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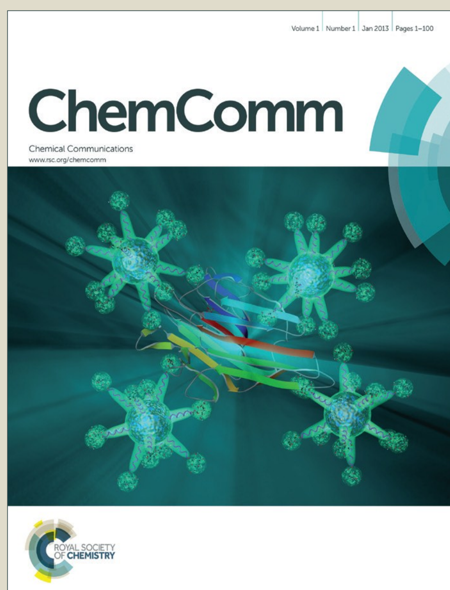

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COMMUNICATION

Protein stabilization by an amphiphilic short monodisperse oligo(ethylene glycol)

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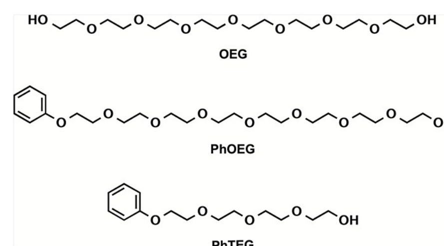
A short, monodisperse additive (octa(ethylene glycol) monophenyl ether) functions to suppress aggregation of thermally and chemically denatured lysozyme. Control studies with shorter and non-amphiphilic derivatives revealed that the amphiphilic structure is essential, and octa(ethylene glycol) is nearly the minimum chain length for amphiphilic poly(ethylene glycol)s to stabilize proteins.

Protein aggregation has been increasingly recognized as a serious problem that limits the applicability of proteins to synthetic reactions, functional foods and medicines. Use of additives is one of the versatile methods to suppress aggregation and assist refolding of proteins.[1] Indeed, a variety of compounds, such as kosmotropes and chaotropes, including amino acids, sugars and polymers, have been reported as effective additives, which are typically considered to work by preferential hydration of proteins and preferential exclusion of solutes from the protein surface. However, most of these additives require quite high concentrations for efficient stabilization of proteins, and eventually cause technical problems by increasing the viscosity and density of solutions. Poly(ethylene glycol) (PEG), a nonionic polyether, is one of the popular additives functioning as a thermo-protective agent for proteins. For example, PEG is able to decrease heat-induced aggregation of urokinase, at concentrations larger than 10 wt%.[2] Recently, Mueller *et al.* reported that an amphiphilic PEG bearing a hydrophobic unit at a terminus allows aggregation suppression of proteins at lower concentrations.[3] Indeed, PEGs with molecular weights of 2000 and 5000 Da, bearing cholesteryl group at the termini, could be employed at the concentrations around 1 wt%.[3] In contrast to macromolecular PEGs with the molecular weight larger than 1000 Da, fewer studies on protein aggregation suppression have been reported using smaller PEGs that are easily separable from the proteins due to a large difference in molecular weight.[4] Also, they have advantages due to possible availability of monodisperse species to allow quantitative analyses and fine tuning of properties by structural modifications. Here, we report that even a quite short monodisperse PEG such as octaethylene glycol (OEG, 370 Da) can acquire the capability to suppress protein aggregation by substitution with a phenyl group at a terminus, while phenyl-appending tetraethylene glycol (PhTEG) hardly suppress the aggregation. To the best of our knowledge, OEG is nearly the minimum PEG-chain length that could acquire this function.



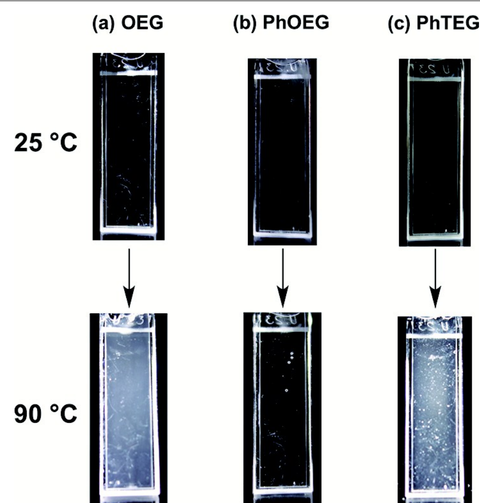


Fig. 1 Photographs of chicken egg white lysozyme (0.037 mM) in PBS buffer (pH 7.4) containing 1.5 mM of (a) OEG, (b) PhOEG and (c) PhTEG in a 1.0-mm-thick quartz cell taken at (top) 25 and (bottom) 90 °C.

