Helix formation of poly(L-glutamic acid) and poly(L-lysine) in the polyion complex prepared in aqueous alcohol solutions

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The total helix content has been estimated by circular dichroism measurements for sodium poly(L-glutamate)/poly(L-lysine) hydrobromide (PLGNa/PLLHBr) mixed systems in aqueous alcohols. The polyion complexes were soluble in the mixed solvents, on account of the rather low total polymer concentration (6.2×10^{-4} (base) mol l^{-1}), except when a polymer mixing ratio of 1:1 equivalents was used. It has been observed that the total helix content significantly decreases with polymer mixing, when compared to that observed in single polymer systems, while by the addition of ClO_4^-, or a short-chain PLGNa, both of which stabilize the helix conformation of PLL, the respective helix conformations are almost completely retained. These experimental findings, together with those concerning the dependence of the helix content on the alcohol species, are discussed, and possible structures for the polyion complexes are proposed.

(Keywords: polyion complex; helix formation; conformation)

INTRODUCTION

It has been known for some time that charged polypeptides, such as poly(L-glutamic acid) (PLG) and poly(L-lysine) (PLL), change their secondary conformation upon complexation with other charged polypeptides and synthetic polyions of opposite charge[1–3]. For example, Nakajima *et al.*[1] found that the helix conformation of PLL in an alkaline medium was destroyed in its polyion complex with PLG, while that of PLG in an acidic medium appeared to be sustained by 'super-helical' PLL. In the latter case, a regular secondary structure seems to be maintained in the complex, although the latter cannot really be called a 'polyion complex', because in the relevant pH region the PLG should lose most of its charges. If a genuine polyion complex, consisting of oppositely charged polypeptides, were prepared with their regular secondary structure kept intact, this species might have potential applications as a useful separation material for ions, chiral molecules, etc.

In this present study, we have attempted the preparation of a polyion complex of PLGNa and PLLHBr, in which the two components are in a helical conformation, as a preliminary step towards the development of the above mentioned material. Polyion complexes were prepared by mixing the two polypeptide solutions in various water/alcohol (methanol (MeOH), ethanol (EtOH), 2-propanol (2PrOH), t-butanol (tBuOH)) mixed solvents. The choice of a mixed solvent system instead of a simple aqueous one is made because both of the polypeptides assume a helical conformation, even in their fully charged states, in the former solvent systems, due to contact ion-pair formation between the polymer charges and counterions. This behaviour has been demonstrated in our previous studies[4–8]. In the case of single polymer systems, marked dependences of the helix content on the alcohol species that are present have been observed and interpreted in terms of three solvent properties, i.e. the dielectric constant (D), the water activity (a_w) and the Gutmann–Mayer acceptor number (AN)[9]; these have been utilized as critical factors in the contact ion-pair formation[6,8]. A significant dependence on the alcohol species has also been observed for the helix formation in this present polyion complex. Therefore, we will consider the experimental findings, including the solvent dependency, concerning the helix formation in this polyion complex by referring to the proposed mechanism for helix stabilization in the single polymer systems.

EXPERIMENTAL

Materials

Sodium poly(L-glutamate) was obtained by saponification of poly(γ-methyl L-glutamate) (Seiko Kasei Co.) in an aqueous alcohol mixture[10]. Poly(L-lysine) hydrobromide was obtained by ion exchange of poly(L-lysine) hydrochloride (Peptide Institute, Inc.) by the use of ultrafiltration in the presence of excess NaBr. The molecular weights of these polymer samples were estimated by viscometry as 7.2×10^4 (degree of polymerization (DP)=470) and 2.5×10^4 (DP=120), respectively. A detailed description of the purification of the polymer samples is given elsewhere[6,8].

Alcohols (MeOH, EtOH, 2PrOH, tBuOH) of analytical grade purity were purchased from Kanto Chemical Co.

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Ion-exchanged and distilled water was used in the sample preparations. NaBr, $NaClO_4$, $MgSO_4$, and $CaSO_4$ (Kanto Chemical Co.), and PLGNa of low *DP* (=30) (PLG30), which was a kind gift from Ajinomoto Co., were used as salts for addition to the polymer solutions.

Circular dichroism (*c.d.*) *measurements*

Polymer solutions were prepared as follows: the required volume of aqueous PLGNa solution, with a concentration (C_p) of 6.2×10^{-3} (base) mol l^{-1}, an aqueous PLLHBr solution of the same C_p, plus salts if necessary, and finally water, were added in that order to alcohol of the required volume, in order to obtain a water/alcohol mixed solution of PLGNa and PLLHBr with a total C_p of 6.2×10^{-4}. In this sample preparation, the molar ratio of the two polypeptides was varied between 0:1 and 1:0 for various mixed alcohol solvent compositions between 40 and 90 vol%. It should be noted here that the respective alkali hydroxides were not added to these present sample solutions, although they were used to prevent protonation of the PLG in our previous studies on the coil–helix transition[6]. Therefore, a marked increase in the helix content of PLGNa with time, which is ascribed to a slight protonation of the PLG charged groups, was inevitably observed in this present study, as shown in the following section. To examine the time dependence of this system, c.d. measurements were made after 10 min, 1 day, 3 days, 1 week, 2 weeks, and finally 4 weeks after each sample preparation.

