Photocontrol of Vesicular Adhesion and Functions by a Photoresponsive Polypeptide

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ABSTRACT: A photoresponsive polypeptide, azobenzene-modified poly(γ-methyl L-glutamate-*co*-L-glutamic acid) of molecular weight 5000, has been prepared and incorporated into the bilayer membrane of the vesicles composed of distearyldimethylammonium chloride. UV light (<330 nm) irradiation to the vesicles induced a transfer of the polypeptide from the hydrophobic bilayer membrane interior to the hydrophilic surface, owing to the photoisomerization of the azobenzene moieties from the trans to cis configuration, which resulted in a decrease of ion permeability through the bilayer membrane of the vesicle and a formation of intervesicular adhesion. Visible light irradiation (>390 nm) or keeping the vesicle in the dark after the UV irradiation did not cause any changes of the joining vesicles structure and the ion permeability; however, the original permeability and dispersed vesicle structure could be recovered by increasing temperature above the gel–liquid crystal-phase transition temperature, T_c = 40 °C, of the vesicle.

Introduction

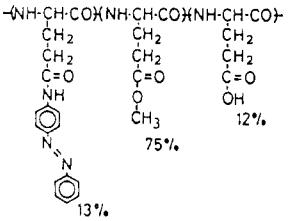
Studies of the interaction between polypeptides and bilayer membranes of synthetic amphiphilic molecules are important for understanding the structural and functional properties of biological membranes. The membrane protein is roughly divided into integral and extrinsic membrane proteins. The former has less hydrophilic amino acid content than the latter, indicating that the localization of the membrane proteins in biological membranes is mainly determined by the hydrophobic amino acid/hydrophilic amino acid ratios in the proteins. It has been shown that a photoreceptor protein[1,2] changes its location in a membrane when exposed to light, and that this response is closely correlated with the function of the membrane in the transfer of light energy or information.

In a previous study,[3] we investigated the influence of relatively low molecular weight polypeptides on the structure of the bilayer membrane of distearyldimethylammonium chloride (DSACl) by means of fluorescence spectroscopic techniques. A hydrophobic polypeptide, poly(γ-methyl L-glutamate) (PMG), was shown to be readily incorporated into the hydrophobic interior of the membrane. On the other hand, a negatively charged poly(L-glutamic acid) (PGA) could hardly penetrate through the membrane and formed cross-linking bridges between the positively charged DSACl vesicles. In addition, we have shown that the polarity of poly(L-glutamic acid) with azobenzene groups in the polymer side chains can be reversibly controlled by photoirradiation.[4]

We report here on UV light-induced permeability changes and intervesicular adhesion of the DSACl vesicles containing γ-methyl L-glutamate–L-glutamic acid copolypeptide with 13 mol % azoglutamine residue (Copoly azo-MG/GA). The results can be explained in terms of the transfer of Copoly azo-MG/GA from the hydrophobic interior of the bilayer membrane to the hydrophilic surface via the photoinduced polarity increase of the azobenzene moieties.

Experimental Section

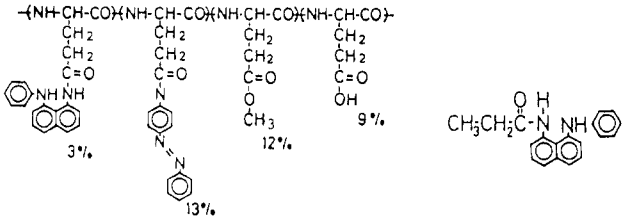
Materials. Poly(γ-methyl L-glutamate) (PMG) was obtained by the polymerization of the *N*-carboxyanhydride of L-glutamic acid γ-methyl ester in 1,2-dichloroethane solution with *n*-hexylamine as initiator.[5] The molar ratio of anhydride to initiator was 75, and the polymerization occurred at room temperature over 24 h. The PMG was precipitated by adding dry methanol. A molecular weight of 4400 was estimated from viscosity measurements in dichloroacetic acid. Poly(γ-methyl L-glutamate-*co*-L-glutamic acid) (Copoly MG/GA) with 25 mol % L-glutamic acid residues was prepared by saponification of PMG.[6] The degree of polymerization did not decrease during saponification. Azobenzene-modified Copoly MG/GA (Copoly azo-MG/GA) was



Copoly azo-MG/GA

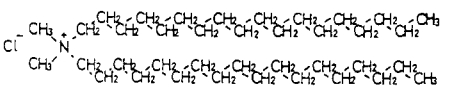
obtained by the condensation of Copoly MG/GA with *p*-aminoazobenzene in dimethylformamide, containing dicyclohexylcarbodiimide (DCC) and *N*-hydroxybenzotriazole (HOBt) for 24 h at room temperature.[7] The Copoly azo-MG/GA obtained was a terpolymer which consisted of azobenzylglutamine (13 mol %), γ-methyl L-glutamate (75 mol %), and L-glutamic acid (12 mol %).

