Simple coacervate of pullulan formed by the addition of poly(ethylene oxide) in an aqueous solution

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Pullulan was phase separated in an aqueous phase as coacervate by adding poly(ethylene oxide) (PEO). The coacervate formation required concentrated (>0.85 unit mol l^{-1}; final) PEO solution, and the amount of the coacervate formed was almost proportional to the pullulan content in the mixture. The coacervates were in a semi-stable state and tended to fuse with each other to reach a complete liquid–liquid two-phase separation. PEO was not detected in the coacervate on analysing the phase-separated coacervate phase using differential scanning calorimetry and infra-red measurements. The coacervate formed by the addition of PEO was concluded to be a simple coacervate composed of only pullulan. The concentration of pullulan in the coacervate was calculated using the hypothesis that all the pullulan remained in the coacervate. The concentration of pullulan in the coacervate phase was ~ 160 g l^{-1}, which is almost the maximum solubility of pullulan. Similar coacervate formation was carried out by adding other synthetic non-ionic polymers such as poly(vinyl alcohol) or poly(*N*-vinyl-2-pyrrolidone) to the pullulan solution. Coacervate formation is concluded to be induced by the dehydration of pullulan after the addition of non-ionic polymers which have higher affinity for water molecules than pullulan.

(Keywords: coacervate; phase separation; pullulan: poly(ethylene oxide); dehydration; Hofmeister's series; starch)

INTRODUCTION

The phase separation of macromolecules in an aqueous solution is usually induced by adding other macromolecules. This is especially true for liquid–liquid phase separation to form coacervates. Coacervates are generally classified into two groups; simple and complex. Typical examples of the simple coacervate are those of nucleic acids or proteins induced by the addition of inorganic salts and/or organic solvents[1]. Most of the simple coacervates are known to be in a semi-stable state. Complex coacervates are formed by mixing aqueous solutions of oppositely charged polyelectrolytes[2]. In such a complex coacervate system, the segmental motion of the component polyelectrolytes is reduced by crosslinking through interpolymer electrostatic interaction and a lot of water molecules are strongly bound to the chains as non-freezing water[3]. Similar complex formation between polysaccharides and synthetic non-ionic polymers through hydrogen bonding has also been analysed[4]. Although a considerable contribution from interpolymer hydrogen bonding was expected, there was only a little interaction. However, competitive hydrogen bonding by the solvent should be taken into account in the complexation[5]. As the component polymers were concentrated in the coacervate, the coacervate was expected to provide a unique chemical environment for applications such as microcapsules.

It is known that mixing of aqueous solutions of poly(ethylene oxide) (PEO) and dextran forms liquid–liquid two-phase separation[6]. This phenomenon has already been applied to food technology and biotechnology. It is, however, expected that the coacervate formation in this system is in the very initial stage of the two-phase separation.

The present paper analysed the conditions and mechanism for phase separation of pullulan as coacervate by the addition of PEO and other non-ionic polymers.

EXPERIMENTAL

Materials

Pullulan (edible, average molecular weight of 200 000) was purchased from Hayashihara Co. Ltd and starch (soluble starch) was purchased from Kokusan Kagaku Co. Ltd. Both polysaccharides were used after complete drying *in vacuo*.

Poly(ethylene oxide)s with average molecular weights of 200, 300, 400, 600, 1000, 2000, 4000, 6000, 20 000 and 50 000 were purchased from Kanto Chem. Co. Ltd, and were used after complete drying *in vacuo*.

Poly(vinyl alcohol) (PVA) with an average degree of polymerization of 2000 was purchased from Wako Pure Chem. Co. Poly(*N*-vinyl-2-pyrrolidone) (PVP) with an average molecular weight of 40 000 was purchased from Tokyo Kasei Kogyo Co. Ltd. Both polymers were dried *in vacuo* before use.

Reagent grade inorganic salts were purchased from Kanto Chem. Co. and used without any purification except for drying *in vacuo*.