The total helix content, θ (%), was estimated from the relationship, $\theta = [\theta]_{222}/400$, where $[\theta]_{222}$ is the molar ellipticity at 222 nm[11], as measured with a Jasco 20A spectropolarimeter at 25 ± 0.1°C.

Infra-red (*i.r.*) *spectroscopic measurements*

The secondary structure of the complexed PLG/PLL (at a molar ratio of 1:1), obtained as a precipitate, was examined by i.r. spectroscopy (JEOL JIR-AQS20) by using the KBr pellet method.

RESULTS AND DISCUSSION

Time dependence of total helix content

Figures 1a–d show the time dependence of the total helix content, θ, for a PLGNa/PLLHBr mixture in aqueous solutions of 80 vol% MeOH, EtOH, 2PrOH and tBuOH, respectively. An increase in θ of the PLGNa with time is most apparent for the MeOH system, followed by the EtOH and 2PrOH systems, with the least appreciable effect shown for tBuOH. This time dependence and its alcohol specificity are ascribed to a slight protonation of the PLG carboxyl groups caused by the protic solvents. (Although this time dependence can be suppressed in the presence of a small amount of alkali hydroxide, as mentioned above, this was not added in this present study so as to assure retention of the fully charged state of PLL.) It was confirmed that the θ values, measured 3 days after sample preparation, were maintained, even after periods of 4 weeks.

One common feature that can be observed in *Figures 1a–d* is the abrupt decrease of θ at a polymer mixing ratio of 1:1. This decrease is the only apparent one due to precipitation. In fact, according to i.r. measurements on the precipitate, the secondary structure of the polypeptides consists of an α-helix, in addition to a β and random coil conformations. The fact that precipitation only occurs at a 1:1 molar ratio, may be ascribed to the otherwise rather low total polymer concentration (6.2×10^{-4} (base) mol l^{-1}) in the systems, and suggests that the polyion complex is formed in such a way that an overall stoichiometry is also maintained on each polymer chain. For example, in the case where PLGNa:PLLHBr = 2:1, half of the carboxyl anions on each PLG chain should be neutralized by the ammonium cations of the PLL, but the possibility that half of the PLG chains are completely neutralized may be excluded. Here it seems to be appropriate to mention various types of 'neutralization', namely $-COO^{-} \cdot {}^{+}H_3N-$ or $-COOH \cdot H_2N-$. For the following reasons, we believe that the latter type of complexation can be safely neglected, or, at least, is only present as a minor component in the whole complex:

1. If the uncharged type is the main component in the complex that is formed, one should be able to observe a remarkably high helix content, even in low-alcohol-content systems.
2. A small difference between the pK_a values of the two polyelectrolytes (PLGNa ~8.0[12], PLLHBr ~7.0[13], in their fully charged states in aqueous solution) seems to explain the apparent difficulty in forming a complex in the uncharged state. As a matter of fact, in a system with a large pK_a difference, i.e. PLGNa/poly(allylamine)hydrobromide (pK_a ~5.5[14]), a remarkably high helix content for the PLG has been observed; typically, $\theta = 80\%$, for mixing ratios of ~1:1, even in a 60 vol% tBuOH system[15].

In the following discussion, therefore, we shall pay attention only to complexation in the charged state.

Dependence of θ *on alcohol composition*

The dependence of θ on the EtOH/water composition is shown as a typical example in *Figure 2*. All of the data shown here were obtained 10 min after the samples were prepared. First, one should note that solvent-induced coil–helix transitions occur for the respective single polymer systems, i.e. at 0 and 1.0 on the horizontal axis. The increase in θ for PLGNa, and the less significant increase for PLLHBr, with increasing EtOH content, have been interpreted as being caused by contact ion-pair formation between the polymer charges and the relevant counterions, which becomes increasingly favourable with increasing alcohol content[6,8]. In this present study, however, our main interest is in the characteristic dependence of θ on the EtOH content for the polyion complex. In this case, this varies from a maximum at the lower EtOH content, then shows a continuous decrease with increasing PLL composition, to finally reach a minimum at the highest EtOH content.

We consider these three patterns of the dependence of θ on the polymer mixing ratio in terms of a combination of the respective conformations taken in the single polymer systems, i.e. coil PLG/coil PLL, (partial) helix PLG/coil PLL and helix PLG/partial helix PLL. As the simplest case, we adopt the helix PLG/coil PLL combination first, and consider the conformations in the complex. *Figure 3a* shows typical results for this combination: θ decreases linearly with an increasing PLL content to a value of 0 for pure PLL. The continuous line (A) in the figure is a hypothetical one for the case in which the respective conformations of the single polymer systems are also maintained intact in the complex. Since