Copoly azo-MG/GA containing 3 mol % 1-amino-8-anilinonaphthalene (AN) in the side chain (side-AN-Copoly azo-MG/GA), and AN-modified propionic acid (Pro-AN) were synthesized by



side-AN-Copoly azo-MG/GA Pro-AN

condensation using DCC and HOBt. 1,6-Diphenyl-1,3,5-hexatriene (DPH) and disteryldimethylammonium chloride (DSACl) were purchased from Tokyo Kasei Co., Ltd., and were used without further purification.



DSACl

The DSACl vesicle containing polypeptide was prepared as follows. Polypeptide (Copoly azo-MG/GA or side-AN-Copoly azo-MG/GA) and DSACl (mol ratio 8.3×10^{-3}) were each dissolved in chloroform. These solutions were mixed and poured into a glass flask, and then a thin film was formed in the inner surface of the flask by evaporating the solvent. Buffer solution (Tris–HEPES, pH 6.8) was added to this flask, and it was sonicated at 40 W by a Branson Sonifier 185 for 3 min in the dark to prepare the vesicle.

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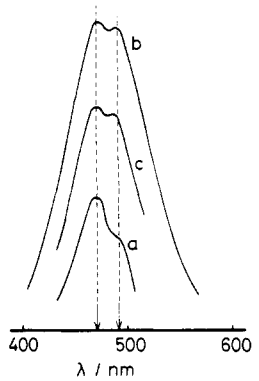

Figure 1. Fluorescence spectra of side-AN-Copoly azo-MG/GA in DSACl vesicle: (a) before UV irradiation at 20 °C and at 20 °C after visible light irradiation and heating to 60 °C; (b) after UV light irradiation at 20 °C; (c) after visible light irradiation at 20 °C; [side AN-Copoly azo-MG/GA]/[DSACl] = 8.3×10^{-3}, [DSACl] = 0.5 wt%.

Methods. Spectroscopic Measurements. Absorption spectra of the Copoly azo-MG/GA in the DSACl vesicle solution were measured with a spectrophotometer (Jasco, UVDEC-670) to elucidate the photoisomerization of the azobenzene moiety of Copoly azo-MG/GA. The concentration of DSACl in the aqueous solution was 0.2 wt %.

Fluorescence spectra of Pro-AN and side-AN-Copoly azo-MG/GA were obtained with a spectrofluorophotometer (Shimadzu, RF-540). The excitation wavelength of AN was 350 nm, and the concentration of DSACl was 0.5 wt %. The degree of polarization, $P = I_{\parallel} - I_{\perp}/I_{\parallel} + I_{\perp}$, of DPH in the bilayer of the DSACl vesicles was determined with a fluorescence polarization spectrophotometer (Union Giken, FS-501). $I_{\parallel}$ and $I_{\perp}$ are the fluorescence intensities polarized parallel and perpendicular to the direction of the polarized excitation beam. The concentration of DSACl was 0.2 wt %. The concentration of DPH used as a fluorescence probe for the fluorescence polarization measurements was 2.2×10^{-5} M. The excitation wavelength of DPH was 357 nm, and the fluorescence was observed at 430 nm.

Microscopic Measurements. The shape of the DSACl vesicles containing Copoly azo-MG/GA was directly observed with a phase-contrast microscope (Olympus Optical, BHR, ×1000). The concentration of DSACl was 0.2 wt %.