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Recently, we reported that amphiphilic monodisperse PEGs, PhOEG and PhTEG, exhibit thermo-responsive conformational changes and reduced dehydration temperatures compared with simple unmodified PEGs.[5] Namely, while OEG remains hydrated over the temperature range from 30 to 80 °C, PhOEG dehydrates around 50 °C, and PhTEG having a shorter chain than PhOEG dehydrates around 40 °C. Thus, the dehydration temperature is likely dependent on the PEG chain length. Since the dehydration temperatures of these amphiphilic PEG derivatives are close to the protein-denaturation temperature, in the present study, we investigated the capabilities of OEG, PhOEG and PhTEG to suppress aggregation of a thermally- and chemically-denatured protein and to assist refolding.

When chicken egg white lysozyme is heated up to 90 °C in PBS buffer (pH 7.4), it readily precipitates out due to thermal denaturation.[6] Likewise, lysozyme in the presence of OEG and PhTEG in PBS buffer ([lysozyme] = 0.037 mM, [OEG] = [PhTEG] = 1.5 mM, pH 7.4; Figs. 1a and c) caused precipitation upon heating to 90 °C, indicating thermal aggregation of lysozyme. In sharp contrast, PBS buffer solution of lysozyme containing PhOEG ([PhOEG] = 1.5 mM) remained clear during the heating (Fig. 1b), suggesting that PhOEG has a function to suppress thermal aggregation of lysozyme. Interestingly, we found that with slightly lower concentration of PhOEG, lysozyme forms aggregation upon heating ([PhOEG] = 1.0 mM, Fig. S1, ESI†). Since the critical aggregation concentration (CA) of PhOEG is 1.3 mM,[5] it is likely that the self-assembled form of PhOEG is necessary for suppressing the aggregation of lysozyme. Dynamic light scattering and transmission electron microscopy experiments indicated the formation of spherical aggregates of PhOEG above the CAC (Fig. S2, ESI).[5]

Structural change of lysozyme in the presence of the additives during the heat treatment process was monitored by variable temperature circular dichroism (VT-CD) spectropolarimetry. Lysozyme in PBS buffer (0.037 mM, pH 7.4) displays a negative CD signal at 207 nm at 20 °C (Fig. 2a left side, blue line). By elevation of the temperature to 90 °C, the signal shifted to 203 nm with a reduction of the intensity (Fig. 2a left side, red line), suggesting a conformational change to a random coil state and aggregation. The CD spectrum hardly recovered after cooling to 20 °C (Fig. 2a right side).[4b] A similar spectral change was observed in the presence of OEG and PhTEG in PBS buffer[§] ([OEG] = [PhTEG] = 1.5 mM; Fig. 2b and 2d, respectively). In contrast, in the presence of PhOEG, although the decrement of the CD intensity was observed during the heating process, the peak of the negative CD signal hardly shifted between 20 and 90 °C (20 °C: 207 nm, 90 °C: 208 nm; [PhOEG] = 1.6 mM; Fig. 2c left side), suggesting that the partial secondary structures of lysozyme were retained even at high temperatures. Importantly, a backward CD spectral change was observed upon cooling to 20 °C to recover a CD spectrum close to the original one before heating (Fig. 2c right side), indicating that the thermal aggregation of lysozyme was mostly suppressed in the presence of PhOEG and the secondary structures of lysozyme was considerably recovered after the cooling.