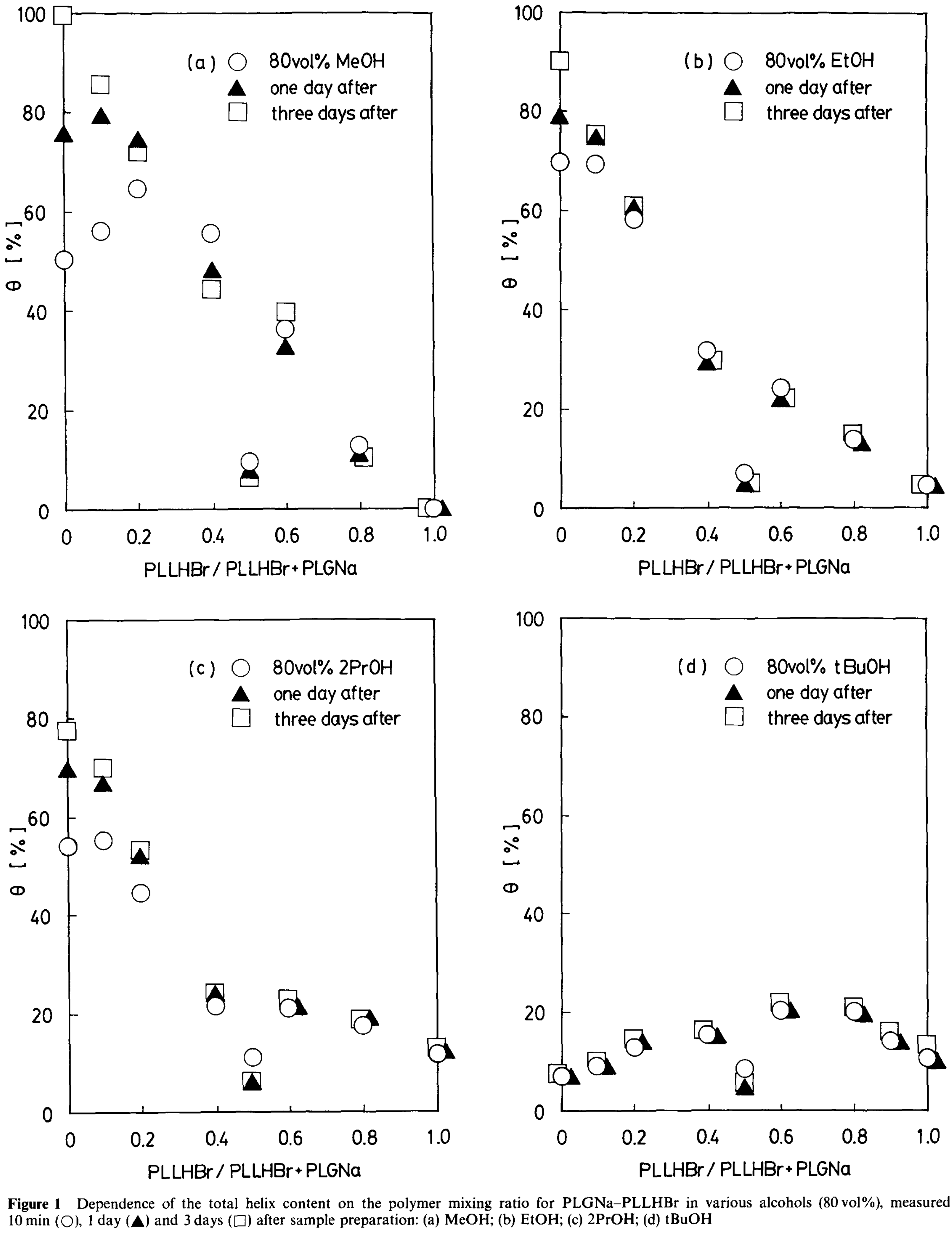


Figure 1 Dependence of the total helix content on the polymer mixing ratio for PLGNa–PLLHBr in various alcohols (80 vol%), measured 10 min (○), 1 day (▲) and 3 days (□) after sample preparation: (a) MeOH; (b) EtOH; (c) 2PrOH; (d) tBuOH

the data points fall almost on this line, the conformations in the complex may be assumed to be as shown in *Scheme 1a*. In the proposed structure, a helical PLG chain is surrounded by a coil PLL, in which the latter can stabilize the helix through charge neutralization.

On the other hand, in the case of a helix PLG/partial helix PLL combination (*Figure 3b*), the θ values fall well below the hypothetical line (B) which represents intact conformations, suggesting that a substantial part of the helix conformation of PLG and/or PLL is destroyed to

