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Molecular Conformation-Dependent Complexation between Acidic- and Basic-Polypeptides *via* Hydrogen Bonding in Solution

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Interpolymer complex formation between basic polypeptide poly(L-proline) Form II (PLP(II)) and acidic polypeptides poly(L-glutamic acid) (PLGA) and poly(L-aspartic acid)(PLAA) has been studied in water-methanol (1 : 2 v/v) mixed-solvent by viscometry, potentiometry, light scattering and circular dichroism (CD) measurements. It has been found that polymer complexes between PLP(II) and PLGA (or PLAA) are formed *via* hydrogen bonding with a stoichiometric ratio of PLP(II)/PLGA (or PLAA)=1 : 2 (in unit mole ratio) and that PLP(II) forms polymer complex more favorably with PLGA than with PLAA. In addition, the minimum (for pH 5.0) and the maximum (for pH 3.2) in reduced viscosity of dilute PLP(II)-PLGA mixed solutions are observed at 0.67 unit mole fraction of PLGA (*i.e.*, [PLP(II)]/[PLGA]=1/2). These findings could be explained in terms of molecular structure (or conformation) of the complementary polymers associated with the complex formation.

Introduction

Polymer complexes are formed, almost stoichiometrically, by the association of two or more complementary polymers *via* electrostatic forces, hydrophobic interactions, hydrogen (H) bonding, van der Waals forces or combinations of these interactions.[1-3] Due to the long-chain character of the polymers, the complex formation process is usually cooperative. Especially, the formation of polymer complexes between a proton-accepting (or Lewis base) polymer [*e.g.*, poly(ethylene oxide) (PEO), poly(N-vinyl pyrrolidone) (PVP), poly(L-proline) (PLP), etc.] and a proton-donating (or Lewis acid) polymer [*e.g.*, poly(methacrylic acid) (PMAA), poly(glutamic acid) (PGA), poly(aspartic acid) (PAA), etc.] *via* H-bonding in organic or aqueous media has attracted a continuing interest as a model of biological systems.[4-10] The interpolymer complexation *via* H-bonding in solution is highly sensitive to such factors as pH, ionic strength, temperature, solvent, concentration, structure and molecular weight of the component polymers, hydrophobic interaction, etc.[11] Hence, most studies have been performed on the polymer complex systems based on H-bonding, mainly focusing on the effects of these factors on complexation.

However, only a few studies[12-14] have been reported so far on the H bonding complexation between acidic- and basic- biopolymers with different conformations (*e.g.*, one with a helical structure and the other with a coiled structure) and on the conformational change of the complementary polymer upon complexation. Hence, for a model study on the interpolymer complexation *via* H bonding between acidic- and basic- biopolymers we have chosen PLP Form II (PLP (II)) as a basic polypeptide and L-forms of PGA and PAA

(*i.e.* PLGA and PLAA) as acidic polypeptides. As is well known, PLP(II) is a unique polymer having a 3_1 left-handed helical structure (with all the peptide bonds in trans conformation) due to the steric restriction about the N-C bond of the pyrrolidone ring without intramolecular hydrogen bonds, and readily soluble in water.[15,16] On the other hand, PLGA and PLAA, being weak polyelectrolytes, may assume a helical and/or random-coiled conformation, depending on pH of the surrounding medium.[17-19]

In this paper, we will report on viscosity, potentiometric (pH), light scattering and circular dichroism (CD) measurements of dilute mixture solutions of PLP(II) with PLGA (or PLAA) of various compositions in water-methanol (1 : 2 v/v) mixed solvent, which will lead to an evidence for the formation of an interpolymer complex with a definite stoichiometry *via* H-bonding. In addition, the influences of molecular structure and conformation of the component polymers upon complexation will be investigated from the experimental results.