Quantitative evaluation of the aggregation suppression and refolding assistance efficiency of the additives was carried out by monitoring the residual enzymatic activity of lysozyme. After the heat treatment of lysozyme in PBS buffer (pH 7.4, 0.037 mM) at 90 °C for 30 min followed by cooling to 25 °C in the presence of the additives, bacteriolytic activity of lysozyme was evaluated following the reported procedure.[7] Fig. 3 displays the percentages of the residual activity of lysozyme as a function of concentrations of the additives. Addition of OEG resulted in almost no residual activity of lysozyme after the heat treatment (Fig. 3, red triangles and solid line), while only 4 ± 2% recovery of the enzymatic activity was observed in the presence of PhTEG (Fig. 3, orange squares and solid line). In sharp contrast, PhOEG showed significant recovery of lysozyme activity up to 78 ± 9% (Fig. 3, blue circles and solid line). These results correspond well to the visual observation of thermal aggregation of lysozyme in the presence of these additives (Fig. 1). It should be mentioned that the lysozyme enzymatic activity hardly recovered after the thermal treatment at [PhOEG] = 1.6 mM, where CD spectroscopy showed the significant recovery of the secondary

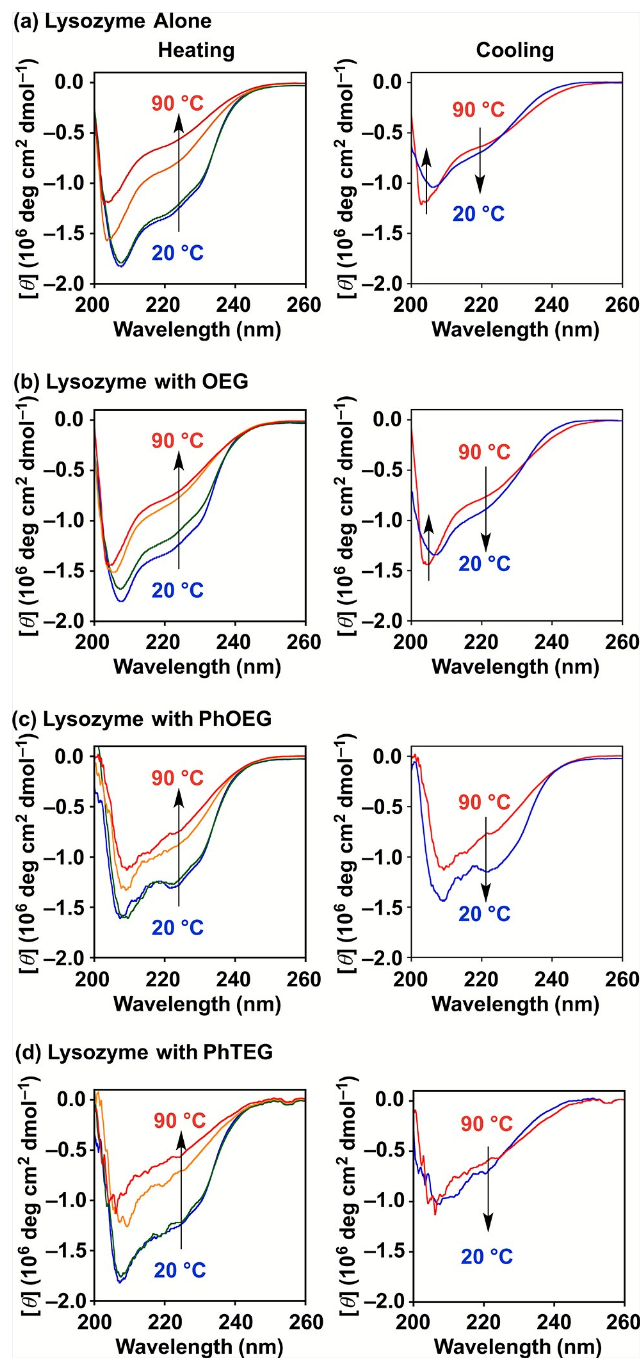

Fig. 2 Circular dichroism spectral changes of chicken egg white lysozyme (0.037 mM) in PBS buffer (pH 7.4) with (a) no additive, (b) OEG (1.5 mM), (c) PhOEG (1.6 mM) and (d) PhTEG (1.5 mM). Arrows indicate the directions of spectral changes upon heating (left; 20, 60, 80 and 90 °C; blue, green, orange, and red, respectively) and cooling (right; 90 and 20 °C; red and blue, respectively).

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structures of lysozyme. This indicates that the native tertiary structure was not fully recovered to exert the original enzymatic activity under this condition. Since OEG showed little recovery of lysozyme activity, amphiphilicity is essential for the aggregation suppression and refolding assistance capability. The significant difference in the aggregation suppression capability between PhOEG and PhTEG suggests that not only amphiphilicity but also the chain length of the PEG part is also an important factor, where TEG is likely too short to attain an ideal hydrophilicity/hydrophobicity balance with phenyl group for these functions.