The concentration of the polymers was normalized (unit mol l^{-1}). As the repeating unit of PEO is considered to be the smallest unit for interaction through hydrogen bonding[3–5], the unit molecular weight of PEO is defined

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as 44.1 (for $(-CH_2-CH_2-O-)$). Similarly, the unit molecular weight of the polysaccharides (pullulan and starch) was defined as 54.0 which was the molecular weight of the repeating unit divided by the number of hydroxyl groups.

Methods

Coacervate formation. Aqueous solutions of concentrated pullulan (2.0 unit mol l^{-1}) and concentrated PEO (2.0 unit mol l^{-1}) were mixed to form the coacervates. The concentration dependence was analysed by changing the initial concentrations of the component polymers.

Amount of coacervate. The coacervate suspension was centrifuged (3000 rev min^{-1}, 30 min) to completely phase separate. The coacervate phase was measured and the amount of coacervate was expressed as a volume fraction.

Isolation and dehydration of coacervate. The coacervate suspension was centrifuged as mentioned above and the supernatant solution was discarded by decantation. The coacervate phase was dried *in vacuo* at 60°C for 5 days.

Infra-red (i.r.) measurements. I.r. spectra were measured on KBr pellets using an Hitachi i.r. spectrometer (model 270-30).

Differential scanning calorimetry (d.s.c.). Measurements were carried out using DSC model CN8059 L1 (Rigaku Denki Co.). Sample (~5–10 mg) was put into the sample pan, which was mechanically sealed for measurement.

Optical microscopy. The coacervates were observed using an optical microscope (Olympus, Polarized Microscope model POM).

RESULTS AND DISCUSSION

Pullulan coacervate by PEO

Typical coacervates were formed by mixing an aqueous solution of pullulan and PEO. *Figure 1a* shows an optical micrograph of a freshly formed coacervate. A size distribution was obtained for the coacervate; the average diameter was ~80 μm. As the coacervates were in a semi-stable state, they fused with each other to form larger coacervates (*Figures 1b* and *c*) and reached complete liquid–liquid phase separation after overnight incubation. The amount of coacervates was measured as a volume fraction of the liquid–liquid phase separation with the aid of centrifugation as mentioned in the Experimental section. *Figure 2* shows the effect of pullulan content in the mixed solution on the amount of coacervate formed. Aqueous solutions (2.0 unit mol l^{-1}) of both pullulan and PEO were mixed at various mixing volume ratios. The amount of coacervate was proportional to the pullulan content in the mixed solution. A small portion of PEO was sufficient to generate the coacervate, but no phase separation was found in the system when the PEO fraction was <2 unit mol%. It is known that the maximum amount of coacervate should be found with equimolar (unit molar) mixing of two-component polymers for the case of a typical polyelectrolyte complex formation only when there was a certain interaction force[2]. For example, the maximum amount of coacervate was obtained with equimolar mixing of polycation and polyanion[2]. In the present case, intermacromolecular interaction such as hydrogen bonding was not expected to induce phase separation.

The complex coacervate or precipitate, formed through some secondary binding forces, should be stable and not dissociate into components even after dilution. The dilution of a complex solution is a convenient method to check the existence of a positive interaction force between component polymers in the complex. The effect of concentration of component polymers on the amount

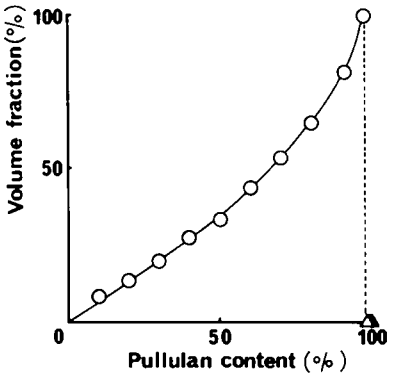

Figure 2 Effect of initial composition of the mixed solution of pullulan and PEO on the volume fraction (yield) of coacervates: $[pullulan]_o$ = 2.0 unit mol l^{-1}; $[PEO_{50000}]_o$ = 2.0 unit mol l^{-1}

