Dependence of ethanol effects on protein charges

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Highlights

1. Ethanol affects structure of BSA pH dependently.
2. Ethanol causes aggregation of BSA and RNase A in a different manner depending on pH.
3. BSA and RNase A are stable up to 50-60 % ethanol.
4. Ethanol can cause protein precipitation without affecting the structure.

Abstract

Ethanol is used as a conventional disinfectant solution. It is highly effective against enveloped viruses due to its effects on virus membranes. It also confers inactivation of non-enveloped viruses, which can be ascribed to conformational changes or changes in association state of the viral proteins induced by ethanol. We have examined here the effects of pH and hence the charged state of proteins on the ethanol-induced conformational changes and self-association of model proteins, i.e., bovine serum albumin (BSA) and ribonuclease A (RNase A). Both proteins showed qualitatively different aggregation behavior and structure changes by ethanol at pH 4.0 and 7.0, at which BSA has opposite charges and RNase A has different degree of net positive charges.

Keywords: Protein, ethanol induced aggregation, charged state

1. Introduction

Ethanol is routinely used as a disinfectant [1–3]. As it is highly effective against enveloped viruses, the effects of ethanol are due to disruption of membrane structures, leading to virus inactivation [4,5]. Such effects are explained in terms of hydrophobic properties of ethanol [6]. However, ethanol can also inactivate non-enveloped viruses pH dependently [7], although at higher concentrations than normally required for enveloped viruses [5], indicating that disruption of membrane structure may not be a sole factor responsible for virus inactivation by ethanol. One possible mechanism of such inactivation by ethanol is its effects on proteins. Ethanol affects proteins in aqueous solution. It can denature proteins [8–11], often accompanied by transition in secondary structure [12,13] and reduce their solubilities [14,15]. Thus, ethanol effects on virus infectivity could be due to pH-dependent denaturation or altered protein-protein interactions of virus proteins by ethanol. Virion structure of viruses is composed of major coat proteins and spike proteins. Viruses have been shown to have widely different isoelectric points [16], reflecting in part different charged states of these viral proteins [17]. Such differences in charged state may affect the sensitivity to ethanol, as ethanol can alter the free energy of charged groups [6]. The structure analysis of viral proteins at different pH values and ethanol concentration would reveal potential mechanism of virus inactivation by ethanol, though accompanied by great difficulty. Alternatively, we here have investigated the effects of ethanol on the structure and aggregation of two model proteins, bovine serum albumin (BSA) and ribonuclease A (RNase A), at pH 4.0 and 7.0. These pH values were chosen to alter the charged state of the proteins. BSA is oppositely charged at these pH values [18], while RNase A is positively charged at different

degrees [19]. Such studies should show potential impact of pH and ethanol that might simulate the structure changes or changes in inter-molecular interactions of viral proteins.

2. Materials and Methods

2.1 Materials

Anhydrous citric acid, trisodium citrate, sodium dihydrogen phosphate dihydrate, disodium hydrogenphosphate and ethanol (99.5% pure) were obtained from Wako Pure Chemical Ind., Ltd. (Osaka, Japan). BSA and RNase A were obtained from Sigma-Aldrich Co. (St Louis, MO, USA). All chemicals used were of reagent grade and used as received.

2.2 Sample preparation

A stock protein solution containing 3.0 mg/ml protein in 5 mM citrate, 5 mM phosphate, pH 4.0 or 7.0 was prepared. A 130 μl aliquot of the stock protein solution was mixed with 1820 μl of a mixture comprising a buffer containing 5 mM citrate, 5 mM phosphate buffer and a 75 % ethanol solution in the same buffer at respective pH. The ratio of the buffer and the 75 % ethanol solution was varied to cover 0-70 % ethanol concentration. The final protein concentration was 0.2 mg/ml. The protein solutions were incubated at room temperature in a turnover motion for 1 hour and centrifuged at 18,800 × g for 15 min. After centrifugation, the supernatant was used for the following measurements.