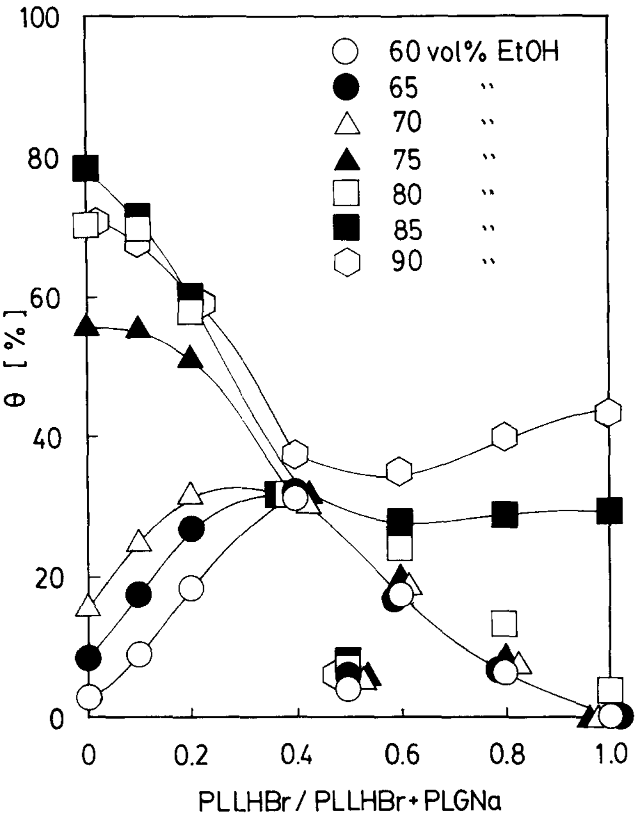
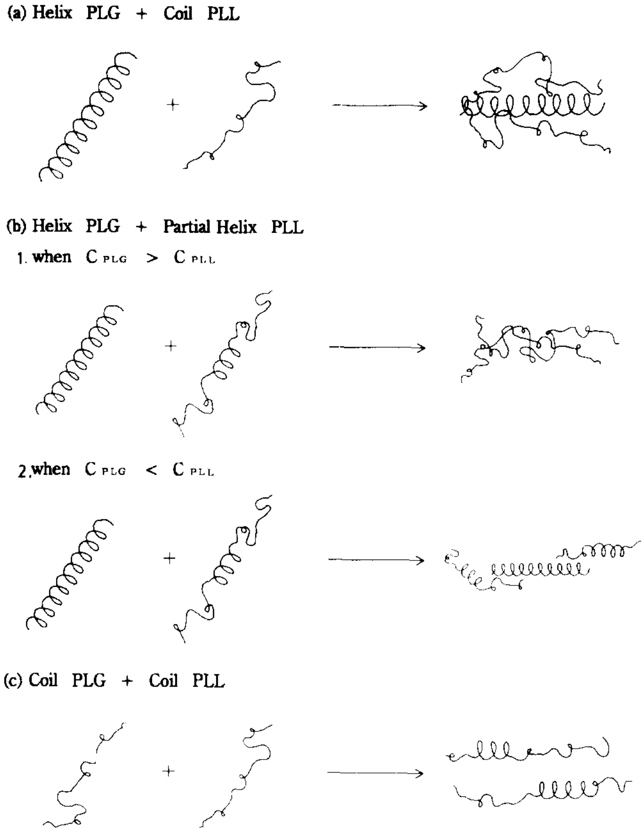


Figure 2 Dependence of the total helix content on the polymer mixing ratio for PLGNa–PLLHBr in aqueous EtOH of various compositions; all data were obtained 10 min after sample preparation



Scheme 1 Structures proposed for the polyion complex formed with different combinations of the respective polypeptide conformations