Experimental

Materials. PLGA, PLAA, and PLP(II) were purchased from Sigma Chemical Co., Ltd., and identified by IR and CD spectra. The (viscosity-average) molecular weights ($\bar{M}_v$) of these polypeptides are as follows; PLGA (sodium salt), 54,600; PLAA (sodium salt), 50,300; and PLP(II), 19,000. Triply-distilled water and methanol (99.8%) were used as solvents in this study.

Sample Preparation. PLGA and PLAA were dialyzed against acidic aqueous solution to remove the sodium salt. The 0.5-1.0 wt% aqueous solutions of these polypeptides were put into the cellulose dialysis sack and stirred in water adjusted to pH 3.2 for about two weeks. The dialyzed polypeptides were freeze-dried to obtain the pure solid forms. PLP (II) was used without further purification. Separate solutions of homopolypeptides used for complex experiments were prepared in a mixed-solvent of water-methanol (1 : 2 v/v) with very dilute concentration (*i.e.*, $c = 1.0\text{-}2.0 \times 10^{-3}$ unit mole/L) to avoid the entanglement problem.

Measurements. In order to be sure of the optimal complex behavior throughout the study, all the measurements on mixed solutions of acidic- and basic-polypeptides of different compositions were performed with rigorous stirring for at least 24 hr. The results obtained were highly reproducible within small experimental errors. The pH measurement was made using a pH meter (Cole-Parmer Inst. Co., Model 5985-80). The pH of each peptide solution before mixing was adjusted to 3.2 using HCl. Viscosities on mixed dilute solutions ($c = 1.94 \times 10^{-3}$ unit mole/L) of PLP(II) with PLGA (or PLAA) at various unit mole ratios in water-methanol (1 : 2 v/v) were measured at 20± 0.02 ℃ with an Ubbelohde-type viscometer. The inner dilution capillary was used for viscosity measurements. The light scattering measurement was carried out using the Brookhaven Instrument (Model BI-2030) equipped with a He-Ne laser light source, the scattering angle (θ) and the wavelength (λ) of the incident light employed being 90° and 500 nm, respectively. The CD spectrum, expressed as the molecular ellipticity [θ] (in degrees cm^2 per decimole of the optically active compound), on binary mixed systems of PLP(II) with PLGA (or PLAA) in water-methanol (1 : 2 v/v) was measured at 25± 0.5 ℃ in the range of wavelength 190-250 nm using a JASCO J-20 CD/ORD spectropolarimeter equipped with a quartz cell of path length 1 mm.

Results and Discussion

Generally, the complex formation between polyacids and polybases *via* H-bonding in aqueous media is strongly dependent on pH of the medium, which will affect the charge density of the component polymers, and their molecular conformations responsible for the interpolymer complexation.[4,9] That is, the molecular chains of an ionizable polypeptide (*e.g.*, PGA, PAA) exist in a random coil form when the degree of ionization (α) is high, but in a helical form as α becomes low. The α for weak polyelectrolytes is usually controlled by pH of the medium, as suggested by the following modified Henderson-Hasselbach equation[20] for weak polyacids;

$$\text{pH} = pK_a + n \log [\alpha/(1-\alpha)] \quad (1)$$

where pK_a is the (apparent) dissociation constant of the acid, and n is a constant close to unity, depending on surrounding conditions.

As stated before, the basic polypeptide PLP(II) in aqueous solution takes a helical structure without any intramolecular hydrogen bonds over a broad pH range.[15,16] By contrast, the weak polyacid PGA exists as a helical form in the pH range where the charge density on the peptide chain is low, but assumes a random coil when its ionizable group is charged at higher pH than 7 owing to the mutual repulsion of ionized groups attached to a polypeptide chain (see Eq. (1)).[17-19] Although PAA has a similar property to PGA, the pH range where PAA has a helical conformation is much lower than that of PGA.[17,18]