Permeation Measurements. The DSACl vesicles containing Copoly azo-MG/GA in an aqueous solution of 0.1 M sodium gluconate were prepared by sonication in a similar manner as above. The aqueous suspension of the DSACl vesicles containing sodium gluconate in the interior and Copoly azo-MG/GA in the bilayer was filtered off, and 150 mg of the residue was added to the aqueous solution of potassium gluconate (0.1 M, 40 mL). The resulting vesicles had an ionic concentration gradient between the interior (0.1 M, sodium ion) and exterior (0.1 M, potassium ion) under isotonic condition. The amount of sodium ion transported across the vesicular bilayers was determined with a ion meter (Horiba, N-7ionII).

Irradiation. The light source used for UV and visible light irradiation was a 500-W high-pressure mercury lamp (Ushio, USH-500D) with glass filters D-33S and L-39 (Toshiba).

Results and Discussion

Photoinduced Partition Changes. Azobenzene is known to change its absorption spectrum when the configuration changes from the trans to the cis form. This isomerization is usually acompanied by a large increase in the polarity of the compound. The photoisomerization of Copoly azo-MG/GA in the DSACl vesicle was determined by the changes in the absorption bands at 380 and 450 nm, assignable to the trans π–π^* transition and the cis n–π^* transition, respectively. From the changes in absorbance at 380 nm, it was estimated that 60% of the trans form was converted into the cis isomer in the vesicle when the membrane was irradiated for a few minutes with UV light at 20 °C. On the other hand, the trans form can be photostationarily recovered by a 2-min irradiation with visible light and completely recovered in the dark. To elucidate the effect of the photoisomerization of the azobenzene moiety on the partition of Copoly azo-MG/GA between the bilayer membrane interior and the surface of the DSACl vesicle, the fluorescence spectra of side-AN-Copoly azo-MG/GA in the vesicle were obtained (Figure 1). Two emission maxima were observed at 470 and 490 nm. The intensities of both maxima were sensitively changed by UV light and heat. The emission maxima of AN were strongly dependent on the polarity around the probe (Figure 2). The figure shows the relationship between λ_{max} of Pro-AN, the fluorescent model compound for side-AN-Copoly azo-MG/GA, in different solvents and the empirical solvent polarity Z.[8] The λ_{max} of Pro-AN shifts to higher wavelength with increasing solvent polarity.[9] Z values for the emissions of the side-AN-Copoly azo-MG/GA at 470 and 490 nm correspond to those of dimethylformamide (dashed line a in Figure 2) and between methanol and water (dashed line b in Figure 2), respectively. Fluorescence spectra of side-AN-Copoly MG/GA (without azobenzene groups) in the DSACl vesicle also show two emission maxima at 470 and 495 nm.[3] The former was assigned to the emission of side-AN-Copoly MG/GA in the hydrophobic interior of the bilayer and the latter at the polar surface of the membrane. Accordingly, the two emission maxima at 470 and 490 nm in Figure 1 are also ascribed to the emissions of side-AN-Copoly azo-MG/GA in the hydrophobic interior of the bilayer and at the polar surface, respectively. The partition equilibrium of Copoly azo-MG/GA between the membrane interior and membrane surface is represented by eq 1, with an equilibrium constant K given by eq 2. The concentrations of Copoly azo-MG/GA in both regions (eq 2) can be roughly estimated from the emission intensities at 470 and 490 nm in Figure 1. The emission strengths were corrected by using the calibration curve for the emission strength of Pro-AN at various wavelengths in different solvents, since the quantum yield of AN depends on the polarity around AN. Table I shows the photoinduced and thermoinduced

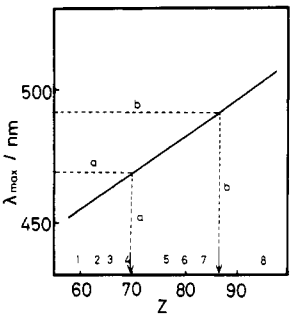

Figure 2. Relation between fluorescent emission maxima of Pro-AN and empirical Z solvent polarity. [Pro-AN] = 3.0×10^{-5} M, (1) octane, (2) chloroform, (3) acetone, (4) dimethylformamide, (5) 2-propanol (6) ethanol, (7) methanol, (8) water.