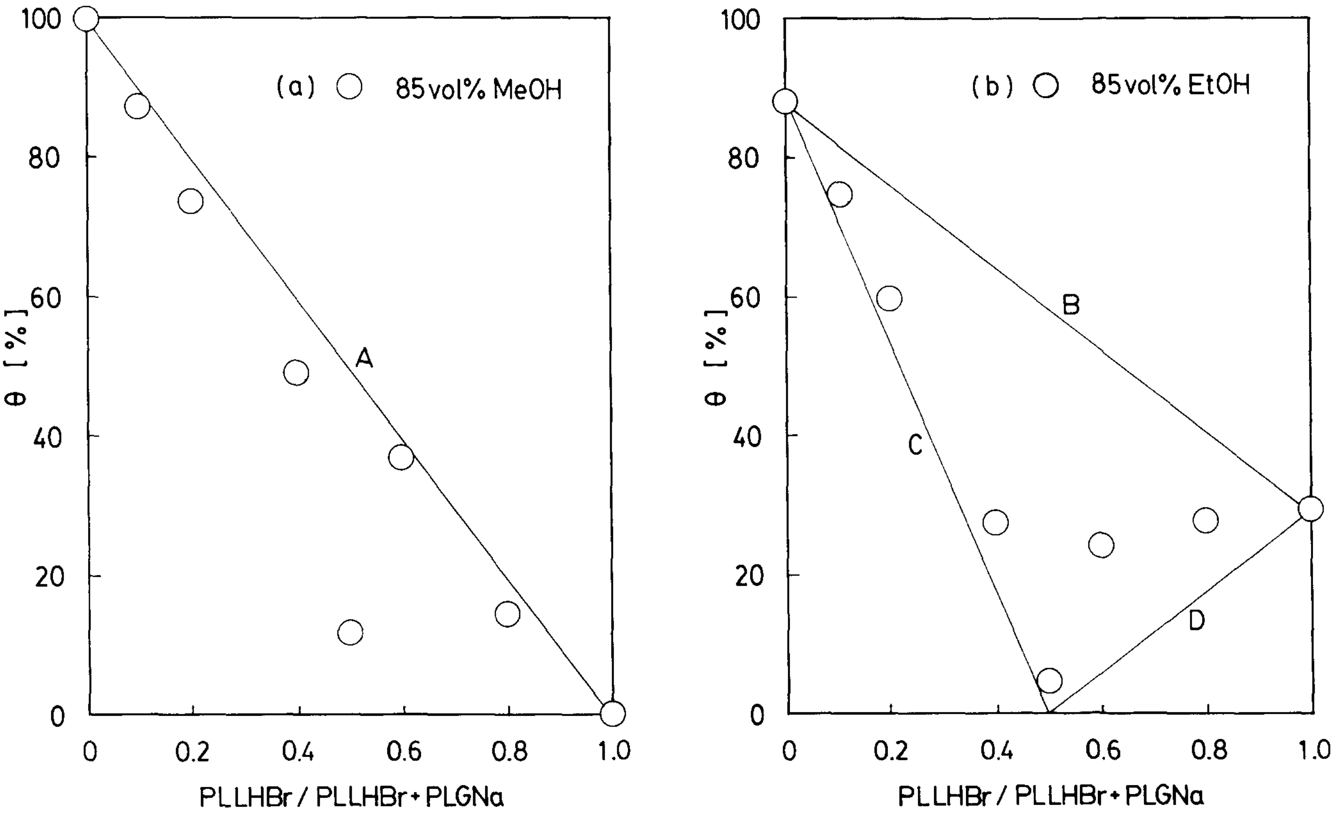


Figure 3 Comparison of the total helix content and hypothetical lines for PLGNa–PLLHBr in 85 vol% MeOH (a) and EtOH (b): experimental data points were obtained 3 days after sample preparation. Lines A and B represent cases in which conformations of the respective single polymer systems are maintained intact, while lines C and D represent cases in which the helix conformations are completely destroyed upon complex formation

give a random coil. In fact, the data points in the PLG-rich region (PLL fraction $\leqslant 0.5$) are close to another hypothetical line (C) that represents a complete transition to a coil conformation of the helix part of the PLG which is complexed with the partial helix PLL conformation, while in the PLL-rich region (PLL fraction $\geqslant 0.5$) the θ values lie between the lines (B) and (D). This suggests that the respective polymer conformations found in the complex formed under specific conditions depend on the relative amounts of the constituent polymers. Therefore, we have assumed that the corresponding conformations in the complex for the PLG-rich and PLL-rich regions are as shown as (1) and (2), respectively, in *Scheme 1b*. In the proposed structure for the polyion complex in the PLG-rich region, the helix part of the PLG is almost completely destroyed to give a coil conformation, as a result of complexation with partial helix PLL. (For the sake of simplicity, the chain lengths are taken as being equal, and a 1:1 complex is shown in the scheme.) Since all of the helical part of PLL must interact with PLG to form a stoichiometric complex in the PLG-rich region, it is inferred that the respective helix parts inevitably approach each other to destabilize the regular conformation and finally attain a conformation in which two random coils are stabilized through one-to-one charge neutralization. On the other hand, in the PLL-rich region, the coil part of PLL may be enough to neutralize the PLG charges, thus keeping the helix part of the PLL out of the interaction. This is shown by case (2) in *Scheme 1b*.

Finally, for the coil PLG/coil PLL combination, which gives a maximum in the plot of θ against the PLL content, we propose a possible structure for the complex which is as shown in *Scheme 1c*. In this, the coil part in the PLG (or PLL) stabilizes the helix of its counterpart. Another likely structure may also be considered, in which a helix is formed only for the PLG through charge neutralization with the positive charges on the random coil PLL. In this present study, however, no conclusions can be drawn as to which is the main structure.

It seems worthwhile to note the results for the helix PLG/partial helix PLL combination again. As stated above, an interaction between the helix parts seems to be unfavourable because a complete one-to-one charge neutralization is impossible to achieve on account of the regular charge array. Therefore, in order to make a polyion complex between a helix PLG and a helix PLL, a screening of the electrostatic interactions between helices with high charge densities must be essential. One of the simplest procedures for reducing electrostatic interactions is the use of a medium with either a higher dielectric constant or a higher ionic strength. In the following, we examine the effects of both of these factors in turn.

Dependence of θ on alcohol species

To examine the effects of the alcohol species on the helix content in the polyion complex, we plotted the values of θ obtained at mixing ratios of 6:4 and 4:6 against the alcohol composition (see *Figure 4*). A notable alcohol-specificity is found for the dependence of θ on the alcohol composition. For the MeOH system, the θ values increase with an increase in the alcohol content, while the values for the other alcohol systems are almost constant, or even decrease. The sharp decreases in θ observed for 90 vol% 2PrOH, and 85 and 90 vol% tBuOH are ascribed to precipitation of the complex. Since the values of θ in the single polymer systems increase with increasing alcohol content, the above results may be interpreted as representing a balance between the inherent helix stabilizing effect in the relevant solvent systems and a destabilization of the helix conformation during complex formation.