For the confirmation of complex formation between PLP (II) and PLGA *via* H-bonding interaction and estimation of the stoichiometric ratio, the pH change upon complexation (ΔpH), *i.e.* the difference in pH between the final (equilibrium) and the initial stage of complexation at a given composition, for mixed solutions ($c = 1.0 \times 10^{-3}$ unit mole/L) of PLP (II) and PLGA in water-methanol (1 : 2 v/v) solvent at 25 ℃ are plotted in Figure 1 as a function of the unit mole fraction of PLGA (x_{PLGA}) in a PLP(II)-PLGA mixture (*i.e.*, [PLGA]/([PLGA]+[PLP(II)]) with respect to their repeating units). (It should be noted that the square bracket denoting the unit mole concentration (mole/L) of the component polymers is usually abbreviated in the text for brevity.) As shown in Figure 1, the maximum value of ΔpH is observed at a PLGA fraction of 0.67 (*i.e.* at a unit mole ratio of PLP(II) /PLGA = 1/2), which can be interpreted as follows. The complexation between poly(carboxylic acids) and polybases *via* H bonds is produced only by carboxyl groups in the undissociated state. Thus, dissociated carboxyl groups in mixed polymer solutions at a certain pH are influenced by the complexation and become undissociated by the extraction of protons from the solution into the domain of polymer chains, leading to an increase in pH. Accordingly, the pH change of mixed solutions of PLP(II) and PLGA increases with x_{PLGA} as a result of complex formation *via* H bonds up to a point of 0.67 (*i.e.* PLP(II)/PLGA ratio of 1/2), beyond which the pH decreases with increasing PLGA fraction due to the dissociation of (excess) uncomplexed PLGA present in the system. Hence, from Figure 1 we can deduce that the complex formation

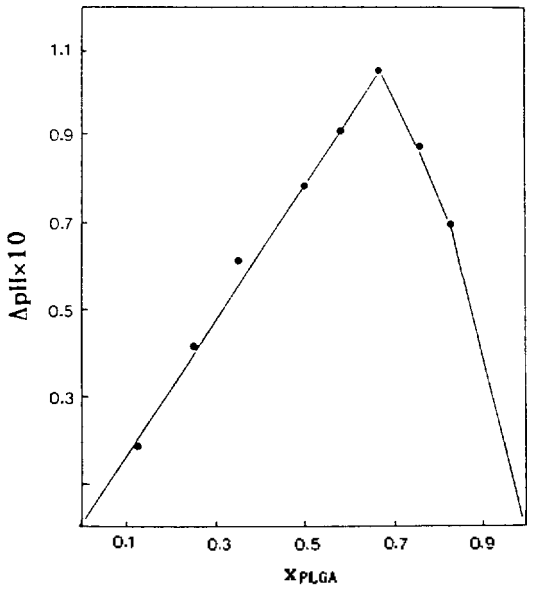

Figure 1. The pH change upon complexation (ΔpH) of PLP(II) /PLGA mixture solutions ($c=1.0\times10^{-3}$ unit mole/L) in water-methanol (1 : 2v/v) at 25 ℃ against the unit mole fraction of PLGA (x_{PLGA}). The pH values of PLP(II) and PLGA solutions before mixing are 6.43 and 5.0, respectively.

between PLP(II) and PLGA in water-methanol (1 : 2 v/v) occurs *via* H bonding with a (most suitable) stoichiometric ratio of PLP(II)/PLGA=1/2. The same thing can be applied to the PLP(II)/PLAA complex system.

It is well known that the CD spectrum (corresponding to the unequal absorption of plane polarized light by a chiral molecule with asymmetric structure as a function of λ of the incident light) is widely used in investigating the conformation or conformational change of optically active biopolymers (*eg.*, polypeptides, proteins) in solution.[17-19,21]