2.3 Absorbance measurement

The absorbance measurements were performed at room temperature using a V-630 UV-vis spectrophotometer (Japan Spectroscopic Co., Ltd., Tokyo, Japan) and a 10 mm path-length quartz cell. The absorbance spectra of the samples in the supernatant were corrected by subtracting the spectra of the respective solvents.

2.4 Circular dichroism measurement

Far-UV circular dichroism (CD) measurements were performed at 25 °C using a J-720 spectropolarimeter (Japan Spectroscopic Co., Ltd., Tokyo, Japan) and a 1 mm path-length quartz cell. The CD spectra of the samples were corrected by subtracting the spectra of the respective solvents and converted to the mean residue ellipticity using the protein concentration of the supernatant.

2.5 Fluorescence measurement

Intrinsic fluorescence spectra of protein were determined at 25 °C using a FP-6500 spectrofluorometer (Japan Spectroscopic Co., Ltd., Tokyo, Japan). The emission spectra (3 nm slit-width) were recorded with excitation at 295 nm (3 nm slit-width) and corrected by subtracting the spectra of the respective solvents.

2.6 Native polyacrylamide gel electrophoresis (native-PAGE)

Native-PAGE was carried out according to the previous study [20] using an 8 % Tris-Gly gel and a Tris-Gly tank buffer (25 mM Tris-HCl, 192 mM glycine, pH 8.3). The samples were mixed with an equal volume of sample buffer containing 50 % sucrose, 0.01 % (w/v) bromophenol blue and 125 mM Tris-HCl, pH 6.8. After gel electrophoresis, the gels were stained with silver nitrate. It should be emphasized that the native-PAGE analysis monitors irreversible changes that occur at pH 4.0 or 7.0 and in the presence of ethanol.

3. Results and Discussion

3.1 BSA

We have examined here the effects of ethanol on the structure of BSA and RNase A at pH 7.0 and 4.0. First, the structure differences at these two pH values were examined for BSA. Fig.1A shows the UV absorbance spectra of BSA at pH 7.0 and 4.0, showing small differences between these two pH values. Such small differences could, however, be simply due to experimental errors, e.g., in protein concentration. Fig.1B shows the CD spectra of BSA at pH 7.0 and 4.0. While the spectral shapes were identical, the intensity appeared to be slightly weaker at pH 4.0. Nevertheless, it should be safe to state that protein structure is not significantly different between these pH values.

Having established a similar structure at pH 7.0 and 4.0, the effects of ethanol on BSA structure were examined at pH 7.0. UV absorbance spectra at pH 7.0 are shown in Fig.2A. There is little change in absorbance spectra between 0 and 40 % ethanol, demonstrating no apparent changes in light scattering and hence associated state up to 40 % ethanol. A large absorbance increase in the entire spectral region was observed at 50 %, an indication of light scattering. Since the sample was spun before absorbance measurement, such light scattering indicates formation of soluble oligomers. The BSA sample used here contained several oligomer bands in addition to main monomeric band, as analyzed by native-PAGE shown in Fig.2E. While no changes in native-PAGE pattern were observed at 50 %, the intensity of each band significantly decreased. It thus appears that 50 % ethanol increased the size of the soluble oligomers so that they no longer enter

the 8 % gel, leading to reduction of each of monomer and oligomer bands. Light scattering does not appear to be present at 60 % ethanol, as seen by no apparent absorbance at 310-320 nm, implying that formation of soluble oligomers was replaced by precipitation: the absorbance was greatly reduced at 60 % after centrifugation. This result was confirmed by native-PAGE showing only a small amount of monomer left: namely, all BSA forms were converted to precipitates. There seems to be no protein left at 70 % ethanol based on absorbance and native-PAGE results.

Fig.2B shows the CD spectra at pH 7.0 as a function of ethanol concentration. Consistent with little changes in absorbance spectra up to 40 %, the CD spectra were identical within experimental errors between 0 and 40 % ethanol. The spectrum at 50 % was also unchanged, different from the observed large increase in absorbance due to light scattering. This suggests that the soluble oligomers formed at 50 % have intact secondary structure. A significant structure change appeared to occur at 60 % ethanol, at which BSA showed considerable precipitation: note that accurate CD and absorbance measurements could not be done due to low protein concentration of the supernatant. Thus, while 60 % ethanol resulted in reduction of protein solubility upon structure alteration, 50 % ethanol enhanced inter-molecular interaction between the intact structures. There is no protein left at 70 % for CD measurement. Tertiary structure of BSA at pH 7.0 was examined by fluorescence spectra.