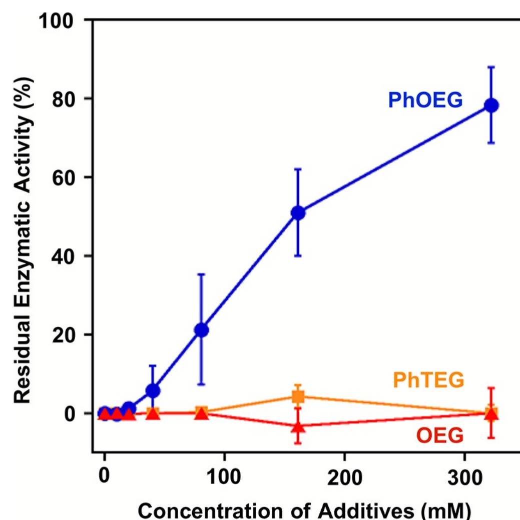

Fig. 3 Residual enzymatic activities of recovered chicken egg white lysozyme (0.037 mM) in PBS buffer (pH 7.4) after heat treatment at 90 °C for 30 min with different concentrations of OEG (red triangles and solid line), PhOEG (blue circles and solid line) and PhTEG (orange squares and solid line).

Differential scanning calorimetry (DSC) measurements[8] visualized the denaturation temperatures (T_m) of lysozyme in the presence of 20 mM and 200 mM additives (Table 1). Lysozyme in PBS buffer (pH 7.4) showed T_m at 72.0 °C. Meanwhile, addition of 20 mM and 200 mM OEG to lysozyme in the PBS buffer showed relatively small change in T_m, indicating the weak interaction between lysozyme and OEG.[8a] Likewise, presence of 20 mM PhOEG and PhTEG hardly influenced T_m (71.3 and 71.1 °C, respectively). In contrast, higher-concentration PhOEG (200 mM) and PhTEG (200 mM) caused decrease of T_m to 64.6 °C and 58.0 °C, respectively. Thus, the amphiphilic OEG derivatives likely have stronger interaction with lysozyme than OEG to stabilize its denatured state.[8,9] Amphiphilic PEG derivatives bearing a hydrophobic head group are known to interact with proteins by hydrophobic interactions.[10] Likewise, the phenyl appendage of PhOEG possibly interacts with the hydrophobic surface of denaturing lysozyme at high temperatures, where strong hydrophilicity of the OEG part likely hampers interactions among the denaturing protein molecules[11] to provide water-solubility (Fig. 1b) and would offer an opportunity for re-hydration of the protein surface to allow for refolding in the cooling process.[12] As for PhTEG, while the interaction with lysozyme is stronger than PhOEG, hydrophilicity is lower than PhOEG due to the shorter PEG chain, which would be disadvantageous to block hydrophobic interactions between the unfolded proteins for suppressing their aggregation at high temperatures as shown in Fig. 1c. Hence, the plausible mechanism of protein stabilization by PhOEG against thermal aggregation is 1) stabilization of lysozyme in the denatured state by hydrophobic interactions between the hydrophobic surfaces of denaturing lysozyme and phenyl appendage of PhOEG and 2) affording sufficient water-solubility to the denatured lysozyme at a high temperature to allow refolding in the cooling process.

Since refolding of proteins is also an important subject in biotechnology, aggregation suppression of chemically unfolded lysozyme under the refolding condition was also studied, which is an essentially different assay from the one on thermal aggregation suppression described above. Chemically unfolded lysozyme was prepared by treatment with dithiothreitol and guanidine hydrochloride, and the refolding was initiated by 60-folds dilution of the resulting lysozyme solution into a degassed renaturation buffer containing the additives under N_2 at 25 °C (3.0 mM glutathione, 0.30 mM glutathione disulfide, 1.0 mM ethylenediaminetetraacetic acid, 40 mM additives, the final concentration of lysozyme: 0.020 mM, 0.10 M Tris•HCl, pH 8.2).[13] For evaluating the efficiency of aggregation suppression by the additives during the oxidative protein refolding, turbidity change was monitored following the procedure reported in the literature,[14] where optical density changes at 600 nm (OD_{600}) were measured for 70 min after mixing the denatured lysozyme with the renaturation buffer in the absence and presence of the additives (40 mM, Fig. 4).[15] When denatured lysozyme was added to the renaturation buffer without an additive, the resultant solution immediately turned turbid due to precipitation (Fig. 4, light blue diamond and solid line). Meanwhile, denatured lysozyme in the presence of OEG showed smaller enhancement of OD_{600}. Interestingly, the presence of PhTEG and PhOEG resulted in further smaller enhancement of OD_{600}, where the smallest enhancement was