We have tried to interpret the experimental results in terms of the three solvent properties, namely dielectric constant (D), water activity (a_w) and acceptor number (AN), that we have previously employed for the elucidation of counterion- and solvent-specific helix formation of charged polypeptides[6,8]. Before discussing the application of these parameters to the present study, we shall briefly explain what each parameter measures with respect to the counterion binding: D determines the strength of the electrostatic interaction, a_w is a measure of preferential hydration and hence of desolvation energy, while AN is also a measure of the desolvation energy of the anions. Therefore, it is expected that in solvent systems with higher alcohol contents, which thus have lower values for these parameters, counterion binding with desolvation (i.e. contact ion-pair formation) should be favourable and hence stabilizes the helix conformation in the respective single polymer systems. In this present study, we apply this reasoning to examining the interaction between two polymers having opposite charges.

We consider that the process of polyion complex formation may be divided into two steps:

1. Association of the polymer chains, and/or binding of oppositely charged groups as a result of strong electrostatic attractive interactions.

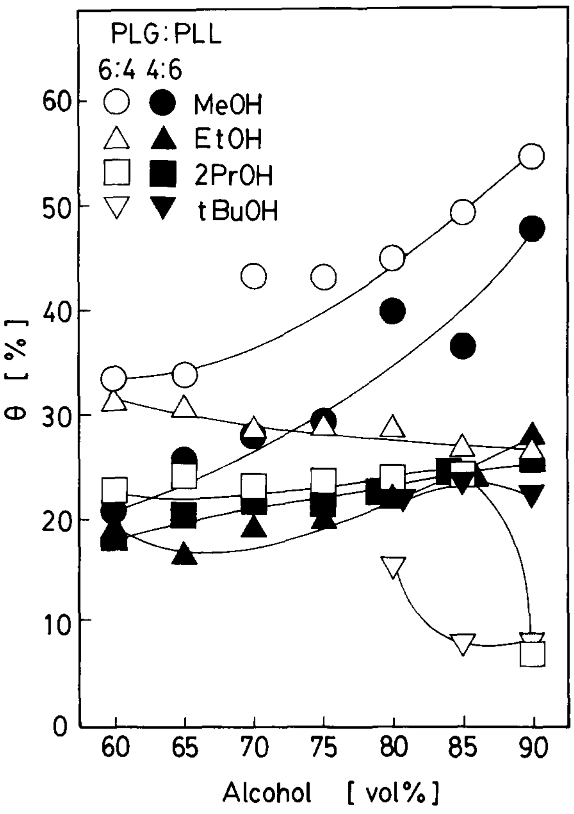


Figure 4 Dependence of the total helix content on alcohol composition for the PLGNa–PLLHBr complex formed at mixing ratios of 4:6 and 6:4; all data were obtained 3 days after sample preparation. For the aqueous tBuOH systems, θ values are plotted only for 80–90 vol% because no helix formation was observed in the polyion complex at lower alcohol compositions

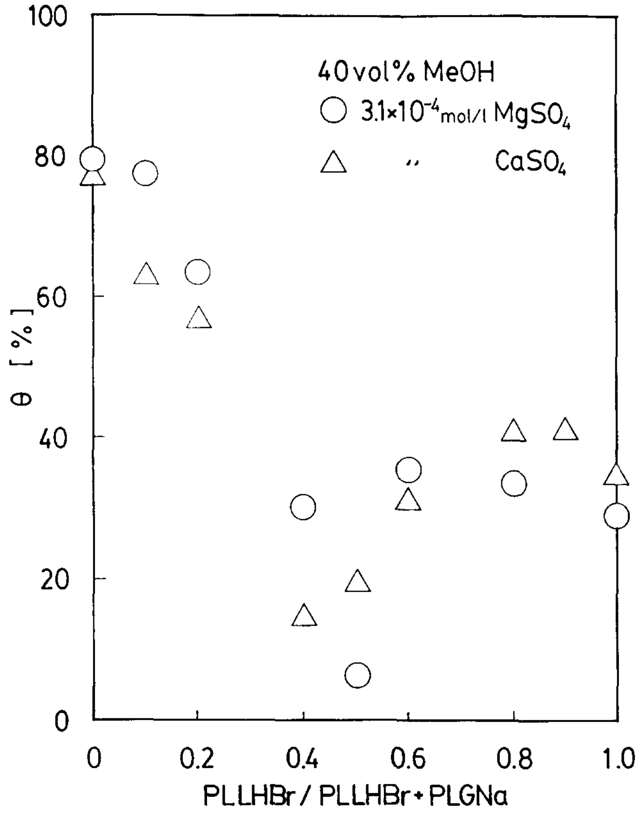


Figure 5 Dependence of the total helix content on the polymer mixing ratio for PLGNa–PLLHBr in the presence of $MgSO_4$ (○) and $CaSO_4$ (△) in 40 vol% MeOH; no helix formation was observed in the absence of the divalent salts

