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Partitioning of proteins and small biomolecules in temperature- and pH-sensitive hydrogels

Alexander P. Sassi, Anita J. Shaw, Sang Min Han, Harvey W. Blanch and John M. Prausnitz*

Chemical Engineering Department, University of California, Berkeley, CA 94720, USA and Chemical Sciences Division, Lawrence Berkeley Laboratory, One Cyclotron Road, Berkeley, CA 94720, USA

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Experimental partition coefficients were measured for the distribution of selected proteins and small biomolecules between aqueous, buffered solutions and hydrogels. Temperature-sensitive hydrogels were prepared by polymerizing *N*-isopropylacrylamide (NIPA) alone and by copolymerizing NIPA and a charged monomer. Temperature- and pH-sensitive hydrogels were prepared by copolymerizing NIPA with weakly acidic and/or weakly basic monomers. The effects of temperature, pH and ionic strength on partitioning were investigated. In general, protein partition coefficients ranged from 0 to 10 in buffers on 0.1 M ionic strength. pH had a significant effect on the partition coefficient for a protein into a weakly ionizable polyelectrolyte gel even at this ionic strength. Published by Elsevier Science Ltd.

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INTRODUCTION

The unique swelling properties of temperature-sensitive gels have led to proposals for gel-based separation processes[1–5]. Temperature-sensitive hydrogels shrink, often by an order of magnitude, at temperatures often not far removed from ambient. Swelling equilibria and transition temperatures of these temperature-sensitive gels can be engineered to some extent by judicious incorporation of a suitable comonomer.

The best known temperature-sensitive gel is the poly-*N*-isopropylacrylamide (poly-NIPA) hydrogel. Both Cussler and co-workers and Prausnitz and co-workers have proposed processes which use poly-NIPA copolymer hydrogels for concentrating or separating dilute protein solutions. Cussler and co-workers have proposed the process illustrated in *Figure 1a*; the solute to be recovered is concentrated in the raffinate phase[1]. The feed solution is brought into contact with deswollen gel, which swells in the feed solution, absorbing water and low-molecular-weight solutes. The swollen gel is separated from the raffinate and collapsed by increasing the temperature. As the gel collapses, it expels imbibed water and solutes. The deswollen gel is then recycled back to the start of the process.

Prausnitz, Blanch and co-workers have proposed the extraction process illustrated in *Figure 1b*; the solute to be recovered is concentrated in the gel, and the gel is deswollen with a temperature and/or pH shift not only for use in another cycle, but also to release the extracted solute[4]. This process requires careful consideration of gel chemistry to choose a gel that is selective for a solute(s) and, in addition, has the required swelling properties.

Both processes are essentially analogous to a two-phase liquid–liquid extraction process with regeneration of one stream. The use of temperature-sensitive gels is advantageous because they are 'gentle' towards solutes and because a small energy investment induces a large change in swelling. The authors know of no attempt to incorporate affinity ligands into temperature-sensitive gels, which would increase selectivity.

Cussler and co-workers have applied their process for the concentration of varous proteins using poly-NIPA, poly-*N*,*N*′-diethylacrylamide (DEA)/sodium methacrylate, and hydrolysed polyacrylamide gels (polyacrylamide gels are not temperature-sensitive; the gel is collapsed with a pH shift)[1,2,6–8]. They report their results in terms of an efficiency of exclusion, η, defined by:

$$\eta = \frac{(C_r/C_f) - 1}{(m_f/m_r) - 1} \times 100$$

where C_f is the concentration of solute in the feed, C_r is the concentration in the raffinate, m_f is the mass of the feed, and m_r is the mass of the raffinate†. They

* To whom correspondence should be addressed

† Cussler's efficiency of exclusion η, depends not only on the partition coefficient of the solute between the gel and solution but also on the amount of gel. For example, an exclusion efficiency of 0% can be achieved with partition coefficients less than unity; a partition coefficient of unity implies equal solute concentrations in the gel and solution phases. If the exclusion efficiency were instead based solely on the ability of the gel to exclude the solute, the efficiency would be 0% only when the partition coefficient was unity. Therefore, although one may initially be tempted otherwise, one must not consider Cussler's η to be a measure solely of the distribution of a solute between a gel and solution

investigated the effects of crosslinking, solute concentration, molecular weight, and solute charge for selected proteins, small biomolecules, dyes and polymers. The effects of crosslinking and molecular weight on the exclusion efficiency were as expected from size-exclusion considerations; η increases with molecular weight and gel crosslinking. The effect of solute charge could be predicted qualitatively with ideal Donnan partitioning equilibria,

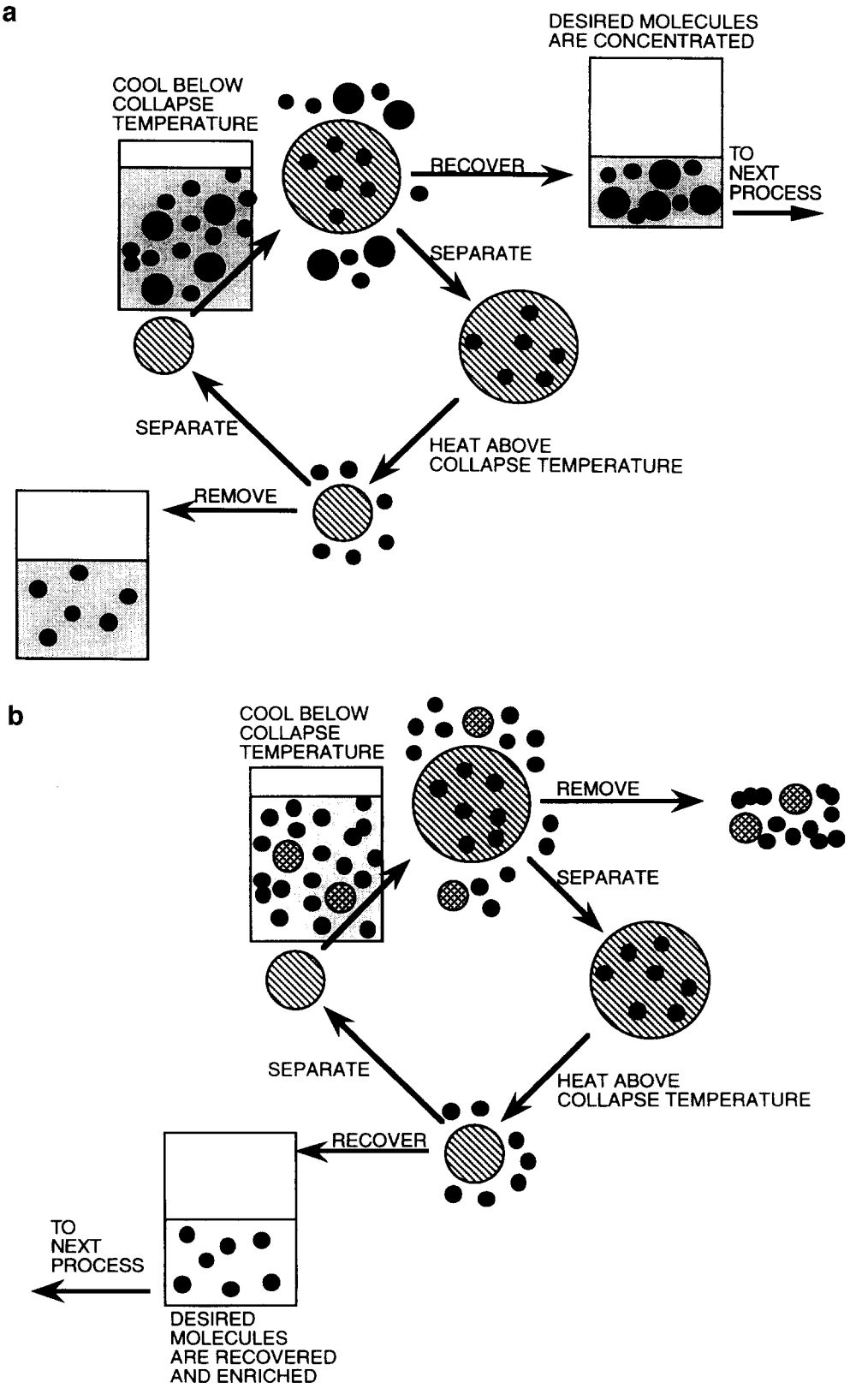

Figure 1 (a) Process proposed by Cussler *et al.* to use thermosensitive gels to concentrate solutes. In this process, the solute of interest is too large to penetrate the gel and is concentrated in the raffinate solution. (b) Process proposed by Prausnitz and Blanch *et al.* to use thermo- and pH-sensitive hydrogels to extract a solute of interest. In this process, the solute of interest partitions preferably into the swelling gel. The gel is removed from the feed solution and collapsed under conditions which favour release of the solute of interest. The solute is thus recovered in the extract solution

but for highly charged dyes, the experimental exclusion efficiency is significantly lower than predicted. The efficiency of exclusion decreases with solute concentration; this was attributed to entrained fluid on the surface of the gel.

Weber and co-workers also investigated the use of hydrogels as extraction solvents[5]. They investigated poly-NIPA gels and polyacrylamide gels copolymerized with sodium acrylate, sodium vinylsulfonate, 2-acrylamido-2-methyl-1-propanesulfonic acid (AMPS) or 3-(methacrylamido)propyltrimethylammonium chloride. Vasheghani-Farahani *et al.* reported results in terms of the partition coefficient, K, defined as:

$$K = C_g/C_r$$

where C_g is the concentration of solute in the gel and C_r is the concentration in the surrounding bath (also termed the raffinate). In experiments with polyethylene glycol (PEG), they report that the partition coefficient decreases with PEG molecular weight, gel crosslinking and monomer concentration at synthesis, as is known from the literature on size-exclusion chromatography[9]. Their results indicate that increasing the monomer concentration at preparation is the most effective way to lower the partition coefficient in a non-ionic gel. They also investigated the partitioning of proteins (β-lactoglobulin, ovalbumin, bovine serum albumin, cytochrome c, α-amylase, lipase and β-galactosidase) into charged gels at pH values above and below the isoelectric point of the protein. The partition coefficient for a protein was not solely a function of net charge; hydrophobic effects and surface adsorption were observed in some cases. Palasis and Gehrke also observed hydrophobic effects in the partitioning of acetaminophen, norethindrone, methyl orange and vitamin B_{12} in poly-NIPA hydrogels[10]. Vasheghani-Farahani *et al.* determined that the biologic activities of the enzymes α-amylase, lipase and β-galactosidase were not affected by the gel[5].

