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Extraction of Human IgG in Thermo-Responsive Aqueous Two-Phase Systems: Assessment of Structural Stability by Circular Dichroism

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The structural stability of human IgG was studied throughout the extraction process with thermo-responsive UCON/dextran aqueous two-phase systems. Systems composed by 8% UCON and 5% dextran were assessed in both acetate pH 5 and phosphate pH 7. The extraction of IgG to the top phase was found to be favored at pH 5 reaching 85% yield with UCON 50HB-3520. During the extraction process conducted at 25°C no alteration in the secondary structure, evaluated by circular dichroism, was observed. The back-extraction step was induced by the thermo-precipitation of the UCON polymers at 59°C. At both pH values, mild changes in the secondary structure of IgG were observed for the 50HB-3520 and 50HB-5100 polymers while 50HB-2000 showed an increase in β-sheet content. The overall yield of the process was as high as 82% determined for the UCON 50HB-3520 system at pH 5.

Keywords aqueous two-phase system; circular dichroism; immunoglobulin G; temperature sensitive polymers

Abbreviations ATPS, aqueous two-phase systems; CD, circular dichroism; PEG, polyethylene glycol

INTRODUCTION

The use of Aqueous Two-Phase Systems (ATPSs) as a liquid–liquid extraction technique for the purification of biopharmaceuticals, including therapeutic monoclonal antibodies, has received considerable attention in the last years (1–2). These systems are spontaneously formed upon mixing two aqueous solutions of two polymers or a polymer and a salt, above a certain critical concentration. Since the phases formed are mainly constituted by water (80–90% w/w) and several of the polymers used are reported to possess a stabilizing effect on the proteins, these systems are viewed as being biocompatible (2). Among the polymers used, smart-polymers, i.e., water soluble polymers that spontaneously precipitate upon a stimulus such as temperature (cloud point), the pH, and ionic strength have been of great interest as they allow an easy back-extraction process. Nevertheless, the use of such polymers might result in protein activity loss if more extreme conditions are needed to promote phase separation. A common smart-polymer used is the thermo-responsive ethylene oxide/propylene oxide copolymer (EOPO) such as the commercially available UCON^{TM}, Pluronic®, and Breox® product lines. Studies conducted by Ferreira et al. already showed the feasibility of UCON ATPSs for the purification of human IgG (3).

Conformational studies of biological macromolecules are suitably performed by CD even though detailed structures are not achievable (4,5). Nevertheless, this technique possesses several advantages over the more detailed methods of NMR and X-ray crystallography which are able to give structural information at atomic resolution. In comparison, CD is a quick and inexpensive method that does not require significant postacquisition data treatment nor sample amount (4–6). Furthermore, since CD experiments are mostly conducted in solution no crystals have to be created, a difficult endeavor that in some cases alters the molecular structure (5). Given the wide variety of sample compositions and experimental parameters usable, physiological conditions can be correctly mimicked and the structural stability assessed in a multitude of conditions. Nevertheless, CD has some limitations regarding the use of organic solvents, salts, and buffer concentrations owing to their excessive absorption at lower wavelength values (6).

The scope of this work is to shed some light on the structural stability of IgG by circular dichroism at different operational conditions (e.g., temperature, pH) and during the purification process with thermo-responsive ATPSs.

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MATERIALS AND METHODS

Materials

Human IgG for therapeutic use (product name: Gammanorm) was obtained from Octapharma (Lachen, Switzerland) as a 165 mg/ml solution with 95% purity.

Polyethylene glycol (PEG) with a molecular weight of 3350 Da was purchased from Sigma. Dextran with a molecular weight of 500 kDa was acquired from Fluka. UCON^{TM} (EOPO) 50-HB-2000, 50-HB-3520, and 50-HB-5100 were a kind gift of Dow Chemical. UltraPure^{TM} guanidine hydrochloride was purchased from Invitrogen. All remaining chemicals were of analytical grade.

Aqueous Two-Phase Extraction

UCON-based ATPS systems were prepared by weighing appropriate amounts of a 25% (w/w) dextran stock solution, 50% (w/w) UCON stock solution, 1 M phosphate pH 7 or 1 M acetate pH 5, water and 1 g/l IgG solution, to a final weight of 5 g, in order to achieve the desired final composition. For PEG-based systems a 50% (w/w) PEG stock solution was used in conjunction with 0.5 M phosphate pH 7 or pH 3. Two different system compositions were investigated, namely, 8% UCON and 5% dextran and 7% PEG and 5% dextran. All systems were prepared with 50% (w/w) of IgG stock solution.

IgG extraction studies were performed in 15 ml graduated centrifuge tubes. The phase components were thoroughly mixed in a vortex shaker and incubated at 25°C overnight. Samples from both upper and lower phases were taken for the determination of IgG concentration. The top phase was further used to assess the stability IgG by circular dichroism. The partition coefficient, K_P, was defined as the ratio of the IgG concentration in the upper phase to that in the lower phase. The extraction yield of IgG, Y_{IgG}, was defined by the ratio between the mass of IgG in the upper phase and the mass of IgG added to the system.