2. Desolvation, after ion-pair formation between the respective charged groups.

D is a relevant measure for step 1, while a_w and AN can be used in the case of step 2. The orders of the parameters are as follows: MeOH > EtOH > 2PrOH > tBuOH for D[16], MeOH < EtOH < tBuOH < 2PrOH for a_w[17] and MeOH > EtOH > 2PrOH > tBuOH for AN[9]. Thus, the increasing trend of θ with an increasing MeOH content may be ascribed to the highest values of D and AN, for which a close and desolvative ion binding between the oppositely charged groups must be relatively unfavourable. In contrast, the decreasing trend in θ and the precipitation observed in the other alcohol systems may be due to lower values of D and AN, since they are able to promote stronger association of the polymer chains with a substantial amount of desolvation. In this present work, a_w seems to have a less important role, when compared with single polymer systems[6]. This may be because the present system deals with the interaction between two charged macromolecules, in which enormous attractive electrostatic forces are exerted, and hence D is more important. Therefore, as expected from the previous section, a medium with a relatively high dielectric constant seems to be a favourable one for forming a stable polyion complex which maintains a helix conformation within the structure.

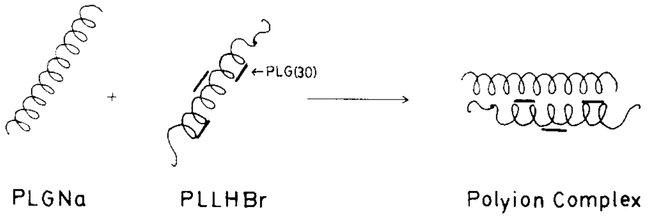
Effects of added salts

Figure 5 shows the effects of simple salts, namely $MgSO_4$ and $CaSO_4$, on the polypeptide conformation in the complex. With NaBr addition, no appreciable change is observed. With the rather low salt concentration (4.0×10^{-4} M), screening of the electrostatic interaction between oppositely charged groups must be insufficient to preserve the respective helix conformations, although with a higher salt concentration the polyion complex will precipitate. On the other hand, for divalent salt systems, the helix conformation in the PLL-rich region is appreciably stabilized. This may be due to partial screening of the electrostatic interaction between the helices by the SO_4^{2-} species bound to PLL. However, a decrease in θ in the PLG-rich region is still significant, suggesting that the Mg^{2+}, Ca^{2+} and SO_4^{2-} counterions bound to the respective polymers are almost completely removed on complex formation. Therefore, addition of simple salts does not seem to be a very effective way of stabilizing the helix conformation in the polyion complex dissolved in the mixed solvents.

We next examined much stronger stabilizers for the PLL helix, namely $NaClO_4$ and short chain PLGNa (PLG30). *Figure 6a* shows the results obtained for $NaClO_4$. It is well known that the ClO_4^- ion is a strong helix inducer for charged PLL, even in aqueous solutions. According to Ebert and Ebert[18], the counterions are firmly bound between two ammonium groups and thus stabilize the PLL helix. This seems to be the case in this present system too, because the substantial helix content in the single PLL system is almost completely maintained in the PLL-rich region. The θ values for the PLG-rich region are also at a rather high level.

Figure 6b shows the results obtained for PLG30. In this case, the total helix content suggests that the respective helix conformations are almost completely retained in the complex. Since we have confirmed in a preliminary experiment that PLG30 does not assume an α-helix conformation in the relevant mixed solvent systems, the apparent conservation of the respective helix conformations may be ascribed to an optimum screening of the electrostatic interactions between the charged helices. *Scheme 2* gives an illustration of the way in which the structureless PLG30 units might intervene between two polypeptide chains to prevent close and direct binding of the oppositely charged groups on the helical polymer chains.

One common feature in these experiments is the fact that no precipitation is observed, even at a 1:1 mixing ratio. This seems to support the proposals of *Scheme 2*, which postulates that the stabilizers bound to PLL are not completely released upon complex formation, thus effecting a diffuse interaction between the oppositely charged polymers, instead of a close, desolvative ion-pair formation between the individual groups. However, dissolution of the complex in the presence of $NaClO_4$ in aqueous alcohols has been observed only for MeOH systems. In the other systems containing EtOH, 2PrOH or tBuOH, the helix conformation in the complex, especially for the PLG-rich region, is much less stabilized, with regard to the formation of precipitates, near the 1:1 mixing ratio. This observation is consistent with results obtained in the absence of the added salt, and may be



Scheme 2 Structure proposed for the polyion complex formed in the presence of a strong helix stabilizer for PLLHBr (e.g. PLG30)