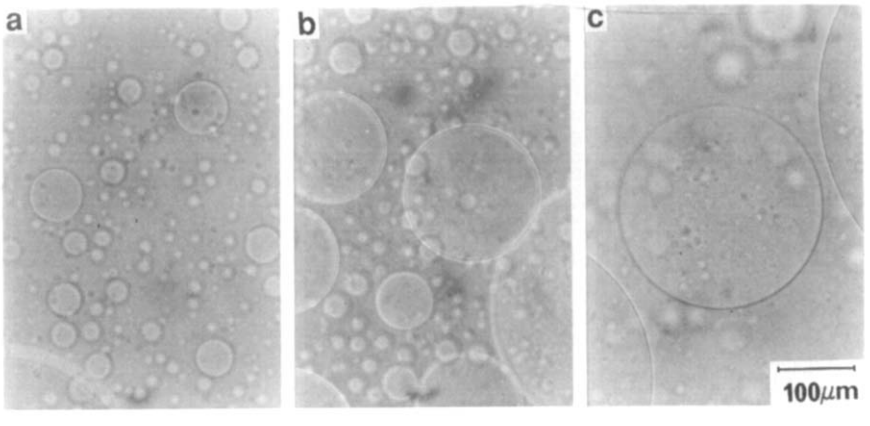

Figure 1 Optical micrographs of coacervates formed by mixing aqueous solutions of pullulan and PEO: (a) immediately after mixing; (b) 15 min after mixing; (c) 20 min after mixing

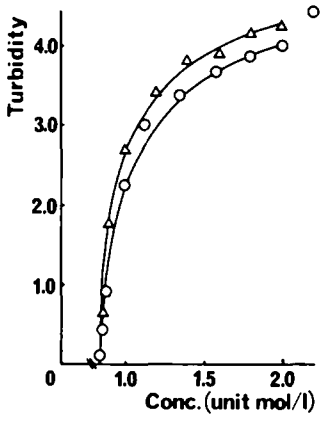

Figure 3 Relationship between the initial polymer concentration and the turbidity of the coacervate suspension: (○) pullulan (2.2 unit mol l^{-1}) and PEO (2.2 unit mol l^{-1}) were mixed to form coacervates, and were then diluted by water: (△) component polymer solutions were diluted and then mixed to form coacervates

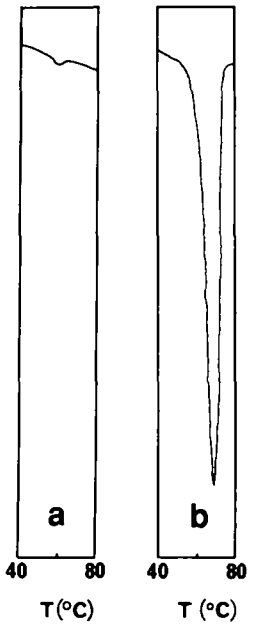

Figure 4 D.s.c. thermograms for (a) dried coacervates and (b) pure PEO_{50000} (reference). Scan rate: 10°C min^{-1}

of coacervate is shown in *Figure 3*. In the present case, the coacervate was formed by equimolar mixing of pullulan and PEO. The coacervate suspension formed was then diluted with pure water. Alternatively, the component polymer solution was diluted first and then mixed to form coacervate. If the coacervate formed was dependent on the intermacromolecular interaction force, the coacervate should be stable even after dilution. There was, however, little difference between the two methods suggesting no positive interaction force between pullulan and PEO. The minimum concentration (final) of pullulan and PEO for coacervate formation was ~0.85 unit mol l^{-1} as seen in *Figure 3*. This value is considerably higher (concentrated) than that for polyelectrolyte complex formation[2]. This also suggested that the coacervate should be a simple coacervate.

After sufficient incubation or centrifugation, coacervates were completely phase separated. A supernatant was pipetted out and the remaining coacervate phase was then dried *in vacuo* at 60°C for 5 days. The d.s.c. measurement on the dried coacervate sample was carried out but no phase separation was detected (*Figure 4*). The d.s.c. thermogram for pure PEO is also shown in *Figure 4*; it showed a sharp endothermic peak at 66°C which was attributed to the melting of the PEO. These d.s.c. results also suggest that the coacervate obtained by the present method does not contain PEO as a component polymer. The very small endothermic peak, seen in the thermogram for the coacervate sample (*Figure 4*), was considered to be the contaminated PEO remaining on separation of the supernatant. The i.r. measurement also did not show peaks for PEO in the coacervate (*Figure 5*). These results strongly support the coacervate being a simple coacervate composed of only pullulan. The PEO was considered to reduce the solubility of the pullulan by taking free water molecules away as solvated water molecules for PEO. No direct interaction between pullulan and PEO was detected in all the data obtained.