Here, we expand on the previous work by Cussler, Weber, and Palasis *et al.* It is clear from previous work that in the absence of significant surface effects, efficient exclusion of a solute (low partition coefficients) can be achieved by increasing the polymer volume fraction of the gel, ensuring that electrostatic interactions between the solute and gel are repulsive and hydrophobic interactions are minimized. We are interested in determining the feasibility of temperature-sensitive gels in the extraction process illustrated in *Figure 1b*. We therefore studied solutes which we expected would not be excluded simply because of their size. In such a process, the partition coefficient of the solute between the collapsed gel and the extract determines to a great extent the process efficiency. Therefore, we have examined the effect of temperature on the partition coefficients for proteins and other biomolecules. We have also examined in more detail the effects of pH and the ionic strength of the feed solution on the partitioning of small proteins into neutral, weakly basic, weakly acidic or weakly amholytic gels, and we compare the abilities of poly-NIPA copolymer gels to select for one of two proteins in solution through a combination of electrostatics and size-exclusion. The qualitative results of our experiments are relevant to liquid chromatography, drug delivery and gel membrane filtration processes. Ref. 11 is concerned with prediction of equilibrium partition coefficients via molecular thermodynamics.

EXPERIMENTAL

Materials

N-isopropylacrylamide (NIPA), 2-dimethylaminoethyl methacrylate (DMA), *N,N'*-methylene bisacrylamide (BIS) (electrophoresis grade), ammonium persulfate (APS), sodium citrate, citric acid, and sodium azide were purchased from Eastman Kodak. [(Methacrylamido)-propyl]trimethylammonium chloride (MAPTAC) was purchased as a 50% solution in water from Monomer-Polymer and Dajac Laboratories. Sodium metabisulfite (SMB), cytochrome c, lysozyme, catalase, hemoglobin, ovalbumin, bovine serum albumin and vitamin B_{12} were purchased from Sigma. Sodium acrylate (SA) was purchased from Polysciences. *N, N, N', N'*-tetramethyl-ethylenediamine (TEMED) was purchased from Aldrich. Sodium phosphates were purchased from Fisher Scientific. All reagents were used as received. Distilled water was filtered (0.2 μm) and deionized (17.9 MΩ cm resistivity) with a Barnstead Nanopure II unit.

Hydrogel synthesis

Uncharged hydrogels were synthesized by free-radical, solution copolymerization of NIPA and BIS, the crosslinking agent. Strongly ionized, cationic hydrogels were synthesized by the copolymerization of NIPA, BIS and MAPTAC initiated by the redox couple APS/SMB. Weakly ionizable hydrogels were synthesized by the copolymerization of NIPA, BIS and SA and/or DMA, initiated by APS and the accelerator TEMED.

All poly-NIPA/MAPTAC gels were prepared in aqueous solution according to methods in ref. 12. For each gel, the desired amounts of NIPA and BIS were dissolved in 96 ml water. Separate solutions of APS and SMB were also made (0.1 g/20 ml each). The solutions were degassed under a 686 mmHg vacuum for 90 min and subsequently transferred to a nitrogen-containing glove box. The appropriate volume of 50% MAPTAC solution was added to the monomer solution. Finally, 2 ml SMB solution and 2 ml APS solution were added. The solution was stirred using a magnetic stir bar for 1 min and then injected quickly with a syringe into moulds chilled in the freezing compartment of a normal refrigerator. The moulds were made of two glass plates (10 × 10 cm) separated by 1.57 mm Teflon spacers and held together using Teflon tape, duct tape and binder clips. The moulds were then placed in a refrigerator at 10°C so that the reaction temperature would not exceed the phase-transition temperature of the forming polymer. After 48 h, discs 1 cm in diameter were cut from the resulting gel slabs using a punch. The gels were soaked in water for one week at 10 °C. The water was changed daily to leach out unreacted monomers

All weakly ionizable gels were prepared in a similar manner except that the reaction was initiated using APS and TEMED in the concentration 0.05 g and 0.4 ml, respectively, per 100 ml of water. The liquid comonomer (DMA) was added to the reaction solution inside the glove box, while the solid comonomer (SA) was added before the degassing step.

The following variables were used to characterize the gels:

% T = mass of all monomers (g)/volume of water (ml) × 100

% C = moles of crosslinking monomer/total moles of monomer × 100

% CM = moles of comonomer in feed/total moles of monomer × 100

The gels synthesized for this work were all 1% C. The poly-NIPA/MAPTAC gels were all 15% T. The weakly acidic poly-NIPA/SA, weakly basic poly-NIPA/DMA and ampholytic poly-NIPA/SA/DMA gels were all 11.8% T. These parameters were chosen such that gels would be highly swollen yet durable enough not to break apart from the handling and agitation necessary for the partitioning experiment. Using MAPTAC as the comonomer, gels were made at 0, 1, 2 and 3% CM to produce a series of gels with varying cationic charge density. Using SA or DMA, gels were made at 10% CM to produce gels where the anionic or cationic charge density varied with pH. Using SA and DMA, gels were made at 5% CM (SA) and 5% CM (DMA) to produce ampholytic gels, i.e. gels with both positive and negative charges.

Partitioning measurements

Partitioning experiments were conducted by one of two methods. In the first method, gels which had been air-dried at ambient conditions were weighed and placed in beakers containing a known mass of buffered solution of known solute concentration. The beakers were incubated with gentle shaking in a Blue M Electric Company Shaker Bath (model MSB-3222A-1) at the desired temperature. After swelling and partitioning equilibria were reached, the gels were removed from the beakers and weighed again. The pH of the external solution was measured before and after the experiment using a Sargent/Welch Ion/pH meter (model 8400) with a Fisher Scientific Standard Combination Electrode (Ag/AgCl reference) probe. The absorbance of the remaining solution in each beaker was measured with a Shimadzu UV-160 Spectrophotometer. The reference solution was aqueous buffer of the same pH as the sample. The concentration of solute was determined by the relation of the absorbance to solute concentration in standard solutions at the same pH. Solution concentrations were dilute enough such that Beer's law was valid.

In the second method, gels were removed from pure water at 10°C, placed in beakers containing aqueous buffer solutions at the desired pH and incubated in the shaker bath until swelling equilibrium had been reached. The gels were then weighed according to the method in ref. 13 and transferred to beakers containing aqueous buffer solutions containing the desired solute. The beakers were returned to the constant-temperature bath, and the experiment proceeded as outlined above. At the conclusion of the experiment, the gels were dried at ambient conditions to constant weight. The mass of buffer salt and protein in the gel was subtracted to determine the dry mass of the polymer. The swelling ratio (SR) was determined as ratio of the mass of swollen gel to that of the dried polymer.

In experiments where more than one protein was present, protein concentrations were determined by cationic exchange chromatography using a Hewlett-Packard 1090 High Pressure Liquid Chromatography system with a 50 by 7.8 mm BIO-RAD HRLC® MA7S Cation Exchange Column (donated courtesy of Dr Wai-Kin Lam of Bio-Rad) with u.v. detection by a diode-array detector. In all cases, a gradient of 0–50% of 1 M NaCl in 20 mM bis-tris buffer (pH 7.2) was found to be an appropriate eluant. Protein concentrations were determined by comparing the peak areas to those for solutions of known concentration.

The concentration of solute in the gel at equilibrium was calculated knowing the initial and final mass of the gel, the initial and final solute concentration in solution and the initial mass of solution. The equilibrium distribution of solute between the gel and solution phases was defined by the partition coefficient, K, where

$$K = \frac{\text{mg solute/g swollen gel}}{\text{mg solute/g external solution}}$$

In this work, reported K values are the arithmetic average of those determined for three to six replicate experiments (beakers). The standard deviation is calculated using the following formula:

$$\sqrt{\frac{n\sum x^2 - \left(\sum x\right)^2}{n(n-1)}}$$

where n is the number of replicates and x is the specific value.

This definition of the partition coefficient does not distinguish between adsorption and true partitioning as modes of solute uptake. By true partitioning we mean that the solute can diffuse freely between the solvent fraction of the gel and the solution surrounding the gel; in other words, the gel is permeable to the solute. By adsorption we mean that the solute has a specific (reversible or irreversible) affinity for the polymer network which is characterized by a finite equilibrium constant or maximum loading capacity.

It is difficult to measure the individual contributions of adsorption and true partitioning. Vasheghani-Farahani *et al.* reported the effect of length-to-diameter (L/d) ratios of cylindrical gels on the partition coefficient for β-lactoglobulin, ovalbumin and bovine serum albumin in anionic poly-NIPA/AMPS hydrogels (L/d = 5 or 0.5)[5]. The pH of the solution where there was a strong effect of the length-to-diameter ratio was rather acidic: pH 2.9–3.8. Our partitioning data were taken at substantially more alkaline conditions (pH 5–8), where Vasheghani-Farahani *et al.* observed little effect of L/d on the partition coefficient. We investigated a procedure involving extensive leaching, acid hydrolysis and amino-acid analysis to determine the amount of irreversibly adsorbed proteins on hydrogels, but we were not able to obtain reproducible and accurate results[14]. Adsorption may contribute to protein uptake in our experiments; however, our data are insufficient for quantitative analysis or reversible adsorption (such as the examination of sorption isotherms and capacity).

We verified that proteins permeate into the gel (as opposed to adsorbing solely to the surface) by slicing gels from partitioning experiments involving haemoglobin, catalase and cytochrome c, which are coloured because of chelated metal ions. If the protein has adsorbed only on the surface of the gel, only the perimeter of the new surface would have been coloured. In fact, the new surface was visually homogeneous in colour, indicating that the proteins did indeed penetrate the gel.