Hence, we tried to use the CD spectroscopic measurement in order to identify a 1 : 2 (base to acid) repeating unit stoichiometry and to understand the conformational change of the component polymers upon complexation *via* H-bonding for the PLP(II)/PLGA(PLAA) complex system. Figure 2 shows the CD spectra for three homopolypeptides used for this complex study, *i.e.* PLP(II), PLGA and PLAA, in water-methanol (1 : 2 v/v) at 25 ℃ and pH 3.2. Since the $n\rightarrow\pi^*$ and $\pi\rightarrow\pi^*$ transitions of amino acids composing the polypeptide chains occur at about 225 nm and 195 nm, the range of the wavelength covered in this study is 190-250 nm. The CD spectra given in Figure 2 are well consistent with those reported by other authors,[19,21] who have assigned the left-handed helix, the right-handed helix, and the random-coiled conformation to PLP(II), PLGA and PLAA, respectively, at this pH range. Figure 3 shows the CD spectra for the PLP (II)-PLGA mixed solutions ($c=1.0\times10^{-3}$ unit mole/L) at various unit mole ratios in water-methanol (1 : 2 v/v) at 25 ℃ and pH 3.2. Curves C, D, E, and F exhibit the actual CD spectra ($[\theta]_m$) of binary mixtures (after equilibrium) for the respective compositions whereas curves c, d, e, and f exhibit the "ideal" CD spectra ($[\theta]_m^{id}$) of the corresponding mixtures calculated from the simple additive rule (Eq. (2)) with respect to unit mole fractions of the component polymers

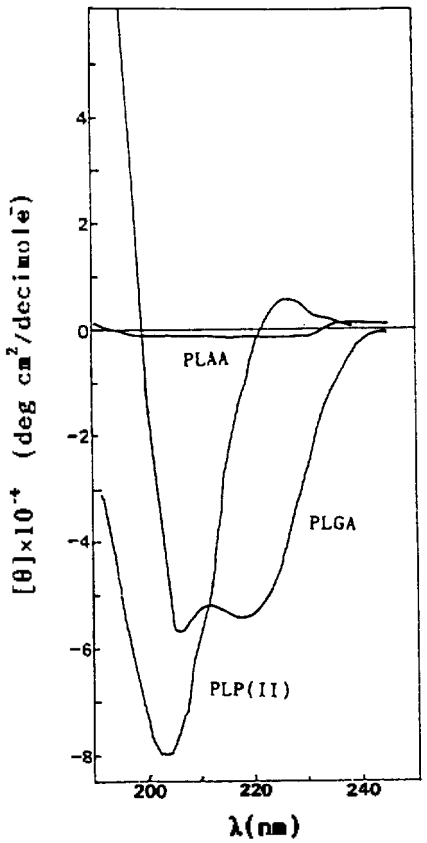

Figure 2. CD spectra of homopolypeptides PLP(II), PLGA and PLAA in water-methanol (1 : 2 v/v) ($c=1.0\times10$ unit mole/L) at 25 ℃ and pH 3.2.

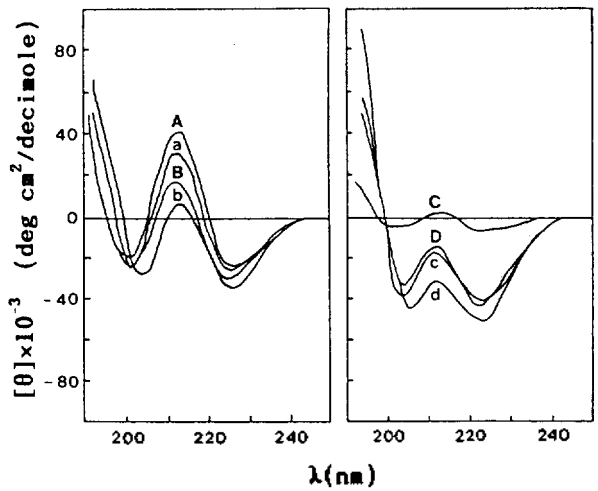

Figure 3. CD spectra for PLP(II)-PLGA mixture of verious compositions ($c=1.0\times10^{-3}$ unit mole/L) in water-methanol (1 : 2 v/v) at 25 ℃ and pH 3.2. The unit mole ratios of PLP(II) to PLGA for the mixture are C, c (2 : 1); D, d (1 : 1); E, e (1 : 2); F, f (1 : 3). Curves C, D, E, and F are actual spectra upon complexation while curves c, d, e, and f are "ideal" spectra calculated by Eq. (2) in the text.