If all the pullulan chains existed as coacervate, their concentration in the coacervate could be calculated from volume fraction data (*Figure 2*). The calculated apparent concentration of pullulan in coacervate was plotted against the pullulan content in the mixed solution as seen in *Figure 6*. The concentration of pullulan in coacervate was expected to be the same regardless of the mixing ratio, if the reduction in solubility was the only factor involved in coacervate formation. The apparent concentration was almost the same (~160 g l^{-1}) when the initial fraction of pullulan in the mixture was <50 unit mol%. However, it decreased gradually with increasing pullulan content as seen in *Figure 6*. This concentration change can be understood by two explanations. One explanation involves the pullulan remaining in the supernatant solution which was ignored in the calculations. Quantitative analysis of the remaining pullulan could not be carried out due to technical limitations. The other explanation is the effectiveness of PEO on the phase

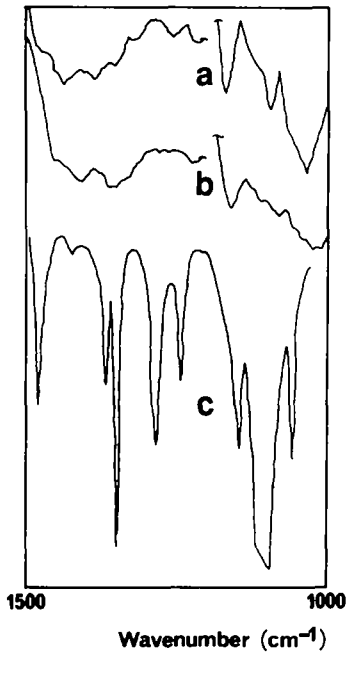

Figure 5 I.r. spectra for dried coacervates or component polymers on a KBr pellet: (a) pullulan; (b) dried coacervates; (c) PEO

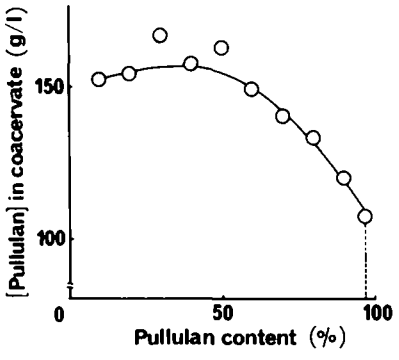

Figure 6 Relationship between pullulan content in a mixed solution and the calculated apparent concentration of pullulan in the coacervates (see text)

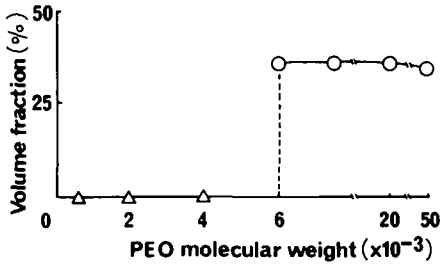

Figure 7 Effect of PEO molecular weight on the volume fraction of coacervates formed by equimolar mixing: $[\text{pullulan}]_0 = 2.0$ unit mol l^{-1}; $[\text{PEO}]_0 = 2.0$ unit mol l^{-1}

separation of pullulan. In the mixed solution containing a small amount of PEO, pullulan was not completely phase separated but remained to some extent in the supernatant solution.