For the enzymes they concentrated using poly-NIPA-based hydrogels, Vasheghani-Farahani *et al.* determined

that biologic activities were unaffected by the presence of the gel[5]. We confirmed that the proteins in our experiments did not unfold significantly during the course of the experiment by monitoring the ultraviolet/visible absorbance spectrum of the protein in solution in the absence of a gel. For cytochrome c, we also confirmed that the protein was not hydrolysed during the experiment by performing gel electrophoresis on samples of the raffinate. Coomassie blue was used to stain the proteins. For every studied combination of pH and temperature, the location of the band from the raffinate samples was identical to that for cytochrome c from a sample of molecular-weight standards for electrophoresis. No bands corresponding to degradation products were found in the lanes where raffinate samples were loaded. We do not know to what extent, if any, the protein in the gel is denatured. We would expect a significant portion of the protein in the gel phase to be denatured only if irreversible adsorption were the mechanism of protein uptake[15,16] in which case the proposed extraction process (*Figure 1b*) is not feasible.

RESULTS

The Appendix presents tables of experimental results for the partition coefficients and swelling capacities for all systems studied. The partition coefficient for any solute is influenced by steric, electrostatic and short-range non-electrostatic interactions. We have discussed the steric (size) exclusion behaviour of temperature-sensitive gels in ref. 9. We now discuss the effects of gel charge density, solution ionic strength and temperature, using examples from the tables in the Appendix. We stress that, more often than not, no single effect dominates.

Effect of gel charge density

Figure 2a illustrates the effect of gel charge density on partition coefficients for poly-NIPA/MAPTAC where the percentage of MAPTAC varied from 0 to 3%. *Figure 2a* presents experimental partitioning data for tryptophan, caffeine, vitamin B_{12}, haemoglobin and catalase as a function of gel charge density at constant solution ionic strength, temperature and feed concentration. *Figure 2b* presents the swelling equilibria of the gels corresponding to the data in *Figure 2a*. The partition coefficient and swelling ratio have been normalized with respect to their values for the uncharged poly-NIPA gel (15% *T*, 1% *C*) to indicate more clearly the relative effect of gel charge density. The charge density was calculated using the nominal molar percentage of MAPTAC.

We expect negatively charged solutes to partition more into a positively charged poly-NIPA/MAPTAC gel than into an uncharged poly-NIPA gel because the electrostatic interactions between the polyelectrolyte gel and the solute are attractive. The partition coefficient may also rise because the charged gels swell more than the uncharged gels, even in 0.01 M buffer, allowing more solute to enter. The two proteins, catalase (pI = 6.7) and haemoglobin (pI = 7.4) have a net negative charge at pH 8 (refs 17 and 18), and their partition coefficients do rise (even if slightly) with increasing cationic charge density.

For neutral solutes, we expect no electrostatic interactions between solute and polymer but, because charged gels swell more than uncharged gels, partition coefficients of neutral solutes should increase with charge density. The partition coefficients of caffeine in poly-NIPA gels with 1–3% MAPTAC do increase with MAPTAC concentration, but are curiously lower than the partition coefficient in the less swollen, but neutral poly-NIPA gel. The higher partition coefficient in neutral poly-NIPA may be a result of hydrophobic interactions between caffeine and the polymer.

We expect the electrostatic interactions between positively charged solutes and the poly-NIPA/MAPTAC gels to be repulsive, causing the partition coefficient to decline with increasing percentage of MAPTAC. However, the increase in swelling of the polyelectrolyte gels with percentage MAPTAC may counteract this effect. The partition coefficients of vitamin B_{12} (cyanocobalamin), where the cobalamin is positively charged, decrease with charge density, but only slightly. An example of an exception to these

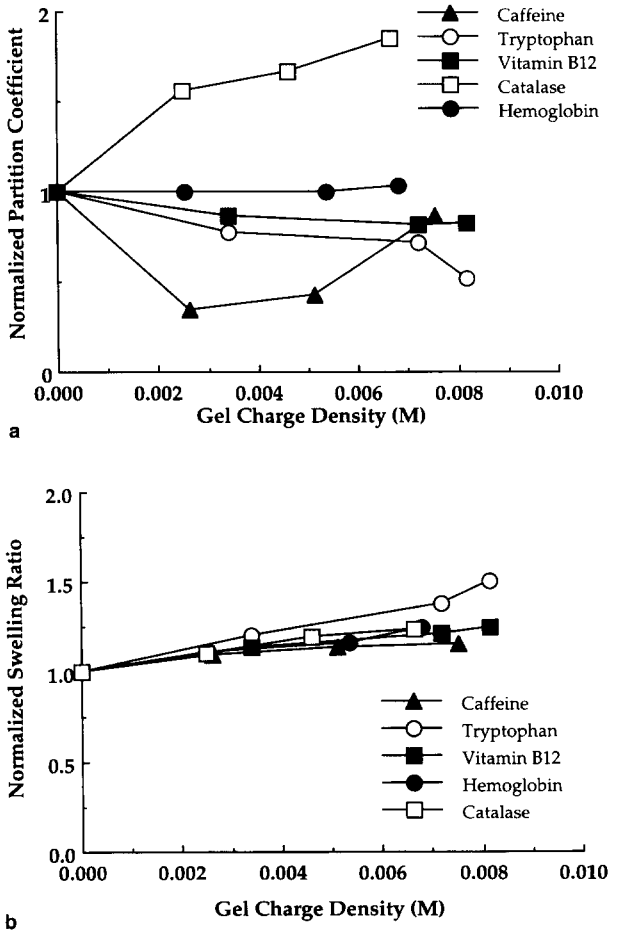

Figure 2 (a) Effect of charge density of groups on the polymer backbone on the partition coefficients for various biomolecules into temperature-sensitive poly-NIPA/MAPTAC gels of 0–3% MAPTAC. Experiments were conducted at 10°C in 0.01 M ionic strength sodium phosphate/citrate buffer with 0.1 g l^{-1} sodium azide. Data are from tables in the Appendix and are normalized by the partition coefficient in the neutral gel. Lines are drawn to guide the eye. (b) Normalized swelling ratios in the experiments of (a). In all cases, the swelling increases with charge density. Swelling ratios are normalized with respect to that of the uncharged gel. Lines are drawn to guide the eye

generalities is provided by the amino acid tryptophan, whose partition coefficient drops with increasing cationic charge density, despite tryptophan's net negative charge at pH 7. Tryptophan is a hydrophobic amino acid, and we surmise that the decrease in partition coefficient with charge density is related to the dilution of favourable hydrophobic interactions with *N*-isopropylacrylamide. Similar effects with the hydrophobic dye methyl orange have been observed by Palasis and Gehrke[10].

Effect of solution ionic strength

Figure 3a presents experimental partitioning data for cytochrome c and bovine serum albumin in various poly-NIPA, poly-NIPA/3% MAPTAC, or poly-NIPA/10% SA hydrogels. *Figure 3b* presents the corresponding swelling equilibria for comparison. As in *Figures 2a* and *b*, the partition coefficients and swelling ratios have been normalized, this time with respect to the partition coefficient or swelling ratio at the lowest ionic strength studied. Ionic strengths below 0.01 M were not studied because of insufficient buffering capacity at extremely low ionic strength. At constant pH, salt reduces the magnitude of solute–polyelectrolyte electrostatic interactions and causes a polyelectrolyte gel to shrink because of charge shielding. This results in a decline of the partition coefficient for solutes oppositely charged with respect to the gel, as is observed experimentally (*Figure 3a*). Data for cytochrome c in neutral poly-NIPA gels demonstrate that ionic strength has essentially no effect on partitioning of a solute in neutral gels, as expected.

Effect of temperature

Figure 4a presents experimental partitioning data for catalase at two temperatures (10 and 35°C) in 0.01 M phosphate-citrate buffer with 0.1 g l^{-1} sodium azide at pH 8. At 10°C, the gels are highly swollen, and this

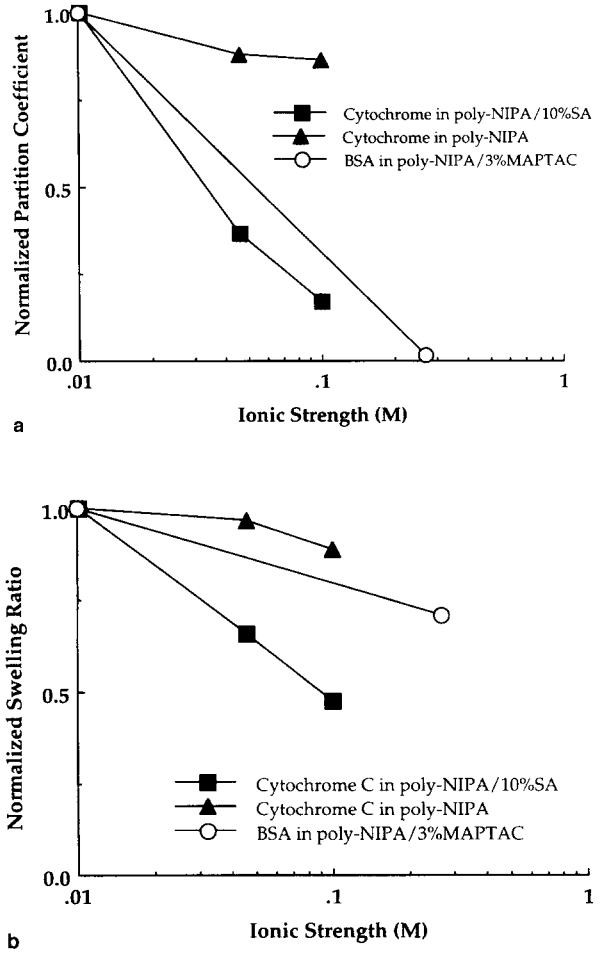

Figure 3 (a) Effect of solution ionic strength on partition coefficients for positively charged cytochrome c in neutral poly-NIPA gels and negatively charged poly-NIPA/SA gels and for negatively charged bovine serum albumin in positively charged poly-NIPA/3% MAPTAC gels. Data for cytochrome c were taken at 22°C in sodium phosphate buffer at pH 8, and data for bovine serum albumin were taken at 10°C in 0.01 M sodium citrate/phosphate and 0.1 M sodium sulfate at pH 8. Data are from tables in the Appendix and are normalized to the partition coefficient at 0.01 M ionic strength. Lines are drawn to guide the eye. (b) Normalized swelling ratios in the experiments of (a). The swelling ratio of the polyelectrolyte gels decreases with ionic strength. Swelling ratios are normalized with respect to the swelling ratio at 0.01 M ionic strength. Lines are drawn to guide the eye