at a given λ using the CD spectra for the pure components as given in Figure 2, assuming no appreciable interactions between the component polymers;

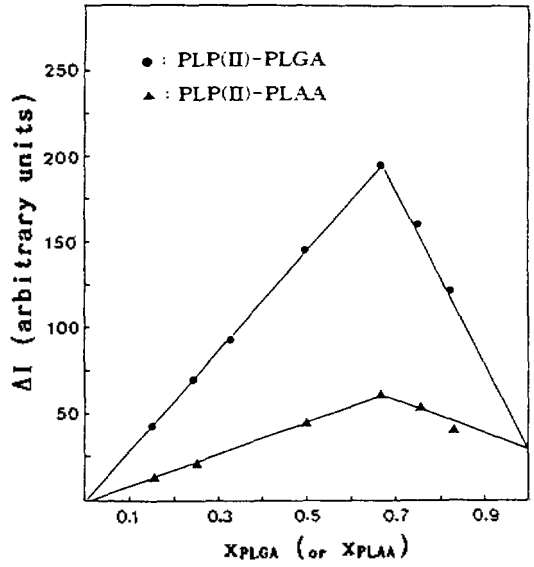

Figure 4. Changes (in excess) scattered intensities of the light ΔI (set at $\theta=90°$ and $\lambda=500$ nm) upon complexation for dilute PLP(II)/PLGA (or PLAA) mixtures ($c=1.0\times10^{-3}$ unit mole/L) in water-methanol (1 : 2 v/v) at 25 ℃ against the unit mole fraction of PLGA (or PLAA).

$$[\theta]_m^{id}=x_1\,[\theta]_1+x_2\,[\theta]_2 \qquad (2)$$

where x_1 and x_2 are unit mole fractions of polymers 1 and 2, respectively, and $[\theta]_1$ and $[\theta]_2$ are the corresponding molecular ellipticities. Here, let numbers 1 and 2 denote PLP(II) and PLGA (or PLAA). In principle, we can predict the occurrence of complexation (or complex stoichiometry) and the conformational change of the component polymers upon complexation by comparing the actual CD spectra with the ideal ones for polyacid/polybase mixtures in solution. The more conformational change (as a result of strong intermolecular interaction) each component polymer has, the more deviation (from the ideal one) the actual CD curve has. From Figure 3, we can notice that the most deviation from the ideal curve is observed for the PLP(II)/PLGA mixed solution at a 1 : 2 repeating unit mole ratio. This result can be another evidence of PLP(II)/PLGA=1 : 2 complex system, and also indicates that the conformational change of the component polymers occurs most strongly in the vicinity of a 1 : 2 unit mole ratio due to the formation of most stable complexes between PLP(II) and PLGA *via* H bonding in a hydroalcoholic medium. A similar result has also been observed for the PLP(II)/PLAA system.

Since the excess scattered intensity of the light for a dilute polymer solution (defined as the difference between the scattered intensities of the sample solution and of the solvent) at a given scattering angle is proportional to the product of the concentration and the (weight-average) molecular size of polymer molecules in solution, light scattering as well as viscosity measurements can provide useful information on complex formation between biopolymers in solution.[22,23] In general, the complex formation between the component polymers brings about the increase in (excess) scattered light intensity of the original binary mixed solution due to the increase in molecular size and/or due to the aggregation