Fig.2C shows the fluorescence spectra as a function of ethanol concentration. No significant differences were observed up to 50 %, except for the effects of ethanol itself on the fluorescence properties of the tryptophans present in BSA. Such ethanol effects are clearly seen in Fig.2D, in which both fluorescence intensity and wavelength maximum are plotted against ethanol

concentration. The wavelength maximum gradually decreased with ethanol concentration due to reduced polarity of aqueous ethanol solution [21]. On the contrary, the fluorescence intensity sharply dropped at 50 % and further at 60 %. Although far UV CD spectrum at 50 % showed little changes, the fluorescence intensity was significantly reduced. Such reduction may be related to the observed light scattering at 50 %, perhaps due to fluorescent tryptophan involved in enhanced oligomer formation. The observed loss of fluorescence intensity at 60 % is due to precipitation and hence loss of the protein. Since there is still protein left at 60 % (see absorbance spectrum), however, nearly complete loss of fluorescence intensity suggests conformational changes. Thus, BSA at pH 7.0 undergoes no secondary structure changes up to 60 % ethanol, but there may be changes in tertiary structure above 60 % ethanol.

A contrasting picture emerges at pH 4.0, at which BSA is positively charged. Fig.3A shows the UV absorbance spectra of BSA at pH 4.0, in which no spectral changes were observed up to 60 % (compare with the precipitation at 60 % at pH 7.0, Fig.2A). Consistent with no changes in absorbance spectra up to 60 %, native-PAGE pattern and band intensities appeared to be unchanged, as shown in Fig.4E. A large light scattering was observed at 70 %, at which extensive precipitation occurred at pH 7.0. It thus appears that BSA at pH 4.0 was more resistant to ethanol than at pH 7.0 against precipitation, but formed more soluble oligomers or aggregates: compare with the absorbance spectrum at 50 % ethanol, pH 7.0. Native-PAGE (Fig.4E) showed decreased intensity of each band without appearance of new oligomers bands at 70 %, suggesting that the oligomers formed are too large to enter the gel. Consistent with the UV absorbance data, CD spectra were unchanged up to 60 %. CD spectrum at 70 % was significantly different, similar to the result at 70 % ethanol, pH 7.0. Such different secondary structure at 70 % may be related

to the observed extensive light scattering at pH 4.0, in contrast to the structure changes at pH 7.0 that resulted in precipitation. Similar to the far UV CD spectra, the fluorescence properties were little affected by ethanol at pH 4.0. Only significant change was a sharp blue shift at 10-20 % ethanol. It is not clear why 10 % ethanol caused a shift in wavelength maximum at pH 4.0 to the extent that required 60 % ethanol at pH 7.0. In any case, it is evident that the response of BSA to the addition of ethanol is pH-dependent: namely, the charged state of BSA may be related to the way ethanol affects its structure and self-association.

3.2. RNase A

A similar experiment was performed with RNase A, which, unlike BSA, is positively charged at both pH 4.0 and 7.0, with more net positive charges at pH 4.0. Fig.4A shows the absorbance spectra at pH 4.0 and 7.0, with no significant differences. Consistent with the absorbance spectra, the CD spectra were identical at pH 4.0 and 7.0. Thus, there appear to be no significant structure differences at two pH values for RNase A as well. Even though RNase A structure appears to be independent of the charged state and is positively charged at both pH values, the response to ethanol was pH dependent. Fig.5 shows the results at pH 7.0, while Fig.6 shows those at pH 4.0. At pH 7.0, the absorbance spectra were unchanged up to 60 % and significant precipitation was observed at 70 % ethanol as seen by the decreased absorbance of the supernatant. Interestingly, CD spectra were identical even at 70 % (Fig.5B), suggesting that 70 % ethanol simply enhanced protein-protein interactions in the native state, resulting in precipitation. At pH 4.0, the results of UV absorbance were essentially identical to those at pH 7.0 at 0-60 % ethanol, as well as the CD spectra at 0-70 %. No changes in secondary structure up to 70 % and no absorbance changes up