For UCON polymers the top phase was further submitted to a back-extraction process by thermoprecipitation of the polymer. Samples were heated for 5 min at 59°C in a water bath followed by centrifugation for 1 min at 4400 rpm. The water rich top phase was recovered and used in CD studies.

IgG concentration was determined by the Bradford method using a protein assay kit from Pierce. Bovine gamma globulin (BGG) from Pierce was used as a standard. To avoid interferences, all samples were analyzed against blanks containing the same phase composition but without proteins.

Circular Dichroism

Circular dichroism studies were conducted with an Applied Photophysics spectropolarimeter (Leatherhead, United Kingdom), model PiStar-180 with a Peltier temperature control unit (Melcor MTCA). Suprasil Quartz cells from Hellma with an optical path of 1 mm were used.

Far-UV (200–250 nm) measurements consisted of an accumulation of 10 scans with a time constant of 1 second and a scan rate of 1 nm/s. The monochromator bandwidth was set to 2 nm and the Peltier set point to 25°C. Each spectrum was corrected with the corresponding control. Except for the aqueous two-phase system, samples were prepared in 10 mM phosphate with a protein concentration of 0.165 g/l.

The mean residue ellipticity was calculated considering a mean residue weight of 113.16 Da.

Chemical Stability

Samples containing concentrations of guanidine hydrochloride (Gdn.HCl) up to 6.3 M were prepared from a 7 M Gdn.HCl, 10 mM phosphate pH 7 stock solution. After Gdn.HCl addition, samples were incubated for 2 hours to ensure that equilibrium was reached. Gdn.HCl induced unfolding was followed at 220 nm with CD measurements being restricted to 212 nm due to the high absorption of the salt.

Data was analyzed by non-linear least square fit assuming a two state behavior according to Equation (1) (7):

$$Y=\frac{Y_f+m_f[Gdn.Hcl]+(Y_u+m_u[Gdn.Hcl])\exp\left(-m(D_{1/2}+[Gdn.Hcl])/RT\right)}{1+\exp\left(-m(D_{1/2}+[Gdn.Hcl])/RT\right)} \quad (1)$$

where Y is the measured ellipticity, m is the dependence of free energy of unfolding on the denaturant concentration, and $D_{1/2}$ is the denaturant concentration at the midpoint of unfolding. R is the ideal gas constant, and T the temperature in Kelvin. The pre-transition slope and intercept are given by m_f and Y_f, respectively, while m_u and Y_u are the corresponding post-transition analogs. The data was fitted to this model using SigmaPlot software version 11.0 from Systat Software Inc.

Thermal Stability

Thermal unfolding was used as a standard protocol to evaluate IgG stability under different solvent conditions. Different pH values, from 2 to 7, were tested as well as the effect of a solubilizing agent (guanidine hydrochloride).

Temperature scans were performed with a heating rate of 1°C/min in steps of 0.3°C. Following manufacturer recommendations a tolerance of 0.1°C and a time constant of 12 s were used (8). Ellipticity was recorded at 216 nm (2 nm resolution) while the sample temperature was directly measured with a thermopar. After denaturation, the sample was immediately cooled down to the initial temperature and a CD spectrum was taken to assess the reversibility of the reaction.

Thermal unfolding data was analyzed by a non-linear least square fit according to Equation (2), assuming a two-state behavior and considering the difference in heat capacity between the native and unfolded protein (ΔCp) neglectable (7):

$$Y=\frac{Y_f+m_fT+(Y_u+m_uT)\exp(-\Delta H_m/R(1/T_m-1/T))}{1+\exp(-\Delta H_m/R(1/T_m-1/T))} \quad (2)$$

where Y is the measured ellipticity, ΔH_m is the enthalpy at the unfolding transition, T_m is the melting temperature, T the temperature in Kelvin, and R is the ideal gas constant. The pre-transition slope and intercept are given by m_f and Y_f, respectively, while m_u and Y_u are the corresponding post-transition analogs. The data was fitted to this model using SigmaPlot software version 11.0 from Systat Software Inc.

RESULTS AND DISCUSSION

Chemical Stability

The chemically induced unfolding of IgG in 10 mM phosphate buffer at pH 7 was followed at 220 nm since a greater variation in ellipcitiy was seen at this wavelength value (Figure 1A). At low Gdn.HCl concentrations (below 2 M), a typical β-sheet type spectrum is observed with a clear minimum at 216 nm. By contrast, high concentrations of the chaotropic agent (above 3 M) resulted in the loss of the minimum at 216 nm with the CD spectra exhibiting a steady increase of the CD signal below 225 nm. This shift in the CD spectra is consistent with the formation of a random coil structure.