The effect of the average molecular weight of PEO on coacervate formation was studied. *Figure 7* shows the relationship between the amount of coacervate (volume fraction) and the average molecular weight of the applied PEO. The initial concentration of the component polymer was fixed to be 2.0 unit mol l^{-1} and coacervate was formed by equimolar mixing as mentioned above. It was clearly observed that PEOs with molecular weight >6000 were effective for coacervate formation. A molecular weight dependence on coacervate yield was frequently found in complex coacervate formation such as polyelectrolyte complex formation. This is known as the intrinsic polymer chain length for the effective complex formation[7]. This molecular weight dependence does not agree with the results for simple coacervate formation as mentioned above. The ability of PEOs with molecular weights of <6000 to form coacervates was then studied. Such PEOs were able to form coacervates but needed more concentrated conditions (*Figure 8*). *Figure 8* shows the required minimum concentration of PEO for pullulan coacervate formation when the pullulan concentration was fixed at 2.0 unit mol l^{-1}. PEOs having smaller chain length require more concentrated conditions for phase separation of pullulan as the coacervate.

Pullulan coacervate by inorganic salts

It is well-known that the addition of inorganic salts to an aqueous solution of biopolymer also induces simple coacervate formation[8]. A series of inorganic salts was studied for their ability to form coacervates. Pullulan was phase separated as a coacervate by sodium sulphate (>1.2 mol l^{-1}). No phase separation was found when other salts were used such as sodium citrate, sodium chloride, potassium sulphate, potassium chloride, magnesium sulphate or calcium chloride, even in concentrated salt solutions. The salting-out ability of inorganic salts for proteins such as albumin has been empirically summarized in the Hofmeister's series. This can be understood in terms of differences in dehydration ability. Sodium sulphate was one of the most powerful salts for the salting-out of proteins[8]. Pullulan was phase separated by dehydration of the chains, but this was only effectively induced by sodium sulphate.

Starch coacervate by PEO

The effectiveness of PEO on coacervate formation of other polysaccharides was also studied. Starch was dissolved in hot water (>80°C), and all the experiments were carried out in the water bath. Starch was found to form coacervate by the addition of PEO but under more concentrated conditions, and concentrated starch solution (4.0 unit mol l^{-1}) was used as a standard component solution. Aqueous solutions of starch and PEO were heated and then mixed together to form coacervate. Freshly formed coacervates were in a semi-stable state and they tended to fuse with each other. The initial average diameter of the coacervates was $\sim 30\ \mu$m, and the size increased with time because of the fusion of the initial coacervates. The average diameter of starch coacervates increased up to $\sim 180\ \mu$m during 20 min incubation at 80°C as shown in *Figure 9*.

The effect of starch content in the mixed solution on

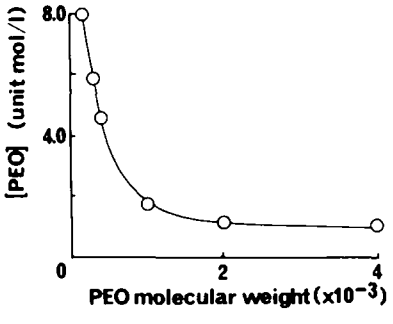

Figure 8 Effect of PEO molecular weight on the required minimum concentration of PEO to induce phase separation of pullulan as coacervates

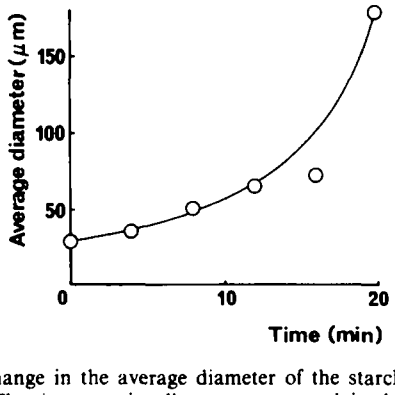

Figure 9 Change in the average diameter of the starch coacervates with time. The increase in diameter was explained in terms of fusion of the coacervates at 85°C: $[\text{starch}]_0 = 4.0$ unit mol l^{-1}; $[\text{PEO}_{50000}]_0 = 4.0$ unit mol l^{-1}