to 60 % have occurred. However, a large increase in absorbance was observed at 70 % due to light scattering. At pH 7.0, 70 % ethanol resulted in precipitation of RNase A, while it resulted in formation of soluble aggregates at pH 4.0. The results with RNase A clearly show salting-out effects of ethanol [22]. Namely, it can enhance protein-protein interaction without altering the structure. The observed differences in the type of protein-protein interaction between pH 4.0 and 7.0 may simply be the degree of self-association. RNase A is more positively charged and thereby has stronger charge repulsion at pH 4.0, which may prevent the protein from forming lager aggregates to precipitate.

A question is then how ethanol enhances protein-protein interaction in the first place, while enhancing electrostatic repulsions. It has been shown that there is unfavorable interaction between charged solutes and low dielectric organic solvents, such as ethanol [22–25]. When such unfavorable interaction reaches a threshold value at certain organic solvent concentration, the charged solutes like protein phase-separate as a form of either soluble aggregates or precipitation [22,25]. There appears to be fine balance between inter-molecular charge repulsion and unfavorable solute-solvent interaction, which determines the extent of protein-protein interaction, i.e., soluble aggregates vs. precipitation.

How do these results relate to inactivation of non-enveloped viruses? Non-enveloped viruses are composed of nucleic acids and proteins. This study showed that ethanol at high concentrations, above 50-60 %, alters the structure or the association state of BSA and RNase A pH dependently and suggested a possibility that ethanol may inactivate non-enveloped viruses through its effect on proteins. Roberts and Lloid showed that 70 %, but not 20 %, ethanol inactivated poliovirus at

pH 4.5 [5]. Requirement of such high ethanol concentration is consistent with the observation in this paper. Namely, high ethanol concentration may irreversibly change the structure or the intermolecular interaction of viral proteins, thereby leading to loss of infectivity. It would be of great interest to study the pH dependent effects of ethanol on additional model proteins and their impacts on viral proteins.

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Figure Captions

Figure 1 Absorbance (A) and far-UV CD spectra (B) of BSA at pH 7(solid line) and pH 4(dotted line)

Figure 2 The effects of ethanol on the spectroscopic properties of BSA at pH 7. Absorbance (A), far-UV CD (B) and intrinsic emission fluorescence spectra (C) as a function of ethanol concentration. Each fluorescence spectrum was shifted upward by 30 A.U. for visual comparison. (D) Fluorescence peak maximum and peak intensity as a function of ethanol concentration. In (D), the measurements were performed three times, and the error bars depict the standard deviation of the mean. (E) Native-PAGE.

Figure 3 The effects of ethanol on the spectroscopic properties of BSA at pH 4. Absorbance (A), far-UV CD (B) and intrinsic emission fluorescence spectra (C) as a function of ethanol concentration. Each fluorescence spectrum was shifted upward by 30 A.U. for visual comparison. (D) Fluorescence peak maximum and peak intensity as a function of ethanol concentration. In (D), the measurements were performed three times, and the error bars depict the standard deviation of the mean. (E) Native-PAGE.

Figure 4 Absorbance (A) and far-UV CD (B) spectra of RNase A at pH 7 (solid line) and pH 4 (dotted line).

Figure 5 The effects of ethanol on the spectroscopic properties of RNase A at pH 7. Absorbance (A) and far-UV CD spectra (B) of RNase A as a function of ethanol concentration.

Figure 6 The effects of ethanol on the spectroscopic properties of RNase A at pH 4. Absorbance (A) and far-UV CD spectra (B) of RNase A as a function of ethanol concentration.