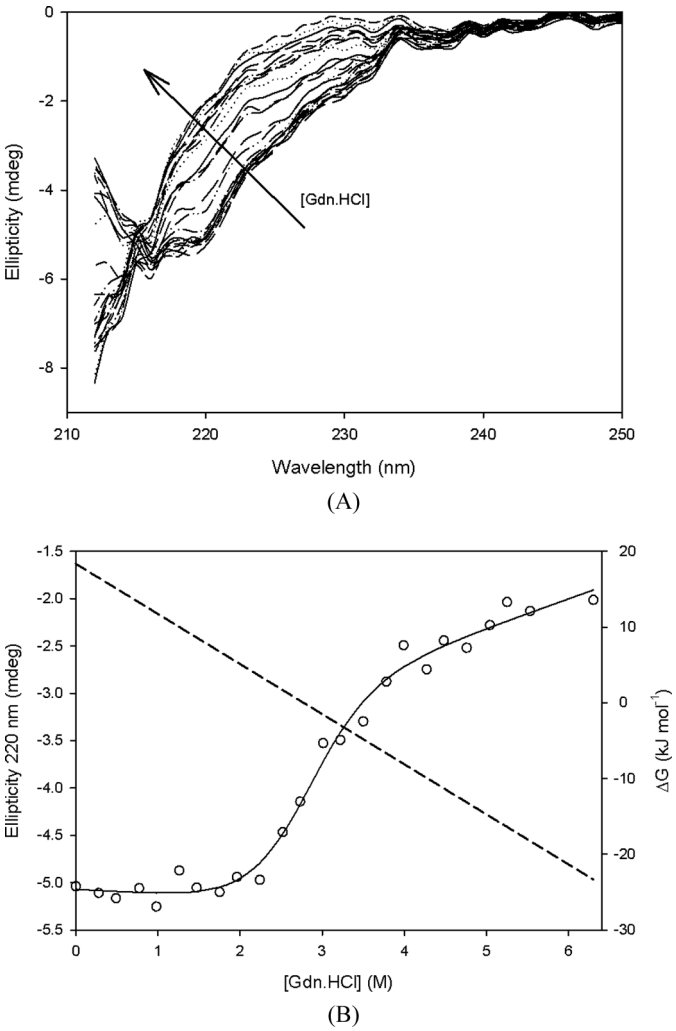


FIG. 1. Guanidine hydrochloride (Gdn.HCl) induced unfolding of IgG in 10 mM phosphate buffer at pH 7. Effect of Gdn.HCl concentration in: A) Far-UV CD spectra. B) Free energy of unfolding (ΔG; dashed line) and ellipticity (θ; solid line) at 220 nm. Lines result from the best non linear least-squares model. Experimental ellipticity values (○).

Non-linear least square fit adequately described experimental data (Fig. 1B) with a root mean square error of 0.14 mdeg ($R^2 = 0.99$). The denaturant concentration at the midpoint of unfolding ($D_{1/2}$, when $\Delta G = 0$) was determined to be 2.7 ± 0.1 M (as seen in Fig. 1B) which is in agreement with the value determined (2.56 M), under analogous conditions, by Park et al. for a humanized monoclonal antibody against pre-S2 surface antigen of hepatitis B virus (9).

Thermal Stability

The thermal stability of human IgG was evaluated through unfolding curves or by the time-dependent evolution of its structure at different incubation temperatures. In both cases, these studies were conducted in aqueous solution and were primarily focused on determining the direct effect of several experimental conditions relevant to UCON based aqueous two-phase extraction systems. Among such parameters, pH, buffer type, incubation time, and temperature were considered.

pH Induced Changes in the Secondary Structure

Human IgG in aqueous solution was incubated at room temperature at different pH values in order to determine the presence of altered structures prior to the thermal unfolding studies. At 25°C, except for the sample at pH 2, a typical β-sheet CD spectrum was observed with a minimum at 216 nm and a maximum near 200 nm. The ellipticity values at the minimum were found to be more negative as the pH decreased, which is in agreement with an augment in β-sheet content (Fig. 2A). In regard to the sample submitted to pH 2, the shift to negative ellipticity values below 208 nm denotes an increase in the random coil content. The exposure of immunoglobulins to low pH values ($pH < 3$) has been reported to give rise to a stable folded structure termed A-state which is characterized by a high degree of secondary structure, increased hydrophobicity, and a tendency towards slow aggregation (10–12). Incubation periods up to 5 hours at pH 3 and at room temperature revealed, within the experimental error, no alteration in the protein secondary structure (Fig. 2B). Similar findings were reported by Ejima et al. for a humanized monoclonal antibody exposed to pH 2.7 at 4°C for up to 24 h (10). Conversely, for higher incubations periods tested, 27 hours and 5 days, a loss of ellipticity below 208 nm was observed (Fig. 2B). Due to the large time gap in the incubation periods tested the maximum

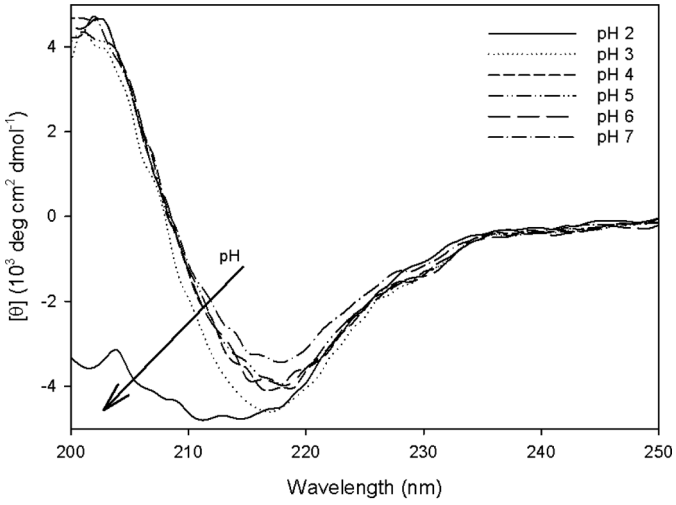


FIG. 2. A) IgG far UV CD spectra at different pH values. B) Influence of the incubation period towards the secondary structure of IgG at pH 3 and at room temperature.

