

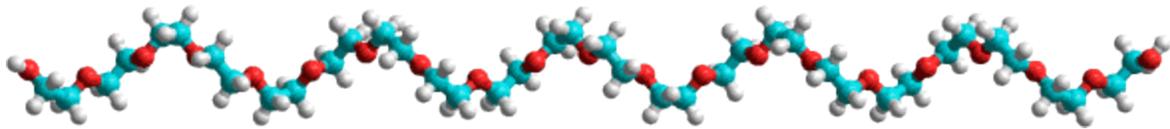
Aqueous Biphasic Systems

▼ [Associative phase separation](#)

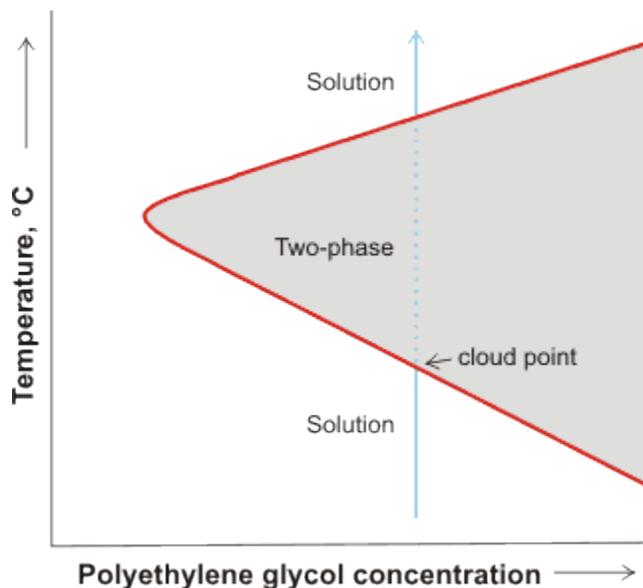
Addition of salts to water will segregate into their preferred [water structuring](#) [1994]. Aqueous biphasic systems occur when certain solutes cause an aqueous solution to fully separate into two aqueous phases. Beijerinck first noted, in 1896, the 'incompatibility' of certain polymers in aqueous solution. In this case, two phases were formed when [agar](#) was mixed with soluble [starch](#) or [gelatin](#). Since then, many immiscible biphasic aqueous systems have been found utilizing hydrophilic polymers in aqueous solution; for example, poly(ethylene glycol) (PEG, HO-(CH₂-CH₂-O)_n-H where n is the degree of polymerization)⁶. The most thoroughly investigated of these is the aqueous dextran-PEG system, where dextran forms the more hydrophilic, denser, lower phase and PEG the more hydrophobic, less dense, upper phase. Extensive examinations of *in vitro* [171] and *in vivo* [264] aqueous phase separations are available.

Phases form when limiting concentrations of the polymers are exceeded. Both phases contain mainly water (typically 70-90% w/w water) and are enriched in one of the polymers. The limiting concentrations depend on the type and molecular weight of the polymers and on the pH, ionic strength and temperature of the solution. Some polymers form a two-phase system by themselves; PEG forming the upper more-hydrophobic phase in the presence of fairly concentrated solutions of citrates, phosphates or sulfates or at higher temperatures (see [below](#)). Such aqueous liquid-liquid two-phase systems are finding increasing use in the extractive separation of labile biomolecules such as proteins, offering mild conditions due to the low interfacial tension between the phases (that is, about 400-fold less than that between water and an immiscible organic solvent) allowing small droplet size, large interfacial areas, efficient mixing under very gentle stirring and rapid partition. The polymers also have a stabilizing influence on most proteins. A great variety of separations have been achieved, by far the most important being the separation of enzymes from broken crude cell material. Separation may be achieved in a few minutes, minimizing the harmful action of endogenous proteases. The systems have also been used successfully for the separation of different types of cell membranes, organelles and actinide ions, the purification of enzymes, extractive bioconversions. Although sometimes perceived as due to polymer incompatibilities, the properties of these biphasic systems can be mainly attributed to incompatibility between aqueous pools of low and higher density water. Each phase may be considered as a different, although aqueous, solvent with properties determined by its structuring (see for example, [171]).