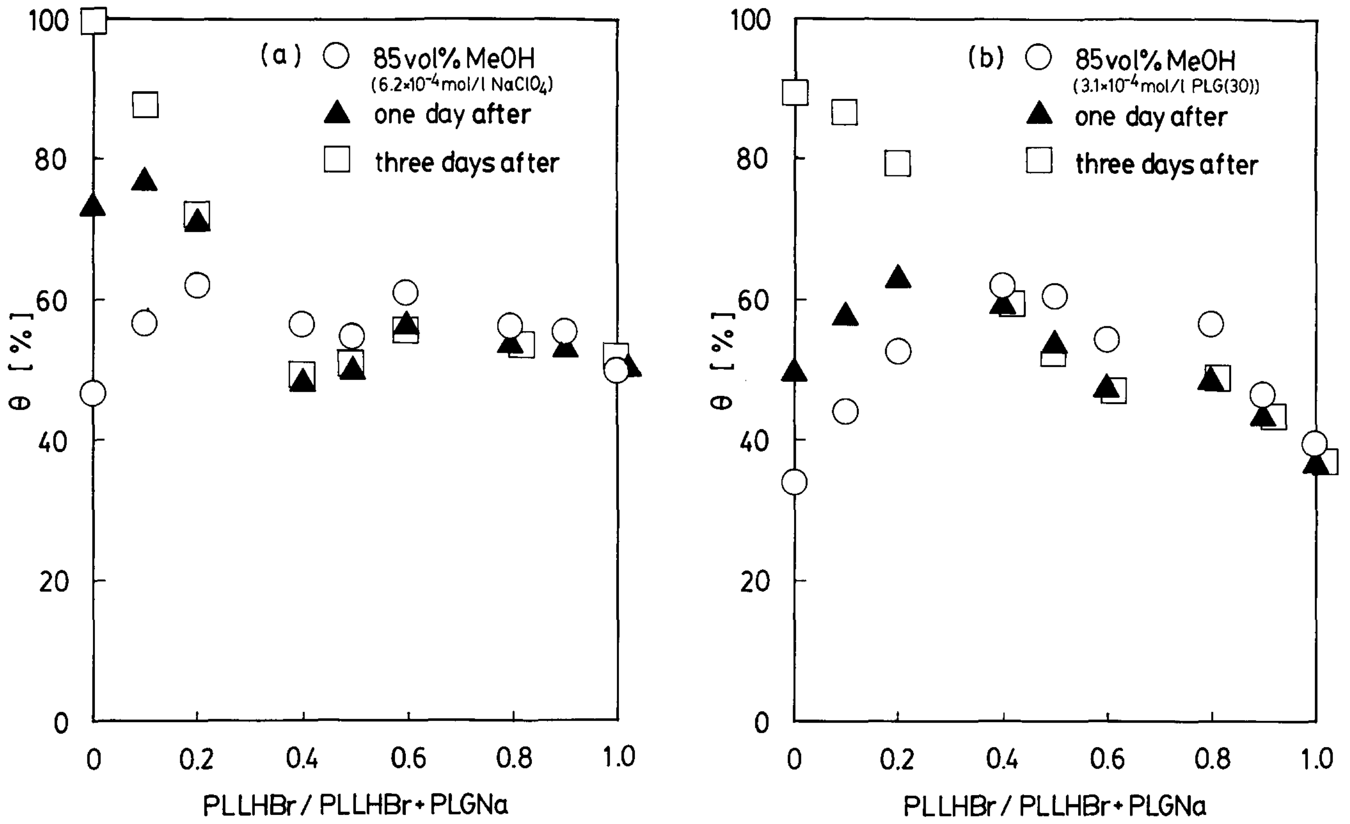


Figure 6 Dependence of the total helix content on the polymer mixing ratio for PLGNa–PLLHBr in the presence of $NaClO_4$ (a) and PLG30 (b) in 85 vol% MeOH

attributed to the lower dielectric constants (higher electrostatic attractive forces between the polymers) for the latter alcohols.

Finally, we examined the secondary structures of the constituent polypeptides in the precipitated complex in the presence of $NaClO_4$; amide I and II bands for complexes that were formed in 85 vol% EtOH at a 1:1 mixing ratio, both in the absence and presence of $NaClO_4$, were compared with those of helical PLGA. (Amide V bands that should appear at $\sim 610\,cm^{-1}$ were obscure.) Amide I and II bands were observed at 1654 and $1542\,cm^{-1}$ in the presence of $NaClO_4$, and these were compared to the bands at 1654 and $1548\,cm^{-1}$ found for PLGA. In the absence of $NaClO_4$, the peak shifts are rather small (1649 and $1542\,cm^{-1}$, respectively), suggesting that both complexes contain substantial amounts of helical polypeptides. However, a strong and sharp band at $697\,cm^{-1}$ was observed for the complex formed in the absence of $NaClO_4$. A corresponding band was also observed for the corresponding complex with $NaClO_4$, although it was weaker and less sharp. Since it was not observed for PLGA, this band may be ascribed to a β or a random coil conformation. Therefore, the presence of $NaClO_4$ seems to be effective to some extent in the stabilization of the α-helix of the charged polypeptides in the precipitated complex.

CONCLUSIONS

In conclusion, the present study demonstrated that it is possible to prepare a polyion complex between the fully charged polypeptides, PLGNa and PLLHBr, each of which maintains the α-helix conformation in the complex. Although the complex formation seems to be confined to a solvent medium that has a high dielectric constant and also contains strong helix stabilizers such as $NaClO_4$ and PLG30, it may be potentially useful for preparing certain functionality materials such as gels and membranes that enable selective sorption and/or transport for ions, chiral substances etc.

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