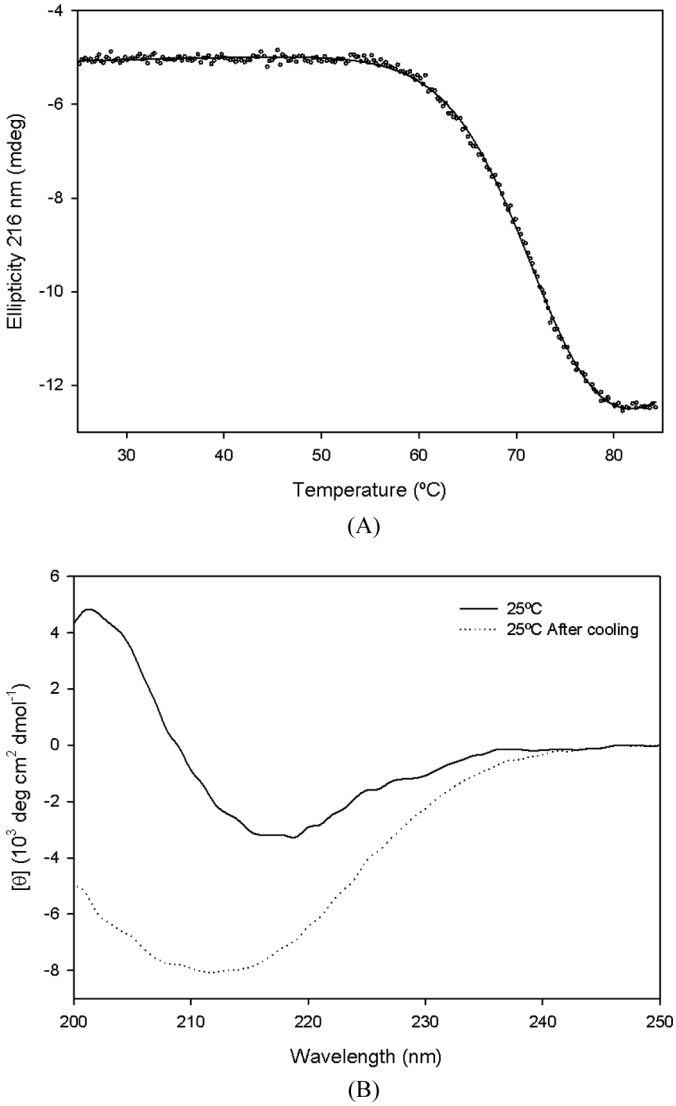


FIG. 3. Human IgG in 10 mM phosphate at pH 5. A) Thermal unfolding curve at 216 nm. Solid line results from the best non linear least-squares model. B) Effect of thermal unfolding in the secondary structure of IgG.

temporal limit ensuring structural stability at room temperature is 6 hours.

Thermal Unfolding

The effect of the pH in the heat-induced denaturation of human IgG was investigated. Figure 3A depicts a typical unfolding curve which is characterized by one transition temperature despite the polyclonal nature of the sample and the multi-domain structure of IgG. Therefore, part of the two-state model validity is fulfilled. In order to determine the reversibility of the process, samples were immediately cooled down to the initial temperature and the far UV spectrum was compared to the native protein. For all the samples, irreversible unfolding of IgG was observed. Figure 3B illustrates the typical far UV spectrum of the unfolded protein. The ellipticity minimum broadens and shifts to lower wavelength values while the maximum disappears. The more negative ellipticity values observed between 210–230 nm and 200–210 nm are consistent with increase in the α-helix and random coil, respectively. Studies conducted with a monoclonal mouse anti-rat antibody of isotype 2b revealed an identical CD spectrum at the denaturated state with the concomitant increase in the α-helix and random coil content (12).

The melting temperature (T_m), which corresponds to the temperature at which half of the IgG molecules are denaturated, was calculated from the unfolding curve by nonlinear regression with the previously mentioned model (2). The model adequately described experimental data

TABLE 1
Melting temperature, enthalpy at the unfolding transition and corresponding fitting parameters for samples at different pH values in phosphate solution and supplemented with 0.75 M Gdn.HCl. Predicted values and corresponding standard deviations. * – Ellipticity recorded at 215 nm. † – 7% PEG 3350, 5% Dextran 500,000 ATPS

pH	T_m (°C)	ΔH_m (kJ mol^{-1})	RMSE	R^2
2	41.1 ± 0.7*	180 ± 10*	0.0449	0.9972
3	59.9 ± 0.5	175 ± 8	0.1047	0.997
3.5	65.0 ± 0.5	168 ± 4	0.0944	0.9982
(Gdn.HCl)	50.1 ± 0.1	159 ± 3	0.0729	0.9995
4	69.7 ± 0.3	189 ± 4	0.095	0.999
4.3†	71.2 ± 0.2	192 ± 2	0.1355	0.9993
5	73.4 ± 0.3	224 ± 3	0.08	0.9991
(Gdn.HCl)	61.4 ± 0.3	177 ± 4	0.0955	0.9989
7	–	–	–	–
(Gdn.HCl)	64.0 ± 0.2	185 ± 4	0.0747	0.999

with R^2 values higher than 0.99. The fitted parameters are summarized in Table 1.