PEG usually has a far higher concentration in the upper (low-density) phase of such solutions in spite of its inherent density being greater than water. This, together with the properties of this PEG phase, encourage the belief that it creates a predominantly [low-density water](#) environment due to its partially [hydrophobic character](#), in turn mainly determined by the methylene groups. Further proof of this may be seen by use of microwave dielectric measurements, which show the water surrounding PEG to be ordered, whereas that surrounding more hydrophilic polymers is disordered [338]. Also, the dissolution of PEG is exothermic (and increasingly exothermic with PEG size), in line with a shift in the [ES](#) ⇌ [CS equilibrium](#) towards the more ordered [ES](#) structure. It is interesting and perhaps not simply fortuitous that the diameter (4.9 Å) of the favored PEG helix (formed by trans, gauche, trans links across the C-O-C-C, O-C-C-O, C-C-O-C bonds) is the same as the diameter of the [spines](#) of the [ES water cluster](#) (4.7 Å) formed by [pentagonal boxes](#), the ether (O-C-C-O) distances (2.88 Å) are close to the O···O distances (2.84 Å) in water and the next ether (O-C-C-O-C-C-O) (5.6 Å) distances are close to the next vertex distance on opposite sides of the [pentagonal boxes](#) (5.4 Å).^a



Model building shows that optimum hydrogen bonding would tend to distort this PEG helix, however. The strongly-held hydration, as determined by viscosity, increases from two molecules of water per PEG monomer at very low polymerization (tetramer) to 5 molecules of water per PEG monomer for 45-mer [576a],^b showing that the extent of water clustering increases with PEG size. The total number of water molecules associated with each PEG monomer is about 32 [1958], or out to about 2-3 nm radius. The partitioning of proteins into the hydrophobic PEG phase shows great sensitivity to the protein's surface hydrophobicity (partition increasing with surface hydrophobicity) and also depends on the PEG size; increasing with PEG molecular mass [597], in line with the extent of water clustering. Increasing PEG size and concentration both increase the proteins' effective hydration as the PEG is excluded from the proteins' surface [953]. However, when the PEG phase becomes too ordered (for example, at higher PEG size) partitioned proteins are excluded [598] due to the reduced available water content.



An interesting and revealing phenomenon occurs in PEG solutions as the temperature is raised; the solution at low temperatures separates into two phases (PEG-rich and PEG-poor) at higher temperature (separating at the cloud-point) and reverts to a single phase at even higher temperatures. This is shown schematically opposite and may be explained as the PEG creating a [low-density water](#) environment with decreased entropy [451]. At low temperatures a solution is formed due to the enthalpy of hydrogen bonding between the PEG and the water more than compensating for the entropy lost in forming the low-density water. This entropy loss is required, due to the hydrophobicity of the methylene groups, but is not great as the water is somewhat ordered already at lower temperatures. At the cloud point, the entropy cost is greater as the water is no longer naturally as structured, and two phases develop; probably involving extended chains in the one continuous phase and aggregated chains in the other cloudy phase [1085]. The stronger hydrogen bonding in D₂O, relative to H₂O, is expected to raise this cloud point. At higher temperatures still, the water possesses excess energy and cannot be structured by the PEG. This reduces the entropic cost, so allowing a solution to form once more. When small PEG molecules are used, for example, in PEG400 [1131], the PEG may aggregate rather than form a second aqueous

phase but the behavior otherwise is similar. Other work, however, indicates that water competes successfully for hydrogen bonding to ethylene glycol compared with other ethylene glycol molecules [1244], so the situation may be finely balanced and depend on temperature, concentration and molecular size. This phenomenon has been made use of in reverse osmosis where non-ionic glycol polymers are used as the osmotically-active draw agent and regenerated by use of the cloud point [2106].