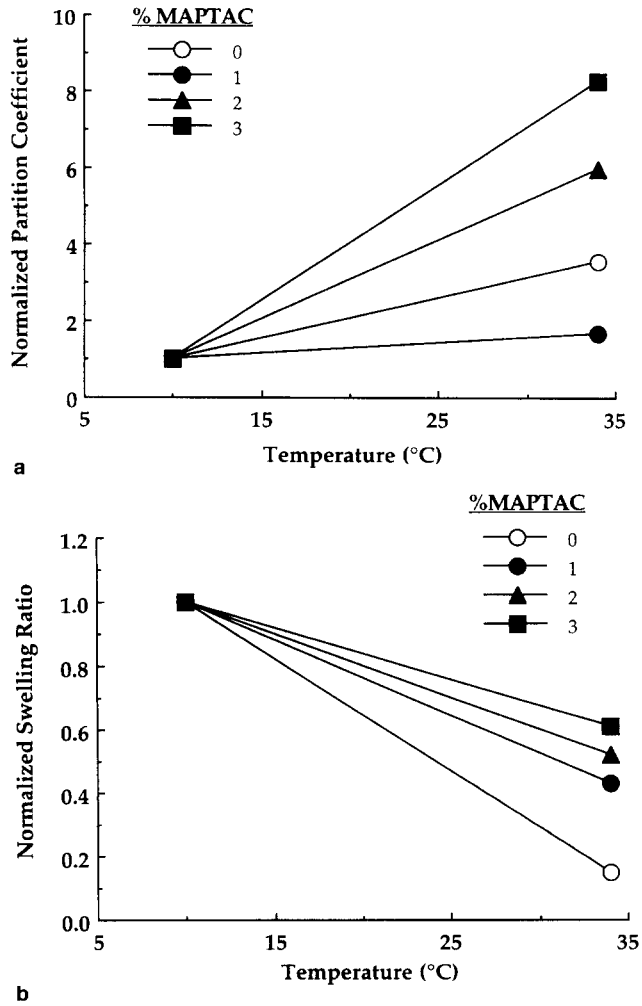

Figure 4 (a) Effect of temperature on partition coefficient of the protein catalase in poly-NIPA/MAPTAC gels of 0–3% MAPTAC in 0.01 M sodium citrate/phosphate buffer, pH 7, with 0.1 g l^{-1} sodium azide as a bacteriocide. The partition coefficient increases upon increasing the temperature above the collapse temperature of poly-NIPA. Data are taken from tables in the Appendix. Lines are drawn to guide the eye. (b) Normalized swelling ratios in the experiments of (a). The swelling decreases when temperature is increased. Swelling ratios are normalized with respect to the swelling ratio at 10°C. Lines are drawn to guide the eye

temperature is in the same range as the temperatures at which previous studies of swelling equilibria for similar gels were conducted[12]. The temperature 35°C was chosen because it is slightly above the collapse temperature for poly-NIPA but not so high as to destabilize protein structure. The gels were poly-NIPA/MAPTAC (15% *T*, 1% *C*, 0–3% MAPTAC). *Figure 4b* presents the corresponding swelling equilibria. The partition coefficient and swelling ratio were normalized to those at the lower temperature. Poly-NIPA hydrogels shrink as temperature increases, more efficiently excluding solutes. The charge density of a charged poly-NIPA hydrogel increases with temperature because the gel shrinks. These two effects counteract each other for solutes oppositely charged with respect to the gel (such as catalase). On the other hand, attractive solute–polymer, non-electrostatic interactions should be more important as temperature increases because the volume fraction of the polymer increases with temperature. In the case of catalase, attractive solute–polymer (electrostatic and possibly also non-electrostatic) effects appear to be relatively more important because the partition coefficients increase significantly with temperature.

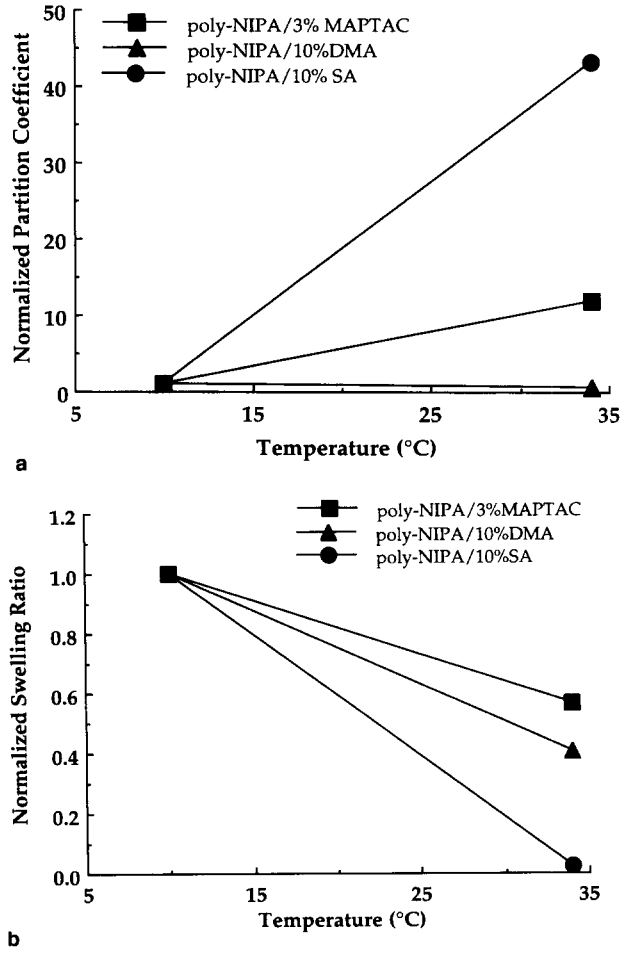

Figure 5 (a) Effect of temperature on partition coefficient of negatively charged bovine serum albumin into positively charged poly-NIPA/10% DMA and poly-NIPA/3% MAPTAC gels and into negatively charged poly-NIPA/10% SA gels. Experiments were conducted in 0.01 M sodium citrate/phosphate buffer, pH 8, with 0.1 g l^{-1} sodium azide as a bacteriocide. The partition coefficient is normalized to that at 10°C. The partition coefficient increases with temperature. Lines are drawn to guide the eye. (b) Normalized swelling ratios in the experiments of (a). The swelling decreases with increasing temperature. Swelling ratios are normalized with respect to the swelling at 10°C. Lines are drawn to guide the eye

Table 1 Effect of pH on partitioning of bovine serum albumin into poly-NIPA/MAPTAC gel (15% *T*, 1% *C*, 3% *CM*) (0.01 M citrate-phosphate buffer, 0.1 g l^{-1} sodium azide at 10°C)

pH	Partition coefficient, *K*
3	0.2 ± 0.2
8	12.8 ± 1.4

Figure 5a presents experimental partitioning data for bovine serum albumin (BSA) at two temperatures (10°C and 35°C) in 0.01 M phosphate-citrate buffer with 0.1 g l^{-1} sodium azide at pH 8 in poly-NIPA/3% MAPTAC, poly-NIPA/10% SA and poly-NIPA/10% DMA gels. *Figure 5b* presents the corresponding swelling equilibria. The normalized partition coefficient decreases with temperature for poly-NIPA/DMA gels, where the solute is charged oppositely to the gels, and increases dramatically for the other gels, where the solute is charged oppositely (poly-NIPA/MAPTC) or the same (poly-NIPA/SA) as the gels. The swelling ratio for all gels is smaller at 35°C because of the temperature sensitivity of the gels.

Effect of pH

Table 1 presents experimental partition coefficients for BSA in 0.01 M phosphate-citrate buffer (0.1 g l^{-1} sodium azide) at pH 3 and 8 at 10°C in poly-NIPA/3% MAPTAC gels. The swelling equilibria and charge density of poly-NIPA/MAPTAC gels do not change with pH because MAPTAC is a strongly ionized monomer. The net charge on the protein, however, depends on pH. Because the pI of BSA is 4.8, the net charge of the protein is positive at pH 3 and negative at pH 8 (refs 17 and 18). We therefore expect the partition coefficient at pH 8 to be greater than that at pH 3, assuming that the only effect of pH is to change the charge of the protein. The experimental partition coefficient at pH 8 is 12.8, significantly greater than 0.2 at pH 3.

For weakly ionizable gels such as poly-NIPA/DMA and poly-NIPA/SA, the extent of ionization of the ionizable monomers varies with pH. This variation of ionization results in pH-dependent swelling equilibria. Weakly basic poly-NIPA/DMA gels swell more at acidic pH; weakly acidic poly-NIPA/SA gels swell more at alkaline pH. Thus, pH influences the partitioning of a protein through electrostatics (by changing the net charge on the protein and on the gel) and through size exclusion (by causing the gel to swell or shrink). The forces between the gel and a protein can be either net-attractive or net-repulsive, depending on whether the pH is less than or greater than the pI. The simultaneous effects of pH and temperature on the partitioning of a protein into a weakly ionizable hydrogel can be imagined to be quite complex.

Figure 6a presents experimental partition coefficients for horse-heart cytochrome c in poly-NIPA, poly-NIPA/10% SA and poly-NIPA/10% DMA at 22.2°C as a function of pH. The solution was buffered with sodium phosphates such that the ionic strength was 0.1 M.