Overall, the thermal stability of IgG was found to be severely affected by the pH. Figure 4A represents the melting temperature as function of the pH value. The general tendency shows higher T_m values to be achieved as pH increased which agrees with the studies conducted by Welfle et al. for a murine anti-p24 monoclonal antibody (13). Nevertheless, at pH 6 or higher protein precipitation was observed above 62°C, with lower temperatures being attained at higher pH values (Fig. 4B). Protein precipitation was easily experimentally verified by the sudden increase in absorbance and concomitant decrease of the magnitude of ellipticity. The higher tendency towards aggregation as pH increases results from the proximity to the IgG isoelectric point (p$I = 9.05$) (14). In these cases, the decreased net charge favors aggregation as less repulsion is felt between molecules leading to an easier interaction of the hydrophobic regions as the molecules unfold. Szenczi and coworkers also report a higher stability of human polyclonal IgG at pH 7 but with increased tendency towards aggregation due to the proximity to the isoelectric point of the major portion of the molecules (15).

To overcome aggregation and resulting protein precipitation, guanidine hydrochloride was added as a solubilizing agent in order to infer a general tendency of T_m at higher pH values. The lowest concentration found to suppress protein precipitation while maintaining the native IgG structure was determined to be 0.75 M. Samples containing Gdn.HCl showed a similar T_m tendency as those in phosphate buffer (Fig. 4A). Nonetheless, the presence of

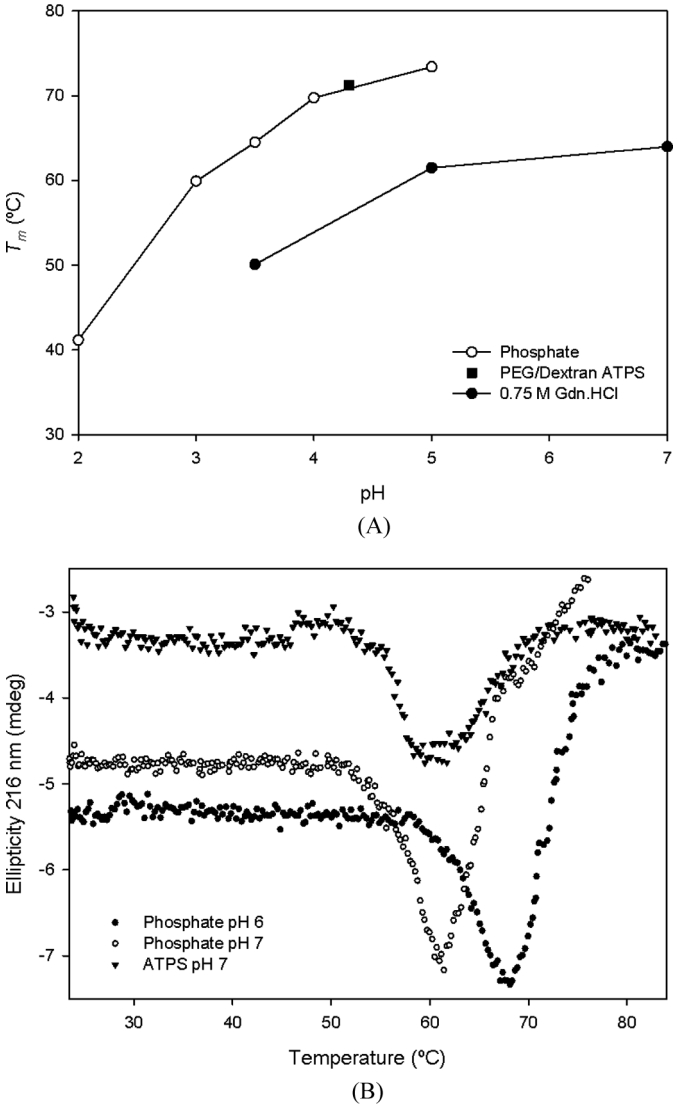


FIG. 4. A) IgG melting temperature at different pH values in phosphate solution and supplemented with 0.75 Gdn.HCl. Effect of phase forming polymers from a 7% PEG 3350, 5% Dextran 500,000 ATPS. B) Thermal unfolding curve at pH 6, 7 and for an ATPS top phase of 7% PEG 3350, 5% Dextran 500,000 at pH 7. Ellipticity values measured at 216 nm. Sudden increase in ellipticity at the minimum results from protein aggregation.

Gdn.HCl led to a decrease of roughly 14°C in the melting temperature value for both samples at pH 3.5 and 5.