Table I
Photo- and Thermoinduced Changes in the Partition Equilibrium Constant K of Copoly azo-MG/GA between the Membrane Interior and Membrane Surface

	dark	UV irra.	vis irra.	Δ
K	2.5	0.48	0.50	2.5

Copoly azo-MG/GA at membrane surface ⇄ Copolyazo-MG/GA in membrane interior (1)

K = [Copoly azo-MG/GA] in membrane interior/[Copoly azo-MG/GA] at membrane surface (2)

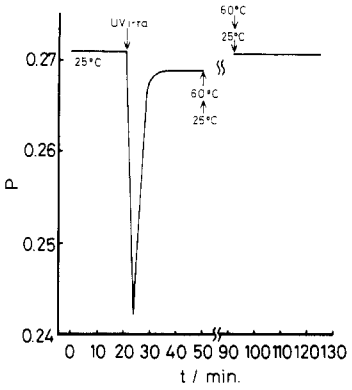

Figure 3. Photo- and thermoinduced changes in the degree of polarization of DPH in the bilayer membrane of DSACl vesicle containing Copoly azo-MG/GA: [Copoly azo-MG/GA]/[DSACl] = 8.3×10^{-3}, [DSACl] = 0.2 wt %, [DPH] = 2.2×10^{-5} M.

changes in the equilibrium constant K. Before UV irradiation, K was 2.5, indicating that 71 mol % of the total amount of Copoly azo-MG/GA is incorporated in the membrane interior. The value of K decreased on UV irradiation, and the polypeptide in the membrane interior decreased to 32 mol %. This means that Copoly azo-MG/GA, which was solubilized mainly into the hydrophobic membrane interior before UV irradiation, was moved to the hydrophilic surface of the membrane by UV irradiation, since irradiation induced the increase in polarity of the azobenzene moiety in Copoly azo-MG/GA, which also resulted in the acid dissociation of neighboring L-glutamic acid residue.[4] The emission spectrum of side-AN-Copoly azo-MG/GA in the vesicle, however, did not change on irradiation with visible light, thus confirming the irreversibility of the photoinduced transfer of Copoly azo-MG/GA from the membrane interior to the surface. This irreversibility can be explained as follows. The degree of dissociation of the L-glutamic acid moiety of Copoly azo-MG/GA is increased by UV irradiation via the polarity increase of the neighboring azobenzene side chains.[4] Furthermore, the negatively charged PGA can form cross-linking bridges between the positively charged DSACl vesicles.[3] It appears therefore that Copoly azo-MG/GA, even though the cis-to-trans isomerization of azobenzene moiety occurs at the membrane surface on visible light irradiation, cannot be resolubilized into the hydrophobic membrane interior at a finite rate because of electrostatic interaction between the L-glutamic acid moieties in Copoly azo-MG/GA and the ammonium cation of DSACl. In addition, the value of K was increased to 2.5 by increasing the temperature above T_c (40 °C),[3] reflecting the resolubilization of Copoly azo-MG/GA in the membrane interior. This result is also consistent with the fact that the transfer of partially charged hydrophobic poly(glutamates) from the membrane surface to the membrane interior is accelerated by increasing temperature, reflecting a large positive entropy change.[3]

Figure 3 shows the photoinduced changes in the degree of polarization, P, of DPH in the bilayer of the DSACl vesicles containing Copoly azo-MG/GA at 25 °C. The P value of DPH is known to reflect the microscopic viscosity of the bilayer membrane interior. After UV irradiation, the local viscosity of the membrane interior immediately decreased and then increased to the equilibrium value within ca. 9 min. This implies that UV light-induced transfer of Copoly azo-MG/GA was completed in 9 min, giving rise to disorder in the membrane interior. The equilibrium P value under UV irradiation was slightly lower than that in the dark, indicating that Copoly azo-MG/GA perturbed the membrane surface more than the membrane interior. This result is consistent with the fact that PGA at a membrane surface can cause more packing faults via electrostatic interaction with DSACl head groups than PMG does in the membrane interior.[3] In addition, the microscopic viscosity in the membrane interior is increased to the original value by increasing the temperature above T_c.