Dependence of ethanol effects on protein charges

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Figure 1

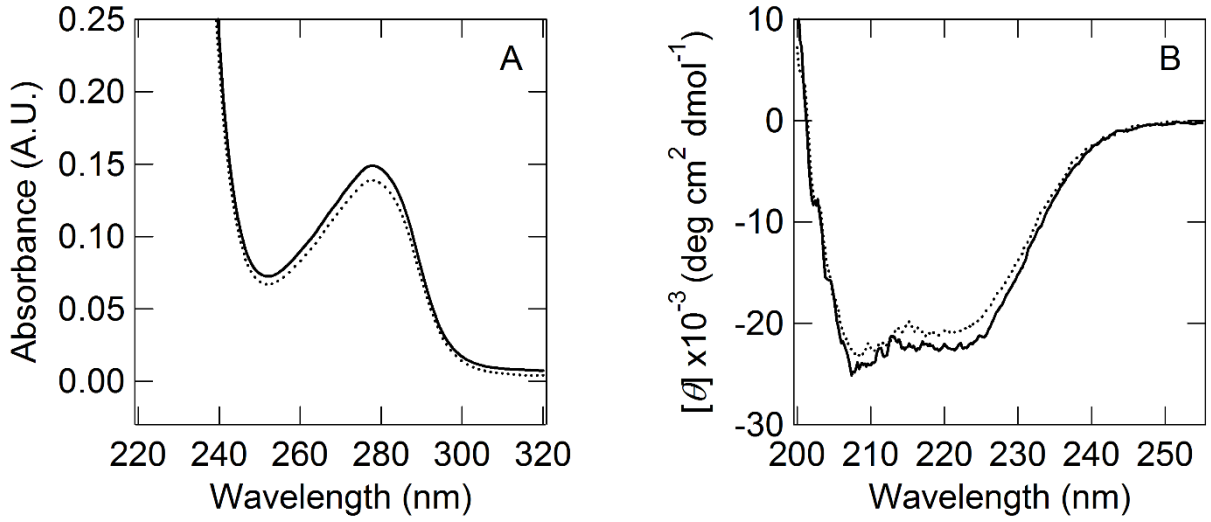

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Figure 2

