lysozyme changes into a random coil state, in which the secondary structures are no longer retained. At 90 °C, intensities of all the signals significantly decreased as a result of the thermal

aggregation mentioned above, and the disappeared signals did not recover even after cooling to 40 °C.[13] On the other hand, a mixture of lysozyme (1.0 mg mL^{-1} = 0.070 mM) and **1** (33 mg mL^{-1} = 37 mM) also showed many signals in a wide range of chemical shifts at 40 °C (Figure 5a), with a slightly different spectral profile from intact lysozyme in the aromatic region. Thus, **1** is likely to interact with lysozyme at this temperature while preserving lysozyme's ternary structure. It is of great importance that several signals in the upfield and downfield regions persisted at 80 °C and even at 90 °C with significant intensities (Figure 5a, filled circles), thus strongly indicating that lysozyme remained dissolved in the buffer at such high temperatures and retained partial higher-order structures. Moreover, the original signals mostly recovered after cooling to 40 °C (Figure 5a, filled squares). The five signals observed in the high magnetic field region can be assigned to the alkyl protons of Leu8, Leu17, Thr51, Leu56, Ile98, Met105, and Lys116 according to a literature,[12] where the original secondary and ternary structures around those residues are deemed to be recovered after the heating process. As shown in Figure 5b, these residues are located in the secondary structures such as α-helix (Leu8, Ile98 and Met105), β-sheet (Thr51), and loop (Leu56) or close to α-helix (Leu17 and Lys116), and are distributed in the whole molecule. Of importance, Thr51, Leu56, Ile98, and Met105 are located beside a large cleft, that is, the active site.[14] Thus, **1** stabilizes lysozyme to preserve the partial higher-order structures as well as the solubility in the buffer at elevated temperatures, which in turn results in the suppression of thermal aggregation and the recovery of the native conformation after cooling.

Here, it is of importance to investigate the relevancy of the thermal *gauche*/*anti* conformational change of **1** and **2** to their capability to suppress aggregation. Fluorescence anisotropy (FA) measurement of lysozyme displayed aggregation features of lysozyme upon an increase in temperature (Figure 6). A slowly tumbling molecule, namely a large molecule or an aggregate, exhibits a large FA value compared with small molecules. Actually, lysozyme, in the absence of additives, showed a monotonic increase of FA values upon elevation of temperatures above 30 °C (Figure 6, blue curve and squares),[15] thus indicating that heating encourages aggregation of lysozyme, and the size of the aggregates increases with increasing temperature. The mixture of lysozyme and **2** displayed a similar tendency (Figure 6, orange curve and diamonds), that is, as expected, **2** hardly influences the aggregation of lysozyme at temperatures above 30 °C. In clear contrast, the mixture of lysozyme and **1** exhibited a gradual decrease of FA values from 20 °C to 50 °C, followed by a steep increment at 60 °C and a subsequent decrement (Figure 6, red circles and curve). The decrease of FA values from 20 °C to 50 °C is deemed to result from the increased tumbling rate of lysozyme, without aggregation. The increment of the FA value around 60 °C is likely due to complexation of lysozyme with **1**, and not to protein aggregation as is suggested by the subsequent FA values above 70 °C. As mentioned above, dehydration of **1** accompanied by a *gauche*-to-*anti* conformational change occurs around 60 °C, which coincides with the temperature at which complexation occurs.