Table II
Photo- and Thermoinduced Specific Viscosity Changes of Aqueous Solution of the DSCl Vesicle Containing Copoly azo-MG/GA

	dark	UV irra.	Δ
η_{sp}	0.032	0.042	0.031

Photoinduced Intervesicular Adhesion. The adhesion and fusion of vesicles caused by charged polypeptides have been reported by many investigators.[10-12] Kriuijff et al.[13] have shown that the adhesion of negatively charged cardiolipin liposome to positively charged poly(L-lysine) is based on Coulombic interaction. We have attempted to demonstrate photocontrol of intervesicular adhesion. The structural changes of the DSACl vesicle caused by photo- and thermoinduced transfer of Copoly azo-MG/GA described above could be directly observed with a phase-contrast microscope. The shape of the vesicle obtained by sonication was found to be close to spherical before UV irradiation at 25 °C (Figure 4A). UV irradiation produced intervesicular adhesion (Figure 4B). Furthermore, visible light irradiation or keeping the vesicle in the dark did not induce any structural changes in the joining vesicles. The original structure of the DSACl vesicle can be re-formed by increasing the temperature above T_c (Figure 4C). This structural change was confirmed by the responses of macroscopic viscosity of an aqueous solution of the DSACl vesicle containing Copoly azo-MG/GA (Table II). That is, the specific viscosity, η_{sp}, of the solution was increased by UV irradiation, reflecting photoinduced intervesicular adhesion. Although the value of η_{sp} was not changed by visible light irradiation, it returned to its original value on increasing the temperature above T_c. Schematic pictures for the structural responses of the vesicles are shown in Figure 5. The photoinduced intervesicular adhesion can be explained in terms of the UV light-induced irreversible transfer of Copoly azo-MG/GA from the membrane interior to the surface (Figure 1 and Table I) and the formation of cross-linking bridges of L-glutamic acid moieties between the positively charged DSACl vesicles (paths 1 and 2, Figure 5). The resolubilization of the polypeptide to the membrane interior above T_c (Figure 1 and Table I) results in the re-formation of the original dispersed vesicles (path 3, Figure 5).

Photoinduced Ion Permeation Changes through the Bilayer Membrane. Figure 6 shows the rate of sodium ion permeation through the bilayer membrane from the vesicular interior to the aqueous phase. The permeability of sodium ion through the bilayer membrane was relatively high before UV irradiation. On UV irradiation, the permeability was decreased by a factor of 4.7. After UV irradiation, however, visible light irradiation or keeping the vesicle in the dark did not induce any permeability changes, but the permeability returned to almost its original value on increasing the temperature above T_c. Such responses of the permeability to photo- and thermostimulations seem to be closely related to those of the Copoly azo-MG/GA partition between the interior of the membrane and the surface. That is, the localization changes of Copoly azo-MG/GA in the bilayer membrane system play an important role in photo- and thermosensitive