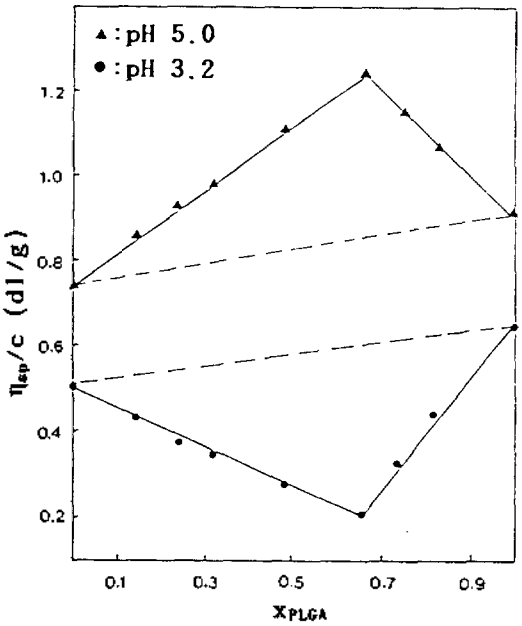

Figure 5. The effect of pH on the relationship between the reduced viscosity, η_{red}, of PLP(II)-PLGA mixture solutions ($c=1.94\times10^{-3}$ unit mole/L) in water-methanol (1 : 2 v/v) at 20 ℃ and unit mole fraction of PLGA. The dashed line for each pH denotes the "ideal curve" obtained from the simple additivity of the component viscosities with respect to unit mole fractions.

effect involved.

With a view to investigating the side chain effect of the acidic homopolypeptides on interpolymer complexation between acidic- and basic-polypeptides of interest, we have attempted to compare the light scattering experimental result of PLP(II)-PLGA system with that of PLP(II)-PLAA system. Light scattering measurements were performed on the mixtures of PLP(II) with PLGA and PLAA of various mixed compositions ($c=1.0\times10^{-3}$ unit mole/L) in water-methanol (1 : 2 v/v) at 25 ℃ and pH 3.2. Figure 4 illustrates the changes (ΔI) in excess scattered intensities of the light (using $\theta=90°$ and $\lambda=500$ nm) for dilute PLP(II)/PLGA(PLAA) mixture solutions ($c=1.0\times10^{-3}$ unit mole/L) resulting from interpolymer complexation as a function of unit mole fraction of PLGA (PLAA). The increase of ΔI with increasing molar fraction of PLGA(PLAA) is considered to be caused by the interpolymer complex formed between PLP(II) and PLGA(PLAA) *via* H bonding interaction. The observation of the maximum ΔI value at x_{PLGA} (x_{PLAA})=0.67 for both systems is again indicative of PLP(II)/PLGA(PLAA)=1/2 (in unit mole ratio) complex system. From Figure 4 we can also see that the higher ΔI value is observed for the PLP(II)-PLGA system than for the PLP(II)-PLAA system at a given mixed composition, suggesting that PLGA with longer side chain has a greater ability of to form H-bonded complex with PLP(II) than PLAA does probably because of more binding sites available for the complexation.

Finally, in order to further sustantiate the results obtained above and to investigate the effect of conformational change of the complementary polymer caused by variations in environmental conditions on H-bonded complexation between acidic- and basic-polypeptides viscosity measurements were

made on dilute solutions of PLP(II)-PLGA mixtures ($c = 1.94 \times 10^{-3}$ unit mole/L) at two different pH values, *i.e.* pH 3.2 and 5.0, in water-methanol (1 : 2 v/v) at 20 ℃, whose results are displayed in Figure 5 as the plot of reduced viscosity η_{red} ($=\eta_{sp}/c$ with η_{sp} being specific viscosity) *vs.* unit mole fraction of PLGA. The (reduced) viscosities of mixed polymer solutions exhibit the maximum for pH 3.2 and the minimum for pH 5.0 at a composition of $x_{PLGA} = 0.67$, implying that the maximum manifestation of interpolymer complexation occurs for a PLP(II)/PLGA = 1/2 unit molar ratio, irrespective of the pH value of the medium, in agreement with the previous results on pH, light scattering, and CD measurements. In addition, we can notice from Figure 5 that the aspects of viscosity changes with x_{PLGA} for PLP(II)-PLGA mixture solutions at pH 3.2 and 5.0 are quite different from each other. The measured η_{red} values of dilute PLP(II)-PLGA mixture solutions exhibit the positive deviation at pH 3.2 but the negative deviation at pH 5.0 from the "ideal" values (dotted lines in Figure 5) obtained from the simple additivity of the component viscosities based on their unit mole fractions, similarly to the case of CD measurement (Eq. (2)).