Figure 6b presents the swelling equilibria for the gels in *Figure 6a* as a function of pH. Cytochrome c is a small and highly stable protein (molecular weight (MW) 12400)[19,20]. Partitioning experiments were carried out using the method whereby the gels were pre-equilibrated in buffer (the second method under Partitioning measurements section above). The pI of cytochrome c is approximately 10.4 (ref. 21). Over the pH range of the experiments (pH 5–8) the protein has a net positive charge. As expected, the partition coefficient in the neutral poly-NIPA gel is not sensitive to pH, with the exception of the data for pH 8. The partition coefficient for cytochrome c at pH 8 is unexplainably about twice that for pH 5. The partition coefficient in the negatively charged poly-NIPA/SA gels depends on pH and is highest at pH 7. The trend in partition coefficient follows the trend in swelling: both swelling and partition coefficient increase with pH. The partition coefficient in the positively charged poly-NIPA/DMA gels is surprisingly independent of pH, although both protein and gel charge vary. For each pH, the partition coefficient is higher than in the neutral poly-NIPA gel. This can be understood as primarily a size-exclusion effect, as the poly-NIPA/DMA gels swell more than the poly-NIPA gels. It appears that the opposing effects of size exclusion and electrostatics cancel each other with the poly-NIPA/DMA gels; while the gel shrinks with rising pH, the electrostatic repulsion between the positively charged solute and positively charged polymer also decreases. The effect of electrostatic repulsion can be observed most clearly at pH 6 where the swelling capacities for the weakly acidic and weakly basic gels coincide. The contribution of size exclusion to the partition coefficients should therefore be equal in both gels. It appears then, that the attractive electrostatic interactions between the protein and weakly acidic gels cause the partition coefficient to be higher in the poly-NIPA/SA gels as opposed to that in the poly-NIPA/DMA gels.

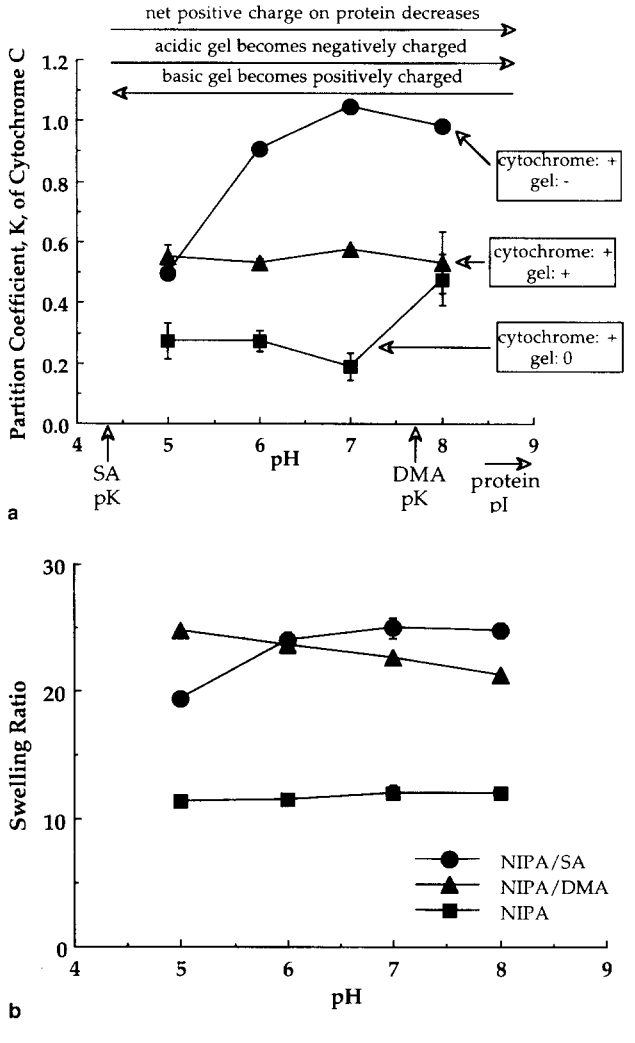

Figure 6 (a) Effect of solution pH on partition coefficient of positively charged cytochrome c in neutral poly-NIPA, negatively charged poly-NIPA/SA, and positively charged poly-NIPA/DMA gels. Experiments were conducted in 0.1 M ionic strength sodium phosphate buffer at 22.2°C. Partition coefficients are highest for the case where the protein and gel are oppositely charged (poly-NIPA/SA). Lines are drawn to guide the eye. (b) Swelling ratios in the experiments of (a). The swelling ratios of the weakly basic and weakly acidic gels are higher than those of the neutral gel. The weakly acidic gel (poly-NIPA/SA) shrinks as the solution becomes more acidic; the weakly basic gel (poly-NIPA/DMA) gel shrinks as the solution becomes more basic. Lines are drawn to guide the eye

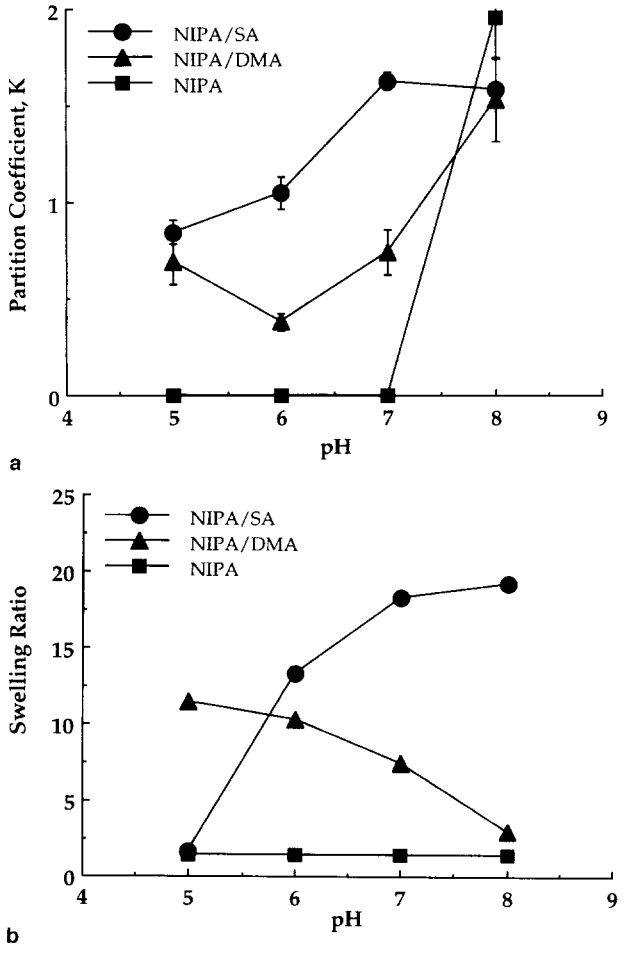

Figure 7 (a) Effect of solution pH on partition coefficient of cytochrome c at 36.4°C in the same gels as in *Figure 6a*. Partitioning is slightly higher for the protein in the polyelectrolyte gels. Lines are drawn to guide the eye. (b) Swelling ratios in the experiments of (a). The swelling ratios here (measured at 36.4°C) are lower than those in *Figure 6b* (measured at 22.2°C) because of the thermosensitivity of poly-NIPA. The trends in swelling with pH are the same at both temperatures. Lines are drawn to guide the eye

Figure 7a presents partitioning data and *Figure 7b* presents swelling data for the same system at 36.4°C, above the transition temperature for uncharged poly-NIPA. Because the swelling ratio for all gels is less at 36.4°C than at 22.2°C, we expect lower partition coefficients (considering size exclusion only), much as Walther *et al.* found for the partitioning of polyethylene glycols and polyethylene oxides in poly-NIPA gels. This expectation holds for the poly-NIPA gels at pH 5–7, where the partition coefficient at 36.4°C is nearly zero. For all other cases, partition coefficients increase with temperature, a phenomenon we have observed in other protein-partitioning experiments. The trends with pH with the poly-NIPA and poly-NIPA/SA are the same as before. However, the partition coefficient with the poly-NIPA/DMA gels now depends on pH; it is a minimum at pH 6.

Figure 8a presents partitioning data for hen egg-white lysozyme in poly-NIPA/SA gels as a function of pH at 22.2 and 36.4°C. *Figure 8b* presents swelling equilibria for the gels. The solution was buffered with sodium phosphates at 0.1 M ionic strength. Lysozyme is also a small protein (MW 14 100), only slightly larger than cytochrome c, with a pI of approximately 11 (refs 22 and 23). Like cytochrome c, lysozyme has a net positive charge at pH 5–8. Lysozyme partitions to a much greater extent into poly-NIPA/SA gels than does cytochrome c; in fact, lysozyme always 'prefers' the gel. Like those of cytochrome c, partition coefficients for lysozyme increase with pH at 22.2°C in accord with swelling equilibria. Partition coefficients are again higher at 36.4°C despite the lower swelling equilibria at this temperature. At pH 6, the partition coefficient of lysozyme at 36.4°C reaches an unexpected maximum. Because significant sorption of lysozyme (as compared to other proteins) by neutral and anionic hydrogels has been reported[24], we are not surprised by the relatively high partition coefficients observed at 0.1 M ionic strength.

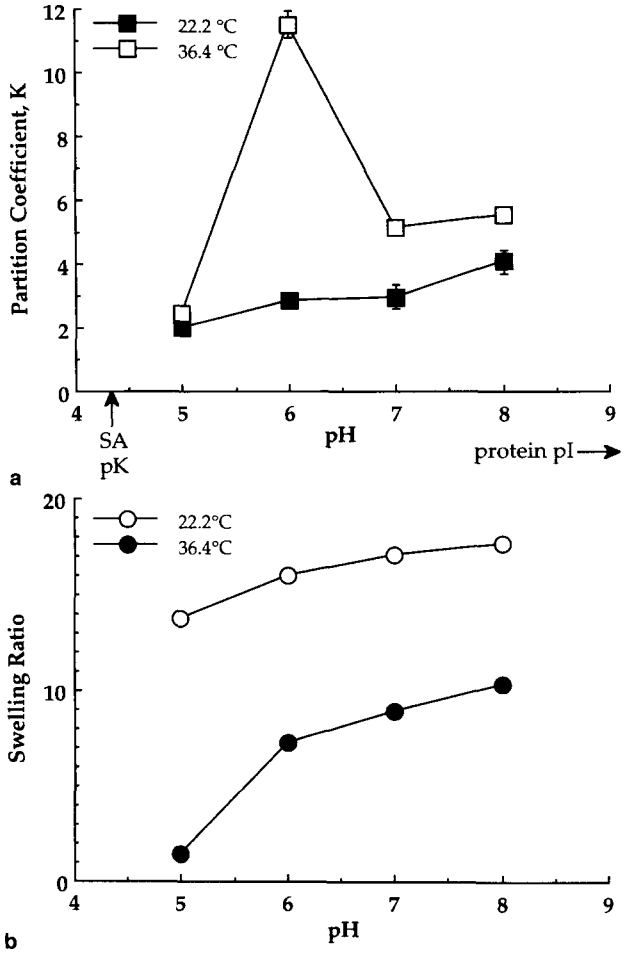

Figure 8 (a) Partition coefficients for positively charged lysozyme in poly-NIPA/10% SA gels at 22.2 and 36.4°C. Experiments were conducted in 0.1 M ionic strength sodium phosphate buffer. The partition coefficients increase slightly with pH, except for pH 6 where the partitioning is a maximum. Partition coefficients are slightly higher at 36.4°C than at 22.2°C. Lines are drawn to guide the eye. (b) Swelling ratios in the experiments of (a). The shrinkage of the gel with decreasing pH is more dramatic at 36.4°C than at 22.2°C. Lines are drawn to guide the eye