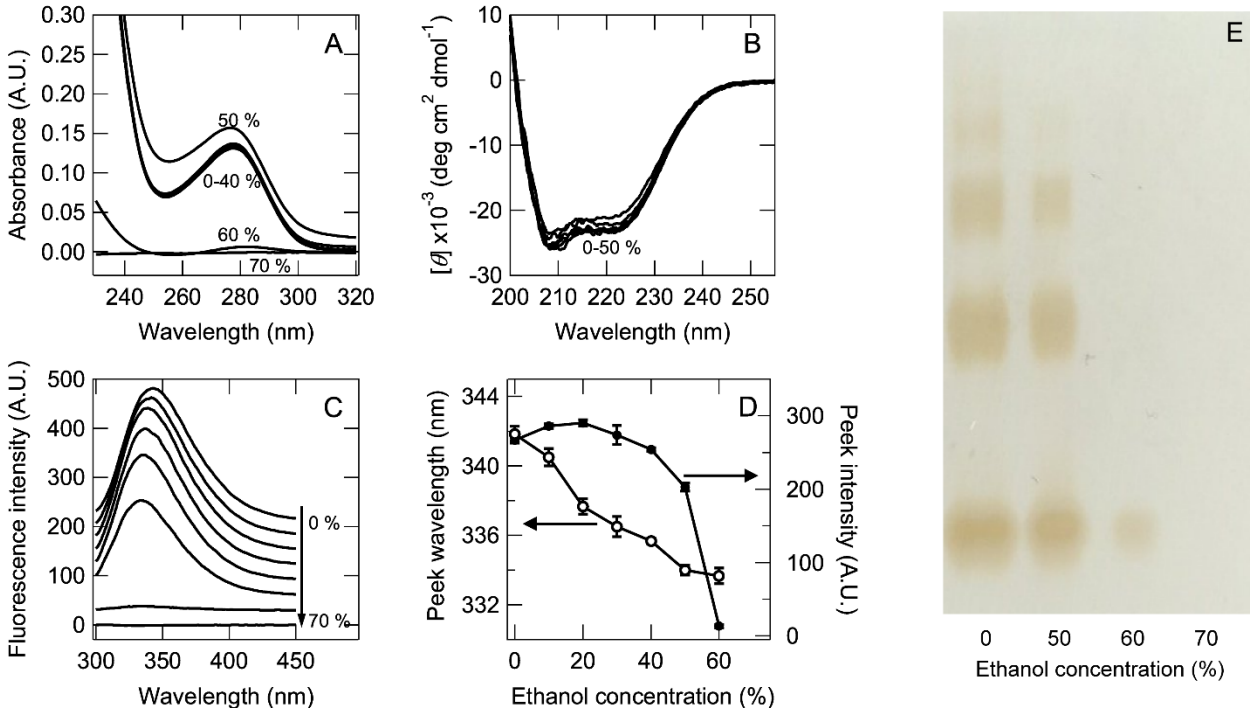

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Figure 3

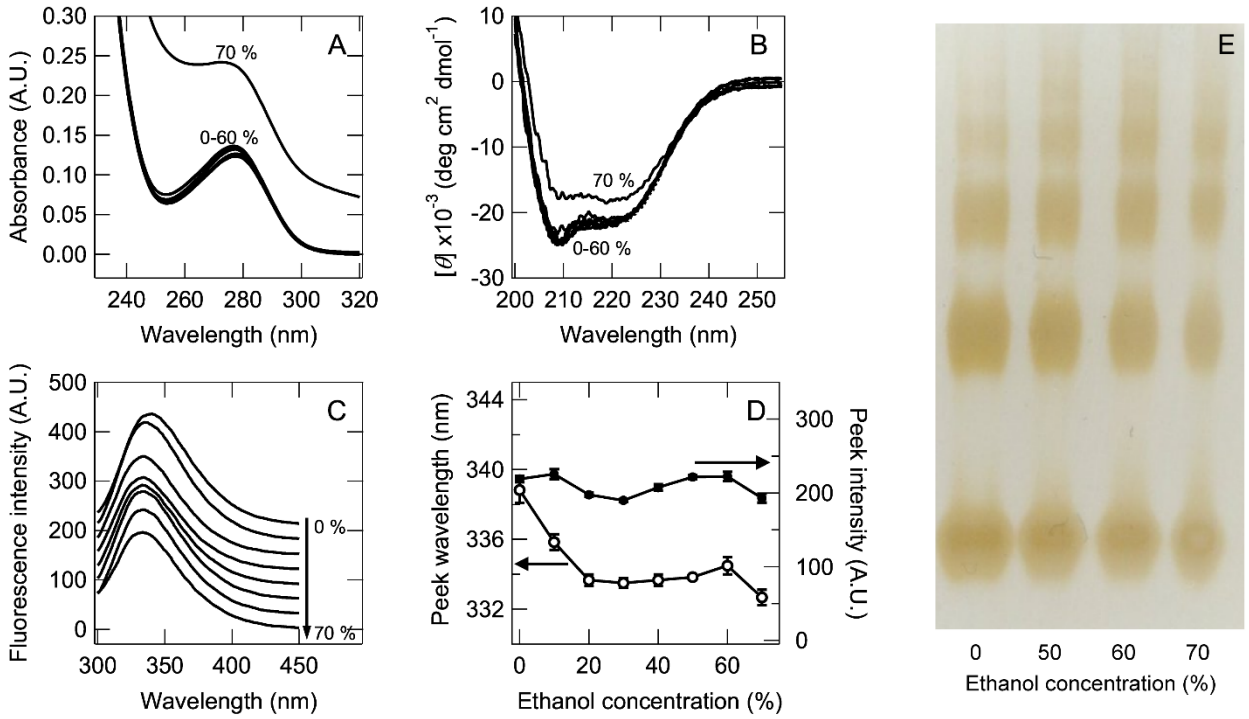

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Figure 4