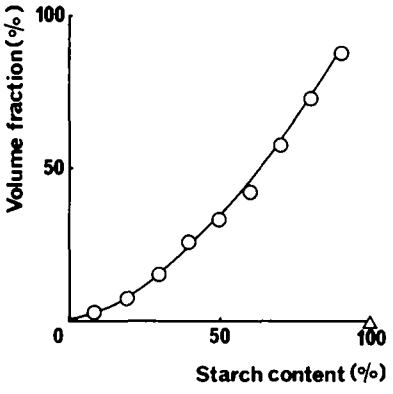

Figure 10 Effect of the initial mixing ratio of starch to PEO on the volume fraction (yield) of coacervates at 85°C: $[starch]_0$ = 4.0 unit mol l^{-1}; $[PEO_{50000}]_0$ = 4.0 unit mol l^{-1}

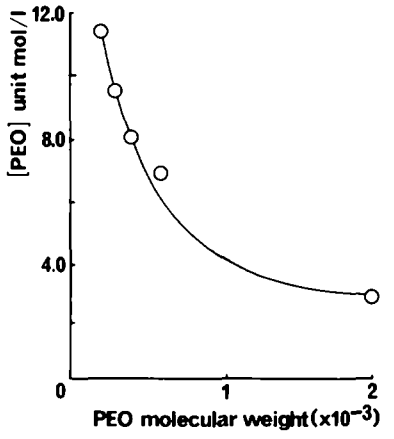

Figure 11 Effect of PEO molecular weight on the required minimum concentration of PEO to induce phase separation of starch as coacervates at 85°C

the amount of coacervate formed is summarized in *Figure 10*. The amount of coacervate was almost proportional to the starch content in the mixed solution. This tendency is quite similar to that for pullulan (cf. *Figure 2*). This clearly shows that the coacervate, formed by mixing aqueous solutions of starch and PEO, is also a simple coacervate. There was a relationship between the average molecular weight of PEO and the minimum concentration for the formation of starch coacervate. The required minimum concentration of a series of PEOs for coacervate formation is plotted in *Figure 11*. PEOs with average molecular weights of >2000 show almost the same ability for coacervate formation as in the present case. On the other hand, PEOs having molecular weights of <1000 require higher concentrations for starch to phase separate as the coacervate.

Neither d.s.c. nor i.r. measurements showed the existence of PEO in the coacervate as in the previous experiments. These data all suggest that starch was phase separated as the coacervate by the addition of PEO in a sufficient concentration.

Coacervate was also formed by the addition of sodium sulphate (>1.0 mol l^{-1}) to an aqueous solution of starch. No other salts were found to be effective for coacervate formation from starch.

Polysaccharide coacervate by non-ionic synthetic polymers

The dehydration process in an aqueous solution of polysaccharides is critical for phase separation to form the coacervate. From the viewpoint of the dehydration ability of PEO, similar coacervate formation was expected by the addition of other non-ionic synthetic polymers which are highly soluble in an aqueous solution. In the present experiments, PVA and PVP were used as typical examples. As expected, coacervates were formed by the addition of such polymers to an aqueous solution of pullulan or starch. In particular, PVP has excellent ability to phase separate pullulan. This may be explained in terms of the hydrophobicity of the polymer; PVP is more hydrophobic than PEO[9]. The solubility of such non-ionic polymers should also be effective for coacervate formation because of the strength of hydration which directly affected the dehydration of the polysaccharides.

Recently, Sjoberg *et al.*[10] reported the efficient separation of pullulan-modified liposomes on PEO treatment. The results obtained here suggest that phase separation of polysaccharides by the addition of non-ionic polymers should be more widely applied.

CONCLUSIONS

Coacervates were formed by the addition of PEO to an aqueous solution of polysaccharides, such as pullulan or starch. Coacervate formation was explained in terms of the phase separation induced by the dehydration of polysaccharides. This ability was observed for not only PEO but also PVA and PVP. Such synthetic polymers were considered to employ free water molecules as solvated water molecules for the synthetic polymers. Other non-ionic polymers may be effective in forming coacervates. It should be noted that the polymers, which dissolved in an aqueous medium easily and accordingly showed high solubility, had a strong potential to induce phase separation of polysaccharides to the coacervate.

This kind of coacervate formation should be useful for designing a new type of microcapsules for several industrial fields and a new separation method for target materials with non-toxic macromolecules.

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