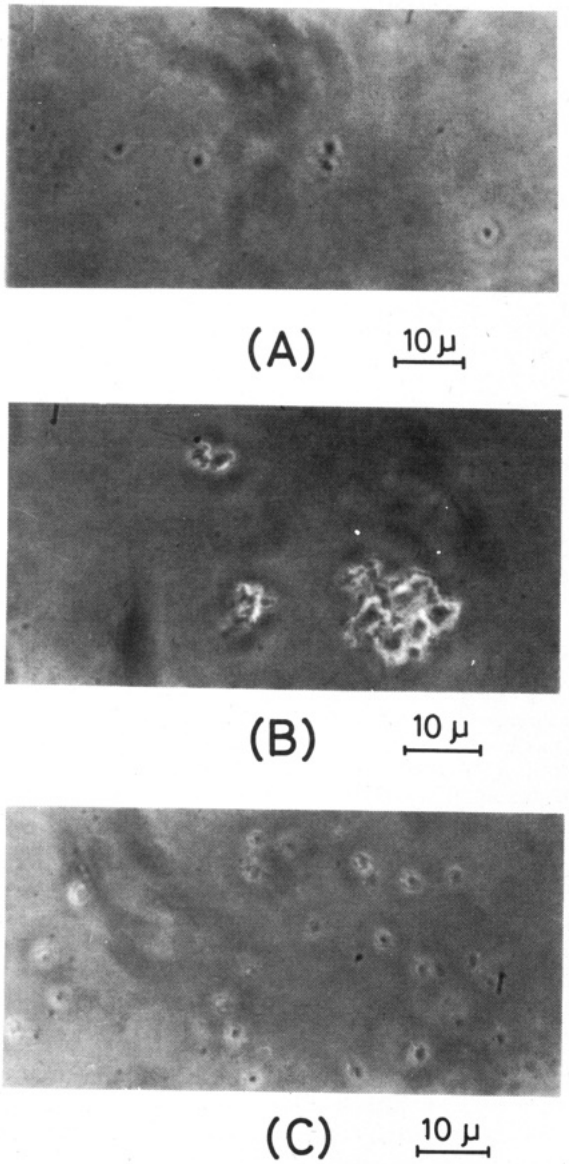

Figure 4. Optical micrographs of a DSACl vesicle containing Copoly azo-MG/GA: (A) before UV irradiation at 25 °C; (B) after UV irradiation at 25 °C; (C) at 25 °C after visible light irradiation and heating to 60 °C; [Copoly azo-MG/GA]/[DSACl] = 8.3 × 10^{-3}, [DSACl] = 0.2 wt %.

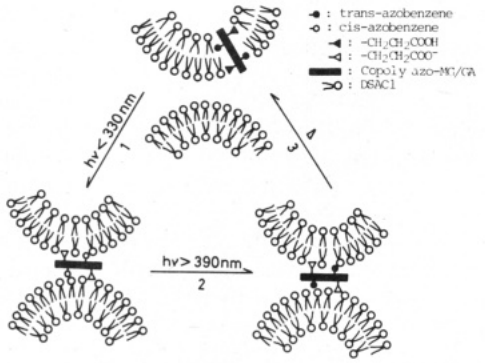

Figure 5. Schematic picture of photo- and thermoinduced changes in DSACl vesicle structure.

permeation. In addition, it has been confirmed that relatively low molecular weight poly(glutamates) can be incorporated into the DSACl membrane interior to form membrane-spanning helixes accompanying their side-by-side aggregation.[3] Therefore, when Copoly azo-MG/GA is in the membrane interior, the polypeptide may act as

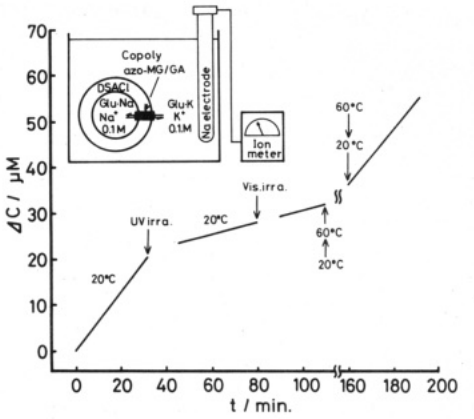

Figure 6. Photo- and thermoinduced sodium ion permeability changes across a DSACl bilayer membrane containing Copoly azo-MG/GA.

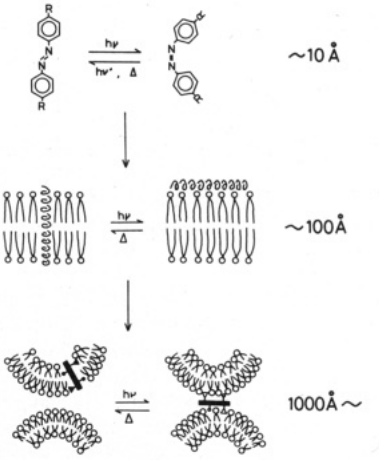

Figure 7. Dimensions in the structural responses of the Copoly azo-MG/GA-DSACl system to photo- and thermostimulations.

a channel for sodium ion, reflecting the high permeability of the membrane in the dark. The photoinduced decrease in the permeability, on the contrary, can be explained in terms of the loss of the channel via the induced transfer of the Copoly azo-MG/GA from membrane interior to the surface. Furthermore, by increasing temperature above T_c, the Copoly azo-MG/GA-DSACl vesicle system recovered its original structure with a polypeptide channel through the bilayer membrane.

The dimensions of the structural responses of the Copoly azo-MG/GA-DSACl system to photo- and thermostimulations are illustrated in Figure 7. The photostimulated configuration change of the azobenzene moieties in the molecular dimension can induce a macroscopic structural change in the DSACl vesicle: intervesicular adhesion.

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Registry No. L-Glutamic acid γ-methyl ester *N*-carboxyanhydride, 1663-47-4; poly(γ-methyl L-glutamate), 25086-16-2; poly(γ-methyl L-glutamate) (SRU), 25036-43-5; *p*-aminoazobenzene, 60-09-3.