The effect of ATPS phase forming polymers on the thermal stability of IgG was addressed by analyzing the corresponding top phases. Two systems composed of 7% PEG 3350 and 5% dextran 500,000 at pH 4.3 and 7 were used. In general, the presence of the polymers did not influence the thermal stability of IgG. At pH 7, IgG aggregated at the same temperature as the sample in aqueous solution (Fig. 4B) while at pH 4.3 the melting temperature was between the values recorded at pH 4 and 5 in aqueous solution (Fig. 4A).

Operational Stability

On a different set of experiments, IgG in 10 mM phosphate at pH 5 was incubated at different temperatures: 50, 55, and 60°C. The secondary structure was assessed in a time-dependent fashion by compiling up to 200 consecutive far UV CD spectra (approximately up to 3 h). After the data acquisition process was started, the sample, initially at room temperature, was heated by a step increase in the Peltier set-point. In every case, thermal equilibrium, assessed by directly measuring the temperature with a thermopar, was reached within the first 10 scans (data not shown). All the samples showed a decrease in ellipticity throughout the incubation period. Furthermore, this decrease was found to be more significant at lower wavelength values and as temperature increased (exemplified by the slope of the linear regression in Fig. 5 and Table 2).

In some cases, a 5°C increase in temperature led to the doubling of the decrease rate. In regard to the structure per se, a decrease in the β-sheet content with the corresponding increase in random coil is supported by the lower ellipticity values below 208 nm. However, even at the longest incubation periods tested, a β-sheet type CD spectrum is observed (data not shown). Considering the thermal unfolding curve of IgG in phosphate at pH 5 (Figure 3A), where the protein shows to be in its native form at temperatures below 53°C, the importance of understanding the time-dependent unfolding proves to be crucial as shown by the decrease in ellipticity of the sample incubated at 50°C.

Thermo-Responsive Aqueous Two-Phase Extraction

ATPSs composed by 8% UCON and 5% dextran 500,000 were analyzed after both the extraction and back-extraction steps.

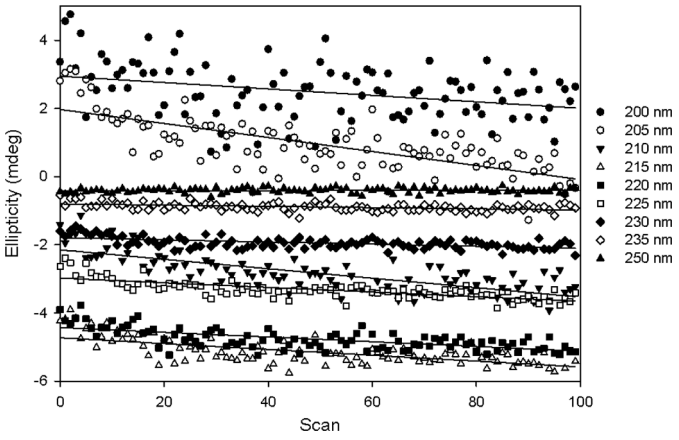


FIG. 5. Time dependent ellipticity variation of IgG in 10 mM phosphate at pH 5 incubated at 60°C. Thermal equilibrium was reached within the 10 first scans.

TABLE 2
Ellipticity decrease rate of IgG in 10 mM phosphate at pH 5 incubated at 50, 55 and 60°C. Values correspond to the slope of the linear regression of the experimental data. The sample incubated at 60°C was only submitted to 100 scans (approximately 1.5 h incubation period)

Wavelength (nm)	50°C ($\times 10^{-3}$)	55°C ($\times 10^{-3}$)	60°C ($\times 10^{-3}$)
200	−5.64	−7.53	−5
205	−3.85	−6.79	−14.74
210	−2.06	−6.21	−11.48
215	−2.22	−4.64	−5.48
220	−1.87	−3.39	−5.18
225	−1.04	−2.76	−4.68
230	−0.87	−1.83	−1.7
235	−0.63	−1.52	−1.23
250	−0.78	−0.57	−0.11

Extraction

IgG partition was found to vary significantly for the two pH values tested. Table 3 summarizes the performance parameters of the various systems. Systems in acetate at pH 5 showed preferential partition of IgG to the top phase ($\log Kp > 0$) while in phosphate at pH 7 only the system composed by UCON 50HB-2000 showed this behavior. The IgG yield in the top phase was always higher at pH 5 reaching up to 85% for the UCON 50HB-3520. Despite the higher *Kp* values observed for the UCON 50HB-2000 at both pH, IgG was found to precipitate at the interface as it can be proven by the low total yield (around 73%).

According to Fig. 6, the extraction process proved to retain the structural integrity of the IgG with all the samples exhibiting identical CD spectra to the corresponding samples in 10 mM acetate pH 5 and phosphate at pH 7, as shown in Figs. 6A and 6B, respectively. Due to the higher protein concentration in the top phase and also in some samples to the presence of carboxylic groups from acetate, the wavelength range had to be decreased to ensure that the total absorbance was kept within the acceptable limits.