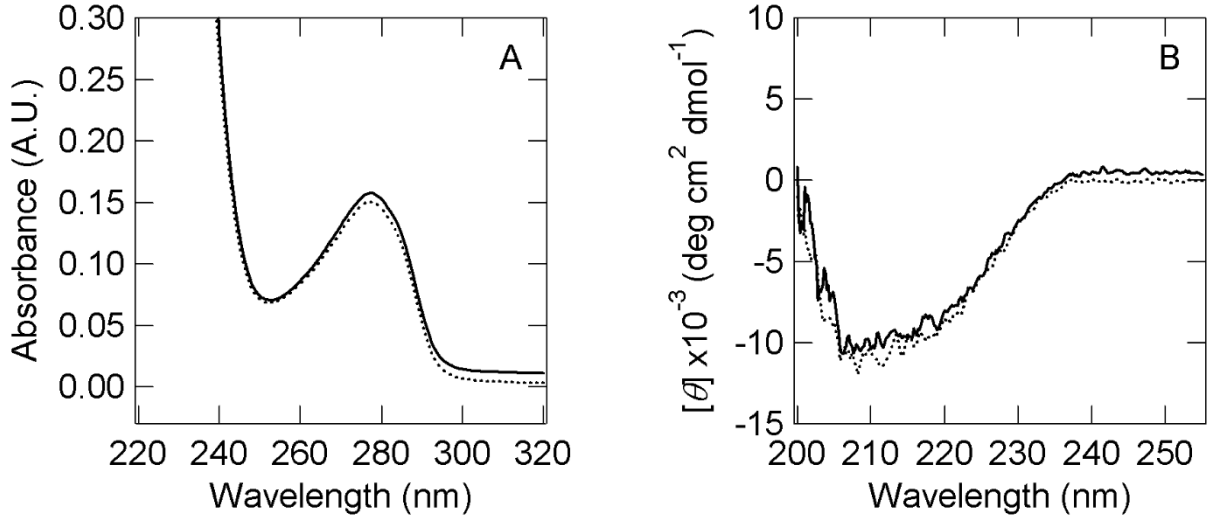

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Figure 5

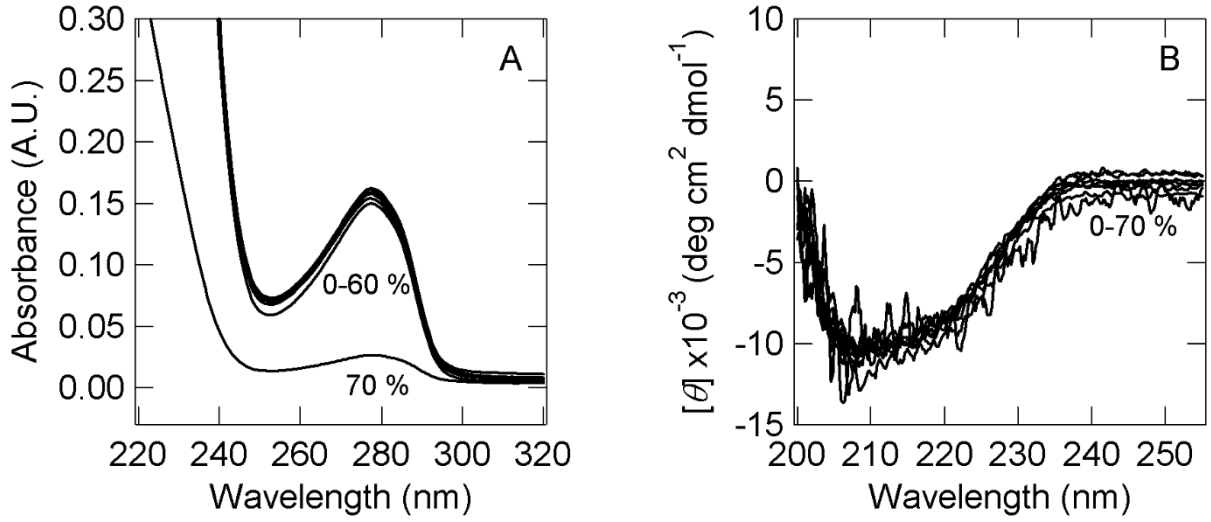

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Figure 6