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Interpretation of Stress-Strain Isotherms for Elastomers Cross-Linked in Solution

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ABSTRACT: Stress-strain isotherms exhibited by elastomeric networks in elongation differ in form from the predictions of the simplest molecular theories. Specifically, the reduced stress $[f^*]$ generally shows an unexpected large decrease with increase in elongation α, and, in the approximation that $[f^*]$ is linear with α^{-1}, the decrease is characterized by the ratio $2C_2/2C_1$ of phenomenological elasticity constants, where $2C_1$ is an approximate measure of the high-deformation modulus. Experimental studies have shown that cross-linking polymers in solution gives networks having greatly reduced values of $2C_2/2C_1$. The more recent and more realistic constraint theory of rubberlike elasticity is found to give a good account of these results when the important constraint parameter κ is varied with volume fraction v_{2C} of polymer present during the cross-linking. However, the dependence of κ on v_{2C} is significantly stronger than that suggested by theory. The values of κ thus obtained are within the range of values obtained in other comparisons of theory and experiment, as are the values of the heterogeneity parameter ζ. The values of κ generally decrease with decrease in v_{2C} and with increase in degree of cross-linking ($2C_1$), according to the relation $\kappa = 2(2C_1)^{-1/2}v_{2C}^{4/3+m}$. Treatment of experimental data by two independent methods led to $m = {}^2/_3$ and ${}^8/_9$, in contrast to a value of $m = 0$ expected from theory. Deviation of m from zero indicates a particularly strong effect of dilution on the degree of network chain interpenetration.

Introduction

The quantity of foremost importance in characterizing the elastomeric properties of rubberlike materials in elongation is the reduced stress of modulus. It is defined by[1,2]

$$[f^*] \equiv fv_2^{1/3}/[A^*(\alpha - \alpha^{-2})] \quad (1)$$

where f is the equilibrium retractive force, v_2 the volume fraction of polymer in the stretched network, A^* the undeformed cross sectional area of the dry sample, and α its elongation (ratio of length in the stretched state to the length in the unstretched state at the same volume). In the simplest molecular theories it is given by [2-6]

$$[f^*] = \nu kTv_{2C}^{2/3} \quad (2)$$

where ν is the number density of network chains relative to the dry network, k the Boltzmann constant, T the absolute temperature, and v_{2C} the volume fraction of polymer chains in the system being cross-linked which are successfully incorporated in the network structure. These theories thus predict that once a network is formed and a temperature chosen for its characterization, its reduced stress should be independent of elongation.

Experimental values of $[f^*]$, however, generally show a strong dependence on α, with $[f^*]$ decreasing with increase in α.[2,7] Over the typical range of elongations studied the relationship can be represented by the simple phenomenological equation[2,7-9]

$$[f^*] = 2C_1 + 2C_2\alpha^{-1} \quad (3)$$

within experimental error, where $2C_1$ and $2C_2$ are independent of α. If $2C_1$ represents the molecular contribution given in eq 1, then the normalized quantity $2C_2/2C_1$ is a measure of the discrepancy between experiment and the simplest molecular theories.[7]

A number of experimental studies[6,10-14] have shown that cross-linking polymers in solution gives networks which, even when dried, have very small and frequently negligible values of $2C_2/2C_1$. Typical results from one of the most extensive studies of this type, on poly(dimethylsiloxane) (PDMS), are shown in Figure 1.[13] They were obtained by interpolations or limited extrapolations of the results given in the first four columns of Table I. The decreases in $2C_2/2C_1$ are seen to be particularly pronounced at lower values of v_{2C} (higher dilutions). Unfortunately, very little is known about the origin of this interesting effect.

In one study[13] it was suggested that solution cross-linked networks have "simpler topologies", without any elaboration beyond the assumption that the degree of entangling among network chains was diminished. An alternative explanation[15] was based on the fact that if a network is formed in the undiluted state but studied swollen, its value of $2C_2/2C_1$ is also unusually small.[2,7] In this interpretation, imperfections even in an unswollen network would act as diluent, thus decreasing $2C_2/2C_1$ in the usual manner. Such imperfections would be expected to be more numerous in networks formed in solution because of the increased probability of intramolecular cross-linking, which leads to loops that can be elastically ineffective.[15] If the cross-linking was carried out by using high-energy radiation, as is frequently the case, then chain scission[16,17] could cause the formation of dangling chains, and such irregu-

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