This could be explained from the structural point of view, *i.e.* in terms of difference in molecular conformation (or hydrodynamic dimension) of PLGA at two different pH conditions with the help of the well-known Flory[24] equation relating the (intrinsic)viscosity to molecular dimension in dilute solution;

$$[\eta] = \Phi \langle r^2 \rangle^{3/2} / M \tag{3}$$

where $[\eta]$ is the intrinsic viscosity obtained by extrapolation of η_{red} to infinite dilution, Φ Flory's universal constant, M the molecular weight, and $\langle r^2 \rangle$ the mean-square end-to-end distance of the polymer in solution. By Eq. (3) it is meant that $[\eta]M$ is a relative measure of the hydrodynamic volume $\langle r^2 \rangle^{3/2}$ of a polymer molecule in solution. Hence, the result shown in Figure 5 can be qualitatively interpreted on the basis of Eq. (3), as has been done previous paper. Both PLP(II) and PLGA molecules in aqueous media assume helical conformations at pH 3.2, as stated before. Therefore, the complex formation between PLP(II) and PLGA at pH 3.2 corresponds to the so-called "order-order complexation", yielding a larger hydrodynamic volume, and hence increased viscosity, as compared to that of each (uncomplexed) complementary polymer, leading to a maximum at $x_{PLGA} = 0.67$, *i.e.* at [PLP(II)]/[PLGA] = 1/2 (in unit mole ratio). Consequently, the viscosity behavior for the PLP(II)-PLGA system at pH 3.2 may exhibit the positive deviation from the simple additive rule. On the other hand, at a condition of pH 5.0 the dissociation of carboxyl groups attached to PLGA is considerably increased as compared to the case of pH 3.2 (see Eq. (1)), thereby causing the (partial) destruction of the helical structure of PLGA. In fact, PLGA in this pH range is reported to coexist as helical and random conformation.[17-19] Thus, the complex formation between PLP(II) and PLGA at pH 5.0 corresponds to the "order-disorder complexation", yielding a smaller hydrodynamic volume, and hence decreased viscosity, as compared to the pure state of each complementary polymer, leading to the viscosity minimum at a 1 : 2 unit mole ratio. Accordingly, the viscosity behavior for the PLP(II)-PLGA system at pH 5.0 may exhibit the negative deviation from the simple additive rule.

In conclusion, it has been found from pH, viscosity, light scattering and CD measurements on the PLP(II)/PLGA (or PLAA) mixed systems in water-methanol (1 : 2 v/v) that polymer complexes between PLP(II) and PLGA (or PLAA) are formed *via* hydrogen bonding with a 1 : 2 repeating unit stoichiometry of PLP(II)/PLGA (or PLAA) and that PLP(II) forms polymer complex more favorably with PLGA than with PLAA. In addition, the minimum (for pH 5.0) and the maximum (for pH 3.2) in reduced viscosity of dilute PLP(II)-PLGA mixed solutions are observed at a PLGA unit mole fraction of 0.67 (*i.e.*, [PLP(II)]/[PLGA] = 1/2). These findings could be explained in terms of molecular structure (or conformation) of the complementary polymers associated with the interpolymer complexation.