Table 1 Thermal denaturation temperatures (T_m) of lysozyme in PBS containing OEG, PhOEG or PhTEG by DSC measurements[a]

Additives	Concentrations (mM)	T_m (°C)
None	–	72.00 ± 0.03
OEG	20	71.42 ± 0.05
	200	69.07 ± 0.05
PhOEG	20	71.34 ± 0.03
	200	64.57 ± 0.01
PhTEG	20	71.10 ± 0.03
	200	58.00 ± 0.05

[a]Condition: [lysozyme] = 0.037 mM, PBS (pH 7.4), Heating rate: 1.0 K min^{-1}

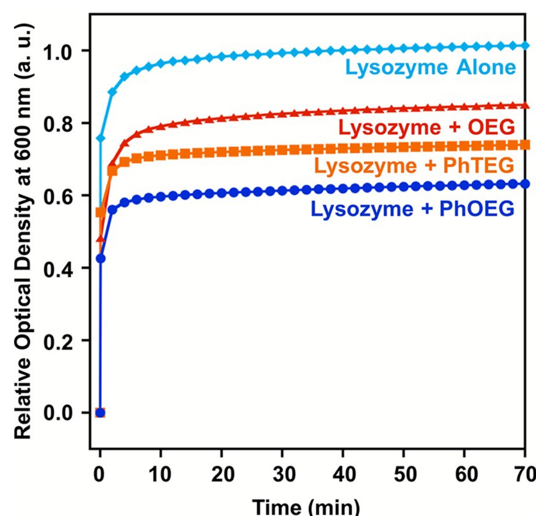

Fig. 4 Optical density changes of lysozyme (0.020 mM, 600 nm) in the absence (light blue diamonds and solid line) and presence of additives (40 mM, OEG: red triangles and solid line, PhOEG: blue circles and solid line, PhTEG: orange squares and solid line) in a renaturation buffer (pH 8.2. 3.0 mM glutathione, 0.30 mM glutathione disulphide, 1.0 mM ethylenediaminetetraacetic acid, 0.10 M Tris•HCl) at 25 °C for the duration of 70 min.

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achieved by PhOEG. It is again likely that both the amphiphilicity and the length of PEG chain of the additive are important factors for suppressing the aggregation of lysozyme. After the refolding process in the presence of OEG, PhTEG and PhOEG, 2, 7 and 15% bacteriolytic activities of lysozyme were recovered, respectively.† The order of the recovery efficiency is in good agreement with the trend of optical density enhancement shown in Fig. 4, and the same trend was observed in the presence of a higher concentration of the additives (480 mM; recovered bacteriolytic activities of lysozyme: OEG: 0.47%, PhTEG: 1.0% and PhOEG: 38%).

In this communication, we demonstrated the efficacy of a short monodisperse PEG derivative, PhOEG, for protein aggregation suppression of thermally and chemically denatured proteins. While OEG hardly suppresses lysozyme aggregation, it was suggested that the amphiphilicity plays a pivotal role in the aggregation suppression and refolding assistance functions. Moreover, sharp differences in the biologically-relevant functions were observed between PhOEG and PhTEG, where PhTEG showed less efficacy. Thus OEG is likely the almost minimum ethylene glycol chain required for the protein aggregation suppression by amphiphilic PEG derivatives. We believe that the present paper contributes to encourage the utility of short monodisperse PEGs for biological applications.

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Notes and references

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§ Because of a baseline disturbance due to strong absorption of the phenyl group, CD measurements of lysozyme in the PBS buffer with PhOEG and PhTEG at higher concentrations than 1.6 mM were unsuccessful.

† Electronic Supplementary Information (ESI) available: Photographs and VT-CD spectra of lysozyme in the presence of PhOEG (1.0 mM). See DOI: 10.1039/c000000x/

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