Back-Extraction

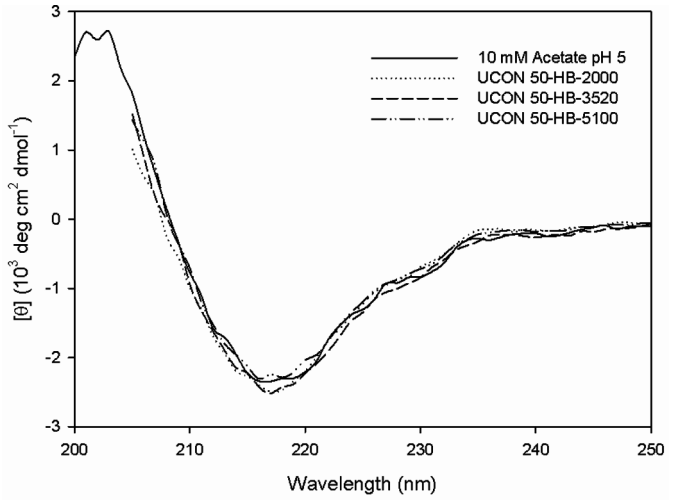
The yield of the back-extraction process was found to be high (>90%) and roughly identical for all the systems tested. Table 3 summarizes the performance parameters of the various systems. Overall, taking into account the integrated process, the 50-HB-3520 system in acetate at pH 5 was found to be the best, achieving a global IgG yield of approximately 82%.

The back-extraction step for the samples at pH 5, revealed no structural changes for the 50-HB-3520 and 50-HB-5100 polymers while an increase in ellipticity for

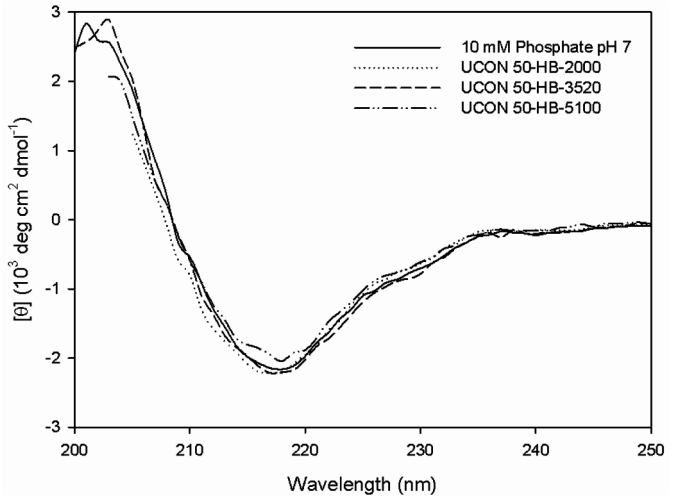
TABLE 3
Performance parameters (partition coefficient, IgG yield in the top phase, total IgG yield, back-extraction yield and global IgG yield) in 8% UCON, 5% Dextran 500,000 ATPS

	Acetate pH 5					Phosphate pH 7				
System	Log *Kp*	Y_{Top} (%)	Y_{Total} (%)	$Y_{Back\text{-}extraction}$ (%)	Y_{Global} (%)	Log *Kp*	Y_{Top} (%)	Y_{Total} (%)	$Y_{Back\text{-}extraction}$ (%)	Y_{Global} (%)
50-HB-2000	0.75	67.1	72.5	99.0	66.4	0.23	57.1	72.2	91.8	52.4
50-HB-3520	0.33	84.8	100.2	96.5	81.8	−0.41	46.1	105.9	109.6	50.5
50-HB-5100	0.34	78.5	96.3	98.9	77.6	−0.23	56.7	98.3	98.2	55.7

the 50-HB-2000 was observed, consistent with an increase in β-sheet content (Fig. 7A). The same behavior was seen for the 50-HB-2000 polymer at pH 7 but with a greater

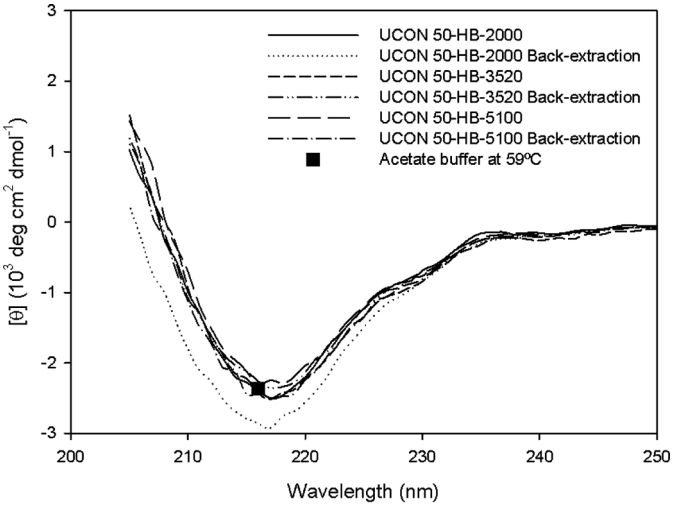


(A)