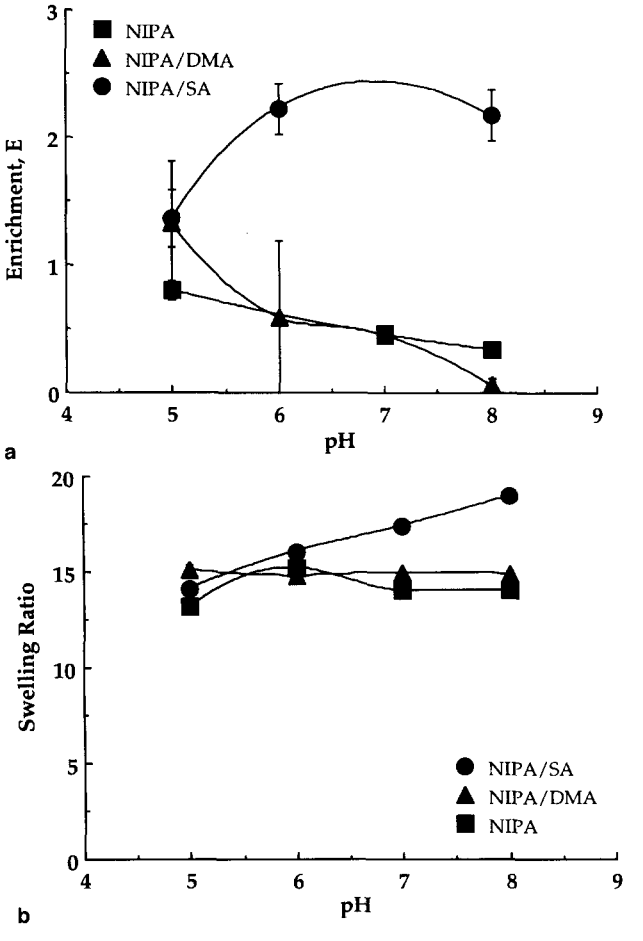

Figure 9 (a) Effect of solution pH on enrichment of cytochrome c relative to ovalbumin by poly-NIPA, poly-NIPA/DMA and poly-NIPA/SA gels. Between pH 5 and 8, cytochrome c is positively charged and ovalbumin is negatively charged. Experiments were conducted at 22.2°C in 0.1 M ionic strength sodium phosphates. Cytochrome is enriched relative to ovalbumin by the weakly acidic, negatively charged poly-NIPA/SA hydrogels at all pH values and by the positively charged NIPA/DMA hydrogels at pH 5. Lines are drawn to guide the eye. (b) Swelling ratios in the experiments of (a). The addition of ovalbumin has depressed the swelling ratios for the polyelectrolyte gels as compared to the swelling ratios in *Figure 6b*, where only cytochrome was present in addition to the buffer. Lines are drawn to guide the eye

Separation of proteins by pH- and temperature-sensitive gels

Hydrogels can separate small solutes efficiently from very large solutes as long as size exclusion is the dominant effect. Therefore, pH- and temperature-sensitive gels might separate proteins of similar size on the basis of pH-dependent electrostatic interactions as well as size. *Figure 9a* presents experimental data for the selectivity of poly-NIPA, poly-NIPA/SA and poly-NIPA/DMA gels for cytochrome c relative to ovalbumin in 0.1 M ionic strength phosphate buffer at 25°C. *Figure 9b* presents the corresponding swelling equilibria. The initial concentration of each protein was 0.05 mg ml^{-1}. The molecular weight of ovalbumin is 44 000, and its isoelectric point is 4.8 (refs 23 and 25). Between pH 5 and 8, ovalbumin has a net negative charge while cytochrome c has a net positive charge. The enrichment, E, is defined relative to the ratio of cytochrome to ovalbumin concentrations in the feed solution:

$$E = \frac{[\text{cytochrome}]_{\text{gel}}/[\text{ovalbumin}]_{\text{gel}}}{[\text{cytochrome}]_{\text{feed}}/[\text{ovalbumin}]_{\text{feed}}}$$

If the relative concentration of cytochrome is greater in the gel than in the feed, E is greater than one. If E is less than one, the relative concentration of cytochrome in the gel is less than that in the feed.

Surprisingly, for neutral poly-NIPA gels, the enrichment, E, is less than one and decreases slightly with pH, contrary to our expectation that the size-exclusion effects of the matrix should lead to an enrichment of the smaller protein. The enrichment for weakly basic poly-NIPA/DMA gels also decreases with pH, again contrary to expectation. The enrichment for weakly acidic poly-NIPA/SA gels increases with pH, in accord with expectation, to a value slightly over 2. At 22.2°C, then, only the poly-NIPA/SA gels are selective for cytochrome, and selectivity increases with solution pH. *Figures 10a* and *10b* present experimental data for E and the swelling ratio for the same system at 36.4°C. The trends in E with pH are similar qualitatively to those in *Figure 9a*, except that E is significantly greater at pH 8 than at pH 7 for both the poly-NIPA and poly-NIPA/DMA gels, and E is a maximum at pH 6 for the poly-NIPA/SA gels. The upturn in E at pH 8 is a manifestation of increased partitioning of cytochrome c at pH 8

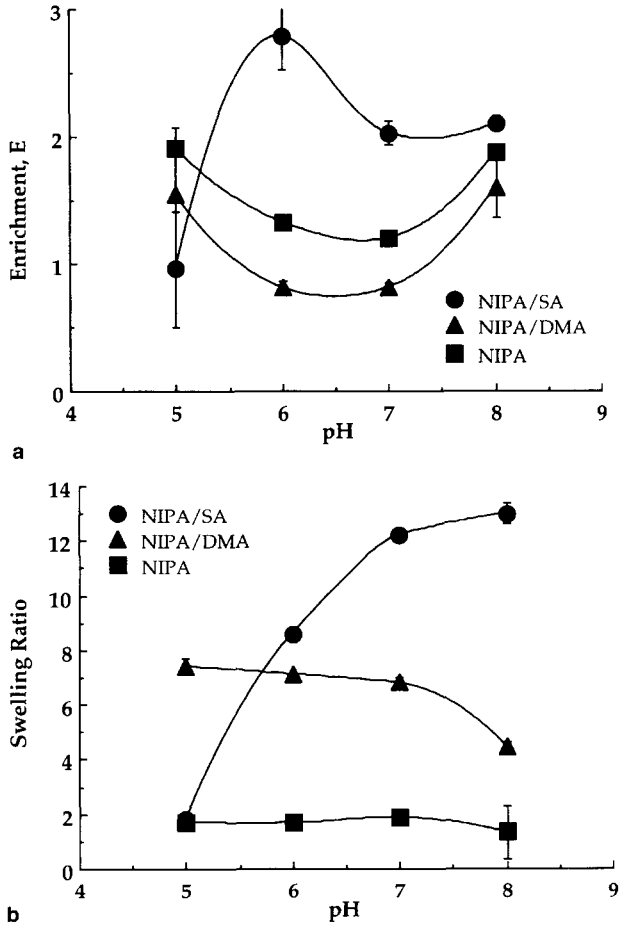

Figure 10 (a) Effect of solution pH on enrichment of cytochrome c relative to ovalbumin by poly-NIPA, poly-NIPA/DMA and poly-NIPA/SA gels at 36.4°C. Experiments are analogous to those in *Figure 9a*, but at a temperature above collapse temperature for poly-NIPA. Cytochrome can be enriched relative to ovalbumin for most combinations of gel type and pH. Lines are drawn to guide the eye. (b) Swelling ratios in the experiments of (a). As in *Figure 9b*, the swelling ratios for the polyelectrolyte gels in the presence of proteins are depressed as compared to the swelling ratios in *Figure 7b*, where only cytochrome was present in addition to the buffer. Lines are drawn to guide the eye

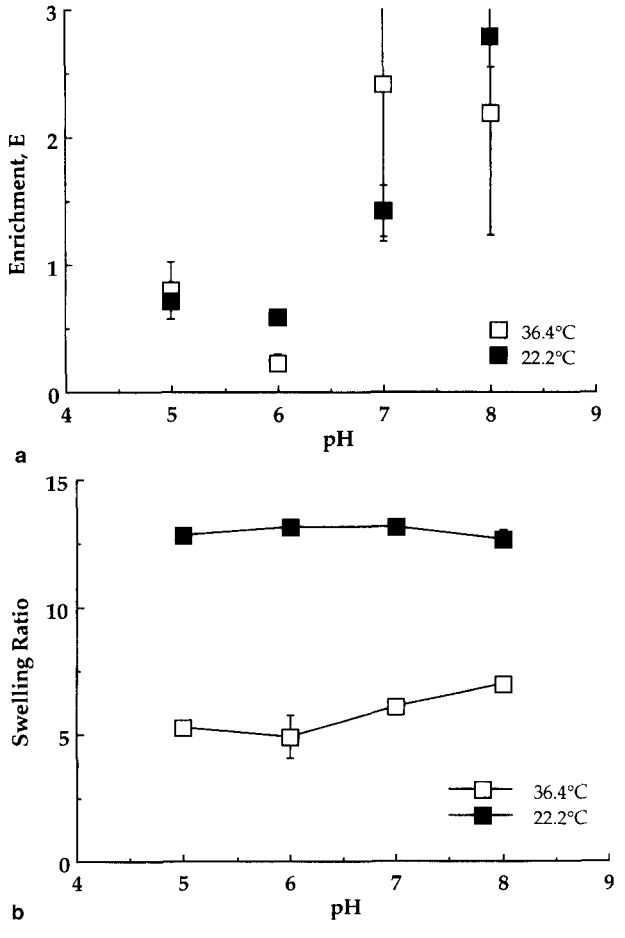

Figure 11 (a) Effect of solution pH on enrichment of cytochrome c relative to ovalbumin by polyampholyte, poly-NIPA/5% SA/5% DMA copolymer hydrogels in 0.1 M ionic strength sodium phosphate buffer at 22.2 and 36.4°C. Cytochrome c is enriched relative to ovalbumin at pH values above 7. Lines are drawn to guide the eye. (b) Swelling ratios for the polyampholyte gels in the experiments of (a). At 22.2°C, the swelling is insensitive to pH, whereas at 36.4°C, the swelling rises slightly between pH 6 and 8. Lines are drawn to guide the eye

compared to that at pH 7, as indicated also in *Figures 6a* and *7a*.