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Photodecomposition Mechanism of 2-Methoxy-1,2-diphenyl Diazoethane

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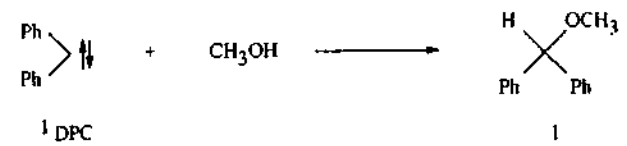
The mechanism of the photodecomposition of 2-methoxy-1,2-diphenyl diazoethane has been investigated in methanol and isoprene using time-resolved laser flash photolysis techniques. The reaction of triplet carbene which is generated from 2-methoxy-1,2-diphenyl diazoethane wth methanol is believed to proceed *via* thermal excitation to the singlet state. The activation energy and enthalpy are conistent with a mechanism involving thermal equilibrium between the triplet and singlet state followed by the reaction of the singlet with methanol to give ether.

Introduction

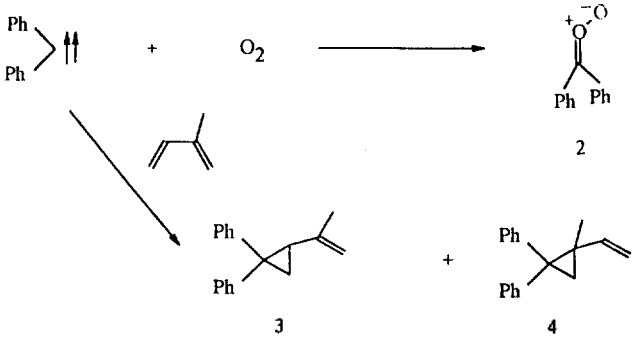
The reactivity of carbene is determined by their spin multiplicity.[1] Carbenes have singlet and triplet electronic states. Generally triplet carbenes react by two-step radical processes, whereas singlet carbenes can undergo singlet-step bond insertion. Methylene is well known as a simple carbene.[2] It is well appreciated that there are two chemically important states of methylene.[3] Their two states can each be detected spectroscopically as $^1A_1$ and $^3B_2$.[4] Organic chemistry have also been eager to measure the singlet-triplet energy gap in large systems such as phenyl carbene, diphenyl carbene and naphthyl carbene.[5] However, in these systems, it has only proven possible to detect the triplet ground state by spectroscopic methods. It is also questionable whether gas phase spectroscopy and high-level calculation can be applied to molecules of such size and complexity to give accurate values of enthalpy between the singlet and triplet states. Many results are focused on the points of combination of product analyses and kinetics as a tool which can be interpreted the reactivity for the molecules of aromatic carbenes. For example, the quenching of a triplet aromatic carbene is used for the standard reaction of probing the singlet-triplet energy gap.[6] It is pointed out that this treatment would make an error for application to some aromatic carbene.[7] Recently carbenes have been detected by electron paramagnetic resonance (EPR) spectroscopy. It is known that diphenylcarbene has a triplet at the ground state[8-11] and the coincidence of recent opinion supports the results that the triplet and singlet states are in thermal equilibrium at room temperature.[5,12] However, evidence supporting this conclusion is based on the assumption that singlet and triplet states of the carbene carry out certain stereotypical reactions. In particular it has been assumed that only the singlet state of the carbene reacts with alcohols to give ethers.[5,12] It is widely believed that it is possible to obtain spin-specific products from diphenylcarbene.

The diagnostic reaction of diphenylcarbene of singlet state (^{1}DPC) with methanol gives ether 1:



^{1}DPC 1

and that of the triplet state (^{3}DPC) with oxygen or isoprene gives 2 or 3 and 4.



The reaction mechanism on which these conclusions are described above can be augmented with measurements made by flash photolysis. In the standard approach,[5,12] an absolute rate constant for the reaction of a triplet carbene with a diene is determined by laser flash photolysis.

Competition studies are then carried out in which the carbene is generated in mixtures of the diene and alcohol. It is assumed that only the singlet state of carbene will react with alcohol, and thus information about the relationship between the singlet and triplet states can be discernible from the experimental results.

Closs and Rabinow[13] were the first to measure an absolute rate constant for a carbene reaction in solution. Flash photolysis of diphenyldiazomethane in an inert solvent such as