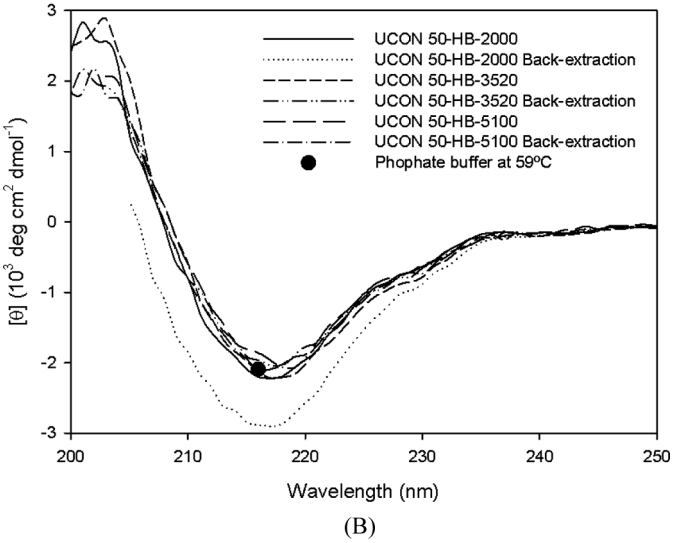


(B)

FIG. 6. Far UV CD spectra of IgG in the top phase of 8% UCON 5% Dextran 500,000 ATPSs. Effect of the extraction step in samples containing acetate buffer pH 5 (A) and phosphate buffer pH 7 (B).



(A)



(B)

FIG. 7. Far UV CD spectra of IgG in the top phase of 8% UCON 5% Dextran 500,000 ATPSs. Effect of the thermally induced back-extraction process in samples containing acetate buffer pH 5 (A) and phosphate buffer pH 7 (B). Ellipticity value at 59°C in the unfolding curve of IgG in acetate pH 5 (■) and phosphate pH 7 (●).

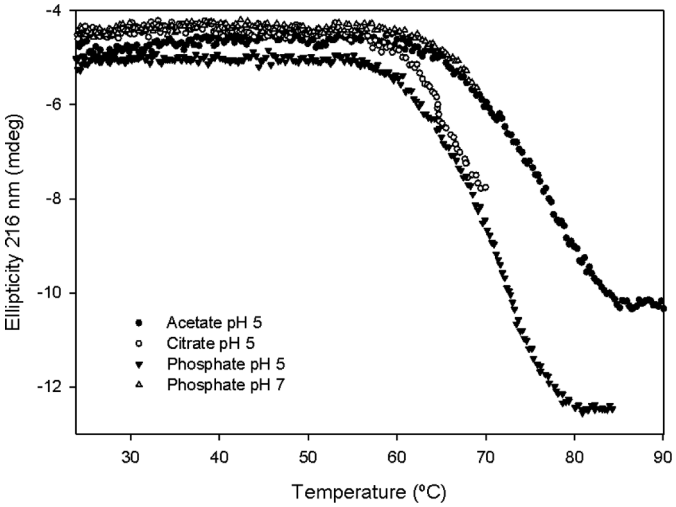


FIG. 8. Thermal unfolding curves of IgG in 10 mM acetate pH 5, citrate pH 5 and phosphate pH 5 and 7.

increase in ellipticity (Fig. 7B). For the other polymers, 50-HB-3520 and 50-HB-5100, the systems at pH 7 recorded a decrease in ellipticity below 208 nm, consistent with an increase in the random coil content.

Considering the thermal stability of IgG, the sample in acetate at pH 5 showed a higher melting temperature than in phosphate at the same pH value, increasing from 73.4 to 78.1°C (Fig. 8). In fact, the thermal unfolding curve of IgG in acetate was identical to that of phosphate at pH 7 before protein precipitation occurred (Fig. 8). Conversely, the use of 10 mM citrate at pH 5 showed the same tendency of phosphate at the same pH value but in this case protein precipitation occurred close to 70°C owing to the higher salting out effect of citrate. Considering the temperature at which phase separation was promoted, 59°C, no significant changes in the secondary structure are expected at both pH values, as at this temperature, the recorded ellipticity value at 216 nm was the same of the native structure (Fig. 8).

CONCLUSION

Within the experimental limitations, CD proved to be a useful technique to assess the protein stability throughout the purification process with aqueous two-phase systems. Initial studies conducted in aqueous buffers provided an insight on the overall behavior of IgG at different operational conditions. Although immediate structural losses were only seen at pH values below 3, long incubation periods and the temperature at which the process is carried out proved to be crucial. For thermo responsive ATPSs, the thermal stability of IgG at a given pH value should be the starting point for a rational process design. As an example, if more acidic conditions are required the cloud point of the polymer has to be lower since the thermal stability of IgG was shown to increase with the pH. However, the influence of the phase forming polymer cannot be predicted. For the extraction process, ATPSs were shown to be fully biocompatible with no influence from the phase forming polymers. Conversely, for the back-extraction step a polymer dependent behavior was observed. Although no predictions can be made on the final outcome of the purification process, CD allowed an overall assessment of the protein structure. In this way, standard methods like ELISA and protein A which only determine the integrity of the Fab and Fc region, respectively, could be circumvented. Furthermore, due to the polyclonal nature of the IgG used, ELISA and protein A were inherently limited as no general antigen can be envisaged and protein A is not capable of binding human IgG3. According to product specifications, IgG3 represents 4.9% of the total IgG content.

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