Figure 11a presents experimental data for E for cytochrome c and ovalbumin in novel, temperature-sensitive polyampholytic gels, poly-NIPA/5% DMA/5% SA at 22.2 and 36.4°C. *Figure 11b* presents corresponding swelling data. These gels have both positive and negative charges, and the ionization of each type of monomer depends on pH. Unlike for polyelectrolyte gels, the pH does not have a dramatic effect on swelling between pH 5 and 8 at ambient temperatures. The buffer was 0.1 M ionic strength sodium phosphates. The data for the two temperatures are nearly the same, within experimental error. The enrichment at pH 6 is somewhat less than at pH 5. For poly-NIPA/SA gels, the enrichment rises from pH 6 to 8 to a value between 2 and 3.

While the poly-NIPA-based hydrogel can be selective for cytochrome over ovalbumin, simple partitioning experiments do not yield information regarding percentages of recovery in the gel-based separation processes described in *Figures 1a* and *1b*. In a process, the actual recovery and enrichment of the desired solute depends also on the amount of gel added to the feed solution. The major trade-off is between concentration and recovery of the desired solute. We discuss the concentration and recovery abilities of the processes in *Figures 1a* and *1b* in ref. 26.

CONCLUSIONS

The partition coefficient of a protein in a charged, thermosensitive hydrogel is influenced by solution properties such as pH, temperature and ionic strength, and material properties such as gel composition, charge density, crosslinking (% C) and monomer fraction at synthesis (% T). Changes in each one of these parameters affects the three major mechanisms which contribute to partitioning into a charged hydrogel: size exclusion, electrostatics and short-range interactions such as hydrophobicity. Higher swelling favours higher partition coefficients because more of the gel phase is accessible (steric effects). High gel charge density and opposite charge of solute and gel favour higher partition coefficients because of favourable electrostatic interactions. Low degrees of swelling favour higher partition coefficients based on hydrophobic interactions because the polymer concentration is increased. The experimental partition coefficient depends on a balance of these effects.

For proteins, partition coefficients up to 10 can be realized for temperature- and pH-sensitive gels based on poly-NIPA on 0.1 M ionic strength solution. Decreasing the ionic strength dramatically increases the partition coefficient of a solute partitioning into an oppositely charged hydrogel because the charges are not highly shielded from each other. Even at 0.1 M ionic strength, changing pH has a strong influence on electrostatics. The partition coefficient of a protein may be a maximum or minimum at a pH other than the isoelectric point. Increasing temperature often results in a slight increase in the partition coefficient. Weakly ionizable thermosensitive gels exhibit selectivity towards a series of solutes based on electrostatics as well as size-exclusion effects. For example, the concentration of cytochrome c relative to ovalbumin in a gel can be up to two-and-a-half times the relative concentration in the feed solution.

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NOMENCLATURE

C_f Concentration of solute in feed (mol m^{-3})
C_g Concentration of solute in gel (mol m^{-3})

C_r	Concentration of solute in raffinate (mol m^{-3})
% C	Percentage of crosslinking monomer
% CM	Percentage of comonomer
E	Enrichment
K	Partition coefficient
m_f	Mass of feed (kg)
m_r	Mass of raffinate (kg)
% T	Ratio of monomer to diluent at synthesis (g ml^{-1})
η	Cussler's dimensionless efficiency of solute exclusion

APPENDIX

Experimental partition coefficients and swelling equilibria

The partition coefficient, K, reported here is the arithmetic average of K determined for three to six replicate experiments. The standard deviation follows the ± symbol and was calculated using the following formula:

$$\sqrt{\frac{n\sum x^2 - \left(\sum x\right)^2}{n(n-1)}}$$

where n is the number of replicates and x is the value.

Table A1 Bovine serum albumin: MW 66 000; pI 4.8; buffer: sodium citrate/phosphate, 0.01 M ionic strength, sodium azide, 0.1 g l^{-1} (0.0077 M ionic strength); feed concentration: 1.0 g ml^{-1}

pH	Temperature (°C)	Gel	Partition coefficient, K	Swelling ratio, SR	Notes
8	10	NIPA/0% MAPTAC	0.0 ± 0.2	19.2 ± 0.4	
8	10	NIPA/3% MAPTAC	12.8 ± 1.4	23.5 ± 0.1	
8	10	NIPA/DMA	8.5 ± 0.5	27.2 ± 0.1	
8	10	NIPA/SA	0.00 ± 0.003	64.0 ± 0.8	
3	10	NIPA/0% MAPTAC	0.0 ± 0.3	18.2 ± 0.2	
3	10	NIPA/3% MAPTAC	0.2 ± 0.2	23.9 ± 0.4	
3	10	NIPA/DMA	0.6 ± 0.2	40.9 ± 0.7	
3	10	NIPA/SA	1.9 ± 0.1	21.8 ± 0.3	
8	35	NIPA/3% MAPTAC	151 ± 7	13.3 ± 0.3	
8	35	NIPA/DMA	6 ± 1	11.0 ± 0.8	
8	35	NIPA/SA	47 ± 20	1.644 ± 0.004	
8	10	NIPA/3% MAPTAC	0.19 ± 0.02	16.7 ± 0.1	with 0.1 M sodium sulfate
8	10	NIPA/DMA	0.48 ± 0.06	22.53 ± 0.06	with 0.1 M sodium sulfate
8	35	NIPA/3% MAPTAC	95 ± 2	12.3 ± 0.3	with 0.2 g l^{-1} sodium azide
8	35	NIPA/3% MAPTAC	71 ± 14	11.70 ± 0.01	with 0.3 g l^{-1} sodium azide
8	35	NIPA/3% MAPTAC	30.9 ± 0.7	11.1 ± 0.3	with 0.5 g l^{-1} sodium azide
8	35	NIPA/DMA	4.3 ± 0.3	10.8 ± 0.3	with 0.2 g l^{-1} sodium azide
8	35	NIPA/DMA	5.9 ± 0.4	10.6 ± 0.2	with 0.3 g l^{-1} sodium azide
8	35	NIPA/DMA	4.7 ± 0.6	10.74 ± 0.05	with 0.5 g l^{-1} sodium azide

Table A2 Caffeine: MW 194.2; buffer: sodium citrate/phosphate, 0.01 M ionic strength; feed concentration: 0.0025 M
[a]Deionized water (no buffer); feed concentration: 0.0005 M

pH	Temperature (°C)	Gel	Partition coefficient, K	Swelling ratio, SR
7	10	NIPA/0% MAPTAC	4.1 ± 0.9	17.7 ± 0.1
7	10	NIPA/1% MAPTAC	1.41 ± 0.05	19.3 ± 0.2
7	10	NIPA/2% MAPTAC	1.8 ± 0.2	19.9 ± 0.1
7	10	NIPA/3% MAPTAC	3.6 ± 1.1	20.3 ± 0.1
NA[a]	10	NIPA/0% MAPTAC	1.1 ± 0.1	11.5 ± 0.2
NA[a]	10	NIPA/1% MAPTAC	1.02 ± 0.02	27.4 ± 0.2
NA[a]	10	NIPA/2% MAPTAC	1.01 ± 0.08	42.7 ± 0.3
NA[a]	10	NIPA/3% MAPTAC	0.98 ± 0.01	54 ± 1

Table A3 Catalase: MW: 245 000; pI: 6.7; buffer: sodium citrate/phosphate, 0.01 M ionic strength, sodium azide, 0.05 g l^{-1}; feed concentration: 0.02 mg ml^{-1}

pH	Temperature (°C)	Gel	Partition coefficient, K	Swelling ratio, SR
7	10	NIPA/0% MAPTAC	1.21 ± 0.1	18.5 ± 0.1
7	10	NIPA/1% MAPTAC	1.9 ± 1.4	20.4 ± 0.2
7	10	NIPA/2% MAPTAC	2 ± 1	22.0 ± 0.1
7	10	NIPA/3% MAPTAC	2.2 ± 0.3	22.9 ± 0.1
7	35	NIPA/0% MAPTAC	4.28 ± 0.03	2.8 ± 0.2
7	35	NIPA/1% MAPTAC	3.14 ± 0.2	8.8 ± 0.03
7	35	NIPA/2% MAPTAC	12 ± 1	11.51 ± 0.07
7	35	NIPA/3% MAPTAC	18.5 ± 0.2	13.96 ± 0.06