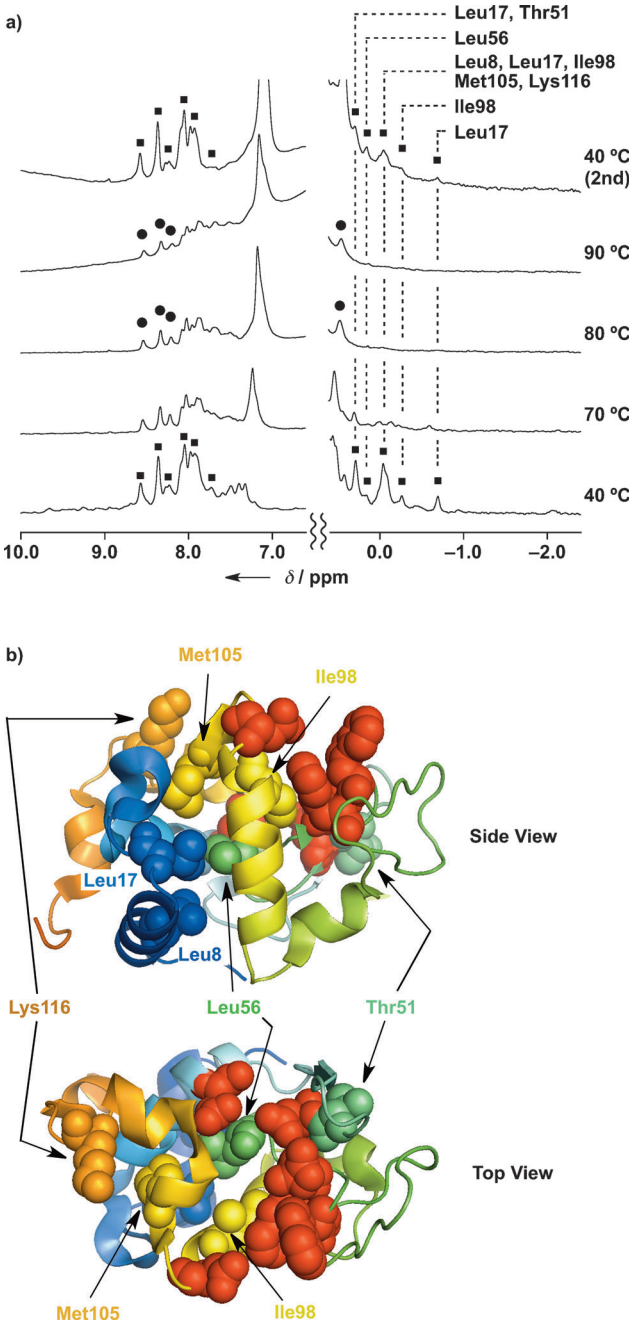


Figure 5. a) ^{1}H NMR spectra of a mixture of lysozyme (1.0 mg mL^{-1} = 0.070 mM) and **1** (33 mg mL^{-1} = 37 mM) in PBS at 40 °C, 70 °C, 80 °C, and 90 °C, followed by cooling to 40 °C. The high-magnetic-field region ($\delta < 0.6$ ppm) of the spectra are magnified three times compared to those in the low-magnetic-field region ($\delta > 6.6$ ppm). Filled circles represent the detectable proton signals of lysozyme at 80 °C and 90 °C, and suggest that partial higher-order structures of lysozyme were retained. Proton signals recovered after cooling to 40 °C are marked with filled squares. b) Side and top views of the crystal structure of lysozyme (PDB ID: 2HUB). The residues for which the corresponding ^{1}H NMR signals were recovered after cooling to 40 °C are labeled on the space-filling representation. The red parts represent the binding site residues (Glu35, Asp52, Asn59, Trp62, Trp63, Asp101).

Therefore, it is likely that the dehydration of **1** allows for interaction with lysozyme at high temperature to suppress

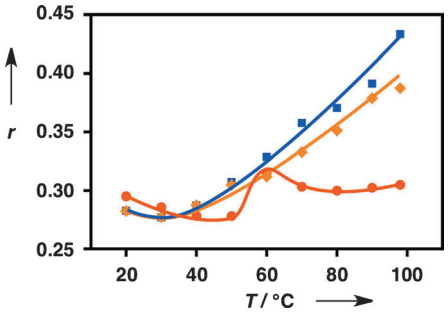


Figure 6. Fluorescence anisotropy changes of lysozyme (0.70 μM) in the absence (blue) and presence of **1** (red, 0.20 mM) or **2** (orange, 0.20 mM) in PBS buffer (pH 7.4) upon an increase in temperature from 20 °C to 98 °C. Excitation and emission wavelengths are 295 and 335 nm, respectively.

aggregation. It should be noted here that because **2** stays hydrated below 80 °C, denatured lysozyme is likely to aggregate before being bound by **2**.

In summary, we have demonstrated that the structured monodisperse PEG analogue **1**, having a triangular structure, has a low dehydration temperature compared with the corresponding linear PEGs, and suppresses protein thermal aggregation with high efficiency. Thus, the structuring of PEG from a linear geometry into higher-dimensional ones affects the physicochemical nature of PEGs to bring about the distinctive biochemical effect of **1**. We believe that detailed studies using monodisperse PEG analogues with diverse structures offer a better and reliable understanding of the characteristics of PEGs and these characteristics can be exploited to explore new applications of PEG-related compounds. Further studies on the capability of **1** to suppress the aggregation of other proteins and on other structured PEGs are now in progress.

Received: August 14, 2012
Published online: January 30, 2013

Keywords: enzymes · macrocycles · polyethylene glycol · protein manipulation · supramolecular chemistry

[1] a) K. Knop, R. Hoogenboom, D. Fischer, U. S. Schubert, *Angew. Chem.* **2010**, *122*, 6430 – 6452; *Angew. Chem. Int. Ed.* **2010**, *49*, 6288 – 6308; b) S. Jevševar, M. Kunstelj, V. G. Porekar, *Biotechnol. J.* **2010**, *5*, 113 – 128; c) M. J. Joralemon, S. McRae, T. Emrick, *Chem. Commun.* **2010**, *46*, 1377 – 1393; d) D. da Silva Freitas, J. Abrahão-Neto, *Int. J. Pharm.* **2010**, *392*, 111 – 117; e) J.-B. Park, Y. M. Kwon, T.-Y. Lee, R. Brim, M.-C. Ko, R. K. Sunahara, J. H. Woods, V. C. Yang, *J. Controlled Release* **2010**, *142*, 174 – 179; f) S. M. Ryan, G. Mantovani, X. Wang, D. M. Haddleton, D. J. Brayden, *Expert Opin. Drug Delivery* **2008**, *5*, 371 – 383; g) C. S. Fishburn, *J. Pharm. Sci.* **2008**, *97*, 4167 – 4183; h) G. Kochendoerfer, *Expert Opin. Biol. Ther.* **2003**, *3*, 1253 – 1261; i) Y.-S. Wang, S. Youngster, M. Grace, J. Bausch, R. Bordens, D. F. Wyss, *Adv. Drug Delivery Rev.* **2002**, *54*, 547 – 570; j) P. Bailon, A. Palleroni, C. A. Schaffer, C. L. Spence, W.-J. Fung, J. E. Porter, G. K. Ehrlich, W. Pan, Z.-X. Xu, M. W. Modi, A. Farid, W. Berthold, *Bioconjugate Chem.* **2001**, *12*, 195 – 202; k) F. M. Veronese, P. Caliceti, O. Schiavon, *J. Bioact. Compat. Polym.* **1997**, *12*, 196 – 207.