Anions have a distinct effect on the cloud point in line with the [Hofmeister Series](#) (cloud point lowering: $\text{SCN}^- < \text{I}^- < \text{Br}^- < \text{Cl}^- < \text{F}^- < \text{OH}^- < \text{SO}_4^{2-} < \text{HPO}_4^{2-} < \text{CO}_3^{2-} < \text{PO}_4^{3-}$); the greater lowering of the cloud point is in line with greater surface charge density [752], stronger hydration, greater tendency to avoid low-density water and the greater destruction of the natural structuring of the water. An oppositely-ordered compensating effect on the cloud point has been recognized due to binding of the anions to the polymer surface [896]. This tends to raise the cloud point at lower salt concentrations as the bound salt increases the polymer net charge and, hence, solubility. The relative effect of the ions is the reverse of the Hofmeister series just given with weakly hydrated ions binding best, i.e. SCN^- having the greatest effect and ionic kosmotropes below Cl^- having negligible effect. The Hofmeister effects on the phase separation process of poly(propylene oxide) has been described by a balance between these interactions [1922].

Cations have a lesser but opposite effect to anions with [chaotropes](#) (for example, NH_4^+) tending to lower the cloud point but [kosmotropes](#) (for example, Li^+) raising it. Exceptionally, however, some di- and trivalent cations such as Mg^{2+} and Zn^{2+} act counter to their normal Hofmeister behavior, due presumably to their specific chelation to oxygen atoms in the PEG molecules [752].

Anions and cations distribute themselves differently between the phases depending on their affinity for low or higher density water but with the requirements that the phases be electrically neutral and iso-osmotic, so producing an interfacial potential difference, which may aid the partitioning of charged biomolecules. Thus sulfate and phosphate ions prefer the bottom phase and, as a consequence, negatively charged proteins are partitioned into the upper PEG phase, so allowing more sulfate or phosphate ions to partition into their preferred lower phase. Preference for the PEG-rich or PEG-poor phase is related to the [Hofmeister Series](#) for the structuring ability of the salts, particularly the anions (for example, preference for PEG-rich phase: $\text{I}^- > \text{Br}^- > \text{Cl}^- > \text{F}^- > \text{SO}_4^{2-}$; $\text{Cs}^+ > \text{Na}^+ > \text{Ba}^{2+} > \text{Ca}^{2+}$; preference for PEG-poor phase: $\text{SO}_4^{2-} > \text{F}^- > \text{Cl}^- > \text{Br}^- > \text{I}^-$) [171]. A similar [Hofmeister Series](#) effect is noticed intensifying the incompatibility between two polymers such as polyethylenimine-PEG, or dextran-PEG, by increasing the concentration of strongly hydrated (CS-forming) anions, such as sulfate [340].

Recently, ionic liquids^d have proven to be alternative to polymers in the formation of aqueous biphasic systems. Addition of common aqueous salt solutions induce the formation of ionic-liquid-based aqueous biphasic systems, with the [salting-outability](#) of the anions and cations following their [Hofmeister Series](#) [1911].

Associative phase separation

A different form of aqueous biphasic system can be formed by associating molecules. This occurs where a water soluble molecule self associates but remains soluble, thus forming a soluble polymeric mass that behaves as a distinct aqueous phase [1696].

Footnotes

^a Note that the polymers formed with either one (-O-C-O-C-O-) or three (-O-C-C-C-O-C-C-C-O-) methylene groups between the oxygen atoms are both insoluble in water. The reason however is not so much that the O···O distances (2.18 Å and 4.79 Å respectively) fit less well with the water cluster spacing but rather that the molecules form almost-linear extended (rather than helical) chains with a pronounced hydrophobic character that have strong intra-molecular attraction.

^b Dielectric studies show 3.7 hydration water molecules per monomer residue [576b].

^c Poly-(ethylene glycol) (PEG, $n \text{ HOCH}_2\text{-CH}_2\text{OH} \longrightarrow \text{PEG} + n-1 \text{ H}_2\text{O}$) and poly-(ethylene oxide) (PEO, $n \text{ C}_2\text{H}_4\text{O} \longrightarrow \text{PEO}$) are often used interchangeably as both consist of repeating CH₂-CH₂-O- units with the only difference being at the polymer ends.

^d Ionic liquids are salts with large ions with low surface charge density that form only short-lived ion pairs and have difficulty crystallising so forming liquids at low temperatures. Examples are ethylammonium nitrate (C₂H₅)NH₃⁺·NO₃⁻ (m.pt. 12 °C) and 1-butyl-3-methylimidazolium tetrafluoroborate (m.pt. -80 °C).

Source: <http://www1.lsbu.ac.uk/water/biphasic.html>