Table A4 Cytochrome c: MW 12 400; pI 10.4; buffer: sodium phosphate, 0.1 M ionic strength; feed concentration: listed under Notes in table

pH	Temperature (°C)	Gel	Partition coefficient, K	Swelling ratio, SR	Notes
5	22.2	NIPA	0.27 ± 0.06	11.4 ± 0.3	0.384 mg ml^{-1} initial concentration
6	22.2	NIPA	0.27 ± 0.04	11.5 ± 0.1	0.326 mg ml^{-1} initial concentration
7	22.2	NIPA	0.19 ± 0.05	12.1 ± 0.5	0.327 mg ml^{-1} initial concentration
8	22.2	NIPA	0.48 ± 0.08	12.1 ± 0.2	0.358 mg ml^{-1} initial concentration
5	22.2	NIPA/10% DMA	0.55 ± 0.04	24.7 ± 0.2	0.376 mg ml^{-1} initial concentration
6	22.2	NIPA/10% DMA	0.53 ± 0.01	23.6 ± 0.1	0.430 mg ml^{-1} initial concentration
7	22.2	NIPA/10% DMA	0.57 ± 0.01	22.7 ± 0.2	0.516 mg ml^{-1} initial concentration
8	22.2	NIPA/10% DMA	0.53 ± 0.1	21.2 ± 0.2	0.470 mg ml^{-1} initial concentration
5	22.2	NIPA/10% SA	0.496 ± 0.006	19.4 ± 0.4	0.690 mg ml^{-1} initial concentration
6	22.2	NIPA/10% SA	0.91 ± 0.01	24.0 ± 0.7	0.587 mg ml^{-1} initial concentration
7	22.2	NIPA/10% SA	1.05 ± 0.02	25.0 ± 0.9	0.443 mg ml^{-1} initial concentration
8	22.2	NIPA/10% SA	0.980 ± 0.007	24.7 ± 0.7	0.617 mg ml^{-1} initial concentration
5	36.4	NIPA	0.0 ± 0.0	1.49 ± 0.03	0.384 mg ml^{-1} initial concentration
6	36.4	NIPA	0.0 ± 0.0	1.41 ± 0.04	0.326 mg ml^{-1} initial concentration
7	36.4	NIPA	0.0 ± 0.0	1.47 ± 0.01	0.327 mg ml^{-1} initial concentration
8	36.4	NIPA	1.961 ± 0.2	1.5 ± 0.2	0.358 mg ml^{-1} initial concentration
5	36.4	NIPA/10% DMA	0.7 ± 0.1	11.5 ± 0.3	0.376 mg ml^{-1} initial concentration
6	36.4	NIPA/10% DMA	0.38 ± 0.05	10.3 ± 0.2	0.430 mg ml^{-1} initial concentration
7	36.4	NIPA/10% DMA	0.7 ± 0.1	7.46 ± 0.4	0.516 mg ml^{-1} initial concentration
8	36.4	NIPA/10% DMA	1.5 ± 0.2	3.0 ± 0.1	0.470 mg ml^{-1} initial concentration
5	36.4	NIPA/10% SA	0.85 ± 0.06	1.7 ± 0.2	0.690 mg ml^{-1} initial concentration
6	36.4	NIPA/10% SA	1.05 ± 0.08	13.4 ± 0.4	0.587 mg ml^{-1} initial concentration
7	36.4	NIPA/10% SA	1.63 ± 0.05	18.3 ± 0.1	0.443 mg ml^{-1} initial concentration
8	36.4	NIPA/10% SA	1.582 ± 0.002	19.2 ± 0.1	0.617 mg ml^{-1} initial concentration

Table A5 Haemoglobin; MW 67 000; pI 7.4; buffer: sodium citrate/phosphate, 0.01 M ionic strength, sodium azide, 0.05 g l^{-1}; feed concentration: 0.04 mg ml^{-1}

pH	Temperature (°C)	Gel	Partition coefficient, K	Swelling ratio, SR
8	10	NIPA/0% MAPTAC	0.3 ± 0.1	18.1 ± 0.1
8	10	NIPA/1% MAPTAC	0.23 ± 0.06	20.0 ± 0.3
8	10	NIPA/2% MAPTAC	0.021 ± 0.005	19.0 ± 0.8
8	10	NIPA/3% MAPTAC	0.34 ± 0.03	22.5 ± 0.4
5	10	NIPA/SA	43 ± 4	42.6 ± 0.4
5	10	NIPA/DMA	0.16 ± 0.07	27.7 ± 0.2

Table A6 Tryptophan: MW 204.3; buffer: sodium citrate/phosphate, 0.01 M ionic strength, sodium azide, 0.1 g l^{-1}; feed concentration: 0.005 M

pH	Temperature (°C)	Gel	Partition coefficient, K	Swelling ratio, SR
8	10	NIPA/0% MAPTAC	5 ± 2	12.4 ± 0.2
8	10	NIPA/1% MAPTAC	4.0 ± 0.7	14.9 ± 0.1
8	10	NIPA/2% MAPTAC	3.6 ± 0.6	17.15 ± 0.08
8	10	NIPA/3% MAPTAC	1.88 ± 0.2	60.9 ± 0.6
8	10	NIPA/DMA	4.2 ± 0.8	19.13 ± 0.05
8	10	NIPA/SA	2.6 ± 0.2	18.7 ± 0.3

Table A7 Vitamin B12 (cyanocobalamin): MW 1355.4; buffer: sodium citrate/phosphate, 0.01 M ionic strength, sodium azide, 0.1 g l^{-1}; feed concentration: 0.338 mg ml^{-1} (except where noted below)

pH	Temperature (°C)	Gel	Partition coefficient, K	Swelling ratio, SR	Notes
8	10	NIPA/0% MAPTAC	1.15 ± 0.01	12.4 ± 0.8	
8	10	NIPA/1% MAPTAC	1.00 ± 0.01	14.9 ± 0.3	
8	10	NIPA/2% MAPTAC	0.943 ± 0.007	14.2 ± 0.4	
8	10	NIPA/3% MAPTAC	0.950 ± 0.007	18.7 ± 0.9	
3	10	NIPA/10% DMA	0.88 ± 0.3	55.9 ± 0.3	0.179 mg ml^{-1} initial concentration
3	10	NIPA/10% SA	0.75 ± 0.1	23.2 ± 0.3	0.179 mg ml^{-1} initial concentration
5	10	NIPA/10% DMA	1.07 ± 0.01	30.8 ± 0.65	0.244 mg ml^{-1} initial concentration
5	10	NIPA/10% SA	1.01 ± 0.01	77.5 ± 0.9	0.244 mg ml^{-1} initial concentration
4.5	8.4	NIPA	1.6 ± 0.1	16.1 ± 0.5	
5	8.4	NIPA	1.2 ± 0.1	16.4 ± 0.9	
5	8.4	NIPA	1.0 ± 0.1	17.1 ± 0.5	

Table A8 Enrichment experiments: Ovalbumin: MW 44 000; feed concentration: 0.05 mg ml^{-1} (except where noted). Cytochrome c: MW 12 400; feed concentration: 0.05 mg ml^{-1} (except where noted); buffer: sodium phosphate, 0.1 M ionic strength

pH	Temperature (°C)	Gel	Enrichment, E	Swelling ratio, SR	Notes
5	22.2	NIPA	0.80 ± 0.07	13.1 ± 0.1	
7	22.2	NIPA	0.46 ± 0.03	15.2 ± 0.6	
8	22.2	NIPA	0.34 ± 0.04	14.0 ± 0.2	
5	36.4	NIPA	1.9 ± 0.7	1.7 ± 0.2	
6	36.4	NIPA	1.3 ± 0.2	1.7 ± 0.1	
7	36.4	NIPA	1.2 ± 0.2	1.9 ± 0.02	
8	36.4	NIPA	1.9 ± 0.2	1.9 ± 0.1	
5	22.2	NIPA/10% DMA	1.35 ± 0.5	15.1 ± 0.2	
6	22.2	NIPA/10% DMA	0.58 ± 0.5	14.8 ± 0.1	
7	22.2	NIPA/10% DMA	0.45 ± 0.03	14.9 ± 0.03	
8	22.2	NIPA/10% DMA	0.057 ± 0.05	14.9 ± 0.1	
5	36.4	NIPA/10% DMA	1.5 ± 0.5	7.4 ± 0.2	
6	36.4	NIPA/10% DMA	0.81 ± 0.05	7.13 ± 0.04	
7	36.4	NIPA/10% DMA	0.81 ± 0.04	6.8 ± 0.2	
8	36.4	NIPA/10% DMA	1.6 ± 0.2	4.5 ± 0.2	
5	22.2	NIPA/10% SA	1.4 ± 0.2	14.1 ± 0.1	
6	22.2	NIPA/10% SA	2.2 ± 0.2	16.0 ± 0.1	
8	22.2	NIPA/10% SA	2.2 ± 0.2	18.9 ± 0.1	
5	36.4	NIPA/10% SA	0.9 ± 0.5	1.80 ± 0.09	
6	36.4	NIPA/10% SA	2.8 ± 0.3	8.6 ± 0.3	
7	36.4	NIPA/10% SA	2.0 ± 0.1	12.2 ± 0.1	
8	36.4	NIPA/10% SA	2.1 ± 0.1	13.0 ± 0.4	
5	22.2	NIPA/5% SA/5% DMA	0.71 ± 0.06	12.8 ± 0.2	cytochrome: 0.62 mg ml^{-1}; ovalbumin: 2.6 mg ml^{-1}
6	22.2	NIPA/5% SA/5% DMA	0.59 ± 0.06	13.1 ± 0.2	cytochrome: 0.62 mg ml^{-1}; ovalbumin: 2.7 mg ml^{-1}
7	22.2	NIPA/5% SA/5% DMA	1.4 ± 0.2	13.1 ± 0.3	cytochrome: 0.65 mg ml^{-1}; ovalbumin: 2.5 mg ml^{-1}
8	22.2	NIPA/5% SA/5% DMA	2.8 ± 0.2	12.7 ± 0.3	cytochrome: 0.63 mg ml^{-1}; ovalbumin: 2.6 mg ml^{-1}
5	36.4	NIPA/5% SA/5% DMA	0.8 ± 0.2	5.2 ± 0.2	cytochrome: 0.62 mg ml^{-1}; ovalbumin: 2.6 mg ml^{-1}
6	36.4	NIPA/5% SA/5% DMA	0.23 ± 0.07	4.9 ± 0.9	cytochrome: 0.62 mg ml^{-1}; ovalbumin: 2.7 mg ml^{-1}
7	36.4	NIPA/5% SA/5% DMA	2.4 ± 1.2	6.0 ± 0.2	cytochrome: 0.65 mg ml^{-1}; ovalbumin: 2.5 mg ml^{-1}
8	36.4	NIPA/5% SA/5% DMA	2.2 ± 0.9	6.9 ± 0.1	cytochrome: 0.63 mg ml^{-1}; ovalbumin: 2.6 mg ml^{-1}