[2] a) B. Scrosati, J. Garche, *J. Power Sources* **2010**, *195*, 2419 – 2430; b) P. Arora, Z. Zhang, *Chem. Rev.* **2004**, *104*, 4419 – 4462; c) J. Y. Song, Y. Y. Wang, C. C. Wan, *J. Power Sources* **1999**, *77*, 183 – 197.

[3] a) A. C. French, A. L. Thompson, B. G. Davis, *Angew. Chem.* **2009**, *121*, 1274 – 1278; *Angew. Chem. Int. Ed.* **2009**, *48*, 1248 – 1252; b) S. A. Ahmed, M. Tanaka, *J. Org. Chem.* **2006**, *71*, 9884 – 9886.

[4] The molecular design of **1** based on molecular modeling is explained in the Supporting Information, Figure S1.

[5] For examples of amphiphilic polymer assemblies for the manipulation of proteins, see: Y. Sasaki, K. Akiyoshi, *Chem. Rec.* **2010**, *10*, 366 – 376.

[6] Interestingly, without any template, the reaction between **5** and **6** preferentially gave cyclic products **13** and **14** (40 % yield), and the polymerized products were obtained only in < 3 % yield, see the Supporting Information.

[7] a) M. J. Hey, S. M. Ilett, G. Davidson, *J. Chem. Soc. Faraday Trans.* **1995**, *91*, 3897 – 3900; b) M. Björling, G. Karlström, P. Linse, *J. Phys. Chem.* **1991**, *95*, 6706 – 6709; c) H. Matsuura, K. Fukuhara, *J. Mol. Struct.* **1985**, *126*, 251 – 260; d) G. Karlström, *J. Phys. Chem.* **1985**, *89*, 4962 – 4964; e) S. Saeki, N. Kuwahara, M. Nakata, M. Kaneko, *Polymer* **1976**, *17*, 685 – 689; f) H. Matsuura, T. Miyazawa, *J. Polym. Sci. Part A* **1969**, *7*, 1735 – 1744.

[8] N. E. Jacobsen in *NMR Spectroscopy Explained: Simplified Theory, Applications and Examples for Organic Chemistry and Structural Biology*, Wiley-InterScience, Hoboken, **2007**, pp. 489 – 550.

[9] As ^{13}C NMR signals corresponding to C2 and C4 of **1** overlap at 80 °C, the VT HMBC experiment was performed at 60 °C.

[10] a) C. Lange, R. Rudolph, *Curr. Pharm. Biotechnol.* **2009**, *10*, 408 – 414; b) T. Arakawa, D. Ejima, K. Tsumoto, N. Obeyama, Y. Tanaka, Y. Kita, S. N. Timasheff, *Biophys. Chem.* **2007**, *127*, 1 – 8; c) K. Shiraki, M. Kudou, S. Nishikori, H. Kitagawa, T. Imanaka, M. Takagi, *Eur. J. Biochem.* **2004**, *271*, 3242 – 3247; d) T. Arakawa, K. Tsumoto, *Biochem. Biophys. Res. Commun.* **2003**, *304*, 148 – 152.

[11] J. Cavanagh, W. J. Fairbrother, A. G. Palmer III, M. Rance, N. J. Skelton, *Protein NMR Spectroscopy: Principles and Practice, 2nd ed.*, Academic Press, San Diego, **2006**.

[12] Y. Wang, T. C. Bjorndahl, D. S. Wishart, *J. Biomol. NMR* **2000**, *17*, 83 – 84.

[13] White precipitates were observed in the NMR tube.

[14] a) J. C. Cheetham, P. J. Artymiuk, D. C. Phillips, *J. Mol. Biol.* **1992**, *224*, 613 – 628; b) N. C. J. Strynadka, M. N. G. James, *J. Mol. Biol.* **1991**, *220*, 401 – 424.

[15] An FA value greater than 0.4 is likely due to the presence of scattered light by the precipitates of lysozyme. J. R. Lakowicz, in *Principles of Fluorescence Spectroscopy, 3rd ed.*, Springer, New York, **2006**.